Improved detection of cystic fibrosis mutations in infertility patients with DNA sequence analysis

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BACKGROUND: Accurate determination of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene is critical for genetic counselling and treatment of obstructive azoospermia. Of concern is that detection rates with routine CFTR mutation panels vary widely depending on patient ancestry; and such panels have limited value for azoospermic patients, who are more likely to carry rare mutations. An alternative approach offers comprehensive, CFTR mutation analysis by a DNA sequence method. We investigated whether this method could improve CFTR detection rates in men with obstructive azoospermia in a prospective study of men with obstructive azoospermia and their partners who were referred for genetic counselling and testing at one of two institutions.

METHODS: Sixteen patients with congenital absence of the vas deferens (CAVD, n = 14) or idiopathic obstructive azoospermia (n = 2) were studied. DNA from all patients was analysed for mutations by the DNA sequence method. In addition to this method, six men underwent CFTR analysis by a common 25 or 31 mutation panel coupled with poly T analysis. In 10 subjects, common mutation panel findings were inferred from DNA sequence method results.

RESULTS: Overall, 12/16 (75%) azoospermic patients had one or more CFTR mutations and/or 5T alleles, including 12 mutations in 10 patients (two compound heterozygotes) and seven 5T alleles in six patients (one homozygote). The sequence method detected all mutations and three variants of unknown significance. By comparison, the common mutation panels detected only 3/12 mutations (25%) and 0/3 variants. CONCLUSION: The DNA sequence method detects more CFTR mutations than common mutation panels. Given the serious, clinical consequences of transmitting such mutations, this study underscores the importance of accurate, CFTR mutation detection in men with obstructive azoospermia and their partners.

Key words: azoospermia/congenital absence of the vas deferens/cystic fibrosis/genetic testing/male infertility

Introduction

Obstructive azoospermia describes male infertility due to congenital absence of the vas deferens, or idiopathic epididymal or ejaculatory duct obstruction. Roughly 1–2% of subfertile men have congenital absence of the vas deferens (CAVD) as a cause of infertility (Jequier et al., 1985). Men with CAVD are classified as having either unilateral (CUAVD) or bilateral (CBAVD) absent vasa deferentia. CAVD is associated with mutations in the cystic fibrosis gene, termed the cystic fibrosis transmembrane regulator gene (CFTR; 7q31.2). Indeed, patients with obstructive azoospermia (including those with CAVD) have ≤80% risk of being carriers of at least one CFTR mutation (Casals et al., 1995; Costes et al., 1995; Mercier et al., 1995; Mickel et al., 1995; Rave-Harel et al., 1995; De Braekeleer and Ferec, 1996; Dork et al., 1997; Mak et al., 1999; Wang et al., 2002). Unlike patients with classic CF, who are more likely to have common, severe, homozygous or compound heterozygous CFTR mutations, CAVD patients are more likely to exhibit rare mutations (Chillon et al., 1995; Costes et al., 1995; Zielenski et al., 1995; Dork et al., 1997; Kanavakis et al., 1998). In addition, alterations in the 5-thymidine variant of the polythymidine tract (IVS8-5T) in intron 8 of the CF gene are frequently found in men with CAVD (Chillon et al., 1995; Costes et al., 1995; Zielenski et al., 1995; Bienvenu et al., 1997). Practice guidelines set forth by the American Society for Reproductive Medicine (ASRM), the American College of Medical Genetics (ACMG) and the American Urological Association (AUA) advise practitioners to offer CF genetic testing to patients with obstructive azoospermia to determine possible genetic causes (Grody et al., 2001; Gangel, 2002). Testing is also strongly advised for the patient’s partner to assess carrier status, since this knowledge allows for the correct calculation and counselling
of genetic risk for offspring. This information is often useful for reproductive decision-making because couples who are carriers may elect prenatal genetic testing. Furthermore, they may also be potential candidates for preimplantation genetic diagnosis (PGD), which allows for selection of unaffected embryos after in vitro biopsy and genotyping.

