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Original Article

Identification of the second *CFTR* mutation in patients with congenital bilateral absence of vas deferens undergoing ART protocols

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Abstract

Congenital bilateral absence of vas deferens (CBAVD) is a manifestation of the mildest form of cystic fibrosis (CF) and is characterized by obstructive azoospermia in otherwise healthy patients. Owing to the availability of assisted reproductive technology, CBAVD patients can father children. These fathers are at risk of transmitting a mutated allele of the CF transmembrane conductance regulator (*CFTR*) gene, responsible for CF, to their offspring. The identification of mutations in both *CFTR* alleles in CBAVD patients is a crucial requirement for calculating the risk of producing a child with full-blown CF if the female partner is a healthy CF carrier. However, in the majority of CBAVD patients, conventional mutation screening is not able to detect mutations in both *CFTR* alleles, and this difficulty hampers the execution of correct genetic counselling. To obtain information about the most represented *CFTR* mutations in CBAVD patients, we analysed 23 CBAVD patients, 15 of whom had a single *CFTR* mutation after screening for 36 mutations and the 5T allele. The search for the second *CFTR* mutation in these cases was performed by using a triplex approach: (i) first, a reverse dot-blot analysis was performed to detect mutations with regional impact; (ii) next, multiple ligation-dependent probe amplification assays were conducted to search for large rearrangements; and (iii) finally, denaturing high-performance liquid chromatography was used to search for point mutations in the entire coding region. Using these approaches, the second *CFTR* mutation was detected in six patients, which increased the final detection rate to 60.8%.

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1 Introduction

Cystic fibrosis (CF, OMIM No. 219700), also known as mucoviscidosis, is one of the most common autosomal recessive diseases in Caucasians. It has a

reported incidence of 1 in 2 500 [1]. CF is caused by more than 1 600 different mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which encodes a chloride-channel protein (Cystic Fibrosis Mutation Database, http://www.genet.sickkids.on.ca/cftr). Classic CF is characterized by obstructive chronic pulmonary disease, pancreatic dysfunction and elevated concentrations of electrolytes in the sweat. The clinical consideration of CF is dominated by progressive lung disease including recurrent episodes of infection and reduced pulmonary function [1]. The related pancreatic insufficiency (PI), which characterizes the most severe form of CF (PI-CF), is precocious and causes difficulty in the digestion and assimilation of fats. In addition to classic CF, several 'atypical' or 'mild' forms exist. These forms are characterized by a less severe phenotype, ranging from monosymptomatic diseases (for example, idiopathic pancreatitis or disseminated bronchiectasis) to CF with pancreatic sufficiency [2]. The phenotype variability is related to the presence of different *CFTR* mutations, which can be classified as 'severe' (CF) or 'mild' (CF^m) mutations [3]. Classic PI-CF is associated with the presence of two severe mutations (the 'CF/CF' genotype), whereas CF^m/CF^m or CF/CF^m genotypes produce atypical CF [2].

One of the less severe forms of CF is represented by the congenital bilateral absence of vas deferens (CBAVD), which is the anatomical cause of the sterility observed in 98% of CF patients [4]. This condition can also be present in an isolated form in patients; in this case, it exclusively shows infertility on an obstructive basis [5]. The most frequent *CFTR* change detected in CBAVD patients is the presence of 5T allele, which is characterized by the presence of five thymidines within intron 8. This change results in a reduction of the splicing efficiency, which induces high levels of exon 9 skipping [6, 7]. In 30%–45% of CBAVD patients, molecular analysis identifies a compatible genotype with this condition (CF/CF^m; CF/5T; $CF^m/5T$; CF^m/CF^m; and 5T/5T); in the remaining patients, only one *CFTR* mutation can be detected (CF, CF^m or 5T allele). The fact that only one mutation can be detected is likely due to a second mutation in these CBAVD patients that is representative of rare variants (that is, mostly private mutations or changes that have a regional impact) [8, 9]. This second mutation can also be representative of large rearrangements of the *CFTR* gene: these rearrangements are not detectable by conventional mutation screening [10–12].

Identification of the second *CFTR* mutation in CBAVD patients is of crucial relevance for patients undergoing assisted reproduction technology (ART) protocols. Because spermatogenesis is normal in CBAVD patients, ART can easily be performed by retrieving sperm from the testis. Consequently, one *CFTR* mutation can be transmitted to the offspring, and there is a risk of producing an affected child when the female partner is a healthy CF carrier. Thus, in CBAVD patients showing only a mild mutation or a 5T allele after the first-level screening, identification of the second mutation by using a second-level analysis should always be performed when the female partner is a CF carrier. This testing scheme can rule out the risk of a severe *CFTR* mutation in the second allele.

