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Original Article

A novel homozygous mutation in the meiotic gene *MSH4* leading to male infertility due to non-obstructive azoospermia

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Abstract: Non-obstructive azoospermia (NOA) is the most severe form of male infertility. Although some causes have been established, including genetic causes, the etiology in most cases remains idiopathic. Mutations in *MSH4* (OMIM: 602105), an important gene involved in meiosis, may be related to female infertility due to primary ovarian insufficiency (POI) and male NOA. Here, we report a novel homozygous stop-gain mutation of *MSH4* associated with NOA. Whole exome sequencing (WES) and bioinformatic analysis were performed in a patient with NOA from a consanguineous family (F1 II-1). A rare homozygous *MSH4* stop-gain mutation (c.1552C>T:p.Q518X) was observed in the patient, and his parents were heterozygous carriers, as verified by Sanger sequencing. Testicular biopsy and hematoxylin and eosin staining of testicular tissue suggested meiotic arrest (MA), and no sperm were observed. *MSH4* was detected in other 50 separate cases with same pathological results of MA using the same procedures, but only one heterozygous mutation was observed. Subsequent real-time quantitative polymerase chain reaction and immunohistochemistry were performed to examine mRNA expression levels and the localization of the MSH4 protein in the testicular tissue. Furthermore, the expression of MSH4 mRNA was significantly decreased compared with normal control. MSH4 protein was highly expressed in spermatocytes in the seminiferous tubules of the normal control, while no obvious expression was observed in F1 II-1. In this present study, *MSH4* was identified as a candidate gene of male infertility causing NOA. A novel mutation of MSH4 (c.1552C>T:p.Q518X) is associated with the MA phenotype during spermatogenesis.

Keywords: Male infertility, non-obstructive azoospermia, meiotic arrest, gene, *MSH4*

Introduction

As a globally prevalent problem, infertility affects 8-12% of the couples of a child-bearing age, and male factors nearly account for half of these cases [1-3]. Severe sexual dysfunction and sperm abnormality, such as oligospermia, asthenozoospermia, and terotospermia, are common causes of male infertility [4]. Among the male factors of infertility, azoospermia, which is defined as an absence of spermatozoa in centrifuged semen in at least three cycles of semen analysis at different times, is considered to be the most clinically severe form [5, 6]. Azoospermia is commonly classified as obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) according to testicular spermatogenic function [7, 8]. Although many infertile couples have benefited from assisted reproductive technology, numerous patients with NOA require donor sperm due to a relatively low sperm retrieval rate [9, 10]. Therefore, it is extremely important to explore the pathogenesis of spermatogenic failure in NOA patients and provide a theoretical basis for the treatment of NOA.

Although several causes of NOA have been established, including some genetic causes (e.g., chromosomal aberrations and Y-chromosome microdeletion), the etiology of the majority of the cases with NOA remains idiopathic [6,

11]. With the development and applications of whole genome sequencing (WGS) and whole exome sequencing (WES), some genes have been identified to be associated with spermatogenesis in humans. For example, *TEX11* (OMIM: 300311), located on the X chromosome, is highly expressed in spermatocytes. It is an important regulatory factor in the process of meiosis and plays a key role in the repair process of DNA double-stranded breaks. In a group of azoospermia patients undergoing testicular pathology, 15% patients with meiotic arrest (MA) were found to carry *TEX11* mutations, and no mutations were presented in the other 63 patients with Sertoli cell-only syndrome (SCOS) [12-14]. In addition to the genes on the sex chromosomes, mutations in genes on autosomes, including *TEX14* (OMIM: 605792), *FANCM* (OMIM: 609644), *SOHLH1* (OMIM: 610224), and *SPINK2* (OMIM: 605753), may also cause spermatogenic failure [15-20]. Notably, mutations in some genes, such as *STAG3* (OMIM: 608489) and *DMC1* (OMIM: 602721), can not only cause spermatogenic failure in males but also cause ovarian failure in females [21-24]. *STAG3* is located on chromosome 7 and encodes a meiosis-specific protein. It is one of the genetic causes of female primary ovarian failure (POI), and its mutations were also found to lead to NOA [21-23]. Similarly, He et al. found the *DMC1* gene on chromosome 22 to cause NOA in a male patient and POI in a female patient from a Chinese consanguineous family [24].

