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ORIGINAL ARTICLE

The *CFTR* polymorphisms poly-T, TG-repeats and M470V in Chinese males with congenital bilateral absence of the vas deferens

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Congenital bilateral absence of the vas deferens (CBAVD) is a frequent cause of obstructive azoospermia, and mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene have also been frequently identified in patients with CBAVD. However, the distribution of the *CFTR* polymorphisms M470V, poly-T, TG-repeats and F508del mutation in the Chinese CBAVD population with presumed low cystic fibrosis (CF) frequency remains to be evaluated. Samples obtained from 109 Chinese infertile males with CBAVD and 104 normal controls were analyzed for the presence of *CFTR* (TG)m(T)n, M470V and F508del by PCR amplification followed by direct sequencing. Our study showed that the F508del mutation was not found in our patients. The 5T mutation was present with high frequency in Chinese CBAVD patients and IVS8-5T linked to either 12 or 13 TG repeats was highly prevalent among CBAVD patients (97.22% of 72 cases and 96.91% of 97 alleles with IVS8-5T). Moreover, a statistically significant relationship between TG12-5T-V470 haplotype and CBAVD was detected. This study indicated that the *CFTR* polymorphisms poly-T, TG-repeats and M470V might affect the process of CBAVD in the Chinese population.
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Keywords: *CFTR*; congenital bilateral absence of the vas deferens; IVS8-5T; male infertility; M470V

INTRODUCTION

Cystic fibrosis (CF) is one of the most common autosomal recessive diseases among the Caucasian population and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.[1] It has an incidence of 1 : 2500 live births among Caucasians. It is estimated that about one in 20–25 Caucasians carries a mutation of the *CFTR* gene.[2] In contrast, CF is very rare in Asian populations including Chinese.[3–5] However, congenital bilateral absence of the vas deferens (CBAVD) is not uncommon in Asian populations.[6] CBAVD accounts for approximately 6% of cases of obstructive azoospermia[7] and is responsible for 1%–2% of all infertility in males.[6] It is well known that CBAVD is also present in nearly 95% of all CF males.[6] To date, more than 1000 mutations have been identified in the *CFTR* gene in classic and atypical CF patients worldwide.[8] Mutations of the *CFTR* gene have also been frequently reported in patients with CBAVD.[6] In the majority of cases, CBAVD can be considered to be an atypical, genital phenotypic presentation of CF, presenting without other clinical manifestations of CF.[9]

The 5T allele, R117H and F508del, are the most common *CFTR* mutations in Caucasian CBAVD patients.[2] The poly(T) sequence located in the splicing acceptor site of intron 8 (IVS8 poly(T)) has three variants, with five, seven or nine thymidines (the 5T, 7T and 9T alleles, respectively).[10] The 7T or 9T allele generate a predominantly normal mRNA transcript, whereas the 5T variant affects splicing efficiency and results in reduced levels of normal mRNA due to deletion of exon 9.[11] The protein product of the *CFTR* transcript lacking exon 9 is devoid of cyclic adenosine monophosphate-activated chloride conductance, and therefore, the 5T allele is now considered as a mild mutation with an incomplete penetrance.[11,12] Prior studies have demonstrated that the disease penetrance of 5T depends on the copy number of its adjacent TG repeats.[13,14] The number of TG repeats immediately adjacent to 5T is associated with a variable efficiency of exon 9 splicing.[15,16] The different alleles at (TG)m(T)n polymorphic loci at the 3′ end of human *CFTR* intron 8 determine the exon 9 splicing efficiency.[17,18]

In other respects, the M470V (1540A/G in exon 10) polymorphism is one of the most common polymorphisms in the *CFTR* gene.[19] By *in vitro* studies, it was shown that the *CFTR* gene carrying the V allele yielded a lower functional *CFTR* protein rate than those carrying the M allele, independently of the intron 8 Tn genotype.[15] de Meeus *et al.*[19] reported that strong linkage disequilibrium was observed between the 5T allele and the V allele of the M470V polymorphism in the CBAVD population, but not in the normal population.

In China, the 5T allele, F508del mutation and M470V polymorphism in the CBAVD population remain to be evaluated. In the present study, we have analyzed the distribution of the *CFTR* polymorphism M470V, poly-T, TG-repeats and F508del mutation in 109 Chinese infertile males with CBAVD and 104 normal controls. The aim of this

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study was to explore the role of *CFTR* gene polymorphisms or mutations in the occurrence of patients with CBAVD in the Chinese population.

