Non-invasive genetic diagnosis of male infertility using spermatozoal RNA: \textit{KLHL10} mutations in oligozoospermic patients impair homodimerization

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Infertility affects an estimated 7% of men worldwide, nearly a quarter of whom are diagnosed as idiopathic. The genetic etiologies of idiopathic male infertility are unknown, partly due to lack of simple diagnostic techniques. Moreover, the transmission risk of such genetic defects to offspring born from assisted reproductive techniques is increasingly becoming a concern for physicians and infertile couples. We explored the feasibility of obtaining full-length mRNAs from transcriptionally inert human spermatozoa in semen as a non-invasive diagnostic tool for identifying germline mutations in candidate infertility-associated genes. The efficacy of reverse-transcription PCR on spermatozoal RNA from infertile patients with wide-ranging sperm concentrations varied between 91 and 99% for multiple haploid germ cell-expressed genes. Using this methodology, we identified seven oligozoospermic patients with missense and splicing mutations in the germ cell-specific gene, \textit{KLHL10}. Three of 270 (1.1%) severely oligozoospermic patients (<10^6 sperm/ml) harbor \textit{KLHL10} alterations that were absent in 394 controls and exhibited significant association ($P \leq 0.02$). Two \textit{KLHL10} missense mutations (A313T and Q216P) resulted in impaired homodimerization with the wild-type protein in yeast interaction assays, suggesting a functional deficiency. This study demonstrates the utility of this approach for analysis of haploid germ cell-expressed genes regulating post-meiotic events including sperm maturation, motility and fertilization. The development of non-invasive techniques to analyze genetic defects of human spermatogenesis, previously possible only with invasive testis biopsies, provides important diagnostic and therapeutic implications for reproductive medicine.

## INTRODUCTION

Infertility is a major health problem of multifactorial etiology, with an estimated world-wide prevalence of 15% (1). In ~50% of infertile couples, a male factor etiology is demonstrable (2), based upon abnormal semen characteristics (i.e. sperm concentration, motility, morphology, etc.). Although sperm concentrations between fertile and subfertile populations overlap extensively (3), oligozoospermia is the most common semen abnormality in infertile males (4). Male infertility has a genetic basis, as evidenced by numerous mouse models created by targeted gene disruptions (reviewed in 1) as well as by association studies designed to detect familial segregation of subfertility (5); however, ~25% of infertile males are diagnosed as idiopathic, reflecting our poor understanding of the basic mechanisms regulating spermatogenesis and sperm function and hence our inability to properly diagnose the etiology.

Unlike other genetic disorders, linkage analyses of genes associated with infertility has been hindered by the absence of large affected pedigrees (6). In addition, the contribution of each locus to the overall disease load in complex disorders is believed to be modest, making linkage an ineffective tool. Detecting an association between male infertility and single
loci defects through candidate-gene approaches is moderately successful; however, for larger scale studies of the association of multiple susceptibility genes with infertility, an efficient and cost-effective screening strategy is necessary. In addition, oligozoospermic and normozoospermic infertile men are never candidates for invasive testis biopsies; accordingly, genetic investigation of these men must rely on somatic DNA screening, precluding the identification of de novo germline defects.

By using germline RNA from human semen samples, we overcame these limitations. Although transcription is repressed in mature sperm, many mRNAs expressed during meiosis and a population of microRNAs persist in human ejaculate spermatozoa (7–11) and are stable up through fertilization (12). Spermatozoal RNAs are proposed to provide an imprint of testicular gene expression based on microarray experiments on fertile donors (10). Herein, we describe a method to use mRNA transcripts from mature sperm in the semen ejaculate for a novel diagnostic strategy. We show that multiple full-length spermatozoal mRNAs that encode candidate infertility-associated proteins can be efficiently screened for mutations by reverse transcriptase–polymerase chain reaction (RT-PCR) and demonstrate the utility of this approach to diagnose unrecognized genetic defects in severely oligozoospermic men. Moreover, as this study demonstrates, limitations of conventional genomic DNA-based screening, such as the inability to detect splicing defects in vivo, can also be overcome by using an RNA-based screen, thereby expanding the spectrum of mutations identifiable.

