Original Article

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

Dongdong Tang1,2,3*, Chuan Xu1,2,3*, Hao Geng1,2,3*, Yang Gao1,2,3, Huiru Cheng1,2,3, Xiaqing Ni1,2,3, Xiaojin He1,2,3, Yunxia Cao1,2,3

1Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, No 218 Jixi Road, Hefei 230022, Anhui, China; 2NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract (Anhui Medical University), No 81 Meishan Road, Hefei 230032, Anhui, China; 3Key Laboratory of Population Health Across Life Cycle (Anhui Medical University), Ministry of Education of The People’s Republic of China, No 81 Meishan Road, Hefei 230032, Anhui, China. *Equal contributors.

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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 teratospermia, 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 azo-
MSH4 mutation leads to NOA

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 spermatogenetic 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**.
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.

Discussion

Although male MSH4- mice appeared to NOA have been presented in 2000, and MSH4 mutations have been verified as one of the

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://swissmodel.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).
MSH4 mutation leads to NOA

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

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xiaojin He and Yunxia Cao, Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, No. 218 Jixi Road, Hefei 230022, China. Tel: +86-551-65908446; E-mail: hxj0117@126.com (XJH); Tel: +86-551-65161002; E-mail: caoyunxia6@126.com (YXC)

References


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

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<tr>
<th>Primer Names</th>
<th>Primer Sequences (5'→3')</th>
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<td>F1 II-1 M-Forward</td>
<td>AGTTTGAAAGAGCAACAAGACCTCT</td>
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<tr>
<td>M-Reverse</td>
<td>CTGTTGAAAACACTGAGTTTGTTCTTTC</td>
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**Supplementary Table 2.** Primers used for QRT-PCR assay of MSH4 and β-actin

<table>
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<td>Reverse</td>
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<td><strong>β-actin</strong></td>
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<tr>
<td>Reverse</td>
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