MATERIALS AND METHODS

Samples

Blood samples were collected from 109 unrelated Chinese males with CBAVD at the Reproductive Medicine Center, The First Affiliated Hospital of Wenzhou Medical College, China, with informed consent. The diagnoses of CBAVD were initially suggested by palpable scrotal vas on physical examination and transabdominal/rectal ultrasonography and subsequently confirmed by cytobiochemical characteristics in accordance with the World Health Organization criteria (World Health Organization, 1999). Each patient had a zero sperm count. Clinical examination for CF symptoms was performed for each patient. However, no classic CF symptoms were identified in any of the patients. As controls, 104 healthy subjects with normal routine sperm parameters from the general population in East China (Zhejiang province) were also evaluated for *CFTR* gene polymorphisms.

Analyses of *CFTR* gene polymorphisms

Genomic DNA was isolated from peripheral blood using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). The intron 8–exon 9 junction and exon 9 region that contain the polymorphic polythymidine tract and polymorphic TG dinucleotide repeats were PCR-amplified with primers E9-F (5′-CAT AAA ACA AGC ATC TAT TG-3′) and E9-R (5′-AGA GAC ATG GAC ACC AAA TT-3′),[6] and these PCR products were then sequenced to determine the length of the IVS8 poly(T) and TG repeats. Detection of the M470V polymorphism and F508del was carried out by PCR amplification of exon 10 with primers E10-F (5′-GCA GAG TAC CTG AAA CAG GA-3′) and E10-R (5′-CAT TCA CAG TAG CTT ACC CA-3′),[20] and followed by direct sequencing.

Statistical analyses

Differences between percentages were tested with the chi-square statistics or Fisher's exact test. *P* values of less than 0.05 were considered to be of statistical significance.

RESULTS

The study of IVS8 poly(T) splicing variants revealed 72 out of 109 (66.06%) cases with the 5T variant, 25 out of 109 (22.94%) with homozygous (5T/5T) and 47 of 109 (43.12%) with heterozygous (5T/–). Normal controls showed 24 out of 104 (23.08%) cases with the 5T variant, four (3.85%) cases with a homozygous (5T/5T) and 20 (19.23%) cases with a heterozygous 5T variant (**Table 1**). In CBAVD patients, the frequency of T5 allele was significantly increased when compared to control population. Overall, the frequency of the 5T allele was observed 97/218 (44.50%) in the 109 CBAVD patients (218 alleles), whereas 28/208 (13.46%) in the 104 control males (208 alleles).

A significant 19 fold increase in TG13 frequency (**Table 2**) was observed in CBAVD patients. The frequency of TG12 allele was also statistically significantly higher in patients with CBAVD (55.05%) than in the control males (44.23%). The frequency of TG13 allele was observed 20/218 (9.17%) in the 109 CBAVD patients (218 alleles) and 1/208 (0.48%) in the control males, whereas the frequency of the TG11 allele (35.78%) was significantly decreased in the CBAVD patients in comparison with controls (55.29%; $P<0.0001$).

Table 1 The frequency of (T)n polymorphism of intron 8 alleles in Chinese CBAVD and controls

	Alleles	
	*Chinese CBAVD (n=109)**	*Chinese controls (n=104)*
5T	97/218 (44.50%)	28/208 (13.46%)
7T	121/218 (55.50%)	179/208 (86.06%)
9T	0/218 (0%)	1/208 (0.48%)
	Population frequency	
5T/5T	25/109 (22.94%)	4/104 (3.85%)
5T/–	47/109 (43.12%)	20/104 (19.23%)

Abbreviation: CBAVD, congenital bilateral absence of the vas deferens.
**P*<0.0001 *vs.* the normal control group.

M470V in Chinese infertile males with CBAVD is not significantly different from that in the control males (**Table 3**). The mutation F508del, the most frequent mutation reported in CBAVD infertile males from the European and American populations, was not found in all Chinese males with CBAVD and controls.

The comparison of TG-T haplotypes revealed the significant 2.5-fold increase of the TG12-5T haplotype in males with CBAVD (**Table 4**). TG11-5T and TG13-5T haplotypes were found 3/218 (1.38%) and 20/218 (9.17%) in the CBAVD patients and were not found in the control males. However, significant increases of TG11-7T and TG12-7T haplotypes were observed in the control males (**Table 4**). One case with the TG13-7T and TG12-9T genotype was found in the control males. To the best of our knowledge, the haplotypes (TG13-7T and TG12-9T) were never reported previously. The TG12-5T haplotype was found in 33.94% of CBAVD patients and 76.29% of CBAVD patients with IV8-5T. Our finding suggests that the haplotype TG12-5T might be the most common disease-associated combination and impair the process of CBAVD.

