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GENETICS

# Targeted next-generation sequencing panel screening of 668 Chinese patients with non-obstructive azoospermia

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## Abstract

**Purpose** We aimed (1) to determine the molecular diagnosis rate and the recurrent causative genes of patients with non-obstructive azoospermia (NOA) using targeted next-generation sequencing (NGS) panel screening and (2) to discuss whether these genes help in the prognosis for microsurgical testicular sperm extraction (micro-TESE).

**Methods** We used NGS panels to screen 668 Chinese men with NOA. Micro-TESE outcomes for six patients with pathogenic mutations were followed up. Functional assays were performed for two *NR5A1* variants identified: p.I224V and p.R281C.

**Results** Targeted NGS panel sequencing could explain 4/189 (2.1% by panel 1) or 10/479 (2.1% by panel 2) of the patients with NOA after exclusion of karyotype abnormalities and Y chromosome microdeletions. Almost all mutations detected were newly described except for *NR5A1* p.R281C and *TEX11* p.M156V. Two missense *NR5A1* mutations—p.R281C and p.I244V—were proved to be deleterious by *in vitro* functional assays. Mutations in *TEX11*, *TEX14*, and *NR5A1* genes are recurrent causes of NOA, but each gene explains only a very small percentage (less than 4/668; 0.6%). Only the patient with *NR5A1* mutations produced viable spermatozoa through micro-TESE, but other patients with *TEX11* and *TEX14* had poor micro-TESE prognoses.

**Conclusions** A targeted NGS panel is a feasible diagnostic method for patients with NOA. Because each gene implicated explains only a small proportion of such cases, more genes should be included to further increase the diagnostic rate. Considering previous reports, we suggest that only a few genes that are directly linked to meiosis can indicate poor micro-TESE prognosis, such as *TEX11*, *TEX14*, and *SYCE1*.

**Keywords** Male infertility · Microsurgical testicular sperm extraction · Next-generation sequencing · Non-obstructive azoospermia · Spermatogenesis

## Introduction

Up to 1 in 10 couples have infertility problems, and male factors account for 20–25% of these cases [1, 2]. Azoospermia is defined as the absence of spermatozoa in the ejaculate after centrifugation of semen specimens. Non-obstructive azoospermia (NOA) is an extreme phenotype of quantitative spermatogenic impairment, affecting approximately 10–15% of infertile men [3]. The etiology of NOA includes both acquired and congenital factors. Genetic analyses such as karyotyping and evaluating azoospermia factor (AZF) microdeletions have become routine clinical practice in andrology [4, 5]. About 13.7% of patients with azoospermia have an abnormal karyotype [6], and about 10.04% of patients with NOA have AZF microdeletions [7]. However, many patients with NOA are still diagnosed as having idiopathic infertility. Without a clear diagnosis, it is hard to counsel such patients about the causes of their infertility, their prognosis for microsurgical testicular sperm extraction (micro-TESE), and the health risk to the man and his offspring.

Some causative genes for NOA have been identified in recent years [8]. Unlike previous genome-wide association studies, next-generation sequencing (NGS)-based studies mainly focus on rare variants with high penetrance, also known as monogenic variants [8]. Genetic defects of more than 400 genes can affect male mouse fertility [9], and at least 14 human genes have been confirmed to have pathogenic effects, including *TEX11*, *TEX14*, *SYCE1*, *SYCP3*, *MEIOB*, *AR*, *NR5A1*, *KLHL10*, *FANCM*,

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*SOHLH1*, *STAG3*, *TEX15*, *TDRD9*, and *ZMYND15* [10–23]. Because most of them are inherited as recessive forms, the interpretation rate is expected to be very low for each gene. As NGS becomes more affordable, it has the potential to become a routine diagnostic method for NOA [24, 25].

Although this approach is promising, there are still a few issues of concern. First, genes in an NGS panel must be selected carefully to avoid misdiagnosis. Only about 18% of all described male genes associated with infertility are at least moderately linked to the phenotype [26], as evaluated by an existing gene-disease scoring system [27]. Second, knowing the diagnostic rate and hot-spot genes or variants can help optimize NGS panels and provide priorities for the development of targeted therapies. Third, a detailed mutational and phenotypic spectrum is needed for diagnosis and for predicting the prognosis of micro-TESE. Here we carefully selected monogenic genes for targeted NGS panels (Table 1), supported by mouse modeling and clinical cases, and used them to screen 668 Chinese patients with NOA to clarify the following issues: (1) the mutational landscape for these genes among such patients with NOA and (2) whether those gene mutations correspond to or can help predict the outcomes of micro-TESE.

## Methods

### Study subjects

This study included 668 Chinese patients with NOA who presented to the Department of Urology and Andrology of Renji Hospital, Shanghai Jiao Tong University, School of Medicine from November 2017 to June 2020. All patients had undergone a comprehensive andrological examination including semen analyses; serum hormone analyses for the levels of follicle-stimulating hormone (FSH) luteinizing hormone (LH), testosterone (T), prolactin (PRL), and estradiol (E2); testicular volume measurement via B-mode ultrasonography; karyotyping analysis; and Y chromosome microdeletion screening (sY84, sY86, sY127, sY134, sY254, sY255) [5]. Patient A4524 had undergone bilateral orchiopexy for undescended testes. Patients with obstructive azoospermia characterized by physical obstruction of the post-testicular genital tract were excluded. Other exclusion criteria include histories of orchitis or parotitis, chemotherapy for neoplastic disease, chromosomal anomalies, or Y chromosome microdeletions (AZFa, AZFb, AZFbc, and AZFc). All study participants signed informed consents.

