article

Molecular characterization of the cystic fibrosis transmembrane conductance regulator gene in congenital absence of the vas deferens

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Purpose: Approximately 20% of patients with congenital absence of the vas deferens remain without two mutations identified. We applied a strategy of serial screening steps to 45 patients with congenital absence of the vas deferens and characterized cystic fibrosis transmembrane conductance regulator gene mutations in all cases. **Methods:** DNA samples of 45 patients with congenital absence of the vas deferens were screened by successive different molecular genetics approaches. **Results:** Initial screening for the 31 most frequent cystic fibrosis mutations, IVS8 poly(TG)m, poly(T)n, and M470V polymorphisms, identified 8 different mutations in 40 patients (88.9%). Extensive cystic fibrosis transmembrane conductance regulator gene analysis by denaturing gradient gel electrophoresis, denaturing high-performance liquid chromatography, and DNA sequencing detected 17 further mutations, of which three were novel. Cystic fibrosis transmembrane conductance regulator gene rearrangements were searched by semiquantitative fluorescent multiplex polymerase chain reaction, which detected a CFTRdele2,3 (21 kb) large deletion and confirmed two homozygous mutations. Overall, 42 patients (93.3%) had two mutations and 3 patients (6.7%) had one mutation detected. **Conclusions:** The present screening strategy allowed a higher mutation detection rate than previous studies, with at least one cystic fibrosis transmembrane conductance regulator gene mutation found in all patients with congenital absence of the vas deferens. ***Genet Med* 2007:9(3):163–172.**

Key Words: *CAVD, CBAVD, CUAVD, CFTR mutations, CFTR rearrangements, cystic fibrosis, male infertility*

Congenital absence of the vas deferens (CAVD) in otherwise healthy males is responsible for 1% to 2% of male infertility and 6% of obstructive azoospermia cases. The disease was classified as a primary genital form of cystic fibrosis (CF) because 67% to 85% of patients with CAVD have mutations and/or splicing variants in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Patients with CAVD with upper urinary tract malformations (20% of cases) have a normal frequency of *CFTR* mutations and are thus considered a distinct clinical entity with a different cause.[1–8]

CFTR genotypes in patients with CAVD are markedly different from those found in patients with CF. Patients with CAVD are usually heterozygous for a mild mutation on one allele and a severe mutation on the second allele (88%), or for mild mutations on both *CFTR* alleles (12%). On the other hand, homozygosity or compound heterozygosity for two severe mutations is responsible for the classic CF phenotype. The most common *CFTR* mutations found in CAVD are T5 allele, deltaF508, and R117H, with the combination between the T5 allele and a severe CF mutation in the other allele being the main cause of CAVD.[9]

The polymorphic polythymidine locus (Tn) is located within the 3′ splice acceptor site of intron 8 (IVS8 poly[T]n). The length of the IVS8 poly(T)n tract (T5/T7/T9) is inversely correlated with the degree of alternative splicing of exon 9. Because the protein product of the *CFTR* transcript lacking exon 9 is devoid of cyclic adenosine monophosphate-activated chloride conductance, the T5 allele is considered a mild mutation with incomplete penetrance.[10–13] Intronic (TG)m and exonic (M470V) *cis*-acting elements additionally influence the alternative splicing of exon 9. The polymorphic IVS8 poly(TG)m tract consists of a variable number (9–13) of repeats and is located immediately upstream of the IVS8 poly(T)n tract. Because of self-base pairing and RNA hairpin formation, which interfere with splicing factor interactions and the recognition of the intron 8/exon 9 junction, longer (TG)m tracts (12/13 repeats) favor the penetrance of the IVS8(T)5 allele and lead to a higher number of transcripts lacking exon 9. The M470V missense polymorphism is located in exon 10 and influences the penetrance of the T5 allele, because the V470 variant increases the proportion of mRNAs lacking exon 9 and thus contributes to even lower levels of normal transcripts.[14–20]

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Screening for the most common CF mutations does not allow the diagnosis of a significant proportion of patients with CAVD. Analysis of the whole *CFTR* gene increases the mutation detection rate, but mutations still will not be detected in a subset of patients with CAVD. This could be attributed to the presence of intronic mutations, the contribution of another gene, or large *CFTR* gene rearrangements.[21–25] In the present study, we determined the type and frequency of *CFTR* gene mutations/variations in 45 Portuguese patients with CAVD. Panel CF screening, whole *CFTR* gene screening by denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (dHPLC) and DNA sequencing, IVS8 poly(T)n analysis, (TG)m and M470V polymorphism evaluation, and *CFTR* gene rearrangement analysis by semiquantitative fluorescent multiplex polymerase chain reaction (QFM-PCR) enabled us to detect mutations in all cases (two mutations in 93% of the patients), with description of three novel mutations.

