Mutation spectrum of the CFTR gene in Taiwanese patients with congenital bilateral absence of the vas deferens

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BACKGROUND: Clinically affected cystic fibrosis (CF) patients present a spectrum of genital phenotypes ranging from normal fertility to moderately impaired spermatogenesis and congenital bilateral absence of vas deferens (CBAVD). Little is known about the CF incidence in the Taiwanese population. It has been shown that the CBAVD in men without clinical evidence of CF is associated with a high incidence of mutated CFTR (cystic fibrosis transmembrane conductance regulator) alleles. In order to understand the involvement of the CFTR gene in the aetiology of Asian/Taiwanese male infertility, we screened the entirety of the CFTR gene in 36 infertile males with CBAVD. METHODS: Temporal temperature gradient gel electrophoresis (TTGE) followed by direct DNA sequencing was used. RESULTS: Five mutations, p.V201M, p.N287K, c.-8G > C (125G > C), p.M469I and p.S895N, were found in five of the patients. p.N287K occurred in the first transmembrane-spanning domain, p.M469I in the first ATP-binding domain and p.S895N in the second transmembrane-spanning domain, were novel. In addition, seven homozygous and seven heterozygous 5T alleles in the intron 8 poly(T) tract were found. The overall frequency of CFTR mutant alleles in Taiwanese CBAVD males was 26 out of 72 = 36%. This finding was lower than the published frequency of CFTR mutations in other ethnic CBAVD patients (ranging from 50 to 74%). The frequency of p.M470V in Taiwanese CBAVD patients is not significantly different from that in the general population ($P = 0.12$). CONCLUSIONS: The results of this study add to the short list of Taiwanese/Asian CFTR mutations. Unlike Caucasian patients, the CFTR mutations cannot account for the majority of Taiwanese CBAVD. This is consistent with the low incidence of CF in the Asian/Taiwanese population. Furthermore, the mutation spectrum of CFTR in CBAVD patients does not overlap with the Caucasian CFTR mutation spectrum.

Key words: CBAVD/CFTR/IVS8-5T/male infertility/Taiwanese CF

Introduction

Congenital bilateral absence of vas deferens (CBAVD; OMIM 277180) was shown to occur in almost all male patients affected with cystic fibrosis (CF; OMIM 219700) (Holsclaw et al., 1971; de la Taille et al., 1998). CBAVD occurs in 1–2% of infertile but otherwise healthy men (Holsclaw et al., 1971). It also accounts for as much as 25% of infertile males with obstructive azospermia (Chillon et al., 1995; Patrizio and Salameh, 1998). Most infertile males with CBAVD carry mutations in the CFTR (cystic fibrosis transmembrane conductance regulator) gene (Anguiano et al., 1992; Osborne et al., 1993; Culard et al., 1994; Jaffe and Oates, 1994; Oates and Amos, 1994; Dumur et al., 1996; Dohle et al., 1999, 2002). To overcome the male infertility, IVF or surgical procedures are usually used. Since CBAVD is associated with mutations in the CFTR gene and the offspring of the infertile males are at an increased risk for CF, mutational analysis of CFTR is recommended for infertile couples (Patrizio et al., 1993; Meschede et al., 1998; Spurgeon, 1999). However, standard screening methods testing for 23–87 CFTR common mutations, including the mutation panel recommended by the American College of Medical Genetics, detect only a small portion of the mutations in CBAVD men depending on the patient’s ethnic background (Mak et al., 1999; Danziger et al., 2004; Dayangac et al., 2004).

Although CF is one of the most common autosomal recessive diseases in Caucasians, it is very rare in Asian populations (Welsh et al., 2001; Wong et al., 2003). Little is known about the mutation spectrum and frequency of CFTR gene mutations in Asian populations. A recent survey on
a small number of the Asian CFTR mutations revealed mostly private mutations that have never been reported in Caucasian CF patients (Wong et al., 2003). Screening of the CFTR gene for 17 common Caucasian mutations, including the polymorphic polythymidine tract in intron 8 (IVS8 poly T), detected only the presence of the IVS8-5T mutation in Taiwanese CBAVD patients (Wu et al., 2004). The frequency of the IVS8-5T allele was found to be significantly higher in CBAVD patients than in normal controls (Wu et al., 2004).