To test the utility of our non-invasive strategy for the diagnosis of infertile men, we selected the human KLHL10 gene to screen for mutations in oligozoospermic patients. The mouse ortholog is an essential gene for spermiogenesis, which acts in a dosage-sensitive manner; we have shown that heterozygosity for a null mutation in Klhl10 is characterized by germ cell loss and defective morphology of spermatids (13). Defective Klhl10 represents one of the few known models of autosomal dominant male infertility. In this study, we demonstrate that analysis of sperm RNA reveals novel mutations in KLHL10 associated with reduced sperm count in infertile men.

RESULTS

Efficient amplification of post-meiotic germ cell-expressed genes from spermatozoal RNA

In brief and as shown in Figure 1, our non-invasive screening strategy to identify mutations in male infertility-associated genes involves the following steps: (i) isolation of total RNA and genomic DNA from spermatozoa and germ cells in semen; (ii) immediate reverse-transcription of the RNA to cDNA; (iii) gene-specific PCR of each open reading frame (ORF) and direct sequencing. We hypothesized that mRNAs for genes expressed in haploid germ cells could be detected in ejaculated spermatozoa from patients with either reduced or normal sperm counts. To test the efficiency of our protocol, we amplified seven genes known to be pre- and/or post-meiotically expressed in germ cells. All were previously characterized using mouse models (Table 1, Fig. 2), including glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (GAPDHS), Golgi-associated PDZ and coiled-coil motif containing protein (GOPC), izumo sperm–egg fusion 1 (IZUMO1), kelch-like 10 (KLHL10), sperm mitochondria-associated cysteine-rich protein (SMCP) and serine/threonine/tyrosine interacting protein (STYX). In addition, we amplified human cyclin A1 (CCNA1), a testis-specific gene expressed in pre-meiotic pachytene spermatocytes (14,15). Two groups of 48 cDNA samples (n = 96) prepared from randomly selected men undergoing evaluation for infertility were chosen as a representative population for amplification of all seven genes. We efficiently amplified the full-length ORFs representing all six haploid-expressed genes in 90% of the patients (Table 1); significantly, the pre-meiotically expressed gene, CCNA1, was amplified, albeit at a lower efficiency, in 73% of patients (Table 1), suggesting that our methodology was highly sensitive and robust.

For optimal amplification across samples, fragments spanning 600–700 bp of the cDNA yielded the highest efficiency and quality of amplification as a function of amplicon size, with the efficiency decreasing to 77–80% when amplicons >750 bp were attempted (data not shown). This is also an optimal size for bidirectional sequencing. These results indicate that genes with an ORF of ~700 bp can be screened...
Table 1. RT–PCR amplification efficiency of candidate infertility-associated germ cell-expressed genes from infertile male patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant phenotype in mouse (References)</th>
<th>Exons</th>
<th>ORF (bp)</th>
<th>RT–PCR amplicons (size in bp)</th>
<th>Amplification efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDHs</td>
<td>Asthenozoospermia (36)</td>
<td>11</td>
<td>1230</td>
<td>3 (315/675/660)</td>
<td>97</td>
</tr>
<tr>
<td>GOPC</td>
<td>Globozoospermia (37)</td>
<td>9</td>
<td>1392</td>
<td>2 (727/775)</td>
<td>91</td>
</tr>
<tr>
<td>IZUMO1</td>
<td>Sperm penetration defect (38)</td>
<td>10</td>
<td>1056</td>
<td>2 (650/611)</td>
<td>91</td>
</tr>
<tr>
<td>KLHL10</td>
<td>Azoospermia in HPC (13)</td>
<td>5</td>
<td>1948</td>
<td>3 (667/673/680)</td>
<td>95</td>
</tr>
<tr>
<td>SMCP</td>
<td>Asthenozoospermia (39)</td>
<td>2</td>
<td>354</td>
<td>1 (600)</td>
<td>99</td>
</tr>
<tr>
<td>STIX</td>
<td>Oligoasthenoteratozoospermia (40)</td>
<td>11</td>
<td>675</td>
<td>1 (837)</td>
<td>97</td>
</tr>
<tr>
<td>CCNA1</td>
<td>Meiotic arrest (41)</td>
<td>9</td>
<td>1401</td>
<td>2 (758/820)</td>
<td>73</td>
</tr>
</tbody>
</table>

NS, normozoospermia; OS, oligozoospermia, n = 96; HPC, high percentage chimera.

