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Original Article

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) in Chinese patients with congenital bilateral absence of vas deferens

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Abstract

Background: Genetic testing of the cystic fibrosis transmembrane conductance (*CFTR*) gene is currently performed in patients with congenital bilateral absence of vas deferens (CBAVD). This study was conducted to investigate the role of mutations in the *CFTR* gene in CBAVD-dependent male infertility.
Methods: 73 Chinese patients diagnosed with CBAVD were studied. The entire coding regions and splice sites of 27 exons of the *CFTR* gene were sequenced in 146 chromosomes from the 73 CBAVD patients. Screening was carried out using PCR, gel electrophoresis and DNA sequencing to identify novel variants of the entire coding regions and boundaries of the 27 exons.
Results: Five novel nonsynonymous mutations, three novel splice site mutations and one deletion were identified by sequencing. Apart from the novel variants, we also found 19 previously reported mutations and polymorphism sites. Thirty-four patients (46.57%) had the 5T variant (6 homozygous and 28 heterozygous) and in two of them it was not associated with any detectable mutation of the *CFTR* gene. All potential pathogenic mutations are not contained in the 1000 Genome Project database. In total, the present study identified 30 potential pathogenic variations in the *CFTR* gene, 9 of which had not previously been described.
Conclusions: Most patients with CBAVD have mutations in the *CFTR* gene. A mild genotype with one or two mild or variable mutations was observed in all the patients. These findings improve our understanding of the distribution of *CFTR* alleles in CBAVD patients and will facilitate the development of more sensitive *CFTR* mutation screening.
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Keywords: Cystic fibrosis transmembrane conductance regulator (*CFTR*); Congenital bilateral absence of vas deferens (CBAVD); Sequencing; Mutation

1. Introduction

Congenital bilateral absence of the vas deferens (CBAVD) [MIM #277180] is an autosomal recessive disease and occurs in 1–2% of infertile men [1]. Male infertility as a result of CBAVD is a recognized primary genital form of cystic fibrosis (CF) [MIM #219700]. CF is a common severe disease in Caucasians populations, but is very rare in Asian populations [2–4]. About 2–6% of male infertility is caused by the mutations in

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the cystic fibrosis transmembrane conductance regulator (*CFTR or ABCC7*) [MIM #602421] gene [5]. Over 1500 CF-causing *CFTR* mutations have been identified (Cystic Fibrosis Mutation Database, 2007). The majority of adult males with CF have CBAVD. The first *CFTR* mutation in CBAVD patients was identified in 1990 [6]. However, it has been shown that mutations in the *CFTR* gene are present in 85% of men with CF where CBAVD is not associated with renal malformations [7].

CBAVD patients typically carry one or more mutation of the gene. About 35% of Iranian males with CBAVD have a single detectable *CFTR* mutation or polymorphism, about 40% have 2 mutations (common *CFTR* mutations or IVS8-5T polymorphism) [8]. The most common *CFTR* gene mutation in many populations is a deletion named ΔF508, resulting in the loss of the amino acid phenylalanine in the mature *CFTR* protein [9–11]. The 5T variant in the polymidine tract of intron 8 (IVS8-5T) is common in the general population of many countries, but a higher than normal frequency of this allele has been found in CBAVD patients [12]. About 27% of patients with CBAVD from the Croatian population had the polythymidine variant 5T found at the polymorphic Tn locus in intron 8 of the *CFTR* gene. The 5T polymorphism can be found in combination with a TG11, TG12, or TG13 allele [13–15]. The skipping of exon 9 is regulated by the polythymidine tract in intron 8 [16]. It has been shown that the clinical symptoms and their severity are correlated with the level of abnormal *CFTR* transcripts [17,18]. A TG repeat, located upstream of the polymidine tract, could modulate the effect of the 5T [15,19,20].

The *CFTR* gene contains 27 exons encompassing 180 kb of DNA on chromosome 7q31.2. The *CFTR* protein is a glycosylated transmembrane protein, which functions as a chloride channel. It regulates the outwardly rectifying chloride channel [21], it inhibits the amiloride sensitive epithelial sodium channel [22], and it influences extracellular ATP delivery [23] and HCO_3 transport [24]. The detection of *CFTR* mRNA in human fetuses suggests that *CFTR* plays an important role in the early development of the reproductive tissues [25]. Apart from its chloride channel function it also functions as a regulator to interact with other proteins. *CFTR* is expressed in epithelial cells of exocrine tissues, such as the pancreas, sweat glands, lungs, and vas deferens [21]. While *CFTR* is expressed throughout the body, *CFTR* abnormalities predominantly impact the pulmonary, digestive, and male reproductive systems, as well as the sinuses and sweat glands [26].

