Cystic Fibrosis Transmembrane Conductance Regulator Gene Mutations in Infertile Males with Congenital Bilateral Absence of the Vas Deferens

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UZUN, S., GÖKÇE, S. and WAGNER, K. Cystic Fibrosis Transmembrane Conductance Regulator Gene Mutations in Infertile Males with Congenital Bilateral Absence of the Vas Deferens. Tohoku J. Exp. Med., 2005, 207 (4), 279-285 —— Congenital bilateral absence of the vas deferens (CBAVD) is characterized by azoospermia and male infertility. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are associated with cystic fibrosis (CF), the most common autosomal recessive disorder in Caucasians. Recent publications on CBAVD raised the question whether CFTR gene mutations are responsible for CBAVD occurrence or not. This study was conducted to explore the role of CFTR gene mutations in the occurrence of CBAVD-dependent male infertility. Forty-four chromosomes of 22 CBAVD patients from Austrian ancestry were studied. For detection of the most common mutation ΔF508, a deletion of phenylalanine at the 508th position of mature CFTR chloride channel protein, the 10th exon of the gene was screened by heteroduplex analysis. In order to identify non-ΔF508 mutations, we also analyzed the entire coding regions, exon/intron boundaries of 27 exons and the 5′- and 3′-untranslated regions of the gene by denaturing gradient gel electrophoresis (DGGE) after polymerase chain reaction. All exons showing different banding patterns on the DGGE gels were sequenced to define existing DNA sequence variations. Among the analyzed 44 chromosomes of 22 patients, disease producing mutations were found in 31.8% (14/44). The most common mutation was ΔF508 with a frequency of 43% (6/14), followed by R117H with 29% (4/14). Our results indicate that CFTR gene mutations are common but not the only reason for the occurrence of CBAVD-dependent male infertility. We recommend screening of the CFTR gene in these patients. ——— CFTR gene mutations; CBAVD; male infertility; cystic fibrosis; CF
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Congenital bilateral absence of the vas deferens (CBAVD) is characterized by azoospermia and male infertility (Anguiano et al. 1992; Oates and Amos 1993). It is found in 6% of obstructive azoospermia cases and it is present in 1-2% of healthy males showing infertility (Wang et al.)
Genetic analysis of 150 males with infertility identified about 10% abnormal karyotypes, 5% AZFc (azoospermia factor c region) deletions and 10% cystic fibrosis transmembrane conductance regulator (CFTR) mutations (Dohle et al. 2002). A high number of cystic fibrosis (CF) gene mutations were also reported previously in CBAVD patients (Casals et al. 1995; Mercier et al. 1995; Wang et al. 2002).

CF is a multi-system disease caused by a defective function of CFTR protein. More than thousand mutations in all domains of the protein have been described, resulting in CFTR dysfunction and producing a wide range of effects contributing to the clinical phenotype. The encoded protein is a cyclic adenosine monophosphate (cAMP) regulated chloride channel mainly found in exocrine glands and secretory epithelia. Chronic obstructive pulmonary disease (COPD), pancreatic exocrine insufficiency (PI), elevated sweat electrolytes and CBAVD dependent male infertility are the most prominent clinical features of this common, autosomal recessive inherited disorder. The loss of CFTR chloride channel function leads to an imbalance of secretory and absorptive ion transport processes that initiate a cascade of events beginning with salt and water depletion and rapidly progress to depressed clearance of mucus in the lungs, bacterial colonization, chronic inflammation, bronchiectasis and ultimately respiratory failure (Boucher 2002; Powell and Zeitlin 2002). Population analysis showed a 1/2500 occurrence risk and 1/25 carrier frequency in Caucasians, but frequencies are different in various European populations. The most common mutation in almost all populations is a deletion of 3 base pairs named ΔF508, resulting in the loss of the amino acid phenylalanine at position 508 in mature CFTR protein (Giovanni and Devoto 1990; Wagner et al. 1992).

In CBAVD patients, ΔF508 is the most common CFTR gene mutation and the second common mutation is a missense change, R117H (Gervais et al. 1993; Casals et al. 1995; Mercier et al. 1995). Other CF mutations, G542X, G551D, D1152H, M470W, R334W, R74W, M952I, W1282X, N1303K, and G85E, are known to be involved in CBAVD etiology (Wang et al. 2002; Danziger et al. 2004).

