

ONLINE MUTATION REPORT

Rapid detection of *CFTR* gene rearrangements impacts on genetic counselling in cystic fibrosis

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Cystic fibrosis (CF) (MIM 219700) is one of the most common autosomal recessive diseases in Caucasians.¹ It affects about 1 in 2500 births and approximately 1 in 25 individuals are heterozygotes, with marked regional variations² (www.genet.sickkids.on.ca/cftr). It is caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR* or *ABCC7*) gene (MIM 602421), which is also involved in a broad spectrum of phenotypes, including male infertility by congenital bilateral absence of the vas deferens (CBAVD),³⁻⁵ disseminated bronchiectasis (DB),^{6,7} and chronic pancreatitis.^{8,9} So far, over 1000 *CFTR* gene mutations have been described throughout the gene, along with geographic and ethnic variations in their distribution and frequency (www.genet.sickkids.on.ca/cftr).^{2,10} Of these variations, 99% consist of point mutations or micro-deletions/insertions (www.genet.sickkids.on.ca/cftr) but account for 33-98% of CF alleles, depending on the population.¹⁰ A number of cases remain unsolved after extensive and laborious screening of the 27 exons, thus making genetic counselling difficult for the patients and their families, particularly when the CF diagnosis is not certain. Unidentified CF mutations may lie in introns or in regulatory regions which are not routinely investigated, or correspond to gene rearrangements such as large deletions at the heterozygous state which escape detection using current PCR based techniques. Deletions have been suspected in a very few situations: upon failure of PCR amplification to target particular exons when the deletions were present in CF patients in the homozygous state, or in cases of abnormal segregation of a mutation or polymorphisms in a family. Two such deletions, *CFTR*dele2-3¹¹ and *CFTR*dele17a-18,¹² which are now routinely tested for by conventional PCR using specific primers, were found in about 5% and 13% of CF chromosomes in Slav and Arab populations, respectively.

Screening for unknown *CFTR* deletions still remains a challenge and quantitative PCR based methods represent an attractive approach.¹³ Very recent data have shown that 16% of unidentified CF alleles in a mainly French population consist of large *CFTR* gene rearrangements.¹⁴ Analysis of a larger and heterogeneous population using a semi-quantitative fluorescent PCR assay targeting the 27 *CFTR* exons, led us to identify undescribed *CFTR* gene anomalies and a similar but higher proportion of rearrangements. We discuss here the place of our assay in the strategy to diagnose CF and related diseases, and its implications for genetic counselling and care of CF patients and their families.

METHODS

Patients

We investigated a total of 78 unrelated French patients or parents of deceased patients from mixed ethnic/geographic origins and subjected them to a complete *CFTR* gene screening. The subjects were divided into three groups according to the results of a previous screening: (i) 43 CF patients who fulfilled the diagnostic criteria of CF¹⁵ and who

Key points

- Cystic fibrosis (CF), one of the most frequent hereditary diseases in the Caucasian population, is mainly due to point mutations scattered over the whole cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *CFTR* gene deletions are rare, but their frequency may be underestimated as they remain undetected when using conventional PCR techniques.
- We studied 78 patients, including 62 CF patients or parents of CF patients bearing one or two unidentified CF alleles after an extensive *CFTR* gene study, and 16 patients apparently homozygous for a *CFTR* mutation.
- Making use of an assay based on semi-quantitative fluorescent PCR targeted on the 27 *CFTR* exons in a three multiplex format, we characterised gene rearrangements in 11 patients, including a recurrent deletion, a complete gene deletion, and the first *CFTR* gene duplication. Taking into account three known deletions previously characterised in other CF patients, gene rearrangements thus accounted for 20% of unidentified CF alleles and for 1.3% of all CF mutations in our population.
- The method is both simple and reliable, and is able to detect large rearrangements and single nucleotide deletions/insertions as well. Together these account for almost 24% of the CF mutations described, and makes this the method of choice for second line screening when frequent mutations are not found. This strategy has notable implications for genetic counselling and care of CF patients and their family.