Mutations in the *CFTR* are common in men with CBAVD and it has been suggested that this syndrome represents a mild form of CF. We hypothesized that men with CBAVD also have mutations in the CFTR which, in addition to causing infertility may lead to subclinical bacterial pulmonary infection and inflammation consistent with mild CF [27,28]. In contrast to CF patients, when *CFTR* is involved, at least one of the mutant *CFTR* genes of CBAVD patients harbors a mild mutation [29–31].

It has been shown that 30%–40% of the chromosomes of males with CBAVD carry a splice variant, the 5T allele, in the site of intron 8 [12]. The studies vary in their methods for detecting mutations in the *CFTR* gene, and include restriction-enzyme based methods, use of specific primers, semi-quantitative fluorescent multiplex PCR (QFM-PCR), temporal temperature gradient gel electrophoresis (TTGE) and sequencing. In our study, 27 sets of oligonucleotide primers were designed to amplify and sequence the individual exons and the immediate flanking sequences in the introns for detection of *CFTR* gene mutations.

2. Materials and methods

2.1. Samples

We investigated a total of 73 male patients with azoospermia and congenital bilateral absence of vas deferens from the Urological Department, Peking Union Medical College Hospital, Chinese Academy of Medical Science, Beijing, China, during the period 2005–2010. The patients were between 21 and 44 years old. They were all given a complete physical examination by the same physician, with particular attention to the testes, vasa deferentia, epididymides, and prostate gland. The diagnosis of CBAVD was initially suggested by impalpable scrotal vas on physical examination and ultrasonography, subsequently confirmed by cytobiochemical characteristics, namely azoospermia with low semen volume (<2.0 mL) and decrease of fructose and carnitine concentrations, followed by hormonal analysis according to World Health Organization criteria (1999). None of the patients had any of the classic symptoms of CF. Informed consent for the studies had been obtained from the patients at the time of referral to the laboratory. The clinical variables are listed in Table 1.

2.2. CFTR mutation scanning

Genomic DNA was extracted from peripheral blood leukocytes using a commercial DNA isolation kit (Qiagen, Hilden, Germany). The 27 coding regions of the *CFTR* gene were amplified by polymerase chain reaction (PCR) using primers shown (Table 2). PCR products were sequenced on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA) for mutation analysis. Sequencing results were compared with the wild-type *CFTR* gene sequence published in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/all.php). The CLC DNA workbench 5 system was used to analyze the conversion of the *CFTR* protein sequence.

3. Results

A total of 30 mutations were identified in 77 of the 146 alleles, as listed in Table 3.

Twenty-two of the 30 mutations were found only once. Among the mutations identified, the 5T variant was the most common, accounting for 20.5% of CBAVD alleles (30/146). ΔF508 and R117H are the most common CBAVD mutations in Northern European population, but none of these was found in this study.

Table 1
The clinical variables of 73 patients.