Several variables make genetic testing and counselling of CAVD patients complex. First, routine cystic fibrosis screening panels consist of a variable number of the most commonly detected mutations, currently ranging from 25 to 87. Second, detection rates vary widely depending not only on the number of mutations analysed, but also on patient ancestry, ranging from 30% in Asians (Macek et al., 1997) to >90% in Northern European Caucasians (Cystic Fibrosis Genetic Analysis Consortium, 1994). Third, there are many possible mutations to consider for analysis as >1000 CF mutations are documented in the literature (Cystic Fibrosis Mutation Database: http://www.genet.sickkids.on.ca/cftr). Finally, men with CAVD have an atypical CF phenotype and are more likely to have mutations not detectable on standard mutation panels. For these reasons, there are inaccuracies in the current practice for counselling and testing of CAVD patients.

Because of the low detection rate of CF mutations in CAVD men and the inherent, uninformative nature of the standard mutation panel for non-Caucasian patients, we sought to determine whether a more extensive screening method involving modified temporal temperature gradient gel electrophoresis (mTTGE) and gene sequence analysis (The Ambry Test) might prove more informative for detecting CF mutations. In particular, we sought to determine whether this method might improve CF mutation detection rates in men with obstructive azoospermia by reducing the variables introduced by limited mutation spectrum and patient ancestry. In this study, we report our initial findings with this CF mutation screening approach in a cohort of patients.

### Materials and methods

#### Patient selection

Study patients were prospectively selected from a population of infertile men attending the male infertility clinic at the University of California San Francisco (UCSF) or the California Pacific Medical Center (CPMC) over a 3 year period. A detailed history and physical examination was performed on all patients by an experienced urologist. The diagnosis of congenital unilateral (CUAVD), bilateral (CBAVD) absence of the vas deferens or idiopathic obstruction was based on physical examination findings. Testis volume was estimated with a Prader orchiometer (ASSI, USA). In addition, a semen analysis, centrifuged pellet of ejaculate and post-ejaculate urine were examined. Renal ultrasonography was recommended to all patients to assess renal morphology and organ presence. Hormone evaluation of the pituitary–gonadal axis was also routinely performed. Affected men were referred to PROGENI (Program in the Genetics of Infertility) at UCSF or the Genetic Risk Assessment Program at CPMC for further genetic assessment and counselling regarding their diagnosis, genetic risks for offspring, and consideration of infertility treatment. Genetic counselling was provided by two experienced genetic counsellors (L.D.B., K.L.D.) Appropriate genetic testing was discussed with each patient and consent was obtained.

CFTR mTTGE and DNA sequencing was offered to patients with obstructive azoospermia based on the following criteria: (i) new diagnosis in an individual with no prior genotyping, or (ii) individuals who were members of an ethnic group with an assumed low detection rate by common mutation panel, with or without a prior, negative, CFTR mutation panel result. Other patients chose this specific method of CFTR analysis for personal, financial or other reasons. CFTR analysis was offered to all patients’ female partners.

#### Description of CFTR genotyping methods

##### Common mutation panel (25 or 31 mutations)

CFTR mutation analysis was performed on blood by DNA extraction, PCR amplification and hybridization to allele-specific oligonucleotides in the reverse dot blot format. Testing included the 25 most common cystic fibrosis mutations as recommended by the American College of Medical Genetics (Grody et al., 2001). At UCSF, an additional six mutations and one polymorphism were included in the mutation panel (Table I). In addition, CFTR variable 5T, 7T and 9T repeats of intron 8 were analysed on leukocyte DNA, amplified by PCR, and hybridized to allele-specific oligonucleotides in the reverse dot blot format.

