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Research Article

Novel NLRP14 Mutations Induce Azoospermia

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Background. LR family pyrin domain-containing 14 (NLRP14 or NALP14) is one of the important members of the NLR family and was mainly expressed in testis. It is reported that deficiency in the NALP14 gene in mice can cause spermatogenic failure, and several NALP14 mutations have been found in oligospermia and infertile men. *Case Presentation*. This study reported two novel NALP14 mutations (c.2076delC: p.L697X and c.T2963C: p.F988S) in our patients with azoospermia. The exonic deletion mutation (c.2076delC) and one missense mutation (c.T2963C) were firstly screened out by whole-exome sequencing (WES) and further verified by amplifying and sequencing the specific exons 5 and 10. Histological analysis of testicular biopsy revealed that NALP14 expression was detected strongly in spermatogonia and weakly in early spermatocytes. Additionally, mutations in this gene caused meiotic arrest, and no postmeiotic round spermatids and mature spermatozoa were observed in the seminiferous tubules. *Conclusions*. This study and previous literatures showed that NLRP14 mutations are closely related to male infertility; we discovered two novel NALP14 mutations and summarized the kinds of literatures on NLRP14 mutations and male infertility. This is the first report that deletion mutation (c.2076delC) and one missense mutation (c.T2963C) in NALP14 all lead to azoospermia, which is still significant to the clinical auxiliary diagnosis of male infertility.

1. Background

In recent years, affected by various factors, more and more people are suffering from infertility, and nearly half of them are caused by male infertility [1]. A considerable part of male infertile patients cannot produce sperm in their testis. This phenomenon is commonly referred to as nonobstructive azoospermia (NOA) [2]. Several studies have shown that NOA is genetically related [3–5]. Therefore, the research on mutation or genetic polymorphism of male infertility-related genes has gradually become a research hotspot. But so far, the research on the molecular mechanism of spermatogenesis is still very limited. An in-depth study of the regulation mechanism of spermatogenesis-related genes can not only help explain the causes of certain idiopathic infertility but also lay a certain foundation for the development of targeted therapy for male infertility and male contraception.

NOD-like receptors (NLRs) have been considered to be involved in the regulation of innate immune response. The pyrin domain- (PYD-) containing NLRP family contains 14 members (NLRP1-NLRP14) [6]. Several NLRs show a highly conservative expression in mammalian germline; however, little is known about the physiological functions, which may play a role in apoptosis (by activation of caspases) and in proinflammation signaling processes. NALP1, NALP3, and NALP6 had been characterized to form inflammasome complexes [7, 8]; NALP2 and NALP12 had been reported to regulate NF-κB signaling and NLRP4 regulate autophagy [9–12], while NALP5 and NALP7 exhibited highly restricted expression in mammalian germline and acted as maternal effect genes were found to play an

important role in reproduction and development [13–15]. NLR family pyrin domain-containing 14 (NLRP14) is one of the important members of the NLR family and was mainly expressed in the testis and secondarily in the ovary. It has been reported that mutations in the NALP14 gene can cause spermatogenic failure [16].

In this literature review, we mainly discuss the dominant effects of NLRP14 on spermatogenesis and male infertility. Testicular-specific high expression may suggest a role of NALP14 in spermatogenesis. There are other studies showing that NLRP14 regulates the differentiation of primordial germ cell-like cell (PGCLC); and interference the expression of NALP14 may result in the substantial decrease in PGCLC differentiation [17]. And in male mice, NLRP14 deficiency resulted in severe sperm count decline and increased abnormality. Further study shows that abnormal differentiation of spermatogonial stem cells (SSC) and aberrant meiosis was observed in NLRP14 KO animals.

Single nucleotide polymorphisms (SNPs) have a major impact on the stable expression of proteins. With the continuous development of next-generation sequencing, SNP assay has been used to detect infertility-related genetic defects. Westerveld et al. and Abe et al. performed a mutation screen of NALP14 gene in 157 men with azoospermia or severe oligozoospermia by direct sequencing; the most striking mutation is the p.K108X nonsense mutation, resulting a shortened protein from normal 1093 amino acids to only 107, and lacks the functional domains of NACHT and LRR [16, 18]. The other four single nucleotide mutations were all missense mutation, resulted in the change of the amino acid. However, the inheritance pattern of these mutations was not determined for that no family members can be used for the analysis. Taken together, these data indicate that mutations of NLRP14 may lead to spermatogenic failure and infertility.