No.	age	Ultrasonographic status kidney	Seminal vesicles	Semen analyses volume (mL)	pH	Fructose	ART
1	29	N	BHy	1.1	7.4		
2	25	N	BHy	0.5	6.4	Neg	
3	28		BHy	0.5	6.4	Neg	
4	33	N	BHy	0.1	7	Pos	
5	44	N	BHy	0.7	7.4	Neg	
6	37	N	BHy	0.9	6.7	Neg	
7	35		BHy	0.7	6.4	Neg	
8	30	N	BHy	0.8	6.4	Neg	
9	32		BHy	1.6	6.4	Neg	
10	27	N	BHy	0.8	7	Neg	
11	30		BHy	1	6.5	Neg	
12	39		BHy	1.7	6.4	Neg	
13	28			0.3	6.4	Neg	
14	23	N		0.5	6.4	Neg	
15	28			0.5	6.4	Neg	
16	30		LA+RHy				ICSI
17	21	N	BHy	1	6.4	Neg	
18	32	N	BHy	0.3	6.7	Neg	
19	32	N	BHy	0.5	6.4	Neg	
20	27	N	BHy	1	6.4	Neg	
21	24	N	BHy	1.5	6.4	Neg	
22	33		BHy	1	6.4	Neg	
23	26	N	BHy	0.7	6.4	Neg	MESA
24	28	N	BHy	1	6.4	Neg	
25	28	N	N	0.5	6.4	Neg	
26	25	N	BHy	0.8	6		
27	40	N	BHy	0.1	6.4		
28	32	N	BHy	0.6	7.4		
29	26		BHy	0.4	6.4	Neg	
30	34	N	RA			Neg	
31	31	N	BHy	1	6.4	Neg	
32	35		Hy	0.2	6.4	Neg	
33	30		BHy	1.5	6.4	Neg	
34	26	N	BHy	0.1	6.4		
35	35	N		0.5	6.4	Neg	
36							
37	25	N	BHy	1	6.4	Neg	
38	34		BHy	1	6.4	Neg	
39	32	N	BHy		6		
40	28	N	RA+LA	0.4	6		
41	35	N	BHy	0.2	6.4	Neg	
42	28	N	LA				
43	30	N	BHy	1.8	6.5	Neg	
44	31	N	BHy	0.8	5.5		
45	26	N	BHy	0.5	7		
46	28	N	BHy	0.2	6.7	Pos	
47	32	N	BHy	<1	7.7	Neg	MESA
48	24	N	BHy	0.2	6.4	Neg	
49	24	N	BHy				
50	25	N	BHy	0.2	6.7	Pos	
51	27	N	BHy	0.2	6.4		
52	24		BHy	0.5			
53	27		BHy	1	6.4	Neg	
54	33	N	BHy	0.5	6	Neg	
55	22	N		0.5	6.4	Neg	
56	30	N	BHy	0.2	6.7	Neg	
57	39	N	BHy	0.1	6	Neg	
58	25	N	BHy				
59	26	N	BHy	0.5	6.4	Neg	
60	28	N	BHy	0.1	6	Neg	
61	29		BHy	0.2	6.4	Neg	
62	43	N	BHy	1	6.4	Neg	
63	31	N	BHy	0.5	7	Neg	
64	26	N	BHy	2	6.5	Neg	
65	27			2	6.4	Neg	
66	30	N	BHy	1	6.4	Neg	
67	28	N	BHy	0.3	7	Neg	
68	27	N	BHy	0.4	6	Neg	
69	26	N		0.2		Neg	
70	28	N	BHy	1			
71	26	N	BHy	0.05	6.4	Neg	
72	27	N	BHy	0.2	6.4	Neg	
73							

Pos, positive; Neg, negative; N, normal; B, bilateral; R, right; L, left; Hy, hypoplasia; A, aplasia; ART, assisted reproductive technology; MESA, microscopic epididymal sperm aspiration; ICSI, intracytoplasmic single sperm injection.

Missense and splice site alterations were also predominant among the identified mutations. First, 5 novel heterozygous missense mutations were detected (Table 4). None of these mutations was found among the general population and the mutations observed in CFTR gene are all conserved in many species (*Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, *Oryctolagus cuniculus*) except for the mutation T338K (Fig. 1). Second, three novel splice site mutations (870−1 G–C, 1209+1 G–C, and 1209+2 T–G) were identified. Furthermore, one novel deletion (3635delT) was detected (Supplement Fig. 1). Lastly, we have observed previously reported mutations and polymorphisms (p.E217G, p.R347H, p.V470M, p.R553X, p.I556V, p.T854T, p.G970D, p.P1290P, p.Q1352H, p.Q1643Q, 744-5delGATT, IVS8-T5) (Supplementary Table 1). All potential pathogenic mutations have not been previously reported in the NCBI dbSNP and are not contained in the 1000 Genome Project database (http://browser.1000genomes.org/).

On screening of the poly T alleles in intron 8, six of the 73 CBAVD patients were shown to be homozygous for the 5T/5T genotype, twenty-eight were heterozygous for the 5T/7T genotype, thirty-seven were homozygous for the 7T/7T genotype and two were heterozygous for the 7T/9T genotype. The frequency of 5T alleles was 34.2%.

There was one patient homozygous for the missense mutation (I556V/I556V), but most other patients carried a missense or splicing mutation on at least one allele. Although a few mutations are not classic CF mutations, we cannot exclude the possibility that they may contribute to a CBAVD phenotype.

In summary, this study led to the identification of 30 *CFTR* gene mutations and presumably pathogenic alterations on 52.7% of chromosomes from the 73 Chinese CBAVD patients. No mutations in the regions sequenced were found in 29 patients (39.7%).

4. Discussions

For couples seeking genetic testing before assisted reproduction if the male infertility is caused by obstructive azoospermia,

Table 2
Primers for *CFTR* in PCR and sequencing.