### Next-generation sequencing

Genomic DNA was extracted from blood samples using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The samples have been screened successively in two NGS panel versions (panel 1 and panel 2, see Table 1). The first 189 samples were screened using panel 1 (5 genes), and the rest 479 samples were sequenced by panel 2 (14 genes). Target capture was performed for coding exons and flanking introns (± 10 bp) using IDT xGen Lockdown Probes (Integrated DNA Technologies, Coralville, IA, USA). The extent of each library was assessed using a Qubit 2.0

**Table 1** Non-obstructive azoospermia causative genes in targeted next-generation sequencing panel

| Gene | Panel 1 | Panel 2 | Gene function | Inheritance | OMIM |
|---|---|---|---|---|---|
| *TEX11* | √ | √ | Chromosome synapsis and formation of crossovers | XLR | 300311 |
| *AR* | √ | √ | Androgen receptor | XLR | 313700 |
| *NR5A1* | √ | √ | Transcription factors involved in sex determination | AD | 184757 |
| *KLHL10* | √ | √ | Mediate protein ubiquitination during spermiogenesis | AD | 608778 |
| *SYCP3* | √ | √ | Component of the synaptonemal complex | AD | 604759 |
| *FANCM* | | √ | DNA repair | AR | 609644 |
| *MEIOB* | | √ | Meiosis specific with OB domain | AR | 617670 |
| *TEX14* | | √ | Intercellular bridges | AR | 605792 |
| *SOHLH1* | | √ | Important regulators of spermatogenesis | AD | 610224 |
| *STAG3* | | √ | Cohesion of sister chromatids, DNA repair | AR | 608489 |
| *SYCE1* | | √ | Component of the synaptonemal complex | AR | 611486 |
| *TEX15* | | √ | Chromosome synapsis and DNA repair | AR | 605795 |
| *TDRD9* | | √ | piRNA-mediated retrotransposon silencing | AR | 618110 |
| *ZMYND15* | | √ | Transcriptional repressor in spermiogenesis | AR | 614312 |

*XLR* X-linked recessive, *AR* autosomal recessive, *AD* autosomal dominance

fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality and size of libraries were measured using a 2100 Bioanalyzer High Sensitivity DNA Assay (Agilent Technologies, Santa Clara, CA, USA) according to the reagent kit guide. The libraries were applied to 2 × 75 bp paired-end sequencing on the Illumina NextSeq 500 platform (Illumina Inc., San Diego, CA, USA).

### Data analysis

FASTQ format raw data were filtered and aligned to the human reference genome (hg19/GRCh37) using BWA v. 0.7.13 [28]. Variants including single nucleotide variants (SNVs) and short insertions and deletions (InDels) were genotyped from recalibrated BAM files by VarDict [29]. Benign or likely benign variants identified by InterVar [30] were filtered, and the remaining variants were classified as pathogenic (P), likely pathogenic (LP), or a variant of unknown significance (VUS) according to the guidelines from the American College of Medical Genetics and Genomics (ACMG) [31]. Copy number variants (CNVs) were first called using the DNAcopy R package [32] and checked based on sequencing depth using the Integrative Genomics Viewer [33]. Nonpolymorphic CNVs were classified as P, LP, or VUS by applying the ACMG guidelines [34]. Patients were considered to achieve a definitive diagnosis when any P/LP variant was found in genes under autosomal dominant or X-linked inheritance, or P/LP homozygous variant were found in autosomal recessive genes, or at least one P/LP variant was found among compound heterozygous mutations under autosomal recessive genes.

### Variant validation

All variants reported in this study were checked manually by the Integrative Genomics Viewer [33] followed by Sanger sequencing validation to avoid false positives. Sanger sequencing was used to distinguish the cis/trans relationship of pairs of heterozygous mutations in autosomal recessive genes using saliva samples from the parents.

### Hematoxylin and eosin (HE) staining

Testicular tissues acquired by diagnostic biopsy or during micro-TESE were fixed in 4% paraformaldehyde solution for 12 h, embedded in paraffin wax, and sectioned at 5 μm thickness. Sections were stained with HE solution, and the images were captured by optical microscopy under ×40 magnification (evos FL auto 2, Thermo Fisher Scientific, Waltham, MA, USA).

### *In vitro* functional assays of *NR5A1* mutations

Western blot analysis and gene expression assays were performed for two *NR5A1* missense variants—p.I224V and p.R281C—identified in this study. Analysis of three additional variants—p.G35E, p.R191C, and p.D238N—described in a previous study [35] were also repeated to increase credibility to our experiment. Wild-type (WT) human *NR5A1* cDNA was cloned into a pCDNA3.0 vector to allow expression of Flag-tagged protein. Based on this vector, five *NR5A1* mutations were generated by site-directed mutagenesis kits (Vazyme Biotech Co., Nanjing, China). The entire coding sequences of all mutant plasmids were confirmed by direct sequencing. Plasmids (2 μg/well) were transfected into HEK293T cells using the lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Cells were collected 48 h later, and whole cell extracts were prepared in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer. Protein products were separated on SDS–PAGE gels and blotted onto nitrocellulose membrane. The blot was probed with anti-Flag and anti-β-actin antibodies.

The *NR5A1*-regulated genes *CYP11A1*, *CYP17A1*, and *CYP19A1* [36] were used for quantitative reverse transcription polymerase chain reaction (RT–qPCR) analysis. Total RNA was isolated from the HEK293T cells containing WT or mutant *NR5A1* cDNA using TRIzol reagent (Invitrogen). Total RNA (1 μg) for each sample was converted into cDNA by RT with oligo(dT) primers. Three replicates were used for each qPCR analysis using a QuantStudio™ 7 Flex Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Empty vector served as a control. The primer sequences used for plasmid construction and qPCR are listed in Supplementary Table S1.

## Results

### Gene variants

For all 668 patients with NOA, the first 189 samples were screened using panel 1 (5 genes) to a mean depth of 1137.73 ± 429.48 ×, and the remaining 479 samples were sequenced using panel 2 (14 genes) to a mean depth of 533.20 ± 54.36×. In all,14 patients were considered to achieve a definitive diagnosis. The positive molecular diagnosis rates were 4/189 (2.1%) for panel 1 and 10/479 (2.1%) for panel 2. The gene variants detected contained 14 SNVs, 1 InDel, and 1 CNV. For four sequenced autosomal dominant genes *NR5A1*, *KLHL10*, and *SYCP3* (both included in panels 1 and 2) and *SOHLH1* (only included in panel 2), only *NR5A1* (4/668; 0.6%) and *KLHL10* (1/668; 0.2%) were found to have causative mutations. For eight of the autosomal recessive genes—*FANCM*, *MEIOB*, *TEX14*, *STAG3*, *SYCE1*, *TEX15*, *TDRD9*,

and *ZMYND15* (included in panel 2 only)—compound heterozygous mutations, homozygous mutations, or homozygous deletions were found in *TEX14* (3/479, 0.6%), *ZMYND15* (1/479, 0.2%), and *SYCE1* (1/479, 0.2%). For two X-linked recessive genes, *AR* and *TEX11* (included in both panel 1 and 2), four SNVs were found in *TEX11* (4/668, 0.6%). In addition, strong candidate mutations were found in four patients including four SNVs in *AR* and *KLHL10* (Table 2).