MSH4, a member of the mammalian mismatch repair (MMR) gene family, plays a vital role in the regulation of chromosome pairing during meiosis [25, 26]. Mutations in *MSH4* have been found to be one of the causes of female infertility due to POI [27]. Similarly, Krausz et al. also reported that *MSH4* mutations were likely responsible for NOA [28]. Furthermore, *MSH4*$^{-/-}$ mice appeared to exhibit NOA in males and POI in females [29]. In this study, WES was performed for a patient with NOA from a consanguineous family, and a novel homozygous *MSH4* mutation was identified as a possible mechanism underlying spermatogenic failure.

Materials and methods

Subjects

One Han Chinese patient with azoospermia from a consanguineous family was admitted to the Reproductive Center of the First Affiliated Hospital of Anhui Medical University (Hefei, China) for fertility counseling after undergoing semen analysis that showed an absence of spermatozoa twice using centrifuged semen in other hospitals. Previous examinations also presented normal somatic karyotypes (46, XY) and no Y chromosome microdeletions. Another semen analysis was performed in our center, and the same result of no spermatozoa was revealed. Subsequent sexual hormone tests were within normal ranges. A routine testicular biopsy was performed for histopathological examination. A cohort of 50 separate cases with idiopathic NOA due to meiotic arrest (MA) was chosen to perform genetic evaluation.

Ethical approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. This patient, his parents, and two controls with anejaculation or OA signed written informed consents before participating in this research.

Histopathology

Testicular tissue obtained from testicular biopsy was fixed using Bouin's fixative immediately. After fixation for 12 h, the tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Whole-exome sequencing (WES), bioinformatic analysis, and Sanger sequencing

Whole peripheral blood was used to extract genomic DNA for performing WES. The sequencing data underwent further bioinformatic analysis. Potentially pathogenic mutations were identified according to the following criteria: presenting low allele frequencies of less than 1% in the ExAc_all, 1KGP, and gnomAD databases; stop-gain, start-gain, splice site, frameshift or nonsynonymous mutation with potentially high pathogenicity predicted by Sorting Intolerant From Tolerant, PolyPhen-2, and Mutation Taster; extremely high expression or specific expression in the testis. The genome of the patient and his parents was subjected to Sanger sequencing to verify the identified possible mutations and parental origins. The primers are listed in Supplementary Table 1.

Table 1. Clinical features and genetic information of *MSH4* mutations of F1 II-1

Individual	F1 II-1
Clinical features	
Age	38 years old
Secondary sexual characteristics	Normal
Testicular volume (Left/Right, ml)	12/12
Somatic karyotype	46, XY
Y Chromosome microdeletions	No
Sex hormone	
Follicle-stimulating hormone (IU/L)	4.34
Luteinizing hormone (IU/L)	5.39
Testosterone (nmol/L)	11.95
Estradiol (pmol/L)	83.00
Prolactin (ng/ml)	6.39
Information of MSH4 mutation	
cDNA mutation	c.1552C>T
Mutation type	Stop-gain
Protein alteration	p.Q518X
Allele frequency in human population	
1KGP	0
ExAc_all	0
gnomAD	0
Functional prediction	
SIFT	N/A
PolyPhen-2	N/A
MutationTaster	Disease_causing Automatic

RefSeq accession number of DNAH9 is NM_001372.3. Abbreviations: 1KGP, 1000 Genomes Project; ExAc_all, all the data of Exome Aggregation Consortium; gnomAD, the Genome Aggregation Database; N/A: Not applicable.

Quantitative real-time PCR (QRT-PCR) and immunohistochemistry

QRT-PCR of MSH4-mRNA and immunohistochemistry of MSH4-protein in testicular issues were performed in accordance with a previous study [30]. The primary antibody was Anti-MSH4 antibody (ab236753, ABCAM). The PCR primers are presented in Supplementary Table 2.

