Journal of Cystic Fibrosis 7 (2008) 347–351

www.elsevier.com/locate/jcf

Epidemiology and a novel procedure for large scale analysis of CFTR rearrangements in classic and atypical CF patients: A multicentric Italian study

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Received 21 November 2007; received in revised form 7 December 2007; accepted 15 December 2007
Available online 14 February 2008

Abstract

Background: Mutation epidemiology in each ethnic group is a crucial step of strategies for cystic fibrosis (CF) diagnosis and counselling. To date, the scanning of the whole coding region of the cystic fibrosis transmembrane conductance regulator (CFTR) gene permits to identify about 90% of alleles from patients bearing CF and a lower percentage in patients bearing atypical CF. CFTR rearrangements in heterozygosis elude current techniques for molecular analysis, and some of them have been reported with a frequency up to 6% in various ethnic groups.
Methods: Using quantitative PCR analysis of all coding regions, we assessed the occurrence of CFTR rearrangements in 130 alleles from classic CF patients and in 198 alleles from atypical CF patients (all unrelated and from Italian descent) bearing unidentified mutations after the scanning of CFTR.
Results: Seven rearrangements (i.e., dele1, dele2, dele2_3, dele 14b_17b, dele17a_18, dele22_23, and dele22_24) were identified in 34/131 (26.0%) CF alleles bearing undetected mutations (which means about 2.5% of all CF alleles) and in none of the 198 alleles from atypical CF. The CFTR haplotype and the sequence analysis of the breakpoints confirmed the common origin of all the rearrangements. Thus, we set up a novel duplex PCR assay for the large-scale analysis of the seven rearrangements. The procedure was rapid (all PCR amplifications were obtained under the same conditions), costless and repeatable.
Conclusions: It is useful to select the CFTR rearrangements more frequent in specific ethnic groups and to set up procedures for large-scale analysis. Their study can be performed in cases in which a high detection rate is required (i.e., partners of CF carriers/patients). On the contrary, the analysis of rearrangement is useless in atypical CF patients.

Keywords: Large-scale analysis; Epidemiology; Large rearrangements; Classic CF; Atypical CF

1. Introduction

Cystic fibrosis (CF) is the most frequent severe autosomal recessive disorder in Caucasian populations, with a frequency of about 1:2500 live births. Thus far, more than 1600 CF-causing mutations have been identified in the cystic fibrosis transmembrane conductance regulator (CFTR) disease-gene [1]. Mutation epidemiology in each ethnic group is a crucial component of strategies for CF diagnosis and counselling, because it permits to select the panel of mutations with the highest detection rate to be routinely tested, and to calculate the residual risk of being a CF carrier. To date, the scanning of the whole coding region of the gene

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doi:10.1016/j.jcf.2007.12.004

Table 1
Number and (percentage) of CF alleles bearing CFTR rearrangements

CF patients (n)	CF alleles bearing undetected mutations	Alleles bearing CFTR rearrangements
Southern Italy (55)	62	19
Central Italy (30)	32	8
North-eastern Italy (36)	37	7
Total (121)	131	34

with DGGE, DHPLC or direct sequencing identifies about 90% of CF alleles [2–4].

More recently, atypical CF has been described, expressing with a mild, monosymptomatic phenotype and usually associated to normal sweat chloride and to a benign prognosis. These forms include congenital bilateral absence of vasa deferentes (CBAVD), recurrent pancreatitis, bronchiectasis and others [5,6]. The detection rate of molecular analysis in these forms is lower as compared to classical CF [7].

Large rearrangements of the CFTR gene may be identified fortuitously either by noting a uniparental inheritance pattern, or through failure to amplify target sequences if such mutations are present in homozygosis. In fact, these mutations in heterozygosis elude current techniques for molecular analysis, including scanning procedures. Southern blotting, traditionally used for detecting large genomic rearrangements, is rather laborious and, therefore, scarcely useful for large-scale analysis. A macrodeletion involving CFTR exons 17a_18 has a high incidence among Palestinian Arabs [8] and another involving CFTR exons 2_3 spreads in different ethnic groups [9]. The development of a rapid technique of comparative dosage analysis permits to found such gene rearrangements and a number of these mutations has been identified in the French CF population [10]; thus, reports on large gene deletions also in Italian patients appeared [11,12]. These studies refer to patients bearing CF, while rearrangements in patient bearing atypical CF are more rare [13–15].

