Original Article

Establishment and clinical significance of genetic factor screening method for patients with nonobstructive azoospermia based on whole exon sequencing technology

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Background: Non-obstructive azoospermia (NOA) is a severe form of male infertility, affecting 10–20% of azoospermic men. Although some NOA genes have been identified, the genetic causes of spermatogenesis failure in NOA remain unclear. This study aimed to identify and characterize genes and mutations associated with NOA.

Methods: Thirty NOA patients were selected for whole-exome sequencing (WES). Patients with chromosomal abnormalities, chromosome copy number issues, or Y chromosome microdeletions were excluded. Relevant genes and mutations in NOA patients were comprehensively screened using WES, MutationTaster software, and related databases. Sequencing results were analyzed for allele screening, mutation deleteriousness, and mutation site prediction to identify potential NOA-associated genes. The study also predicted altered gene function due to mutations and assessed pathogenicity from DNA sequence alterations.

Results: The study screened 37 genes with 56 variant loci, identifying 27 genes with 34 variant loci related to NOA, including *CFAP65*, *SEPTIN12*, *ZMYND15*, *DNAH2*, *CEP112*, *SHOC1*, *DNAH10*, *ACTL9*, *CFAP43*, *DNAH17*, *DNAH1*, *ARMC2*, *AK7*, *PMFBP1*, *FSIP2*, *SPATA16*, *TSGA10*, *SPEF2*, *CFAP69*, *TTC21A*, *NDNF*, *ADCY10*, *GATA4*, *CYP17A1*, *CHD7*, *CHD7*, *SEMA3A*, and *CFTR*. Notable findings included the variant c.1223C>A p.S408* in the *CFAP65* gene and potential associations of genes such as *CFAP43*, *CFAP69*, *ZMYND15*, *DNAH17*, and *DNAH2* with spermatogenic disorders.

Conclusions: The study identified genes related to spermatogenic disorders in azoospermia, providing a reference for clinical genetic diagnosis and basic NOA research.

Keywords: Non-obstructive azoospermia (NOA); whole-exome sequencing (WES); gene; mutation

Submitted Nov 25, 2024. Accepted for publication Feb 27, 2025. Published online Apr 27, 2025.
doi: 10.21037/tau-2024-676
View this article at: https://dx.doi.org/10.21037/tau-2024-676

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Introduction

The inability to conceive in 10–15% of couples worldwide is a significant issue in human health, with male factors contributing to approximately 50% of infertility cases (1). Non-obstructive azoospermia (NOA) is considered the most severe form of male infertility. Unlike azoospermic patients with normal spermatogenesis, individuals with NOA experience spermatogenesis failure, leading to the absence of sperm in the ejaculate. This condition accounts for approximately 10% to 20% of infertile men with azoospermia (2,3).

NOA can be caused by various factors, including abnormal hormone levels, immune factors, and genetic abnormalities. Genetic abnormalities, which include sex chromosome aberrations and single gene defects, account for approximately 25% of NOA cases (4). Spermatogenesis, the process of sperm production, involves the intricate interaction of over 2,000 genes (5). Dysfunction at any stage of spermatogenesis can result in spermatogenesis block and azoospermia. Currently, around 40 genes are known to be associated with NOA (6). Among these, 14 genes, such as *TEX11*, *MSH4*, and *STAG3*, have been identified as pathogenic genes in NOA (7). However, there is still a need to employ efficient and reliable methods to continue the search for additional NOA-related genes and new gene mutations.

The advent of next-generation sequencing (NGS) technology, including whole-genome sequencing (WGS) and whole-exome sequencing (WES), has revolutionized clinical practice, providing a powerful tool for diagnosis and treatment. WES, in particular, has proven to be useful in identifying new candidate genes associated with NOA. By employing high-throughput sequencing, WES enables efficient detection of genetic variations that may contribute to NOA. In recent years, several studies have utilized WES to discover new genes associated with NOA phenotypes. For example, variations in the germ cell-specific genome integrity factor GCNA have been found to be related to spermatogenesis and male infertility (8). Additionally, three new TEX11 mutations (exon 5, c.313C>T: p.R105*, exon 7, c.427A>C: p.K143Q, and exon 29, c.2575G>A: p.G859R) have been identified through WES, expanding the spectrum of mutations observed in NOA patients (9). Genetic identification provides precise diagnosis and treatment strategies for patients with NOA, avoids ineffective medical interventions, reduces genetic risks, and advances research in reproductive medicine. As gene sequencing technology becomes more widespread, genetic analysis is increasingly incorporated into the routine diagnosis and treatment of male infertility.

