A comprehensive gene mutation screen in men with asthenozoospermia

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Objective: To find novel genetic causes of asthenozoospermia by comprehensively screening known candidate genes derived from mouse models.
Design: Case-control study.
Setting: A fertility center based in an academic hospital.
Patient(s): Thirty men with isolated asthenozoospermia.
Intervention(s): Screening nine candidate genes for mutations: *ADCY10, AKAP4, CATSPER1, CATSPER2, CATSPER3, CATSPER4, GAPDHS, PLA2G6,* and *SLC9A10.* To account for a possible effect of heterozygous mutations, assessing imprinting of all candidate genes by studying the expression pattern of heterozygous SNPs in testis biopsies of five unrelated men.
Main Outcome Measure(s): Mutations found in patients only.
Result(s): We identified 10 heterozygous asthenozoospermia-specific mutations in *ADYC10* (n = 2), *AKAP4* (n = 1), *CATSPER1* (n = 1), *CATSPER2* (n = 1), *CATSPER3* (n = 1), *CATSPER4* (n = 3), and *PLA2G6* (n = 1). These mutations were distributed over six patients. In silico analysis showed that 8 of the 10 mutations either had a negative BLOSUM score, were located in conserved residues, and/or were located in a functional domain. Expression analysis demonstrated that *CATSPER1* and *CATSPER4* are imprinted.
Conclusion(s): Given their putative effect on protein structure, their location in conserved sequences or functional domains, and their absence in controls, the identified mutations may be a cause of asthenozoospermia in humans. (Fertil Steril® 2011;95:1020–24. ©2011 by American Society for Reproductive Medicine.)

Key Words: Asthenozoospermia, gene, male infertility, spermatogenesis

Subfertility, the inability to conceive within 1 year of unprotected intercourse, affects one in eight couples in the Western world (1). It therefore constitutes an important clinical problem with psychological, social, and economical consequences. In up to half of subfertile couples, semen quality is the only or a contributory etiologic factor. Low semen quality is diagnosed when the number of sperm cells produced, their morphology, or their motility is below the World Health Organization cutoffs for normal spermatogenesis (2). Reduced motility, that is, asthenozoospermia, is found in approximately 18% of subfertile couples (3, 4) and is the most important factor negatively affecting natural conception (5, 6).

Mouse models have provided a number of candidate genes for isolated asthenozoospemia: in knockout models, genes that produce a phenotype with no other abnormalities apart from asthenozoospermia (7–14). These genes include genes encoding sperm specific ion channels (*CATSPER1* [NM_053054.2], *CATSPER2* [NM_172095.1], *CATSPER3* [NM_178019.1], *CATSPER4* [NM_198137.1], *SLC9A10* [NM_183061.1]), enzymes (*ADCY10* [NM_018417.4], *GAPDHS* [NM_014364.4], *PLA2G6* [NM_003560.2]), and a structural protein (*AKAP4* [NM_003886.2]) (Supplemental Table 1, available online).

The four homologous male germ cell–specific *CATSPER* (cation channel of sperm) genes, *CATSPER1–4*, appear to encode subunits of sperm calcium channels that are present in the principal piece of the sperm tail and are required for sperm cell hyperactivated motility and fertilization. It is currently unknown if and how the four *CATSPER* proteins interact. *CATSPER1–4* null sperm all lack the spermatozoal $I_{Catsper}$ calcium current that is present in wild-type animals (15, 16). It is interesting that *CATSPER1*$^{-/-}$ mice also lack the *CATSPER2* protein, whereas the CATSPER1 protein is also absent in *CATSPER2*$^{-/-}$ mice, with knockout sperm showing identical phenotypes (17).

GAPDHS, *PLA2G6,* and *ADCY10* encode sperm specific enzymes. In the absence of GAPDHS, a glycolytic enzyme, sperm lack progressive motility (18). *Pla2g6* male knockouts have greatly reduced fertility and produce spermatozoa with impaired motility (12). Bicarbonate ions present in the female genital tract activate the adenylate cyclase ADCY10. Mice deficient for this enzyme are infertile because of a severe sperm motility defect (13).

SLC9A10 is a member of the sodium-hydrogen exchanger (*NHE*) family. *Slc9a10*-null males have normal testis histology, normal sperm numbers and morphology, but are completely infertile with severely diminished sperm motility (14).

The *Adcy10*-knockout and *Slc9a10*-null mice demonstrate a similar phenotype, with altered sperm motility, absence of

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hyperactivation, and absence of tyrosine phosphorylation. In addition, for both models, sperm fertilizing potential can be restored by removing the oocyte zona pellucida, whereas motility can be rescued by the addition of cell-permeable cyclic adenosine 3′:5′ monophosphate (cAMP) analogs (19), which suggests that the two proteins act together. Indeed, in vivo, *Slc9a10* associates with the *Adcy10*, forming a complex involved in bicarbonate signaling (20).

AKAP4 encodes testis-specific fibrous sheath protein that functions as a scaffold for signal transduction and enzymatic molecules. In mice, its disruption results in infertility associated with reduced sperm motility, whereas sperm count and morphology are unaffected (7).

In humans, only few of these genes have been related to impaired sperm motility. The *AKAP4* gene has been studied in relation to disrupted sperm motility caused by anatomic defects of the sperm tail known as dysplasia of the fibrous sheath. A single case report describes mutations in *AKAP4* in a man with dysplasia of the fibrous sheath (21). The *CATSPER2* gene is one of two genes that are deleted in 15q15.3 deletion syndrome [MIM:611102], characterized by deafness and male infertility resulting from astheno-teratozoospermia (22, 23).