2. Case Presentation

2.1. Patients and Medical Examinations. This study was approved by the Ethics Committee of Xiamen Women and Children's Hospital, and written consent was obtained from our patients, a questionnaire was completed by the patient, and factors affecting fertility such as their lifestyle, habits, occupation, marriage, family history, physical information (height and body weight), and chromosome and hormone levels were all recorded to exclude (listed in Table 1), and informed consent was obtained from the patients. There was no history of genetic diseases or infertility in their family. Their parents had a nonconsanguineous marriage, their wives had regular menstrual cycles, and all parameters of the female medical check-up were normal. To verify their azoospermia status, three semen analyses (1 week interval) were carried out after 3 days of sexual abstinence, according to WHO guidelines. Semen analysis indicated normal ejaculate volume, and no sperm was found in each round of analyses, so the patients were diagnosed as having azoospermia.

2.2. Cytogenetic and Molecular Genetic Analyses. Peripheral blood lymphocytes (PBL) were collected to confirm the chromosomal status and cytogenetic chromosomal karyotype. PBL were treated with 20 mg/mL colcemid for 1 h to stay at metaphase. G-banding of metaphase chromosomes was performed by Giemsa staining. A total of 20–100 metaphase cells were counted and described by the G-banding method according to the international system for chromosome nomenclature [19]. We applied FISH for the detection of chromosomal X and Y karyotypes, and FISH analyses were carried out as described previously [20]. FISH analysis was conducted with the combination of SRY and DYZ3 probes [21]. Moreover, AZF was used to detect Y chromosome microdeletions; according to the European Academy of Andrology and the European Molecular Genetics Quality Network guidelines, three selected sequence-tagged sites within specific AZFa, AZFb, and AZFc regions were analyzed by PCR method as described previously [22]. The SRY region was also duplicated, and four hydrolysis probes were designed to detect the four amplicons, respectively.

According to the results of the karyotype and FISH analyses, the karyotype of all the patients was normal (46, XY) (Figure. S1), and no gonadal mosaicism was observed in the patients. Moreover, we did not observe any Y chromosome microdeletions in either patient (Figure. S2). To determine possible mutations causing azoospermia, whole-exome sequencing (WES) in patients and their parents was conducted. Briefly, genomic DNA from patients' semen and their parents' blood cells was prepared in Illumina paired-end libraries and sequenced by using the Illumina HiSeq 2000 platform. The data were processed and analyzed, according to previous protocols [16, 23]. To screen specific mutations, all variants of the genome-wide data were compared to external publicly available databases including the 1000 Genomes Project (http://www.1000genomes.org) and other large-scale exome sequencing projects.

Mutations (c.2076delC and c.T2963C, GenBank accession number, NM_176822.4) in exons 5 and 10 of the NLRP14 gene in the chromosome 11 were identified by WES. To further verify the results of WES, exon 10 of the NALP14 gene was amplified using conventional end-point polymerase chain reaction (PCR), and the following primers were used for detection of the mutation of c.2076delC: forward, 5′-TTATCCCTC CTGAGTTGCTT-3′; reverse, 5′-ATTGGTGACCTGCCTA GAAT-3′. And primers used for detection mutation of c.T2963C in exon 5 were listed as follows: forward, 5′-TTAG ACCCTCCCATGTATGT-3′; reverse, 5′-AGCACAATTCT TGACCCTGA-3′. The sequences of PCR products were verified by the Sanger sequencing. Consistent with the results of WES, the mutations (2076delC and 2963T→C) were verified in patients and their family (Figure 1). Accordingly, it is determined that the mutation (2076delC) in exon 5 was predicted to result in a premature stop codon in exon 5, causing a truncated protein lacking of LRR domains, and the mutation (2963T→C) in exon 10 results in a substitution of the amino acid from phenylalanine to serine. All the mutations were inherited from their mother (Figure 2).

Table 1: The clinicopathological variables of infertile patient with NLRP14 mutations.

