The heterozygous mutations of SLC26A8 are not the main actors but might be the guest players for male infertility

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Abstract

Male infertility has become a serious health and social problem troubling approximately 15% of couples worldwide; however, the genetic and phenotypic heterogeneity of human infertility poses a substantial obstacle to effective diagnosis and therapy. A previous study reported that heterozygous mutations in solute carrier family 26 member 8 (SLC26A8, NG_033897.1) were causatively linked to asthenozoospermia. Interestingly, in our research, three deleterious heterozygous mutations of SLC26A8 were separately detected in three unrelated patients who were suffered from teratozoospermia. These three heterozygous mutations resulted in the reduce of SLC26A8 expression in transfected cells, while no disrupt expression of SLC26A8 was observed in sperm from the affected individuals. Noticeably, two of the three SLC26A8 heterozygous mutations detected in the patients were inherited from their fertile fathers. Thus, we suggested that male infertility associated with SLC26A8 mutations should be involved in a recessive-inherited pattern, considering the infertile homozygous Slc26a8 KO male mice. Given that SLC26A8 heterozygous mutations were detected in the infertile patients, and SLC26A8 is predominantly expressed in the various germ cells during spermatogenesis, the heterozygous mutations in SLC26A8 may not be the direct genetic cause but contribute to male infertility to a certain degree.

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Abstract

Male infertility has become a serious health and social problem troubling approximately 15% of couples worldwide; however, the genetic and phenotypic heterogeneity of human infertility poses a substantial obstacle to effective diagnosis and therapy. A previous study reported that heterozygous mutations in *solute carrier family 26 member 8*(*SLC26A8* , NG_033897.1) were causatively linked to asthenozoospermia. Interestingly, in our research, three deleterious heterozygous mutations of*SLC26A8* were separately detected in three unrelated patients who were suffered from teratozoospermia. These three heterozygous mutations resulted in the reduce of SLC26A8 expression in transfected cells, while no disrupt expression of SLC26A8 was observed in sperm from the affected individuals. Noticeably, two of the three *SLC26A8* heterozygous mutations detected in the patients were inherited from their fertile fathers. Thus, we suggested that male infertility associated with*SLC26A8* mutations should be involved in a recessive-inherited pattern, considering the infertile homozygous *Slc26a8* KO male mice. Given that *SLC26A8* heterozygous mutations were detected in the infertile patients, and SLC26A8 is predominantly expressed in the various germ cells during spermatogenesis, the heterozygous mutations in*SLC26A8* may not be the direct genetic cause but contribute to male infertility to a certain degree.

KER WORDS: *SLC26A8* , heterozygous mutation, recessive inheritance, male infertility, WES

INTRODUCTION

World Health Organization (WHO) has deemed that infertility is a global health problem that affects about 15% of couples in the world, and male infertility accounts for about 30%˜50% (Jiao et al., 2021). Spermatogenesis is regulated by multiple gene expression strictly, and gene variations disrupting protein function often lead to defective sperm development (Noveski et al., 2016). Therefore, male infertility has a strong genetic basis. In spite of some disease-related genes that have been investigated, each gene is possibly responsible for only a small fraction of pathogenic factors. Due to the high genetic heterogeneity in male infertility (Kasak et al., 2021), the pathogenesis and mechanisms of male infertility have not been comprehensively studied.