In this study, a specific group of NOA patients with chromosomal abnormalities and Y chromosome microdeletions were excluded. The remaining patients underwent WES to identify potential pathogenic genes and mutations. To further analyze and predict the significance of these findings, various software tools such as Sorting Intolerant from Tolerant (SIFT), Polyphen2, and MutationTaster databases were employed. This comprehensive approach allowed for the screening and enrichment of relevant genes, expanding the gene pool associated with NOA. This study will also enrich the gene database to provide theoretical and experimental basis for the diagnosis and treatment of NOA patients and genetic counseling. We present this article in accordance with the STREGA reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-2024-676/rc).

Highlight box

Key findings

- Whole-exome sequencing (WES) of 30 patients with non-obstructive azoospermia (NOA) identified 27 potentially associated genes and 34 putative pathogenic variants, suggesting novel molecular insights into NOA pathogenesis.

What is known and what is new?

- Genetic factors are established contributors to NOA, with genes such as *TEX11*, *MSH4*, and *STAG3* implicated in its etiology. However, the diversity of genetic variants and their unpredictable effects on clinical phenotypes remain incompletely characterized.
- This study advances the field by applying WES integrated with bioinformatics tools to systematically screen NOA-associated genes and mutations. Our approach demonstrated high reliability in identifying novel candidate genes and pathogenic variants, providing a comprehensive framework for genetic exploration in NOA.

What is the implication, and what should change now?

- These findings underscore the need to optimize genetic diagnostics for NOA to guide clinical decision-making, including testicular sperm extraction and assisted reproductive strategies, and to enhance genetic counseling. Future priorities include functional validation of candidate genes, family-based studies to clarify inheritance patterns, and incorporation of these genetic insights into clinical screening panels. Collaborative efforts to translate genomic discoveries into actionable diagnostic and therapeutic pathways are critical for improving patient outcomes.

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Methods

Study participants

In this study, the researchers selected male patients with azoospermia who sought treatment at The Affiliated Reproductive Hospital of Jiangxi University of Chinese Medicine as the research subjects. The study protocol was approved by the ethics review committee of The Affiliated Reproductive Hospital of Jiangxi University of Chinese Medicine (No. 2021-007). The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. Given the retrospective nature of the study and the use of de-identified data, the requirement for individual informed consent was waived. The inclusion criteria for the study were as follows: (I) meeting the diagnostic criteria for NOA (10); (II) no history of mumps, orchitis, or other conditions that could cause azoospermia; (III) no abnormalities detected in chromosome karyotype analysis, and no microdeletions observed in the Y chromosome staining.

The exclusion criteria were as follows: (I) azoospermia caused by radiotherapy, chemotherapy, orchitis, cryptorchidism, or other known factors; (II) azoospermia caused by retrograde ejaculation, erectile dysfunction, or other functional issues; (III) azoospermia caused by congenital or acquired absence of the fallopian tubes, traumatic infection, or other specific conditions; (IV) obstructive azoospermia (OA).

The diagnostic criteria for NOA were as follows: (I) no sperm are observed under the microscope after three consecutive semen centrifugations (≥3,000 g for 15 minutes), and ejaculatory dysfunction is excluded; (II) non-obstructive causes are identified through physical examination (evidenced by reduced testicular volume and normal vas deferens), seminal plasma biochemistry (normal levels of fructose and α-glucosidase), and imaging studies (ultrasonography excluding obstruction); (III) the etiology is further clarified through endocrine assessments [e.g., significantly elevated follicle-stimulating hormone (FSH) indicating spermatogenic dysfunction] and genetic analyses (e.g., Y chromosome microdeletions, chromosomal karyotype abnormalities); (IV) spermatogenic dysfunction is confirmed via testicular biopsy or microdissection testicular sperm extraction (micro-TESE), with pathology revealing spermatogenic arrest or Sertoli cell-only syndrome.

WES and bioinformatics analysis

Peripheral blood was taken from the patients and tested for karyotype, copy number variations and Y chromosome microdeletions. To perform WES, DNA was extracted from the peripheral blood samples. The extracted DNA underwent quantification and quality testing to ensure its suitability for sequencing. And then, DNA libraries were constructed by fragmentation, junction ligation, and amplification and purification. Subsequently, post-capture libraries were prepared, and the target regions were captured after library concentration and hybridization. The exon and adjacent intron regions of human genes were detected in the whole-exome genes, and the data obtained were compared with the reference sequences; and the coverage of the target regions and the quality of the sequencing were assessed.