MATERIALS AND METHODS

Patients

Forty-five patients with CAVD were diagnosed during the period 1999 to 2004 (43 of Portuguese descent, 1 of Ukrainian descent, and 1 of Indian descent). Forty-two patients had congenital bilateral absence of the vas deferens (CBAVD), and three patients had congenital unilateral absence of the vas deferens (CUAVD). Patients had a good general condition with no clinical manifestations or family history suggestive of CF, including pancreatic insufficiency, other gastrointestinal symptoms, or chronic airways disease. Diagnosis was based on azoospermia with low seminal fluid volume ($<$1.5 mL), pH ($<$7.2) and/or fructose levels, and physical examination of the scrotum (presence of globus major and absence of palpable vas deferens). Abdominal ultrasonography was performed to rule out renal abnormalities. Transrectal and scrotal ultrasonography showed hypoplasia/aplasia of the seminal vesicles and a dilated epididymis head. Patients had normal serum hormone levels and a normal 46, XY karyotype, and tested negative for Y-chromosome AZF and *DAZ* microdeletions. At the diagnostic testicle biopsy, all patients showed conserved spermatogenesis with germ cell sloughing, suggestive of tubular obstruction. Sperm retrieval for infertility treatment was performed by microsurgical epididymal sperm aspiration or open testicular sperm extraction, depending on the presence of enough motile sperm in the dilated epididymis. Absence of the vas deferens and of distal epididymis, and presence of epididymis globus major were confirmed during the surgical procedure.[26] All patients were diagnosed and treated by a single experienced urologist. Genetic analyses were performed under patient-informed consent.

CFTR mutation panel testing

DNA samples were obtained from peripheral blood lymphocytes using a salting-out method. Samples were screened for the 31 most common *CFTR* gene mutations within the white population with the Cystic Fibrosis Diagnostic System kit (Abbott, Wiesbaden, Germany).[13]

IVS8 poly(T)n, poly(TG)m, and M470V polymorphisms

Characterization of the IVS8 poly(TG)m(T)n polymorphic sequence was carried out by PCR amplification of the junction between intron 8 and exon 9 (forward primer: 5′TGAAAATATCTGACAAACTC3′; reverse primer: 5′ATTTGGGTAGTGTGAAGGG3′), followed by DGGE analysis and direct DNA sequencing of the amplified DNA fragments using the Big Dye Terminator v1.1 Cycle Sequencing kit in an ABI PRISM 310 DNA automated sequencer with the appropriate sequencing analysis software (Applied Biosystems, Foster City, CA).[12,13,27] M470V was studied by PCR amplification (forward primer: 5′TCCTGAGCGTGATTTGATAA3′; reverse primer: 5′ATTTGGGTAGTGTGAAGGG3′) (CFTR DGGE, Ingeny, The Netherlands), followed by direct DNA sequencing as above.

Whole *CFTR* gene analysis

The 27 *CFTR* exons and their boundaries were studied by DGGE and dHPLC, which currently achieves a 95% mutation detection rate in European populations with CF. DNA samples with abnormal migration patterns were amplified by PCR with the same primers and sequenced for identification of the mutations as described above.[13,27–29]

CFTR gene rearrangements

CFTR gene rearrangements were screened by QFM-PCR. QFM-PCR is based on the simultaneous amplification of short *CFTR* gene fragments followed by comparison of the fluorescent profiles with control DNAs and quantification of the fluorescence of each amplicon. The amplification step of the PCR assay was stopped at the exponential phase, allowing the quantification of the number of alleles amplified. Three fluorescent-labeled multiplex PCR assays were developed to amplify all 27 *CFTR* exons, the promotor region, and the region containing the polyadenylation signal sequence. Primers were labeled with the fluorescent phosphoramidite 6-FAM dye. The detailed procedure is described elsewhere.[22] Multiplex PCR-1 (MP1) amplified the promotor, the polyadenylation signal sequence, and exons 1–6a and 11. MP2 amplified 10 exons, 7–10 and 12–16. MP3 amplified 10 exons, 6b and 17a–24. Each MP assay contained control primer pairs (internal controls) that amplified short exonic fragments from other human genes located on different chromosomes, exon 4 of *DSCR1* (chromosome 21) and exons 5, 7, or 8 of *F9* (X chromosome), depending on the MP used. Each experiment also included negative controls (samples with absence of deletions or duplications) and positive controls (samples with a known *CFTR* deletion). MP was performed in a 25-μL reaction mixture using the Qiagen Multiplex PCR Kit (Qiagen, Courtaboeuf, France), with 300 ng of genomic DNA and 0.1 to 0.8 μM of primers. PCR cycling conditions were as follows: initial denaturation (15 minutes at 95°C) followed by 21 cycles (30 seconds at 95°C; MP1/MP2: 30 seconds at 55°C; or MP3: 30 seconds at 50°C; extension: 45 seconds at 72°C) and a final extension step (10 minutes at

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72°C). Primer concentrations and the number of amplification cycles were optimized to ensure that each PCR was still in the exponential phase of amplification. Purified PCR products (2 μL) were added to a mixture containing 9.8 μL of formamide and 0.2 μL of GeneScan-500 Rox size standards (Applied Biosystems) and then separated on an ABI PRISM 310 DNA automated sequencer and analyzed using the GeneScan 3.1 software (Applied Biosystems) to obtain the electrophoregrams from each sample. Data were analyzed by superimposing fluorescent profiles of test and control DNAs followed by visual comparison of the peak heights of the corresponding amplicons, normalized against the F9 and/or DSCR1 peaks. The presence of a deletion was indicated by a decrease of approximately 50% in the height of the corresponding peak, whereas duplication showed an increase in signal intensity of approximately 50%. To determine the copy number of each exon amplified, the peak height values from a sample were compared with one another and with those from controls, which gave a series of dosage quotients. Peak height data from samples and controls were imported into an Excel (Microsoft Corp, Redmond, WA) spreadsheet, and dosage quotients for pairs of exons in a sample were then calculated by dividing the ratio of the two exons' peak height from the sample by the corresponding ratio obtained from the controls.