Amplification site	PCR primer	Product length	Sequencing primer
CFTR-Exon 1	Forward: 5′-AGGTCTTTGGCATTAGGA-3′ Reverse: 5′-AAACAAGTGCATAGTAGCG-3′	394 bp	5′-AGGTCTTTGGCATTAGGA-3′
CFTR-Exon 2	Forward: 5′-GCTGCTATGGAGTGAGGAGACA-3′ Reverse: 5′-CCTGGTCTCAAGCAATCCTCTC-3′	909 bp	5′-TGTAAGAGATGAAGCCTGGT-3′
CFTR-Exon 3	Forward: 5′-TCTGGCTGAGTGTTTGGTGTTG-3′ Reverse: 5′-TTGCTGAGCCCATTGAGATTG-3′	504 bp	5′-GTGGTTTCTTAGTGTTTGGAGT-3′
CFTR-Exon 4	Forward: 5′-TAAACTTGTCTCCCACTG-3′ Reverse: 5′-TCAGCATTTATCCCTTA-3′	538 bp	5′-TAAACTTGTCTCCCACTG-3′
CFTR-Exon 5	Forward: 5′-ACTCCCGCACAATATCAATGG-3′ Reverse: 5′-TGCCTTTGAGTTGGGTGGTTC-3′	597 bp	5′-AAACATTTATGAACCTGAGAAG-3′
CFTR-Exon 6a	Forward: 5′-AAACAGCATTGGACCCACAGC-3′ Reverse: 5′-TGCGCCTCTAATCCCAGCTAC-3′	912 bp	5′-AGAACCACGAAGTGTTTGAT-3′
CFTR-Exon 6b	Forward: 5′-ATACCCACCGCTCATAG-3′ Reverse: 5′-TAACACCCTGGACCAAC-3′	578 bp	5′-CATAGGAGGCATTTACCAA-3′
CFTR-Exon 7	Forward: 5′-TGCCCAAGGTCACACAGGTC-3′ Reverse: 5′-CATTTGCAACCTCTGGCCTAG-3′	890 bp	5′-TGCCCAAGGTCACACAGGTC-3′
CFTR-Exon 8	Forward: 5′-TGAATCCTAGTGCTTGGCAA-3′ Reverse: 5′-TCGCCATTAGGATGAAATCC-3′	359 bp	5′-ATAAGATGTAGCACAATGAGAGT-3′
CFTR-Exon 9	Forward: 5′-GCCATGTGCTTTTCAAACTAAT-3′ Reverse: 5′-AAAAAGAGACATGGACACCAAA-3′	352 bp	5′-AAAAAGAGACATGGACACCAAA-3′
CFTR-Exon 10	Forward: 5′-GAATCCTGAGCGTGATTTGA-3′ Reverse: 5′-GATCCATTCACAGTAGCTTACCC-3′	384 bp	5′-GATCCATTCACAGTAGCTTACCC-3′
CFTR-Exon 11	Forward: 5′-GCTGAGGATGGACGAGGAAGA-3′ Reverse: 5′-CACCAAGATACGGGCACAGATT-3′	890 bp	5′-AAAATGGACCTATGGATGAT-3′
CFTR-Exon 12	Forward: 5′-AAACCCAACATCTTCAA-3′ Reverse: 5′-CATTCTGCCATACCAAC-3′	666 bp	5′-CATTCTGCCATACCAAC-3′
