Cystic fibrosis mutation screening in healthy men with reduced sperm quality

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The majority of men with cystic fibrosis (CF) are infertile due to a bilateral congenital absence of the vas deferens (CBAVD). However, clinically affected CF patients present a spectrum of genital phenotypes ranging from normal fertility to severely impaired spermatogenesis and CBAVD. Recently, it has become apparent that CF can manifest itself as isolated CBAVD in the absence of other clinical symptoms. The present study was undertaken to test the possible involvement of the CF gene in the aetiology of male infertility other than CBAVD. Semen specimens from 127 unrelated healthy males with various diagnoses of reduced sperm quality were screened for a panel of 13 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Fourteen of 80 (17.5%) healthy men with infertility due to reduced sperm quality and 3 of 21 (14.3%) men with azoospermia had at least one CF mutation (one azoospermic male was a compound heterozygote). The frequency of mutations in our sample of infertile males was significantly higher than the expected CF carrier frequency in the local population (P = 0.00139). No mutations were found in a control group of 26 individuals with normal semen parameters. This increased frequency of CF mutations in healthy men with reduced sperm quality and in men with azoospermia without CBAVD suggests that the CFTR protein may be involved in the process of spermatogenesis or sperm maturation apart from playing a critical role in the development of the epididymal glands and the vas deferens.

Key words: CFTR mutations/cystic fibrosis/infertility/reduced sperm quality

Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disease in Caucasians, with a reported incidence of 1 in 2500 (Boat et al., 1989). Statewide newborn screening programmes in the USA have yielded CF incidences of 1 in 2521 in Colorado (Hammond et al., 1990) and 1 in 3431 in Wisconsin (Gregg et al., 1993). Based on these data, carrier frequencies can be estimated to be between 1 in 25 and 1 in 30 in the USA. Since the discovery of the CF gene (called cystic fibrosis transmembrane conductance regulator, or CFTR) in 1989 (Kerem et al., 1989), >400 'disease causing' mutations and 80 DNA sequence variants have been identified. Although one mutation, called ΔF508, accounts for the majority of mutations on CF chromosomes, its frequency varies among populations of different geographical and ethnic backgrounds. The distribution of other CF mutations is also highly heterogeneous among populations, with other 'common' mutations occurring on 0.1-2.4% of CF chromosomes (Tsui, 1992).

The CFTR gene product has been characterized as a transmembrane protein that forms a cyclic AMP-regulated chloride channel (Miller, 1993). The loss or reduction of chloride channel function as well as defective acidification of intracellular compartments in CF epithelia likely account for the majority of the classical clinical symptoms in CF. Abnormal electrolyte transport leads to the thickening and reduced clearance of mucous secretions in various organs, resulting in recurrent pulmonary infections, pulmonary and pancreatic insufficiency and infertility in the majority of male patients. However, wide variations in disease manifestation and disease severity exist among CF-affected individuals, probably due to the multitude of disease-causing mutations in the CFTR gene that differentially affect the function of the CFTR protein.

Infertility in males with CF typically originates in developmental abnormalities in the vas deferens or the distal half of the epididymis. In 97-98% of men with CF, a bilateral congenital absence of the vas deferens (CBAVD) blocks the transport of spermatozoa from testicular or epididymal structures to the outer genital tract, resulting in azoospermia. However, pulmonary or pancreatic manifestations of CF do not necessarily coincide with infertility due to CBAVD, because cases of normal fertility in clinically affected CF patients have been described (Taussig et al., 1972; Barreto et al., 1991). Furthermore, histological studies of testicular tissues of males with CF and CBAVD yield a spectrum of results, from normal spermatogenesis (Gottlieb et al., 1991) to severely decreased spermatogenesis with abnormal sperm forms and reduced sperm numbers (Kaplan et al., 1968). These data suggest that the CFTR protein may play a critical role in spermatogenesis or sperm maturation that is independent of its function in the development of epithelial ducts such as the vas deferens.

Isolated CBAVD has long been understood as a disease on its own. The discovery of mutations in the CFTR gene in
approximately half of those otherwise healthy men with CBAVD suggests that isolated CBAVD represents a primarily genital form of CF (Dumar et al., 1990; Angiulano et al., 1992; Patrizio et al., 1993a).