Since the haplotype TG12-5T was found 81/96 (84.38%) in males carrying the 5T allele (57/72 (79.17%) in CBAVD and 24/24 (100%) in normal controls), the association of TG12-5T and M470V was analyzed in CBAVD patients and normal controls (**Table 5**). Statistical analysis showed that the TG12-5T-V470 genotype was significantly associated with CBAVD (30/57 or 52.63%) as compared to normal controls (5/24 or 20.83%) ($\chi^2=6.959$, $P<0.01$; OR=4.22, 95% CI=1.39–12.86).

DISCUSSION

CBAVD is characterized by bilateral absence of the vas deferens that leads to azoospermia and male infertility.[21] It accounts for 6% of cases of obstructive azoospermia.[7] On the other hand, a large number of reports have described the *CFTR* mutation in infertile patients with CBAVD, but their results were not completely consistent.[22] The main objectives of this study were to determine the possible involvement of *CFTR* dysfunction in CBAVD males from China, a country with a

Table 2 The frequency of (TG)m Polymorphism of intron 8 alleles in Chinese CBAVD and controls

	Alleles	
	*Chinese CBAVD (n=109)**	*Chinese controls (n=104)*
TG11	78/218 (35.78%)	115/208 (55.29%)
TG12	120/218 (55.05%)	92/208 (44.23%)
TG13	20/218 (9.17%)	1/208 (0.48%)

Abbreviation: CBAVD, congenital bilateral absence of the vas deferens.
**P*<0.0001 *vs.* the normal control group.

Table 3 The distribution of the M470V allele in Chinese CBAVD and controls

	*Chinese CBAVD (n=109)**	*Chinese controls (n=104)*
Genotype distribution		
M/M	30/109 (27.52%)	19/104 (18.27%)
M/V	42/109 (38.53%)	50/104 (48.08%)
V/V	37/109 (33.94%)	35/104 (33.65%)
Allele frequencies		
M470	102/218 (46.79%)	88/208 (42.31%)
V470	116/218 (53.21%)	120/208 (57.69%)

Abbreviation: CBAVD, congenital bilateral absence of the vas deferens.
**P*>0.05 *vs.* the normal control group.

presumed low frequency of CF. We analyzed the *CFTR* gene in 109 Chinese CBAVD patients for the genotype of TG-repeat and polythymidine tract (TG)m(T)n in intron 8, M470V and F508del in exon 10. The 5T variant in the intron 8 polythymidine tract was the most frequent *CFTR* gene alteration identified in Caucasian individuals with CBAVD.[23] This nonfunctional *CFTR* mRNA accounts for up to 92% of the total mRNA when the 5T allele is found on both *CFTR* genes.[11] We observed a significant proportion of Chinese CBAVD males who have the 5T allele (44.50%), as compared with the control males (13.46%). Also, a high frequency of homozygous (5T/5T) was found in the Chinese CBAVD patients (**Table 1**). The frequency of IVS8-5T in our patients was higher than in Portuguese (27.4%),[24] Iranian (25.94%),[25] Chinese residents of Taiwan (29.2%)[26] and Turkish patients (19.6%).[27] The compound heterozygote with a major mutation of *CFTR* such as F508del or R117H, which causes the development of CBAVD in Caucasian populations, determined the pathogenic 5T allele.[10] However, no major *CFTR* mutations, such as F508del, were found in Japanese and Chinese residents of Taiwan subjects with CBAVD.[6] In our study, we also found no F508del mutation in Chinese population with CBAVD. Therefore, our results suggest that this allele may not be a critical mutation in the Chinese CBAVD population.

Previous studies suggested that IVS8-5T shows higher disease penetrance when it is combined with the higher number of IVS8-(TG)m.[15] It was found that 5T was in *cis* with three different TG repeats (TG11-5T, TG12-5T, TG13-5T).[13] In 72 cases with 97 alleles of IVS8-5T present in 109 Chinese CBAVD patients, 70 cases (97.22%) and 94 alleles (96.91%) had 12 or 13 TG repeats adjacent to 5T. TG12-5T was the most common disease-associated combination, and was found in 33.94% of the Chinese CBAVD patients *versus* 13.46% of controls (**Table 4**). It is interesting to note that TG11-5T and TG13-5T were found exclusively in the Chinese CBAVD patients. Prior studies have

Table 4 The frequency of (TG)m(T)n haplotypes in Chinese CBAVD and controls

Linkage haplotypes	*Alleles*	
	*Chinese CBAVD (n=109)**	*Chinese controls (n=104)*
TG11-5T	3/218 (1.38%)	0/208 (0%)
TG12-5T	74/218 (33.94%)	28/208 (13.46%)
TG13-5T	20/218 (9.17%)	0/208 (0%)
TG11-7T	75/218 (34.40%)	115/208 (55.29%)
TG12-7T	46/218 (21.10%)	63/208 (30.29%)
TG13-7T	0/172 (0%)	1/208 (0.48%)
TG12-9T	0/172 (0%)	1/208 (0.48%)

Abbreviation: CBAVD, congenital bilateral absence of the vas deferens.
**P*<0.0001 *vs.* the normal control group.