Sequencing results were subjected to allele screening, pathogenicity assessment of variant sites, and both screening and prediction of variant sites to identify genes or variants potentially associated with NOA. These genes or variants were categorized according to Online Mendelian Inheritance in Man (OMIM), American College of Medical Genetics and Genomics (ACMG) ratings, and clinical variant databases to evaluate genetic variability. The pathogenicity of genetic variants was predicted using mainstream software such as SIFT, Polyphen2, and MutationTaster, with annotation information classified in the InterVar database based on variant site pathogenicity, statistical data, and clinical relevance. The Rare Exome Variant Ensemble Learner (REVEL) is a key tool in gene screening. Splicing predictions were conducted using MaxEntScan and Spidex_dpsi for variant sites that may lead to NOA, with a focus on spermatogenic failure.

SIFT predicts the impact of an amino acid change on protein function by considering sequence homology and the physical properties of the amino acid. It can be applied to both naturally occurring non-synonymous mutations (polymorphisms) and laboratory-induced missense mutations. Polyphen2 predicts the potential deleteriousness of a mutation on a scale of 0 to 1, where higher scores indicate a greater likelihood of the mutation being deleterious. MutationTaster assesses the functional consequences of various DNA sequence alterations, including amino acid substitutions, insertion and deletion mutations, intronic and synonymous changes, and mutations that span intron-exon boundaries, to determine their pathogenicity. REVEL is an integrated method for predicting missense mutations, utilizing the random forest algorithm to improve the prediction of rare missense mutations. Additionally, this study utilized the

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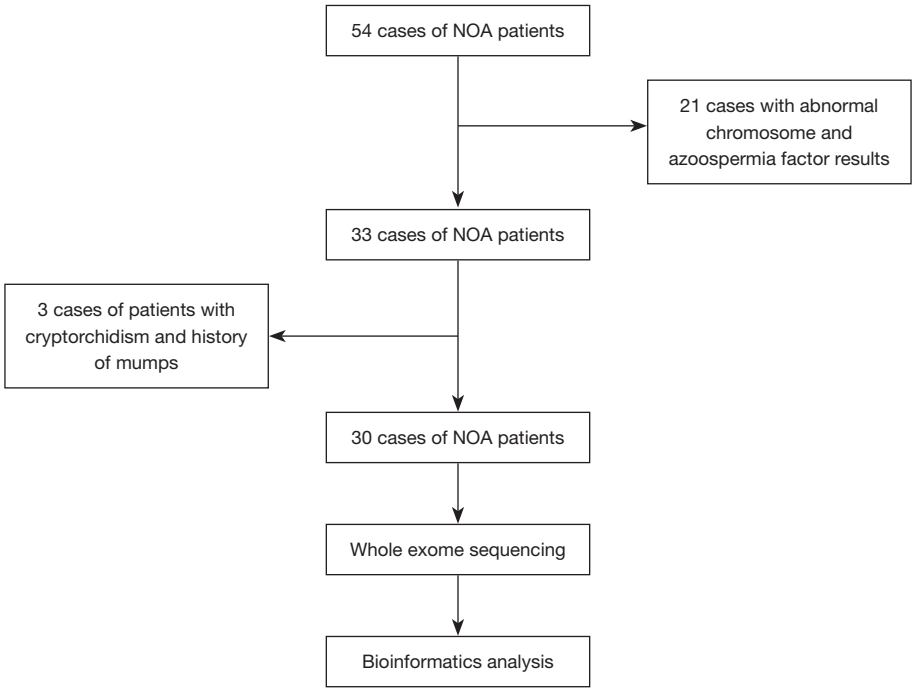

Figure 1 The flow chart of patient selection. NOA, non-obstructive azoospermia.

MaxEntScan platform, which is based on the Maximum Entropy Principle and predicts the fitness of sequence motifs as donor or acceptor splice sites. The study also incorporated predictions from the SPIDEX database (11) and followed the ACMG standards. The ACMG standards provide criteria for assessing evidence of pathogenicity and benignity of variant sites, as outlined in the ACMG summary statement of evidence of pathogenic/benign (12).

Results

General information

A total of 54 cases of NOA patients were collected in this study, and after excluding 21 cases with abnormal chromosome and azoospermia factor (AZF) results, there were 33 cases remaining, including 3 cases of patients with cryptorchidism and history of mumps, so that a total of 30 cases of NOA patients who fulfilled the inclusion criteria of the present study were finally analyzed (*Figure 1*).