RESULTS

CFTR mutations and genotypes

Genomic DNA from 45 patients with CAVD was extensively studied for *CFTR* mutations in the context of male infertility caused by CBAVD (42 patients) and CUAVD (3 patients). All patients had normal karyotypes and absence of Y-chromosome microdeletions, abnormalities of the urinary system, and clinical manifestations of CF. Analysis of the 31 most common CF mutations using a commercial kit (OLA, Abbott, Wiesbaden, Germany) identified seven different *CFTR* mutations in 32 patients (71.1%). Study of the IVS8 poly(TG)m(T)n polymorphic loci identified at least one T5 allele in 25 patients (55.6%). In total, the initial study allowed the identification of eight different mutations (seven *CFTR* mutations and the T5 allele) in 40 patients (88.9%). Mutations were found in both alleles of 25 patients (55.6%) (5 *CFTR*/*CFTR*, 17 *CFTR*/T5, 3 T5/T5) and in one allele of 15 patients (33.3%) (10 *CFTR*/-, 5 T5/-), whereas 5 patients (11.1%) remained with no mutations identified (Tables 1 and 2).

In a second step, extensive analysis of the *CFTR* gene by DGGE and dHPLC was performed in DNA samples of those 40 patients with CAVD who after the initial screening had only one or no *CFTR* mutations detected. This revealed 17 further different *CFTR* mutations, thus increasing to 25 the number of different mutations detected. All 45 patients had at least one *CFTR* mutation or one T5 allele identified. Mutations were found in both alleles of 41 patients (91.1%) (20 *CFTR*/*CFTR*, 19 *CFTR*/T5, 2 T5/T5) and in one allele of 4 patients (8.9%) (3 *CFTR*/-, 1 T5/-) (Tables 1 and 2).

DNA samples of 12 patients with CAVD, still without a genotype that could explain the CAVD phenotype after DGGE/dHPLC screening, were then selected to proceed for *CFTR* gene rearrangements analysis by QFM-PCR. These included two patients with one mild *CFTR* mutation, one patient with one T5 allele, seven patients whose *CFTR* mutations or T5 alleles could probably not explain the clinical phenotype (two cases with mild/mild *CFTR* mutations, three cases with one mild *CFTR* mutation and a T5 allele, and two cases with two T5 alleles), and two apparently homozygous patients for a mild *CFTR* mutation but in whom the homozygous status was not possible to confirm by familial studies (Table 2). Patients carrying one severe CF mutation in one allele were not selected for the *CFTR* rearrangements study, because it was not expected that another severe mutation would be detected (large deletion/insertion) in the absence of CF clinical manifestations. A large *CFTR* gene rearrangement CFTRdele2,3 (21 kb) was detected in a Ukrainian patient carrying the T5 allele. A duplex PCR assay was performed using specific primer pairs that annealed at both sides of the breakpoint junction (introns 1 and 3 of the *CFTR* gene) and at intron 3. A 243-base pair junction product was amplified, confirming the deletion of exons 2 and 3. This deletion spanned 21.08 kb of the *CFTR* gene and included approximately 25% of intron 1, exon 2, intron 2, and exon 3, and approximately 45% of intron 3. *CFTR* gene rearrangements were thus found in 1 of 12 patients (8.3%), which represents 1 of 45 of the total patients studied (2.2%).

Overall, 26 different mutations were found in 45 patients with CAVD, 42 (93.3%) with mutations in both alleles and 3 (6.7%) with one mutation. Of the 26 different mutations found, 17 (65.4%) were missense, 4 (15.4%) were splice site, 2 (7.7%) were nonsense, 2 (7.7%) were deletions of a single amino acid, and 1 (3.8%) was a large deletion (Table 1). Patients with two detected mutations were mostly compound heterozygotes (38/42, 90.5%), whereas four patients (9.5%) were homozygotes for T5 allele (two cases), R334W, and S1235R (Table 2). The true homozygosity of the last two cases was confirmed by QFM-PCR. With the exception of one patient carrying two severe CF mutations (DeltaF508del/E831X), all other patients with two detected mutations (97.6%) had a mild missense/splicing site mutation in one of the alleles. The most frequent genotype of compound heterozygotes was the association of a CF/*CFTR* mutation with the T5 allele, accounting for 20 of 38 (52.6%) of the cases. Of them, deltaF508/T5 was the most frequent (12/20, 60%) genotype (Table 2).