Because data on the genetic background of asthenozoospermia in humans are extremely limited, we set out to evaluate the association of nine candidate genes, *ADCY10, CATSPER1, CATSPER2, CATSPER3, CATSPER4*, *GAPDHS, SLC9A10*, *PLA2G6* with asthenozoospermia. Our case control study included 30 men with isolated asthenozoospermia and 90 normozoospermic controls.

MATERIALS AND METHODS

Participants

As part of our ongoing research into genetic causes of low semen quality, we included men who had attended our center and given informed consent from January 1998 until September 2005. From January 1998 until January 2000, we included only men with low semen quality, that is, one or more semen parameters below the World Health Organization cutoffs based on at least two semen analyses. From January 2000 until September 2005, we consecutively included all men before semen analyses. Men with a history of orchitis, surgery of the vasa deferentia, bilateral orchidectomy, chemotherapy or radiotherapy, obstructive azoospermia, retrograde ejaculation, bilateral cryptorchidism, numerical or structural chromosome abnormalities, or Y-chromosome deletions were excluded.

As cases, we included all men with isolated severe asthenozoospermia, defined as a normal sperm concentration ($>20 \times 10^6$/mL) with less than 10% fast progressive motile sperm (grade A) in at least two semen samples. For each case, we randomly selected three controls from the same cohort that had a normal sperm concentration and more than 40% fast progressive motile sperm in two semen samples. The institutional review board of the Academic Medical Centre approved this study.

Mutation Analysis

We extracted DNA from peripheral blood leucocytes according to standard procedures. We amplified all coding exons and intron/exon boundaries of *ADCY10, AKAP4, CATSPER1–4, GAPDHS, PLA2G6,* and *SLC9A10*. Primer pairs (Supplemental Table 2, available online) were designed with the aid of Primer3 using the available genomic sequence information from the U.S. National Center for Biotechnology Information (NCBI) (24). In cases in which several isoforms were known, we selected as a reference sequence either the isoform that is expressed in the testis or else the longest known isoform. Large exons and promoter regions were covered by overlapping polymerase chain reaction (PCR) products of maximally 500 base pairs (bp). The PCR was performed in a total volume of 25 μL and contained 50 ng DNA, 3 μL of 10x PCR buffer (Roche, Woerden, the Netherlands), 0.2 mM dNTPs, 12 pmol forward and reverse primer, 2 mM $MgCl_2$ and 0.5 IU Super*Taq* polymerase. We used a touchdown PCR program with a temperature range of 62° to 50°C with a 2°C decrement per cycle, a 1-cycle increment per temperature step, and a final amplification for 20 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 5 minutes.

Direct sequencing of both sense and antisense strands was performed using the same primers as those used for PCR, and an automated ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). All sequences were analyzed with the CodonCode Aligner software (CodonCode Corporation, Dedham, MA).

For *AKAP4*, *CATSPER1-3*, *SLC9A10*, *GAPDHS,* and *PLA2G6*, encountered mutations were analyzed using the Alamut mutation interpretation software (Interactive Biosoftware, Rouen, France). For *CATSPER4* and *ADCY10,* which are not included in Alamut, mutations were evaluated using the following Web-based interfaces. To predict the possible effect of silent exonic variants on splicing activity, we used the ESE finder (Exonic Splicing Enhancer) program (25). Similarly, to predict the possible effect of intronic variants at the intron/exon boundaries and the branch site sequence on splicing activity, we used the Human Splicing Finder (26). We applied the BLOSUM62 Substitution Scoring Matrix to describe the putative impact of identified amino acid changes (27).

To determine the conservation throughout species, the aligned amino acids of gene orthologues were analyzed using the ClustalW program (www.ebi.ac.uk/clustalw/index.html). Publicly available UniProt data were used to assess whether variants were located in a functional protein domain (www.uniprot.org). All missense mutations or single-nucleotide polymorphisms (SNPs) as well as those intronic variants that were predicted to alter splice site activity were analyzed in controls.

Statistical analysis of genotype frequencies and distributions in patients and controls was performed using chi-square tests and R-code software (www.r-project.org). $P<.05$ was considered statistically significant.

TABLE 1

Age and semen characteristics of asthenozoospermic patients and normozoospermic controls.

Characteristic	Patients (n = 30)	Controls (n = 90)
Age	36.1 ± 5.5	36.4 ± 6.0
Semen analyses[a]		
Volume	3.3 ± 1.3	3.9 ± 1.3
Concentration	32 (27–60)	87 (58–114)
Fast progressive motility (grade A) %	5 ± 3	49 ± 7
Normal morphology %	20 (15–32)	50 (46–56)

[a] Based on at least two semen analyses per individual. Data are presented as mean ± standard deviation or median (25th–75th percentile).

Visser. Mutation screening in asthenozoospermia. Fertil Steril 2011.

Imprinting Analysis

Imprinting was verified by studying the expression pattern of heterozygous SNPs in testis biopsies from five unrelated men who had undergone elective testicular surgery as part of the treatment for prostate cancer or for suspected malignant testicular disease. Only tumor-free material was used.

Tissue samples were homogenized with the use of a Magna Lyser (Roche Diagnostics, Basel, Switzerland). DNA and RNA were extracted from biopsies by trizol digestion. For the first-strand complementary DNA (cDNA) synthesis, we used M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) with random primers on total RNA extracted from biopsies. At the DNA level, samples were screened for the presence of known SNPs. With the aid of Primer3, intron-spanning primers were designed to evaluate the expression pattern of these SNPs in cDNA samples (Supplemental Table 3, available online). Potential DNA contamination of RNA samples was controlled for by incorporating a DNAse treatment in the extraction protocol as well as using intron-spanning primers for cDNA PCR.