Application of this technology to detect mutations in KLHL10 in oligozoospermic patients

We applied this basic screening strategy to detect an association between mutations in candidate genes and oligozoospermia in infertile males. For complex traits, the gene candidates must exhibit biological plausibility for the given phenotype. To test for the biological utility of our approach, we sequenced the entire intron 3 and exons 3–5 of this patient. Direct sequence analysis revealed that seven out of 556 (1.3%) patients have heterozygous alterations in both mRNA and genomic DNA in exons 2 and 3 of KLHL10 (Fig. 3). Four patients (DL57488, DL58175, DL60143 and DL60322) with oligozoospermia were found to have a missense mutation 647A>C (Q216P) that substitutes a highly conserved glutamine for a proline in the BACK domain of the protein (Fig. 3). Two patients (DL60949 and DL61463) with severe oligozoospermia have another missense mutation 937G>A (A313T) that results in the substitution of a highly conserved alanine for threonine within the first kelch repeat motif of the protein (Fig. 3). We genotyped 394 patient controls who had completely normal sperm counts for these alleles. None of the 394 controls had the A313T substitution and only one out of 394 harbored the Q216P substitution.

One severely oligozoospermic patient (DL59031) had a splicing defect in the cDNA amplicon that represents exons 3–5, resulting in a frameshift. Although cDNA amplicons encompassing exons 1–3 could be normally amplified from this patient, amplicon 3 representing exons 3–5 was aberrantly spliced due to a cryptic splice site activation in intron 3. This resulted in partial retention of intron 3 in the mature mRNA, but skipping of exon 4 and part of exon 5. The aberrant transcript had a termination codon immediately following exon 3, which theoretically leads to a truncation of the KLHL10 protein at the end of kelch repeat 3 (Fig. 3). To identify a potential splice site mutation in this patient, we sequenced the entire intron 3 and exons 3–5 of this patient. We identified a 4 bp heterozygous deletion in intron 3 (IVS3_121delTCTT), located 121 bp downstream of the exon–intron junction (Fig. 3).
Figure 3. KLHL10 mutations identified in oligozoospermic patients. (A) The position of missense mutations in KLHL10. Sequence chromatograms representing missense mutations Q216P (Gln216Pro) in the BACK domain and A313T (Ala313Thr) in kelch repeat 1 and their evolutionary conservation among mammalian orthologs are shown. The mutant alleles are shaded in gray. (B) The splicing defect associated with the IVS3_121delTCTT was observed in DL59031. The altered splice product shows partial retention of intron 3 (in yellow) and skipping of exons 4 and part of 5 (in gray) and truncation of the protein product (see Results). The chromatogram depicts a 4 bp TCTT deletion (framed) resulting in a frame shift. Hs, Homo sapiens; Mf, Macaca fascicularis; Bt, Bos taurus; Cf, Canis familiaris; Rn, Rattus norvegicus; Mm, Mus musculus.