Patient characteristics													
Sample	Age (years)	Semen volume (mL)	Semen pH	Karyotype	Height (cm)	Weight (kg)	Mutation	Testis volume (mL)[a]		FSH (IU/L)	LH (IU/L)	Testosterone (ng/mL)	PRL (ng/mL)
								Left	Right				
Patient 1	40	2.5-3.1	7.3	46, XY	165	74	c.2076delC	10	10	43.67	10.3	3.7	13.05
Patient 2	38	2.8-3.2	7.5	46, XY	166	76	c.2076delC	12	12	46.81	9.56	4.37	12.28
Patient 3	35	2.5-3.2	7.5	46, XY	171	78	c.2076delC	12	12	48.26	10.56	4.75	16.38
Patient 4	31	2.3-3.5	7.5	46, XY	NA	NA	c.T2963C	10	10	27.23	5.97	1.83	20.42

FSH: follicle-stimulating hormone; LH: luteinizing hormone; NA: not available. [a]Volume as determined by scrotal ultrasound.

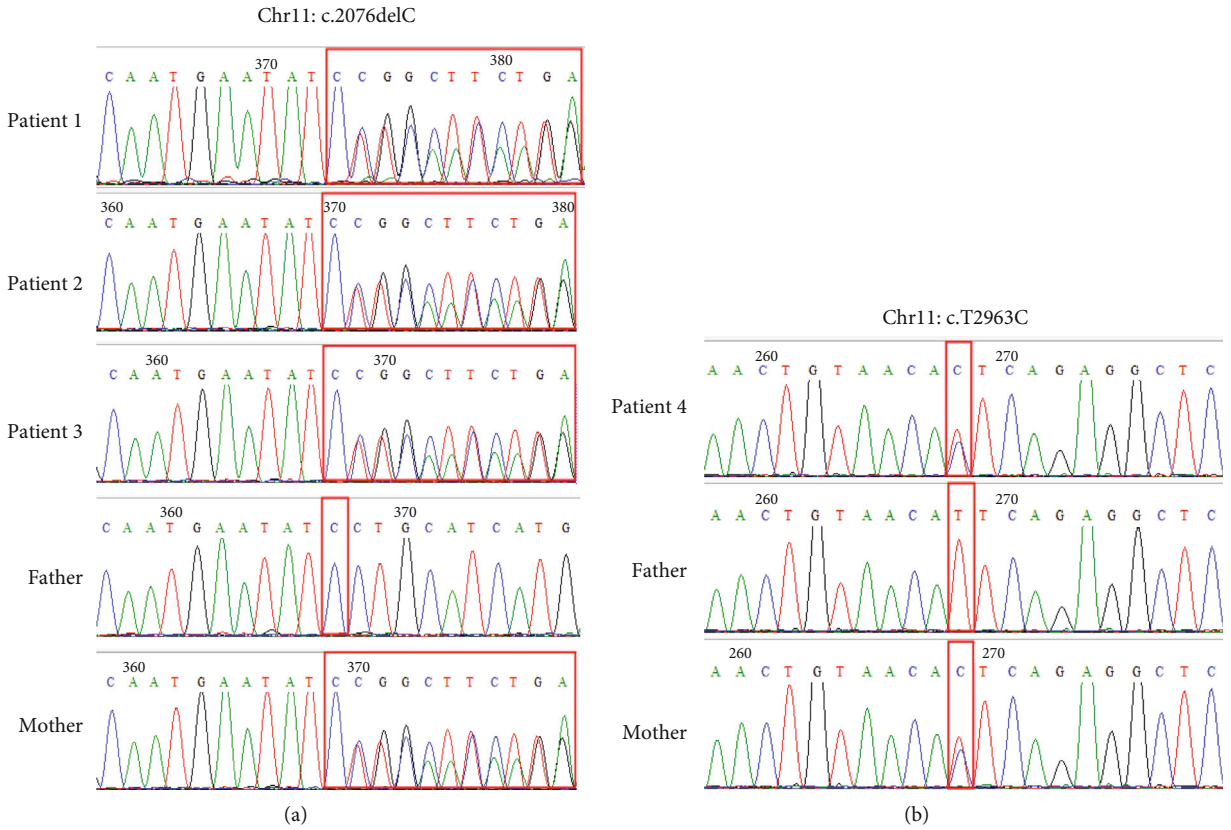


Figure 1: Identification of the NLRP14 mutation. (a) Conventional end-point PCR was performed to amplify exon 5 of the NLRP14 gene from genomic DNA, and PCR product sequences from the three brothers and their parents were verified by the Sanger sequencing and aligned to human NLRP14 cDNA. (b) Conventional end-point PCR was performed to amplify exon 10 of the NLRP14 gene from genomic DNA, and the PCR product sequences from the patient and his parents were verified by the Sanger sequencing and aligned to human NLRP14 cDNA.