Three mutations were found as complex alleles (two or three sequence alterations associated in *cis* on the same allele), each in two patients, G576A-R668C, D443Y-G576A-R668C, and S1235R-T5 (one patient with T5 in homozygosity) (Table 2).

Three novel *CFTR* missense mutations were identified in three patients with CBAVD who were compound heterozygotes. P439S was identified in a 34-year-old patient who carried a R334W mutation in the other chromosome. It results in a proline substitution by a serine at position 439, located in the nucleotide-binding domain (NBD) 1 of the CFTR protein. V1108L was found in a 37-year-old patient who also carried the T5 allele. It causes the substitution of a valine by a leucine at position 1108, located in the carboxyterminal transmembrane domain of the CFTR protein.

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Table 1
CFTR gene mutations and polymorphisms in patients with congenital absence of the vas deferens

	Mutation	Location	Nucleotide alteration	Effect	Method
1	**CFTRdele2,3**	Exons 2–3	Deletion of exons 2 and 3	**Frameshift**	QFM-PCR
2	R117H	Exon 4	G→A at 482	AA substitution	31 mutation panel
3	**P205S**	Exon 6a	C→T at 745	**AA substitution**	DGGE/dHPLC
4	L206W	Exon 6a	T→G at 749	AA substitution	DGGE/dHPLC
5	R258G	Exon 6b	A→G at 904	AA substitution	DGGE/dHPLC
6	R334W	Exon 7	C→T at 1132	AA substitution	31 mutation panel
7	T5 allele	Intron 8	Deletion of 2T at 1342-12 to -6	Aberrant splicing	DGGE/DNA sequencing
8	*P439S*	Exon 9	C→T at 1447	AA substitution	DGGE/dHPLC
9	D443Y[a]	Exon 9	G→T at 1459	AA substitution	DGGE/dHPLC
10	**I507del**	Exon 10	Deletion of 3 bp at 1648-1653	**AA deletion**	31 mutation panel
11	**DeltaF508**	Exon 10	Deletion of 3 bp at 1652-1655	**AA deletion**	31 mutation panel
12	**G542X**	Exon 11	G→T at 1756	**Truncation**	31 mutation panel
13	V562I	Exon 12	G→A at 1816	AA substitution	DGGE/dHPLC
14	G576A[a]	Exon 12	G→C at 1859	Aberrant splicing	DGGE/dHPLC
15	D614G	Exon 13	A→G at 1973	AA substitution	DGGE/dHPLC
16	R688C[a]	Exon 13	C→T at 2134	AA substitution	DGGE/dHPLC
17	V754M	Exon 13	G→A at 2392	AA substitution	DGGE/dHPLC
18	**E831X**	Exon 14a	G→T at 2623	**Truncation**	DGGE/dHPLC
19	3272-26A>G	Intron 17a	A→G at 3272-26	Aberrant splicing	DGGE/dHPLC
20	2789+5G→A	Intron 14b	G→A at 2789+5	Aberrant splicing	31 mutation panel
21	*V1108L*	Exon 17b	G→C at 3454	AA substitution	DGGE/dHPLC
22	L1227S	Exon 19	T→C at 3812	AA substitution	DGGE/dHPLC
23	S1235R	Exon 19	T→G at 3837	AA substitution	DGGE/dHPLC
24	P1290S	Exon 20	C→T at 4000	AA substitution	DGGE/dHPLC
25	**N1303K**	Exon 21	C→G at 4041	**AA substitution**	31 mutation panel
26	*E1401K*	Exon 23	G→A at 4333	AA substitution	DGGE/dHPLC
	Polymorphisms				
1	TG repeats	Intron 8	9–13 copies at 1342-12 to -35	Sequence variation	DGGE/DNA sequencing
2	M470V	Exon 10	A or G at 1540	Sequence variation	DNA sequencing
3	125G/C	Exon 1	G→C at 125	Sequence variation	DGGE/dHPLC
4	1001+11T/C	Intron 6b	C→4T at 1001+11	Sequence variation	DGGE/dHPLC
5	1716G/A	Exon 10	G→A at 1716	Sequence variation	DGGE/dHPLC
6	1899-136T/G	Intron 12	T→G at 1899-136	Sequence variation	DGGE/dHPLC
7	T854T	Exon 14a	T→G at 2694	Sequence variation	DGGE/dHPLC
8	3601-65C/A	Intron 18	C→A at 3601-65	Sequence variation	DGGE/dHPLC
9	4521G/A	Exon 24	G→A at 4521	Sequence variation	DGGE/dHPLC

QFM-PCR, semiquantitative fluorescent multiplex polymerase chain reaction; bp, base pair; DGGE, denaturing gradient gel electrophoresis; dHPLC, denaturing high-performance liquid chromatography.
[a] Missense mutations linked on the same allele, G576A-R688C and D443Y-G576A-R688C, were present in two patients with CBAVD each.
CFTR mutations: severe (bold); novel (italics).

E1401K was found in a 37-year-old patient who carried a deltaF508 mutation in the other chromosome. It leads to the substitution of a glutamic acid by a lysine in (NBD) 2 at its carboxy-terminal side (Tables 1 and 2). None of these mutations were detected in 200 chromosomes from 100 healthy individuals and in 10 chromosomes from 5 patients with CF.