In this study, we report a research designed to identify the second *CFTR* mutation in CBAVD patients using a triplex approach based on the analysis of: (i) mutations with regional impact; (ii) large genomic rearrangements; and (iii) point mutations within the entire coding region.

2 Materials and methods

The study evaluated 23 CBAVD patients (pats. 1–23), with an average age of 32 years (range 29–37 years). The patients did not have a family history of CF, and they were enroled in ART programme. All patients gave their written, informed consent for DNA analysis. Diagnosis of CBAVD was based on semen analysis performed according to World Health Organization [13] recommendations (azoospermia with low seminal fluid volume), physical examination of the scrotum (presence of globus major and absence of palpable vas deferens) and transrectal and scrotal ultrasonography (hypoplasia/aplasia of the seminal vesicles and dilated epididymis head). Laboratory testing included measurements of gonadotrophins (luteinizing hormone and follicle-stimulating hormone) and biochemical semen analysis (volume, pH and concentration of fructose) (Table 1). All patients had a normal diploid karyotype. A first-level screening of the *CFTR* gene was performed by analysis of 36 mutations and the 5T allele by using a reverse dot-blot approach (Inno-LiPA *CFTR*19 and Inno-LiPA *CFTR*17 + Tn Update Kits; Innogenetics, Pomezia, Italy). These analyses were performed according to a previously reported protocol [14, 15]. In all cases, the same analysis was also performed on the female partners of the CBAVD patients.

Table 1. Summary of the clinical findings in a population of 23 CBAVD patients .

Pat.	Age (years)	Fertility status	Reproductive hormones			Semen analysis			Clinical symptoms
			FSH (IU L^{-1})	LH (IU L^{-1})	T (nmol L^{-1})	Vol. (mL)	pH	Fr. (mmol per ej)	
1	29	A	4.5	5.5	12.5	0.4	6..9	2.0	No symptoms
2	30	A	NA (VU)	NA (VU)	NA (VU)	0.7	6..3	0.9	No symptoms
3	35	A	6.7	8.2	17	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
4	36	A	5.3	5.1	10.5	0.8	6.5	0..5	Asthma
5	30	A	NA (VU)	NA (VU)	NA (VU)	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
6	30	A	NA (VU)	NA (VU)	NA (VU)	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
7	37	A	6.2	7.4	11.5	0.6	6.8	0.4	Recurrent bronchial infections
8	37	A	4.4	5.3	20.2	0.8	6.8	0.9	No symptoms
9	31	A	NA (VU)	NA (VU)	NA (VU)	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	Nasal polyposis
10	34	A	NA (VU)	NA (VU)	NA (VU)	0.8	6.9	1.9	No symptoms
11	32	A	NA (VU)	NA (VU)	NA (VU)	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
12	34	A	8.2	6.5	2.3	0.9	6.7	1.5	No symptoms
13	33	A	7.5	6.0	19.7	0.9	< 7.0 (VU)	< 8 (VU)	No symptoms
14	34	A	NA (VU)	NA (VU)	NA (VU)	0.8	6..9	2.8	No symptoms
15	30	A	NA (VU)	NA (VU)	NA (VU)	0.9	< 7.0 (VU)	< 8 (VU)	No symptoms
16	31	A	6.6	9.9	23.2	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
17	36	A	NA (VU)	NA (VU)	ND (VU)	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
18	32	A	8.2	5.3	15.5	0.8	6.7	0.4	No symptoms
19	29	A	8.8	8.3	ND	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
20	31	A	NA (VU)	NA (VU)	NA (VU)	< 1 (VU)	< 7.0 (VU)	< 8 (VU)	No symptoms
21	35	A	NA (VU)	NA (VU)	NA (VU)	0.8	6.6	1.5	No symptoms
22	37	A	7.3	6.7	19.9	0.9	6.8	2.0	No symptoms
23	32	A	ND	NA (VU)	NA (VU)	0.9	< 7.0 (VU)	< 8 (VU)	No symptoms

Abbreviations: A, azoospermia; ej, ejaculation; fr., fructose; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NA, no abnormalities; ND, not determined; Pat., patient; T, testosterone; vol., semen volume; VU, values unspecified.

In cases in which a single mutation was detected, an analysis for identification of the second change was performed by using a triplex approach based on the search for rare mutations with 'regional impact,' large genomic rearrangements and point mutations within the entire *CFTR* coding region.