CFTR-Exon 13	Forward: 5′-TGCTAAAATACGAGACATATTGCA-3′ Reverse: 5′-TACACCTTATCCTAATCCTATGAT-3′	906 bp	5′-AGACCTTACACCGTTTCTCATT-3′ 5′-GCCATTCATTTGTAAGGGAGT-3′
CFTR-Exon 14a	Forward: 5′-GGAGCCCTTCTTTGTGATTCAT-3′ Reverse: 5′-CCCACCTCAGCCTCCTTAGTTA-3′	1551 bp	5′-ATTCTGGCTATAGAATGACATCA-3′
CFTR-Exon 14b+15	Forward: 5′-GGCATGGGAGGAATAGGTGAAG-3′ Reverse: 5′-GAGGTTCAACAAAGGGCACATG-3′	1141 bp	5′-TGCGTCAGCCTGTGGAG-3′ 5′-GTAGCCACCGCACTCCA-3′
CFTR-Exon 16	Forward: 5′-GGGTTTCGGTAGAGGTA-3′ Reverse: 5′-TGAGGCTAGTTTCAAGTGTA-3′	947 bp	5′-TGAGGCTAGTTTCAAGTGTA-3′
CFTR-Exon 17a	Forward: 5′-CACCGCATATTCTCACT-3′ Reverse: 5′-TAGCAGCACTATCCTTGT-3′	838 bp	5′-TAGCAGCACTATCCTTGT-3′
CFTR-Exon 17b	Forward: 5′-GGAGCTGCCATTTCGTGTG-3′ Reverse: 5′-GCTAGGACCCTTCACCAATATG-3′	1110 bp	5′-ATTAGCCAGAAAACTCCCAG-3′
CFTR-Exon 18	Forward: 5′-TGTGCCCTAGGAGAAGTGTGAA-3′ Reverse: 5′-AGCCAAGCCACTGACTGAGG-3′	807 bp	5′-GAATCCTATGACCCAGCAAT-3′
CFTR-Exon 19	Forward: 5′-GCCCGACAAATAACCAAGTGAC-3′ Reverse: 5′-TGCTAACACATTGCTTCAGGCT-3′	455 bp	5′-GCCCGACAAATAACCAAGTGAC-3′
CFTR-Exon 20	Forward: 5′-TGTTTATGGCATGGTAC-3′ Reverse: 5′-ATTGCTAACTTGGAGGT-3′	534 bp	5′-TGTTTATGGCATGGTAC-3′
CFTR-Exon 21	Forward: 5′-CACAAGGGACTCCAAAT-3′ Reverse: 5′-ACAGCAAGAAAGCACCA-3′	837 bp	5′-CAGTTAGGGGTAGGTCCAGT-3′
CFTR-Exon 22	Forward: 5′-TGGCAGGTAGTGGGGGTAGA-3′ Reverse: 5′-CACGCAGACATGACAGCCTAAT-3′	407 bp	5′-TGGCAGGTAGTGGGGGTAGA-3′
CFTR-Exon 23	Forward: 5′-CCCATGGTTGAAAAGCTGATTG-3′ Reverse: 5′-ACCTCACCACATGGCTCAGATC-3′	465 bp	5′-CCCATGGTTGAAAAGCTGATTG-3′
CFTR-Exon 24	Forward: 5′-GCTCTGGACATTGCATTCTTTG-3′ Reverse: 5′-TGGGGAGAGGAGAGAAAAAGG-3′	800 bp	5′-GCTCTGGACATTGCATTCTTTG-3′