The aim of our study was to define the incidence of CFTR gene rearrangements in a large number of alleles from unrelated Italian patients bearing CF or atypical CF, and to set up a novel procedure for large-scale analysis.

2. Materials and methods

2.1. Patients

We studied 121 unrelated patients bearing CF selected from three geographical areas: Southern (i.e., Campania and Basilicata regions, 55 cases); Central (i.e., Lazio and Molise regions: 30 cases); North-eastern Italy (Veneto region: 36 cases). All patients had a classic form of the disease (i.e., altered sweat test, pancreatic insufficiency and a different degree of respiratory involvement), and were regularly in follow-up to the regional Centre for CF of each region. These patients had been selected among all patients followed to these Centres because after scanning analysis of the whole coding regions of CFTR, one (111 patients) or both (10 patients) mutations resulted undetected. Globally, 131 alleles had CFTR undetected mutations.

Furthermore, we studied 144 patients bearing atypical CF forms, i.e., 84 congenital bilateral absence of vas deferens (CBAVD); 26 recurrent pancreatitis; and 34 bronchiectasis. Also these patients had been selected among all patients bearing atypical CF from our Centre because after scanning analysis of the whole coding regions of CFTR, one (90 patients) or both (54 patients) mutations resulted undetected. Globally, 198 alleles had undetected mutations.

2.2. Methods

A sample of DNA from the above mentioned patients, collected at the time of diagnostic molecular analysis, was used for our study once received the informed consent from the patient (or tutor for minors). In 85 CF patients and in all 144 patients bearing atypical CF, the analysis of CFTR rearrangements was performed using a quantitative PCR followed by capillary electrophoresis (MLPA SALSA, MRC-Holland, The Netherlands). Deletions of probe recognition sequences appear as a 35–50% reduced relative peak area of the amplification product of that probe. Copy number of target sequences is determined by a comparison to control samples. For each run, three control samples were used, i.e., sample from a heterozygote subject for a CFTR rearrangement, a wild-type sample and a negative control (no DNA). Each rearrangement was confirmed by gene sequencing analysis of the junction (protocol available on request) with an automated procedure (3100 Genetic Analyzer, Applied Biosystem). The analysis of CFTR intragenic STR IVS8CA, IVS17bTA and IVS17bCA was performed by PCR followed by capillary electrophoresis set up on 3130 Genetic Analyzer (Applied Biosystem) (protocol available on request); the IVS8 TG repeat and diallelic polymorphisms XV2c, KM19, J44 and M470V were analyzed by direct gene sequencing.

The remaining 36 CF patients were analyzed using a quantitative multiplex PCR amplification of short fluorescent

Table 2
CFTR haplotype associated to each rearrangement

CFTR rearrangement	CF alleles	XV2c	KM19	J44	IVS8 TG	IVS8 CA	M470V	T854	IVS17b CA	IVS17b TA
Dele1 c.4_53+69del; 53+4192_53+4489inv; insG	2 (2, 0, 0)	1/2	1/2	N.T.	7	16	2	1	13	30
Dele2 c.54-5811_164+2186del;273+6780_237+6961inv	8 (1, 2, 5)	1/2	1	2	7	16	2	1	13	30
Dele2_3 c.54-5490_273+10250del	1 (0, 1, 0)	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Dele14b_17b c.2620-674_3367+198del	2 (0, 0, 2)	2	1	2	7	17	2	1	13	Partially deleted
Dele17a_18 c.2988+1173_3468+2111del	14 (3, 3, 8)	2	2	1	9	23	1	1	Deleted	Deleted
Dele22_23 c.3964-78_4242+577del	2 (0, 1, 1)	2	2	1	9	23	1	1	12	30
Dele22_24 c.3964-3890_*3143delinsTAACT	5 (1, 1, 3)	2	1	2	7	16	2	1	12	30

N.T.: not tested. The numbers of CF alleles in parenthesis refer to North-eastern, Central and Southern Italy, respectively.

