Nonobstructive azoospermia (NOA) is a common cause of infertility and is defined as the complete absence of sperm in ejaculation due to defective spermatogenesis. The aim of this study was to identify the genetic etiology of NOA in an infertile male from a Chinese consanguineous family. A homozygous missense variant of the membrane-bound O-acyltransferase domain-containing 1 (MBOAT1) gene (c.770C>T, p.Thr257Met) was found by whole-exome sequencing (WES). Bioinformatic analysis also showed that this variant was a pathogenic variant and that the amino acid residue in MBOAT1 was highly conserved in mammals. Quantitative polymerase chain reaction (Q-PCR) analysis showed that the mRNA level of MBOAT1 in the patient was 22.0% lower than that in his father. Furthermore, we screened variants of MBOAT1 in a broader population and found an additional homozygous variant of the MBOAT1 gene in 123 infertile men. Our data identified homozygous variants of the MBOAT1 gene associated with male infertility. This study will provide new insights for researchers to understand the molecular mechanisms of male infertility and will help clinicians make accurate diagnoses.

Asian Journal of Andrology (2022) 24, 186–190; doi: 10.4103/aja202160; published online: 24 September 2021

Keywords: male infertility; membrane-bound O-acyltransferase domain-containing 1; nonobstructive azoospermia; whole-exome sequencing

INTRODUCTION

Infertility affects approximately 50 million couples worldwide, with male infertility accounting for nearly half of these cases. Azoo spermia, found in approximately 10%–20% of infertile men, is a major cause of infertility and is thought to be the most severe phenotype of male infertility. Azoo spermia is divided into two main groups, obstructive azoospermia (OA) and nonobstructive azoospermia (NOA). The latter, NOA, refers to the complete absence of sperm during ejaculation due to a defect in spermatogenesis. To date, genetic defects have been reported as the most common cause of azoospermia. The most well-known genetic factors are Y chromosome microdeletions of the azoospermic factor locus and chromosomal abnormalities such as Klinefelter (47,XXY) and cystic fibrosis transmembrane conductance regulator (CFTR) variants (associated with congenital OA). Currently, approximately 1000 genes have been identified as male germ cell-specific genes, and any variant in these genes may lead to male infertility. Although good progress has been made, the underlying cause of NOA is still largely unknown.

In recent years, technological progress, such as whole-exome sequencing (WES), has provided a powerful method to discover the potential pathogenic variation from a single candidate gene to the coding portion of the whole genome. To date, researchers have studied the blood lineage of NOA sons by the WES method and found a number of candidate gene variations. Some of them are meiotic control genes, such as testis expressed 11 (TEX11), testis expressed 15 (TEX15), SPO11 initiator of meiotic double-stranded breaks (SPO11), DNA meiotic recombinase 1 (DMC1), meiosis specific with OB-fold (MEIOB), and synaptonemal complex central element 1 (SYCE1). Among these genes, TEX11 is an X chromosome gene that plays an important role in meiotic recombination and chromosomal synopsis. TEX11 variants account for 1%–2% of male azoospermia cases and 15% of male azoospermia cases with meiotic arrest. SYCE1 is one of the components of the synaptonemal complex, which is essential for homolog interactions and crossover formation during meiosis. Maor-Sagie et al. found a splice site variant in SYCE1 in two affected siblings with NOA by WES. These siblings were born to a consanguineous Iranian–Jewish couple. A recessive variant in TEX15, which is required for DNA double-strand break repair and homologous chromosome synopsis during male meiosis, has also been reported in three siblings affected with NOA in a Turkish family. These findings suggest that meiotic genes are potential genetic candidates for male infertility.