Gene variants site screening

After performing whole-exome sequencing on 30 patients, a comprehensive analysis revealed a total of 34 variants in 27 genes, including *CFAP65*, *SEPTIN12*, *ZMYND15*, *DNAH2*, *CEP112*, *SHOC1*, *DNAH10*, *ACTL9*, *CFAP43*, *DNAH17*, *DNAH1*, *ARMC2*, *AK7*, *PMFBP1*, *FSIP2*, *SPATA16*, *TSGA10*, *SPEF2*, *CFAP69*, *TTC21A*, *NDNF*, *ADCY10*, *GATA4*, *CYP17A1*, *CHD7*, *CHD7*, *SEMA3A*, and *CFTR*. These variants resulted in both base changes and amino acid changes, with the exception of the mutant loci in the ADCY10 gene. All the identified mutant loci had heterozygous allele types. The observed mode of inheritance included 8 cases of autosomal dominant and 26 cases of autosomal recessive inheritance (*Table 1*).

The mutational deleteriousness assessment revealed that 20 genes and 26 variant loci were associated with disorders of spermatogenesis. Additionally, the analysis suggested that the remaining 5 genes and 6 variant loci could potentially be linked to abnormalities in gonadotropin secretion, vas deferens deficiency, and testicular abnormalities. However, the cause of azoospermia in the remaining two genes with two loci remains unclear.

According to the SIFT prediction analysis, 12 mutation sites were identified as having the potential to affect protein function. Additionally, the prediction results from Polyphen2 indicated that 7 mutation sites had the potential to cause deleterious mutations. MutationTaster predicted the presence of 18 mutation sites that could potentially

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Table 1 Information on 34 variants in 27 genes related to NOA

ID	Gene name	Location in chromosome	Transcript ID	Base substitution	Amino acid substitution	Zygote type	Mode of inheritance
1	*CFAP65*	chr2:219894869	NM_194302.4	c.1223C>A	p.Ser408*	Heterozygote	AR
2	*SEPTIN12*	chr16:4835850	NM_144605.4	c.332C>A	p.Thr111Lys	Heterozygote	AD
	SEPTIN12	chr16:4836010-4836011	NM_144605.5	c.262_263del	p.Q88Dfs*35	Heterozygote	AD
3	*ZMYND15*	chr17:4646747	NM_001136046.3	c.1294G>A	p.Gly432Ser	Heterozygote	AR
	ZMYND15	chr17:4647532	NM_001267822.1	c.1543G>A	p.Val515Met	Heterozygote	AR
4	*DNAH2*	chr17:7707605	NM_020877.5	c.9004G>A	p.Ala3002Thr	Heterozygote	AR
5	*CEP112*	chr17:63898415	NM_001199165.4	c.2018C>T	p.Thr673Met	Heterozygote	AR
6	*SHOC1*	chr9:114489955	NM_001378211.1	c.1792T>C	p.Cys598Arg	Heterozygote	AR
7	*DNAH10*	chr12:124343737	NM_001372106.1	c.6671G>A	p.Arg2224His	Heterozygote	AR
8	*ACTL9*	chr19:8808481	NM_178525.5	c.571G>A	p.Val191Ile	Heterozygote	AR
9	*CFAP43*	chr10:105905237	NM_025145.5	c.3941C>T	p.Pro1314Leu	Heterozygote	AR
10	*DNAH17*	chr17:76424773	NM_173628.3	c.12421A>G	p.Ile4141Val	Heterozygote	AR
	DNAH17	chr17:76482203	NM_173628.3	c.7114C>T	p.Arg2372*	Heterozygote	AR
	DNAH17	chr17:76480981	NM_173628.3	c.7618C>T	p.Leu2540Phe	Heterozygote	AR
	DNAH17	chr17:76457681	NM_173628.3	c.9284G>T	p.Ser3095Ile	Heterozygote	AR
11	*DNAH1*	chr3:52398962	NM_015512.4	c.5445G>T	p.Glu1815Asp	Heterozygote	AR
	DNAH1	chr3:52381946	NM_015512.4	c.2062G>A	p.Asp688Asn	Heterozygote	AR
12	*ARMC2*	chr6:109190159	NM_032131.4	c.424C>T	p.Arg142Trp	Heterozygote	AR
13	*AK7*	chr14:96904193	NM_152327.3	c.631G>A	p.Val211Ile	Heterozygote	AR
14	*PMFBP1*	chr16:72188294	NM_031293.2	c.230A>G	p.Gln77Arg	Heterozygote	AR
15	*FSIP2*	chr2:186667643	NM_173651.2	c.13877A>T	p.Glu4626Val	Heterozygote	AR
16	*SPATA16*	chr3:172766778	NM_031955.5	c.719G>A	p.Arg240Gln	Heterozygote	AR
17	*TSGA10*	chr2:99636821	NM_025244.2	c.1739A>C	p.Gln580Pro	Heterozygote	AR
18	*SPEF2*	chr5:35667268	NM_024867.3	c.1262G>A	p.Arg421His	Heterozygote	AR
19	*CFAP69*	chr7:89929272	NM_001039706.2	c.1949A>G	p.Gln650Arg	Heterozygote	AR
20	*TTC21A*	chr3:39166934	NM_001105513.2	c.1180A>T	p.Ile394Phe	Heterozygote	AR
21	*NDNF*	chr4:121958150	NM_024574.4	c.976G>A	p.Ala326Thr	Heterozygote	AD
22	*ADCY10*	chr1:167849351	NM_018417.6	c.1216+2T>C	p,?	Heterozygote	AD
23	*GATA4*	chr8:11566308	NM_002052.3	c.487C>T	p.Pro163Ser	Heterozygote	AD
24	*CYP17A1*	chr10:104592955	NM_000102.3	c.764G>A	p.Arg255Gln	Heterozygote	AR
25	*CHD7*	chr8:61765855	NM_017780.3	c.6571G>A	p.Glu2191Lys	Heterozygote	AD
	CHD7	chr8:61655481	NM_017780.3	c.1490C>A	p.Pro497His	Heterozygote	AD
26	*SEMA3A*	chr7:83764184	NM_006080.2	c.196C>T	p.Arg66Trp	Heterozygote	AD
27	*CFTR*	chr7:117267766	NM_000492.3	c.3659C>T	p.Thr1220Ile	Heterozygote	AR