We have noticed that Dirami T *et al* . reported that three heterozygous missense mutations(c.260G>A [p.Arg87Gln], c.2434G>A [p.Glu812Lys] and c.2860C>T [p.Arg954Cys]) in *solute carrier family 26 member 8*(*SLC26A8* , NG_033897.1) could result in male infertility associated with asthenozoospermia (Dirami et al., 2013). The authors further demonstrated heterozygous mutations in *SLC26A8* might impair the formation of SLC26A8-CFTR complex, thus disrupting the capacity to activate CFTR-dependent anion transport (Dirami et al., 2013). These events finally damaged the CFTR-dependent sperm-activation in sterile patients (Dirami et al., 2013). However, *Slc26a8*$^{-/-}$ mice presented infertile phenotype but*Slc26a8* $^{+/-}$ mice showed normal fertility (Touréet et al., 2007; Rode et al., 2012), indicated *Slc26a8* is involved in reproduction through recessive-inheritance manner. The disparate inherited pattern between humans and mice makes us confused. Does *SLC26A8* play diverse roles in the reproductive process of humans and mice? More importantly, what kind of genetic model is*SLC26A8* participating in spermatogenesis? Therefore, a more comprehensive interrogation of the function of *SLC26A8* in male infertility is needed to further boost clinical diagnosis.

Interestingly, it was a remarkable fact that we found three heterozygous mutations of *SLC26A8* respectively in three unrelated infertile males with teratozoospermia in this study, and the aberrant sperm morphology

and ultrastructure were confirmed by electron microscope. Surprisingly, two of these heterozygous mutations of *SLC26A8*detected in the patients were inherited from their fathers who have no reproductive barriers. The deleterious effect of the three heterozygous mutations on SLC26A8 expression was confirmed by western blotting in vitro. However, no significant expression difference of SLC26A8 was exhibited in sperm between patients and normal control. Therefore, we suggested that the heterozygous mutations in *SLC26A8* might not be the immediate cause of asthenozoospermia but participate in spermiogenesis to a certain extent.

2. METHODS

2.1 Study Participants

The infertile patients were enrolled at the West China Second University Hospital of Sichuan University. These patients all had normal somatic karyotypes (46, XY). This study was conducted following the tenets of the Declaration of Helsinki, and ethical approval was obtained from the Ethical Review Board of West China Second University Hospital, Sichuan University. Informed consent was obtained from each study participant.

2.2 Genetic Analysis

Peripheral blood samples were obtained from all subjects, and the genomic DNA was isolated by DNeasy Blood and Tissue Kit (QIAGEN), according to the manufacturer's protocol. Next-generation sequencing was carried out using Agilent SureSelectXT Human All Exon Kit and Illumina HiSeq X-TEN. Functional annotation was done using ANNOVAR through a series of databases including 1000Genomes Project, dbSNP, HGMD and ExAC. Next, PolyPhen-2, SIFT, MutationTaster and CADD were used for functional prediction. Targeted testing of the potentially pathogenic variants in the patients' parents was performed by Sanger sequencing. The primers used in the PCR analysis were as follows: F1, 5'-GACATGAGCTGGCAGAGACAAG-3'; R1, 5'-CAAGGCTCTGGTGGCCTAG-3'; F2, 5'- TGTCATGGCACCAGTCCATG-3'; R2, 5'-GGAGAATTGCTTGAACCTGGCAG-3'; F3, 5'-AATGTGGGAATGTGTAGAGACG-3'; R3, 5'- GCAGATAAGGGAACAAATGGT-3';

2.3 Western blotting

The proteins of the cultured cells and sperm samples were extracted using a universal protein extraction lysis buffer (Bioteke) containing a protease inhibitor cocktail (Roche). Then, denatured proteins were separated on 10% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) for immunoblot analysis. The primary antibodies used were anti-SLC26A8 (1:1000, Atlas antibody) and GAPDH (1:5000, Abcam).

2.4 Immunofluorescence staining

The spermatozoa samples were washed with Sperm Washing Medium (CooperSurgical Inc.) and then fixed onto slides with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 5% BSA. Next, the slides were sequentially incubated with primary antibodies (SLC26A8, 1:50) overnight at 4 °C. The slides were washed in 1× PBS, incubated with DyLight 488- or DyLight 594-labeled secondary antibodies (1:1000, Thermo Fisher) for 1 h at room temperature, and then counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma- Aldrich).