Both CF and CBAVD are known to be inherited autosomally recessively. Autosomal recessive inheritance required at least one disease producing mutation on each allele of the gene, leading to homozygosity or compound heterozygosity, to develop clinical phenotype of the particular disease. And the carriers (heterozygotes) do not show clinical manifestations of the disease (Dean et al. 1990).

According to the phenotypic effects there are severe and mild alleles in CF genotype. While severe alleles lead to recurrent COPD and PI, mild alleles mostly cause mild pulmonary disease or CBAVD-dependent infertility without other clinical signs of CF disease. ΔF508 and G542X are known as severe alleles and R117H as a mild allele. Homozygosity of a mild allele or compound heterozygosity of mutant alleles, such as R117H/D1152H (mild/mild) or R117H/ΔF508 (mild/severe), could cause a mild form of CF (Dean et al. 1990; Kerem et al. 1990; Pignatti 1994) or male infertility without other clinical signs (Gervais et al. 1993; Oates and Amos 1993). The aim of this study was to explore the role of CFTR gene mutations in the occurrence of patients with CBAVD.

**MATERIALS AND METHODS**

Forty-four chromosomes of 22 adult infertile males from Austrian ancestry clinically diagnosed with CBAVD have been analyzed to identify the presence of point mutations in the CFTR gene. None of 22 patients showed clinical manifestations of cystic fibrosis, renal malformations, absence of epididymis or seminal vesicles. For the study procedures involving human materials, the approval from Ethic Committee of Medical University of Graz and written informed consent from each individual have been obtained. Ten ml of venous blood samples of patients were obtained. Genomic DNA was extracted according to phenol-chloroform extraction method (Maniatis et al. 1982). To determine the most common mutation ΔF508, exon 10 of the CFTR gene was scanned by heteroduplex analysis (Rommens et al. 1990). To identify other non-ΔF508 mutations, the entire coding region composed of 27 exons, exon/intron splice regions, and 5’- and 3’-untranslated region of the gene were
scanned for possible sequence variations by PCR followed by denaturing gradient gel electrophoresis (DGGE) (Saiki et al. 1988; Fanen et al. 1992; Macek et al. 1997).

Both normal and positive controls have been used (double controlled). Internal primers were used by PCR amplification of exons for DGGE, in order to provide analysis of 3’ and 5’ exon-intron splice regions simultaneously (Zielenski et al. 1991; Fanen et al. 1992). To enhance the resolution of PCR products on the DGGE gel, one of the primers carried a GC clamp (Myers et al. 1988; Fanen et al. 1992). Reaction conditions were provided as 10 mM Tris HCl pH 8.8, of 25°C, 50 mM KCl, 1.5 mM MgCl$_2$, 0.1% Triton X-100, 100 mM dNTP’s, 4 pmol of each primer and 0.4 unit of Taq polymerase. To enhance the reaction specificity and avoid non-specific amplifications 0.5% of tetramethylammonium chloride (TMACl) was added to reaction mixture. Annealing temperatures were varied between 48-68°C depending on the exons and 45 PCR cycles were performed.

Based on our optimization results, PCR products were analyzed overnight on denaturing polyacrylamide gel electrophoresis containing a denaturant gradient between 0-100% resulting in different band patterns. Coding and flanking regions of each exon showing different band pattern, in comparison to normal controls, on the DGGE gel were sequenced (Zielenski et al. 1991; Greil 1995). Sequencing reaction was performed after nested PCR with biotin labeled primer followed by M-280 streptavidin (Dynal Biotech, Oslo, Norway) solid phase separation process (Uhlen 1989). Thermo Sequenase Version 2.0 kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and IRD-41 dye labeled primers (MWG Biotech, Ebersberg, Germany) were used for sequencing.

Samples were analyzed automatically with LI-COR Model 4000 Sequencer (MWG Biotech) (Middendorf et al. 1992) and results were evaluated according to the Cystic Fibrosis Mutation Database.