2.1 Identification of mutations with regional impact

The search for rare *CFTR* mutations with regional impact was performed by using reverse dot blots with the Inno-LiPA *CFTR* Italian Regional Kit (Innogenetics), which assays the presence of 21 rare *CFTR* mutations that have a high prevalence in the Italian population (Table 2).

2.2 Identification of large CFTR rearrangements

Searches for deletions and/or duplications of the *CFTR* gene were performed by using the multiple ligation-dependent probe amplification (MLPA) assay, which is based on the simultaneous hybridization of several probe pairs to the target gene followed by their ligation and subsequent PCR amplification. This technique permits the copy number of the investigated target to be determined [16–19]. For the purposes of this study, we used the 'P091 *CFTR*' kit (MRC-Holland, Amsterdam, The Netherlands), which is able to analyse the promoter and all *CFTR* exons in a single reaction. This probe mix contains two probes each for exons 6, 14, 17 and 24. It also contains one probe for each of the other exons, and it contains 12 control probes [20]. After the MLPA reaction, samples were run on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and were analysed by using the GeneScan software (Applied Biosystems). The presence of copy number variations in the *CFTR* gene exons was assessed by means of the Coffalyzer software, version 9.4 (MRC Holland, Amsterdam, The Netherlands), which is able to calculate the relative peak area associated with each single reaction. The presence of deletions or duplications is determined based on correlations with the other peaks.

2.3 Identification of point mutations within the CFTR coding region

Screening of *CFTR* point mutations was performed by using denaturing high-performance liquid chromatography (DHPLC). Each *CFTR* exon was amplified by PCR using specific primers [21] and was run on a Transgenomics WAVE 3500 system (Transgenomics Inc., Omaha, NE, USA). Runs showing a different profile compared with the normal controls were analysed by direct sequencing using an ABI PRISM 3100 genetic analyzer.

3 Results

First-level screening on the basis of the analysis of 36 *CFTR* mutations showed the presence of two alterations in eight out of 23 patients (34.8%) (pats. 1–8). The detected genotypes consisted of [delta]F508/5T in five cases (pats. 1–5), G542X/5T in two cases (pats. 6 and 7) and W1282X/5T in the last patient (pat. 8). In the remaining 15 cases (65.2%), only a

Table 2. Mutations that were analysed by the different INNO-LiPA CFTR kits.

INNO-LiPA *CFTR*19	INNO-LiPA *CFTR*17	INNO-LiPA *CFTR* Italian regional
[delta]F508	621+1G>T	1259insA
G542X	3849+10kbC>T	4016insT
N1303K	2183AA>G	4382delA
W1282X	394delTT	852del22
G551D	2789+5G> A R1162X	D579G
1717-1G>A	3659delC	G1244E
R553X	R117H	G1349D
CFTRdele2,3 (21 kb)	R334W	I502T
[delta]I507	R347P	L1065P
711+1G>T	G85E	R1158X
3272-26A>G 3905insT	1078delT	T338I
R560T	A455E	S549R(A>C)
1898+1G>A S1251N	2143delA 711+5G>A	991del5
I148T	E60X	D1152H
3199del6 3120+1G>A	2184delA	1898+3A>G, R1070Q
Q552X	*Poli-T tract variations*	R1066H
		R347H
		621+3A>G
		R334Q
		E217G

Abbreviation: CFTR, cystic fibrosis transmembrane conductance regulator.

single mutation was detected. That mutation was [delta] F508 in two patients (pats. 9 and 10) and a 5T allele in the remaining 13 patients (pats. 11–23).

Analyses were performed by using reverse dot blotting, MLPA and DHPLC in these 15 cases. These tests detected a second mutation in six patients (40%). For two cases (pats. 9 and 11), who first exhibited the [delta]F508 and 5T mutations, respectively, the second mutation was detected by reverse dot blotting using the Italian Regional kit. In both cases, the second detected mutation was T338I, which consists of a T-to-C change in exon 7, nucleotide 1145. This mutation leads to a threonine-to-isoleucine substitution in codon 338 of the CFTR protein.

In the other two CBAVD patients (pats. 12 and 13), who both exhibited a 5T allele after the first analysis, genomic rearrangements of the *CFTR* gene were detected by the MLPA assay. In patient 12, the mutation consisted of a deletion of the promoter and exon 1. In patient 13, a deletion of exon 19 was detected.

Finally, in the remaining two patients (pats.14 and 15), who both exhibited a 5T allele after the first analysis, a *CFTR* sequence change was detected by DHPLC analysis. In patient 14, the change consisted of the allelic variant 2811G/T in exon 15. Patient 15 exhibited a T-to-A change in exon 4, nucleotide 446, which leads to the substitution of an isoleucine with an asparagine in position 105 of the CFTR protein (I105N) .