Considering the variety of genital manifestations in males with CF and the wide range of disease severity in CF-affected individuals, we postulated that mutations in the CFTR gene may be associated with male infertility due to factors other than CBAVD. The major objective of this study was to evaluate the frequency and nature of mutations in the CFTR gene in healthy men with reduced sperm quality and in men with azoospermia without CBAVD.

Materials and methods

Sample composition

Our sample consisted of semen specimens from 127 men attending clinics in the Section of Endocrinology and Infertility at the University of Chicago and Andrology Laboratory Services Inc., Chicago, IL, USA because of marital infertility. Samples were encountered consecutively. The majority of patients with reduced sperm quality (>80%) had seen a urologist prior to presenting at the infertility clinics. According to the referring physicians, none of the subjects had any history of CF or showed clinical signs or symptoms of CF. However, sweat tests were not performed in these subjects and it was not possible to obtain objective measurements of pancreatic status or pulmonary function retrospectively.

For purposes of confidentiality, semen specimen containers were labelled only with the code number that had been assigned to the sample in the referring andrology laboratory. Samples were given a second code number in our laboratory before DNA extraction was started and CF mutation studies were initiated. Thus, all mutation analyses were performed without knowledge of the semen diagnosis or the patient's identity. All semen specimens were obtained for the andrological diagnosis and semen volumes higher than 7.0 ml (n = 3), the classification was determined by a combination of two or more pathological parameters; in no case could a large semen volume have compensated for low sperm counts as the only reason for infertility.

The combination of small volume of ejaculate, acid pH and low or absent fructose content can be found in ~89% of patients with obstructions of the ejaculatory ducts (Pryor and Hendry, 1991). Therefore, seminal plasma fructose concentration was measured in all azoospermic samples to exclude unidentified cases of CBAVD. For fructose determination, a colorimetric assay was used (WHO, 1992). Fructose concentrations of ≥13 µmol per ejaculate were considered normal according to WHO guidelines. All samples included in the study had fructose concentrations in the normal range; furthermore, all semen volumes in this azoospermic group were >1.0 ml. Thus, based on these data and an estimated frequency of ejaculatory duct obstructions among azoospermic men of 4.8% based on vasography (Hendry et al., 1990), the existence of a large number of individuals with undetected CBAVD among our study population seems unlikely. However, because fructose may be present in a minority of cases with unilateral obstructions of the vas deferens or obstructions at higher sites than the orifices of the seminal vesicles, the presence of ejaculatory duct obstructions in our sample, although unlikely, cannot be ruled out completely. No further evaluation of the anatomy or presence of potential structural abnormalities of the male genital organs was performed in this group of patients.

Mutation analysis

DNA was obtained from spermatozoa and, in case of azoospermic samples, from seminal plasma leukocytes and epithelial cells. After ejaculates were centrifuged at 5000 rpm for 5 min, the supernatant was removed and the sperm or cell pellet was washed twice in 1× phosphate-buffered saline. In the case of azoospermic samples, a standard cell lysis protocol using 0.06 mg/ml proteinase K with

Table I. Sample composition by semen variables. Nomenclature is modified from the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (1992)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Sperm concentration</th>
<th>Motility</th>
<th>Morphology</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermia</td>
<td>&gt;20 × 10^6/ml</td>
<td>&gt;25% rapidly</td>
<td>&gt;30% normal</td>
<td>26</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>reduced</td>
<td>normal</td>
<td>normal</td>
<td>4</td>
</tr>
<tr>
<td>Asthenozoospermia</td>
<td>normal</td>
<td>reduced</td>
<td>normal</td>
<td>27</td>
</tr>
<tr>
<td>Teratozoospermia</td>
<td>normal</td>
<td>normal</td>
<td>&gt;30% normal</td>
<td>4</td>
</tr>
<tr>
<td>Oligoasthenoteratozoospermia</td>
<td>reduced</td>
<td>reduced</td>
<td>&gt;30% normal</td>
<td>16</td>
</tr>
<tr>
<td>Oligoteratozoospermia</td>
<td>reduced</td>
<td>normal</td>
<td>&gt;30% normal</td>
<td>17</td>
</tr>
<tr>
<td>Oligoasthozoospermia</td>
<td>reduced</td>
<td>reduced</td>
<td>normal</td>
<td>5</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>no spermatozoa</td>
<td>not</td>
<td>applicable</td>
<td>21</td>
</tr>
</tbody>
</table>

Semen samples were collected by masturbation after 2-5 days of abstinence into sterile containers. Patients were instructed to keep the samples at body temperature and to deliver them to the andrology laboratory within 1 h. Semen analysis was performed in the andrology laboratories of the participating institutions and specimens were classified according to World Health Organization (WHO, 1992) guidelines, using the 1992 normal values for diagnosis. Assessment of sperm count and sperm motility was performed on a Hamilton-Thorn HTM-S analytical system. In some cases (n = 3), sperm count and motility were assessed light microscopically by means of a Makler chamber (Sefi-Medical, Haifa, Israel).

