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ORIGINAL ARTICLE

Male Infertility

MBOAT1 homozygous missense variant causes nonobstructive azoospermia

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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.

INTRODUCTION

Infertility affects approximately 50 million couples worldwide, with male infertility accounting for nearly half of these cases.[1] Azoospermia, 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.[2] Azoospermia 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).[3] Currently, approximately 1000 genes have been identified as male germ cell-specific genes, and any variant in these genes may lead to male infertility.[4] 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.[4] 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*),[5–7] testis expressed 15 (*TEX15*),[8,9] SPO11 initiator of meiotic double-stranded breaks (*SPO11*),[10,11] DNA meiotic recombinase 1 (*DMC1*),[12] meiosis specific with OB-fold (*MEIOB*),[13] and synaptonemal complex central element 1 (*SYCE1*).[14] Among these genes, *TEX11* is an X chromosome gene that plays an important role in meiotic recombination and chromosomal synapsis. *TEX11* variants account for 1%–2% of male azoospermia cases and 15% of male azoospermia cases with meiotic arrest.[5,7] SYCE1 is one of the components of the synaptonemal complex, which is essential for homolog interactions and crossover formation during meiosis.[15] Maor-Sagie *et al.*[14] 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 synapsis during male meiosis, has also been reported in three siblings affected with NOA in a Turkish family.[8,9] 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.[16] 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*),[17] zinc finger MYND-

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type containing 15 (*ZMYND15*),[17] neuronal PAS domain protein 2 (*NPAS2*),[18] dynein axonemal heavy chain 6 (*DNAH6*),[13] tudor domain containing 9 (*TDRD9*),[19] 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 (*MAGEB4*),[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'-GGAGCAACGTTGTCCTTAAC-3', and the reverse primer was 5'-GCTTTGAGGCTTGCATGACA-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'-CGTCCAGCCTTTCCTACCG-3', and the reverse primer was 5'-GCACAGAGTACCAGCCGAAA-3'). The fold change in gene expression was quantified by the relative quantification method ($2^{-\Delta\Delta CT}$) using the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as a reference (the forward primer of *GAPDH* was 5'-GTCAAGGCTGAGAACGGGAA-3', and the reverse primer of *GAPDH* was 5'-AAATGAGCCCCAGCCTTCTC-3'). Data are 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
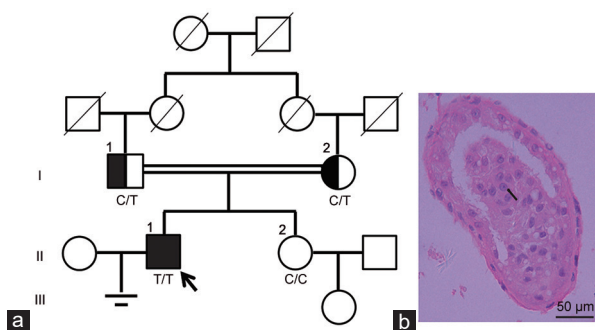
Figure 1: Details of a patient with NOA in a consanguineous family. (**a**) Family tree of consanguineous Chinese family. I, II, and III refer to the generation of the family; 1 and 2 refer to the individual number of this generation. Arrow refers to the proband. (**b**) Pathological section of testicular tissue of the patient. Scale bar = 50 μm. NOA: nonobstructive azoospermia.
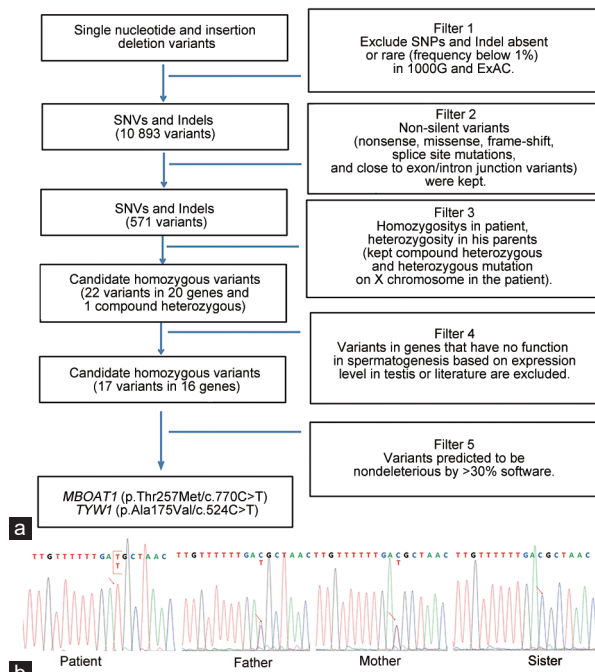
Figure 2: Exome sequencing reveals a biallelic variant in the *MBOAT1* gene. (**a**) Gene filter strategies. (**b**) Sanger sequencing was performed to validate the biallelic variant in the patient and his parents. The arrow shows the base at which the variation occurs. *MBOAT1*: membrane-bound *O*-acyltransferase domain-containing 1; SNV: single-nucleotide variation; Indels: insertion/deletion; SNP: single-nucleotide polymorphism; *TYW1*: tRNA-yW synthesizing protein 1 homolog.

exhibited no expression level in the testis,[25] (ii) excluding variants in genes that had no function in spermatogenesis according to the data obtained from model organisms, and (iii) excluding variants predicted to be nondeleterious by >30% of the software covering them (SIFT, PolyPhen-2, CADD, MutationAssessor, MutationTaster, REVEL, MetaLR, LRT, MetaSVM, and FATHMM[26,27]). We found two missense variants (*MBOAT1* and tRNA-yW synthesizing protein 1 homolog [*TYW1*]; **Supplementary Table 2**). Through a literature search, we found that *MBOAT1* is important for normal spermatogenesis in *Drosophila*.[28] Thus, we considered that the homozygous missense variant in *MBOAT1* was the only candidate pathogenic variant causing NOA in this family.

