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中文摘要

先天性兩側無輸精管（congenital bilateral absence of vas deferens; CBAVD），被認為是纖維囊腫(cystic fibrosis, CF)的一種輕微表現。CF 在台灣屬於一種罕見疾病，但是在我們臨床執業中，卻也不乏 CBAVD 的病人前來尋求人工生殖的幫忙。為了瞭解台灣 CBAVD 病人的 *CFTR* 突變情形，在我們之前初步針對高加索民族常見的突變基因進行篩檢的結果顯示，27 位台灣 CBAVD 的病人，都沒有檢測出高加索民族所常見的ΔF508 及 R117H 的突變，而 IVS8-5T 出現的的頻率（44.4%）則比高加索民族高出許多。台灣在文獻上有報告的 CF 病例非常稀少，在最近的一篇文獻中，報告了兩個個案，發現了兩種新的突變型(E7X 和 989-992insA)。因此我們強烈地懷疑，台灣 CBAVD 病人的 *CFTR* 突變基因圖像(mutation spectrum)與高加索民族是不同的。有鑑於此，在本研究中，我們針對 36 位台灣 CBAVD 的病人，以溫度梯度膠體電泳（Temporal temperature gradient gel electrophoresis, TTGE）及基因定序（sequencing）的方法進行全面性的 *CFTR* 突變基因的篩檢。結果顯示，分別在五位病人的 DNA 定序中，發現到五種不同的 *CFTR* 基因突變：p.V201M、p.N287K、c.-8G>C (125G>C)、p.M469I 和 p.S895N。其中 p.N287K 發生在 *CFTR* 基因的第一組 transmembrane spanning domain，p.M469I 發生於第一組 ATP binding domain，以及 p.S895N 發生於第二組 transmembrane spanning domain，是新發現的 novel mutation 或是 polymorphism。除此之外，在 IVS8-Tn 的定序結果顯示有 7 位病人帶有 5T/5T，另有 7 位病人則是 5T/7T 的表現。因此，台灣 CBAVD 病人的 *CFTR* 基因突變的整體出現頻率為(7x2+7+5)/72=36%。從這個結果看來，台灣的 CBAVD 病人所帶的 *CFTR* 突變基因圖像與高加索民族是截然不同的，*CFTR* 的突變也無法解釋大多數台灣 CBAVD 的成因，這與 CF 在亞洲／台灣的低發生率是一致的。我們的研究，雖不至於牽涉到 *CFTR* 與 CBAVD 之間相關的機轉，但是針對 CBAVD 病人，乃至於他們的配偶所做的 *CFTR* 突變基因的篩檢，提供作為不孕症遺傳諮商的參考，尤其在異國婚姻愈趨普遍的今天，是相當重要而且必要的。

關鍵詞：纖維囊腫基因突變、先天性無輸精管症、纖維囊腫、男性不孕症

英文摘要

Congenital bilateral absence of the vas deferens (CBAVD) was recognized to be a mild form of Cystic fibrosis (CF). CF is a rare disease in Taiwan, nevertheless in our clinical practice, there are CBAVD patients visiting and searching for the help of artificial reproductive techniques. In order to understand the involvement of the *CFTR* gene, we have had screened for the most common mutations of *CFTR* gene and looked for clinical correlations in 27 patients with clinically diagnosed CBAVD. No mutations of ΔF508 or R117H were identified in any of the samples analyzed. In the screening of IVS8-Tn, the frequency of 5T alleles was 44.4%, which was much higher than that of Caucasian CBAVD patients. There was little CF case report in Taiwan, however in a recent document, two novel CF mutations (E7X and 989-992insA) were reported. Therefore we strongly suspected that the mutation spectrum of *CFTR* mutation in Taiwanese patients with CBAVD is different from that of Caucasian population. Accordingly in this study, we screened the entirety of the *CFTR* gene by TTGE (temporal temperature gradient gel electrophoresis) mutation analysis followed by direct DNA sequencing in 36 infertile males with the anomalies of the vas deferens. 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, 7 homozygous and 7 heterozygous 5T alleles in intron 8 polyT tract were found. The overall frequency of *CFTR* mutant alleles in Taiwanese CBAVD males was (7x2+7+5)/72=36%. Unlike the Caucasian patients, the *CFTR* mutations cannot account for the majority of Taiwanese CBAVD. This is consistent with the low incidence of CF in Asian/Taiwanese population. Our study, although not involved in the mechanism about the relationship between *CFTR* mutation and CBAVD, it is most important and necessary that comprehensive analysis of the *CFTR* gene in its entirety for both the infertile male and his partner is essential for those who are considered for the IVF procedure.

