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Case Report

Identification of Polymorphisms in the Hrb, GOPC, and Csnk2a2 Genes in Two Men With Globozoospermia

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Case Report

Globozoospermia, or round-headed sperm syndrome, is a unique form of male infertility characterized by sperm possessing a round head, lack of an acrosome, and a disorganized midpiece and tail. It is a rare disorder, affecting approximately 0.1% of male infertility patients (Schirren et al, 1971; Aitken et al, 1990). Two forms of globozoospermia have been described: the more severe type I, with complete absence of the acrosome and acrosomal enzymes; and type II, with acrosomal remnants remaining and the presence of other morphology types in the ejaculate (Singh, 1992). Because of the lack of a functional acrosome, the primary fertility defect connected with globozoospermia is severely diminished fertilization.

A specific mode of inheritance has not been identified for the syndrome, although several modes, including autosomal dominant, autosomal recessive, monogenic, and polygenic, have all been suggested (Trokoudes et al, 1995). While the specific etiology of globozoospermia is unclear, a genetic component appears certain, as the rare syndrome has been identified in multiple members of the same family on several occasions (Carrell et al, 1999, 2001; Kilani et al, 2004). Patients with globozoospermia demonstrate a broad range of semen characteristics, and it is probable that multiple genetic components contribute to the disorder either independently or together.

Several studies have investigated genetic factors in globozoospermic patients. At least 15 patients with globozoospermia have been karyotyped, and with the exception of one individual with mosaic Down syndrome (Kim et al, 2001), all have been normal (Kullander and Rausing, 1975; Baccetti et al, 1977; Lalonde et al, 1988; Stone et al, 2000; Viville et al, 2000; Zeyneloglu et al, 2002; Martin et al, 2003; Kilani et al, 2004; Morel et al, 2004). The chromatin structure in globozoospermic patients has also been studied, with conflicting results. One group found low levels of DNA fragmentation in their patient (Larson et al, 2001), consistent with highly fertile men, while two other groups both found significantly increased rates of fragmentation compared with fertile men (Baccetti et al, 1996; Vicari et al, 2002). These differences may be due to the tests used (sperm chromatin structure assay and Comet vs terminal deoxynucleotidyl transferase mediated dUTP nick end labeling), or they may simply reflect differences in globozoospermic patients. Protamine levels and ratios, for instance, can vary widely between globozoospermic men and fertile controls and are known to correlate with DNA fragmentation (Carrell et al, 1999).

Aneuploidy rates have also been analyzed in 9 globozoospermic men, including 2 pairs of brothers. A subset of chromosomes 1, 7, 9, 12, 13, 15, 18, 21, X, and Y were evaluated by fluorescent in situ hybridization in 1 of 6 different studies (Carrell et al, 1999; Viville et al, 2000; Carrell et al, 2001; Vicari et al, 2002; Martin et al, 2003; Morel et al, 2004). Significant aneuploidy increases were found for at least one chromosome of 4 patients, and in one of the patients, the nullisomy rates for chromosomes 13 and 21 were exceptionally high, at 33.8% and 37.9%, respectively (Carrell et al, 1999). With the exception of this individual, sperm aneuploidy rates reported for globozoospermic men are similar to those found for infertile men, in general, with poor sperm morphology.

Over the past few years knockout studies investigating the role of specific genes have identified several male infertility candidate genes, including some that alter the sperm head morphology. Three genes in particular, HIV-1 Rev-binding protein (Hrb) (Kang-Decker et al, 2001), Csnk2a2 (Xu et al, 1999), and Golgi-associated PDZ- and coiled-coil motif-containing protein (GOPC) (Yao et al, 2002), are of particular interest because of phenotypes in the null mutant mice that are very similar to human globozoospermia. In addition to being infertile, the sperm from these 3 knockouts are primarily round, spherical, and lack an acrosome.

The human Hrb gene is located on chromosome 2 at position q36.3 and comprises 13 coding exons. In wild-type mice, Hrb associates with the cytosolic surface of proacrosomic transport vesicles that fuse to create a single large acrosomic vesicle in step 3 of mouse spermiogenesis. In mutant mice, proacrosomic vesicles form, but fail to fuse, blocking acrosome development (Kang-Decker et al, 2001). In addition to many sperm in knockout mice having a classic globozoospermic appearance, a recent report also indicates that Hrb-/- mice have multiple centriolar anomalies causing deformed or multiple flagella (Juneja and van Deursen, 2005).