Table 5 The association of TG12-5T and M470V in Chinese CBAVD and controls

Phenotype	*M470V*		
	M470/M470	*M470/V470*	*V470/V470*
Chinese CBAVD (TG12-5T)	2/57 (3.51%)	25/57 (43.86%)	30/57 (52.63%)
Chinese controls (TG12-5T)	2/24 (8.33%)	17/24 (70.83%)	5/24 (20.83%)

Abbreviation: CBAVD, congenital bilateral absence of the vas deferens.

demonstrated that a 5T variant, along with the preceding higher repetitive dinucleotide TG sequences such as (TG)13 or (TG)12, produces more mRNA transcripts with inframe deletion of exon 9.[11,15,28] Groman *et al.*[13] suggested that TG repeat number is a reliable predictor of penetrance for pathogenic 5T alleles. Those individuals carrying TG12-5T or TG13-5T were substantially more likely to exhibit an abnormal phenotype than those carrying TG11-5T.[13] TG11-5T was by far the most common in United States, Czech Republic, Poland, Italy and Germany populations, followed by TG12-5T and TG13-5T.[13] However, our study showed that TG11-5T was not found in the normal Chinese population (**Table 4**). Ethnic differences in the incidence of *CFTR* (TG)m(T)n around the world are quite striking.[14]

Another common variant in *CFTR*, a methionine or valine at codon 470, was suggested to influence the penetrance of 5T.[15] The quantity and quality of *CFTR* transcripts and/or proteins was affected by (TG)m(T)n and M470V polymorphic loci.[15] Previous studies have demonstrated that the 5T-V470 haplotype showed a higher disease association than the 5T-M470 haplotype.[19,25] Cuppens *et al.*[15] demonstrated that the V470 allele yielded a lower functional CFTR protein rate than the M470 allele, independently of the intron 8 genotypes, using *in vitro* studies. In the present study, the V allele frequency is not increased in the Chinese CBAVD population (**Table 3**). However, we found a significant association between the TG12-5T-V470 haplotype and CBAVD (**Table 5**), in agreement with a previous report.[29] Thus, although a particular allele by itself might have no deleterious consequences, the combination of specific alleles at several polymorphic loci might lead to less functional or even insufficient CFTR protein.[15,30]

In conclusion, this study indicates that the 5T allele in intron 8 of *CFTR* or IVS8-5T linked to either 12 or 13 TG repeats exhibit a high prevalence among the CBAVD patients tested. Moreover, a statistically significant relationship between the TG12-5T-V470 haplotype and CBAVD was detected for the Chinese population. These data provide a better characterization of CBAVD patients in China. However, the search for other mutations in the *CFTR* gene should be continued. There are many possible mutations to consider for analysis as more than 1000 mutations in the *CFTR* gene are documented in the literature (Cystic Fibrosis Mutation Database: http://www.genet.sickkids.on.ca/cftr).[31,32] The 5T allele is associated with different phenotype, which is presumably due to variable penetrance.[31] CBAVD patient carriers of the 5T allele are more likely to have a severe or a mild mutation in the second allele.[33] The combination of a severe mutation on one chromosome and a 5T variant on the other seems to be one of the major causes of CBAVD.[33] Clinically, intracytoplasmic single sperm injection can be used to provide fertility for men with cystic fibrosis or CBAVD.[34] However, the CFTR mutation can be transmitted to the next generation by intracytoplasmic single sperm injection, even in a population with a low prevalence of CF,[35] so the risk of having children with CF or CBAVD will be increased.[33] At present, a careful clinical examination of children seems to be mandatory when the father has CBAVD. The major reasons for this are derived from the variable phenotypic expression of

combinations of *CFTR* genes and the fact that *CFTR* mutations may have remained undetected in these couples.[33] Cystic fibrosis mutation analysis is required in CBAVD patients and their partners.[36] Thus, further counseling is suggested for patients with CBAVD, or with a family history of CBAVD.[37]

AUTHOR CONTRIBUTIONS

WHN carried out the molecular genetic studies, participated in data analysis and drafted the manuscript. LJ participated in the sequence alignment and designed of the study and drafted the manuscript. QJF participated in the patients sample collection and data analysis, JYJ participated in the sequence alignment and patients sample collection. XY participated in the patients sample collection and data analysis, XFH conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

All authors declare that there are no competing financial interests.

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