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Frequent Occurrence of the CFTR Intron 8 $(TG)_n$ 5T Allele in Men with Congenital Bilateral Absence of the Vas Deferens

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Abstract

Isolated congenital bilateral absence of the vas deferens (CBAVD) is an autosomal recessive disorder which has recently been shown to be associated with cystic fibrosis (CF) mutations. As part of an effort to understanding the genetic basis of this disorder, we have analysed the entire coding sequence and all the intron/exon boundaries of the cystic fibrosis transmembrane conductance regulator (CFTR) gene from 45 azoospermic individuals with this phenotype. We were able to detect a CFTR gene defect in 86% of chromosomes from these subjects. In addition to identifying 9 novel CFTR gene mutations, we found that a surprisingly high proportion (84%) of men with CBAVD who are heterozygous for a CF mutation carry the intron 8 polypyrimidine 5T CFTR allele on one chromosome. We hypothesise that this tight and significant ($p < 10^{-6}$) linkage reflects the very mild impact of this mutation on CFTR gene expression. Although genetic heterogeneity cannot be excluded, CBAVD patients in whom no CFTR mutation has been detected are likely to harbour additional unidentified mild mutations. These observations have implications for the genetic counselling of CBAVD patients and CF families, and couples undergoing in vitro fertilisation procedures.

Key Words
CFTR mutations
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Introduction

Male infertility due to isolated congenital bilateral absence of the vas deferens (CBAVD), an autosomal recessive disorder (McKusick 27718), has been shown in many instances to be associated with mutations in the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene. Although CBAVD is present in the majority of males with CF [1–3], some male patients present with isolated CBAVD. These individuals exhibit none of the clinical symptoms characteristic of CF [4]. The striking observations that the same mutations are found in CF and in isolated CBAVD [5–11] and that certain associations of mutations produce alternative phenotypes, either typical cystic fibrosis or isolated CBAVD, have prompted the genetic analysis of the CFTR gene in men with isolated CBAVD to determine the spectrum of mutations involved in this syndrome. These studies have initially revealed that an unexpectedly high proportion of men with CBAVD carry the most common CF mutation, ΔF508 [5], in one of their CFTR genes. Other CFTR defects were subsequently found in about 35% of chromosomes from males with CBAVD. Forty to sixty percent of these subjects are heterozygous for one known CF mutation, whereas around 10–20% harbour CF mutations in both CFTR genes [10, 12].

It has recently been observed that a particular mutation, R117H, can confer either a CF or isolated CBAVD clinical phenotype depending upon whether or not it is associated with different lengths of a polypyrimidine tract within intron 8 of the CFTR gene [13]. On the basis of this observation, we have surveyed the complete coding sequence and intron/exon boundaries of the CFTR genes from 45 patients with isolated CBAVD to determine the spectrum of CFTR mutations. We have also focused our analysis on the intron 8 polypyrimidine tract, of which one form, the 5T length variant, affects splicing efficiency of exon 9 [14, 15], and therefore diminishes CFTR protein production. Interestingly, R117H, in conjunction with the 5T length variant, results in CF when it is associated with another CF mutation [13]. In addition to the identification of 9 novel CFTR mutations, we show here that a high proportion (84%) of CBAVD men heterozygous for one CF mutation carry the 5T variant on one chromosome. Moreover, 2 of the 5 individuals in whom no CF defect could be detected carry one copy of this allele.

Materials and Methods

Patients

Forty-five male subjects suffering from infertility with azoospermia were examined. The diagnosis of congenital bilateral absence of the vas deferens and epididymis (cauda, corpus) was established by clinical examination. In doubtful cases, surgical exploration confirmed the diagnosis. In all subjects, semen analysis showed a reduced volume of ejaculate, complete absence of spermatozoa and the typical biochemical changes found in vas deferens agenesis: increased semen acidity (in most cases pH < 7), absence or decreased levels of fructose (a biochemical marker of seminal vesicle secretion), normal levels of citric acid (a prostatic marker) [16] and decreased levels of *L*-carnitine, confirming the absence of epididymal secretion [17]. Ultrasonography of the genito-urinary tract showed absence of the seminal vesicles in 2 subjects, and the presence of one or two hypoplasic or cystic vesicles in the others. Kidneys were normal in all but 1 subject who had unilateral hydronephrosis. Plasma follicle-stimulating hormone (FSH), luteinising hormone (LH) and testosterone levels were normal in all subjects.