The IVS8-5T of the CFTR gene is found in 5–10% of individuals in the general population (Groman et al., 2004). When found in trans with a severe CFTR mutation, IVS8-5T can result in male infertility, non-classic CF or a normal phenotype (Chillon et al., 1995; Zielenski et al., 1995). The incomplete penetrance is due to the number of TG repeats adjacent to 5T (Groman et al., 2004). A recent study found that those individuals with 5T adjacent to either 12 or 13 TG repeats were substantially more likely to exhibit a disease phenotype (Groman et al., 2004). In order to understand the molecular aetiology of CF and CBAVD and to determine the CFTR gene mutations in the Taiwanese population, we analysed the whole CFTR gene in 36 infertile males with CBAVD using the newly developed temporal temperature gradient gel electrophoresis (TTGE) (Wong et al., 2001, 2003; Wong and Alper, 2004).

### Materials and methods

#### Patients and DNA extraction

Male patients with infertility were referred to us for diagnosis at Taipei Medical University Hospital, Taipei, Taiwan from 1994 to 2004. The diagnosis of CBAVD was based on physical examination of the scrotal content showing the absence of a palpable vas deferens on both sides, but with normal testes size (long axis > 2 cm). Twenty cases were confirmed by surgical exploration, including 15 cases of microscopic epididymal sperm aspiration (MESA) and five cases of testicular sperm extraction (TESE) for subsequent ICSI.

We performed clinical examination for CF symptoms on every patient. However, no classic CF symptoms were identified in any of the patients. Clinical characteristics and laboratory results are summarized in Table I.
the patients. Every patient provided detailed clinical and family history. In addition to routine semen analysis, special examination for semen pH and fructose content was carried out to confirm CBAVD diagnosis. Eighteen patients received transrectal ultrasonography for the evaluation of morphology and size of the seminal vesicles, prostate and ejaculatory ducts. To detect any renal anomaly, we carried out renal ultrasonography to assess the existence and outline of both kidneys, and also hormonal assays and chromosomal analyses to rule out testicular azoosperma. Table I lists their clinical variables.

A total of 37 patients donated blood for complete CFTR gene mutational analysis. Fifty-three age- and gender-matched healthy, fertile males were used as controls for p.M470V polymorphism analysis. Total genomic DNA was extracted from peripheral blood lymphocytes using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene, Sunnyvale, CA) following the manufacturer’s recommended procedures and specifications. The study was performed according to the Taipei Medical University Hospital-approved Institutional Review Board protocol.

**TTGE (temporal temperature gradient gel electrophoresis) mutational analysis**

Patients’ DNA was analysed by TTGE for unknown mutations in the exons and intron–exon junctions of the entire CFTR gene (Wong et al., 2001, 2003; Wong and Alper, 2004). The primer sequences used for the amplification of the 27 coding exons and their flanking intron–exon junctions, as well as PCR and TTGE conditions have been described in detail previously (Wong et al., 2001). The size of the PCR product varies from 260 bp for exon 23 to 862 bp for exon 13 (Wong et al., 2001). Briefly, 5 μl of denatured and renatured PCR products were loaded onto a polyacrylamide gel containing 6 mol/l urea. The electrophoresis was carried out at 130 V at constant temperature increments of 1–2°C/h over a range of temperatures suitable for each exon (Wong et al., 2001). The temperature range of TTGE for each PCR fragment was determined empirically with the aid of computer simulation (MacMelt, Bio-Rad Laboratories) (Wong et al., 2001; Wong and Alper, 2004). The gels were stained in 2 μg/ml ethidium bromide for 5 min and imaged with a digital charged-coupled device (CCD) gel documentation system. TTGE analysis reveals homozygous change as a bandshift and heterozygous change as multiple bands (Wong et al., 2001; Wong and Alper, 2004).