##### mTTGE and DNA sequencing methodology

Patient genomic DNA was isolated from whole cell blood according to the manufacturer’s recommendations using the GFX genomic blood isolation kit (Amersham Pharmacia Biotech, USA). gDNA quality and quantity were assessed by gel agarose electrophoresis. The Ambry Test CF includes a full mutation scan of the CFTR gene by modified temporal temperature gradient electrophoresis analysis (mTTGE) followed by dye terminator DNA sequencing of suspect regions. All CFTR exons as well as relevant intronic regions were amplified using PCR and proprietary primers. Standard PCR amplification was performed using HotStarTaq Master Mix (Qiagen, USA) with 100–150 ng input gDNA per reaction. Typical PCR conditions were: one cycle: 95°C for 15 min; 35 cycles: 94°C for 30 s, 54°C for 30 s, 72°C for 30 s; 1 cycle: 72°C for 10 min. Annealing temperatures differ depending on primer pair. Prior to Ambry gel analysis the PCR products were denatured and slowly cooled to allow for maximal heteroduplex formation. For a subset of CFTR regions, DNA was mixed with known wild type DNA to facilitate detection of homozygous mutations. PCR products were then processed for mTTGE on DCode gels (BioRad, USA) in accordance with the Ambry Test technology. Polyacrylamide gels were analysed for the presence of mutations following staining in ethidium bromide (EtBr) and image capture under UV using the Gel Doc 1000 system.

### Table I. List of CFTR mutations included in common mutation panels

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Description</th>
<th>Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508</td>
<td>R553X</td>
<td>A455E</td>
</tr>
<tr>
<td>G542X</td>
<td>1717G→A</td>
<td>1898G→A</td>
</tr>
<tr>
<td>G551D</td>
<td>621G→A</td>
<td>2148GΔIa</td>
</tr>
<tr>
<td>R117H</td>
<td>R560T</td>
<td>711G→T</td>
</tr>
<tr>
<td>W1282X</td>
<td>3658GΔC</td>
<td>2789G→A</td>
</tr>
<tr>
<td>N1303K</td>
<td>3210G→A</td>
<td>R347P</td>
</tr>
<tr>
<td>R1162X</td>
<td>I148T</td>
<td>1078GΔIe</td>
</tr>
<tr>
<td>3849+10kbC→T</td>
<td>G85E</td>
<td></td>
</tr>
<tr>
<td>ΔI507</td>
<td>R334W</td>
<td></td>
</tr>
<tr>
<td>Y1092X</td>
<td>3905insT</td>
<td></td>
</tr>
<tr>
<td>R347H</td>
<td>5549N</td>
<td></td>
</tr>
<tr>
<td>3849+4</td>
<td>F508C (polymorphism)</td>
<td></td>
</tr>
</tbody>
</table>

Six additional mutations and one polymorphism in UCSF panel (31 mutations)

Y1092X   | 3905insT    |       |
R347H    | 5549N       |       |
3849+4   | F508C (polymorphism) |       |
Gel analysis was performed by two individuals and fragments were scored against known controls. Amplified regions indicating the presence of a mutation were subsequently processed for sequencing. Relevant exons were first amplified with a unique primer set using Taq PCR Master Mix (Qiagen, USA). Typical PCR conditions were: one cycle: 95°C for 5 min; 35 cycles: 94°C for 30 s, 54°C for 30 s, 72°C for 30 s; one cycle: 72°C for 10 min. Annealing temperatures differ depending on primer pair. PCR products were analysed via agarose gel electrophoresis, followed by treatment with Exosap-It (USB, USA) according to the manufacturer’s recommendations. Standard dye terminator cycle sequencing (DTCS; Beckman Coulter, USA) was conducted followed by loading onto a CEQ2000 sequencer. Exons were always sequenced in both sense and antisense directions. In rare instances, only one direction may give adequate sequence due to repeat regions such as the poly T site upstream of CFTR exon 9. Sequence analysis was performed by two individuals and data matched back to the TTGE data. The Ambry test covers all CFTR exons (OMIM 602421), >20 bases 5’ and 3’ into each intron, as well as selected deep intronic mutations.

**Results**

**Subject characteristics**

Among 16 male patients (labelled nos. 1–16) with obstructive azoospermia, 13 were diagnosed with CBAVD, one with CUAVD, and two with idiopathic epididymal obstruction. Of four CBAVD patients who underwent renal ultrasounds, two were found to have unilateral renal agenesis. Male patient ethnicities included: nine Asian or Asian-Indian, four Caucasian, two Hispanic, one mixed Caucasian/Asian/Ashkenazi Jewish. Female partner ethnicities included: nine Asian/Asian-Indian, two Caucasian, one Hispanic, one mixed Caucasian/Asian, and two mixed Caucasian/Ashkenazi Jewish.