mutation analysis of the *CFTR* gene has become a standard procedure. However, the spectrum and frequency distribution of *CFTR* gene mutations in most patients of European descent or other countries may not be the same for all countries or ethnicities. Therefore, it is important to clearly define the relevant mutation parameters in the Chinese population.

The large cohort of patients studied in this work can help to identify the frequency and significance of *CFTR* mutations when screening these patients. The *CFTR* gene, encoded a 1480-amino acid protein, acts as a cyclic adenosine monophosphate (cAMP)-regulated chloride channel in the apical membrane of epithelial cells. The *CFTR* protein is composed of two repeated regions; each containing a transmembrane domain (TMD1 or TMD2) and two putative nucleotide-binding folds (NBF1 and NBF2). The two parts are linked by a cytoplasmic hydrophilic regulatory (R) domain, which is required for protein kinase A sensitivity of channel gating [32,33]. Depending on the effect at the protein level, *CFTR* mutations can be

Table 3
CFTR genotypes in 73 patients with congenital bilateral absence of the vas deferens.

△F508	R117H	Mutation genotypes	IVS8-Tn	n (%)
Two mutations detected				
Neg	Neg	I556V/I556V	7T/7T	1(1.3)
Neg	Neg	I556V/1209+2 G–C	5T/7T	1(1.3)
Neg	Neg	I556V/726delATT	5T/5T	1(1.3)
Neg	Neg	I556V/−	5T/5T	1(1.3)
Neg	Neg	I556V/−	5T/7T	1(1.3)
Neg	Neg	G970D/−	5T/7T	1(1.3)
Neg	Neg	C592F/−	5T/5T	1(1.3)
Neg	Neg	1209+1 G–C/−	5T/7T	1(1.3)
Neg	Neg	R553X/−	5T/7T	1(1.3)
Neg	Neg	Q1352H/−	5T/7T	1(1.3)
Neg	Neg	S485C/−	5T/7T	1(1.3)
Neg	Neg	A357T/−	5T/7T	1(1.3)
Neg	Neg	E217G/−	5T/7T	1(1.3)
Neg	Neg	R347H/−	5T/7T	1(1.3)
Neg	Neg	G451K/−	5T/7T	1(1.3)
Neg	Neg	L558S/−	5T/7T	1(1.3)
Neg	Neg	3635delT/Q1352H	7T/7T	1(1.3)
Neg	Neg	A1136T/G970D	7T/7T	1(1.3)
Neg	Neg	870−1 G–C/−	5T/7T	1(1.3)
Neg	Neg	520−2 A-G/−	5T/7T	1(1.3)
Neg	Neg	R419I/−	5T/7T	1(1.3)
Neg	Neg	C491F/Q1643Q	7T/7T	1(1.3)
Neg	Neg	Q1352H/−	5T/7T	1(1.3)
Neg	Neg	R851X/−	5T/7T	1(1.3)
Neg	Neg	P750L/G970D	7T/7T	1(1.3)
One mutation detected				
Neg	Neg	−/−	5T/7T	2(2.7)
Neg	Neg	−/−	5T/7T	3(4.1)
Neg	Neg	−/−	5T/7T	5(6.8)
Neg	Neg	−/−	5T/5T	2(2.7)
Neg	Neg	−/−	5T/5T	1(1.3)
Neg	Neg	G970D/−	7T/7T	2(2.7)
Neg	Neg	D993Y/−	7T/7T	1(1.3)
Neg	Neg	I556V/−	7T/7T	1(1.3)
Neg	Neg	T388R/−	7T/7T	1(1.3)
No mutation detected				
Neg	Neg	−/−	7T/7T	8(10.9)
Neg	Neg	−/−	7T/7T	15(20.5)
Neg	Neg	−/−	7T/9T	2(2.7)
Neg	Neg	−/−	7T/7T	4(5.5)

Neg: Negative.

divided into at least 5 classes. Class I mutations result in no *CFTR* synthesis because of mutations affecting splice sites and nonsense mutations resulting in truncated *CFTR* protein, which is mostly unstable and therefore degraded, and in mutations shifting the coding frame in the gene (frameshift deletions and insertions). Class II mutations, which was include the most common mutation ΔF508, result in *CFTR* proteins that fail to mature and are degraded. Class III mutations resulted in *CFTR* proteins that mature and therefore reach the apical membrane of the cell but exert abnormal regulatory properties on the chloride channel. Class IV mutations result in *CFTR* channels with abnormal conductive properties because of mutations in the conductivity pore. Finally, Class V mutations result in some functional *CFTR* proteins. Class I, II, and III mutations are severe mutations, while class IV and V mutations are known as mild mutations [34,35].

Table 4
The novel mutations found in the present study.

Mutation	Location	Nucleotide change	Codon change
A357T	Exon8	G–A at 1069	Ala-Thr
T388K	Exon9	C–A at 1163	Thr-Lys
R419I	Exon10	G–T at 1256	Arg-Ile
G451K	Exon10	G–A at 1351	Gly-Lys
C592F	Exon14	G–T at 1775	Cys-Phe
870−1 G–C	Intron7	Splice error	–
1209+1 G–C	Intron8	Splice error	–
1209+2 T–G	Intron8	Splice error	–
3635delT	Exon22	Frameshift	–

In the present study, nine mutations were described for the first time: p.A357T, p.T388K, p.R419I, p.G451K, p.C592F, 870−1 G–C, 1209+1 G–C, 1209+2 T–G, 3635delT. They were all found in the heterozygous state (Table 3). The mutations (p.T388K, p.R419I, p.G451K) are located in the NBF1 domain, which contains a number of highly conserved motifs predicted to bind and hydrolyse ATP. An exon 16 mis-sense mutation in TMD-M9, p.D993Y [36], and an exon 18 mis-sense mutation in TMD-M12 p.A1136T [37], two previously described mutations were found in two CBAVD phenotypes. Because the mutations are in highly conserved regions, the changes in the amino acid in the transmembrane domain of *CFTR* may cause defective channel formation in the resulting protein (Fig. 2).

The CFTR mutation p.I556V has been reported in the project of 1000 Genomes, and identified in Japanese infertility patients and French patients with asthma-like bronchopathy and chronic diarrhea [38,39]. However, our study detected an I556V gene mutation on both alleles in one Chinese CBAVD patient; this was located in the domain which functions as the ATP-binding domain. We suggest that this could be a CBAVD-causing mutation.