T5 was the most frequent mutation found, accounting for 28 of 90 (31.1%) of the alleles (Table 3). (TG)12T5 was the most frequent associated haplotype (18/28, 64.4%), followed by the (TG)11T5 (5/28, 17.8%) and (TG)13T5 (5/28, 17.8%) alleles (Table 2). DeltaF508 was the second most common mutation, representing 21 (23.3%) of total alleles, followed by R334W (6,

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Table 2
CFTR genotypes identified in patients with congenital absence of the vas deferens

CFTR mutation genotypes		[(TG)mTn] genotype		M470V		Patients N	Patients %
DeltaF508		(TG)10T9	(TG)**12T5**	M	**V**	11	24.4
DeltaF508		(TG)10T9	(TG)**11T5**	M	M	1	2.2
DeltaF508	R117H	(TG)10T9	(TG)10T7	M	M	2	4.4
G542X		(TG)10T9	(TG)**12T5**	M	**V**	2[a]	4.4
DeltaF508	R334W	(TG)10T9	(TG)11T7	M	**V**	1	2.2
DeltaF508	D443Y-G576A-R668C	(TG)10T9	(TG)10T7	M	M	1	2.2
DeltaF508	D614G	(TG)10T9	(TG)11T7	M	**V**	1	2.2
DeltaF508	**E831X**	(TG)10T9	(TG)11T7	M	**V**	1	2.2
DeltaF508	L1227S	(TG)10T9	(TG)11T7	M	M	1	2.2
DeltaF508	*E1401K*	(TG)10T9	(TG)11T7	M	**V**	1	2.2
I507del	D614G	(TG)11T7	(TG)10T7	M	**V**	1	2.2
N1303K	L206W	(TG)10T9	(TG)9T9	M	M	1	2.2
R117H	**P205S**	(TG)11T7	(TG)10T7	M	**V**	1	2.2
R117H	R334W	(TG)10T7	(TG)11T7	M	**V**	1	2.2
R334W	*P439S*	(TG)11T7	(TG)11T7	M	**V**	1	2.2
R334W	R334W[b]	(TG)11T7	(TG)11T7	**V**	**V**	1	2.2
R334W	V562I	(TG)11T7	(TG)**11T5**	**V**	M	1	2.2
D443Y-G576A-R668C	3272-26A→G	(TG)10T7	(TG)10T7	M	M	1	2.2
G576A-R668C	V754M[b]	(TG)10T7	(TG)11T7	M	M	1	2.2
S1235R	S1235R[b]	(TG)**13T5**	(TG)**13T5**	M	M	1	2.2
2789+5G→A	S1235R[b]	(TG)10T7	(TG)**13T5**	M	M	1	2.2
3272-26A→G	P1290S	(TG)11T7	(TG)10T7	M	**V**	1	2.2
P205S		(TG)11T7	(TG)**12T5**	**V**	**V**	1	2.2
G576A-R668C	[b]	(TG)10T7	(TG)**11T5**	M	M	1	2.2
V1108L	[b]	(TG)11T7	(TG)**11T5**	**V**	M	1	2.2
N1303K		(TG)10T9	(TG)**12T5**	M	**V**	1	2.2
3272-26A→G	[b]	(TG)10T7	(TG)**12T5**	M	**V**	1	2.2
CFTRdele2,3	[b]	(TG)11T7	(TG)**13T5**	**V**	M	1	2.2
	[b]	(TG)**11T5**	(TG)**12T5**	M	**V**	1	2.2
	[b]	(TG)**13T5**	(TG)**12T5**	M	**V**	1	2.2
DeltaF508	—	(TG)10T9	(TG)11T7	M	**V**	1[a]	2.2
L206W	—[b]	(TG)9T9	(TG)11T7	M	**V**	1	2.2
R258G	—[b]	(TG)11T7	(TG)11T7	**V**	**V**	1	2.2

[a]CUAVD.
[b]Subjected to QFM-PCR analysis.
CFTR mutations: detected by panel CF screening (underlined); severe (bold); novel (italics).

6.7%). These three mutations led to the identification of 55 of 90 (61.1%) of the total alleles (Tables 2 and 3). The allelic frequency of the other mutations was 4.4% for R117H, G576A, and R668C, 3.3% for S1235R and 3272-26A→G, and 2.2% for P205S, L206W, D443Y, G542X, D614G, and N1301K, whereas the remaining 12 mutations were present in single patients (Table 3).

CFTR polymorphisms

The extensive *CFTR* gene screening performed in 40 DNA samples by DGGE/dHPLC revealed the presence of seven polymorphisms, of which one is novel. The allelic frequencies of these sequence variations were 21.3% (17/80) for 2694T/G, 20% (16/80) for 1001 + 11T/C, 18.8% (15/80) for 4521G/A, 6.3% (5/80) for 1899-136T/G, and 1.3% for 125G/C, 1716G/A, and 3601-65C/A[1] (Table 1). The novel polymorphism 1899-136T/C was detected by DGGE analysis in five heterozygous patients with CBAVD. Analysis of 114 alleles from 57 healthy individuals showed that the C variant was present in 30 (26.3%) of the total alleles studied (6 cases in homozygotes and 18 cases in heterozygotes).

