ORIGINAL RESEARCH

Clinical hallmarks and genetic polymorphisms in the *CFTR* gene contribute to the disclosure of the A1006E mutation

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Abstract

Since the identification of the Cystic Fibrosis transmembrane conductance regulator (*CFTR*) gene in 1989, many genetic mutations have been found in cystic fibrosis (CF) patients. Dysfunctions of the *CFTR* gene are responsible for the highly variable clinical presentation ranging from severe CF, disseminated bronchiectasis, idiopathic chronic pancreatitis and congenital bilateral absence of vas deferens (CBAVD). Linkage disequilibrium studies have shown that some mutations are stringently coupled with polymorphisms in a genetic complex called haplotype. From a familial study of a patient with CBAVD, carrier of the A1006E mutation, we have observed its strict association with the polymorphism 5T-TG11. In order to speed up the genetic diagnosis and to correlate the clinical setting to this genetic feature, we have directly investigated the exon 17a, where the A1006E mutation is located, of five cystic fibrosis patients belonging to two unrelated families. All patients had the 5T-TG11 tract, F508del and one unknown mutation. One more family with two affected individuals carrying the Q220X/A1006E mutations was investigated for the poly-T polymorphism. All the members were found to have the A1006E mutation and the 5T-TG11 in the same DNA strand, demonstrating that this strategy is a reliable and inexpensive method for genotyping the *CFTR* gene. A detailed description of the clinical presentation and follow-up are provided in order to highlight common phenotypic

features useful to improve the management of cystic fibrosis patients.

Severe dysfunction of the Cystic Fibrosis transmembrane conductance regulator gene (*CFTR*) causes cystic fibrosis (CF), a life-shortening disorder in which progressive lung disease is unfortunately common. The diagnosis is easy in patients presenting typical symptoms and multiple organ involvement; however, the age of onset of symptoms and the clinical presentation may be highly variable, making the diagnosis difficult especially for individuals with partial phenotypes.¹ To date more than 1500 sequence variations have been identified in the *CFTR* gene and their listing is continuously updated within the Cystic Fibrosis Genetic Analysis Consortium database (<http://www.genet.sickkids.on.ca/cftr/app>). Moreover, molecular diagnosis of CF, particularly for non-classical CF or *CFTR*-related diseases, involves rare mutations and requires extensive gene sequencing, a time consuming and costly approach.² In order to overcome this problem, many attempts have been

made to use linkage analysis of polymorphic loci to known mutations because intragenic marker haplotype analysis has been shown to facilitate mutation screening.³ In fact, it is well known that F508del mutation is in linkage disequilibrium with the polymorphic tract 9T⁴ and the I148T sequence variation is frequently associated with the 3199del6 mutation.^{5,6}

The poly T tract, a string of thymidine bases located in intron 8 of the *CFTR* gene, may be associated with non-classical CF or *CFTR*-related disorders depending on its size.⁷ The 5T variant decreases the efficiency of intron 8 splicing when the associated TG sequence is 12 or 13 repeats in length, while 11 repeats seems to be irrelevant.^{8,9} 5T-TG analysis is routinely performed in our lab and we have investigated many patients, including some cases of Congenital Bilateral Absence of Vas Deferent (CBAVD). Among these, due to familial segregation studies, we have found that the A1006E mutation is in cis with the 5T-TG11 polymorphism. Based on these observations we have decided to use the 5T-TG11 haplotype to trace the A1006E mutation in five CF patients with incomplete genotyping, belonging to two unrelated families. Two additional related patients, both compound heterozygotes carrying the A1006E, were investigated for the Tn-TGm locus to implement the finding of this association. A detailed clinical presentation and follow-up is provided in order to obtain a genotype-phenotype correlation.

Clinical History

Family 1

A 36-year-old, well-nourished (BMI 22.2 kg/m²), female had been referred to our Centre one year earlier, complaining of recurrent respiratory symptoms. She had a negative family history of CF and no significant episodes of pulmonary exacerbation and/or gastrointestinal symptoms. She has a daughter in good clinical condition. CF was then diagnosed (chloride sweat test: 84 mmol/L), with pancreatic insufficiency (positive

fecal elastase test: 42 µg/g feces), mild lung failure (FEV1: 70%) and diffuse bronchiectasis as evidenced on a CT scan. First level screening genetic analysis revealed a genotype F508del/Un, 5T/9T. Sputum culture showed a chronic colonization by *Staphylococcus aureus* and an intermittent appearance of *Pseudomonas aeruginosa*. Recently, numerous colonies of *Aspergillus fumigatus* have been detected. She takes oral supplementation with digestive enzymes and no abdominal symptom has been reported; however, in the last year she suffered from multiple pulmonary exacerbations that required several intravenous antibiotic courses.