The DNA fragments that showed abnormal banding patterns on TTGE analysis were sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and analysed on an ABI Prism 377 DNA Sequencer (Applied Biosystems) according to the manufacturer’s protocols. The sequencing data were analysed using ABI DNA sequencing analysis software (version 3.0) and compared with the GenBank sequence by using Mac Vector (version 7.0). The mRNA (GenBank NM_000492.2) sequence of the CFTR gene is used as the reference sequence. DNA mutational numbering is based on the cDNA sequence that uses the A of the ATG translation initiation start site as nucleotide +1. The traditional nomenclature is also included using nucleotide position 133 as the translation start site. Mutation nomenclature follows journal and human genome variation society (HGVS) guidelines. Exon 9 and its 5’ upstream intron 8 region that contains the polymorphic polythymidine tract and polymorphic TG dinucleotide repeats were sequenced to determine the length of IVS8 poly(T) and TG repeats.

**Genotyping of M470V polymorphism by allele-specific oligonucleotide (ASO) dot blot hybridization**

The p.M470V polymorphism was assayed using PCR/ASO dot blot analysis. Briefly, exon 10 of the CFTR gene containing the p.M470V (c.1408A > G) polymorphic site was amplified with forward primer 5’gcaaatctgaaacagag3’ and reverse primer 5’cattacagttacacc3’ located in the flanking intron regions of exon 10. A 2 μl aliquot of PCR products was dotted onto positively charged nylon zeta membrane. Two blots were prepared, one for normal probe M470 and the other one for mutant probe 470V (DeMarchi et al., 1994; Wong and Senadheera, 1997). Hybridization and wash were carried out according to published procedures (DeMarchi et al., 1994; Wong and Senadheera, 1997).

**Results**

**Mutations identified**

All 27 exons of the CFTR gene were analysed by TTGE analysis. In addition, exon 9 including the flanking polymorphic intron 8 region of each sample was sequenced. A total of 21 IVS8-5T (seven homozygotes and seven heterozygotes) and five other mutations were found (Table I). The IVS8-5T mutation accounts for 81% (21 out of 26) of all identified CFTR mutant alleles. Three novel mutations (Figure 1) were identified in three heterozygous patients who were all homozygous for 7T in intron 8 (Table I). These novel mutations include p.M469I (1539G > T or c.1407G > T) in the first ATP-binding fold, p.N287K (993C > G or c.861C > G) in the first transmembrane-spanning domain and p.S895N (c.2684 G > A or 2816G > A) in the second transmembrane-spanning domain. The p.N287K mutation which changes a non-charged amino acid asparagine to a highly positively charged lysine in the hydrophobic transmembrane span is predicted to cause some structural-functional effect. Although the p.M469I mutation has never been reported, mutation at the same amino acid, p.M469V, has been found in CBAVD patients (http://genet.sickkids.on.ca). The novel missense p.S895N mutation is predicted to be a mild change. Another mutation was p.V201M (733G > T or c.601G > T) in the first transmembrane domain. The p.V201M mutation has been reported in other patients with CBAVD (http://genet.sickkids.on.ca) (Danziger et al., 2004). Its clinical significance is not known. A polymorphism 125G > C (or c.-8G > C) in the 5’-untranslated region of exon 1 was found in one patient who did not carry any other mutations. Whether this polymorphism is affecting the translational efficiency is not known. Overall, the mutations in the CFTR gene account for only 36% (26 out of 72) of the CF chromosomes in Taiwanese CBAVD patients.

A Caucasian patient who resided in Taiwan was also referred to us for molecular analysis due to CBAVD and infertility. Two mutations, ΔF508 and p.L375F, were found (Table I, patient 37). Both have been reported in Caucasian CBAVD patients. This patient is not included in the analysis of allele frequency.

