The I510V mutation in KLHL10 in a patient with oligoasthenoteratozoospermia

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Abstract. Oligoasthenoteratozoospermia is a human infertility syndrome caused by defects in spermatogenesis, spermiogenesis, and sperm maturation, and its etiology remains unclear. Kelch-like 10 (KLHL10) is a component of ubiquitin ligase E3 10 (KLHL10) and plays an important role in male fertility. Deletion or mutation of the Klhl10 gene in Drosophila or mice results in defects in spermatogenesis or sperm maturation. However, the molecular mechanisms by which KLHL10 functions remain elusive. In this study, we identified a missense mutation (c.1528A→G, p.I510V) in exon 5 of KLHL10, which is associated with oligoasthenoteratozoospermia in humans. To investigate the effects of this mutation on KLHL10 function and spermatogenesis and/or spermiogenesis, we generated mutant mice duplicating the amino acid conversion using the clustered regularly interspaced palindromic repeat/caspase 9 (CRISPR/Cas9) system and designated them Klhl10I510V mice. However, the Klhl10I510V mice did not exhibit any defects in testis development, spermatogenesis, or sperm motility at ten-weeks-of-age, suggesting that this mutation does not disrupt the KLHL10 function, and may not be the cause of male infertility in the affected individual with oligoasthenoteratozoospermia.

Key words: Clustered regularly interspaced palindromic repeat/caspase 9 (CRISPR/Cas9), KLHL10, Missense mutation, Oligoasthenoteratozoospermia, Spermatogenesis

Infertility is a major health problem worldwide, with a prevalence in developed and developing countries ranging from 3.5% to 16.7%, and male factors account for approximately half of the cases [1, 2]. Disorders in spermatogenesis are the principal causes of male infertility. Spermatogenesis is a process in which spermatogonial stem cells undergo a series of mitosis, meiosis, and morphological changes to form mature sperm, involving more than 2000 genes [3]. In the late stage of spermatogenesis, the process of round spermatozoa forming and growing to sperm for release is called spermiogenesis, including nuclear condensation, acrosome formation, tail formation, and residual body loss [4–6]. Spermiogenesis plays a crucial role in sperm maturation and motility, as well as in final sperm-egg fertilization. To date, dozens of genes have been reported to regulate this process. For example, previous studies have shown that SEPTIN12 regulates the morphogenesis of sperm heads and the elongation of sperm tails, probably implicating its association with α- and β-tubulins [7], and its family is required for the structural integrity and motility of the sperm tail during postmeiotic differentiation [8].

Another study identified a protein located on the plasma membrane of sperm flagellum, CatSper, as a “sperm related cation channel” protein, which plays an important role in sperm motility [9, 10]. However, the mechanisms underlying sperm deformation are still not completely understood.

Ubiquitination plays a key role in eliminating unnecessary proteins in sperm during the middle and late stages of sperm deformation [11, 12]. Kelch-like 10 (KLHL10), characterized by an amino terminal BTB/POZ domain and kelch repeats at the carboxyl terminus, is a member of a large BTB (Bri-a-brac, Tramtrack, and Broad-Complex)-kelch protein superfamily [12]. A previous study showed that male mice with a null mutation in one allele of Klhl10 were infertile, displaying asynchronous spermatid maturation, degeneration of late spermatids, and significant reduction in the number of late spermatids, indicating that normal KLHL10 levels are essential for sperm maturation and male fertility [13]. As a testis-specific protein, KLHL10 is expressed in elongated and elongated sperm. In Drosophila, it interacts functionally and physically with Culi3-testis and Roc1b to form ubiquitin ligase E3 to promote caspase activation and spermatid individualization [14], and specific point mutations altering conserved amino acid residues in the Kelch domain of Klhl10 blocked caspase activation and spermatid individualization, causing excessive sperm death [14, 15]. In addition, a number of point mutations in KLHL10 have been identified in clinical patients with azoospermia or oligoasthenospermia [16, 17]. These studies demonstrated that KLHL10 plays an essential role in spermatogenesis and spermiogenesis. However, the molecular mechanisms by which KLHL10 functions are still largely unknown, except for a model in which KLHL10 complexes with CUL3 act as
a testis-specific ubiquitin E3 ligase, in which KLHL10 is postulated to orientate substrate protein for degradation [12].

In this study, we performed the exome analysis of male infertility-related candidate genes and identified a heterogeneous mutation (c.1528A→G, p.I510V) in exon 5 of the KLHL10 gene in a patient with oligoasthenoteratozoospermia. To explore the clinical and biological significance of this point mutation in male fertility, we duplicated this point mutant in mice; however, the mutant mice did not exhibit any disorder in male fertility at the age of ten weeks.