The missense M470V polymorphism was evaluated in all 45 patients with CAVD (Table 2). The allelic frequency of the M470 variant

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Table 3
Allelic frequencies of *CFTR* mutations in patients with congenital absence of the vas deferens

		CBAVD		CUAVD		Total	
	Patients	42		3		45	
	Alleles	84		6		90	
	Mutations	N	%	N	%	N	%
1	T5 allele	26[a]	31	2	33.3	28	31.1
2	DeltaF508	20	23.8	1	16.7	21	23.3
3	R334W	6[a]	7.1	0	0	6	6.7
4	R117H	4	4.8	0	0	4	4.4
5	G576A	4[b]	4.8	0	0	4	4.4
6	R688C	4[b]	4.8	0	0	4	4.4
7	S1235R	3[a]	3.6	0	0	3	3.3
8	3272-26A→G	3	3.6	0	0	3	3.3
9	P205S	2	2.4	0	0	2	2.2
10	L206W	2	2.4	0	0	2	2.2
11	D443Y	2[b]	2.4	0	0	2	2.2
13	D614G	2	2.4	0	0	2	2.2
14	N1303K	2	2.4	0	0	2	2.2
12	G542X	0	0	2	33.3	2	2.2
15	R258G	1	1.2	0	0	1	1.1
16	P439S	1	1.2	0	0	1	1.1
17	I507del	1	1.2	0	0	1	1.1
18	V562I	1	1.2	0	0	1	1.1
19	V754M	1	1.2	0	0	1	1.1
20	E831X	1	1.2	0	0	1	1.1
21	2789+5G→A	1	1.2	0	0	1	1.1
22	V1108L	1	1.2	0	0	1	1.1
23	L1227S	1	1.2	0	0	1	1.1
24	P1290S	1	1.2	0	0	1	1.1
25	E1401K	1	1.2	0	0	1	1.1
26	CFTRdele2,3	1	1.2	0	0	1	1.1

CBAVD, congenital bilateral absence of the vas deferens; CUAVD, congenital unilateral absence of the vas deferens.
[a]Includes homozygotes. [b]Missense mutations linked on the same allele, G576A-R688C and D443Y-G576A-R688C, were present in two patients with CBAVD each.

(A at nucleotide 1540) was 58.9% (53/90) and of the V470 variant (G at nucleotide 1540) was 41.1% (37/90). Most of the patients with the V470 variant had an associated T5 allele (21/37, 56.8%), especially in combination with the (TG)12T5 haplotype (17/21, 81%).

DISCUSSION

The strategy for mutation detection in the 45 patients with CAVD included an initial screening for the 31 most frequent *CFTR* mutations found in patients with CF and the analysis of the IVS8 poly(T)n alleles with two associated polymorphisms: poly(TG)m and M470V. This allowed the detection of 8 different mutations in 40 patients (88.9%), with 25 (55.6%) showing mutations in both alleles (5 *CFTR*/*CFTR*, 11.1%). In a second step, DNA samples from 40 patients with only one or no *CFTR* mutations were subjected to a complete screening of the 27 exons and their flanking intronic regions by DGGE and dHPLC. This identified 17 further different mutations, thus increasing to 25 the number of different mutations and to 45 the number of patients who had at least one mutation, with 41 (91.1%) showing mutations in both alleles (20 *CFTR*/*CFTR*, 44.4%). Twelve patients who remained without a genotype justificative for CAVD were then selected to proceed for the study of *CFTR* gene rearrangements by QFM-PCR. Large

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CFTR gene rearrangements are not detectable using conventional PCR-based screening techniques, because the amplification of a nondeleted allele masks the lack of a PCR product corresponding to the deletion site.[21,22,24] This detected a CFTRdele2,3 (21 kb) deletion in a Ukrainian emigrant with CBAVD and heterozygous for the T5 allele. This large deletion was previously described in 5% of Slavic patients with CF from Central and Eastern Europe and was predicted to cause a truncated CFTR protein lacking all functional domains.[30,31] In the present case, the combination of CFTRdele2,3 (21 kb) with the T5 allele in *trans* is thus expected to result in reduced *CFTR* channel function. Large *CFTR* gene rearrangements were previously described to account for 1.3% of all CF mutations and for 16% to 20% of unidentified CF mutations, having also been found in approximately 2% of patients with CBAVD.[21–25] Our present results are in accordance with those findings, because *CFTR* gene rearrangements were found in 1 of 45 patients (2.2%) with CAVD. QFM-PCR also confirmed the true *CFTR* homozygosity of two CAVD cases (R334W and S1235R), because the determination of *CFTR* copy numbers by semiquantitative PCR also allows the discrimination between true homozygotes and compound heterozygotes for a *CFTR* mutation.