**RESULTS**

Ten out of 22 patients, who do not have clinical signs of CF except for CBAVD-dependent infertility were found to carry CFTR gene mutations in a homozygote (1/10), compound heterozygotes (3/10) or heterozygotes (6/10) (Table 1). Thus, 14 of 44 chromosomes analyzed contain CFTR gene mutations. The common mutation ΔF508 leading to severe phenotypes was found in 6 cases, 4 heterozygotes and two compound heterozygotes; namely, 6 of 14 mutant alleles (43%) were ΔF508 positive. Second common mutation R117H allele frequency was found in 4/14 CF alleles (29%). No homozygous patient for ΔF508 mutation was found our 22 CBAVD patients. The genotypes of two compound heterozygotes were ΔF508/R117H and ΔF508/M952I. The remaining four patients with ΔF508 were a heterozygote without a second CFTR mutation. The genotypes of 3 patients with R117H mutation were one homozygote R117H/R117H and two compound heterozygotes, R117H/621+1 G- > T and R117H/ΔF508. The remaining two patients were heterozygous for the nonsense mutation G542X and missense mutation D1152H (Table 1). In the 12/22 of CBAVD patients, no CFTR gene mutation was found.

Evaluation of our DGGE results in comparison to controls showed no difference in exons 2, 3, 5, 6a, 7, 8, 12, 13, 14b, 17a, 17b, 19, 22, 23 and 24 and exon/intron splice regions of CFTR gene, in our patients. Different band profiles in exons 1, 4, 6b, 9, 10, 11, 14a, 15, 16, 18, 20 and 21 were further analyzed by DNA sequencing. Disease producing mutations have been found in exons 4, 10, 11, 15 and 18 (Fig. 1). The other sequence variations were evaluated as polymorphisms described already in the Cystic Fibrosis Mutation Database (data is not shown).

The non-ΔF508 mutations detected are the following disease-producing mutations (Fig. 1 and Table 1).

621+1 G- > T. A G to T transversion in the exon/intron splice region at nucleotide position 621+1 in intron 4, leading to a splicing mutation. This mutation was found on the one allele. The patient is a compound heterozygote with a mild missense mutation R117H.

**R117H Mutation.** A G to A transition at nucleotide position 482 in the coding region of exon 4. The mutation causes the substitution of arginine with histidine at amino acid position 117 of the mature CFTR protein. This mutation was found in 4 of 44 alleles in 3 patients; one case is homozygote and other two are compound heterozygotes of R117H/621+1 G- > T and R117H/ΔF508.
Fig. 1. A schematic diagram of exon/intron organization of CFTR gene and locations of ΔF508 and non-ΔF508 mutations were identified in our study (for details see text). The CFTR gene is 250 kb in size and composed of 27 exons. The diagram was adapted from Zielenski et al. (1991). Exons; introns, DNA strand.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Exon 10</th>
<th>Exon 4</th>
<th>Exon 11</th>
<th>Exon 15</th>
<th>Exon 18</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1440</td>
<td>ΔF508 mut. het</td>
<td>R117H Missense mutation, het.</td>
<td></td>
<td></td>
<td></td>
<td>ΔF508 / R117H</td>
</tr>
<tr>
<td>1474</td>
<td></td>
<td>R117H Missense mutation, hom.</td>
<td></td>
<td></td>
<td></td>
<td>R117H / R117H</td>
</tr>
<tr>
<td>1628</td>
<td></td>
<td>R117H Missense mut., het. 621 + 1 G- &gt; T Splice site mutation, het.</td>
<td></td>
<td></td>
<td></td>
<td>R117H / 621 + 1 G- &gt; T</td>
</tr>
<tr>
<td>1635</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>D1152H Missense mutation, het.</td>
</tr>
<tr>
<td>1725</td>
<td>ΔF508 mut. het</td>
<td></td>
<td></td>
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<td>ΔF508 / --</td>
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<tr>
<td>1827</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G542X Nonsense mutation, het.</td>
</tr>
<tr>
<td>1879</td>
<td>ΔF508 mut. het</td>
<td></td>
<td></td>
<td></td>
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<td>2097</td>
<td>ΔF508 mut. het</td>
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<td>ΔF508 / --</td>
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<tr>
<td>2162</td>
<td>ΔF508 mut. het</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M952I Missense mutation, het.</td>
</tr>
<tr>
<td>2448</td>
<td>ΔF508 mut. het</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ΔF508 / --</td>
</tr>
</tbody>
</table>

het, heterozygote; hom, homozygote; mut, mutation.
**G542X Mutation.** A G to T transversion at nucleotide position 1756 in exon 11 generates a nonsense mutation. Glycine at amino acid position 542 is changed to a stop codon. This mutation was found on one allele; the patient is heterozygous for the mutation.