2.5 Electron Microscopy

For scanning electron microscopy (SEM), the sperm cells were fixed onto slides using 4% glutaraldehyde refrigerated overnight at 4°C. After washing the slides with PBS three times, the slides were gradually dehydrated with an ethanol gradient (30%, 50%, 75%, 95%, and 100% ethanol) and dried by a CO_2 critical-point dryer. After metal spraying by an ionic sprayer meter, the samples were observed by SEM (S-3400, Hitachi). For transmission electron microscopy (TEM), the sperm cells were washed three times and fixed routinely. After embedded in Epon 812, ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a TEM (TECNAI G2 F20, Philips) with an accelerating voltage of 80 kV.

RESULTS

3.1 Identification of three novel mutations of *SLC26A8* in infertile patients with teratozoospermia

In our study, three individuals (patient A, patient B, and patient C) were consulted for primary infertility, and then carried out semen analysis (Table 1). The sperm count of three individuals was basically normal, but of a high percent of morphological abnormalities in sperm. Then we performed WES on the three patients to evaluate the potential genetic causes for their sterile phenotype. Consequently, three latently detrimental heterozygous mutations of *SLC26A8* strikingly attracted our attention (Figure 1). The heterozygous frameshift mutations of c.1570_1571del [p.A524*] detected in patient A and c. 306del [p.G103Afs*9] detected in patient C were absent in the general population databases (Table 1). A heterozygous missense mutation of c.2191G>A [p.V731I] was found in patient B and is estimated extremely low allele frequency in public databases (Table 1). Moreover, the site of this missense variant is 100% conserved across several species (Figure S1a).

According to the report of Dirami T *et al.* , the heterozygous alterations of *SLC26A8* contributed to human asthenozoospermia (Dirami et al., 2013). To confirm the patients' phenotype in detail, we collected their sperm samples to carry out the exhausting morphologic examination. We observed serious frequencies of pyriform-head sperm in patient A, and round-head anomalies in sperm from patient B as well as coiled-tail sperm from patient C under Papanicolaou staining (Figure 2a) and SEM further confirmed that sperm of the patients possessed aberrant head or irregular flagella (Figure 2b). Furthermore, irregular ultrastructure either on the head or flagella was also observed in the spermatozoa of the three patients by transmission electron microscopy (TEM) (Figure 2c). The nucleus in most sperm of patient A was irregular, and patient B showed the larger sperm head with unconsolidated chromatin (Figure 2c). And disorganization of mitochondria helices was detected in the sperm flagella of patient C (Figure 2c). Together, the three patients carrying the heterozygous *SLC26A8* mutations showed typical teratozoospermia.

3.2 Heterozygous *SLC26A8*-mutated spermatozoa show normal SLC26A8 expression

To validate the putative contribution of three heterozygous mutations to the infertility of the affected individuals, we investigated these*SLC26A8* mutations via Sanger sequencing in the three families (Figure 1). Surprisingly, two of the harmful mutations (c.1570_1571del [p.A524*] and c.2191G>A [p.V731I]) in two patients were inherited from their unaffected fathers who presented that they possess the normal reproductive capability. With the striking findings noticed, the result that the sterile phenotype associated with teratozoospermia in our patients caused by the three mutations was questionable. Considering the previous observation that decreased expression of SLC26A8 resulting from those three missense mutations detected in the transfected eukaryotic expression vectors (Dirami et al., 2013), we also used eukaryotic expression vectors for each variant and wild-type*SLC26A8* to transiently transfect HEK-293Tcells, respectively. As expected, the western blotting showed that the expression of SLC26A8 protein encoded by c.2191G>A [p.V731I] mutation was significantly decreased when compared to the wild-type SLC26A8 protein (Figure 3a). No SLC26A8 expression was detected in the cells transfected with the vector carrying c.1570_1571del [p.A524*] mutation and c. 306del [p.G103Afs*9] mutation respectively (Figure 2a). Nevertheless, using immunostaining, we confirmed that there was no difference in SLC26A8 amounts in sperm between the three infertile individuals and normal control (Figure 2c). In addition, western blotting results of spermatozoa lysates further confirmed the similar expression of SLC26A8 between patients and normal control (Figure 2b). Thus so, we deduced that no differential expression of SLC26A8 between the patients and normal control might be explained by the compensation of the maintenance of one normal *SLC26A8* copy, although another copy is mutated. Moreover, the*Slc26a8* $^{-/-}$ mouse further proofed that*Slc26a8* participated in spermatogenesis is linked to a recessive-inheritance but not a dominant-inheritance (Touréet et al., 2007; Rode et al., 2012). All findings demonstrated*SLC26A8* mutations in male infertility is a recessive-inheritance, and heterozygous mutations of*SLC26A8* might exhibit a certain degree of determination towards male infertility.