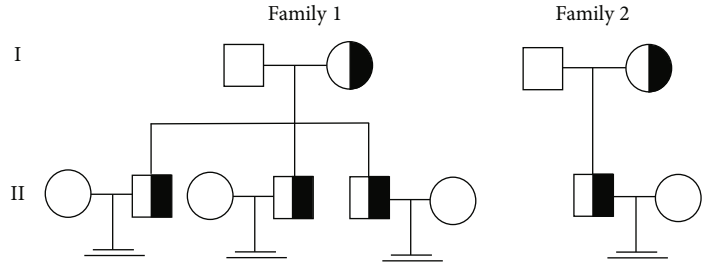


Figure 2: Family pedigree of the proband affected by NOA with a NALP14 mutation. The dotted square symbols indicate the patient who is the heterozygous carrier of the NALP14 mutation, and the dotted circle symbols indicate the patient's mother who is the heterozygous carrier of the NALP14 mutation.

2.3. Histological Analysis. Testicular biopsies were performed to determine the relationship between the histological change and azoospermia; testicular tissues were taken from the patient and immediately fixed in the Bouin fixative at 4°C overnight, dehydrated in graded ethanol, embedded in paraffin, and cut into 4 μm thick sections. To examine the testicular histology, the sections were deparaffinized in the xylene, rehydrated in graded ethanol, and stained with hematoxylin and eosin (H&E); stained sections were examined microscopically. Spermatogenesis was scored according to Johnsen's scoring system [24]. The seminiferous tubules of fertile testes contained a full spectrum of spermatogenic cells including round spermatids and mature spermatozoa. To investigate the effect of NLRP14 mutation on protein expression, we examined the protein expression of NLRP14 in the testicular biopsies from the patient and a normal testis by immunohistochemistry. Immunostaining of NLRP14 was carried out using NLRP14 primary antibody; after washing, the slides were reincubated with the secondary antibody. The stained sections were counterstained lightly with

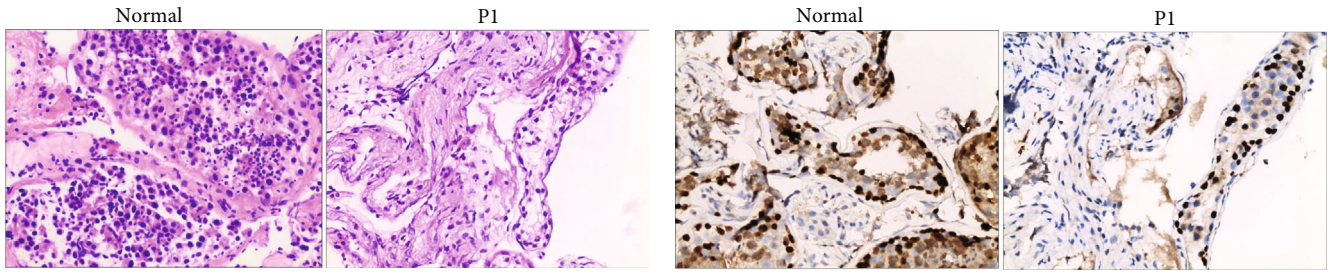


Figure 3: Histological examination and NALP14 staining in testicular biopsies. (a) Representative image of testicular histology from the older brother with azoospermia by hematoxylin and eosin staining. Testicular tissue from a healthy fertile man was used as a control. (b) Representative image of testicular histology from patient 1. Immunostaining of NALP14 in a testicular biopsy from the patient with azoospermia.

Table 2: The cases of azoospermia male with NLRP14 mutation.