A nomenclature for semen variables is given in Table I. Andrological diagnosis was based on at least two semen analyses. Among the 127 semen specimens analysed, 26 samples were classified as normozoospermic, 21 as azoospermic, and 80 showed various degrees of reduced sperm quality (Table I).

Semen volumes lay between 0.5 and 8.5 ml. In all cases of an andrological diagnosis and semen volumes higher than 7.0 ml (n = 3), the classification was determined by a combination of two or more pathological parameters; in no case could a large semen volume have compensated for low sperm counts as the only reason for infertility.

The combination of small volume of ejaculate, acid pH and low or absent fructose content can be found in ~89% of patients with obstructions of the ejaculatory ducts (Pryor and Hendry, 1991). Therefore, seminal plasma fructose concentration was measured in all azoospermic samples to exclude unidentified cases of CBAVD. For fructose determination, a colorimetric assay was used (WHO, 1992). Fructose concentrations of ≥13 µmol per ejaculate were considered normal according to WHO guidelines. All samples included in the study had fructose concentrations in the normal range; furthermore, all semen volumes in this azoospermic group were >1.0 ml. Thus, based on these data and an estimated frequency of ejaculatory duct obstructions among azoospermic men of 4.8% based on vasography (Hendry et al., 1990), the existence of a large number of individuals with undetected CBAVD among our study population seems unlikely. However, because fructose may be present in a minority of cases with unilateral obstructions of the vas deferens or obstructions at higher sites than the orifices of the seminal vesicles, the presence of ejaculatory duct obstructions in our sample, although unlikely, cannot be ruled out completely. No further evaluation of the anatomy or presence of potential structural abnormalities of the male genital organs was performed in this group of patients.

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Table II. Cystic fibrosis transmembrane conductance regulator (CFTR) mutations by andrological diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of mutations per no. of chromosomes (%)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermia</td>
<td>0/52 (0)</td>
<td></td>
</tr>
<tr>
<td>Abnormal diagnosis</td>
<td>18/202 (8.9)</td>
<td></td>
</tr>
<tr>
<td>Azoospermia</td>
<td>4/42 (9.5)</td>
<td></td>
</tr>
<tr>
<td>Asthenozoospermia</td>
<td>5/54 (9.2)</td>
<td></td>
</tr>
<tr>
<td>Teratozoospermia</td>
<td>1/8 (12.5)</td>
<td>1. G551D/R117H</td>
</tr>
<tr>
<td>Oligoteratozoospermia</td>
<td>1/4 (7.1)</td>
<td>2. G542X/-</td>
</tr>
<tr>
<td>Oligoteratozoospermia</td>
<td>5/72 (15.6)</td>
<td>3. 621 + 1G-»T</td>
</tr>
<tr>
<td>Asthenoteratozoospermia</td>
<td>1/34 (2.9)</td>
<td>4. AF508/-</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>0/8 (0)</td>
<td>5. G542X/-</td>
</tr>
</tbody>
</table>

denaturation at 58°C over 60 min and a subsequent heat inactivation at 95°C over 10 min was used. For sperm DNA lysis, a protocol using a lysis buffer containing 1.7 μM sodium dodecyl sulphate/20 mM diethylethanol with 0.05 mg/ml proteinase K was applied (Gyllensten, 1990).

Approximately 25 μl of the cell or sperm lysate was subjected to amplification by polymerase chain reaction (PCR) using standard amplification conditions. If not otherwise indicated, each 100 μl reaction tube contained 1X PCR reaction buffer (10X stock solution = 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 1 μM of each primer, 800 μM of mixed dNTPs and 2.0 U Taq DNA polymerase.