Results

Results of testicular histopathology

Histopathological analysis by HE revealed no round spermatids and spermatozoa in the seminiferous tubules of the patient, whereas similarly abundant spermatogonia and spermatocytes compared with the control. The testicular histopathological results suggested an NOA phenotype of MA.

A homozygous mutation in MSH4 was identified in the patient with NOA

To detect the possible genetic causes of spermatogenic failure in this patient with NOA, WES and bioinformatic analyses were performed to search for meaningful mutations. Because of the consanguineous family history, we particularly focused on homozygous mutations. After careful bioinformatic analysis and screening, a homozygous mutation in *MSH4*, the only gene related to spermatogenesis which met the above criteria, was identified. Subsequently, Sanger sequencing was performed to verify the identified mutation site of *MSH4* in the patient and his parents. The patient was confirmed to be carrying a homozygous mutation of *MSH4* (c.1552C>T:p.Q518X), and his parents were heterozygous carriers (**Table 1**; **Figure 1**). The stop-gain mutation resulted in a truncated protein, whose three-dimensional structure was predicted by SWISS-MODEL software (https://swiss-model.expasy.org/) and is presented in **Figure 2**. Additionally, only one heterozygous mutation (c.652G>T:p.V218L) in MSH4 was observed in the other 50 separate cases with same pathological results of MA.

Expression of MSH4 was absent/decreased in patient's testicular tissue

In the seminiferous tubules of the healthy control, MSH4 protein was found to be highly expressed in spermatocytes using immunohistochemistry. However, no obvious expression was observed in the seminiferous tubules of this patient. To detect the expression levels of MSH4 mRNA, qRT-PCR was performed. Furthermore, significantly decreased levels of MSH4 mRNA in the testicular tissue of this patient were observed compared with the control (**Figure 3**).

Discussion

Although male *MSH4*$^{-/-}$ mice appeared to NOA have been presented in 2000, and *MSH4* mutations have been verified as one of the

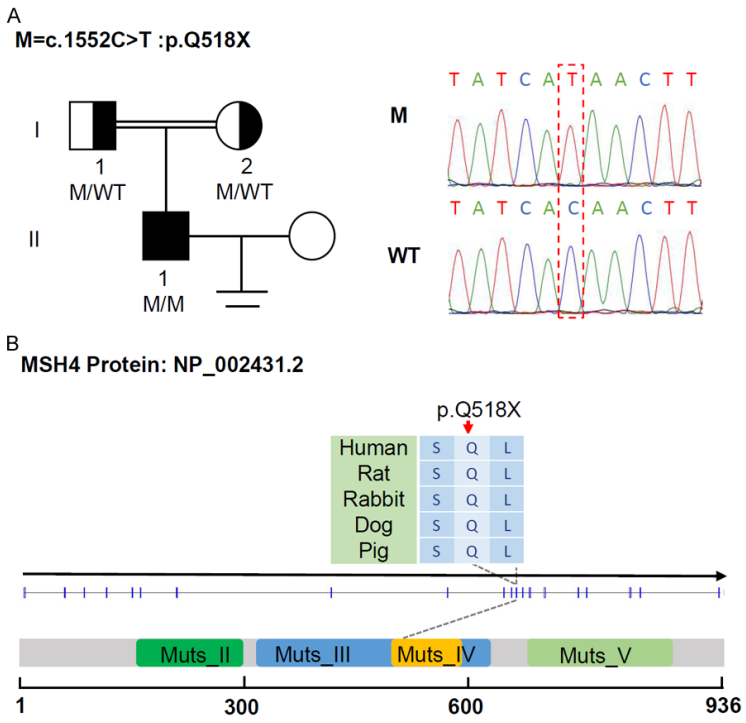

Figure 1. Mutations of *MSH4* in F1 II-1. (A) The family affected by the variants in *MSH4*. The red frame indicate mutated positions in the Sanger sequencing results. (B) The mutated positions of *MSH4* are conserved among species (red arrows). And the dotted lines indicate the positions of *MSH4* mutations in MSH4 protein. M, *MSH4* mutation; WT, wild type.