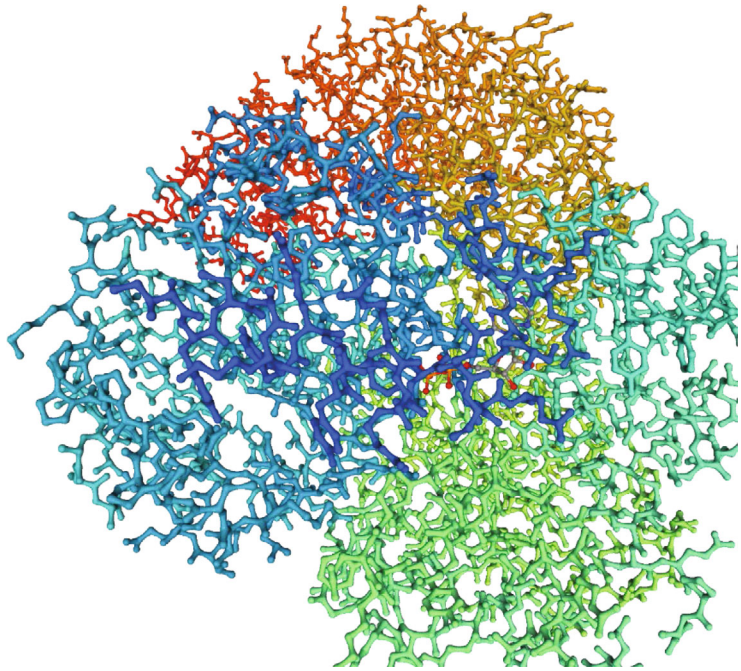
Case	Semen analysis (mL)	Mutation	Karyotype	Reference
1	0.1×10^6	p.D522Q	46, XY	Westerveld et al.
2	2.8×10^6	p.M1019I	46, XY	Westerveld et al.
3	1.5×10^6	p.K108X	46, XY	Westerveld et al.
4	2.7×10^6	p.A375T	46, XY	Westerveld et al.
5	2.0×10^6	p.D86V	46, XY	Westerveld et al.
6	No sperm	p.L697X	46, XY	Present report
7	No sperm	p.F988S	46, XY	Present report

hematoxylin. Compared with normal testicular histology, the testicular histology from the patient (2076delC) showed that poorly developed spermatocytes, no post-meiotic round spermatids, or mature spermatozoa were observed in the seminiferous tubules (Figure 3(a)), which is consistent with the typical characteristics of azoospermia. In the normal testis, NLRP14 was present in spermatogonia, spermatocytes, round spermatids, and mature spermatozoa in the seminiferous tubules, and loss of function in NALP14 may interfere with the differentiation of SSC and miss the spermatocytes; the signal of the deep staining of the tubule is located in SSC on the immunostaining of NALP14 in a testicular biopsy from the patient 1 (2963T→C) (Figure 3(b)).

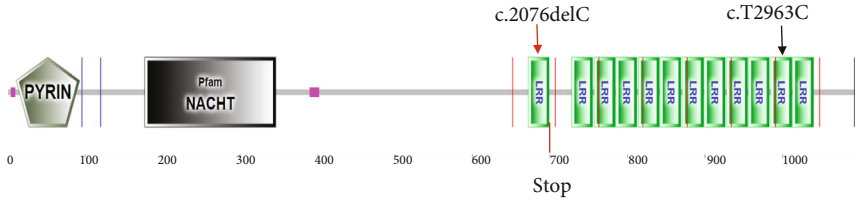
3. Discussion and Conclusions

Infertility affects nearly 15% of couples, and about half of infertility is caused by male factors. The occurrence of whole-exome sequencing helps to identification of genetic mutations leading to male infertility and implement the clinical molecular diagnosis. NALPs have a PYD domain, with functional similarity to the death domain (DD), which is known to be involved in stress signaling pathways, leading either to NF-κB activation or apoptosis [25]. NLRP14 is thought to be a testis-specific gene and highly expressed in human testis, but little is known about the function of NALP14 in spermatogenesis.