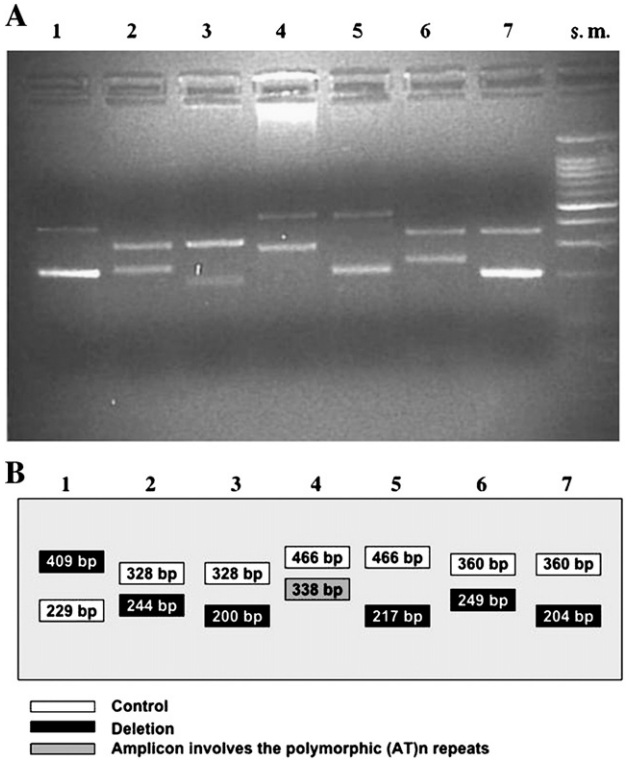

Fig. 1. Duplex PCR assay of the seven rearrangements. A) Gel electrophoresis and B) schematic representation of the pattern in seven different heterozygous patients bearing: 1) dele_1; 2) dele_2; 3) dele2_3; 4) dele14b_17b; 5) dele17a_18; 6) dele22_23; 7) dele22_24 and s.m) 100 bp size marker.

fragments (QMPSF) previously developed [10]. QMPSF is a semiquantitative-PCR: oligonucleotide primer pairs for amplifying short fragments corresponding to the 27 exons of the CFTR gene were used to construct six multiplex PCRs. Each multiplex PCR assay also contained a control primer pair that amplified a short exonic sequence of the hemocromatosis gene (HFE). One primer of each pair was 5' labelled with the 6-FAM fluorochrome. Amplified DNA fragments were separated on an ABI PRISM 3130 (Applied Biosystem). The presence of a rearrangement is indicated by a two-fold reduction in the height of the corresponding peak.

For the CFTR rearrangements we used the nomenclature by Human Genome Variation Society, but within the text and in tables, for each rearrangements we used a simplified nomenclature as follows: exon 1 rearrangement- c.4_53+69del53+4192_53+4489invinsG alias dele1; exon 2 rearrangement- c.54-5811_164+2186del273+6780_237+6961inv alias dele2; exons 2 and 3 deletion- c.54-5490_273+10250del alias dele2_3; exons 14b, 15, 16, 17a and 17b deletions- c.2620-674_3367+198del alias dele14b_17b; exons 17a, 17b and 18 deletion- c.2988+1173_3468+2111del alias dele17a_18; exons 22 and 23 deletion- c.3964-78_4242+577del; exons 22, 23 and 24 rearrangement- c.3964-3890_*3143delinsTAACT alias dele22_24. References [10] and [12] report diagrams showing the proposed mechanisms of dele1 and dele2 mutations.