In the majority of cases, CBAVD can be considered a genital form of CF, presenting without the other clinical features of the disease. There is a core of 25 most common mutations designated by the CF Steering Committee in 2001, which occur in European populations with a frequency of $\geq 0.1\%$. Some mutations are clearly associated with a mild phenotype [40]. Other attempts to link mutations in *CFTR* to disease severity have failed, suggesting an influence of non-*CFTR* gene modifiers and environmental factors [2,41]. A large number of CF mutations have been discovered during the past years, and undoubtedly, mutations often combine to cause disease. The most common mutation in almost all CF populations is a deletion of 3 base pairs named ΔF508, resulting in the loss of the amino acid phenylalanine at position 508 in the mature *CFTR* protein [9,10]. The *CFTR* genotype may contribute to the frequency and severity of Wolffian duct malformations in CBAVD patients [42].

IVS8-5T and ΔF508 were found to be common in Iranian CBAVD patients. The frequency of IVS8-5T in the Iranian patients was similar to data published for Portuguese (27.4%) [43] and Taiwanese (29.2%) [44] patients, but higher than that in Turkish patients (19.6%) [45]. Approximately 27% of patients

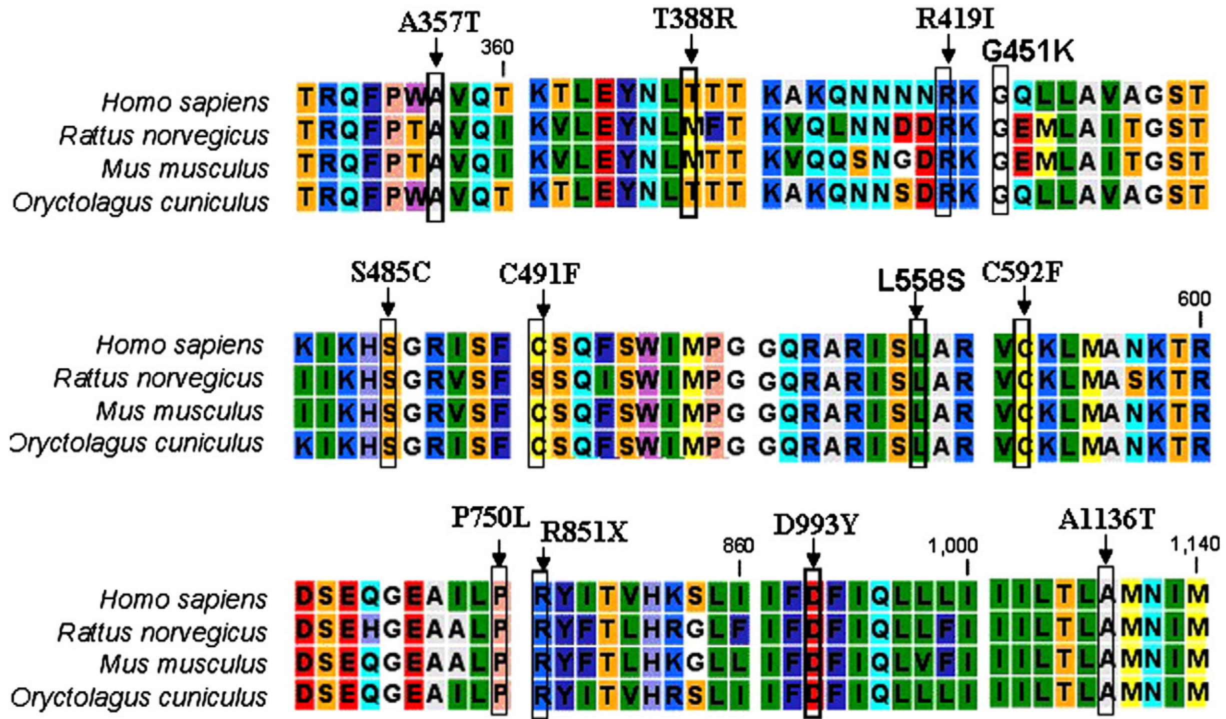

Fig. 1. The conserved analysis in species.