## Clinical characteristics

Eight of the patients had been examined for testicular histopathology: A2799, A961, A4124, A5352, and A5181 showed partial or complete maturation arrest (MA); A3600 and A2664 had Sertoli cell only (SCO) phenotype; and A5197 had normal spermatogenesis (Table 3, Fig. 1). More than half of the patients (10/18; 56%) had small testes (< 10 mL), and half of them (9/18; 50%) had higher than normal FSH values, indicating gonadal dysgenesis (Table 3). Testicular histopathology diagnoses for patients A2664 (SCO with a *KLHL10* mutation), A961 (MA with a *TEX11* mutation), and A5197 (normal spermatogenic function with an *NR5A1* mutation) are shown in Fig. 1.

## Deleterious function of *NR5A1* mutations

Five of the analyzed mutations—p.I224V, p.R281C, p.G35E, p.R191C, and p.D238N—did not decrease the expression of NR5A1 protein (Fig. 2a), but they all affected the expression of downstream genes *CYP11A1*, *CYP17A1*, and *CYP19A1* (Fig. 2b). The p.I224V and p.R281C mutations caused the most severe effects, reducing downstream gene expression to empty vector (EV) levels. The degrees of deleterious effect of p.G35E, p.R191C, and p.D238N were consistent with a previous report [35], indicating robustness of this experiment.

## Outcomes of micro-TESE

Most patients chose not to use medication or micro-TESE treatments after being informed of their genetic diagnosis, and they gave up hope of fatherhood or used donor semen. Three of them (A4524, A1064, and A5352) chose to use 3 months of medication: a combination of vitamin E (300 mg/day) and clomiphene citrate (50 mg/day). Of these, patients A4524 and A1064 with *NR5A1* mutations successfully produced a few spermatozoa by micro-TESE, but patient A5352 with a *TEX14* mutation remained with a diagnosis of NOA after treatment. Only two

**Table 2** Patients with definite diagnosis and strong candidate variants

| Patient ID | Age | Gene | Transcript | Exon/intron | Variants (zygosity) | Pop Freq | Clin Sig |
|---|---|---|---|---|---|---|---|
| Patients with definite diagnosis | | | | | | | |
| A2380 | 34 | *KLHL10* | NM_001329596 | Exon 3 | c.1012G>A, p.A338T, (Het) | 0 | LP (PM1+PM2+PP2+PP3) |
| A4524 | 20 | *NR5A1* | NM_004959 | Exon 2 | c.39C>A, p.C13X, (Het) | 0 | LP (PVS1+PM2) |
| A5197 | 31 | *NR5A1* | NM_004959 | Intron 3 | c.244+1G>A, NA, (Het) | 0 | LP (PVS1+PM2) |
| A1064 | 35 | *NR5A1* | NM_004959 | Exon 4 | c.730A>G, p.I244V, (Het) | 0 | LP (PS3+PM1+PM2+PP2) |
| A2719 | 32 | *NR5A1* | NM_004959 | Exon 4 | c.841C>T, p.R281C, (Het) * | 0 | LP (PS3+PM1+PM2+PP2) |
| A2799 | 30 | *TEX11* | NM_031276 | Exon 26 | c.2240C>A, p.S747X, (Hem) | 0 | P (PVS1+PM2+PP3) |
| A4999 | 30 | *TEX11* | NM_031276 | Exon 16 | c.1337G>T, p.R446M, (Hem) | 0 | LP (PM1+PM2+PP2+PP3) |
| A961 | 31 | *TEX11* | NM_031276 | Exon 7 | c.466A>G, p.M156V, (Hem) * | 0.0006 | LP (PM1+PM2+PP2+PP5) |
| A2153 | 34 | *TEX11* | NM_031276 | Exon 16 | c.1246C>T, p.Q416X, (Hem) | 0 | LP (PVS1+PM2) |
| A4124 | 30 | *TEX14* | NM_001201457 | Exon 10 | c.1113dupG, p.F372fs, (Het) | 0 | LP (PVS1+PM2) |
| | | *TEX14* | NM_001201457 | Exon 10 | c.1102C>T, p.H368Y, (Het) | 8.24E-06 | LP (PM1+PM2+PM3+PP3) |
| A5352 | 22 | *TEX14* | NM_001201457 | Exon 2 | c.76C>T, p.Q26X, (Hom) | 8.25E-06 | LP (PVS1+PM2) |
| A6326 | 26 | *TEX14* | NM_001201457 | Exon 14 | c.1898C>A, p.S633X, (Hom) | 0 | P (PVS1+PM2+PP3) |
| A5343 | 28 | *ZMYND15* | NM_001136046 | Exon 3 | c.827G>A, p.R276Q, (Het) | 0.0001 | VUS (PM2+PM3+PP3) |
| | | *ZMYND15* | NM_001136046 | Intron 11 | c.1837+1G>C, NA, (Het) | 8.24E-06 | LP (PVS1+PM2) |
| A5181 | 33 | *SYCE1* | NM_001143763 | Whole gene | Homozygous deletion | / | P |
| Patients with strong candidate variants | | | | | | | |
| A4640 | 32 | *AR* | NM_000044 | Exon 1 | c.1325A>T, p.E442V, (Hem) | 0 | VUS (PM2+PP2) |
| A2864 | 33 | *AR* | NM_000044 | Exon 1 | c.1157G>T, p.R386L, (Hem) | 5.13E-05 | VUS (PM1+PM2+PP2) |
| A3600 | 28 | *KLHL10* | NM_001329596 | Exon 2 | c.53C>T, p.P18L, (Het) | 8.12E-06 | VUS (PM1+PM2+PP2) |
| A2664 | 36 | *KLHL10* | NM_001329596 | Exon 4 | c.1121T>C, p.V374A, (Het) | 0 | VUS (PM1+PM2+PP2) |