CFTR Gene Analysis

DNA Preparation and PCR Amplification. Genomic DNA was extracted from blood leukocytes or from dried blood stains [18]. PCR amplification was performed as described previously [19,20]. Briefly, 1 μg of DNA was amplified in a 100-μl reaction mixture containing 50 m*M* KCl, 10 m*M* Tris-HCl (pH

8.4), 1.5 m*M* $MgCl_2$, 0.01% gelatin, 200 μ*M* of each dNTP, 0.2 μ*M* of each primer and 2.5 U of Taq polymerase (Perkin-Elmer Cetus). Forty cycles were performed, with 1 min denaturation at 94°C, 1 min annealing at the relevant temperature, and 2 min elongation at 72°C. The reaction was initiated by 5 min incubation at 94°C and ended with 7 min incubation at 72°C.

Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was carried out as described [19, 20]. Each of the 27 exons and surrounding sequences was analysed in each patient using conditions designed to obtain maximum resolution determined with the computer programmes MELT87 and SQHTX provided by L. Lerman [see ref. 21].

$(TG)_n$ Tm Sequence Analysis. Oligonucleotide primers used for amplification of the DNA region containing exon 9 and adjacent intron sequences have been given elsewhere [19]. Amplified DNA samples were run on a 6.5% polyacrylamide gel containing a linear denaturing gradient from 10 to 60%. Electrophoresis was performed at 160 V for 3 h with respect to the melting map of the sequence of interest. The DGGE patterns produced by the variable length of the $(TG)_n$ Tm sequences preceding exon 9 were characterised by direct DNA sequencing [22].

Results

CFTR Mutations Found in CBAVD Patients

Twenty-seven different molecular defects, in addition to ΔF508, were found in the patients studied in this work, and among them, 9 previously undescribed mutations were identified (table 1). Of these mutations, seven are missense mutations: D443Y, I556V (NBF1), V232D (TMD), D1377H (NBF2), R75L, D1154G, and A1067V, one is a nonsense defect (Q1411X) and another is a probable splicing mutation (876-10del8). These sequence variations have not been detected in the 200 non-CF chromosomes nor in the 350 non-ΔF508 CF chromosomes we have examined, and the amino acid substitutions produced by four of them (R75L, I556V, A1067V, and D1377H) are conserved among species (*Xenopus*, dogfish, mouse and cattle) [23]. Except for the ΔF508 mutation, which was observed on 23 chromosomes, no other defect seems prevalent in this cohort of CBAVD males. The mutations are scattered over the whole CFTR gene (fig. 1), and affect the two nucleotide-binding folds (NBFs), the regulatory (R) domain and the transmembrane regions.

Distribution of Intron 8 Variants in Normal, CF and CBAVD Chromosomes

The nucleotide variability of the DNA segment containing exon 9 and the immediately upstream intron 8 $(TG)_n$ Tm polymorphic tract produces several distinct recognisable DGGE patterns. These patterns could be allocated by sequence analysis to nine different alleles defined by the association of 9, 10, 11, 12, or 13 TGs with 5, 7 or 9 consecutive thymidines. Table 2 shows the distribution of these various $(TG)_n$ Tm variants determined in normal (n = 131), CF (n = 50) and CBAVD (n = 90) chromosomes. The 7T variant is the common variant found in normal chromosomes (85.5%) while the 9T and 5T variants account for only 9.2 and 5.3%, respectively. In the CF chromosomes, the 9T variant is predominant (82%) and no 5T variant is observed. In CBAVD chromosomes, there is a 2- to 3-fold higher prevalence of the 9T and 5T variants than in control chromosomes. As previously described by Chu et al. [15], the TG_{10} 9T allele is in absolute linkage disequilibrium with the common ΔF508 mutation.