with CBAVD from the Croatian population had the polythymidine variant 5T [46]. In our study, the frequency of 5T polymorphism is 37.5%. The length of the polythymidine tract in intron 8 (IVS8-Tn polymorphism) of the *CFTR* gene affects the splicing efficiency of intron 8. Three alleles have been identified in IVS8 (9T, 7T and 5T). The 9T allele is associated with the most efficient usage of the intron 8 splice acceptor site and allows normal reading of the gene. Genetic studies have shown that the IVS8-5T variant causes alternative splicing of exon 9 increasing the proportion of *CFTR* transcripts that lack exon 9 whose translation products will not contribute to apical chloride channel activity. When 5T was founded in compound heterozygosity with a severe *CFTR* mutation (or 5T), it may accompanied by pathology. The 5T polymorphism was therefore classified as a disease mutation with partial penetrance [47]. Genetic studies have found that a polymorphic polythymidine (Tn) locus located within the splice site of IVS8 is associated with the variable efficiency of exon 9 splicing [14,48]. The (TG)m locus influences the penetrance of the $(T)_5$ allele, which may be associated with male infertility, in particular in the context of $(TG)_{12}$ and $(TG)_{13}$ [15].

In the present study, the most common (TG)m-Tn Combinations were TG11-T7 and TG12-T7 (the frequencies were 0.34 and 0.36), and the frequencies of TG10-T5, TG11-T5, TG11-T9, TG12-T9 and TG13-T9 were 0 (Table 5).

The most common mutation, ΔF508, reaches frequencies of about 70% in Northern European populations, while lower frequencies are observed in our Chinese population. The mutations in the *CFTR* gene are the most common cause of CBAVD, but it also has other clinical presentations. Our study of *CFTR* mutations in patients with CBAVD indicates that CBAVD may be an extreme form of a wide nosologic spectrum of conditions that have a common molecular basis. These different patterns could share some of the same pathogenic factors. Further studies with common diagnostic criteria are required to confirm this hypothesis. It is also possible that some causative mutations in our patients may lie outside the regions analyzed in our study.

The results of this study reflect the high allelic heterogeneity of *CFTR* gene mutations. In 64.4% of CBAVD patients, only one or no *CFTR* mutation could be found. Unidentified *CFTR* mutations may lie in regulatory regions of the introns, which are not routinely investigated using current PCR-based techniques, or may correspond to gene rearrangements such as large deletions in the heterozygous state which escape

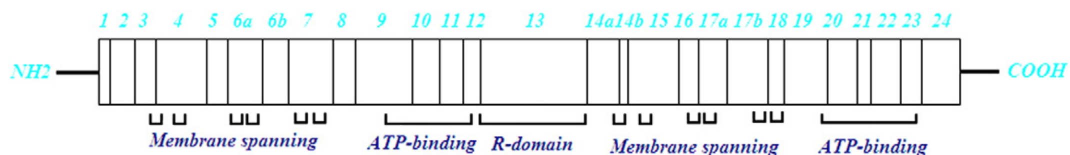

Fig. 2. Schematic diagram showing putative domain-type structure of the *CFTR* gene.

Table 5
Frequency of (TG)m-Tn Combination genotypes.

	TG10	TG11	TG12	TG13	No information
T5	0	0	32(0.22)	4(0.03)	4
T7	1(0.01)	50(0.34)	52(0.36)	2(0.01)	–
T9	1(0.01)	0	0	0	–

detection. Ratbi I et al. have identified four rearrangements using a semi-quantitative fluorescent PCR assay [49].

Although CBAVD patients are able to have children using intracytoplasmic sperm injection (ICSI), they will experience a higher risk than normal of having a child with CF. Assuming a risk of 1/25 of the partner being a CF carrier, and the affected male having a chance of 0.5 of transmitting the mutant *CFTR* gene to the child, the combined risk of a CBAVD-affected couple having a CF child is 1/100. The partner of the CBAVD patients should be tested for *CFTR* mutations and clear genetic counseling should be provided. Our findings provide new important information for predicting the risks of having a CF affected child in China.

5. Conclusion

These findings also improve our understanding of the distribution of *CFTR* alleles in Chinese CBAVD patients and will facilitate the development of more sensitive *CFTR* mutation screening in the future.

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Conflict of interest statement

This statement is to certify that all authors have seen and approved the manuscript being submitted. There is no conflict of interests.

Authors' contributions

Hongjun Li, Xinyu Zhang and Hanzhong Li collected all samples and participated in the design of the study.

Qiaolian Wen, Jing Wang, Jingwen Yang and Si Chen drafted the manuscript, carried out the molecular genetic studies

Longfei Cheng and Hongjun Li participated in manuscript revision and samples collection.

Binbin Wang and Xu Ma conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

Professors Xu Ma (genetic@263.net.cn) and Binbin Wang (wbbahu@163.com) contributed equally to the research project and can be considered co-corresponding authors.

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