**CFTR mutations with obstructive azoospermia/CAVD**

DNA from all 16 male patients was analysed by the DNA sequence method (mTTGE and DNA sequencing). Six male subjects had CFTR mutations and 5T alleles analysed by a common 25 or 31 mutation panel in conjunction with poly T analysis, and these findings were directly compared to those from the DNA sequence method. In 10 subjects, the common mutation panel findings were inferred from the DNA sequence method results.

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Diagnosis/clinical information</th>
<th>Ancestry</th>
<th>5T allele</th>
<th>Common mutation panel</th>
<th>Sequence method</th>
<th>Interpretation</th>
<th>Mutation panel/DNA sequence concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CBAVD</td>
<td>N.E. Cauc.</td>
<td>Negative</td>
<td>Negative</td>
<td>Het. P750L</td>
<td>Mutation</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>CBAVD</td>
<td>Asian</td>
<td>Negative</td>
<td>Negative</td>
<td>Het. V201M</td>
<td>No mutation detected</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>CBAVD</td>
<td>Asian</td>
<td>Negative</td>
<td>Negative</td>
<td>Het. 1717→4A→G</td>
<td>Variant of unknown significance</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>CBAVD</td>
<td>Asian-Indian</td>
<td>Negative</td>
<td>Negative</td>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>CBAVD</td>
<td>Asian</td>
<td>Negative</td>
<td>*</td>
<td>V520I/3601→3C→A</td>
<td>Mutation/variant of unknown significance</td>
<td>No†</td>
</tr>
<tr>
<td>6</td>
<td>CBAVD*</td>
<td>N.E./S.E. Cauc.</td>
<td>Het.</td>
<td>*</td>
<td>ΔF508/R117H</td>
<td>Mutation/mutation</td>
<td>Yes‡</td>
</tr>
<tr>
<td>7</td>
<td>CBAVD</td>
<td>Asian-Indian</td>
<td>Het.</td>
<td>Negative</td>
<td>Het. V456A</td>
<td>Mutation†</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>CBAVD</td>
<td>Asian</td>
<td>Negative</td>
<td>*</td>
<td>Het. Q1352H</td>
<td>Mutation</td>
<td>No†</td>
</tr>
<tr>
<td>9</td>
<td>CBAVD</td>
<td>Asian</td>
<td>Negative</td>
<td>*</td>
<td>Negative</td>
<td>No mutation detected</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>CBAVD</td>
<td>N.E. Cauc./Asian/Ashkenazi</td>
<td>Negative</td>
<td>*</td>
<td>I556V/2752→26A→G</td>
<td>Mutation/mutation</td>
<td>No†</td>
</tr>
<tr>
<td>11</td>
<td>CBAVD</td>
<td>Hispanic</td>
<td>Homozygous</td>
<td>*</td>
<td>Het. W1098C</td>
<td>Mutation</td>
<td>No‡</td>
</tr>
<tr>
<td>12</td>
<td>CBAVD</td>
<td>Asian</td>
<td>Negative</td>
<td>Negative</td>
<td>Het. 3499+25C→G</td>
<td>Variant of unknown significance</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>CUAVD</td>
<td>Hispanic</td>
<td>Negative</td>
<td>*</td>
<td>Negative</td>
<td>No mutation detected</td>
<td>Yes‡</td>
</tr>
<tr>
<td>14</td>
<td>CBAVD</td>
<td>N.E. Cauc.</td>
<td>Negative</td>
<td>*</td>
<td>Negative</td>
<td>No mutation detected</td>
<td>Yes‡</td>
</tr>
<tr>
<td>15</td>
<td>Idiopathic obstruction</td>
<td>Asian-Indian</td>
<td>Negative</td>
<td>*</td>
<td>Het. I807M</td>
<td>Mutation‡</td>
<td>No†</td>
</tr>
<tr>
<td>16</td>
<td>Idiopathic obstruction</td>
<td>N.E. Cauc.</td>
<td>Het.</td>
<td>*</td>
<td>ΔF508</td>
<td>Mutation</td>
<td>Yes‡</td>
</tr>
</tbody>
</table>