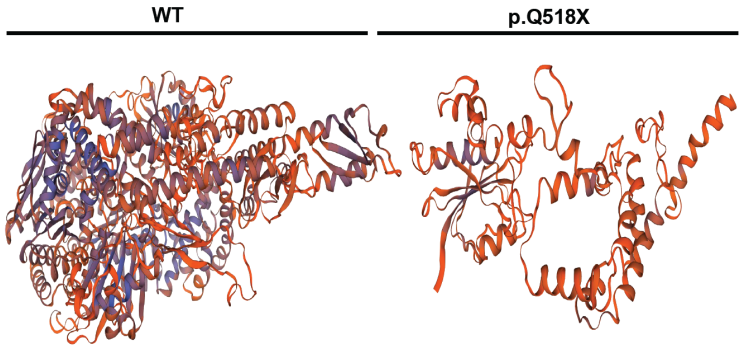

Figure 2. The predicted part three-dimensional structure of mutated MSH4 residues by SWISS-MODEL software (https://swissmodel.expasy.org/); WT, wild type.

causes of female POI [27, 29], mutations in *MSH4* were considered to be responsible for NOA until 2020 [28]. In this present research, one patient with NOA was revealed to carry a novel homozygous stop-gain mutation of *MSH4*. In this context, we showed that *MSH4* mutations are a novel causative genetic etiology of NOA due to MA in humans together with previous studies.

MSH4 is a member of the MMR gene family, which plays a vital role in the repair of post replicative DNA mismatches and meiotic recombination [25, 26]. Furthermore, it was described to be involved in the disjunction of homologous chromosomes in yeast for first time in 1994 by Ross-Macdonald et al. [31]. Similarly, in mammals, it was also found to be required for chromosome pairing during meiosis. The disruption of *MSH4* leads to azoospermia in *Msh4*$^{-/-}$ male mice, and oocyte loss in *Msh4*$^{-/-}$ female mice due to meiotic failure [29]. It was also shown that *MSH4* mutations probably result in POI in female humans [27]. In human males, Stouffs et al. studied the association between the mutations of *MSH4* and the maturation arrest of spermatogenesis in 2011. They enrolled 40 patients with NOA due to MA and detected four *MSH4* mutations altering amino acid sequences. Although several mutations were homozygous in patients with MA, they were not speculated to be pathogenic due to their equal frequencies in males with normozoospermia [32]. Until 2020, two patients with NOA were found to carry two different homozygous missense mutations in *MSH4*. In our present study, a novel homozygous stop-gain mutation of *MSH4* was identified, which was probably the main cause of azoospermia in this patient. It may be considered that all mutations in the study of Stouffs et al. were missense, which may have only mild harmful effects on the structure and function of the MSH4 protein. However, the *MSH4* mutation in this present study was a stop-gain mutation, which resulted in a truncated protein and significantly damaged the structure and function of the MSH4 protein. This disruption might further lead to meiotic failure and NOA.