Table 2. Genotype–phenotype correlation for mutations in KLHL10

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (years)</th>
<th>Sperm concentration (×10^6/ml)a</th>
<th>Motile %</th>
<th>Morphology (% normal)b</th>
<th>Clinical category</th>
<th>Genomic mutation</th>
<th>cDNA mutation</th>
<th>Protein alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL59031</td>
<td>53</td>
<td>1.7 ± 0.2</td>
<td>25</td>
<td>2.0</td>
<td>Severe OS</td>
<td>IVS3_121delTCTT</td>
<td>Splicing</td>
<td>Frameshift</td>
</tr>
<tr>
<td>DL61463</td>
<td>37</td>
<td>4.5 ± 1.2</td>
<td>45</td>
<td>2.5</td>
<td>Severe OS</td>
<td>Exon 3 937G&gt;A</td>
<td>937G&gt;A</td>
<td>A313T</td>
</tr>
<tr>
<td>DL60949</td>
<td>52</td>
<td>5.5 ± 0.9</td>
<td>30</td>
<td>0.0</td>
<td>Severe OS</td>
<td>Exon 3 937G&gt;A</td>
<td>937G&gt;A</td>
<td>A313T</td>
</tr>
<tr>
<td>DL60143</td>
<td>48</td>
<td>16.0 ± 5.8</td>
<td>60</td>
<td>4.5</td>
<td>OS</td>
<td>Exon 2 647A&gt;C</td>
<td>647A&gt;C</td>
<td>Q216P</td>
</tr>
<tr>
<td>DL58175*</td>
<td>35</td>
<td>21</td>
<td>65</td>
<td>ND</td>
<td>OS</td>
<td>Exon 2 647A&gt;C</td>
<td>647A&gt;C</td>
<td>Q216P</td>
</tr>
<tr>
<td>DL60322*</td>
<td>53</td>
<td>26</td>
<td>40</td>
<td>ND</td>
<td>OS</td>
<td>Exon 2 647A&gt;C</td>
<td>647A&gt;C</td>
<td>Q216P</td>
</tr>
<tr>
<td>DL57488*</td>
<td>41</td>
<td>31</td>
<td>55</td>
<td>ND</td>
<td>OS</td>
<td>Exon 2 647A&gt;C</td>
<td>647A&gt;C</td>
<td>Q216P</td>
</tr>
</tbody>
</table>

IVS, intronic variant sequence; ND, not determined.

aValues represent single actual reading closest to the mean ± SEM.

bKruger’s strict criteria.

*Single observation.
extracted from both spermatozoa and peripheral blood, indicating that the deletion was inherited. None of our 394 controls harbored this intronic deletion. Interestingly, the 4 bp intronic deletion (TCTT) is in the middle of a stretch of polypyrimidine residues, which binds the ubiquitous eukaryotic splice regulator polypyrimidine tract-binding protein (PTB). PTB acts through binding to intronic splice silencer elements to negatively regulate splicing and exon definition (17). Therefore, this finding implies that the intronic deletion leads to loss of PTB binding and results in derepression of a cryptic splice site in intron 3, ultimately leading to the aberrant splicing pattern in this patient.

To test our hypothesis that mutant KLHL10 alleles are associated with a reduced sperm count, we first analyzed the ethnic composition of our patients and controls (see Materials and Methods). Two oligozoospermic patients (DL58175 and DL60143) with the Q216P alteration belong to the Y chromosome ‘E’ haplogroup (18). All three severely oligozoospermic cases (DL59031, DL60949 and DL61463) and the remaining two oligozoospermic cases (DL57488 and DL60322) belong to the Y chromosome ‘R1’ haplogroup. Further analyses revealed that 130/270 severely oligozoospermic patients and 175/394 controls belong to the R1 haplogroup. This suggests a significant association of KLHL10 alleles (3/130) with severe oligozoospermia (likelihood-ratio chi-squared test, $G = 5.1$, $P = 0.02$). When matched for the R1 Y haplogroup, the Q216P allele (2/108 in cases; 1/175 in controls) did not show significant association with oligozoospermia; however, this mutation was found in two unrelated haplogroups that are predominant in two divergent ethnic groups, implying that the mutation is not a rare variant with a founder effect. In addition, a novel non-synonymous SNP (886T > C), which causes an I296T substitution, was found at a similar frequency in oligozoospermic men (5/556; 0.9%) and controls (5/394; 1.3%); this SNP probably represents a polymorphic allele.