AD, autosomal dominant; AR, autosomal recessive; NOA, non-obstructive azoospermia.

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cause diseases. Furthermore, both MaxEntScan and ACMG standards predicted two mutation sites each, which were associated with altered gene expression and pathogenicity. The detailed prediction results of the databases or software used are shown in *Table 2*.

Discussion

Infertility is a significant issue affecting human reproductive health, with a prevalence of approximately 15%. Among infertile couples, approximately 50% of cases are attributed to male factors, which primarily include oligozoospermia (low sperm count), dyszoospermia (abnormal sperm morphology), or azoospermia (absence of sperm in the ejaculate) (13). Azoospermia is the most severe form of male infertility and is characterized by the complete absence of spermatozoa in the semen. Azoospermia can be further classified into OA and NOA. NOA occurs when there is a deficiency or disruption in spermatogenesis or sperm maturation, leading to the absence of spermatozoa in the ejaculate. This phenotype poses the greatest risk to reproductive health and is associated with significant challenges in achieving natural conception. Currently, there is a lack of effective treatments to restore spermatogenesis in these patients, and the use of donor sperm is often the primary option for producing offspring (14). Numerous genes have been identified as playing a role in various aspects of spermatogenesis, including sperm motility, capacitation, volume regulation, and chemotaxis.

Unlike single-gene disorders, NOA is a complex condition with diverse phenotypes and pathogenesis. The genetic causes underlying spermatogenesis failure in NOA are still being actively explored, as the genetic mutations involved in this disease are rare. Therefore, large-scale screening of relevant genes and mutations is necessary to gain a better understanding of the condition. With the advancements in sequencing technology, WES has become a reliable and cost-effective method for genetic testing. In this study, WES was performed on a total of 30 azoospermia patients. The obtained results were further analyzed, and 27 genes and 34 mutation loci were identified that may have an impact on spermatogenesis in men. The molecular expression of these genes varies, contributing to the genetic diversity observed in male infertility. Some of the genes with identified variants still require further investigation in the context of NOA, as their potential functions may directly or indirectly influence spermatogenesis. The genes identified in this study were also subjected to prediction and evaluation using several databases, enhancing the reliability of the findings.