Spermatogenesis is a complex process. In addition to meiosis, spermatogenesis also includes spermatogonia proliferation, spermatogonia differentiation, and transformation of round spermatids into the most special cells. Any gene defect in these processes may lead to abnormal spermatogenesis and male infertility. To date, previous reports have identified several key genes associated with these cellular processes that may contribute to male infertility, including TATA-box binding protein associated factor 4b (TAF4B), zinc finger MYND-
type containing 15 (ZMYND15),17 neuronal PAS domain protein 2 (NPAS2),18 dynein axonemal heavy chain 6 (DNAH6),19 tudor domain containing 9 (TDRD9),13 nanos C2HC-type zinc finger 2 (NANOS2),20 KASH domain containing 5 (CCDC155),11 serine peptidase inhibitor Kazal type 2 (SPINK2),21 MAGE family member B4 (MAGEBA),22 and WNK lysine deficient protein kinase 3 (WNK3).11

In the current study, we used WES to identify the genetic variants in a man with NOA from a consanguineous Chinese family. We detected a homozygous missense variant in the MBOAT1 gene coding for membrane-bound O-acyltransferase domain-containing 1. Quantitative polymerase chain reaction (Q-PCR) analysis revealed that the MBOAT1 mRNA level of the patient was 22.0% lower than that of his father. Furthermore, we detected an additional homozygous variant in MBOAT1 in 123 infertile men. Taken together, the results of our study provide the view of the physiological role of the MBOAT1 gene in human male infertility.

PARTICIPANTS AND METHODS

Participants

A patient, who was from a consanguineous family (II-1 in Figure 1a), was referred to the Center for Reproductive Medicine for the treatment of infertility (The First Affiliated Hospital of USTC, Hefei, China). The clinical examination suggested that he was suffering from NOA (Figure 1b) and that he had normally developed male external genitalia, bilaterally normal testicular size, and normal follicle-stimulating hormone (FSH) levels. His parents were first-degree cousins (Figure 1a).

In addition, 123 men with NOA (from January 2015 to December 2018) who had been diagnosed at The First Affiliated Hospital of USTC were recruited for this study. Diagnostic criteria: without vas deferens obstruction, and no sperm was found in two routine semen analyses. Exclusion criteria were as follows: patients diagnosed with obstructive azoospermia or patients who had been diagnosed with mumps and systemic diseases and patients who had genital trauma, radiotherapy, or chemotherapy. In addition, 254 normal sporadic cases, including individuals with normal fertility and males with normal semen examination results, were also included in this study. All participants had no history of unhealthy activity or contacts with adverse chemicals. The chromosomal karyotypes of all participants were normal (46,XY), and no microdeletions were identified in the Y chromosome.

This work was approved by the ethics committee of Anhui Medical University (approve ID: 20190331; Anhui, China). Written informed consent was signed by all participants, and normal individuals were used as controls.

DNA preparation and WES

Genomic DNA samples were obtained from all study members, including the patients and their parents in the pedigree (i.e., I-1, I-2, and II-1 in Figure 1), as well as 123 NOA men and 254 normal sporadic cases in scattered cases, as previously described.23 These genomic DNA samples were then subjected to WES, which was performed by BGI in Shenzhen using the HiSeq2000 sequencing platform (illumina, san diego, CA, USA). This WES raw read analysis was described as previously reported.23 Variants that fulfilled the following criteria were considered candidate genes: (i) variants that were absent or rare (frequency of the minor allele [MAF] <0.01) in the two databases (1000G and ExAC); (ii) nonsense variants removed and missense, frameshift, and splice site variants were retained; and (iii) retained variants that were homozygous in the patient, heterozygous in his parents, and compound heterozygous variants were retained.

Sanger sequencing validation

Variants suspected to cause NOA were validated using Sanger sequencing in the patient as well as his parents. Sanger sequencing was performed according to the literature.24 We amplified the PCR products for exon 8 of the MBOAT1 gene using specific primers (the forward primer was 5’-GGAGCAAGGTTGCTTAAAC-3’, and the reverse primer was 5’-GCTTTGAGCCTTGACATGACA-3’). The PCR products were then sequenced on an ABI 3730XL automated sequencer (Applied Biosystems, Forster City, CA, USA).