The A313T and Q216P mutations adversely affect the homodimerization of KLHL10

To test whether the missense alleles cause any functional deficiencies in the protein, we performed a series of interaction experiments in yeast. Since the N-terminal BTB domain of Drosophila kelch is involved in homodimerization of the protein (19), we first investigated whether human KLHL10 could form homodimers. When yeast bait and prey vectors carrying wild-type (WT) KLHL10 were mated, robust growth of blue colonies were obtained within 48 h on SD-Ade/-His/-Leu/-Trp/X-gal selective plates, indicating that human KLHL10 dimerizes with itself in vivo (Fig. 4A). WT human KLHL10 also interacted strongly with mouse CUL3, similar to the interaction described for mouse KLHL10 (16), but not with human LAMIN A/C (Fig. 4A) or with mouse p53 (data not shown), indicating that the interactions were specific. However, when the WT human KLHL10 protein, either as a bait or as a prey, was tested for interaction with the mutant KLHL10 proteins (A313T and Q216P), no growth of yeast colonies could be seen even after 8 days (Fig. 4A), indicating that the mutant proteins interact very weakly with the WT protein.

To further define the nature of the interactions, we performed a fluorometric assay to test for yeast two-hybrid interactions between the WT and the mutant KLHL10 proteins. Confirming our results from the selective plates, WT KLHL10 was found to interact strongly with itself and with CUL3, as reflected by a strong increase in fluorescence intensity (see Materials and Methods) after 24 h of culture (Fig. 4B); the mutant KLHL10 proteins show a drastically weakened interaction with the WT protein, as reflected by a nominal increase in fold fluorescence ($P < 0.001$; ANOVA), suggesting that the missense mutations, A313T and Q216P, impair the formation of KLHL10 homodimers.
DISCUSSION

Recently, the presence of RNA in mature ejaculated spermatozoa was reported. In this report, we successfully utilized spermatozoal RNA from semen ejaculates as a non-invasive means to diagnose genetic defects associated with oligozoospermia. By targeted amplification of the mature transcripts, we obtained sequence encoding multi-exon genes with up to 11-fold higher throughput and efficiency (see STYX, Table 1). Full-length coding sequences for genes were obtained in >90% of men seeking evaluation of their infertility. Several human genes with essential functions during murine spermatogenesis were efficiently amplified, suggesting the potential for widespread application of this technology in genetic analysis. The higher detection efficiency of post-meiotically expressed genes suggests that the stability of these RNAs in mature sperm might be correlated with post-transcriptional regulation and storage in male haploid germ cells, similar to mechanisms previously described (20). Importantly, our strategy identifies abnormal splice variants as well as point mutations, thus expanding the mutational spectrum for genetic analysis.

Infertility affects ~8 million couples in the USA (2). According to the World Health Organization (WHO), idiopathic oligozoospermia is the most frequent diagnosis of semen abnormality in infertile males (4). Today, investigators suspect that these idiopathic individuals and perhaps as much as 50% of male infertility have a genetic contribution (4,21). To date, however, genetic factors for the majority of these conditions remain to be elucidated. The widespread availability of in vitro fertilization and intracytoplasmic sperm injection has meant that ~1–4% of children born in the developed countries are conceived through assisted reproductive techniques (22,23). Limitations of the current methods for evaluation of idiopathic male infertility means that at least some of these individuals might be transmitting to their offspring the genetic defects that caused their infertility. Accordingly, the development of improved diagnostic approaches is paramount for infertile couples seeking assistance.

The feasibility of an RNA-based approach in diagnosing genetic defects in the germine of infertile males is shown by the analysis of KLHL10. Analysis of sperm RNAs in 556 oligozoospermic and 394 normozoospermic individuals revealed seven (1.3%) missense and splicing mutations in this evolutionarily conserved, spermatid-expressed gene. The A313T mutation affects the kelch domain of the protein believed to interact with substrate proteins destined for 26S proteasomal degradation (24,25). The Q216P mutation affects the BACK domain thought to orient the substrate in a complex (26). Since both alleles impair homodimerization of KLHL10 and presumably cause dimer instability, deficiency of protein function may be either due to a dominant-negative effect or due to decreased levels of functional dimers. More studies are needed to address the physiological role of these homodimers, but it is conceivable that in addition to its putative role in a CUL3–KLHL10 complex, KLHL10 might perform CUL3-independent functions during spermiogenesis, similar to MEL26, an analogous BTB-kelch protein in C. elegans (27).