The GOPC gene, also commonly identified as PDZ domain protein interacting specifically with TC10, is located at 6q21 and contains 9 exons. The protein localizes in the *trans*-Golgi region in round spermatids of mice and appears to play a role in vesicle transport from the Golgi apparatus. The primary defect in mutant mice is fragmentation of the acrosome in early round spermatids and abnormal acrosomal vesicles that fail to fuse. Later-stage spermatozoa also demonstrate nuclear malformation and abnormal mitochondria arrangement (Yao et al, 2002).

The Csnk2a2 gene is preferentially expressed in the later stages of spermatogenesis, and Csnk2a2-/- mice exhibit rounded sperm heads in which the acrosome has detached, is present in remnants or vesicles, or is entirely absent, as is the case in type II globozoospermia (Xu et al, 1999). The

gene product of Csnk2a2 is the catalytic α' subunit of casein kinase II, a constitutively active serine/threonine kinase, with more than 160 different substrates (Pinna and Meggio, 1997).

Each of the 3 genes described generate a phenotype that is consistent with human globozoospermia upon homologous disruption. In the heterozygous state, they can be transmitted without affecting fertility, making them good candidate genes for an autosomal recessive disorder. The purpose of this study was to screen the coding sequence and flanking intronic sequence of these 3 genes in 2 individuals with globozoospermia, for mutations that could lead to a globozoospermic condition.

Materials and Methods

Patients and Controls— After obtaining Institutional Review Board approval, individuals previously diagnosed with globozoospermia, based on morphology evaluations following World Health Organization criteria, were contacted and invited to participate in a study screening globozoospermia candidate genes. Two unrelated individuals elected to participate in the study. Following informed consent, 15 mL of whole blood was taken from each patient using standard phlebotomy techniques, and genomic DNA were extracted using the Puregene DNA extraction kit (Gentra, Minneapolis, Minn). To act as controls, 12 genomic DNA samples were obtained from the Utah Genetic Reference Project from men who had fathered at least 1 child.

Polymerase Chain Reaction and Sequencing— Based on the exons' proximity to each other, polymerase chain reactions (PCRs) were designed to amplify the genomic sequence containing all coding exons in segments no greater than 3 kb, as summarized in Table 1. As different numbering systems exist for the exons, exons were numbered the same as shown in the National Center for Biotechnology Information evidence viewer. In some cases, "non-coding" exons were listed. These exons were not sequenced and are not listed in Table 1. General thermocycling conditions were as follows: hold at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, annealing for 30 seconds, extension at 72 °C (1 min/kb), and a hold for 5 minutes at 72 °C. Additional sequencing primers were designed to allow sequencing of individual exons in the forward and reverse directions. Primary PCR products were purified using a high-salt/guanidium and ethanol wash protocol and were sequenced on an ABI 3700 capillary sequencer. The sequence trace files were analyzed visually for alterations from each gene's consensus sequence, obtained from National Center for Biotechnology Information.

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Table 1. *Primer sequences and annealing temperatures for PCR amplification*

Results

Patients— Two patients with globozoospermia were enrolled for the study. Semen parameters for both patients are listed in Table 2. Patient 1 is a 42-year-old male with a 17-year history of primary infertility. He has 6 brothers, 1 who has also been diagnosed with globozoospermia and has a 22-year history of infertility and 5 who are believed to be fertile. Patient 2 is a 36-year-old male with a history of primary infertility. He has 2 brothers, 1 diagnosed with globozoospermia and another with normal semen parameters and a history of normal fertility.

View this table: Table 2. *Semen characteristics of patients*

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Mutation Screening of the Hrb, GOPC, and Csnk2a2 Genes— The entire coding sequence and flanking intronic sequence of the Hrb, GOPC, and Csnk2a2 genes was analyzed for variations in 2 unrelated men with globozoospermia. In total, 14 single nucleotide variants were identified in the globozoospermic patients, which are listed in Table 3. Six of the identified variants have been reported previously in the single nucleotide polymorphism (SNP) database (www.ncbi.nih.gov), 5 in the introns, and a silent SNP, *T472T,* in exon 12 of Hrb.

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Table 3. *Nucleotide variants detected in globozoospermia candidate genes*

Eight variants were detected that have not been reported previously. Seven of these fall within the intronic sequences of the genes. Of the newly identified variants detected, only one, in the Hrb gene, *V139A,* resulted in a heterozygous amino acid change in patient 2. The *V139A* change falls 5 residues after the conserved N-terminus zinc finger domain and represents a conservative change from valine to alanine. The *V139A* SNP was not observed in any of the 12 control patients screened. A comparison of the published Hrb protein sequences shows that the residue is conserved in the human, mouse, and zebrafish sequences.