*Pop Freq* population allele frequency (ExAC database), *Clin Sig* clinical significance, *LP* likely pathogenic, *P* pathogenic, *VUS* variant of uncertain significance, *Het* heterozygous, *Hom* homozygous, *Hem* hemizygous. Reported mutations are marked as asterisk

**Table 3** Clinical phenotype, hormone profile, testicular volume, and treatment prognosis

| Patient ID | Gene | Histopathology | LH (mIU/mL) | FSH (mIU/mL) | PRL (mIU/L) | E2 (pg/mL) | T (ng/mL) | Testicular volume (L/R, mL) | Medicine treatment (3 months) | Micro-TESE |
|---|---|---|---|---|---|---|---|---|---|---|
| Patients with definite diagnosis | | | | | | | | | | |
| A2380 | *KLHL10* | / | 5.01 | 10.71 | 210.32 | 27 | 2.68 | 9.7/9.9↓ | / | / |
| A4524 * | *NR5A1* | / | 4.51 | 17.91↑ | 182.90 | 35 | 3.94 | 6.6/10.1↓ | Few sperm | / |
| A5197 | *NR5A1* | Normal | 19.04↑ | 50.92↑ | 357.63 | 21 | 2.69 | 4.1/5.4↓ | / | Succeed |
| A1064 | *NR5A1* | / | 7.91 | 9.35 | / | 10 | 5.84 | 13.4/14.1 | Few sperm | / |
| A2719 | *NR5A1* | / | 21.98↑ | 36.85↑ | / | 35 | 4.19 | 0.7/0.7↓ | / | / |
| A2799 | *TEX11* | Complete MA | 4.00 | 6.11 | / | 34 | 2.57↓ | 12.6/14.2 | / | No sperm |
| A4999 | *TEX11* | / | 7.10 | 10.19 | 191.24 | 20 | 2.91 | 10.2/11.1 | / | / |
| A961 | *TEX11* | Complete MA | 3.95 | 16.70↑ | 231.80 | 22 | 2.49↓ | 10.1/10.9 | / | No sperm |
| A2153 | *TEX11* | / | 5.92 | 12.81↑ | 210.81 | 30 | 3.17 | 8.1/7.9↓ | / | / |
| A4124 | *TEX14* | Partial MA | 3.71 | 11.82↑ | 156.38 | 40 | 4.75 | 10.2/10.6 | / | Few sperm |
| A5352 | *TEX14* | Complete MA | 7.90 | 13.85↑ | 209.12 | 31 | 3.90 | 8.2/9.7↓ | No sperm | / |
| A6326 | *TEX14* | / | 6.68 | 7.06 | 283.30 | 37 | 7.00 | 8.2/7.3↓ | / | / |
| A5343 | *ZMYND15* | / | 5.96 | 5.76 | 182.13 | 51 | 9.81 | 9.3/9.8↓ | / | / |
| A5181 | *SYCE1* | Complete MA | 1.96 | 2.65 | 329.36 | 10 | 1.81↓ | 15.4/15.4 | / | / |
| Patients with strong candidate variants | | | | | | | | | | |
| A4640 | *AR* | / | 6.81 | 21.56↑ | 278.20 | 25 | 33.70↑ | 9.5/8.0↓ | / | / |
| A2864 | *AR* | / | 7.05 | 27.02↑ | 185.11 | 15 | 0.96↓ | 4.1/4.8↓ | / | / |
| A3600 | *KLHL10* | SCO | 4.02 | 6.24 | 372.50 | 13 | 1.83↓ | 12.1/11.9 | / | No sperm |
| A2664 | *KLHL10* | SCO | 4.10 | 9.91 | 201.19 | 18 | 2.58↓ | 10.1/11.3 | / | No sperm |

*TV* testicular volume (left/right, normal ≥ 10mL); Reference ranges are marked in brackets as follows: LH (1.80–8.40 mIU/mL), FSH (1.30–11.80 mIU/mL), PRL (86.92–392.20 mIU/mL), E2 (0–56 pg/mL) and T (2.60–7.40 ng/mL); *normal* normal spermatogenic function, *MA* meiotic arrest, *SCO* Sertoli cell only. Abnormal values are all marked with upward (higher than normal) or downward (lower than normal) arrows. Slash means untested or untreated. A4524 has history of cryptorchidism (marked as asterisk)

of six patients with L/LP or VUS mutations produced viable spermatozoa through micro-TESE. Of these, patient IA5197 with an *NR5A1* mutation successfully produced sufficient spermatozoa for intracytoplasmic sperm injection (ICSI). Only very few spermatozoa (1–2 per high power field) that were insufficient for ICSI were obtained for patient A4124 with a *TEX14* mutation. Two patients, A3600 and A2664 with SCO phenotypes (*KLHL10* mutations), failed to obtain any spermatozoa through micro-TESE (Fig. 1, Table 3).

## Discussion

### Diagnostic rate

To date, there have been several studies using NGS or whole exome sequencing for NOA or severe oligozoospermia (SO) in cohort screening, yielding diagnostic rates of 6/314 (1.9%) [37] and 1/314 (0.3%) [38]. In this study, targeted panel sequencing could explain 4/189 (2.1% for panel 1) or 10/479