Keywords: CFTR mutation, congenital bilateral absence of vas deferens, cystic fibrosis, male infertility

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 azoospermia (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). 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. 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 azoospermia. Table 1 lists their clinical variables. A total of 37 patients donated blood for complete CFTR gene mutational 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 ml of denatured and reannealed PCR products were loaded onto a polyacrylamide gel containing 6 mol/l urea. The electrophoresis was carried out at 130V 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 mg/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 Vectore (version 7.0). The mRNA (GenBank NM_000492.2) sequence of the CFTR gene is used as the reference sequence. DNA mutation 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 translational start site. Mutation nomenclature follows journal and Human Genome Variation Society (HGVS) guidelines. Exon 9 and its 50 upstream intron 8 region that contains the polymorphic polythymidine tract were sequenced to determine the length of IVS8 poly(T).

Results

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 1). 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 1). 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 span. 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 1, patient 37). Both have been reported in Caucasian CBAVD patients. This patient is not included in the analysis of allele frequency.

Discussion

The mutations in the CFTR gene account for only 36% of the total CF alleles in Taiwanese CBAVD patients. Studies (Table 2) 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., 1998; Josserand et al., 2001; Wang et al., 2002; Danziger et al., 2004; Dayangac et al., 2004). It has been 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 2). 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 2). 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. 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 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 CFTR 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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Table 1. *CFTR* genotypes and clinical information in Taiwanese CBAVD patients.

	PID	Genotype	Kidney	Seminal Vesicles	Semen analyses	
					pH	Fructose
		Both alleles identified				
1	#10	IVS 8-5T/ IVS 8-5T	N	BHy		
2	#34	IVS 8-5T/ IVS 8-5T	N	BA	6.5	Neg
3	#38	IVS 8-5T/ IVS 8-5T	N	RA+LHy	6	Neg
4	#42	IVS 8-5T/ IVS 8-5T				
5	#49	IVS 8-5T/ IVS 8-5T			8	Pos
6	#50	IVS 8-5T/ IVS 8-5T				
7	#60	IVS 8-5T/ IVS 8-5T				
		One allele identified				
8	#18	N287K/ ?	N	BHy		
9	#40	V201M/?				
10	#52	c.-8G>C(125G>C)/ ?	N	BHy	6	Neg
11	#58	M469I/ ?				
12	#61	S895N/?	N	BHy	6	Neg
13	#1	IVS 8-5T/ ?	N	BHy		
14	#16	IVS 8-5T/ ?	N	N	7.5	Pos
15	#17	IVS 8-5T/ ?	N	BA	6.5	Neg
16	#35	IVS 8-5T/ ?	N	BHy	6	Neg
17	#37	IVS 8-5T/ ?	N	BHy	5	Neg
18	#46	IVS 8-5T/ ?				
19	#47	IVS 8-5T/ ?				
20	#9	?/?				
21	#21	?/?	N	BHy	6	Neg
22	#23	?/?		BHy	6.5	Neg
23	#39	?/?				
24	#44	?/?				
25	#41	?/?	N			
26	#43	?/?				
27	#45	?/?				
28	#48	?/?	N			
29	#51	?/?				
30	#54	?/?		BHy	6.5	Neg
31	#55	?/?	N	RHy+LA	6.5	Neg
32	#56	?/?				
33	#57	?/?	N	BA	6.5	Neg
34	#59	?/?	N			
35	#62	?/?				
36	#63	?/?	N	BHy	6	Neg
37	#53	ΔF508/L375F(Canadian)				