3.3 The special expression pattern of SLC26A8 during spermatogenesis

The previous animal model presented that male *Slc26a8*$^{-/-}$ mice sperm showed lack of motility (Touréet et al., 2007; Rode et al., 2012), while the information of SLC26A8 in spermatogenesis still needs to be explored (Toure et al., 2001). Mouse testicular sections were used for immunofluorescence and the results revealed that SLC26A8 is detectable in the nucleus and cytoplasm of various germ cells and in the flagella of spermatozoa (Figure 3a). Additionally, we evaluated the expression of SLC26A8 in human testis, and the results demonstrated that SLC26A8 was distributed in the cytoplasm of spermatocytes, and in early spermatids, the expression began to drop off (Figure 3b). Moreover, germ cell-typing staining results revealed that SLC26A8 was detectable in the head and cytoplasm of various germ cell types, supporting that SLC26A8 is a major morphogenetic participant in the early spermatogenic process (Figure 3c and Figure S1b). In summary, our results suggest that SLC26A8 may be involved in spermatogenesis and unravels its potential role in regulating sperm morphology.

DISCUSSION

In the present study, we described the monoallelic mutations in*SLC26A8* are not be the causative mutations of male infertility. Especially, although these heterozygous mutations in *SLC26A8* were identified to be all deleterious in transfected cells, immunofluorescence staining and western blotting results depicted the heterozygous mutations didn't impair SLC26A8 expression in the patients' sperm. And two heterozygous mutations were inherited from the fertile fathers. Collectively, we provided evidence that heterozygous mutations in *SLC26A8* were not directly responsible for male infertility.

SLC26A8 has a regulatory effect on cystic fiber transmembrane transduction regulatory factors (CFTR) (Dirami et al., 2013). CFTR is located in the head and mid-flagella of mature sperm and controlled sperm capacitation and motility (Rode et al., 2012). SLC26A8 protein combined with CFTR to form the SLC26A8-CFTR complex regulated the ion flux of sperm and further the movement of sperm (Dirami et al., 2013; El Khouri et al., 2014). As indicated by Dirami T *et al* , the heterozygous mutations of *SLC26A8* were involved in asthenozoospermia (Dirami et al., 2013), which was classified into spermatogenic failure 3 (OMIM 608480) later. Regretfully, the authors did not provide the data about the inheritance of the mutations. However, in our study, it is worth stating the fact that normal fertile males also carried the dangerous heterozygous variants of *SLC26A8* , strongly supporting the heterozygous variants of *SLC26A8* might not be direct etiology for asthenozoospermia. Besides that, no reproductive barriers were performed on the heterozygous *Slc26a8* KO male mice, while the homozygous *Slc26a8* KO male mice occurred in sterility, with completely immotile and malformed spermatozoa. These findings suggest infertility associated with *Slc26a8* mutations was relevant to the autosomal recessive mode of inheritance (Touréet et al., 2007; Rode et al., 2012). What's more, the autosomal dominant inheritance of the heterozygous alterations in *SLC26A8* might be in contrast to the variants previously identified in other members of the *SLC26* family (*SLC26A2, SLC26A3, SLC26A4, SLC26A5*), which follow an autosomal recessive pattern of inheritance (Anwar et al., 2009; Barreda-Bonis et al., 2018; Dawson et al., 2005; Forlino et al., 2005; Höglund et al., 2001; Mutai et al., 2013; Napiontek et al., 2009;). Collectively, we suggested that the biallelic mutations of *SLC26A8* might be the confidential genetic cause for male infertility. Of note, both of our patients and the patients in previous research were harboring the detrimental *SLC26A8* heterozygote, which suggested that the heterozygous mutations of *SLC26A8* might increase the risk of male infertility. Exactly, the heterozygous mutation of the *SLC26A8* is not the main actor but might be a guest player for male infertility. Our findings would provide valuable insights into the molecular mechanism responsible for the male infertility related to *SLC26A8* mutations, which is important for the diagnosis and treatment of male infertility.