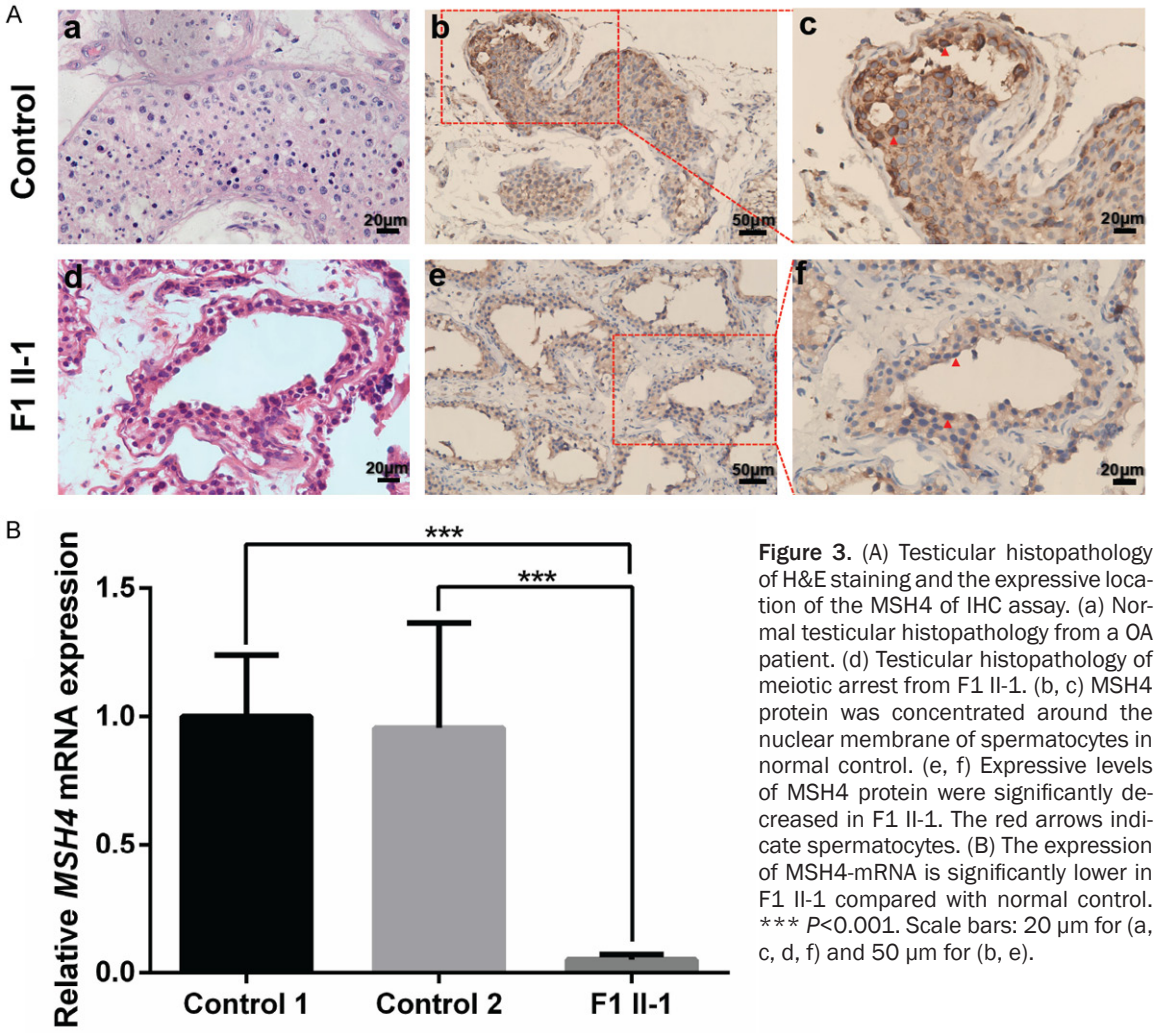

Figure 3. (A) Testicular histopathology of H&E staining and the expressive location of the MSH4 of IHC assay. (a) Normal testicular histopathology from a OA patient. (d) Testicular histopathology of meiotic arrest from F1 II-1. (b, c) MSH4 protein was concentrated around the nuclear membrane of spermatocytes in normal control. (e, f) Expressive levels of MSH4 protein were significantly decreased in F1 II-1. The red arrows indicate spermatocytes. (B) The expression of MSH4-mRNA is significantly lower in F1 II-1 compared with normal control. *** $P<0.001$. Scale bars: 20 μm for (a, c, d, f) and 50 μm for (b, e).

The expression levels of MSH4 mRNA were significantly decreased in the testicular tissue of our patient compared with the normal control. These results further supported the harmful effects of the novel homozygous mutation. Furthermore, it was reported that the transcript levels of *MSH4* in the testis of the patients with NOA were significantly lower than those in the patients with OA [33]. MSH4 protein, through its interaction with RAD51 and DMC1, probably has a function soon after the initiation of DNA strand-exchange [25]. The truncated protein, transcribed and translated by mutated *MSH4,* might lead to the failure of this function.