Each sample was screened for 13 mutations in the CFTR gene: R117H, 621 + 1G-»T, 1609delICA, Δ1507, Δ508, S549N, G542X, G551D, R553X, R1162X, 3849+10kbC-»T, W1282X, N1303K. The methods used for detecting Δ508/Δ1507, S549N, G551D, R553X, R1162X and W1282X have been described previously (Ober et al., 1992). The frequencies of each of the six mutations that were detected in this sample are shown in Table III. The most common mutation in this sample was G542X (2/18, or 44% of mutations), followed by Δ508 (7/18, or 33% of mutations). Four other mutations (G551D, R117H, 621 + 1G-»T, 3849+10kbC-»T) were each detected in a single individual (1/18, or 5.6% of mutations).

Discussion

In this investigation, 14 of 80 (17.5%) healthy men with infertility due to sperm abnormalities had detectable mutations in the Chicago area (C.Ober and K.van der Ven, unpublished data). To compare the mutation frequency in infertile males with the theoretically derived population mutation frequency, we formed a Z-statistic by looking at the difference between the mutation frequency in the study population and 4% of the estimated mutation frequency on Chicago-area CF chromosomes and dividing by its standard error. The statistic was compared to tables of normal distributions to determine the P value.

Results

Eighteen mutations were detected on 202 chromosomes from men with abnormal sperm parameters, yielding a CF mutation frequency of 8.9%; no mutations were detected on 52 chromosomes from men with normal sperm parameters. Among the former group, a single mutation was detected in 16 samples and two mutations (R117H and G551D) were detected in one sample. The distribution of mutations by sperm parameters is shown in Table II.

Among 21 men with azoospermia, four mutations were detected in three men (9.5% of chromosomes). Among 80 men with a variety of diagnoses of abnormal sperm parameters, 14 mutations were detected in 14 individuals (8.9% of chromosomes; range = 0% in four men with oligoasthenospermia to 15.6% in 32 men with oligoasthenoteratozoospermia).

The frequencies of each of the six mutations that were detected in this sample are shown in Table III. The most common mutation in this sample was G542X (8/18, or 44% of mutations), followed by Δ508 (7/18, or 33% of mutations). Four other mutations (G551D, R117H, 621 + 1G-»T, 3849+10kbC-»T) were each detected in a single individual (each 1/18, or 5.6% of mutations).
in the CFTR gene. CF mutations were present in men with a variety of diagnoses of reduced sperm quality and were not confined to the impairment of a single sperm parameter (Table II). No mutation was predominant in the individuals of this study or in subgroups with specific semen characteristics.

Three of 21 (14.3%) healthy men with azoospermia had at least one mutation (one azoospermic male was a compound heterozygote). As indicated before, CBAVD was excluded by measuring seminal plasma fructose concentrations and total semen volume. However, in this subgroup, the rare presence of fructose-positive cases with obstruction of the vas deferens at high sites cannot be ruled out with certainty. Therefore, the increased frequency reported here of CF mutations in men with azoospermia without CBAVD warrants reconfirmation in a sample where potential obstructions of the vas deferens can be definitely excluded.

The frequencies of mutations in infertile men and in Chicago area individuals with clinical CF are shown in Table III, along with the theoretically derived population carrier frequencies. As mentioned before, the ethnic composition of our study group is unknown because of the sampling procedure. Because the semen specimens were obtained consecutively from patients attending infertility clinics in the Chicago area who presumably also live in this region, a comparison of CF mutation frequencies in our study group with the frequencies of CF carriers and affected men found in the local general population seemed appropriate. The frequency of mutations in this sample (0.089) was significantly higher than that expected in the local (Chicago) population (0.029) (Z = 2.99, one-tailed; P = 0.00139). Increased mutation frequencies in infertile men as compared with general population estimates could result if population carrier frequencies were underestimated. However, CF carrier frequencies would need to be as high as 12% in order for mutations to be detected in 5% of the general (local) population (i.e. the lower estimate of the 95% confidence interval of mutation frequencies in infertile men), which we consider to be unlikely. We could detect only one mutation in all but one infertile male using a limited panel of 13 mutations. The fact that CF carrier status has not been associated with infertility could lead to the speculation that a second undetected mutation is present in those men and that those cases present a variant form of CF, where genital manifestations predominate, as has been similarly proposed for CBAVD.