In summary, our work unveiled that the *SLC26A8* heterozygous mutations were not the direct causes for asthenozoospermia but may act as a risk factor to male infertility. This report thus corrected the previous work stating that the heterozygous *SLC26A8* mutations led to asthenozoospermia in a dominant inherited manner. Due to the complexity of the spermatogenetic process, the etiological factors of male infertility are mysterious. We thus should be cautious about the gene mutations discovered in patients, and performed more functional experiments to constitute the relationship between the genotype and phenotype, so that we can provide more strong and accurate evidence for clinical diagnosis of male infertility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

WEB RESOURCE

NCBI:*https://www.ncbi.nlm.nih.gov/homologene*

1000 Genomes Project: http://www.internationalgenome.org

ENCODE: https://www.encodeproject.org

ExAC Browser: http://exac.broadinstitute.org

GnomAD: https://gnomad.broadinstitute.org

OMIM: http://www.omim.org

PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/

SIFT: *http://sift.jcvi.org*

The human protein altas: https://www.proteinatlas.org/

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FIGURE LEGENDS

Figure 1. Three heterozygous mutations in *SLC26A8* were identified in three infertile patients. Pedigrees of three families with heterozygous variants in *SLC26A8* . The c.1570_1571del mutation was exhibited by the dotted triangle in family A. The c.2191G>A mutation was exhibited by the dotted box in family B. The c.306del mutation was exhibited by the dotted triangle in family C.

Figure 2. The teratozoospermia phenotype of the three patients.(a) The Concentrations Papanicolaou staining of sperm in the three patients and normal control. The sperm malformation of pyriform heads, round-heads, and coiled-tail were observed in the three patients. (b) Detailed defects in sperm head and flagella were observed in the patients by SEM (scale bars, 5 μm). (c) The anomaly head and flagellum as well as abnormal organized mitochondria of sperm from the three individuals, compared with the normal control by TEM. (scale bars, 100 nm). ODF: outer dense fiber; OD: peripheral microtubule doublets; CP: central microtubules.

Figure 3. The expression analysis of the three heterozygous *SLC26A8* mutations in transfected cells and sperm from the patients. (a) The western blotting analysis of SLC26A8 expression in cells transfected with plasmids carrying variants of p.A524*, p.V731I, and p.G103Afs*9. (b) The SLC26A8 levels