In total, 26 different mutations were found, with at least one mutation being detected in all 45 patients with CAVD, 42 (93.3%) showing mutations in both alleles (20 *CFTR*/*CFTR*, 44.4%). In comparison with previous studies, this is the highest mutation frequency reported for patients with CAVD[5,6,13,30,32–37] (Table 4). The three patients with CAVD (6.7%) who remained with only one mutation detected might be explained by mutations located outside of the analyzed regions, such as sequence alterations in intronic regulatory regions or mutations in other disease-causing genes. This could result in a CFTR protein with normal structure but with low levels of expression, thus changing the development of susceptible tissues such as the vas deferens and epididymis.[9,38]

Table 4
Comparative analysis of the frequency of patients with congenital absence of the vas deferens with at least one *CFTR* mutation or T5 allele after complete screening of the *CFTR* gene

	Patients			
		At least one mutation		
Countries	N	N	%	References
Iran	113	57	50	37
Brasil	17	10	59	34
Canada	74	49	66	5
Greece	14	10	71	32
Spain	134	103	77	33
Turkey	51	40	78	35
Germany	106	89	84	30
France	327	284	87	6
Portugal	45	45	100	13, 36, PS

PS, present study.

As expected for an incomplete CF phenotype, the majority of the 26 different mutations detected corresponded to amino acid changes (69.2%) and splice site mutations (11.5%), which cause a mild *CFTR* dysfunction. Despite the high heterogeneity of the mutations found in Portuguese patients with CAVD, three were the most frequent mutations (T5, deltaF508, and R334W), being present in 37 of 45 cases (82%) with 61% (55/90) allelic frequency. The T5 allele was the most frequent mutation, with 31% allelic frequency, which is one of the higher frequencies in comparison with previous studies. In Portugal, the T5 allele was found with an allelic frequency of 3% in fertile males and of 5% in the general population.[13] As in other studies, deltaF508 was the second most frequent mutation, with an allelic frequency of 23%. However, and contrary to all other studies, the third most frequent mutation was R334W, with 7% allelic frequency[5,6,13,30,32–44] (Table 5).

Compound heterozygosity was found in 91% (38/42) of the cases with two mutations. The most frequent compound genotype was a combination between a CF/*CFTR* mutation and the T5 allele (20/38, 53%), with deltaF508/T5 being responsible for 60% (12/20) of these cases. The high frequency of mild or very mild *CFTR* mutations in patients with CAVD adds further evidence that the vas deferens is one of the tissues most susceptible to the effect of changes in *CFTR* activity. In fact, previous studies have shown that healthy individuals produce less functional CFTR protein in the reproductive tract than in the respiratory epithelium, because the splicing efficiency of exon 9 is lower regardless of the IVS8 poly(T)n genotype. In the presence of mild *CFTR* mutations, there might be enough CFTR protein to prevent disease in other organs, but the additive effect of the T5 allele specifically affects the reproductive tract and results in the production of a nonfunctional protein, thus explaining why CAVD does not necessarily coincide with pulmonary or pancreatic manifestations of CF.[9,45,46]

Although a particular sequence variation may not have deleterious consequences when present in isolation, the combination of such variations in *cis* might result in a less functional or even insufficient CFTR protein. Studies have shown that intronic IVS8 poly(TG)m and exonic (M470V) *cis*-acting elements affect the splicing efficiency of the *CFTR* gene bearing the T5 allele. The (TG)m tract variant located in intron 8 has a predominant role in the exclusion of exon 9 from *CFTR* transcripts, because higher numbers of TG repeats (12/13) increase the penetrance of the T5 allele.[14,17–19] In accordance with these findings, the present study also showed that the T5 allele was mainly combined with the TG12 and TG13 variants (82%). The M470V polymorphism located in exon 10 has also been reported as a modifier element of the penetrance of the T5 allele, with the V470 variant being involved in lower normal CFTR protein levels.[14,15,19] In the present study, the V470 variant was found in 34 of 45 patients (76%), with an allelic frequency of 41% (37/90). Although the phase of these alleles was not established, most patients with CAVD with T5 allele were associated with the V470 variant (18/28, 64.3%). As expected

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Table 5
Comparative analysis of *CFTR* mutation allelic frequencies (%) in patients with congenital absence of the vas deferens

Countries	Patients	T5 allele	DeltaF508	R334W	R117H	References
Argentina	36	NA	20.8	NA	5.6	43
Austria	22	NA	13.6	NA	9.1	44
Italy	12	8.3	29.2	NA	4.2	39
The Netherlands	21	9.5	19.0	NA	21.4	38
Germany	106	12.3	26.4	0.5	11.3	30
Greece	14	14.3	14.3	NA	NA	32
France	800	16.3	21.8	NA	4.4	6
United States	92	17.9	21.2	NA	2.2	41
Canada	74	18.2	16.9	1.4	6.1	5
Turkey	51	19.6	2.9	NA	NA	35
Brazil	17	20.6	11.7	NA	2.9	34
Spain	134	20.9	16.0	0.4	3.0	33
Iran	113	25.7	12.4	0.9	3.5	37
Egypt	16	43.7	6.2	NA	NA	40
Taiwan	27	44.4	NA	NA	NA	42
Portugal	45	31.1	23.3	6.7	4.4	13, 36, PS

NA, not available; PS, present study.

by previous reports, a strong association was observed among the (TG)12(T)5, V470 allele, and the CAVD phenotype.[14,19] Homozygosity for the T5 allele is a less frequent genotype, causing exon 9 skipping in approximately 90% of the transcripts.[9,10] In the present study, two patients were homozygous for the T5 allele in association with TG12/V470 variants. This suggests that an undetected mutation might be located in *cis* in one allele or the association of other genetic factor could be responsible for the CBAVD phenotype.