The low frequency of KLHL10 mutations in the oligozoospermic population (1.3%) is not unusual in a complex disorder with multifactorial inheritance. Interestingly, in addition to oligozoospermia, we observed teratozoospermia in five of seven patients and moderate asthenozoospermia in four of seven patients with KLHL10 mutations. We speculate that other measures of semen quality are affected because of mutations in KLHL10 in addition to sperm count. Further phenotypic characterization of the semen quality from these patients may help to define the characteristic phenotype of these mutations. Importantly, the Q216P mutant allele was identified in two different ethnic groups, ruling out a founder effect. The fact that one man with a normal sperm count also harbors the Q216P allele suggests that this allele is partially penetrant.

In conclusion, stable mRNAs present in sperm permit efficient genetic analysis of genes expressed in post-meiotic germ cells. Because estimates suggest that ~4% of the mouse transcriptome is dedicated to post-meiotic male germ cells (28), a large number of candidate genes can potentially impair normal fertility. Coupled with a precise clinical diagnosis that allows disassembly of the ‘infertile’ phenotype into specific categories (Fig. 1), our strategy should greatly accelerate cost-effective future genetic studies that aim to simultaneously screen multiple loci in a single individual. Here, we have presented genetic, evolutionary, statistical and functional evidence for the association with the KLHL10 allele with oligozoospermia. The mechanism by which disruption of KLHL10 function leads to oligozoospermia in men is under further investigation.

MATERIALS AND METHODS

Patient recruitment and classification

Male patients enrolled in this study underwent evaluation for male factor infertility in the Division of Male Reproductive Medicine and Surgery in the Scott Department of Urology at Baylor College of Medicine and were recruited with full approval and oversight by the Institutional Review Board (IRB no. H-12083). Patients were classified into various groups (e.g. oligozoospermia, asthenozoospermia) on the basis of their semen analysis. When multiple semen analyses were performed, the semen parameters (i.e. count, motility, etc.) represent the single result closest to the mean value. Although the WHO recommended limits of adequacy for normal sperm concentration (20 × 10^6 sperm count/ml) are widely used, the diagnosis of oligozoospermia must accommodate the inherent biological intra- and inter-individual variability in sperm concentrations. Prospective studies (3,29–31) define a lower limit of the normal range to be 31.2–34 × 10^6 sperm/ml, which is a more sensitive threshold for detecting borderline oligozoospermia (29,31). In our study, we adopted <34 × 10^6/ml as the lower limit of normal for classifying patients with decreased sperm counts. Patients with sperm counts <10 × 10^6/ml (n = 270) were further subclassified as having severe oligozoospermia (32); patients with counts in the 10–34 × 10^6/ml range (n = 286) were subclassified as oligozoospermic. Patients with counts ≥34 × 10^6/ml were classified as having a normal sperm count and served as
controls for this study \( n = 394 \). Procedures for semen analysis (e.g. cell count, motility and strict morphological analysis of spermatozoa) are described in the WHO guidelines (33).

Except in a few cases where the ethnic origin of patients was self-reported, we used a series of non-recombining Y chromosome biallelic markers (M173, M40, M170, M89, M09) to construct Y chromosome haplogroups (18) and determine the paternal ancestry of our sample population as a proxy for resolving the ethnic composition. The M173 marker is an ancient Eurasian marker that defines the ‘R1’ haplogroup (see the Y Chromosome Consortium nomenclature website: http://ycb.biosci.arizona.edu/nomenclature_system/fig1.html), which characterizes ~50% of extant European Y chromosomes. The ‘R1’ haplogroup and its subclades R1a and R1b are the predominant Y haplogroups of Europe (34) and by extension, North Americans of European origin. The M40 marker defines the ‘E’ haplogroup that is predominant among modern-day Africans (18).