*Analysis not done.
*Brother diagnosed with classic CF.
Subject has unilateral renal agenesis.
Unpublished data indicates sequence change is deleterious (Ambry Genetics, personal communication).
Concordance inferred based on known mutations included in the common mutation panel.
Ashkenazi = Ashkenazi Jewish; CBAVD = Congenital bilateral absence of the vas deferens; CUAVD = Congenital unilateral absence of the vas deferens; Het. = Heterozygous; N.E. Cauc. = Northern European Caucasian; S.E. Cauc. = Southern European Caucasian.
common mutation panel detected only 3/12 mutations (25%) and 0/3 variants of unknown significance (Table IV). The nine mutations not found by the common mutation panel were outside the spectrum of mutations included in the test panel. This was also true for the three variants of unknown significance.

**Mutations in females and comparison of methods**

Among the female partners (labelled nos. 1F–16F, Table V), 12 underwent testing with the DNA sequence analysis method, two were analysed by the common mutation panel only, and one was tested by CSGE only. DNA sequence identified two CFTR mutations (I807M, L997F) in two subjects (14F and 15F respectively) and one 5T allele (12F). Neither of the mutation carriers had CFTR screening by the common mutation panel; however, given that both mutations lay outside the spectrum of mutations included in the panel, it is assumed that neither would have been identified by this method. Three additional mutations in three female subjects were identified by two other test methods: two mutations (ΔF508 and G551D) by the common mutation panel and one mutation (R74W) by CSGE. DNA sequence analysis was not done on these three subjects, so a direct comparison of methods cannot be made.

**Discussion**

CFTR analysis performed by the sequence method in obstructive azoospermia identified 75% of patients with at least one mutation or 5T allele. These findings are in agreement with previously published results (Casals et al., 1995; Costes et al., 1995; Mickle et al., 1995; Rave-Harel et al., 1995; DeBraekeleer and Ferec, 1996; Dork et al., 1997; Mak et al., 1999; Wang et al., 2002). The comparison of two distinct methods of CFTR analysis demonstrated that only 25% of mutations identified by the sequence method were detectable by the common mutation panel. While the sample size in this study is small, this detection difference is consistent with several other studies that suggest that more extensive testing will improve CFTR mutation detection rates (Mak et al., 1999; Dork et al., 1997; Claustres et al., 2000; Wang et al., 2002).

Wang et al. found that using mass spectrometry and primer oligonucleotide base extension for 100 mutations increased detection by 12% in those who were previously not thought to have any CFTR mutations and identified a second mutation in >50% of those considered to be heterozygous after 25 mutation panel analysis (Wang et al., 2002). Likewise, Mak et al. (1999) demonstrated that a routine 31 mutation panel failed to detect 54% of mutations found by multiplex heteroduplex shift analysis followed by direct sequencing. Clearly, mutation analysis using a common mutation panel for men with obstructive azoospermia fails to detect genetic mutations in this population. This is understandable given that the common mutation panel was originally designed to detect the most common cystic fibrosis mutations in affected patients of Northern European Caucasian descent.

Data from this study also confirm that the spectrum of mutations in men with obstructive azoospermia or CAVD is different from those found on common mutation panels. Since rare mutations are associated with obstructive azoospermia, a diagnosis that affects men of all ethnicities, it follows that common mutation panels are inadequate for mutation detection in this population. On the contrary, the sequence method has the unique potential to address both of these testing variables.

The most common genetic finding in this series of men was the 5T allele. This is in agreement with other investigated populations that show a prevalence of 5% (Kiesewetter et al., 1993; Chillon et al., 1995). Carriers of the 5T allele would be expected to have reduced levels of normal CFTR mRNA and ultimately CFTR protein product, due to the deletion of exon 9. The high frequency of this relatively mild allele suggests that
In addition to the mutations (i.e. R117H) may exert a more deleterious effect than the 5T allele. These findings illustrate the range of phenotypic effects observed with the 5T allele.