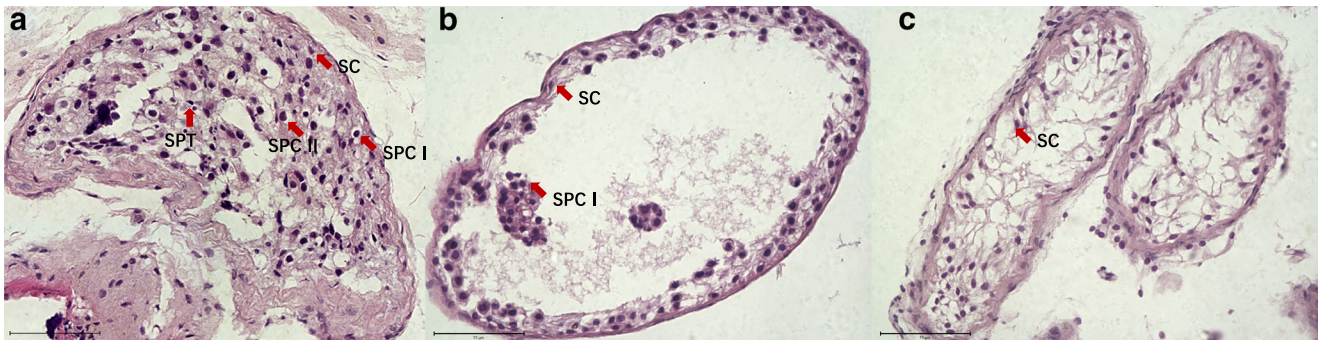


**Fig. 1** HE-stained sections of testicular tissue. Three figures demonstrate testicular phenotypes of varying severity in patients with NOA, including **a** normal spermatogenic function (A5197 with *NR5A1* mutation), **b** maturation arrest (A961 with *TEX11* mutation), and **c** Sertoli cell only (A2664 with *KLHL10* mutation). Bar represents 75 μm. SC, Sertoli cells; SPC I and II, primary and secondary spermatocytes; SPT, spermatids

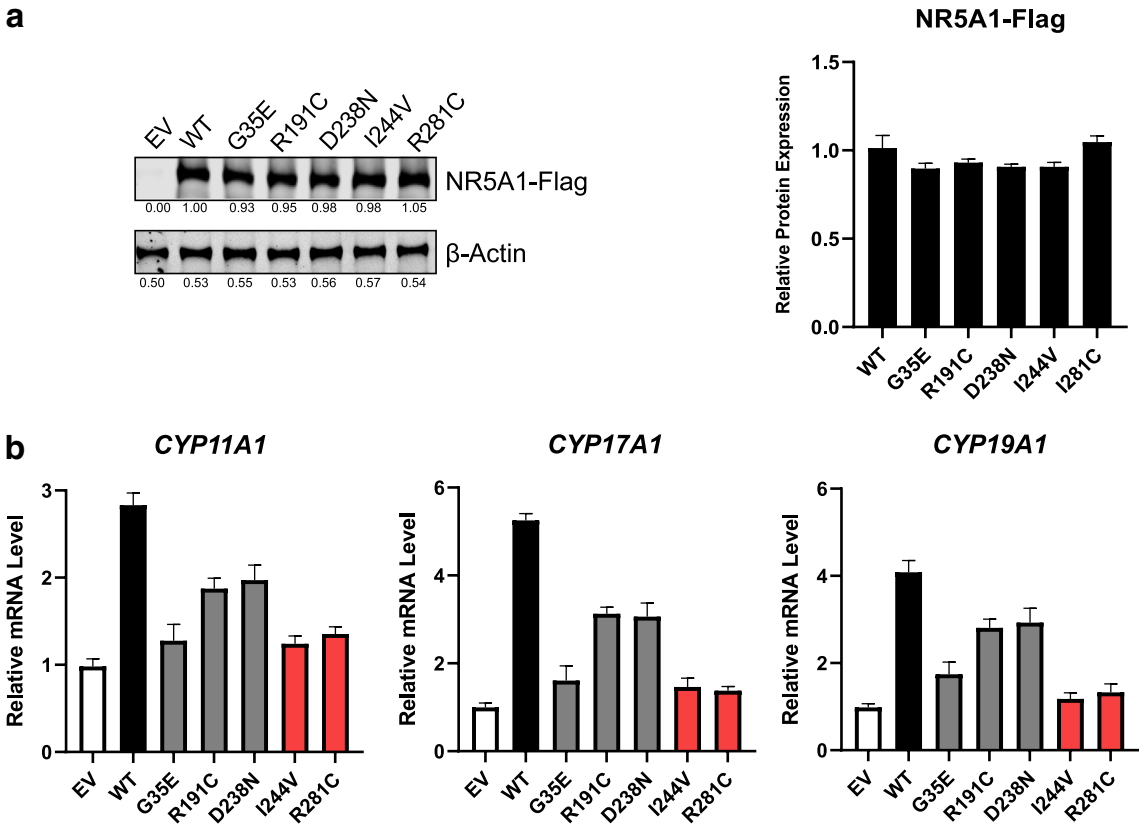


**Fig. 2** *In vitro* functional assay of *NR5A1* mutations. p.I224V and p.R281C were identified in this study. Analysis of three previously described variants p.G35E, p.R191C, and p.D238N were also repeated to increase credibility to our experiment. **a** Western blot analysis. **b** Assays of *NR5A1* transcriptional activity by using downstream gene *CYP11A1*, *CYP17A1*, and *CYP19A1*. EV, empty vector; WT, wild type

(2.1% for panel 2) of the patients with NOA after exclusion of those with karyotypic abnormalities and Y chromosome microdeletions. Some studies have not been discussed here because they did not classify variants properly according to the ACMG guidelines [31]. Fakhro et al. [39] reported two variants of *NANOS2* and *FKBP6* at 5% and 9%, respectively, in 75 infertile man. Araujo et al. [40] conducted genetic screening of 16 Brazilian patients with MA and SCO, and 5/10 (50%) of the reported mutations had a population frequency of more than 1%, which is benign evidence.

Our low diagnosis rates using the NGS panels (2.1% or 2.1%) might be because of the high heterogeneity of NOA. First, NOA can arise from nongenetic causes, and this will lower the genetic diagnostic rates. More stringent categorization into MA or SCO phenotypes by testicular histopathology could certainly increase the genetic diagnosis rate, but this would diminish the noninvasive advantage of genetic testing. Second, the low diagnostic rate by NGS panel is linked to limited number of genes selected for testing. According to the available screening studies [37–39] and this study, it appears that each gene explains only a very small percentage of cases with NOA. Therefore, increasing the diagnostic rate of NGS panels for NOA needs to exclude patients with nongenetic causes as much as possible, at the same time expanding the numbers of genes in NGS panel.