### 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;[26,27] **Supplementary Table 2**), the c.770C>T variant in the *MBOAT1* gene is probably a damaging or disease-causing variant. The conservation of this variation 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
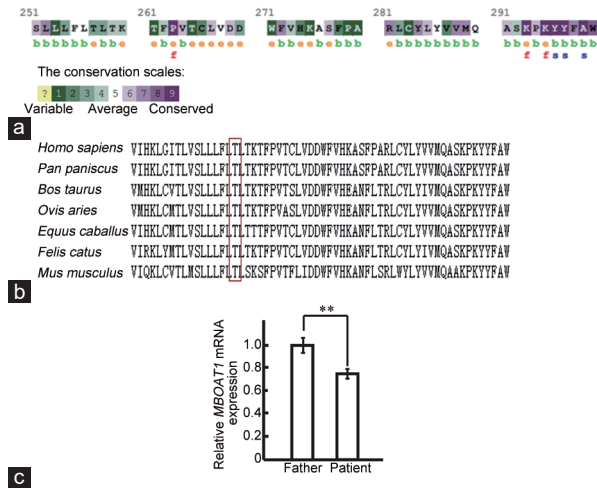
Figure 3: Conservation of the mutated threonine (Thr) residue and expression studies at the mRNA level of *MBOAT1* in the patient and his father. (**a**) Results of ConSurf32 analysis of the Thr257 region. Color intensity denotes conservation, "e" and "b" denote predicted exposed and buried residues, and "f" and "s" denote predicted functional and structural residues. (**b**) The amino acid sequence of Thr257 is highly conserved across mammals. (**c**) Expression studies at the mRNA level of *MBOAT1* by qRT-PCR analysis in the patient and his father. The mRNA expression analyzed by qRT-PCR was quantified as a ratio relative to *GAPDH* and expressed relative to their farther. **P < 0.01. *MBOAT1*: membrane-bound *O*-acyltransferase domain-containing 1; qRT-PCR: quantitative reverse transcription PCR; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

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 (MBOAT) 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.[29] Steinhauer *et al.*[28] reported that *oysnes* (*Drosophila* homologs of *MBOAT1*) double-mutant adult male *Drosophila* are sterile due to specific defects in

Table 1: Homozygous variant identified in an infertility patient

Patient	Gene	cDNA change	Protein change	Variant type	Status	dbSNP ID	SIFT	PolyPhen-2
P78	MBOAT1	c.151C>T	p.R51C	Missense	Homozygous	rs184491612	Deleterious	PD

P78: patient 78; *MBOAT1*: membrane-bound *O*-acyltransferase domain-containing 1; Polyphen-2: polymorphism phenotyping v2; SIFT: sorting intolerant from tolerant; PD: probably damaging; cDNA: complementary DNA; dbSNP: database of single nucleotide polymorphism

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.[28] Our results showed that *MBOAT1* plays a very important role in germline development. Intriguingly, studies have shown that a chromosomal translocation (t[4;6] [q12;p23]) disrupts human *MBOAT1*, resulting in male sterility and brachydactyly,[30] which highlights the important role of *MBOAT1* in mammalian spermatogenesis. However, there are few studies on the role of *MBOAT1* in human spermatogenesis, and there is no report on the relationship between the *MBOAT1* variant and male infertility. Our study finds a homozygous missense variant (c.770C>T) in the *MBOAT1* gene associated with NOA. The C-to-T transition at position 770 in the *MBOAT1* 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 *MBOAT1* expression showed that it was significantly downregulated in NOA patients, suggesting that the variant (p.Thr257Met) in *MBOAT1* may affect the process of *MBOAT1* transcription. These preliminary results revealed a correlation between *MBOAT1* and human spermatogenesis.

To further validate that the *MBOAT1* variant may be pathogenic, we evaluated a broader population. We found a new homozygous *MBOAT1* variant in sporadic infertile men of Han Chinese origin. Patient 78 (P78) carried the *MBOAT1* 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 *MBOAT1* variants to NOA. In adults, it has been reported that *MBOAT1* 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 *MBOAT1* gene. Thus, the role of *MBOAT1* in women's fertility needs to be further studied.

In conclusion, our study found homozygous missense variants in the *MBOAT1* 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 YYW. 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.

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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
CFAP47	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.Arg39Lys/c.116G>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>C
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
TECPR1	missense_variant	NM_015395.2:p.Cys801Ser/c.2401T>A
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