Genotypes of the CBAVD Patients at the CFTR Locus

The CBAVD patients fall into three groups: 15 (33%) harbour a CF defect in both chromosomes, 25 (56%) have one detectable CF mutation, and in the 5 remaining individuals (11%), no CF defect could be detected. Among the patients of the first group, only 3

Table 1. Association of CFTR mutations with the $(TG)_n$ Tm alleles in 45 CBAVD patients

Patient	CFTR mutations	$(TG)_n$ Tm alleles	Sweat test mEq/l	Other clinical features
2 mutations detected (33%)				
1	ΔF508/*A1067V*	$(TG)_{10}9T/(TG)_{10}7T$	ND	
2	ΔF508/P574H	$(TG)_{10}9T/(TG)_{11}7T$	ND	
3	ΔF508/*R75L*	$(TG)_{10}9T/(TG)_{11}7T$	ND	
4	ΔF508/R117H	$(TG)_{10}9T/(TG)_{10}7T$	53	
5	ΔF508/R117H	$(TG)_{10}9T/(TG)_{10}7T$	ND	diarrhoea
6	ΔF508/R117H	$(TG)_{10}9T/(TG)_{10}7T$	72	
7	ΔF508/R117C	$(TG)_{10}9T/(TG)_{10}7T$	ND	
8	ΔF508/*D1154G*	$(TG)_{10}9T/(TG)_{10}7T$	50	
9	ΔF508/R668C+*D443Y*	$(TG)_{10}9T/(TG)_{10}7T$	69	
10	2622+1G→A/*D1377H*	$(TG)_{10}9T/(TG)_{11}7T$	ND	
11	W1282X/3849+10 kbC→T	$(TG)_{10}9T/(TG)_{12}7T$	103	recurrent bronchitis
12	*Q1411X*/R668C+*D443Y*	$(TG)_{10}7T/(TG)_{10}7T$	55	
13	S549N/*D443Y*	$(TG)_{10}7T/(TG)_{11}7T$	65	
14	3272-26A→G/P99L	$(TG)_{10}7T/(TG)_{12}7T$	55	
15	*I556V*/Q1352H	$(TG)_{11}7T/(TG)_{11}7T$	40	
1 mutation detected (56%)				
16	ΔF508/–	$(TG)_{10}9T/(TG)_{11}5T$	63	
17	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
18	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
19	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
20	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
21	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	52	
22	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	36	
23	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	33	
24	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	42	
25	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
26	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
27	ΔF508/–	$(TG)_{10}9T/(TG)_{12}5T$	ND	
28	ΔF508/–	$(TG)_{10}9T/(TG)_{13}5T$	ND	
29	ΔF508/–	$(TG)_{10}9T/(TG)_{11}7T$	ND	
30	*876-10del8*/–	$(TG)_{10}7T/(TG)_{12}5T$	ND	
31	W1282X/–	$(TG)_{10}7T/(TG)_{12}5T$	ND	
32	W1282X/–	$(TG)_{10}7T/(TG)_{12}5T$	ND	
33	G542X/–	$(TG)_{10}9T/(TG)_{9}9T$	ND	
34	E60X/–	$(TG)_{9}9T/(TG)_{11}7T$	64	
35	1717-1G→A/–	$(TG)_{10}7T/(TG)_{12}5T$	ND	
36	*V232D*/–	$(TG)_{10}9T/(TG)_{12}5T$	58	
37	1677delTA/–	$(TG)_{11}7T/(TG)_{12}5T$	ND	
38	1078delT/–	$(TG)_{11}7T/(TG)_{12}5T$	ND	
39	V562I/–	$(TG)_{10}7T/(TG)_{11}5T$	ND	
40	G1069R/–	$(TG)_{10}7T/(TG)_{11}7T$	53	
No mutation detected (11%)				
41	–/–	$(TG)_{11}7T/(TG)_{13}5T$	ND	
42	–/–	$(TG)_{10}7T/(TG)_{11}9T$	ND	
43	–/–	$(TG)_{10}7T/(TG)_{12}5T$	69	pancreatitis
44	–/–	$(TG)_{10}7T/(TG)_{11}7T$	48	
45	–/–	$(TG)_{10}9T/(TG)_{10}7T$	ND	

The previously undescribed mutations are shown in italics; – = no mutations identified.