The frequently reported gene variants in patients with NOA are likely to be pathogenic. As NGS-based studies in NOA are still limited, almost all of the mutations we detected here have not been reported except for *NR5A1* p.R281C [41] and *TEX11* p.M156V (reported as p.M171V of NM_001003811.2) [14]. It should be noted that *SYCE1* deletions are likely to have high prevalence in the Chinese population [42]. Therefore, this study provides important evidence for the clinical diagnosis of NOA in China and highlights the importance of sharing data across different clinical centers.

## Clinical phenotypes

Inferring the phenotype—the prognosis for micro-TESE—by genotyping is currently challenging. Lines of evidence to be considered are as follows: (1) how genes are involved in spermatogenesis; (2) the phenotypes of specific gene knockout mice; and (3) the phenotypes of previously reported cases. For example, several tested genes are directly involved in meiosis, and *TEX14*, *TEX11*, and *SYCE1* were found to have pathogenic mutations in this study. The Tex14 protein localizes to male germ-cell intercellular bridges [11]; *TEX11*

interacts with *SYCP2*, an integral component of lateral elements of the synaptonemal complex [43]; and *SYCE1* encodes central element protein 1 of the synaptonemal complex [44]. In the absence of these key proteins, spermatogenesis would halt at meiotic division [11, 12, 14, 43]. Previously reported cases all showed MA or SCO phenotypes for *TEX14* [39, 40, 45], *TEX11* [14], and *SYCE1* [12]. Here, the outcomes of testicular biopsy or micro-TESE were all poor for patients with *TEX14*, *TEX11* (Fig. 1b), or *SYCE1* mutations. Therefore, we suggest that *TEX11*, *TEX14*, and *SYCE1*, which are directly associated with meiosis, predict a poor micro-TESE prognosis.

The genes *AR* and *NR5A1* affect spermatogenesis by participating in hormonal regulation and action [36, 46]. The *AR* gene encodes the androgen receptor, which mediates the role of androgens in spermatogenesis and sexual development [47]. *NR5A1* encodes a transcription factor involved in the regulation of reproduction, steroidogenesis, and sexual differentiation [48]. As a result, patients with mutations in these two genes can exhibit a wide phenotypic spectrum, ranging from severe to mild sexual developmental abnormalities [36, 49] to isolated azoospermia [46, 50, 51]. *AR* mutations are not predictive of poor micro-TESE prognosis, also supported by case reports [52, 53]. It should be noted that the *AR* variants reported here are two types of VUS lacking functional studies.

The inheritance of *NR5A1* mutations is dose dependent, but is not classical autosomal recessive or autosomal dominant [16]. In our study, heterozygous LOF mutations p.C13X and c.244+1G>A were identified in patients A4524 and A5197, who had a history of cryptorchidism and who both showed small testes (4.1/5.4 mL) Both patients produced spermatozoa following medication (a combination of vitamin E and clomiphene citrate) [54] or micro-TESE. That the patients could be treated medically indicates that *NR5A1* variants do not affect spermatogenesis. A heterozygous missense mutation, p.R281C, was found in A2719 who had severe gonadal dysplasia with 0.7/0.7 mL testicular volumes. This patient has had no further treatment. Buonocore et al. [41] identified p.R281C in a patient with disorders of sex development and speculated that the 280/281 position of the *NR5A1*-encoded protein forms a key region interacting with corepressors. The *in vitro* functional analysis of p.R281C showed an obvious effect on downstream gene expression (Fig. 2b). Although this effect of p.I244V on downstream genes is equivalent to p.R281C (Fig. 2b), patient A1064 presented as isolated NOA with a normal hormone profile, and micro-TESE was successful. This suggests that the effects of *NR5A1* mutations and the severity of clinical phenotypes are not simple correspondences. The three medically treated cases we report here also demonstrate that *NR5A1* mutations are not predictive of a poor micro-TESE prognosis.

As of now, patient A5343 is the second reported case with NOA caused by a *ZMYND15* mutation, while the first such case was from a consanguineous family and had an MA phenotype identified by bilateral testis biopsies [23]. *ZMYND15* encodes a transcriptional repressor, and its inactivation results in early activation of haploid genes and depletion of late spermatids. *Zmynd15*-null male mice also presented with MA [55]. More patients with NOA associated with *ZMYND15* mutations are needed to expand the phenotypic spectrum.

*KLHL10* is expressed in the cytoplasm of elongating and elongated spermatids, and mutations of this gene can disrupt spermiogenesis and lead to reduction in the numbers of late spermatids [56]. Two patients with *KLHL10* variant reported in this study (A3600 and A2664) had SCO phenotypes (Fig. 1b for patient A2664). However, the previously reported cases all had SO phenotypes [17], so whether these two VUS mutants (p.P18L and p.V374A) are causative of the SCO phenotype remains uncertain and requires further study. The phenotypic spectrum of the *KLHL10* mutations also includes SO, which indicates that *KLHL10* mutation is not associated with poor micro-TESE prognosis.

## Limitations

This work had limitations as follows: (1) although the cohort size was relatively large (668), the number of samples used to establish relationships between genotype and phenotype was insufficient because of the low rate of molecular diagnosis of NOA. Moreover, some patients with NOA will choose donated semen instead of micro-TESE and ICSI; this further reduces the opportunity to observe the relationship between mutations and micro-TESE outcomes. (2) The testicular histopathology analysis was not sufficiently detailed to provide a Johnsen's score of spermatogenesis [57]. (3) This study did not perform functional analyses of all VUS mutations. We will continue to enlarge the NOA cohort and refine the histopathology and functional analysis in the future.

## Conclusions

We used NGS panels to screen 668 patients with NOA. Targeted panel sequencing could explain 4/189 (2.1% with panel 1) or 10/478 (2.1% with panel 2) of patients with NOA after exclusion of karyotypic abnormalities and Y chromosome microdeletions. Almost all mutations detected in this study were newly described except for *NR5A1* p.R281C and *TEX11* p.M156V. Two missense *NR5A1* mutations, p.R281C and p.I244V, were proved to be deleterious by *in vitro* functional assays. Mutations in *TEX11*, *TEX14*, and *NR5A1* genes were causes, but each gene explains only a very small percentage (4/668 or 0.6%). Although more genes should be included to increase the diagnostic rate, we suggest that only a few

genes such as *TEX11*, *TEX14*, and *SYCE1* that are directly related to meiosis can indicate a poor micro-TESE prognosis.