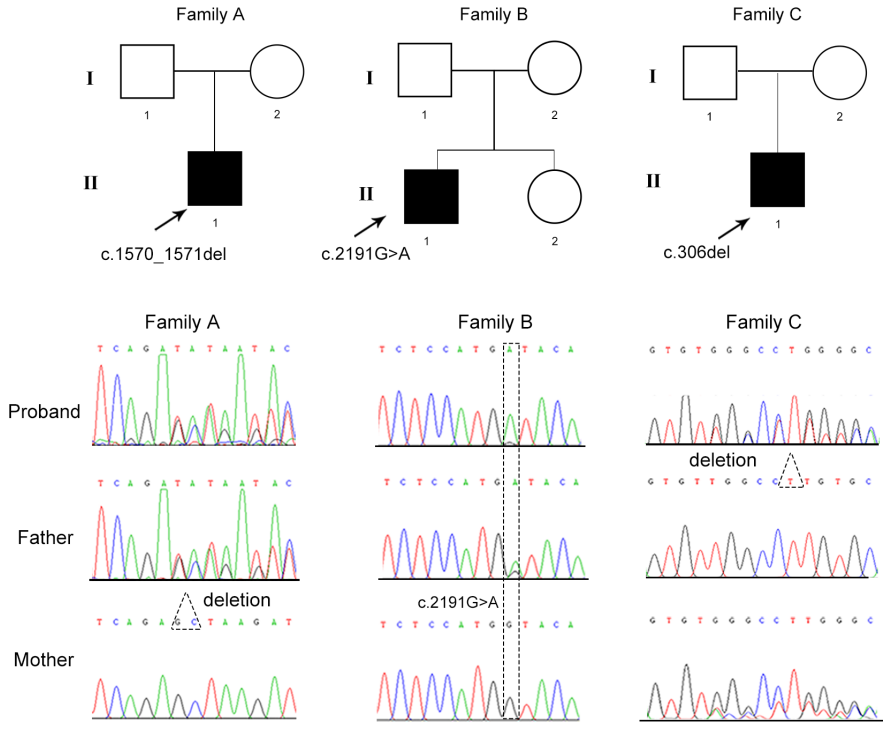
in sperm lysates of patients and control. The SLC26A8 protein was present in the three individuals and normal control without distinction. (c) The immunofluorescence results showed no significant difference in the SLC26A8 expression of the sperm between three individuals and normal control.

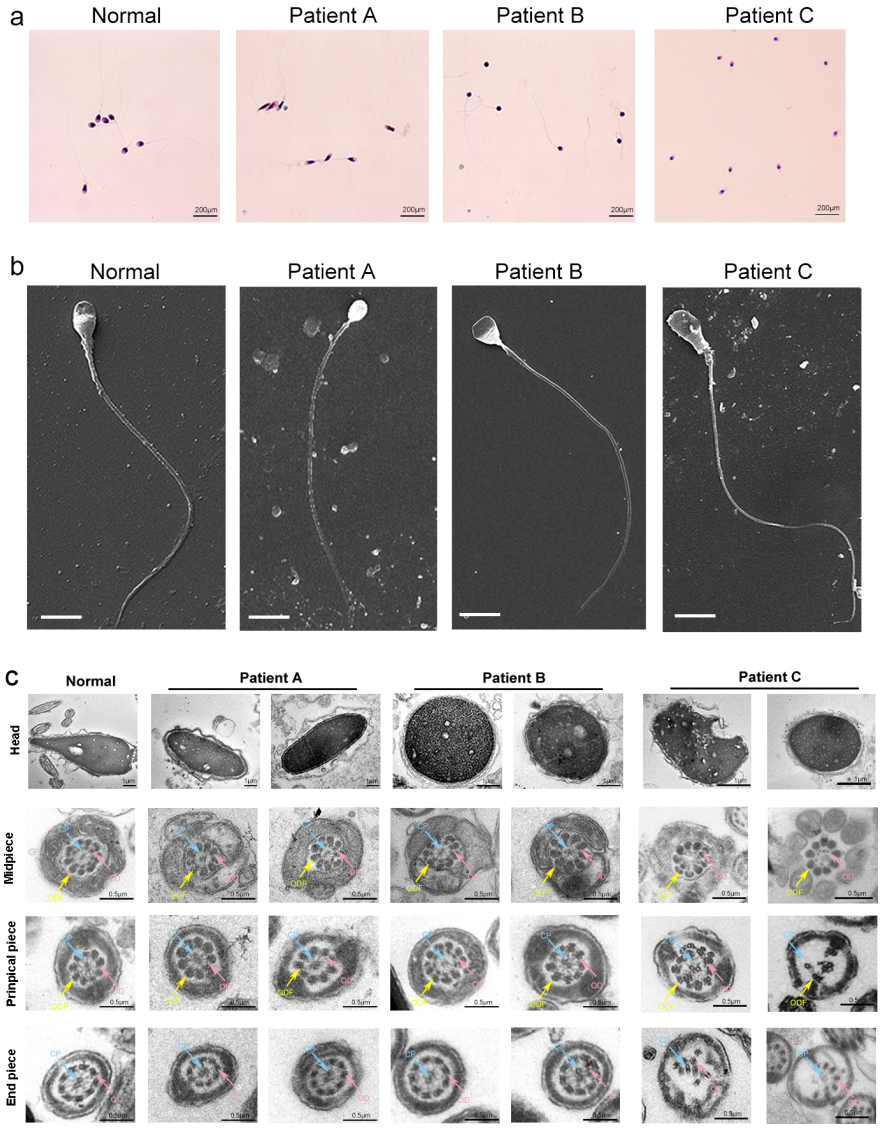
Figure 4. The expression of SLC26A8 in human and mouse testes.(a) Representative images of testicular tubules in a mouse showing that SLC26A8 is principally localized to the cytoplasm of different stages of spermatids (scale bar, 5 μm; red, SLC26A8; blue, DAPI). (b) SLC26A8 was detected in the nucleus and cytoplasm of germ cells at different stages (scale bar, 5 μm; red, SLC26A8; blue, DAPI). (c) Immunofluorescence staining indicated that SLC26A8 was primarily expressed in the head and cilia in human spermatogenic cells (scale bar, 5 μm; red, PNA; green, SLC26A8; blue, DAPI).