The *CFAP65* gene is responsible for encoding proteins associated with cilia and flagella, which play crucial roles in spermatogenesis and motility. Pathogenic variants of the *CFAP65* gene, whether in a pure or compound heterozygous state, can lead to spermatogenic failure 40 (MIM#618664). This condition is inherited in an autosomal recessive pattern and is characterized by infertility, reduced sperm count, decreased sperm motility (including low or no motility), abnormalities in flagellar structure (such as short, absent, curled, or bent flagella), disrupted mitochondrial and ciliary sheaths, malformation of the sperm head, acrosome deletion or malformation, and deletion of the microtubule center pair. In this particular study, the patient's *CFAP65* gene was located at chr2:219894869, and one variant of this gene, c.1223C>A p.S408* (408/1,925), was identified. The MutationTaster predicted that this variant may be harmful. However, the comprehensive analysis resulted in the causative ratings being of unknown significance. Jiang *et al.* discovered that the *CFAP65* gene is involved in the assembly process of sperm flagellar structure, which can lead to polymorphic abnormalities in the sperm flagellum and subsequently infertility, and animal experiments have revealed that the gene is expressed in all levels of germ cells (15). Currently, the study of *CFAP* genes primarily focuses on sperm morphology and flagellum-related abnormalities. Spermatogenesis is a complex process, and any disruption at a certain stage may result in spermatogenic failure and subsequently azoospermia. Further research is needed to determine if this gene is a causative mutation of NOA.

The *ZMYND15* gene has been identified as a causative factor for Spermatogenic failure 14, which can lead to male infertility, particularly azoospermia or oligospermia. In this study, the *ZMYND15* gene was located at chr17:4646747 and chr17:4647532, and two variants were found: c.1294G>A p.G432S and c.1543G>A p.Val515Met. The SIFT/Polyphen2/MutationTaster results for these variants were tolerated/benign/polymorphism and deleterious/probably damaging/polymorphism, respectively. In humans, ZMYND15 has been associated with NOA and severe oligozoospermia (16). Kherraf *et al.* discovered that the presence of a double allele ZMYND15 variant can lead to severe oligozoospermia. They also highlighted the possibility of misdiagnosing the phenotype associated with this gene, which can result in delays in genetic diagnosis (17). Genetic defects in ZMYND15 have been shown to induce

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Table 2 Assessment of harmfulness of gene mutations