The primary limitation of this present study is that the mutation frequency of *MSH4* was not detected in males with normal fertility. Secondly, chromosome spreads from homozygous mutant spermatocytes were not performed to verify the abnormality of the chromosome as the little testicular tissue was obtained by testicular biopsy. So we did not clear whether this mutated *MSH4* might lead to the failure of meiotic recombination. Thirdly, as the lower mutation frequency and relatively small sample size, only one patients was found that the causative genetic etiology of NOA was *MSH4* mutation. A larger sample size is needed to explore mutation frequency of *MSH4* in NOA patients and verify *MSH4* mutation is one of causative genetic etiologies of NOA.

Conclusion

In summary, a novel mutation of *MSH4* (c.1552C>T:p.Q518X) was identified to be related to NOA. Taken together with other pieces of evidence of the association between *MSH4* mutations and NOA phenotypes due to MA, both in human and mice, our results suggest that *MSH4* is one of the candidate causative

genes for NOA, whose mutations may lead to spermatogenetic failure and MA.

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Disclosure of conflict of interest

None.

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References

[1] Vander Borght M and Wyns C. Fertility and infertility: definition and epidemiology. Clin Biochem 2018; 62: 2-10.

[2] Deshpande PS and Gupta AS. Causes and prevalence of factors causing infertility in a public health facility. J Hum Reprod Sci 2019; 12: 287-293.

[3] Sun H, Gong TT, Jiang YT, Zhang S, Zhao YH and Wu QJ. Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990-2017: results from a global burden of disease study, 2017. Aging (Albany NY) 2019; 11: 10952-10991.

[4] Jungwirth A, Giwercman A, Tournaye H, Diemer T, Kopa Z, Dohle G and Krausz C; European Association of Urology Working Group on Male Infertility. European association of urology guidelines on male infertility: the 2012 update. Eur Urol 2012; 62: 324-332.

[5] Gudeloglu A, Brahmbhatt JV and Parekattil SJ. Medical management of male infertility in the absence of a specific etiology. Semin Reprod Med 2014; 32: 313-318.

[6] Chiba K, Enatsu N and Fujisawa M. Management of non-obstructive azoospermia. Reprod Med Biol 2016; 15: 165-173.

[7] Jashnani K, Gundawar R, Kavishwar V and Parameshwar V. Fine-Needle aspiration cytology of the testes for the classification of azoospermia and its value in the assessment of male infertility. Acta Cytol 2020; 64: 216-223.

[8] Huang IS, Huang WJ and Lin AT. Distinguishing non-obstructive azoospermia from obstructive azoospermia in Taiwanese patients by hormone profile and testis size. J Chin Med Assoc 2018; 81: 531-535.

[9] Li P, Yao CC, Zhi EL, Xu Y, Wan Z, Jiang YC, Huang YH, Gong YH, Chen HX, Tian RH, Yang C, Zhao LY and Li Z. Modified stepwise mini-incision microdissection testicular sperm extraction: a useful technique for patients with a history of orchidopexy affected by non-obstructive azoospermia. J Zhejiang Univ Sci B 2020; 21: 87-92.

[10] Caroppo E, Colpi EM, Gazzano G, Vaccalluzzo L, Piatti E, D'Amato G and Colpi GM. The seminiferous tubule caliber pattern as evaluated at high magnification during microdissection testicular sperm extraction predicts sperm retrieval in patients with non-obstructive azoospermia. Andrology 2019; 7: 8-14.

[11] Tournaye H, Krausz C and Oates RD. Novel concepts in the aetiology of male reproductive impairment. Lancet Diabetes Endocrinol 2017; 5: 544-553.

[12] Yatsenko AN, Georgiadis AP, Ropke A, Berman AJ, Jaffe T, Olszewska M, Westernstroer B, Sanfilippo J, Kurpisz M, Rajkovic A, Yatsenko SA, Kliesch S, Schlatt S and Tuttelmann F. X-linked TEX11 mutations, meiotic arrest, and azoospermia in infertile men. N Engl J Med 2015; 372: 2097-2107.