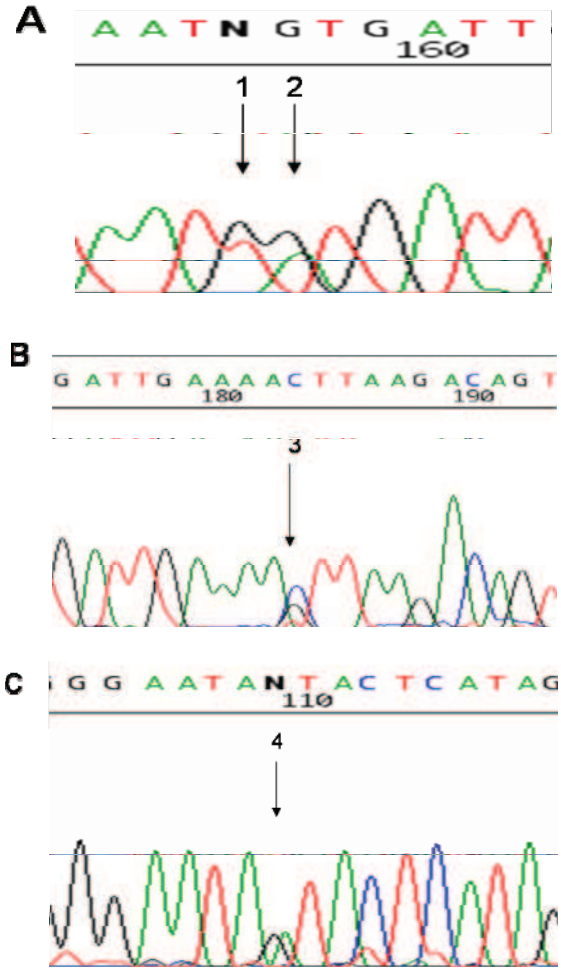
Table 2. Comparison of mutation spectrum in different CBAVD patients groups.

	France	Belgium, France, Spain, and the US	South France	Boston USA	California USA	Turkish	Taiwanese
Method	22 mutations	DGGE sequence	DGGE sequence	MALDI-TOF	sequence	sequence	TTGE sequence
2 mutations known	15 (30)	95 (55)	43 (67)	33 (36)	4 (25)	34 (67)	7 (20)
1 mutation known	26 (52)	40 (23)	9 (14)	29 (31)	8 (50)	6 (12)	12 (33)
Both unknown	9 (18)	37 (22)	12 (19)	30 (33)	4 (25)	11 (22)	17 (47)
Total	50	172	64	92	16	51	36
5T allele	14 (14)	80 (23)	22 (17)	Not done	6 (19)	22 (22)	21 (29)
others	42 (42)	150 (44)	73 (57)	95 (52)	10 (31)	52 (51)	5 (7)
Total known	56 (56)	230 (67)	95 (74)	95 (52)	16 (50)	74 (73)	26 (36)
Unknown allele	44 (44)	114 (33)	33 (26)	89 (48)	16 (50)	28 (28)	46 (64)
Total	100	344	128	184	32	102	72
Reference	Josserand et al., (2001)	Patrizio and Zielenski, (1996)	de Meeus et al., (1998a)	Wang et al., (2002)	Danziger et al., (2004)	Dayangac et al., (2004)	This study

Note: Numbers are number of patient or allele (% of total).

Figure 1. Novel *CFTR* mutations identified in Taiwanese CBAVD patients.

A. DNA sequence of portion of exon 10 of patient 58,
arrow 1: c.1407G>T (p.M469I), and arrow 2: c.1408A>G (p.M470V)

B. DNA sequence of portion of exon 6a, arrow 3: c.861C>G (p.N287K)

C. DNA sequence of portion of exon 15, arrow 4: c.2684G>A (p.S895N)