**Materials and Methods**

**Clinical information**

A couple (both aged 34) suffering from infertility for four years turned to our reproductive center for assistance with reproductive technology. Laboratory examinations showed that the wife had normal hormone levels and regular menstrual cycles, and ultrasonic examinations also indicated normal size and morphology of the uterus, normal follicular development, and ovulation. However, the husband demonstrated oligoasthenoteratozoospermia by sperm quality analysis (Table 1). He had no history of diseases that may affect male fertility and had normal somatic karyotypes. To better diagnose the potential genetic cause, the husband approved to participate in exome analysis of the candidate genes (Table 2) that are known to be associated with male infertility. Informed consent was obtained from the patient to report this case, and the study was approved by the Ethics Committee of Shenzhen Maternity and Child Healthcare Hospital and Southern Medical University.

**Human blood sampling and exome for candidate genes**

Blood samples were obtained from the patient (5 ml taken into ethylenediaminetetraacetic acid (EDTA Tube, 101680720, Improve Medical, Guangzhou, China) by venipuncture) and immediately stored at –80°C until detection. Genomic DNA isolation, polymerase chain reaction (PCR), and subsequent high-throughput sequencing combined with bioinformatics analysis were performed by Yikon Genomics (Shanghai, China) to screen for potential mutations in the male infertility-related genes (Table 2).

**Targeted point mutation of Klhl10 (I510V) in mice using the clustered regularly interspaced palindromic repeat/cas9 system**

*Klhl10* mutant mice (c.1528A→G, p.I510V) were generated by the CRISPR/Cas9 system.