Family 2

A 29-year-old female was diagnosed with CF when she was 14-year-old, following investigation for a recurrent nasal polyposis. Her elder sister, currently aged 36 years, was also investigated. Both sisters had a positive chloride sweat test (>70 mmol/L) and a chronic lung colonization by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. They also carried the same *CFTR* gene mutations (Q220X/A1006E). At the time of diagnosis both sisters were pancreatic sufficient and exclusively referred respiratory symptoms. Subsequently, the elder sister developed pancreatic insufficiency after recurrent bouts of pancreatitis, and was started on oral assumption of digestive enzymes. The younger sister did not develop pancreatic insufficiency but she presented clinical signs of the metabolic syndrome (systemic blood hypertension, obesity and hypercholesterolemia). They both have bronchiectasis, mild lung failure and recurrent episodes of pulmonary exacerbations. Currently, both patients are fertile females each with a healthy child.

Family 3

CF was diagnosed in the youngest son of the family through neonatal screening (ImmunoReactive Trypsinogen test, IRT) and successively confirmed by a positive sweat test (Na⁺: 76 mmol/L). Now he is 18-

year-old. Diagnostic investigation was extended to his three elder sisters, currently aged 34, 32 and 31 years, showing a positive sweat test in all cases (Na^+ : 121, 119 and 97 mmol/L, respectively). First level screening genetic analysis revealed the same genotype (F508del/Un, 5T/9T) in all the family members. All the patients suffer from chronic lung colonization by *Staphylococcus aureus*; chronic colonization by *Pseudomonas aeruginosa* has been detected only in the two eldest sisters. At diagnosis, they had pancreatic and respiratory sufficiency. The patient aged 32 years had just developed an atelectasis of the upper lobe of the right lung; she successively underwent a surgical excision of the lobe because of severe episodes of haemoptysis. The last measured FEV1 value was 74.7% whereas the last lung CT evaluation showed diffuse bronchiectasis. Furthermore, she had recurrent episodes of pancreatitis that led to pancreatic failure. Now she refers to our Centre for a follow-up visit at least every two months.

The other three family members currently maintain pancreatic and respiratory sufficiency. Neither of the two younger sisters show significant episodes of pulmonary exacerbation and lung damage, whereas the eldest sister had two episodes/year, treated with oral antibiotics and initial bronchiectasis at the X-ray assessment.

Materials and Methods

Genomic DNA was extracted from peripheral blood, after informed consent had been obtained, using the commercial kit High Pure PCR Template Preparation (Roche Diagnostics, Mannheim, Germany). Mutations and Tn locus were screened by means of the InnoLipa 17+ Tn update, 19 and Regional assay (Innogenetics N.V. Ghent, Belgium) following the manufacturer's protocol. Exon 9 and its 5' intron junction, containing the TG(m)-Tn loci, were amplified using the following primers: forward 5' TGAAAATATGTGGACA AACT 3'; reverse 5' TTTCTAGTTTGTAGTGCTGG AAG 3'. The different alleles were designated accord-

ing to the number of repeats. For the exon 17a and exon 10 analyses the primers pairs respectively used were:

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Ex-17a F AATCACTGACACACTTTGTCCACTT;
Ex 17a R TCAAATAGCTCTTATAGCTTTTACAAAGATG.
Ex 10 F GTGATTTGATAATGACCTAATAAT;
Ex 10 R CATTACAGTAGCTTACCCA.
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DNA amplification was carried out in a 9600 thermal cycler (Applied Biosystem, Foster City, CA, USA) as reported by Le Maréchal¹⁰ with minor modifications. The PCR product was sequenced on ABI PRISM 310 genetic analyser (Applied Biosystem) using the Big Dye Terminator v.1.1. Data were analyzed with the Sequencing Analysis 5.3.1 and the SeqScape 2.6 software (Applied Biosystem).

Results

Patients were firstly investigated to detect the number of TG repeats associated with the 5T polymorphism. The splicing junction of the exon 9 was sequenced and all the samples showed to have a string of 11 dinucleotide repeats. Family 1 and Family 3 members underwent exon 17a sequencing that revealed the 3149 C>A mutation (A1006E) in all of them. Exon 10 was also analyzed in order to complete the haplotype feature: M470 was homozygous in each individual while the V562I sequence variation was present in all but one patient. A familial study involving all the informative relatives was then carried out showing the linkage of 5T-TG11 with the A1006E mutation.

Discussion

The A1006E is included in the group of the so called "borderline mutations".⁷ It is a missense defect due to the C to A nucleotide variation at 3149, within the exon 17a, resulting in the amino acid change alanine to glutamic acid. The involved proteic region is the second membrane spanning domain that contributes to complete the proper CFTR channel structure. In our CF patient's database the A1006E mutation has been

TABLE 1. Allelic genotyping of the patients with *CFTR* gene

Patients	Haplotype	Mutations	Tn-TGm	Sequence variations	Sequence variations
Pt1	Strand 1	F508del	9T-TG10	M470V	
	Strand 2	A1006E	5T-TG11	M470V	-
Pts2-3	Strand 1	Q220X	7T-TG11	M470V	
	Strand 2	A1006E	5T-TG11	M470V	V562I
Pts4-7	Strand 1	F508del	9T-TG10	M470V	
	Strand 2	A1006E	5T-TG11	M470V	V562I

detected, using the trace of the 5T-TG11 polymorphism, in 3 out of 436 CF chromosomes, accounting for an estimated incidence of about 0.7%. A similar rate has been observed in the Spanish population, while, surprisingly, this mutation was not detected among the Hispanics of the Latin American populations.¹¹ The same study identified its association with the V562I and the IVS8-5T in a complex allele that is now updated including the TG11 polymorphism reported from our investigations. These results have been further confirmed through familial studies of informative relatives that showed the same linkage of the 5T-TG11 polymorphism with the A1006E mutation.