**Past medical history of patients and clinical description**

The medical records of patients DL57488, DL58175, DL60322 and DL60949 were unremarkable. The pituitary–gonadal endocrine axes were assessed by measuring the levels of pituitary gonadotropins, prolactin and testosterone. One moderately oligozoospermic patient with the Q216P substitution (DL60143) and a severely oligozoospermic patient with the A313T substitution (DL61463) had a history of corrected varicocele and recent hormone replacement therapy. Patient DL59031 with the splicing mutation has a complex medical history including primary gonadal failure with azoospermia. He received hormone replacement for the past year, resulting in limited production of sperm.

**Analysis of Y chromosome deletions**

Since ~4% of severely oligozoospermic patients harbor Yq microdeletions (35), we also examined patients DL59031, DL60949 and DL61463 for a panel of 13 sequence-tagged site markers encompassing AZFa, b and c (azoospermia factors a, b and c) regions on the Y chromosome in peripheral blood-derived genomic DNA. The primers were multiplexed into three reactions (6, 4 and 3, respectively) for convenience. Using this approach, no Yq deletions were observed in DL59031; the remaining two patients could not be evaluated because of limited amounts of genomic DNA available. The patients were analyzed using the clinical diagnostic test available in the Laboratory for Male Reproductive Research and Testing at the Scott Department of Urology at Baylor College of Medicine.

**RNA and DNA extraction from semen**

Freshly ejaculated semen was allowed to liquefy for 30 min at 37°C. Semen was thoroughly mixed with equal volume of Sperm Washing Medium (Irvine Scientific, Santa Ana, CA, USA), and the suspension was centrifuged for 10 min at 652g at room temperature. The cells were resuspended and lysed with 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA and DNA were extracted and resuspended in DEPC-treated water and sterile TE solution, respectively. Samples were arranged in 96-well PCR plates and given unique identifiers. The concentration of total RNA was measured after DNase I (Invitrogen) treatment, using a compatible SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA).

**RT–PCR and sequence analysis**

For first-strand cDNA synthesis, we used M-MLV Reverse Transcriptase (Invitrogen) with random primers on total RNA from spermatozoa. RNA–cDNA hybrids were removed from the nascent first-strand reaction with RNase H (Ambion, Austin, TX, USA). PCR was performed with 1 U of JumpStart RedTaq DNA polymerase and reaction mix [1 × PCR buffer, 3.5 mM MgCl2 (Sigma-Aldrich, St Louis, MO, USA)], 400 μM dNTPs, 200 nM of each primer and at least 50 ng of cDNA or 40 ng of DNA. PCR pairs of primers for amplification of the three cDNA fragments of KLHL10 (NM_152467) are as follows: for1—ACCTTAGAAAGCAAGCTCTC, rev1—CTTTTTAAAATGGCCTAATACAGC; for2—GAAAGAT GAGCTCAATGTCAAAC, rev2—GATTGGTCTCTGCT CAATAAC; for3—GCTACGTGCGTCTAAACACTG, rev3— TCTATGCTTAGACTTTTTATGC with \( T_m = 58°C \) for all amplicons. Primer sequences for amplifying the KLHL10 exons, intron 3 deletion interval and the cDNAs of GAPDH, GOPC, IZUMO1, SMCP, STYX and CCNA1 are available upon request. The PCR products were sequenced with BigDye Sequencing reagent V3.1 (Applied Biosystems, Foster City, CA, USA). PCR and sequencing products were purified using Performa Ultra 96-well PCR plates (Edge Biosystems, Gaithersburg, MD, USA). Sequencing was run on ABI Prism Sequencer 3130XL (Applied Biosystems). Sequence analyses were performed with Sequencer 4.2 software (Gene Codes, Ann Arbor, MI, USA). Multiple sequence alignments were made with ClustalW (http://www.ebi.ac.uk/ clustalw). Novel single nucleotide polymorphisms (SNPs) were crosschecked against dbSNP (build 126).