| Table 2. A panel of human male infertility genes |
|------------------------------------------------|
| AR | ARM4 | ARMC4 | AURKC | AZF | BMP15 | CATSPER1 | CCDC103 |
| CCDC114 | CCDC151 | CCDC39 | CCDC40 | DNAFA1 |
| DNAAF2 | DNAH11 | DNAHS | DNAII | DNA12 |
| EIF2B2 | EIF2B4 | EIF2B5 | ERCC6 | FEZF1 |
| FGFR1 | FIGLA | FII | FLRT3 | FMR1 |
| FV | GNRH1 | GNHRH | HFM1 | IL17RD |
| KISS1 | KISS1R | KLHL10 | LHB | LHCGR |
| NANO51 | NOBOX | NRS5AI | POIF1 | POR |
| PSMC3IP | RSPH1 | RSPH4A | SLC26A8 | SPAG1 |
| STAG3 | SYCE1 | SYCP3 | TAC3 | TACR3 |
| TUBB8 | WDR11 | ZMYND10 | ZP1 | ZFY4B |
| AR, Androgen receptor; ARMC4, Armadillo repeat containing 4; AURKC, Aurora kinase C; AZF, Azoozpermia factor 1; BMP15, Bone morphogenetic protein 15; CATSPER1, Cation channel, sperm associated 1; CCDC103, Coiled-coil domain containing 103; CCDC114, Coiled-coil domain containing 114; CCDC151, Coiled-coil domain containing 151; CCDC39, Coiled-coil domain containing 39; CCDC40, Coiled-coil domain containing 40; CFTR, Cystic fibrosis transmembrane conductance regulator; CHD7, Chromodomain helicase DNA binding protein 7; DNAFA1, Dynin, axonomal assembly factor 1; DNAAF2, Dynin, axonomal assembly factor 2; DNAH11, Dynin, axonomal, heavy chain 11; DNAH5, Dynin, axonomal, heavy chain 5; DNAII, Dynin intermediate chain 1; AXN; DNA12, Dynin intermediate chain 2, axonomal; DPY19L2, Probable C-mannosyltransferase DYPY19L2; DUSP6, Dual specificity protein phosphatase 6; EIF2B2, Translation initiation factor eIF-2B subunit beta; EIF2B2, Translation initiation factor eIF-2B subunit beta; EIF2B5, Translation initiation factor eIF-2B subunit epsilon; ERCC6, DNA excision repair protein ERCC-6; FEZF1, Fez family zinc finger protein 1; FGFI7, Fibroblast growth factor 17; FGFI8, Fibroblast growth factor 8; FGFR1, Fibroblast growth factor receptor 1; FIGLA, Factor in the germline alpha; FII, Coagulation factor II; FLRT3, Leucine-rich repeat transmembrane protein FLRT3; FMR1, Synaptic functional regulator FMR1; FOXL2, Forkhead box protein L2; FSHR, Follicle-stimulating hormone receptor; FV, Friend virus susceptibility; GNRH1, Progonadotrophin-1; GNRHR, Gonadotropin-releasing hormone receptor; HFM1, Probable ATP-dependent DNA helicase HFM1; IL17RD, Interleukin-17 receptor D; INSL3, Insulin-like 3; KAL1, Anosmin-1; KISS1, Metastasis-suppressor KiSS-1; KISS1R, KiSS-1 receptor; KLHL10, Kelch-like protein 10; LHB, Lutropin subunit beta; LHCGR, Lutropin-choriogonadotrophic hormone receptor; LRRc6, Protein tIB-hormone; MCM8, DNA helicase MCM8; NANO51, Nanos homolog 1; NOBOX, Homeobox protein NOBOX; NRS5AI, Steroidogenic factor 1; POIF1, Protein POIF1; POR, NADPH-chorochrome P450 reductase; PRO2K, Prokinetin-2; PROK2, Prokinetin receptor 2; PSMC3IP, Homologous-pairing protein 2 homolog; RSPH1, Radial spoke head 1 homolog; RSPH4A, Radial spoke head protein 4 homolog A; SLC26A8, Testis anion transporter 1; SPAG1, Sperm-associated antigen 1; SPATA16, Spermatogenesis-associated protein 16; SPRY4, Protein sprouty homolog 4; STAG3, Cohesin subunit SA-3; SYCE1, Synaptonemal complex central element protein 1; SYCP3, Synaptonemal complex protein 3; TAC3, Tachykinin-3; TACR3, Neumedin-K receptor; TAF4B, Transcription initiation factor TFID subunit 4B; TEX11, Testis-expressed protein 11; TUBB8, Tubulin beta-8 chain; WDR11, WD repeat-containing protein 1; ZMYND10, Zinc finger MYND domain-containing protein 10; ZP1, Zona pellucida sperm-binding protein 1; GAS1, Dynin regulatory complex subunit 4.
Shanghai Model Organisms (Shanghai, China), using CRISPR/Cas9 technology to introduce the target point mutations by homologous recombination repair. Briefly, the Cas9 mRNA and guide RNA were obtained by in vitro transcription, oligo donor DNA was obtained by synthesis, and cas9 mRNA, gRNA, and donor DNA were microinjected into one-cell stage fertilized eggs of C57BL/6J mice (Shanghai Model Organisms, Shanghai, China) to obtain F0 generation mice. A total of 225 eggs were injected, and seven chimeric F0 mice were identified by two-round genotyping by PCR and subsequent sequencing. F1 mice were obtained by mating F0 mice with C57BL/6J mice, followed by mating F1 mice to generate homozygous F2 generation. DNA was isolated from tail biopsies and used to genotype the mice, followed by PCR product sequencing (Sanger sequencing, Sangon, Shanghai, China). The gRNA sequence was 5′-TACGAGGGTTAAACATAGTG-3′. The primer sequences for genotyping were as follows: forward, 5′-ACCTGTACCTGTCTTCTGTCTA-3′, reverse: 5′-ATCCCCTTCCTTACTCCCTCATT-3′. The forward primer was also used as the sequencing primer. Male mice with complete amino acid substitutions were designated as Klhl10\textsuperscript{I510V} mice, and littermate wild-type (WT) males were used as controls and were designated as Klhl10\textsuperscript{WT} mice. All mice were housed in a clean, temperature-controlled environment with a 12-h light/dark cycle, and were given free access to regulate laboratory chow diet and water for housing in regular cages.

All the animal experiments were approved by the Southern Medical University Committee on the Use and Care of Animals, and were performed in accordance with the Committee’s guidelines and regulations.

**Tissue collection and histological analysis**

Following euthanasia, the testes and epididymides of mice were removed, weighed, fixed in modified Davidson fixative, and processed in paraffin according to standard methods. At least three sections from each testis and epididymis (5 μm, taken 100 μm apart) were stained with hematoxylin and eosin (H&E) for regular histological examination.

**Sperm concentration and motility analysis**

Human sperms were collected from the patient via masturbation and incubated at 37°C for 20 min for liquefaction. For mice, one epididymis from each mouse was removed and minced in M2 medium (0.5 ml; M7167-100ML, Sigma-Aldrich, Shanghai, China) at 37°C for 15 min to allow the sperm to be released into the medium. Sperm concentration was assessed with a hemocytometer, and sperm motility was analyzed using a microscope and CASA (Hamilton Thorne IVOS software, Hamilton Thorne, Beverly, MA, USA) under parameters for humans and mice, respectively.