**M952I Mutation.** A missense mutation caused by a G to C transversion at nucleotide 2988 in exon 15. Methionine at the amino acid position 952 is changed to isoleucine. This mutation was found on one allele; the patient is a compound heterozygote of M952I/ΔF508.

**D1152H Mutation.** A missense mutation caused by a G to C transversion at nucleotide 3586 in exon 18. Asparagine at the amino acid position 1152 is changed to histidine. This mutation was found on one allele; the patient is heterozygous.

**DISCUSSION**

CBAVD is characterized by bilaterally absence of the vas deferens leading to azoospermia and male infertility. It appears to be a consequence of CF and 97% of CF male patients show CBAVD. On the other hand, the isolated CBAVD cases without clinical manifestations of CF were found to have CFTR gene mutations (Anguiano et al. 1992; Oates and Amos 1993; Wang 2002).

In this study, we have found that 10 out of 22 isolated CBAVD cases (45%) have at least one disease-producing mutation and 4 of 22 (18%) at least two mutant alleles, in the analyzed region of the CFTR gene. In the remaining 12 patients (55%), no CFTR gene mutation was found in the scanned area. Thus, in the 14 out of 44 (31.8%) chromosomes analyzed, a disease producing mutation was found (Table 1).

Although clinically diagnosed as CBAVD, none of the patients in our group show typically severe clinical manifestations of CF disease. The heterozygous ΔF508 mutation was found in 4 of cases. The genotypes R117H/R117H (homozygosity of mild/mild), ΔF508/M952I and ΔF508/R117H (compound heterozygosity of severe/mild), R117H/621+1 G- > T (compound heterozygosity of mild/mild) (Table 1) are known to cause male infertility with CBAVD but without CF clinical signs (Gervais et al. 1993; Oates and Amos 1993). The compound heterozygous status ΔF508/R117H is a genotype that occurs commonly in CBAVD patients (Wang et al. 2002).

Despite our extensive analysis no sequence variations in the exons 2, 3, 5, 6a, 7, 8, 12, 13, 14b, 17a, 17b, 19, 22, 23, 24 and related exon/intron splice regions have been found. This may be due to the conservation of these exons in CBAVD genotypes during evolution. No mutations or sequence variations were found in the 13th exon, encoding regulatory domain of CFTR protein. This may be a reason of non-occurrence of CF clinical manifestations in CBAVD phenotype. On the other hand, many of sequence variations observed in exon 10 are classified as polymorphisms (data is not shown). More than two sequence variations in the CFTR gene were present in all our CBAVD patients, but only on the 31.8% of chromosomes disease producing mutations were found (Table 1), whereas the remaining sequence variations were polymorphisms (data is not shown).

Comparing our results to population analysis data, carrier frequency raised from 1/25 to 10/22 (from 4% to 45%) and mutations in both alleles rose from 1/2500 to 4/22. These figures give an average 11 fold increase of carrier frequency and about 455 fold increase of homozygous probability of CFTR gene mutations in CBAVD patients. Our findings support data on carrier frequency in patients with CBAVD published previously (Anguiano et al. 1992; Gervais et al. 1993; Oates and Amos 1993; Casals et al. 1995). The increase of CFTR gene mutation frequency in CBAVD patients group suggests a significant association between CBAVD phenotype and CFTR gene mutations. However CFTR gene mutations are not the only cause for CBAVD-dependent male infertility. There may be some secondary effects of yet unknown genes (heterogeneity, polygeneity) or environmental effects impact the occurrence of CBAVD phenotype (Anguiano et al. 1992; Augarten et al. 1994; Mercier et al. 1995; Rave-Harel et al. 1995; Boucher 2002). The CFTR gene polymorphisms may be also the potential enhancers of CBAVD phenotype occur-
ference (Larriba et al. 2001).

In conclusion, CFTR gene mutations are the major but not the only cause for the occurrence of CBAVD-dependent male infertility. Some unknown factors and genes are likely to be involved in the pathogenesis of CBAVD. And infertile males with CBAVD have the possibility of having children by assisted reproduction technologies such as in vitro fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) (Aittomaki et al. 2004). Because of high mutation prevalence in such patients and CFTR gene mutations may also exist in their partners, offspring with CF disease may be born. Therefore we recommend genetic testing and counseling for couples undergoing IVF or ICSI procedure, to avoid having children with CF disease. The karyotype and Y-chromosome microdeletions should be also checked in the donors.

References