The expression of NLRP14 in mid and late pachytene spermatocytes indicates that NALP14 could be involved in the apoptotic processes that occur often during the meiotic divisions. Westerveld et al. have identified five unique mutations of NLRP14 in 157 men with severe oligozoospermia (listed in Table 2), one of which is the p.K108X nonsense mutation; this mutation may inactivate the allele and leads to a reduced protein expression of NALP14 [16]; Abe et al. proposed that NLRP14 induced ubiquitination and degradation of TBK1 through an unknown pathway, the K108X mutation resulted in a truncated protein expression, which did not associate with TBK1 and failed to inhibit TBK1-induced IFNβ and NF-κB promoter activation as well as endogenous IFNβ and IP10 mRNA expression. And the signaling could be partly enhanced by supplementing with the full-length NLRP14, which illustrates that both PYD and NACHT domains are required for NLRP14's inhibitory function [18]. It is reported that excessive type I IFN signaling can interfere with seminiferous tubules in mice, leading to a loss of germ cells and infertility [26], and IFNα intraperitoneal injections can also cause damage to spermatogenesis in rodents [27]; moreover, high levels of IFNα were also detected in semen of infertile men [28]. The other variants are missense mutations and may alter the secondary and tertiary structures of the NALP14 protein [16]. Yin et al. hold different views; their data show that NLRP14 might facilitate spermatid biogenesis and germ cell specification through forming a triple-protein complex NLRP14-HSPA2-BAG2



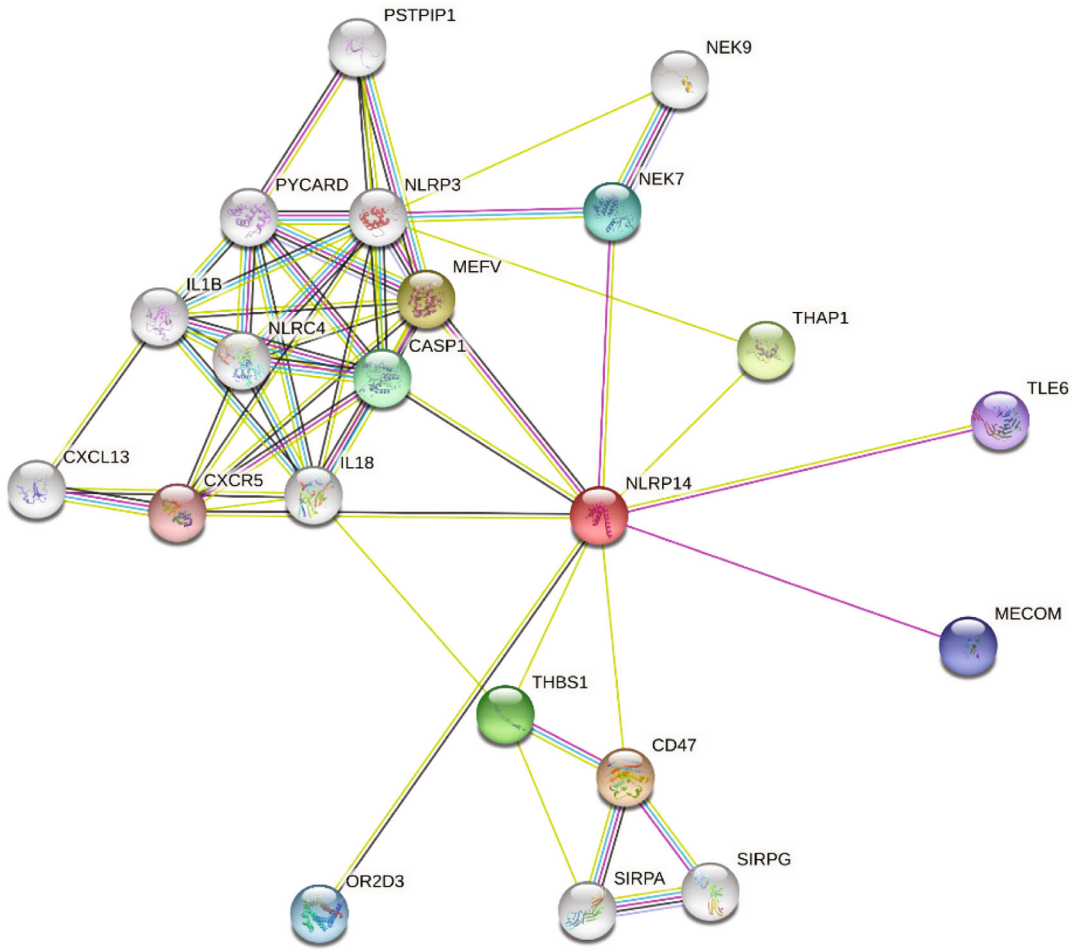
(a)



Name	Start	End	E-Value
Low complexity	4	10	N/A
PYRIN	11	93	2.4e-28
Pfam: NACHT	177	346	1.4e-43
Low complexity	390	402	N/A
LRR	672	699	25.9
LRR	728	755	0.0118
LRR	757	784	13.9
LRR	785	812	0.00295
LRR	814	841	0.0126
LRR	842	869	0.232
LRR	871	898	31.5
LRR	899	926	0.00778
LRR	928	955	172
LRR	956	983	8.27e-7
LRR	985	1012	18.7
LRR	1013	1040	0.692

(b)

Figure 4: Continued.