**Statistical analysis**

All experiments were performed in triplicate. Data were expressed as the mean values ± standard deviation (SD), and the between-group differences were analyzed using the t-test for measurable data and the Chi-square test for percentage data (SPSS 13.0). Statistical significance was set at P < 0.05.

**Result**

**Identification of a missense mutation in KLHL10 in a patient with oligoasthenoteratozoospermia**

Sperm quality analysis showed that the husband exhibited markedly decreased sperm concentration and motility and 100% sperm with abnormal morphology (Table 1). H&E staining of the smeared sperm further confirmed the abnormal morphology in sperm, characterized by round or irregular head, and/or short tail (Fig. 1), indicative of oligoasthenoteratozoospermia in the patient. To explore the potential genetic cause for this affected individual, we performed exome combined with bioinformatics analysis on a panel of 75 candidate genes [18–20], which have been demonstrated to be strongly associated with male infertility (Table 2). Interestingly, we identified a heterogeneous point mutation at nucleotide 1528(A→G) (located in exon 5) in KLHL10, according to GenBank entry NM_152467. The transition generated a missense amino acid substitution, I510V, located in the kelch-5 domain of the KLHL10 protein (Table 3; Fig. 2A). However, due to the patient’s family reasons, it was impossible to trace the hereditary mode. Point mutations in the human KLHL10 gene have been reported in the Genome Aggregation Database (gnomAD) in 0.015% of the normal East Asian individuals tested. However, we hypothesized that this may be the cause of oligoasthenoteratozoospermia in the affected individual.

**Generation of Klhl10 mutant mice duplicating the missense mutation (c.1528A→G, p.I510V) by CRISPR/Cas9**

We then blasted the amino acid sequences of KLHL10 among the species and found that the amino acid sequences adjacent to the mutant

Fig. 1. Morphology of sperm via hematoxylin and eosin (H&E) staining. (A) Normal human sperm; (B) Representative images showing the irregular sperm head or tail in the patient. Scale bars: 10 μm for (A) and 5 μm for (B).
site were highly conserved among species. Thus, to elucidate the effect of this missense mutation on the function of KLHL10 as well as on spermatogenesis in vivo, we duplicated the mutation (c.1528A→G, p.I510V) in mice using CRISPR/Cas9 (Fig. 2B). By breeding the F1 mice, followed by PCR and sequencing, we obtained homozygous Klhl10 I510V mutant mice and their littermate controls (Klhl10 WT) (Fig. 2C–E). These data indicated the successful generation of Klhl10 mutant mice with target point mutations (1528A→G, I510V), and this point mutation did not cause any splicing abnormality in Klhl10.

Klhl10 I510V mice do not exhibit any defects in testis development, spermatogenesis, or sperm motility at ten-weeks-of-age

The Klhl10 I510V mice had no obvious difference in physical appearance or body weight at any age during the experiments, compared with their control littermates (data not shown). Similarly, there was no significant difference in testis weight between the Klhl10 I510V and Klhl10 WT males at ten-weeks-of-age (Fig. 3A). Further morphological examination showed that the Klhl10 I510V and Klhl10 WT males had indistinguishable seminiferous tubular development, manifested as comparable tubular size, seminiferous epithelium thickness, and cell architecture (Fig. 3B). These observations indicated that the I510V transversion in Klhl10 may not disturb tubular or testis development in mice. Accordingly, the Klhl10 I510V and Klhl10 WT males had parallel sperm concentrations (Fig. 3C). In line with the observations of the seminiferous epithelium, the Klhl10 I510V males exhibited normal spermatogenesis (Fig. 3D). Moreover, the Klhl10 I510V mice also showed normal sperm motility and morphology (Figs. 3E and F), and had similar litter sizes compared with Klhl10 WT mice (Fig. 3G). Taken together, these results suggest that the I510V substitution in Klhl10 does not impair testis development, spermatogenesis, or sperm motility in mice at ten-weeks-of-age.