**5T allele and its adjacent polymorphic TG repeats**

Since the disease penetrance of 5T is affected by its adjacent polymorphic TG repeats, the number of TG dinucleotide repeats in intron 8 of each patient was determined by direct sequencing. It was found that in all patients carrying 5T, the mutant allele was associated with either 12 or 13 TG repeats.

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*Page 3 of 6*
Table II. Distribution of 5T/7T, 11/12/13TG and M470V alleles

<table>
<thead>
<tr>
<th></th>
<th>5T</th>
<th>7T</th>
</tr>
</thead>
<tbody>
<tr>
<td>11TG</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>12TG</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>13TG</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>M470</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>470V</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

None of our patients or the normal fertile males had a 9T allele.

The p.M470V polymorphism

Several reports have suggested that the most frequent CFTR polymorphism, p.M470V, played a role in modulating CFTR protein level at both transcriptional and translational levels independent of intron 8 polythymidine genotype (Cuppens et al., 1998; de Meeus et al., 1998b). Our results did not show a significant difference ($P = 0.12$) in genotype and allele frequency distributions of p.M470V in Taiwanese CBAVD patients and matched controls (Table III). There is no evidence of linkage disequilibrium between the 5T allele and p.M470V allele (Table I). The results indicate that the wild-type M470 allele is not the more common allele in Taiwanese population (42.5 versus 57.5% for M to V allele frequency ratio), but it is a more common allele in Taiwanese CBAVD patients (51.4 versus 48.6%) (Table III).

Discussion

The mutations in the CFTR gene account for only 36% of the total CF alleles in Taiwanese CBAVD patients. Studies (Table IV) on Caucasian CBAVD populations using various mutation detection methods found CFTR gene mutations in 50–74% of the alleles (Patrizio and Zielenski, 1996; de Meeus et al., 1998a; Josserand et al., 2001; Wang et al., 2002; Danziger et al., 2004; Dayangac et al., 2004). We have demonstrated in a number of studies that TTGE is a sensitive mutation detection method (Wong et al., 2001, 2003; Wong and Alper, 2004). Using TTGE, 97.5% of Hispanic CF mutant alleles and previously unknown Taiwanese CF mutations were identified (Wong et al., 2001, 2003; Wong and Alper, 2004). Taiwanese CBAVD patients do not carry any of the common CFTR mutations found in Caucasians (Alper et al., 2003; Wong et al., 2003). The results of our study showed that unlike the findings in other studies, the 5T allele accounted for the majority of the mutant alleles in Taiwanese CBAVD (Table IV). In addition to the IVS8-5T
and numerous CFTR mutations in Caucasians, the Turkish study found a p.D1152H mutation that occurred at an unexpected high frequency (15%), suggesting that a specific mutation profile may be responsible for CBAVD patients in a particular population (Dayangac et al., 2004). This finding can be supported further by the study conducted by Danziger et al. (2004), in which investigators analysed a group of infertile male patients with various ethnicities including nine Asian or Asian-Indians, four Caucasians, two Hispanics and one mixed Caucasian/Asian/Ashkenazi Jewish (Danziger et al., 2004). Only 50% of the mutant alleles were detected (Table IV). The low detection rate of CFTR mutations in the Taiwanese CBAVD patient group can also be explained by the rarity of the CF disease in the Asian and Taiwanese population. Despite the extensive analysis of the CFTR gene, the CF mutant chromosomes are so rare in the Taiwanese population that other pathogenic mechanisms may account for the majority of the CBAVD cases. One other possibility is that the most frequent CFTR mutations in the Taiwanese population have not yet been discovered. The mutations may occur in the promoter region, deep in introns or in 3'-untranslated regions, that affect transcription, translation or mRNA splicing and stability.