**Cloning of KLHL10 and site-directed mutagenesis**

Full-length ORF (1848 bp) for human WT KLHL10 was amplified from spermatozoal RNA of donor semen and cloned into the pGEM-Teasy vector (Promega, Madison, WI, USA). Mutant constructs \( \text{pGEM-T-KLHL10C647} \) and \( \text{pGEM-T-KLHL10A937} \) carrying the 647A>C and 937G>A missense mutations were created according to the manufacturer’s instructions, using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with the following oligos: TGGATTTCCTCATGACCCCCCAATAAGAAC GACGC and CATATTGGTACTGGGGA GACAGATGG, respectively. WT KLHL10 and the two mutant constructs were subcloned into the MatchMaker GAL4 two-hybrid pGBK7T7 and pGAD7T7 vectors (Clontech, Mountain View, CA, USA).

**Yeast two-hybrid and biosensor analysis**

For testing two-hybrid interactions, bait (pGBK7T7) vectors carrying the WT and mutant KLHL10 constructs were transformed into the Y187 yeast strain. The corresponding prey
(pGADT7) vectors were transformed into the AH109 yeast strain. Y187 and AH109 cells transformed with the bait and the prey were selected on synthetic dropout (SD) plates lacking either tryptophan (SD/-Trp) or leucine (SD/-Leu), respectively, and the two strains were mated according to the manufacturer’s instructions. Mating mixtures were spread onto double dropout plates (SD/-Leu/-Trp) to select for diploids. To test for interactions in diploid strains, the transformants were streaked onto quadruple dropout plates (SD/-Ade/-His/-Leu/-Trp/X-α-gal) and positive interactions were scored as growth of blue colonies.

For further assessment of yeast two-hybrid interactions, a 96-well format BioSensor fluorometric assay was performed according to the manufacturer’s protocol (Clontech). The BioSensor assay utilizes a fluorophore whose emission is quenched by oxygen in a concentration-dependent manner. Accordingly, metabolism leads to scarcity of oxygen, and this in turn leads to increased fluorescence and vice versa. This assay provides a direct quantitative estimate of the rate of growth of colonies. It also provides an indirect estimate of the strength of yeast two-hybrid interactions required for growth. Briefly, mated strains carrying both the bait and the prey vectors were cultured overnight in SD/-Leu/-Trp media till saturation (OD = 0.8) and diluted 1:10 in fresh SD/-Leu/-Trp medium. Then, 10 µl of diluted cultures were added to 190 µl of SdG medium (1:200 final dilution) and to a 96-well BioSensor plate (t = 0). Fluorometric readings were taken at t = 0, 14, 24 h using recommended wavelengths (excitation, 485 nm; emission, 630 nm) on a Gemini microplate spectrofluorometer (Molecular Devices Corp.).

Statistical analyses
To test for an association between oligozoospermia in patients and the presence of SNP in KLHL10, the frequencies of the alleles were compared between the control group (normal sperm count) and each of the two test groups (oligozoospermia and severe oligozoospermia) separately, using a likelihood ratio χ² test. For each test, the evaluation was performed after matching the test and the control groups for the identical haplogroup. All tests were two-sided, with an alpha level of 0.05 considered to indicate statistical significance, and unadjusted P-values were reported.

For the BioSensor protein interactions, analysis of variance (ANOVA) was used to consider the statistical significance of all possible combinations. To test for significant differences among test groups, a post hoc (Tukey–Kramer honestly significant difference) test was performed, which uses the distribution of the maximum range among all variables. All statistical analyses were performed using JMP Start Statistics software (SAS Institute Inc., Cary, NC, USA).

ACCESSION NUMBERS
GenBank (http://www.ncbi.nih.gov/Genbank) accession numbers for the genes are as follows: GAPDH, NM_014364; GOPC, NM_001017408; IZUMO1, NM_182575; KLHL10, NM_152467; SMCP, NM_030663; STYX, NM_145251; CCNA1, NM_003914.

CONTRIBUTORS
A.N.Y., A.R., D.J.L. and M.M.M. designed the study and prepared the manuscript, to which other authors added their comments. Laboratory work was undertaken by A.N.Y., A.R., R.C., L.M. and L.J.M., R.C. and L.J.M. collected and processed samples. W.Y. contributed reagents and assisted in designing the yeast studies.

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Conflict of Interest statement. We declare that we have no conflict of interest.

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