ID	Genes	OMIM disease information	SIFT[#]	Polyphen2[##]	MutationTaster[¶]	REVEL	MaxEntScan	Spidex_dpsi_zscore	Spidex_dpsi_max_tissue	Maximum frequency	ACMG standards	Variants classify
1	*CFAP65*	Spermatogenic failure 40	–	–	A	–	Consensus not affected	–3.681	–65.19	–	PVS1, PM2	Likely pathogenic
2	*SEPTIN12*	Spermatogenic failure 10	D	P	N	0.408	Consensus not affected	–1.145	–0.5541	0.0173522	PM1, BS1	Uncertain significance
	SEPTIN12	Spermatogenic failure 10	–	–	–	–	Predicted lost	–	–	–	PVS1, PM2	Likely pathogenic
3	*ZMYND15*	Spermatogenic failure 14	T	B	N	0.023	Consensus not affected	–1.417	–0.9103	0.0005449	PM2, BP4	Uncertain significance
	ZMYND15	Spermatogenic failure 14	D	D	N	0.155	Consensus not affected	0.224	0.0348	0.00429488	PM2	Uncertain significance
4	*DNAH2*	Spermatogenic failure 45	T	B	N	0.077	Consensus not affected	0.9	0.3063	0.000978899	BP4	Uncertain significance
5	*CEP112*	Spermatogenic failure 44	D	D	D	–	Consensus not affected	–0.728	–0.222	0.0000545316	–	Uncertain significance
6	*SHOC1*	–	T	–	N	0.141	Consensus not affected	0.065	0.0095	0.000113417	BP4	Uncertain significance
7	*DNAH10*	Spermatogenic failure 56	–	D	D	0.504	Consensus not affected	–0.826	–0.2818	0.0012837	PM1, PP3	Uncertain significance
8	*ACTL9*	Spermatogenic failure 53	T	–	N	0.064	Consensus not affected	–	–	0.0000549451	BP4	Uncertain significance
9	*CFAP43*	Hydrocephalus, normal pressure, 1; spermatogenic failure 19	D	D	D	0.314	Consensus not affected	–2.313	–3.7974	0.00117925	–	Uncertain significance
10	*DNAH17*	Spermatogenic failure 39	–	B	D	0.058	Consensus not affected	0.589	0.1382	0.000217486	–	Uncertain significance
	DNAH17	Spermatogenic failure 39	–	–	A	–	Consensus not affected	–3.862	–73.0385	0.000116259	PVS1	Uncertain significance
	DNAH17	Spermatogenic failure 39	–	–	D	0.295	Consensus not affected	0.797	0.2406	0.00128205	–	Uncertain significance
	DNAH17	Spermatogenic failure 39	–	–	N	0.518	Consensus not affected	0.968	0.3574	0.00176357	PM1	Uncertain significance
11	*DNAH1*	Ciliary yskinesia, primary, 37; spermatogenic failure 18	T	B	D	0.029	Consensus not affected	0.488	0.1016	0.000166889	–	Uncertain significance
	DNAH1	Ciliary dyskinesia, primary, 37; spermatogenic failure 18	T	B	D	0.091	Consensus not affected	–1.369	–0.8363	0.000283158	–	Uncertain significance
12	*ARMC2*	Spermatogenic failure 38	D	B	D	–	Consensus not affected	–1.512	–1.0716	0.000174125	–	Uncertain significance
13	*AK7*	Spermatogenic failure 27	T	P	D	0.269	Consensus not affected	–1.957	–2.1879	0.00192555	PM1	Uncertain significance
14	*PMFBP1*	Spermatogenic failure 31	D	B	D	0.044	Consensus not affected	1.842	1.6482	0.00641026	BS1	Uncertain significance
15	*FSIP2*	Spermatogenic failure 34	D	–	N	0.307	Consensus not affected	–	–	–	PM1, PM2	Uncertain significance
16	*SPATA16*	Spermatogenic failure 6	D	P	N	0.076	Consensus not affected	–0.889	-0.3248	0.000199681	PM2	Uncertain significance
17	*TSGA10*	Spermatogenic failure 26	D	D	D	0.162	Consensus not affected	0.224	0.0348	0.00097858	–	Uncertain significance
18	*SPEF2*	Spermatogenic failure 43	D	D	D	–	Consensus not affected	–1.323	–0.7691	0.0109796	BS1	Uncertain significance
19	*CFAP69*	Spermatogenic failure 24	T	B	N	0.104	Consensus not affected	0.396	0.0739	0.000391324	–	Uncertain significance
20	*TTC21A*	Spermatogenic failure 37	T	B	N	0.089	Consensus not affected	–1.059	–0.4676	0.000111247	–	Uncertain significance
21	*NDNF*	Hypogonadotropic hypogonadism 25 with anosmia	T	P	D	0.314	Consensus not affected	–	–	0.000641026	–	Uncertain significance
22	*ADCY10*	{Hypercalciuria, absorptive, susceptibility to}	–	–	D	–	Predicted lost & splice donor variant	–3.23	–21.9897	–	PM2, PP3	Uncertain significance
23	*GATA4*	Testicular anomalies with or without congenital heart disease; atrial septal defect 2; atrioventricular septal defect 4; tetralogy of Fallot; ventricular septal defect 1	T	P	D	0.692	Consensus not affected	0.161	0.024	0.00066726	PM1, PM2, PP2, PP3, BS2, BP1	Uncertain significance
24	*CYP17A1*	17,20-lyase deficiency, isolated; 17-alpha-hydroxylase/17,20-lyase deficiency	T	B	N	0.13	Consensus not affected	–1.929	–2.0879	0.00192308	PM2, BS2, BP4	Likely benign
25	*CHD7*	CHARGE syndrome; hypogonadotropic hypogonadism 5 with or without anosmia	T	B	D	0.163	Consensus not affected	–0.367	–0.0749	0.00256739	BP1	Uncertain significance
	CHD7	CHARGE syndrome; hypogonadotropic hypogonadism 5 with or without anosmia	D	B	D	0.065	Consensus not affected	0.5	0.1056	–	PM2	Uncertain significance
26	*SEMA3A*	{Hypogonadotropic hypogonadism 16 with or without anosmia}	D	D	D	–	Consensus not affected	–1.795	–1.6949	0.000818849	PM1	Uncertain significance
27	*CFTR*	Congenital bilateral absence of vas deferens; cystic fibrosis; sweat chloride elevation without CF	T	B	A	0.4	Consensus not affected	0.579	0.1345	0.00192555	PM2, BP4	Uncertain significance

[#], D: deleterious (SIFT ≤0.05); T: tolerated (SIFT >0.05). [##], D: probably damaging (score ≥0.909); P: possibly damaging (0.447≤ score <0.909); B: benign (score ≤0.452). [¶], A: disease causing automatic; D: disease causing; N: polymorphism. ACMG, American College of Medical Genetics and Genomics; CF, cystic fibrosis; OMIM, Online Mendelian Inheritance in Man; REVEL, Rare Exome Variant Ensemble Learner; SIFT, Sorting Intolerant from Tolerant.

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complete NOA or severe oligozoospermia, accompanied by severe dysmorphic animal spermatosis. Study in mice has revealed that ZMYND15 encodes a histone deacetylase-dependent transcriptional repressor that is essential for spermatogenesis and male fertility. It controls the temporal expression of genes in haploid cells during spermatogenesis. Inactivation of ZMYND15 in mice leads to early activation of transcription of important haploid genes like *Prm1*, *Tnp1*, *Spem1*, and *Catsper3*. This premature activation results in the loss of late spermatocytes and complete azoospermia (18).