Ultimately, cystic fibrosis mutation testing for the infertility population attempts to answer two important questions: (i) are CFTR mutations the cause for the CAVD or obstructive azoospermia in the individual? and (ii) what are the genetic risks for the patients’ offspring? It is the second question that makes the genetic testing particularly compelling for couples, since reproductive technologies now allow these men to father children. Since the risk of passing on CFTR genetic mutations is a real possibility, it is important that female partners undergo carrier testing for CF so that an accurate assessment of genetic risk is possible. The phenotype for offspring who inherit one or more mutations ranges from normal to CAVD (in sons) to chronic cough, which could represent a mild CF phenotype.

The importance of accurate genetic counselling and assessment of a couple’s risk of having a child with cystic fibrosis cannot be underestimated and is further illustrated by the following example. In one couple, the patient (subject 1), a Northern European Caucasian male with CBAVD and no family history suggestive of CF, was tested for CFTR mutations by the common mutation panel and found to be negative. The test report, which was based solely on his ethnicity and the 90% detection capability of the test, calculated his residual risk to be a carrier for an undetectable mutation (not included in the common panel). With this a priori risk of carrying one or more mutations.

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information, the couple’s risk of having a child with CF was
now calculated to be 1/4 (25%), and the risk of CBAVD
estimated to be as high as 50%, which represent much higher
risks than previously calculated with the panel results. The
additional clarification of their risk led this couple to pursue
PGD to prevent the birth of a child with CF. Thus, improving
CFTR mutation detection in at-risk populations can signifi-
cantly alter the reproductive care options chosen by mutation-
carrier couples.

Finally, although CF mutation detection rates are improved
with DNA sequence analysis, this technique can also introduce
new testing variables. In this study, we observed three
previously uncharacterized sequence alterations, the clinical
significance of which is unclear. Alterations are considered
mutations when evidence suggests that the sequence change
significantly alters the gene or protein. In the absence of
molecular data, there may also be phenotypic or other clinical
data to support the view that the sequence change is deleterious
(Cystic Fibrosis Mutation Database: http://www.genet.sickkid-
s.on.ca/cftr). The relevance of such novel variations poses a
challenge when observed in patients with CAVD or obstructive
azoospermia. Are they mild mutations associated with an
atypical phenotype or simply coincidental findings? If any of
the alterations were inherited along with a known severe
mutation, would the individual have classic CF?

In some cases, it is possible to clarify whether a variant of
unknown significance is truly a novel, deleterious mutation. In
this study, we determined that two previously uncharacterized
alterations were inherited along with a known severe
mutation (Ambry Genetics, personal communication). Mutation V456A (subject 7) has
since been detected in three other symptomatic individuals,
including: a Caucasian fetus with echogenic bowel, who has
one other deleterious mutation, ΔF508; a Pakistani adult
female with pulmonary symptoms and referred for CF testing
who also harbors a ΔF508 mutation; and an Asian female infant
with positive newborn screening and sweat tests, who has one
other mutation, R709X. These clinical data from three
compound heterozygote provides compelling evidence that
V456A is a genuine mutation.

The second alteration involves I807M (subjects, 15 and 15F),
detected in both our Asian-Indian patient and his consangui-
nous wife (they are first cousins). Recently, this alteration has
been found in two other symptomatic individuals: a Caucasian
child with chronic pancreatitis and a positive sweat test, with
a Q1352 mutation, and a newborn suspected of having CF
without another, identified mutation (Ambry Genetics, personal
communication). The suspicion of I807M as a genuine
mutation is also supported by the fact that the majority of
amino acid-changing exonic alterations are deleterious and that
most intronic sequence changes reported as deleterious are
located within 10 base pairs of exons. The challenge of
interpreting sequence variations will certainly continue along
with our experience with the DNA sequence method, but the
goal of improved care for infertile couples is a worthy one.

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of cases only one CFTR allele could be detected. Hum Genet 95,205–211.
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