Since the disease penetrance of 5T depends on its adjacent number of TG repeats, it is important to determine the length of TG repeats linked to 5T. Groman et al. (2004) demonstrated that the odds of pathogenicity are 28 and 34 times greater for 5T–12TG and 5T–13TG, respectively. Although only one 5T allele linked with 11TG was identified in the 23 Taiwanese normal fertile males in our controlled study, our CBAVD cases showed that the 5T allele is linked with either 12 or 13 TG allele, establishing the pathogenicity of 5T in Taiwanese CBAVD patients (Table I).

Previous studies have suggested strong linkage disequilibrium between 5T and p.M470V in Caucasian CBAVD patients but not in the normal population (de Meeus et al., 1998b). Although our sample size was small, the data in our study showed that M470 alleles were associated with 7T (M/V = 24/20) whereas 470V alleles were associated with 5T (M/V = 6/8) (Table II). This could be due to an ethnicity difference that includes the absence of the 9T allele and ΔF508 in the Taiwanese population. Furthermore, in Caucasian populations, 5T–11TG was always found with M470, ~95% of 5T–12TG was found with V470, and 5T–13TG was found to occur only with M470 (Groman et al., 2004). Our data also showed that TG repeat number, rather than M470V status, is the major determinant of penetrance for 5T. However, further studies of a larger sample size will be required in order to confirm this observation.

In conclusion, our studies of the CFTR mutations in Taiwanese CBAVD patients showed that the number of mutations was limited, that the most common mutation IVS8-5T accounted for 81% of the mutations identified, and that most mutant alleles (64%) remained unknown. Those observations are consistent with the finding that the CF incidence is rare in the Taiwanese population. Based on the finding of this study, we suggest that either the mutations in the CFTR gene are yet to be identified, or other novel

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**Table IV.** Comparison of mutation spectrum in different CBAVD patients groups

<table>
<thead>
<tr>
<th>Country</th>
<th>France</th>
<th>Belgium, France, Spain and the USA</th>
<th>South France</th>
<th>Boston, USA</th>
<th>California, USA</th>
<th>Taiwan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>DGGE sequence</td>
<td>TTGE sequence</td>
<td>MALDI-TOF</td>
<td>DGGE sequence</td>
<td>MALDI-TOF</td>
<td>DGGE sequence</td>
</tr>
<tr>
<td>Number of mutations</td>
<td>22</td>
<td>26</td>
<td>30</td>
<td>29</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>Number of patients known</td>
<td>12</td>
<td>18</td>
<td>29</td>
<td>24</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>45</td>
<td>59</td>
<td>53</td>
<td>57</td>
<td>39</td>
</tr>
<tr>
<td>5T allele</td>
<td>14 (14)</td>
<td>22 (22)</td>
<td>26 (22)</td>
<td>16 (24)</td>
<td>26 (22)</td>
<td>21 (29)</td>
</tr>
<tr>
<td>Others</td>
<td>42 (42)</td>
<td>150 (44)</td>
<td>73 (57)</td>
<td>95 (52)</td>
<td>10 (31)</td>
<td>52 (51)</td>
</tr>
<tr>
<td>Total known</td>
<td>56 (56)</td>
<td>230 (67)</td>
<td>95 (74)</td>
<td>95 (52)</td>
<td>16 (50)</td>
<td>74 (73)</td>
</tr>
<tr>
<td>Unknown allele</td>
<td>44 (44)</td>
<td>114 (33)</td>
<td>33 (26)</td>
<td>89 (48)</td>
<td>16 (50)</td>
<td>28 (28)</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>344</td>
<td>128</td>
<td>184</td>
<td>32</td>
<td>102</td>
</tr>
</tbody>
</table>

References:
pathological mechanisms are responsible for Taiwanese CBAVD. Despite the low detection rate, the information is important to facilitate our understanding of CF pathogenesis in the Taiwanese population. Comprehensive analysis of the CTR gene in its entirety for both the infertile male and his partner is essential for those who are considered for IVF (Danziger et al., 2004; Wong et al., 2004).

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References


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