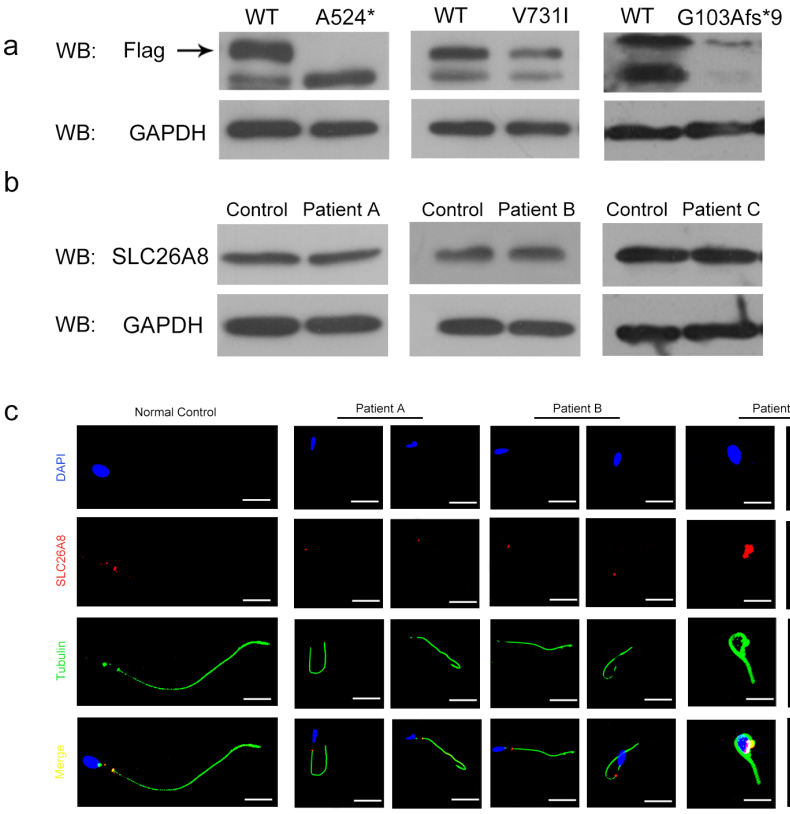
Table 1. Semen and variant analysis in the three patients harboring heterozygous *SLC26A8* mutations.