Complex alleles arise from DNA regions, with low recombination rates, that segregate preferentially together. Some haplotypes contain loci mutually influencing the gene function, such as the R117H-poly-T association;¹²⁻¹⁴ others are neutral or ameliorative. A detailed *in phase* description of the current study haplotypes is shown in Table 1. Among the A1006E part-

ners a possible role in modifying gene activity may be attributed to the poly-5T-TG tract, and indirectly, to M470V. Previous works report the strong association of the valine at 470 and the 5T allele and suggest that the V470 sequence variation could be involved in reducing the 5T expression resulting in lower CFTR product levels.¹⁵ In our families, including the CBAVD patient who gave rise to this study, we always found the M470 in *cis* with 5T-TG11. Moreover, all but one case (Pt1) show the V562I revealing a weaker association between this sequence variation and the previously described haplotype.

The A1006E mutation has been detected in large series of patients with CBAVD or CF but scarce information is available from the clinical setting. Our patient's phenotypic features are summarized in Table 2. All cases show elevated sweat test values; members of Family 3 were tested with a sweat sodium (not chloride) analysis because it was routinely performed at the time of diagnosis (about 18 years ago). Despite the late onset of clinical presentation (mean age at di-

TABLE 2. Phenotypic features of patients with *CFTR* gene

Pts	Age at diagnosis (years)	Sweat test (mmol/L)	Current clinical status				
			Age (years)	X-Ray Lung Assessment	FEV1%	PS	ARP
1	35	Cl: 84	36	Bronchiectasis	61	-	-
2	21	Cl: 74	36	Bronchiectasis	76	-	+
3	14	Cl: 76	29	Bronchiectasis	73	+	-
4	IRT +	Na: 76	18	Bronchial thickening	110	+	-
5	16	Na: 121	34	Initial bronchiectasis	86	+	-
6	14	Na: 119	32	Bronchiectasis; lobe excision	74	-	+
7	13	Na: 97	31	Bronchial thickening	91	+	-

IRT: Immunoreactive Trypsinogen Test

FEV1: Forced Expiratory Volume in 1 second

PS: Pancreatic Sufficiency

ARP: Acute Recurrent Pancreatitis

agnosis of 16.2 ± 10.5 years, median age of 14 years) the course of the disease leads to a complete phenotypic expression. Except for two individuals with less frequent pulmonary exacerbation (pt4 and pt7), lung disease has been the most relevant clinical feature leading to repeated hospitalizations or surgical treatments. Exocrine pancreatic failure was observed at the time of referral only in pt1, who displayed full-blown symptoms of a complete form of CF; this probably reflects the older age at diagnosis (35 years) in comparison with the other patients. Pt2 and pt6, initially pancreatic sufficient, became pancreatic insufficient owing to recurrent episodes of acute pancreatitis. The neonatal screening (IRT) allowed an early detection of CF in the younger patient of Family 3 who, to date, has not developed pancreatic insufficiency and significant symptoms or signs of a lung damage. IRT also permits the rapid investigation of other family members, avoiding further delays in diagnosis. The positive impact of neonatal screening on the diagnosis of CF underlines the relevance of a more comprehensive management strategy for the disease, even if it is currently unclear which kind of management would be suitable.¹⁶ The results of neonatal screening and the first level genetic testing may suggest further investigations and, as in our cases, suggest a shortcut to a faster and less expensive genotyping. On the other hand, the same approach could be used for adult patients who never underwent IRT screening but present symptoms suggestive of CF or a CFTR-related disease.

An interesting observation is the unexpectedly elevated incidence of the A1006E mutation within our data base of CF patients. This result may be attributed to the small sample size; a more exhaustive study, including a large cohort of patients, should be carried out. Moreover, the geographic origin of our families may suggest a possible relationship for the high rate of the A1006E, with the historical Spanish domination along the south-western regions of our country.

In conclusion, the study reports a detailed description of the phenotypic spectrum of the A1006E muta-

tion, providing useful information for a ready and inexpensive genotyping in selected cases. Moreover, our data corroborate the importance of poly-T analysis for effective characterization of different CF forms. For the clinical setting, these results illustrate the importance of an early detection of the disease - before the patients become symptomatic;¹⁷ IRT and sweat test appear to be sensitive diagnostic tests for patients affected by the A1006E mutation. Since there is the possibility of the occurrence of a late, but complete, phenotypic expression of the disease, clinical follow-up is necessary to start adequate preventive therapy, thereby preventing or delaying the onset of lung damage.

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