Discussion

In this study we tested the hypothesis that mutations in the Hrb, GOPC, or Csnk2a2 genes might be responsible for globozoospermia and infertility in 2 unrelated, globozoospermic men with family histories of globozoospermia. We demonstrated the presence of 2 nucleotide changes within the coding region of the Hrb gene, one of which, *V139A,* results in a heterozygous amino acid change at a conserved position. We also identified several other nucleotide changes in the intronic regions flanking the exons of these genes.

Many of the variants found were present in both patients or had been previously identified in the National Center for Biotechnology Information SNP database. This would indicate that they are relatively common and do not contribute to the pathogenesis of globozoospermia. Nevertheless, it remains possible that one of the intronic polymorphisms could lead to a disruption of transcription or translation in some way.

The *V139A* variant was found in only 1 of the 2 patients and in none of the 12 fertile controls screened. While valine and alanine residues are both neutral, hydrophobic amino acids with only 1 methyl group difference, the position shows great cross-species preservation. A basic local alignment search tool analysis showed conservation of the valine residue in multiple species, including zebrafish and xenopus. This evolutionary preservation of the residue and the entire region indicates it may be of importance for the protein.

Patient 2, the heterozygous carrier of the *V139* SNP, has type II globozoospermia, with 76% round heads. While it is known that postmeiotic spermatids continue to share cytoplasm, limiting the effects of haploinsufficiency (Caldwell and Handel, 1991), one possibility is that the SNP

contributes to a deficiency of the Hrb protein in some of this patient's sperm, producing his type II phenotype. Unfortunately, the globozoospermic brother, siblings, and family were not available for genotype-phenotype correlations. The role of the *V139A* SNP cannot be known for certain until more globozoospermic patients and controls are screened or until an assay is designed to test whether the *V139A* SNP introduces a functional change in the Hrb gene.

Spermatogenesis is an involved process, with an estimated 2000 genes participating (Hargreave, 2000). Knockout studies in mice have now identified many genes that produce an infertile male phenotype when disrupted (Christensen and Carrell, 2002). In many cases, these phenotypes are very similar in presentation, making it a challenge to know which genes may be of clinical significance. Because of the large numbers of genes involved, unraveling the pathophysiology of infertility is complex.

Some researchers have suggested that most SNPs involved in complex traits should be found in the regulatory elements of the genome (King and Wilson, 1975; Mackay, 2001). It is possible that one or both of our patients have a mutation in the promoter or in other regulatory elements of the 3 genes we sequenced, which we did not find. However, to date, most of the reported SNPs connected to complex traits have been found in the amino acid-coding exons (Glazier et al, 2002). Furthermore, evidence indicates that SNPs associated with complex traits generate amino acid changes in less-critical areas of the protein. Several of these complex trait-associated SNPs, spread over several genes, then combine to generate a phenotype (Thomas and Kejariwal, 2004). Male infertility in general is certainly a complex trait, and it is as yet unknown whether the etiology of globozoospermia will follow classic Mendelian inheritance, be the result of multiples genes, or a combination of the two.

Although we did not find a definitive mutation with a clear link to globozoospermia, our results should not be taken to mean that Hrb, GOPC, and Csnk2a2 do not play a role in the pathophysiology of globozoospermia. The mouse knockout models for these genes, previously described, show how major disruptions of any of the 3 can clearly lead to an infertile, globozoospermic condition. Because of the rarity of the disorder, adequate sample sizes of patients with globozoospermia are not readily available for screening. Until sufficient numbers of patients have been screened, these genes should continue to be considered as globozoospermia candidate genes. To our knowledge, this is the first report of a search for mutations in Hrb and GOPC in globozoospermic patients and the second for mutations in the Csnk2a2 gene. A recent report screened the exonic sequence of Csnk2a2 in 6 globozoospermic patients, without finding any causative mutations (Pirrello et al, 2005).

The use of intracytoplasmic sperm injection in assisted reproduction allows severely infertile men, including those with globozoospermia, a reasonable chance at biological fatherhood (Lundin et al, 1994; Trokoudes et al, 1995). It also bypasses the mechanisms of natural selection and allows the transmission of disease alleles to a new generation. With up to 50% of male infertility being idiopathic and potentially genetic in nature, it is important that we continue to search out the molecular causes of infertility. The information we find can be used to inform our patients of both the causes of infertility and the risks associated with passing on infertility mutations.

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