A new TEX11 mutation causes azoospermia and testicular meiotic arrest

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There are many unknown genetic factors that lead to infertility in nonobstructive azoospermia men. Here, we performed whole-exome sequencing in blood samples obtained from 40 azoospermia patients with meiotic arrest and found a novel c.151_154del (p.D51fs) frame-shift mutation in exon 3 of the testis expressed 11 (TEX11) gene in one patient. Sanger sequencing analysis of the patient and 288 fertile men was performed to validate the mutation. Immunohistochemical analysis showed TEX11 expression in late-pachytene spermatocytes and in round spermatids in fertile human testes. In contrast, testes of the patient with TEX11 mutation underwent meiotic arrest and lacked TEX11 expression. Western blotting of human embryonic kidney (HEK293) cells transfected with a vector for the p.D51fs TEX11 variant detected no TEX11 expression. In conclusion, we identified a novel frame-shift mutation in the TEX11 gene in an azoospermia patient, emphasizing that this gene should be included in genetic screening panels for the clinical evaluation of azoospermia patients.

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INTRODUCTION
Infertility impacts approximately 15% of couples, and about 50% of infertility is caused by male factors.1–4 Human spermatogenesis comprises a series of precisely regulated, continuous processes, including differentiation of spermatogonia, meiosis of spermatocytes, and sperm maturation.5 Factors known to deleteriously impact spermatogenesis include hormonal regulation, varicocele, cryptorchidism, immune factors, environmental chemicals, and genetic factors, among others.6–8 Spermatogenesis disorders caused by genetic factors represent the most commonly encountered problems, accounting for 15%–30% of male infertility, and these typically manifest as severe oligospermia or azoospermia.2–9

The incidence of azoospermia is about 10%–15% in male infertility,8 which can be divided into obstructive azoospermia (OA; 15%–20%)10 and nonobstructive azoospermia (NOA; 80%–85%).6,11 The many etiologies of NOA include pretesticular factors such as endocrine abnormalities and testicular factors such as testicular torsion, genetic abnormalities, and idiopathic causes,12,13 whereas the posttesticular categories are considered to be the major contributors to OA.14,15 For OA patients, it is sometimes possible to restore reproductive function via surgery or other treatments,4,16 whereas NOA is understood as one of the most difficult diseases to treat in male infertility.17 At present, the etiology and mechanisms of many NOA patients remain unknown, further limiting treatment options.18 Moreover, only limited genetic tests are currently available to help determine the etiology of infertile men, including karyotyping, Y chromosome azoospermia factor (AZF) microdeletion, and cystic fibrosis transmembrane conductance regulator (CFTR) mutation screening.6,8 We are unaware of any established predictive tests to indicate testicular pathology and/ or the possible presence of sperm cells in the testis. In this context, research into the meiotic genetic factors in male infertility, and especially in NOA patients, is very likely to further clarify the causes and mechanisms of spermatogenesis disorders and to improve the capacity of clinicians to provide individualized diagnoses as well as genetic counseling for patients.

Testis expressed 11 (TEX11) is a gene located on the X chromosome that is expressed specifically in germ cells;14,19 it has high sequence identity in mouse and human.10 The incidence of TEX11 gene mutations in male primary infertility is high, at about 1%, and TEX11 dysregulation has been implicated in spermatocyte apoptosis, maturation disorders, and azoospermia.10,20,21 Work with Tex11−/−null mice has shown that the lack of TEX11 causes meiotic arrest, and TEX11 is known to regulate homologous chromosomal synopsis and the repair of DNA double-strand breaks.21,22 In the present study, a novel mutation of the TEX11 gene was found in an azoospermia patient based on whole-exome sequencing,20 and we show that this mutation is causative for azoospermia and testicular meiotic arrest.

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PATIENTS AND METHODS

Ethical approval
This study was approved by the Institutional Review Board for Reproductive Medicine of Shandong University (Jinan, China; ethical committee reference number 2019 #36), and written informed consent was obtained from each patient. All of the DNA and tissue samples were handled in accordance with the National Regulation of Clinical Sampling in China.

Patients and study population
We studied 40 NOA Chinese patients with meiotic arrest. The diagnoses in men with azoosperma were made on the basis of semen analysis, and the pathological type of meiotic arrest was confirmed by means of histologic examination of testicular biopsy specimens according to the guidelines of the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Sperm (5th edition, 2010). Any conditions that result in spermatogenic failure (e.g., chromosomal abnormalities, Y-chromosomal microdeletions, orcsis, cryptorchidism, radiotherapy, or chemotherapy) were not present among the 40 meiotic arrest patients. The patients were recruited from the Center for Reproductive Medicine of the Shandong Provincial Hospital Affiliated with Shandong University (Jinan, China).