[13] Sha Y, Zheng L, Ji Z, Mei L, Ding L, Lin S, Wang X, Yang X and Li P. A novel TEX11 mutation induces azoospermia: a case report of infertile brothers and literature review. BMC Med Genet 2018; 19: 63.

[14] Yang F, Silber S, Leu NA, Oates RD, Marszalek JD, Skaletsky H, Brown LG, Rozen S, Page DC and Wang PJ. TEX11 is mutated in infertile men with azoospermia and regulates genome-wide recombination rates in mouse. EMBO Mol Med 2015; 7: 1198-1210.

[15] Greenbaum MP, Yan W, Wu MH, Lin YN, Agno JE, Sharma M, Braun RE, Rajkovic A and Matzuk MM. TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci U S A 2006; 103: 4982-4987.

[16] Gershoni M, Hauser R, Yogev L, Lehavi O, Azem F, Yavetz H, Pietrokovski S and Kleiman SE. A familial study of azoospermic men identifies three novel causative mutations in three new human azoospermia genes. Genet Med 2017; 19: 998-1006.

[17] Kasak L, Punab M, Nagirnaja L, Grigorova M, Minajeva A, Lopes AM, Punab AM, Aston KI, Carvalho F, Laasik E, Smith LB, Consortium G, Conrad DF and Laan M. Bi-allelic recessive loss-of-function variants in FANCM cause non-