All of the mutations detected by the different approaches are summarized in Table 3.

All of the female partners of the CBAVD patients screened negative for the 36 mutations, as assayed by reverse dot blotting, except for the wife of pat. 9, who

Table 3. *CTFR* mutation detection rate of the different approaches used in this study.

Patient	First-level *CFTR* screening (36 mutations + 5T allele)	*CFTR* Italian regional kit	MLPA analysis	DHPLC analysis	Final genotype
1	[delta]F508/5T	—	—	—	[delta]F508/5T
2	[delta]F508/5T	—	—	—	[delta]F508 /5T
3	[delta]F508/5T	—	—	—	[delta]F508/5T
4	[delta]F508/5T	—	—	—	[delta]F508/5T
5	[delta]F508 /5T	—	—	—	[delta]F508/5T
6	G542X/5T	—	—	—	[delta]F508/5T
7	G542X/5T	—	—	—	[delta]F508/5T
8	W1282X/5T	—	—	—	W1282X/5T
9	[delta]F508/wt	[delta]F508/T338I	—	—	[delta]F508/T338I
10	[delta]F508/wt	[delta]F508/wt	[delta]F508/wt	[delta]F508/wt	[delta]F508/wt
11	5T/wt	5T/T338I	—	—	5T/T338I
12	5T/wt	5T/wt	5T/del ex1	—	5T/del ex1
13	5T/wt	5T/wt	5T/del ex19	—	5T/del ex19
14	5T/wt	5T/wt	5T/wt	5T/2811G/T	5T/2811G/T
15	5T/wt	5T/wt	5T/wt	5T/I105N	5T/I105N
16	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
17	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
18	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
19	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
20	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
21	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
22	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
23	5T/wt	5T/wt	5T/wt	5T/wt	5T/wt
Detection rate	8/23 (34.8%)	2/15 (13.3%)	2/13 (15.3%)	2/11 (18.1%)	14/23 (60.8%)

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; DHPLC, denaturing high-performance liquid chromatography; MLPA, multiple ligation-dependent probe amplification; wt, wildtype.
The first-level screening is composed of two analyses: one for 19 mutations and the other for 17 mutations.

exhibited the presence of a 5T allele.

4 Discussion

It is widely accepted that CBAVD represents the mildest form of CF that is caused either by the presence of two mild CF mutations or by a severe CF mutation on one allele and a mild CF mutation on the second allele [4]. Because spermatogenesis is normal in these cases, sperm can be easily retrieved from the testis and then used for ART protocols. This procedure creates the risk of producing a child with full-blown CF if the female partner is a healthy CF carrier [15]. In this context, precise knowledge of the *CFTR* genotype in CBAVD patients is crucial information for calculating the couple's risk of producing a CF child. In fact, for CBAVD patients with the CF/CF^m or CF/5T genotype, in the presence of a severe *CFTR* mutation in the female partner, there is a 25% risk of producing a child with full-blown CF. There is also a 25% risk of producing a child with atypical CF. In contrast, carriers of the CF^m/5T, CF^m/CF^m or 5T/5T genotype have a 50% risk of producing a child with atypical CF, but they have no risk of producing a child with classic CF. This is true even in the presence of a severe *CFTR* mutation in the female partner [15, 22]. In the majority of CBAVD patients, conventional screening of the *CFTR* gene is not able to detect mutations in both alleles, because the second change is a rare mutation not detectable by first-level analysis. Thus, the use of additional approaches for detection of the second mutation in these cases is of relevance to genetic counselling of CBAVD patients undergoing ART.

In this study, we analysed 23 CBAVD patients by using a first-level screening of the *CFTR* gene on the basis of the analysis of 36 mutations. This approach permits detection of both *CFTR* mutations in only 34.8% of cases (8/23), according to the data in the literature [23, 24]. In our study, in the majority of cases (15/23, 65.2%), screening of 36 mutations permitted detection of a single mutation, and the search for the second mutation was performed by using a sequential approach. The sequential approach consisted of screening for *CFTR* mutations with regional impact by using reverse dot blotting, the analysis of large *CFTR* rearrangements by MLPA and the scanning of point mutations within the *CFTR* coding region by DHPLC. These approaches were performed in the described order because of considerations of the cost/benefit ratio for each method. Reverse dot-blot analysis is an inexpensive and fast approach (1–2 days) that permits analysis of selected mutations on the basis of the regional origin of the investigated patient. MLPA analysis is a more expensive and time-consuming (2–3 days) technique, but it is able to detect large genomic rearrangements that are not detectable by any of the other techniques. Finally, DHPLC analysis is the most expensive and time-consuming approach (several weeks), but it permits detection of any sequence changes in the *CFTR* gene. To the best of our knowledge, this is the first study describing the search for a second *CFTR* mutation by using multiple approaches in CBAVD patients.