Immunohistochemical (IHC) analysis
For histology, testicular biopsy from those azoospermia men was fixed in 4% paraformaldehyde (PFA) solution (Solarbio, Beijing, China), embedded in paraffin, and cut into 5-µm thick sections. After sectioning, the slides were rehydrated and then processed with hematoxylin and eosin staining. Testicular biopsies from a fertile patient (who underwent percutaneous testicular biopsy for oligozoosperma) and from the patient with the TEX11 mutation underwent IHC staining. A mouse-specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Hong Kong, China) was used for IHC staining. Briefly, after rehydration, sections were blocked with Hydrogen Peroxide Block Reagent (Abcam) for 10 min. The sections were then boiled for 15 min in Sodium Citrate Antigen Retrieval Solution (Solarbio). After cooling to room temperature, the rest of the procedure was conducted according to the IHC Kit product protocol. Immunostaining of TEX11 was carried out using a primary polyclonal goat-anti-human TEX11 antibody (1:100 diluted, Abcam), with secondary mouse-anti-goat IgG-B antibody (1:200 diluted, Santa Cruz Biotechnology, Shanghai, China).

Whole-exome sequencing (WES) analyses
We performed WES for the 40 NOA patients with meiotic arrest. Genomic DNA was extracted from peripheral blood samples using QIAamp DNA mini kits (Qiagen, Beijing, China) following the manufacturer’s instructions. Then, the samples were sent to iGeneTech company (Beijing, China). Whole-exome sequencing (WES) analyses
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RESULTS

A novel azoospermia phenotype-associated mutation in the TEX11 gene

We conducted an analysis of 40 azoospermic men using WES. All of the infertile patients of the study were prescreened for the lack of chromosomal abnormalities and Y-chromosomal microdeletions. The WES results revealed a novel deletion mutation from site 151 to 154 of the TEX11 isofrom 2 transcript (NM_031276) in one patient with meiotic arrest (Figure 1a). Table 1 lists all of the previously reported TEX11 mutations as well as the novel mutation we identified in the present study.1 To verify the mutation, the infertile patient with the TEX11 mutation and 288 fertile men were examined via Sanger sequencing. The deletion mutation in the patient was confirmed; none of the 288 control men carried the novel variant (Figure 1b). Since TEX11 is an X-linked germ-cell specific gene, it is likely to be associated with spermatogenic failure. The frame-shift mutation in exon 3 c.151_154del (p.D51fs) was predicted to result in a premature stop codon in exon 4, resulting in a truncated protein comprising only 57 amino acids (Figure 1c).

Figure 1: A novel frame-shift mutation identified in the TEX11 gene. (a) The TEX11 c.151_154del mutation is localized in exon 3 (red arrow), and the premature stop codon is localized in exon 4 of TEX11 transcript isoform 2 (NM_031276.3). White rectangles represent noncoding exons, and black rectangles represent coding exons. The coding sequence of the gene begins a start codon in exon 2 and ends at a stop codon in exon 30. (b) Sanger sequencing validation of the identified deletion mutation in the patient. (c) Predicted TEX11 domains with a SP022 meiosis-specific motif (amino acid positions 175–402) and multiple TPR-containing regions (amino acid positions 402–436 and 441–471). The mutation in the coding region is located in the N-terminal sequence (red arrow). A predicted protein length schematic of truncated protein variant comprising 57 N-terminal amino acids is shown under the predicted protein domains for TEX11. TEX11: testis-expressed 11; SP022: sporulation domain; TPR: tetratricopeptide repeat; WT: wild type; AA: amino acid.

Meiotic arrest in the azoospermia patient bearing the p.D51fs TEX11 mutation

The detailed phenotypes of the azoospermic patient with the p.D51fs TEX11 mutation were ascertained through semen analysis and histologic examination of testicular biopsy specimens using light microscopy. No sperm was found in three separate semen analyses or in testicular biopsy sections. The patient was 25 years old, with normal endocrine hormone levels (Table 2). The karyotype of the patient was normal (46,XY), and no Y chromosome microdeletions were detected (Table 2). Histological analysis of the fertile man’s testis biopsy showed spermatids and different stages of male germ cells (Figure 2a–2c). In comparison, the patient’s testis biopsy revealed meiotic arrest at pachytene stage in most seminiferous tubules; round spermatids were observed only rarely in tubules (arrows; Figure 2d–2f). No mature spermatoozoa were observed in the seminiferous tubules, in accordance with the diagnosis of azoospermia. Postmeiotic cells were not seen in most tubules of the azoospermia patient, although we did detect a few late spermatocytes and round spermatids in rare tubules. Based on the histological analysis, the primary defect caused by the TEX11 deletion mutation of this patient was concluded to be meiotic arrest.