obstructive azoospermia. Am J Hum Genet 2018; 103: 200-212.
[18] Yin H, Ma H, Hussain S, Zhang H, Xie X, Jiang L, Jiang X, Iqbal F, Bukhari I, Jiang H, Ali A, Zhong L, Li T, Fan S, Zhang B, Gao J, Li Y, Nazish J, Khan T, Khan M, Zubair M, Hao Q, Fang H, Huang J, Huleihel M, Sha J, Pandita TK, Zhang Y and Shi Q. A homozygous FANCM frameshift pathogenic variant causes male infertility. Genet Med 2019; 21: 62-70.
[19] Nakamura S, Miyado M, Saito K, Katsumi M, Nakamura A, Kobori Y, Tanaka Y, Ishikawa H, Yoshida A, Okada H, Hata K, Nakabayashi K, Okamura K, Ogata H, Matsubara Y, Ogata T, Nakai H and Fukami M. Next-generation sequencing for patients with non-obstructive azoospermia: implications for significant roles of monogenic/oligogenic mutations. Andrology 2017; 5: 824-831.
[20] Kherraf ZE, Christou-Kent M, Karaouzene T, Amiri-Yekta A, Martinez G, Vargas AS, Lambert E, Borel C, Dorphin B, Aknin-Seifer I, Mitchell MJ, Metzler-Guillemain C, Escoffier J, Nef S, Grepillat M, Thierry-Mieg N, Satre V, Bailly M, Boitrelle F, Pernet-Gallay K, Hennebicq S, Faure J, Bottari SP, Coutton C, Ray PF and Arnoult C. SPINK2 deficiency causes infertility by inducing sperm defects in heterozygotes and azoospermia in homozygotes. EMBO Mol Med 2017; 9: 1132-1149.
[21] van der Bijl N, Ropke A, Biswas U, Woste M, Jessberger R, Kliesch S, Friedrich C and Tuttelmann F. Mutations in the stromal antigen 3 (STAG3) gene cause male infertility due to meiotic arrest. Hum Reprod 2019; 34: 2112-2119.
[22] Riera-Escamilla A, Enguita-Marruedo A, Moreno-Mendoza D, Chianese C, Sleddens-Linkels E, Contini E, Benelli M, Natali A, Colpi GM, Ruiz-Castane E, Maggi M, Baarends WM and Krausz C. Sequencing of a 'mouse azoospermia' gene panel in azoospermic men: identification of RNF212 and STAG3 mutations as novel genetic causes of meiotic arrest. Hum Reprod 2019; 34: 978-988.
[23] Xiao WJ, He WB, Zhang YX, Meng LL, Lu GX, Lin G, Tan YQ and Du J. In-frame variants in STAG3 gene cause premature ovarian insufficiency. Front Genet 2019; 10: 1016.
[24] He WB, Tu CF, Liu Q, Meng LL, Yuan SM, Luo AX, He FS, Shen J, Li W, Du J, Zhong CG, Lu GX, Lin G, Fan LQ and Tan YQ. DMC1 mutation that causes human non-obstructive azoospermia and premature ovarian insufficiency identified by whole-exome sequencing. J Med Genet 2018; 55: 198-204.
[25] Neyton S, Lespinasse F, Moens PB, Paul R, Gaudray P, Paquis-Flucklinger V and Santucci-Darmanin S. Association between MSH4 (MutS homologue 4) and the DNA strand-exchange RAD51 and DMC1 proteins during mammalian meiosis. Mol Hum Reprod 2004; 10: 917-924.
[26] Santucci-Darmanin S, Walpita D, Lespinasse F, Desnuelle C, Ashley T and Paquis-Flucklinger V. MSH4 acts in conjunction with MLH1 during mammalian meiosis. FASEB J 2000; 14: 1539-1547.
[27] Carlosama C, Elzaiat M, Patino LC, Mateus HE, Veitia RA and Laissue P. A homozygous donor splice-site mutation in the meiotic gene MSH4 causes primary ovarian insufficiency. Hum Mol Genet 2017; 26: 3161-3166.
[28] Krausz C, Riera-Escamilla A, Moreno-Mendoza D, Holleman K, Cioppi F, Algaba F, Pybus M, Friedrich C, Wyrwoll MJ, Casamonti E, Pietroforte S, Nagirnaja L, Lopes AM, Kliesch S, Pilatz A, Carrell DT, Conrad DF, Ars E, Ruiz-Castane E, Aston KI, Baarends WM and Tuttelmann F. Genetic dissection of spermatogenic arrest through exome analysis: clinical implications for the management of azoospermic men. Genet Med 2020; 22: 1956-1966.
[29] Kneitz B, Cohen PE, Avdievich E, Zhu L, Kane MF, Hou H Jr, Kolodner RD, Kucherlapati R, Pollard JW and Edelmann W. MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice. Genes Dev 2000; 14: 1085-1097.
[30] Guo YN, Luo B, Chen WJ, Chen X, Peng ZG, Wei KL and Chen G. Comprehensive clinical implications of homeobox A10 in 3,199 cases of non-small cell lung cancer tissue samples combining qRT-PCR, RNA sequencing and microarray data. Am J Transl Res 2019; 11: 45-66.
[31] Ross-Macdonald P and Roeder GS. Mutation of a meiosis-specific MutS homolog decreases crossing over but not mismatch correction. Cell 1994; 79: 1069-1080.
[32] Stouffs K, Vandermaelen D, Tournaye H, Liebaers I and Lissens W. Mutation analysis of three genes in patients with maturation arrest of spermatogenesis and couples with recurrent miscarriages. Reprod Biomed Online 2011; 22: 65-71.
[33] Terribas E, Bonache S, Garcia-Arevalo M, Sanchez J, Franco E, Bassas L and Larriba S. Changes in the expression profile of the meiosis-involved mismatch repair genes in impaired human spermatogenesis. J Androl 2010; 31: 346-357.

Supplementary Table 1. Primers used for verification of *MSH4* mutations

	Primer Names	Primer Sequences (5'-3')
F1 II-1	M-Forward	AGTTTGAAAGAGCAACAAGACCTCCT
	M-Reverse	CTGTGGAAAACACTGAGTTTGGTATTTC

Supplementary Table 2. Primers used for QRT-PCR assay of MSH4 and β-actin

	Primer Names	Primer Sequences (5'-3')	Tm
MSH4	Forward	TTCAGCACTGTCCTAATGGAGG	61.4
	Reverse	TCTATCATGGCTGTCTGTTCACT	60.8
β-actin	Forward	CATGTACGTTGCTATCCAGGC	60.8
	Reverse	CTCCTTAATGTCACGCACGAT	60.2