Patients with CAVD carry *CFTR* genotypes composed of a severe and a mild mutation or two mild mutations.[2,9] However, one Portuguese patient with CBAVD, without clinical manifestation of CF, was found to be a compound heterozygote for two severe *CFTR* mutations, deltaF508/E831X. This genotype was previously associated with a CF phenotype, with pancreatic insufficiency.[8] A Turkish patient with CBAVD was also reported to be a compound heterozygote for the same E831X mutation and the truncating mutation 1677delTA.[35] It is possible that the association of the E831X mutation with a CF mutation confers an unusually mild phenotype, because the disease phenotype may be ameliorated through modulation of the *CFTR* genotype by external/internal factors or modifier genes.[47] In addition, E831X mutation is located at the first nucleotide of exon 14a and may alter exon 14a splicing. The exon 14a skipping may generate a mutant protein that keeps residual function. Therefore, E831X may not be considered as a classic severe mutation.

Three novel missense mutations (E1401K, P439S, and V1108L), not detected in the general population or in patients with CF, were here first described in patients with CAVD. Although the pathogenicity of these mutations is still being assessed by expression and functional in vitro studies, the combination of the amino acid substitutions with other mutations (deltaF508/E1401K, R334W/P439S, V1108L/T5) might be responsible for the CBAVD phenotype. In fact, they occur in highly conserved regions of the CFTR protein, which share 100% amino acid sequence homology between species[48] and affect the NBD1, NBD2, and transmembrane regions of the protein, which are known to regulate chloride conductance and permeability.[49–51] P439S was previously reported in a child with CF with pancreatic insufficiency and mild lung disease, in association with the P439S/R688C genotype.[52] The E1401K mutation occurs at a position in which other mutations, E1401X and E1401A, have been described in patients with CF with pancreatic insufficiency.[8]

Some difficulties in defining CF or CAVD-causing mutations were observed with some missense mutations.[6,27] G576A and R668C have been found independently, in pairs, or combined with the D443Y mutation on the same chromosome in patients with a CF-related syndrome. In accordance with previous studies, we expected that G576A and R668C were located in *cis* in two patients and combined with D443Y in the same chromosome in two patients.[6,9,12] Although initially described as polymorphisms,[27] they were later considered mild mutations associated with the CBAVD phenotype when combined in *trans* with the severe deltaF508 mutation.[53] However, our present results suggest they might also cause the CAVD phenotype when associated with other mild *CFTR* mutations, because three of four patients carrying these complex alleles harbored a mild or very mild mutation in the other chromosome (D443Y-G576A-R668C/3272-26A→G,

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G576A-R668C/V754M, G576A-R668C/T5), and only one case was associated with a severe mutation (DeltaF508del/D443Y-G576A-R668C).

In regard to the S1235R mutation, in this study it was found in homozygosity in one patient (2.2%) and in compound heterozygosity with a mild *CFTR* mutation (2789 + 5G→A/S1235R) in one patient (2.2%). In both cases, S1235R was in *cis* with the haplotype (TG)13T5. Homozygosity for the S1235R mutation is first described here, whereas compound heterozygosity between S1235R and another *CFTR* mutation (DeltaF508del) was previously described with a frequency of 0.08% in patients with variable CF phenotypic manifestations[6] or of 0.12% to 0.98% in patients with CAVD.[2,6] Although there is some controversy whether T5 homozygosity explains the CAVD phenotype, the T5/T5 genotype was found with a frequency of 6.7% (Table 2), similar to that previously reported (1.1%–10.4%).[2,5,30,33,35,37,41] Because the penetrance of the T5 allele might be increased when there is an association between the T5 allele and the (TG)13 tract, the combination of S1235R with (TG)13T5 in homozygosity or in compound heterozygosity with another *CFTR* mutation might be responsible for the CBAVD phenotype, although the primary pathogenic factor, the T5 allele or the S1235R mutation, remains to be evaluated.

In conclusion, when patients display one or no mutations in the initial *CFTR* panel screening, all 27 exons should be analyzed (DGGE, single-strand conformation polymorphism, dHPLC, and DNA sequencing). If still negative, the study of large *CFTR* gene rearrangements should then be considered. In CF mutation/T5 or *CFTR* mutation/T5 compound heterozygotes, analysis of the haplotypes associated with the T5 allele is of interest because (TG)m repeats and M470V variants help in predicting the pathogenesis of the T5 allele. In patients with CAVD with one or two mutations identified, who undergo the assisted reproduction technique, the female partners should also be screened for mutations in the *CFTR* gene. If the female partner has at least one mutation, genetic counseling should be offered and patients informed about the possibility of prenatal or preimplantation genetics diagnosis.

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