TEX11 protein expression in a healthy control and the azoospermia patient

IHC analysis of testis sections from the fertile control man showed that TEX11 was mainly expressed in late-pachytene spermatocytes and in round spermatids; TEX11 was completely absent in surrounding somatic cells including Sertoli cells and interstitial cells (Figure 3a). However, no TEX11 expression was detected in the azoospermia patient with the TEX11 mutation (Figure 3b). We also conducted experiments wherein Western blotting was used to assess TEX11 protein accumulation in HEK293 cells, which were transiently transfected with expression vectors including an empty control (PCMV6), a fusion construct of the wild-type TEX11 coding sequence (CDS) with a terminal His tag (Figure 3c), and a fusion construct of the CDS for the p.D51fs mutant allele with a His tag. Normal His-TEX11 was detected as expected (approximately 107 kDa) from
Table 1: Mutations of TEX11 reported for azoospermia patients in published literature and our data

| Position | Nucleotide change | Protein/RNA change | Testicular sperm extraction | Patients (n) | Reference |
|----------|------------------|-------------------|-----------------------------|--------------|-----------|
| Exon 6   | 405C→T           | Silent mutation, A135sp1 d^b | Few sperm                  | 1            | 20        |
| Exon 7   | 466A→G           | Missense mutation, M156V | No sperm                   | 1            | 20        |
| Exons 9–11 | 607del237bp   | Missense mutation, L249sp1 d^b | No sperm                  | 2            | 20        |
| Intron 10 | 748+1G→A^c       | Missense mutation, V142I | No sperm                   | 1            | 10        |
| Intron 21 | 1793+1G→C^c      | Missense mutation, V142I | No sperm                   | 1            | 10        |
| Exon 24  | 2047G→A          | Missense mutation, V142I | No sperm                   | 1            | 10        |
| Exon 6   | 405C→T           | Silent mutation      | No sperm                   | 1            | 10        |
| Exon 7   | 424G→A           | Missense mutation, V142I | No sperm                   | 1            | 10        |
| Exon 7   | 515A→G           | Missense mutation, Q172R | No sperm                   | 1            | 10        |
| Exon 10  | 731C→T           | Missense mutation, T244I | No sperm                   | 1            | 10        |
| Exon 16  | 2243T            | Intronic alteration  | No sperm                   | 1            | 10        |
| Exon 26  | 2243T→A          | Intronic alteration  | No sperm                   | 1            | 10        |
| Exon 27  | 2319T→C          | Silent mutation      | No sperm                   | 1            | 10        |
| Intron 3 | -17T→C^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 5 | -48G→A^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 10 | +42C→A^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 12 | -28T→C^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 15 | -64G→A^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 21 | -1G→A^c          | Alteration of splicing acceptor site | No sperm | 1 | 10 |
| Intron 22 | -37A→G^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 24 | +119G→A^c        | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 27 | -55A→C^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Intron 28 | -44A→G^c         | Intronic alteration  | No sperm                   | 1            | 10        |
| Exon 29  | 2568G→T          | Missense mutation, W856C | No sperm                   | 1            | 9         |
| Exon 3   | 151_154del       | D51 frame-shift mutation | No sperm                   | 1            | This study |

*TEX11 mutations are mapped to isoform 2 (GenBank accession number, NM_031276). *The term spl d represents the splicing donor site; +1 refers to the first base of a given intron, while -1 denotes the last base. TEX11: testis expressed 11; del: deletion; bp: base pair; Ins: insertion.

Table 2: Clinical data of the analyzed TEX11 mutation-bearing azoospermic patient

| Variable | Patient | Normal range |
|----------|---------|--------------|
| Testosterone (ng dl⁻¹) | 354.3 | 280–800 |
| Luteinizing hormone (IU l⁻¹) | 2.74 | 1.7–8.6 |
| Follicle stimulating hormone (IU l⁻¹) | 3.25 | 1.5–12.4 |
| Prolactin (ng ml⁻¹) | 6.81 | 4.04–15.2 |
| Testis (ml) | | |
| Left | 12 | 12–25 |
| Right | 12 | 12–25 |
| Y chromosome microdeletion | No deletion | No deletion |
| Karyotype | 46,XY | 46,XY |
| Histology | Normal spermatocyte maturation arrest | spermatogenesis |

TEX11: testis expressed 11

consistently with our study. However, Adelman and Petrini²¹ figured out that exon 3 knockout male mice were fertile with the production of normal litter sizes.²³ As was stated above, the phenotypes of Tex11-null mice were not in accordance in different kinds of Tex11-knockout mice, and we suppose this may result from knockout of a different exon.