(c)

Figure 4: Predicted domains, repeats, motifs, features, and interaction proteins within Homo sapiens protein NALP14. (a) Confidently predicted 3D model by SWISS-MODEL of NALP14. (b) Predicted domains, repeats, motifs, and features of NALP14. (c) The interaction proteins analyzed by STRING.

not target TBK1 [17]. HSPA2 has been reported to be indispensable for male germ cell development; loss of function may impede the development of spermatogenic cells, disrupt meiosis, and eliminate apoptosis inhibition of the late-stage pachytene spermatocytes [29, 30]. The phenotypes detected in NLRP14 KO animals were consistent with HSPA2 [17]. The interaction among NLRP14, HSPA2, and BAG2 was conserved among species, and p.K108X nonsense mutation in NLRP14 failed to protect HSPA2 from polyubiquitination and proteasomal degradation by forming the NLRP14-HSPA2-BAG2 complex in cells [17].

The 3D structure of NLRP14 was predicted by SWISS-MODEL as shown in Figure 4(a). Moreover, domains, repeats, motifs, and features of NLRP14 were confidently predicted (Figure 4(b)), and we also analyzed the interaction proteins by STRING (Figure 4(c)). In this study, novel compound homozygous mutation in NALP14 was identified in our patient population of men. Additionally, cases with mutations either in c.2076delC or c.T2963C all suffered from a spermatogenetic failure. The mutations that occurred in this report were all in the leucine-rich repeat (LRR), which consists of 2-45 motifs of 20-30 amino acids in length that generally folds into an arc or horseshoe shape [31]. LRRs were reported to provide a structural framework for the formation of protein-protein interactions [32, 33]; proteins containing LRRs include tyrosine kinase receptors, cell adhesion molecules, virulence factors, and extracellular matrix-binding glycoproteins and are involved in a variety of biological processes, including signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, apoptosis, and immune response [34].

In summary, NLRP14 seems to play a vital role in spermatogenesis; mutations in this gene may cause spermatogenic failure. Through knockout mouse study, NALP14 was found to be crucial for PGCLC specification, differentiation of SSCs, and meiosis. Moreover, NLRP14 interacted with HSPA2 mainly through NACHT and LRR domains; mutations in the two domains may destruct the interaction between NALP14 and HSPA2. There are two novel mutations found in our case report, all of which were located in LRR domains; whether the protein structure has changed is unclear; further study will be necessary to clarify the molecular determinants that control NLRP14 function and the connection between function domain and function. Overall,

this is the first report that deletion mutation (c.2076delC) and a missense mutation (c.T2963C) in NLRP14 all lead to azoospermia, and our results expand the spectrum of NALP14 mutations and provide genetic evidence for male infertility.

Abbreviations

FISH: Fluorescent in situ hybridization
PCR: Polymerase chain reaction
WES: Whole-exome sequencing.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

All procedures involving human participants were performed following the ethical standards of the Ethics Committee of the Maternal and Child Care Hospital of Xiamen, and the approval number is KY-2021-021-H01.

Consent

Written informed consent was obtained from the patient to publish this case report and accompanying images. A copy of the written consent is available for review by the editorial office of this journal. 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.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Jian Song was responsible for the conceptualization, methodology, investigation, and data curation. Xuhui Zeng was responsible for the visualization, reagents, and analytic tools. Xiaoning Zhang was responsible for the data curation and methodology. Yanwei Sha was responsible for the methodology, funding acquisition, and investigation. Xiuling Zhao was responsible for the methodology and writing—review and editing.

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Supplementary Materials

Figure S1: G-banded male karyotype. Figure S2: the results of Y chromosome microdeletions. *(Supplementary Materials)*

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