3. Results

As shown in Table 1, we identified a CFTR gene rearrangements in 34/131 (26.0%) CF alleles bearing undetected mutations (which means about 2.5% of all CF alleles); the occurrence of these mutations ranged between 18.9% (North-eastern Italy) to 30.6% (Southern Italy). Table 2 shows the distribution of these rearrangements in CF alleles from patients of different origin (i.e., North-eastern, Central and Southern Italy). Seven different gene rearrangements all previously described were identified in these 34 alleles (Table 2). One rearrangement (dele2_3) was observed in a single CF allele; three of them (i.e., dele1, dele14b_17b, dele22_23) were identified each in 2 CF alleles; the dele22_24 was identified in 5 CF alleles; the dele2 was identified in 8 CF alleles and finally, dele17a_18 was identified in 14 CF alleles.

The analysis of extragenic (i.e., XV2c, KM19, J44) and intragenic CFTR polymorphisms associated to each allele bearing a rearrangement revealed that each of the six rearrangements identified in more than a single allele is associated to a single haplotype (Table 2). Sequence analysis confirmed that each of

Table 3
Primers pairs for duplex PCR analysis of CFTR rearrangements

CFTR rearrangement	Control			Deletion	
	Exon	Primers pair	Amplicon size (bp)	Primers pair	Amplicon size (bp)
Dele1	1	F: GGCAGGCACCCAGAGTAGTA R: GCTTATTCCTTTACCCCAAACC	229	F: GGCAGGCACCCAGAGTAGTA R: GCTTATTCCTTTACCCCAAACC	409
Dele2	2	F: TTCCATATGCCAGAAAAGTTGA R: GCCACCATACTTGGCTCCTA	328	F: GGAATCAGAGGAGGGGAAAT R: ATCATTCCCTCAGCATCCAA	244
Dele2_3	2	F: TTCCATATGCCAGAAAAGTTGA R: GCCACCATACTTGGCTCCTA	328	F: ATTGACCACTTTAATGGTGTTTACCTA R: TACACTCAGAACCCATCATAGGATAC	200
Dele14b_17b	17a	F: ATGTGAAAATGTTTACTCACCAACA R: ATGAATGTCCTGTACACCAACTGT	466	F: AATGTTTCCACCTTGAGAAAGC R: AATCTGTGTGCATCGGTTTTTA	338*
Dele17a_18	17a	F: ATGTGAAAATGTTTACTCACCAACA R: ATGAATGTCCTGTACACCAACTGT	466	F: CCCAAGTGAACTTTGGCATT R: GCATGGGTGACAGCACTATG	217
Dele22_23	23	F: CCCATGGTTGAAAAGCTGAT R: GCAATTTGCAGGAACTATCACA	360	F: CTGATTCTTTTGAGCTGTCAAGG R: CAGAACTTCCTTGTGACTAAACCAT	249
Dele22_24	23	F: CCCATGGTTGAAAAGCTGAT R: GCAATTTGCAGGAACTATCACA	360	F: TAAAGGATTCTGCTGCCACA R: GGGGAGATATGTGGCCTTCT	204

*Amplicon involves the polymorphic (AT)n repeats.

the six rearrangements was associated to the same breakpoint (data not shown).

The analysis of the 198 alleles bearing undetected CF mutations from the patients with atypical CF revealed that none of them had gene rearrangements.

To analyze the seven rearrangements on large-scale, we developed a novel duplex PCR assay. We designed primers flanking the deletion breakpoint that amplify a product of about 200 bp in the presence of the rearrangement, whereas control primers generate a product of about 400 bp containing an intact CFTR exon involved in the rearrangement. The only exception is the deletion of exon 1. In this case the deletion spans 119 bp and there is an insertion of 229 bp, so only one pair of primers is required to obtain a 229 bp wild type PCR product and a 409 bp mutated product. A standard PCR protocol was followed with primer annealing set at 55 °C (data available on request). PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium-bromide staining (see an example in Fig. 1A). All primers and product size are summarized in Table 3 and in Fig. 1B and also report the size of the fragments obtained from the wild-type and the mutated allele for each rearrangement. The same primers may be used also to detect the junction by direct gene sequencing.