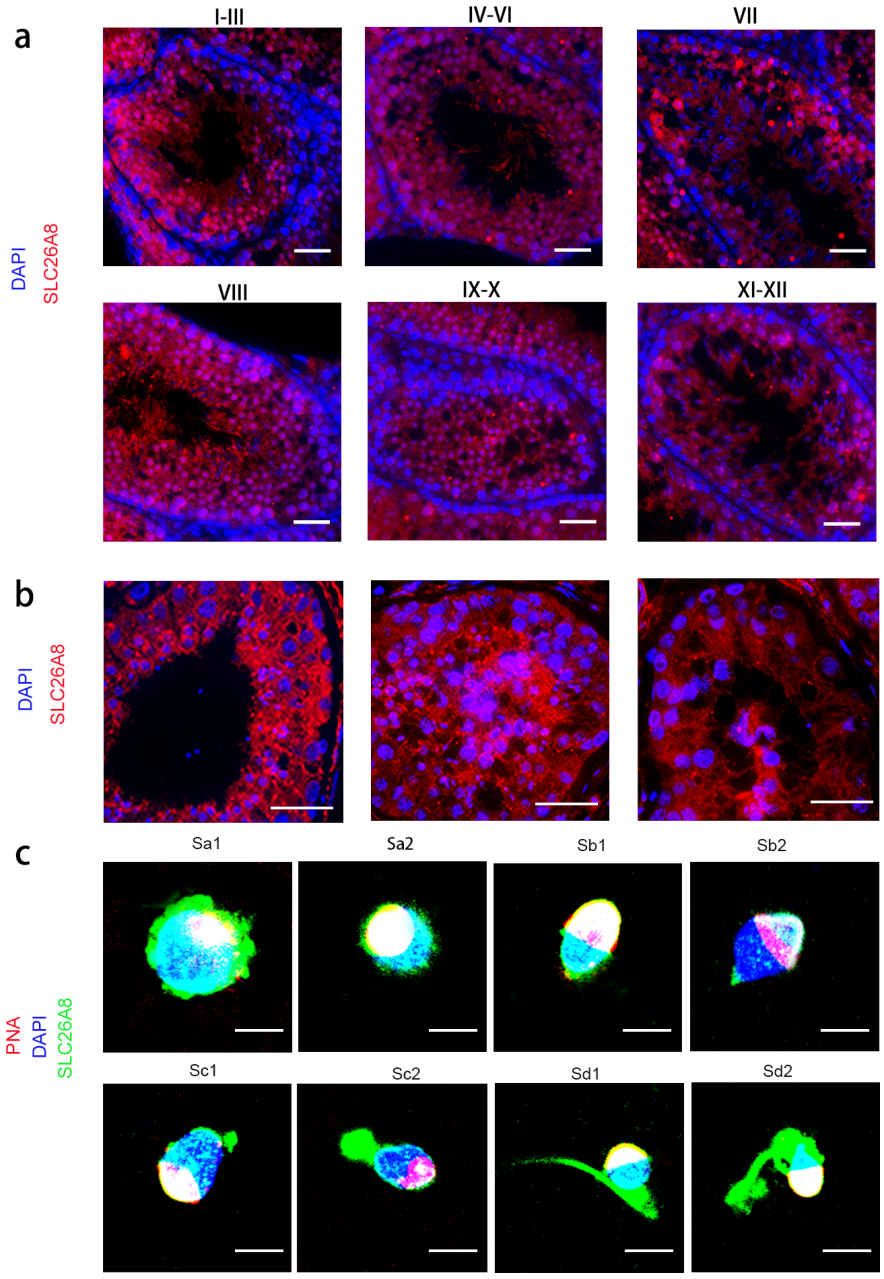
Supplementary Information

Figure S1. (a) Multiple sequence alignment of the SLC26A8 protein among different species. (b) Single-cell sequencing of SLC26A8 expression in human testis (https://www.proteinatlas.org/ENSG00000112053-SLC26A8/celltype).









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