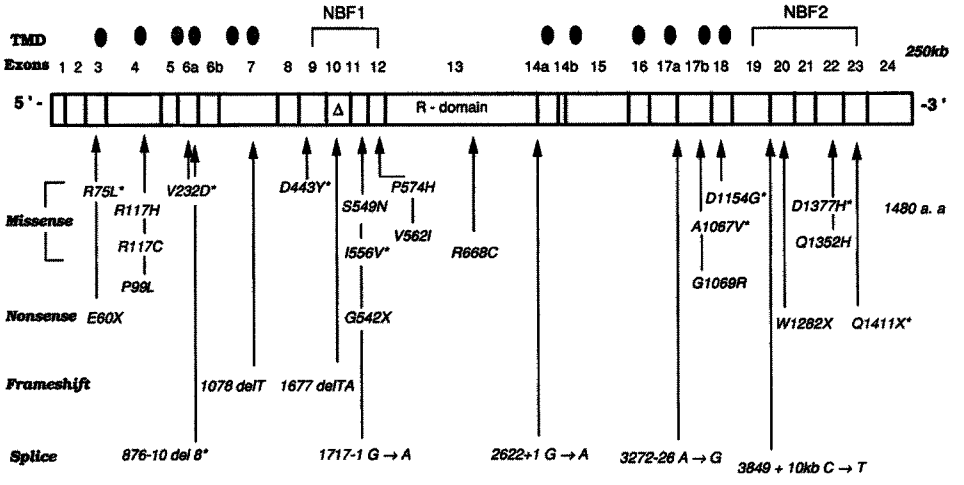

Fig. 1. Diagram of the CFTR gene cDNA showing the location of mutations identified in the CBAVD patients from this study, and their relation with the predicted functional domains of the protein. TMD = Transmembrane domains; NBF1 and 2 = nucleotide-binding domains; R-Domain = putative regulator domain; Δ = ΔF508 mutation; aa = amino acid; * = novel mutations.

Table 2. Distribution of CFTR intron 8 $(TG)_n$ Tm alleles on normal, CF and CBAVD chromosomes

$(TG)_n$ Tm	Normal[a] (non-CF chromosome)	CF[b]	CBAVD (45 subjects)
$(TG)_9$9T	0	0	2
$(TG)_{10}$9T	11	41	27
$(TG)_{11}$9T	1	0	1
Number of 9T	12 (9.2%)	41 (82%)	30 (33%)
$(TG)_{10}$7T	28	5	21
$(TG)_{11}$7T	72	3	14
$(TG)_{12}$7T	12	1	2
Number of 7T	112 (85.5%)	9 (18%)	37 (41%)
$(TG)_{11}$5T	7	0	2
$(TG)_{12}$5T	0	0	19[c]
$(TG)_{13}$5T	0	0	2
Number of 5T	7 (5.3%)	0 (0%)	23 (26%)[d]
Total	131	50	90

[a] The CFTR allele not transmitted to CF patients is considered normal (non-CF chromosome).

[b] CF = Cystic fibrosis alleles (35 ΔF508 chromosomes and 15 non-ΔF508 chromosomes).

[c] $p = 3 \cdot 10^{-5}$ (Fisher test) when compared to the $(TG)_n$ 5T normal alleles.

[d] $p < 10^{-6}$ when compared to the 5T in normal alleles.

(20%) are compound heterozygotes for ΔF508/R117H, and the polypyrimidine tract variants detected are either 7T or 9T. The 5T variant is only observed in the two other groups, in 21 instances (84%) in the CBAVD males bearing one CF mutation, and in 2 (2/5) patients of the third group, in which no CF mutations could be detected.

Discussion

The analysis of the CFTR genes in 45 CBAVD patients confirms the previous observations [5–11] that this syndrome is associated with a high incidence of mutations also observed in CF [24, 25]. None of the subjects studied in this work had pulmonary or gastrointestinal manifestations of CF, nor had they a family history of pulmonary disease or CF. Nevertheless, since 3 of them had suffered sporadically from episodes of bronchitis, diarrhoea or pancreatitis (table 1), long-term follow-up of these patients is necessary since they might develop other symptoms related to a mild form of CF.

In many instances, the patients were reluctant to disclose their medical status to their relatives and opposed our request to perform genetic analysis of their family. To establish which $(TG)_n$ Tm allele was linked with one or the other mutation carried by the patient, we made use of the fact that the ΔF508 mutation has been shown to be associated in all instances with the 9T variant [15]. However, in 8 of 23 cases, the linkage phase remained unknown.