Recent investigations seem to indicate that this point of view might be overly simplistic. An extensive analysis of the entire coding region of the CFTR gene in 67 patients with CBAVD revealed that 24% of patients were compound heterozygotes and another 42% were carriers of one CFTR mutation. In 34% of patients, no CFTR mutations could be identified (Mercier et al., 1995). Based on a 95–98% detection rate of the applied screening methods in CF populations, Mercier et al. concluded that 20–25% of cases with CBAVD reflect mild forms of CF. The presence of just one CF allele in ~42% of CBAVD patients implies a role of CF in CBAVD, although additional factors or genes are necessary for development of CBAVD in those patients (Mercier et al., 1995). The additional analysis of a DNA variant which causes reduced concentrations of CFTR protein (5T allele) in 109 patients with CBAVD showed the presence of the 5T allele in ~63% of patients with mutations in one copy of the CFTR gene and in 24% of patients with no detectable CFTR mutations (Chillon et al., 1995). Thus, the combination of a CFTR mutation on one chromosome and the 5T allele on the other, leading to severely reduced levels of functional CFTR protein, appears to be another common cause of CBAVD.

Aetiological factors contributing to the picture of CBAVD do not, however, seem to be confined to the CFTR gene. Haplotype analysis in 11 families with a history of CBAVD provided evidence for genetic heterogeneity in two families (Rave-Harel et al., 1995), suggesting that mutations at other loci could be the basis of CBAVD in those cases.

A similar explanation might be applicable to the role of the CFTR gene in spermatogenesis. The significantly increased number of CFTR-mutation carriers among patients with reduced sperm quality suggests an involvement of the CFTR protein in spermatogenesis. However, because of the lower rate of CF-mutation carriers when compared to CBAVD patients (17.5 versus 66%), the impact of additional genes might be even stronger than reported for CBAVD. Furthermore, the potential role of CFTR intron 8 DNA variants, which alter the splicing efficiency of the CFTR mRNA in exon 9 (Kiesewetter et al., 1993), warrants investigation in this context.

A direct involvement of the CFTR protein in sperm development and maturation has been suggested by experimental studies. For example, CFTR mRNA was detected in rat testicular tissue, where it was confined to post-meiotic round spermatids (Trezise et al., 1993). During this developmental stage, haploid spermatids are converted into spermatozoa by a number of morphogenetic changes, including nucleus condensation and dramatic decreases in cytoplasmic volume. The latter is thought to be due to a reduction of intracellular water content. Maximal CFTR expression in round spermatids immediately precedes nuclear condensation and simultaneous decreases in cytoplasmic volume, suggesting a physiological role for CFTR as a chloride channel during rat spermatogenesis (Trezise et al., 1993). Although equivalent experiments in humans have not yet been reported, histological descriptions of testicular tissue in men with clinical CF and CBAVD emphasize a high number of cytological abnormalities that preferentially occur in the spermatid stages (Gottlieb et al., 1991; Kaplan et al., 1968). The onset of CF-related impairment of spermatogenesis in humans parallels the rodent data because pre-meiotic spermatogonia in CF patients appear morphologically normal, whereas the post-meiotic spermatogenic stages show malformations or impairment of nuclear division (Denning et al., 1968).

Additional insight into the involvement of the CFTR protein in sperm function can be derived from in-vitro fertilization studies of epididymal sperm aspirated from males with CBAVD. Spermatozoa from individuals with CBAVD that is associated with CFTR mutations showed significantly reduced fertilization rates as compared to subjects with CBAVD in whom CFTR mutations were not detected (P = 0.000003) (Patrizio et al., 1993b). The loss of fertilization potential of spermatozoa from men with CBAVD and CFTR mutations cannot be solely the consequence of the occlusion of the vasa...
deferentia, because the control group showed comparable sperm parameters, but significantly better fertilization results. When spermatozoa from men with CBAVD are used for intracytoplasmic sperm injection (ICSI), however, fertilization rates are not reduced, which implies a specific defect in zona penetration or membrane fusion capacity in spermatozoa from CBAVD males (Silber et al., 1994). The latter observations suggest that mutations in the CFTR gene are associated with intrinsic biochemical defects in spermatozoa and further support the hypothesis of a role of the CFTR protein in spermatogenesis and function in humans.

In conclusion, this is the first report of CFTR mutations in healthy men with infertility due to sperm abnormalities. The data suggest that mutations in the CFTR gene can have specific effects on sperm development. However, further investigations are needed to elucidate the exact role of the CFTR gene in male gametogenesis. In this context, the search for and definition of the nature of potential second CFTR mutations, if present in those individuals, as well as identification of other genes involved in spermatogenesis would be helpful.

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CF mutations in male infertility