This sequential approach permitted detection of the second *CFTR* mutation in six of the 15 analysed patients (40%). It also increased the total detection rate in the analysed samples from 34.8% to 60.8% (Table 2). In two patients, the mutation was detected by using reverse dot-blot analysis (T338I). In two other patients, MLPA analysis detected large rearrangements of the *CFTR* gene (deletion of promoter and exon 1 in one case and of exon 19 in the other case). In the remaining two patients, DHPLC analysis revealed a 2811G/T and an I105N sequence change. Thus, the three approaches showed similar sensitivity and were able to detect the second *CFTR* mutation in two cases each.

To verify the presence of a genotype–phenotype correlation, we analysed the pathogenic role played by each of the detected mutations. The missense T338I mutation, detected in two patients by the Italian Regional kit, is typical of the Sardinia region, where it accounts for about 10% of all the mutated *CFTR* alleles [25]. This mutation has also been detected in other Italian regions. Both of our patients were from the Abruzzo Region in Middle Italy and had no Sardinian ancestor. Because no data are available regarding the frequency of this mutation in the Abruzzo population, it was not possible to exclude that T338I is also a common mutation in this region. When present in compound heterozygosity with a severe *CFTR* mutation, the T338I mutation has been reported to cause a mild form of CF characterized by isolated hypotonic dehydration [26]. However, a more severe phenotype (pulmonary disease) has been reported in Swedish patients [27]. This mutation has also been detected in two CBAVD patients in compound heterozygosity with the G542X mutation [8]. Thus, the T338I mutation can be considered as a mild *CFTR* mutation.

Deletion of the *CFTR* promoter and exon 1, which

were detected in one case in our study by MLPA, has been previously reported in compound heterozygosity with the 3120+1G>A mutation in three siblings with full-blown CF [28]. In addition, the deletion of exon 19, detected in another patient from our study, has been previously reported in CF patients in compound heterozygosity with one severe CF mutation or in a homozygous configuration [29, 30]. Thus, both of these mutations can be considered as severe *CFTR* mutations.

The missense I105N mutation, which was detected in one case by DHPLC, has been previously reported in one patient with full-blown CF in compound heterozygosity with the Q890X mutation (Cystic Fibrosis Mutation Database, Mutation Details) [8]. Thus, it can be considered a severe *CFTR* mutation. Finally, the 2811G/T allelic variant, which was detected in one case, has been previously reported in one patient with an atypical form of CF in compound heterozygosity with one severe CF mutation (Cystic Fibrosis Mutation Database, Mutation Details). However, because this sequence variant does not produce any amino acid changes in the CFTR protein, the pathogenic role of the 2811G/T mutation is debatable, even in CBAVD patients.

In conclusion, our sequential analysis allowed for the detection of three severe *CFTR* mutations, two mild mutations and one sequence variant of unknown pathogenic meaning. When considering the *CFTR* genotypes of our patients, we conclude that four out of the six patients with two mutations were carriers of a severe CF mutation. These patients were thus at risk for producing a full-blown CF child if the female partner was a healthy carrier of a severe CF mutation. Of these four patients, the second mutation had been detected in only one patient by the conventional screening for 36 *CFTR* mutations. The second mutation in the remaining three patients were identified by using the sequential approach that utilized reverse dot blotting, MLPA or DHPLC. We must stress that in clinical practice, this sequential approach should be used only when the presence of a CF mutation in the patient's female partner has been detected by first-level screening. In this study, none of the patients' female partners showed a CF mutation, and the study was performed only for research purposes.

In nine patients, despite use of the sequential approach, the second mutation of the *CFTR* gene was not detected. Because a carrier status is insufficient to cause CBAVD, additional factors or genes are required to clarify the pathophysiology of the disease in these patients. One possibility is that in patients carrying only one mutation, the second mutation could be a deep intronic variant in the *CFTR* gene. Such mutations have recently been described in three unrelated Italian CF patients [31]. Moreover, despite accurate patient selection and the use of three different approaches, the complete genotype rate in this study (60.8%) is lower than that in other recent European studies [12, 32, 33]. This information could suggest that in the Italian population, there are other regional mutations outside the *CFTR* coding region. The identification of such mutations represents a crucial point in the molecular diagnosis of *CFTR* alterations in CBAVD patients.

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