The well-studied TEX11 protein contains a meiosis-specific sporation (SPO22) domain (175–429 AA) and numerous tetratricopeptide repeat (TPR) containing regions (402–436 AA, 441–471 AA) that have been shown to mediate protein–protein interactions.¹⁸,²⁰ In *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, zinc transporter 4 precursor (Zip4), an ortholog of TEX11, physically interacts with ZMM (abbreviation for Zip1/2/3/4, Msh4/5, and Mer3) proteins and with the axial element of the synaptonemal complex (SC), providing a direct physical link between crossover-designated recombination intermediates and SC assembly.²¹,²² Previous studies have reported that full-length mouse TEX11 interacts with synaptonemal complex protein 2 (SYCP2),²² and the C-terminal portion of human TEX11 is known to interact with both shortage in chiasmata 1 (SHOC1, also known as ZIP2) and nijmegen breakage syndrome 1 (NBS1) proteins that are involved in meiotic synopsis, repair, and crossover formation. However, we are unaware of any studies reporting any dysfunction related to the N-terminal region of TEX11. In addition, mutations previously reported in human were either single base replacement or bases deletion and insertion from exon 6 to exon 29.⁵,¹⁰,²⁰ Therefore, the TEX11 c.151_154del mutation in exon 3 found in our study was the first reported mutation on the N-terminal region of human TEX11.

Yang et al.¹⁰ reported that the frequency of rare TEX11 mutations is significantly elevated in azoospermic men, suggesting that TEX11
Figure 3: Expression of wild-type TEX11 and the p.D51fs truncation mutant variant in human testis and HEK293 cells. (a) Immunohistochemical detection of TEX11 in testicular tissue sections using a polyclonal anti-TEX11 antibody. In a control with normal spermatogenesis, TEX11 was highly expressed in late-pachytene spermatocytes and round spermatids. (b) In the patient, germ cells exhibited no staining for TEX11. (c) Schematic for the pCMV6-His-TEX11 vector used for cell transfection as wild-type TEX11-His. (d) Western blotting results stained for anti-His antibody with HEK293 cells transiently transfected with mock vector pCMV6, wild-type TEX11-His, or TEX11-mutant (p.D51fs)-His vectors. Actin served as a loading control, and molecular weight markers are shown on the left. SV40: simian virus 40; CMV: cytomegalovirus; f1: F1 phage; ori: origin; AmpR: ampicillin resistance gene; M13 fwd: enterobacteria phage M13 forward; T7: promoter for bacteriophage T7 RNA polymerase; His: histone; TEX11: testis-expressed 11; FLAG: DYKDDDDK amino acid sequence tag; Myc: EQKLISEEDL amino acid sequence tag; M13 rev: enterobacteria phage M13 reverse; hGH poly(A): human growth hormone polyadenylation; HSV TK poly(A): herpes simplex virus-thymidine kinase polyadenylation; NeoR/KanR: neomycin/kanamycin resistance gene; pCMV6: plasmid CMV6.

may function in human spermatogenesis.\textsuperscript{10} Mutations in this single X-linked gene cause infertility in 1% of azoospermic men.\textsuperscript{16,17} Our study identified a TEX11 mutation in infertile patients with meiotic arrest, representing a 2.5% incidence. Our study therefore supports previously published data that it would be beneficial to include the TEX11 gene in genetic screening panels for the clinical evaluation of azoospermia patients. One deficiency of the present study is that we were unable to explore the inheritance of the mutation, because the proband’s family declined to participate in the study. It will be helpful to further explore the relative incidence of this TEX11 mutation in azoospermia in larger and more diverse cohorts. In conclusion, we identified a novel (p.D51fs) mutation in the TEX11 gene of an azoospermia patient and demonstrated that the genetic disruption of TEX11 is causative for azoospermia.

AUTHOR CONTRIBUTIONS
MJL, HBL, and HBZ carried out the genetic studies and participated in the WES analysis. XCY, FFC, and SJY carried out the histological experiment. XCY and MJL carried out in vitro protein expression experiment. XCY, HBL, and HBZ drafted the manuscript. MJL, HBL, and HBZ conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declared no competing interests.

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