Although difficult to obtain, sweat tests were nevertheless carried out in 19 patients. Seven had sweat chloride values >60 mEq/l (table 1).

Of the 45 CBAVD patients studied, 33% bear two CFTR mutations, 56% have one such mutation, while we could not find any mutation in the remaining 5. This proportion of CBAVD subjects compound heterozygous for two CF mutations is higher than previously reported (10–24%) by others who have also extensively analysed the CFTR genes in their cohort of patients [10, 11, 26, 27]. The detection method we have used to screen the entire CFTR exons and intron/exon boundaries is suited to detect more than 98% of the nucleotide substitutions. Nevertheless, it is possible that the promoter region or other regulatory sites of the CFTR gene may be altered by mutations that escaped the resolution of this method. Although we cannot exclude the possibility that another gene or genes could be involved in this syndrome [26, 28], the men with isolated CBAVD in whom we detected only one, or even no, mutation, are likely to carry additional nucleotide variation(s) that remain to be detected. Linkage analysis of families in which several men display this phenotype could provide insight into a possible genetic heterogeneity of this disease.

Of particular interest, our findings extend those of Osborne et al. [29] and Chillon et al. [27] who have observed a high proportion of the 5T variant in the patients having one or no CF mutation. Furthermore, we show that the 5T variant is found in more than 82% of cases (19/23) within the $(TG)_{12}$ 5T sequence repeat ($p = 3 \cdot 10^{-5}$). This significant excess of the 6T variant could explain the clinical phenotype displayed in a favourable genetic context, since this variant, which is thought to be responsible for inefficient splicing, lowers the levels of the CFTR protein produced from the corresponding chromosome. It has been demonstrated that the presence of the 5T allele sequence in intron 8 is associated with a mRNA lacking exon 9 [15]. This altered mRNA accounts for up to 92% of the total CFTR mRNAs in homozygous 5T individuals, and results in a non-functional CFTR protein [30, 31]. Consequently, the CBAVD pa-

tients with the 5T variant bear in fact two CF mutations, a severe one such as ΔF508 or one of the nonsense or splicing defects, and a very mild CF allele (5T). When 5T occurs in cis to another defect such as R117H in patients who also carry in trans a severe CF mutation, CF results [13]. Although 1 CBAVD patient, homozygous for the 5T allele but with no other detected CFTR mutation has been recently reported [27], whether this sequence alone can account for the CBAVD phenotype remains unclear. Homozygosity for 5T could allow production of an amount of CFTR protein compatible with a normal cellular function. In this hypothesis, the CBAVD phenotype would manifest only when this splicing defect acts in concert with another mutation able to alter in some way CFTR protein function.

If this hypothesis is correct, we can predict that the genotype of the individuals listed in the second group of subjects (table 1), who have one CFTR mutation and the 5T allele, for example ΔF508/5T or W1282X/5T, is sufficient to produce the CBAVD phenotype. Only 4 of the 25 patients in this group (one mutation detected) have CBAVD with no 5T. In these patients, analysis of mature CFTR mRNA using RT-PCR could reveal the existence of a splicing anomaly caused by another yet unknown intronic sequence variation. The same experiments should be performed in the patients of the third group in whom no mutation was as yet found, in addition to looking at the occurrence of possible defects in the promoter region.

Finally, our results are important with regard to the genetic counselling provided to infertile patients treated by in vitro fertilisation and to members of CF families. Patients with CBAVD should be informed of the consequences, for them and their relatives, of being carriers of CFTR gene mutations that have, for the most part, also been observed in patients with CF. An increasing number of couples with infertility choose to be treated by assisted-fertilisation methods such as intracytoplasmic sperm injection; when the male patient has CBAVD, a search for the most frequent CF mutations should be undertaken in his partner. In addition, the occurrence of mutations which, like R117H, have been observed in different phenotypes and on two chromosomal contexts [13], may complicate counselling for prenatal diagnosis and carrier screening. Recent data, including those reported here, show that a more accurate prediction of the type of illness which is at risk in a given individual should rely on genotyping protocols that include determination of CFTR intron 8 polypyrimidine tract length variants.

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