Q-PCR

RNA extraction from whole blood was carried out using TRI REAGENT® BD (Molecular Research Center, Inc., Cincinnati, OH, USA) as per the manufacturer’s protocol as previously described.23 Q-PCR was carried out according to the manufacturer’s protocol (the forward primer of MBOAT1 was 5’-CGGCCAGCGCTTTTCTACTAGC-3’, and the reverse primer was 5’-GACACAGTGAGCAGGAAA-3’). The fold change in gene expression was quantified by the relative quantification method (2−ΔΔCT) using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a reference (the forward primer of GAPDH was 5’-GCTGCGGACCAGCCCTCCTT-3’, and the reverse primer was 5’-GACACAGTGAGCAGGAAA-3’). Data were shown as the average fold increase and standard error of the mean. The statistical analysis was calculated using Student’s t-test with SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). When P < 0.05, the difference was statistically significant.

RESULTS

MBOAT1 gene variant in a man with NOA

To identify a possible genetic variant that could explain NOA, we analyzed the peripheral blood genomic DNA obtained from the patient and his parents using WES. Given the loop of consanguinity in the family (Figure 1a), we supposed that infertility was likely transmitted by a recessive mode of inheritance and was thus caused by homozygous variants. Moreover, hematoxylin and eosin (H&E) staining showed that the number of spermatocytes in the seminiferous tubules decreased, and no sperms were found (Figure 1b).

After the exclusion of frequent variants and the application of stringent technical and biological filters (Figure 2a), we identified a limited list of homozygous variants (Supplementary Table 1). Then, we continued filtering the data by (i) omitting variants in genes that...
Conservation of the mutated threonine (Thr) residue and expression studies at the mRNA level of MBOAT1.

The amino acid sequence of Thr257 is highly conserved across mammals. In silico analysis of the variant

Validation by Sanger sequencing

Sanger sequencing revealed that the patient with NOA was also homozygous for this MBOAT1 variant, whereas his parents harbored the variant in the heterozygous state (Figure 2b).

In silico analysis of the variant

According to online pathogenicity predictive tools (SIFT, PolyPhen-2, MutationAssessor, MutationTaster, MetaLR, and LRT; Supplementary Table 2), the c.770C>T variant in the MBOAT1 gene is probably a damaging or disease-causing variant. The conservation of this variant site was predicted by computational analysis, and the results showed that this amino acid was moderately conserved (Figure 3a), while the threonine (Thr) residue is highly conserved in most mammals (Figure 3b). These results suggest that the Thr residue at site 257 plays an important role in mammals.

Detrimental effects of the identified variant in MBOAT1

To assess the pathogenic impact of the MBOAT1 variant, Q-PCR was performed to analyze the MBOAT1 expression level. Total RNA was isolated from the whole blood of the patient and his father and was reverse-transcribed to complementary DNA (cDNA). This cDNA mix was used for Q-PCR, and the results showed that, compared with his father, the mRNA expression of MBOAT1 in the patient was significantly downregulated (Figure 3c).

Screening the MBOAT1 variant in male infertility patients

To estimate the frequency of the variant in MBOAT1 in broader populations, we performed variant screening for MBOAT1 using WES in a cohort of 123 NOA men and 254 controls who had fathered live offspring. All participants were Han Chinese. We found one additional homozygous variant in MBOAT1 in 123 infertile men (0.8%; Table 1). In patient P78, who carried MBOAT1 homozygous missense variant c.151C>T, the variant resulted in the substitution of an Arg for Cys at position 51. This variant is extremely rare, and the accumulated frequency in the general population is approximately 0.1% (gnomAD and 1000G). Furthermore, no pathogenic variants in MBOAT1 were found in 254 controls.

DISCUSSION

In this study, we analyzed a NOA patient from a consanguineous Chinese family. We identified a single-base homozygous variant (c.770C>T) in the MBOAT1 gene by WES.