### Discussion

Spermatogenesis encompasses a complex network of processes that occur in the seminiferous tubules and culminates in the production of mature male gamete [21]. Thus, spermatogenesis is strictly controlled and requires tight coordination of numerous genes. There are dozens of genes with abnormal expression levels or mutations in humans or mice have been reported to cause male reproductive disorders. Therefore, screening potential mutations of candidate genes in the pedigree of patients with reproductive disorders is important, not only for the etiologic and prenatal diagnosis, but also for the methods used for assisted reproductive technology. In this study, we screened potential mutation(s) in 75 candidate genes in a patient with oligoasthenoteratozoospermia and identified a heterogeneous KLHL10 gene at nucleotide 1528 (A→G) (located in exon 5), which produces a missense amino acid substitution (I510V) located in

| Table 3. Mutation information of the affected individual |
|---------------------------------|
| Gene | Chromosome position | Transcript | Nucleotide change | Amino acid change | Zygote type |
| KLHL10 | chr17: | NM_152467 | c.1528A>G | p.I510V | Heterozygous |
| 40004260 |
Fig. 3. The I510V substitution in Klhl10 does not impair spermatogenesis or sperm motility in mice at ten-weeks-of-age. (A) Similar Klhl10WT and Klhl10I510V testis weight. Data are presented as the mean ± standard deviation (SD), n = 5. (B) Testes morphology by H&E staining. (C) No significantly reduced epididymal sperm (intact spermatozoa plus sperm flagella) concentration in Klhl10I510V mice compared with wild-type (WT) males. Data are presented as the mean ± SD, n = 5. (D) Epididymis tubular morphology by H&E staining. (E) No significantly reduced total motility of Klhl10I510V epididymal headless spermatozoa. Data are presented as the mean ± SD, n = 5. (F) Detailed morphology of the smeared sperm by H&E staining. (G) Analysis of litter size. Comparison of Klhl10WT with Klhl10I510V mice, where no significant difference in the average number of pups per litter. Data are presented as the mean ± SD, n = 15. Scale bars: 50 µm for (B) and (D) 20 µm for (F). Scale bars: 50 µm for (B) and (D) 20 µm for (G).
the kelch-5 domain of KLHL10 protein. Thus, we speculated that this missense mutation in exon 5 of KLHL10 is associated with olo gaonethetoratoozpspermia in humans.

As a testis-specific gene, KLHL10 is expressed in elongating and elongated sperm, and has been demonstrated to be essential for male fertility as hamponsufficiency of Khl10 gene in mice disrupted spermatogenesis and caused male infertility [13]. Subsequent studies showed that deletion or mutations of Khi10 resulted in male infertility in Drosophila [14, 15], and mechanistically, Khi10 regulated spermatogenesis via ubiquitation-mediated target protein degradation [12, 14, 15, 22]. In addition, a high frequency of mutations in KHL10 was also identified in infertile men with azospermia or olo ozoo spermia [16, 17]. Therefore, KHL10 plays a critical role in spermatogenesis and male fertility.

It has been well evidenced that KHL10 and CUL3 form a complex, in which KHL10, specifically, its six kelch repeats, acts to recognize substrate proteins for ubiquitination-dependent degradation during sperm maturation [14, 15, 22]. In the present study, we identified a missense mutation in an infertile male with olo gaonethetoratoozpspermia, which produces an I510V substitution located in the Kelch 5 domain in the KHL10 protein. However, the mutant mice harboring such an amino acid transversion failed to replicate the infertile phenotypes in the patient, without any defects observed in testis development, spermatogenesis, or sperm maturation. These observations are contradictory to a previous study in Drosophila showing that five single mutations located in each kelch domain in Drosophila Khi10 protein blocked spermatid individualization [14]. We hypothesized that this discrepancy may be due to species differences. Another explanation is that the remaining intact five-kelch repeats may compensate for the inactivation of kelch 5, or the I510V conversion was not sufficient to disrupt the function of kelch 5 in mice. Previously, an A313T mutation located in Kelch 1 of KHL10 identified in an oligozoospermic man was found to fail to form a homodimer via a yeast two-hybrid assay; however, the significance of this homodimer formation for KHL10 function was not illustrated in that study [17]. In addition, as both Ile and Val are hydrophobic-aliphatic amino acids, the substitution of Ile with Val may have little effect on protein conformation. Interestingly, chicken KHL10 has V508, which corresponds to I510 of human KHL10 and mouse Khi10. Thus, in light of evolution, replacement of Ile/Val at this residue seems to be acceptable for the function of KHL10 protein. The last explanation is that there may be other genes that are not included in the panel of 75 candidate genes that are responsible for infertility in the affected individual. Our study suggests that generating a mouse model mimicking gene mutations in patients is an important step in evaluating the significance of variants associated with human diseases. Nevertheless, our report does not deny the importance of KHL10 in male fertility, but rather calls for further in-depth studies to unveil the mechanism by which KHL10 recognizes the substrate proteins for degradation as well as its targets.

Conflict of interests: All the authors have no conflicts of interest.

Acknowledgments

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