Using the novel procedure, we analyzed in blind all DNA samples bearing rearrangements and an equivalent number of controls. The analysis was performed in double, by two independent operators, and provided the expected results, i.e., all alleles were correctly classified.

4. Discussion

We observed a CFTR rearrangement in 26.0% of alleles from Italian patients bearing CF with unidentified CFTR mutations after gene scanning. Considering that the percentage of CF alleles bearing undetected mutations after gene scanning analysis is 8 to 12% in different Italian regions [2–4], it means that about 2.5% of alleles from Italian CF patients bear a rearrangement.

Only seven CFTR rearrangements were been observed, i.e., dele1, dele2, dele2_3, dele14b_17b, dele17a_18, dele22_23 and dele22_24, and all these mutations had been previously described in other populations. For example, the dele17a_18, that we observed in 14 CF alleles, had been observed with a high frequency also in Palestinian Arabs due to a founder effect [8]. On the contrary, the dele2_3, observed only in 1 CF alleles in our study had been described with a frequency ranging from 0 to 6% in CF alleles from a large series of ethnic populations from Central and East Europe [9]. Furthermore, we identified 8 patients bearing the new insertion/deletion (dele2) recently reported in 3.4% of CF alleles from Sardinian patients [12]. Even if the number of studied alleles is low, some differences may exist in the distribution of the seven rearrangements in CF alleles from patients of different origin (i.e., North-eastern, Central and Southern Italy), as it has been previously described for other CFTR mutations [4,16].

Each rearrangement observed in more than a single allele in our study is associated to the same haplotype of CFTR polymorphisms and this evidence, together to the observation that the breakpoints coincide for all CF alleles bearing each of the six rearrangements, confirms that each of these mutations has a single origin. For the dele17a_18, the haplotype observed in our population coincide with that reported for the same mutation in Palestinian Arabs [8] with the exception of the IVS8, that in our population is 23 and in the other is 22 due to a slippage event or to a technical mis-typing; this observation, and the concordance of the breakpoint suggests a common origin of the mutation, even if it is difficult to speculate on a founder effect both among Palestinian Arabs and Italians.

In any case, the coincidence of the breakpoint of all alleles bearing the same rearrangement, and the absence of other, novel/rare macrodeletions in our population, suggest that the seven rearrangements identified in our patients may be routinely tested with the PCR of the junction avoiding PCR quantitative procedures of the whole gene. It can be used a first level approach, as those described in the present paper (duplex analysis), which is rapid, repeatable and costless, being based on PCR amplification of all involved traits under the same conditions. Otherwise, the direct gene sequencing can be performed by labs that scan the whole CFTR gene.

From the diagnostic point of view, the analysis of rearrangements increases of about 2.5% the detection rate of molecular analysis, at least in our population, thus, it must be performed in all cases in which a high detection rate is required i.e., the partner of a CF carrier/patient, in which a more accurate estimation of the risk is required.

Finally, in patients bearing atypical CF no rearrangements were evidenced in the present study, confirming that these mutations are rare among atypical CF alleles. Infact, Hantash et al. described a single CBAVD bearing the deletion of exons 22 to 24 and 5T allele [13] and Taulan et al. detected two heterozygous deletion, one encompassing exon 2 and the other removing exons 22 to 24 in two males carrying a typical CBAVD mutation on the other allele [15]. Thus, a strategy can be to test for CFTR rearrangements only in atypical patients that do not bear a “severe” CFTR mutations after first level analysis.

To conclude: seven CFTR rearrangements have a frequency of about 2–3% in alleles from patients bearing CF in Italian regions. The common origin of these mutations permits their large-scale analysis with rapid and simple procedures as that described in the present study. No rearrangements were identified in alleles from patients bearing atypical CF.

Acknowledgments

This study was supported by the “Fondazione per la ricerca sulla Fibrosi Cistica” (Verona, Italy) and by Ministero della Salute (Rome, Italy), D.L. 229/99, 2003-2004 and L. 362/99. We are indebted to Jean Gilder for editing the text.

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