MBOAT1 is a superfamily of enzymes of the membrane-bound O-acyltransferase (MOAT) family and is located on chromosome 6. MBOAT1 plays a crucial role in a series of cellular events, such as membrane synthesis and remodeling, lipid storage, and signaling. It catalyzes the addition of fatty acyl chains to diverse substrates, such as proteins, neutral lipids, and phospholipids. Steinhauser et al. reported that oysnes (Drosophila homologs of MBOAT1) double-mutant adult male Drosophila are sterile due to specific defects in
spermatid individualization. In addition, oysnes double-mutant embryos show defects in the ability of germ cells to migrate into the mesoderm and show an increase in the saturated fatty acid content of several phospholipid species.\textsuperscript{29} Our results showed that \textit{MOB1} plays a very important role in germline development. Intriguingly, studies have shown that a chromosomal translocation ([t[4;6] [q12;p23]) disrupts human \textit{MOB1}, resulting in male sterility and brachydactyly,\textsuperscript{30} which highlights the important role of \textit{MOB1} in mammalian spermatogenesis. However, there are few studies on the role of \textit{MOB1} in human spermatogenesis, and there is no report on the relationship between the \textit{MOB1} variant and male infertility. Our study finds a homozygous missense variant (c.770C>T) in the \textit{MOB1} gene associated with NOA. The C-to-T transition at position 770 in the \textit{MOB1} gene resulted in the substitution of a Met for Thr at position 257. Interspecies comparison does not show significant conservation of this Thr residue (Figure 3a); however, in most mammals, this threonine residue is significantly conserved (Figure 3b). Studies on the effect of the variant on \textit{MOB1} expression showed that it was significantly downregulated in NOA patients, suggesting that the variant (p.Thr257Met) in \textit{MOB1} may affect the process of \textit{MOB1} transcription. These preliminary results revealed a correlation between \textit{MOB1} and human spermatogenesis.

To further validate that the \textit{MOB1} variant may be pathogenic, we evaluated a broader population. We found a new homozygous \textit{MOB1} variant in sporadic infertile men of Han Chinese origin. Patient 78 (P78) carried the \textit{MOB1} homozygous missense variant c.151C>T, which resulted in the substitution of an Arg for Cys at position 51 (Table 1). The cumulative frequency of this variant in the general population is approximately 0.1% (gnomAD and 1000G). These results provide further evidence for the contribution of \textit{MOB1} variants to NOA. In adults, it has been reported that \textit{MOB1} is expressed in the ovary, which suggests that it may also play a role in female reproduction. The elder sister of the patient in this family was reported to have a child. Sanger sequencing showed that she had no variation in the \textit{MOB1} gene. Thus, the role of \textit{MOB1} in women's fertility needs to be further studied.

In conclusion, our study found homozygous missense variants in the \textit{MOB1} gene that were associated with male infertility. These results will extend researchers’ novel understanding of the molecular mechanisms of male infertility and lay the foundation for further research.

**AUTHOR CONTRIBUTIONS**

The results of exon sequencing and bioinformatic analysis of mutation were completed by JH. XSZ participates in the design of the project, the analysis and guidance of the results, and the revision of the paper. All clinical samples were diagnosed and collected by YY, RNA extraction and Q-PCR experiments were completed by LG and YY. The pictures were taken by XSY, BX and HJ wrote and revised the paper. All authors read and approved the final manuscript.

**COMPETING INTERESTS**

All authors declare no competing interests.

**ACKNOWLEDGMENTS**

The authors thank all families and individuals for participating in this research. This research was supported by the National Key Research and Development Project (2019YFA0802600) and the National Natural Science Foundation of China (81971333).

Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

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## Supplementary Table 1: Variants were found by whole-exome sequencing

| Gene       | Change                        | Annotation                                                                 |
|------------|-------------------------------|-----------------------------------------------------------------------------|
| **Homozygous variants**                                                                                           |
| AKAP9      | missense_variant              | NM_005751.4:p.Met3905Thr/c.11714T>C                                        |
| C6orf223   | missense_variant              | NM_153246.5:p.Arg26Trp/c.76C>T                                            |
| CALD1      | missense_variant              | NM_033138.3:p.Ala265Val/c.794C>T                                           |
| CFA47      | missense_variant              | NM_001304548.1:p.Asp2873Asn/c.8617G>A                                      |
| COPG2      | missense_variant              | NM_012133.5:p.Leu203Val/c.607C>G                                           |
| ERC1       | missense_variant              | NM_178040.3:p.Gln971Arg/c.2912A>G                                          |
| FAM47B     | missense_variant              | NM_152631.2:p.Arg391ys/c.1160G>A                                          |
| GRM4       | missense_variant              | NM_000841.3:p.His377Gln/c.1131C>A                                          |
| HDAC6      | missense_variant              | NM_001321225.1:p.Arg849Gln/c.2546G>A                                       |
| KEL        | missense_variant              | NM_000420.2:p.Ala313Thr/c.937G>A                                          |
| MBOAT1     | missense_variant              | NM_001080480.2:p.Thr257Met/c.770C>T                                        |
| MUC12      | missense_variant              | NM_001164462.1:p.Ser498Gly/c.1492A>G                                       |
| MUC12      | missense_variant              | NM_001164462.1:p.Gly32Ser/c.94G>A                                          |
| MUC17      | missense_variant              | NM_001040105.1:p.Thr2355Ile/c.7064C>T                                      |
| NRCAM      | missense_variant              | NM_001037132.2:p.Met846Val/c.2536A>G                                       |
| PLOD3      | missense_variant              | NM_001084.4:p.Val360Leu/c.1078G>C                                         |
| PTPRZ1     | missense_variant              | NM_002851.2:p.His1129Pro/c.3386A>G                                         |
| SSPO       | missense_variant              | NM_198455.2:p.Ser3574Phe/c.10721C>T                                        |
| SSPO       | missense_variant              | NM_198455.2:p.His1173Arg/c.3518A>G                                         |
| STX1A      | missense_variant              | NM_004603.3:p.Gln6Arg/c.17A>G                                              |
| TYW1       | missense_variant              | NM_018264.3:p.Ala175Val/c.524C>T                                           |
| **Compound heterozygous variants**                                                                                   |
| S1PR4      | missense_variant              | NM_003775.3:p.Gly167Ser/c.499G>A                                           |
| S1PR4      | missense_variant              | NM_003775.3:p.Arg192His/c.575G>A                                          |

MBOAT1: membrane-bound O-acyltransferase domain-containing 1

## Supplementary Table 2: In silico analysis of the mutations

| Variants  | REVEL   | Polyphen-2 | SIFT    | Mutation taster | CADD   | Mutation assessor | MetalR  | LRT  | MetaSVM | FATHMM |
|-----------|---------|------------|---------|-----------------|--------|-------------------|---------|------|---------|--------|
| MBOAT1 (c. 770C>T) | 0.59 (LDC) | 0.961 (D) | 0.01 (D) | Damage          | 24     | 0.769 (M)         | 0.518 (D) | 0.000046 (D) | -0.106 (T) | -0.69 (T) |
| TYW1 (c. 524C>T)   | 0.73 (LDC) | 0.982 (D) | 0.003 (D) | Damage          | 25     | 0.769 (M)         | 0.621 (D) | 0.00 (D)  | 0.353 (D) | -1.19 (T) |

SIFT: sorting intolerant from tolerant; MBOAT1: membrane-bound O-acyltransferase domain-containing 1; CADD: Combined Annotation Dependent Depletion; REVEL: Rare Exome Variant Ensemble Learner; LRT: Likelihood Ratio Test; FATHMM: Functional Analysis Through Hidden Markov Models; MetaSVM: Meta-analytic support vector machine; LDC: Likely disease causing; D: deleterious; M: medium; T: tolerance; TYW1: tRNA-yW synthesizing protein 1 homolog