The *DNAH2* gene has been identified as a causative factor for spermatogenesis failure type 45, which is characterized by severely malformed spermatozoa. Spermatozoa from affected individuals display various flagellar morphological abnormalities, including short, curly, irregular caliber, and head abnormalities (19). In this study, the *DNAH2* gene was located at chr17:7707605, and a variant c.9004G>A p.A3002T was found. The SIFT/Polyphen2/MutationTaster results for this variant were tolerated/benign/polymorphism, respectively. Ultrastructural analysis revealed that the axonemal complex and mitochondrial sheaths were severely damaged in patients with DNAH2 mutations. This indicates that DNAH2 plays a critical role in human cilia function. Spermatozoa from these patients exhibited loss of motility, and the gene was found to be highly expressed in the testis. Additionally, patients with DNAH2 mutations primarily showed weak spermatidosis and infertility (20). The relationship between the DNAH family and NOA is still under investigation.

Furthermore, other genes such as CHD7 and SEMA3A have been associated with hypogonadotropic hypogonadism, and studies have found a correlation between hormone levels and the pathophysiology of NOA (21,22). Whether the aforementioned genes indirectly contribute to the development of NOA requires further investigation.

The identification of genes and mutations associated with spermatogenic failure in azoospermia is found to have significant clinical implications. Firstly, the precise identification of pathogenic genes overcomes the limitations of traditional testing, clarifies the classification of etiologies, improves diagnostic rates, and prevents unnecessary treatments. Secondly, genetic testing guides personalized treatment strategies; specific mutations indicate the probability of successful sperm retrieval, thus optimizing surgical decisions. It also provides a foundation for genetic risk assessment in assisted reproductive technologies (such as ICSI), preventing vertical transmission risks to offspring (such as AZF region deletions). Moreover, understanding the genetic model (dominant/recessive/X-linked) enables family screening and preimplantation genetic testing (PGT-M), which blocks the transmission of pathogenic mutations and achieves prenatal intervention. Finally, gene identification advances basic research on spermatogenesis mechanisms, laying the groundwork for future targeted therapies (such as gene editing or drug regulation) and promoting clinical translation.

The study has certain limitations. Firstly, no new genes causing NOA were identified. Secondly, further research is needed to investigate the genes related to spermatogenesis obtained through predictive analysis in order to obtain accurate and reliable gene and mutation information. Additionally, the analysis of patients' family genetic status was not performed, and statistical results for the frequency of certain variants in the population were not available. In future research, the sample size should be increased and multi-center, multi-ethnic cohorts should be included. Long-read sequencing (such as PacBio/Nanopore) and epigenomic techniques [such as assay for transposase-accessible chromatin using sequencing (ATAC-seq)] are combined to systematically screen for new candidate genes associated with NOA. Additionally, clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology is used to create mutant mouse models or to validate the function of candidate genes in the spermatogenesis process through patient-derived testicular organoid models. Furthermore, trio whole-exome sequencing (Trio-WES) of NOA patient families, combined with co-segregation analysis, is conducted to clarify the genetic patterns of mutations (such as *de novo* mutations or recessive inheritance).

Conclusions

Although no new genes were found, multiple variant loci were discovered. By employing mainstream analysis and prediction methods and conducting a comprehensive analysis of multiple databases, we were able to determine causative ratings with a high degree of reliability for the NOA genes associated with male infertility. The study identified genes related to spermatogenic disorders in azoospermia, providing a reference for clinical genetic diagnosis and basic NOA research.

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Acknowledgments

None.

Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-2024-676/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-2024-676/dss

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-2024-676/prf

Funding: This project was funded by Jiangxi Provincial Key Research Laboratory of Traditional Chinese Medicine Andrology (No. KP202102007).

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-2024-676/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the ethics review committee of The Affiliated Reproductive Hospital of Jiangxi University of Chinese Medicine (No. 2021-007). The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. Given the retrospective nature of the study and the use of de-identified data, the requirement for individual informed consent was waived.

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Cite this article as: Yao W, Fu J, Zhang D, Chen S, Zhou Y, Jiang X, Zheng X, Zhang M, Wu F. Establishment and clinical significance of genetic factor screening method for patients with nonobstructive azoospermia based on whole exon sequencing technology. Transl Androl Urol 2025;14(4):1005-1014. doi: 10.21037/tau-2024-676

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