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## References

1. Lotti F, Maggi M. Sexual dysfunction and male infertility. Nat Rev Urol. 2018;15:287–307.
2. WHO. WHO laboratory manual for the examination and processing of human semen. Fifth ed. Geneva: WHO; 2010.
3. Jarow JP, Espeland MA, Lipshultz LI. Evaluation of the azoospermic patient. J Urol. 1989;142:62–5.
4. Salonia A, Bettochi C, Carvalho J, Corona G, Jones TH, Kadioğlu A, et al. EAU guidelines on sexual and reproductive health 2020. In: European Association of Urology Guidelines. 2020 Edition., vol. presented at the EAU Annual Congress Amsterdam 2020. Arnhem, The Netherlands: European Association of Urology Guidelines Office; 2020.
5. Krausz C, Hoefsloot L, Simoni M, Tuttelmann F. European Academy of A, European Molecular Genetics Quality N. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. Andrology. 2014;2:5–19.
6. Van Assche E, Bonduelle M, Tournaye H, Joris H, Verheyen G, Devroey P, et al. Cytogenetics of infertile men. Hum Reprod. 1996;11(Suppl 4):1–24 discussion 25-6.
7. Liu XY, Zhang HY, Pang DX, Xue LT, Yang X, Li YS, et al. AZFa microdeletions: occurrence in Chinese infertile men and novel deletions revealed by semiconductor sequencing. Urology. 2017;107:76–81.
8. Kasak L, Laan M. Monogenic causes of non-obstructive azoospermia: challenges, established knowledge, limitations and perspectives. Hum Genet. 2021;140(1):135–154.
9. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. Nat Med. 2008;14:1197–213.
10. Luo M, Yang F, Leu NA, Landaiche J, Handel MA, Benavente R, et al. MEIOB exhibits single-stranded DNA-binding and exonuclease activities and is essential for meiotic recombination. Nat Commun. 2013;4:2788.
11. Greenbaum MP, Yan W, Wu M-H, Lin Y-N, Agno JE, Sharma M, et al. TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci U S A. 2006;103:4982–7.
12. Maor-Sagie E, Cinnamon Y, Yaacov B, Shaag A, Goldsmidt H, Zenvirt S, et al. Deleterious mutation in SYCE1 is associated with non-obstructive azoospermia. J Assist Reprod Genet. 2015;32:887–91.
13. Miyamoto T, Hasuike S, Yogev L, Maduro MR, Ishikawa M, Westphal H, et al. Azoospermia in patients heterozygous for a mutation in SYCP3. Lancet. 2003;362:1714–9.
14. Yatsenko AN, Georgiadis AP, Ropke A, Berman AJ, Jaffe T, Olszewska M, et al. X-linked TEX11 mutations, meiotic arrest, and azoospermia in infertile men. N Engl J Med. 2015;372:2097–107.
15. Abou Alchamat G, Madania A, Alhalabi M. Mild androgen insensitivity syndrome (MAIS): the identification of c.1783C>T mutation in two unrelated infertile men. BMJ Case Rep. 2017;2017:bcr2017220361.
16. Fabbri-Scallet H, de Sousa LM, Maciel-Guerra AT, Guerra-Júnior G, de Mello MP. Mutation update for the NR5A1 gene involved in DSD and infertility. Hum Mutat. 2020;41:58–68.
17. Yatsenko AN, Roy A, Chen R, Ma L, Murthy LJ, Yan W, et al. Non-invasive genetic diagnosis of male infertility using spermatozoal RNA: KLHL10 mutations in oligozoospermic patients impair homodimerization. Hum Mol Genet. 2006;15:3411–9.
18. Kasak L, Punab M, Nagirnaja L, Grigorova M, Minajeva A, Lopes AM, et al. Bi-allelic recessive loss-of-function variants in FANCM cause non-obstructive azoospermia. Am J Hum Genet. 2018;103:200–12.
19. Choi Y, Jeon S, Choi M, Lee MH, Park M, Lee DR, et al. Mutations in SOHLH1 gene associate with nonobstructive azoospermia. Hum Mutat. 2010;31:788–93.
20. Pezzi N, Prieto I, Kremer L, Perez Jurado LA, Valero C, Del Mazo J, et al. STAG3, a novel gene encoding a protein involved in meiotic chromosome pairing and location of STAG3-related genes flanking the Williams-Beuren syndrome deletion. FASEB J. 2000;14:581–92.
21. Okutman O, Muller J, Baert Y, Serdarogullari M, Gultomruk M, Piton A, et al. Exome sequencing reveals a nonsense mutation in TEX15 causing spermatogenic failure in a Turkish family. Hum Mol Genet. 2015;24:5581–8.
22. Arafat M, Har-Vardi I, Harlev A, Levitas E, Zeadna A, Abofoul-Azab M, et al. Mutation in TDRD9 causes non-obstructive azoospermia in infertile men. J Med Genet. 2017;54:633–9.
23. Ayhan O, Balkan M, Guven A, Hazan R, Atar M, Tok A, et al. Truncating mutations in TAF4B and ZMYND15 causing recessive azoospermia. J Med Genet. 2014;51:239–44.
24. Cannarella R, Condorelli RA, Duca Y, La Vignera S, Calogero AE. New insights into the genetics of spermatogenic failure: a review of the literature. Hum Genet. 2019;138:125–40.
25. Robay A, Abbasi S, Akil A, El-Bardisi H, Arafa M, Crystal RG, et al. A systematic review on the genetics of male infertility in the era of next-generation sequencing. Arab J Urol. 2018;16:53–64.
26. Oud MS, Volozonoka L, Smits RM, Vissers L, Ramos L, Veltman JA. A systematic review and standardized clinical validity assessment of male infertility genes. Hum Reprod. 2019;34:932–41.
27. Smith ED, Radtke K, Rossi M, Shinde DN, Darabi S, El-Khechen D, et al. Classification of genes: standardized clinical validity assessment of gene-disease associations aids diagnostic exome analysis and reclassifications. Hum Mutat. 2017;38:600–8.
28. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754–60.
29. Lai Z, Markovets A, Ahdesmaki M, Chapman B, Hofmann O, McEwen R, et al. VarDict: a novel and versatile variant caller for next-generation sequencing in cancer research. Nucleic Acids Res. 2016;44:e108.
30. Li Q, Wang K. InterVar: clinical interpretation of genetic variants by the 2015 ACMG-AMP guidelines. Am J Hum Genet. 2017;100:267–80.
31. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of

Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17:405–24.
32. Venkatraman ES, Olshen AB. A faster circular binary segmentation algorithm for the analysis of array CGH data. Bioinformatics. 2007;23:657–63.
33. Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform. 2013;14:178–92.
34. Riggs ER, Andersen EF, Cherry AM, Kantarci S, Kearney H, Patel A, et al. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). Genet Med. 2020;22:245–57.
35. Bashamboo A, Ferraz-de-Souza B, Lourenco D, Lin L, Sebire NJ, Montjean D, et al. Human male infertility associated with mutations in NR5A1 encoding steroidogenic factor 1. Am J Hum Genet. 2010;87:505–12.
36. Lin L, Achermann JC. Steroidogenic factor-1 (SF-1, Ad4BP, NR5A1) and disorders of testis development. Sex Dev. 2008;2:200–9.
37. Chen S, Wang G, Zheng X, Ge S, Dai Y, Ping P, et al. Whole-exome sequencing of a large chinese azoospermia and severe oligospermia cohort identifies novel infertility causative variants and genes. Hum Mol Genet. 2020;29(14):2451–2459.
38. Rocca MS, Msaki A, Ghezzi M, Cosci I, Pilichou K, Celeghin R, et al. Development of a novel next-generation sequencing panel for diagnosis of quantitative spermatogenic impairment. J Assist Reprod Genet. 2020;37:753–62.
39. Fakhro KA, Elbardisi H, Arafa M, Robay A, Rodriguez-Flores JL, Al-Shakaki A, et al. Point-of-care whole-exome sequencing of idiopathic male infertility. Genet Med. 2018;20:1365–73.
40. Araujo TF, Friedrich C, Grangeiro CHP, Martelli LR, Grzesiuk JD, Emich J, et al. Sequence analysis of 37 candidate genes for male infertility: challenges in variant assessment and validating genes. Andrology. 2020;8:434–41.
41. Buonocore F, Clifford-Mobley O, King TFJ, Striglioni N, Man E, Suntharalingham JP, et al. Next-generation sequencing reveals novel genetic variants (SRY, DMRT1, NR5A1, DHH, DHX37) in adults with 46,XY DSD. J Endocr Soc. 2019;3:2341–60.
42. Zhe J, Ye D, Chen X, Liu Y, Zhou X, Li Y, et al. Consanguineous Chinese familial study reveals that a gross deletion that includes the SYCE1 gene region is associated with premature ovarian insufficiency. Reprod Sci. 2020;27:461–7.
43. Yang F, Gell K, van der Heijden GW, Eckardt S, Leu NA, Page DC, et al. Meiotic failure in male mice lacking an X-linked factor. Genes Dev. 2008;22:682–91.
44. Dunne OM, Davies OR. Molecular structure of human synaptonemal complex protein SYCE1. Chromosoma. 2019;128:223–36.
45. Gershoni M, Hauser R, Yogev L, Lehavi O, Azem F, Yavetz H, et al. A familial study of azoospermic men identifies three novel causative mutations in three new human azoospermia genes. Genet Med. 2017;19:998–1006.
46. Wang Q, Ghadessy FJ, Trounson A, de Kretser D, McLachlan R, Ng SC, et al. Azoospermia associated with a mutation in the ligand-binding domain of an androgen receptor displaying normal ligand binding, but defective trans-activation. J Clin Endocrinol Metab. 1998;83:4303–9.
47. Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science. 1988;240:324–6.
48. Tremblay JJ, Viger RS. A mutated form of steroidogenic factor 1 (SF-1 G35E) that causes sex reversal in humans fails to synergize with transcription factor GATA-4. J Biol Chem. 2003;278:42637–42.
49. Hiort O, Klauber G, Cendron M, Sinnecker GH, Keim L, Schwinger E, et al. Molecular characterization of the androgen receptor gene in boys with hypospadias. Eur J Pediatr. 1994;153:317–21.
50. Zare-Abdollahi D, Safari S, Mirfakhraie R, Movafagh A, Bastami M, Azimzadeh P, et al. Mutational screening of the NR5A1 in azoospermia. Andrologia. 2015;47:395–401.
51. Hasani N, Mohseni Meybodi A, Rafaee A, Sadighi Gilani MA, Mohammadzadeh R, Sabbaghian M. Spermatogenesis disorder is associated with mutations in the ligand-binding domain of an androgen receptor. Andrologia. 2019;51:e13376.
52. Massin N, Bry H, Vija L, Maione L, Constancis E, Haddad B, et al. Healthy birth after testicular extraction of sperm and ICSI from an azoospermic man with mild androgen insensitivity syndrome caused by an androgen receptor partial loss-of-function mutation. Clin Endocrinol. 2012;77:593–8.
53. Massin N, Bry-Gauillard H, Abirached F, Bassam H, Claessens F, Young J. Androgen receptor gene mutation and partial androgen insensitivity syndrome: birth after testicular sperm extraction and ICSI. Fertil Steril. 2007;88:S387.
54. ElSheikh MG, Hosny MB, Elshenoufy A, Elghamrawi H, Fayad A, Abdelrahman S. Combination of vitamin E and clomiphene citrate in treating patients with idiopathic oligoasthenozoospermia: a prospective, randomized trial. Andrology. 2015;3:864–7.
55. Yan W, Si Y, Slaymaker S, Li J, Zheng H, Young DL, et al. Zmynd15 encodes a histone deacetylase-dependent transcriptional repressor essential for spermiogenesis and male fertility. J Biol Chem. 2010;285:31418–26.
56. Yan W, Ma L, Burns KH, Matzuk MM. Haploinsufficiency of kelch-like protein homolog 10 causes infertility in male mice. Proc Natl Acad Sci U S A. 2004;101:7793–8.
57. Johnsen SG. Testicular biopsy score count–a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones. 1970;1:2–25.