carried a CF mutation, and seven parents of deceased CF patients, a CF mutation having already been identified in the other parent (50 unidentified CF alleles); (ii) 12 CF patients with no identified CF mutation (24 unidentified CF alleles); and (iii) 16 patients apparently homozygous for a *CFTR* mutation and who had CF (F508del -n=6-, 2104insA-2109del10, S945L, 3120+1G→A, N1303K) or a *CFTR* related disease, that is, isolated CBAVD (D110H, R117H, L997F, R74W-D1270N) or DB (R334W, R668C-G576A-D443Y) (0-16 unidentified CF alleles). In these cases, status confirmation was not possible by family analysis. In

Abbreviations: CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; *CFTR* gene, cystic fibrosis transmembrane conductance regulator gene; DB, disseminated bronchiectasis; DGGE, denaturing gradient gel electrophoresis; DHPLC, denaturing high pressure liquid phase chromatography; FISH, fluorescent in situ hybridisation; MP, multiplex PCR; QFM-PCR, semi-quantitative fluorescent multiplex PCR

addition, we considered that the genotype could not explain the observed phenotypes in CBAVD and DB patients.

When available, parents of patients in whom an abnormal pattern had been detected were studied. All the patients were referred to our laboratory between 1989 and 2004. Informed consent to *CFTR* studies had been previously obtained from the patients and/or their parents at the time of referral to the laboratory.

Previous extensive *CFTR* gene analyses with a mutation detection rate of 95% included: (i) screening for 31 frequent mutations (CF OLA assay, Abbott, Rungis, France); (ii) scanning of the 27 exons and their boundaries using denaturing gradient gel electrophoresis (DGGE)^{16,17} or denaturing high pressure liquid phase chromatography (DHPLC), followed by sequencing to characterise the variants; (iii) screening for the intronic splicing 1811+1.6kba→G mutation¹⁸; and (iv) screening for three previously characterised deletions using specific primers: *CFTR*dele2,3,¹¹ *CFTR*dele17a–18,¹² and *CFTR*dele19.¹⁹ This last step led us to identify large deletions in four former patients: *CFTR*dele2,3 (one allele), *CFTR*dele17a–18 (two alleles), and *CFTR*dele19 (two alleles). These patients were thus not included in the cohort of patients studied here, but the data were taken into account to determine the proportion of *CFTR* gene rearrangements among CF alleles.

DNA extraction

Most genomic DNAs were extracted from whole blood samples collected on EDTA using a phenol chloroform reference protocol or a commercial kit (Nucleon, BACC3, Amersham Biosciences, Saclay, France). Some DNAs, received from other laboratories, were extracted with different protocols. DNA concentration and quality were determined for each sample.

Screening for *CFTR* rearrangements by semi-quantitative fluorescent multiplex PCR (QFM-PCR)

The original protocol described by Yau *et al.*²⁰ was adapted to screen for *CFTR* rearrangements. Briefly, the principle is based on comparisons of the fluorescent profiles of multiplex PCR fragments obtained from different samples, the amplification being stopped at the exponential phase. This procedure allows the detection of heterozygous deletions (twofold reduction of fluorescence intensity) and heterozygous duplications (1.5-fold increase). The 27 *CFTR* gene exons, a promoter region (18731_18868, GenBank AC000111.1, or –940 to –803 according to the current *CFTR* gene numbering) and a region containing the polyadenylation signal sequence (58920_59091, GenBank AC000061.1, or 6035_6206 according to the current *CFTR* gene numbering) were amplified in three fluorescently labelled multiplex reactions, denoted multiplex PCR (MP): MP 1 (promoter, polyadenylation signal sequence, exons 1–6a and 11), MP 2 (exons 7–10 and 12–16), and MP 3 (exons 6b and 17a–24). Intronic specific primers (but exonic for the large exon 13) were chosen mostly among those designed for mutation screening. The sequences of the primers used are available upon request. In each set, two external controls were used: *DSCR1* exon 4 (chromosome 21)²¹ and *F9* exon 5 (chromosome X). The forward primers were labelled with the fluorescent phosphoramidite 6-FAM dye and all the primers were HPLC purified (MWG, Courtaboeuf, France). The PCR reactions were performed in duplicates in 25 µl reactions using the QIAGEN Multiplex PCR kit (Qiagen, Courtaboeuf, France), with 300 ng of genomic DNA and a mix of primers (concentration range 0.1–0.8 µM). The reaction started with an initial denaturation of 15 min at 95°C, followed by 19 cycles at 95°C for 30 s, 55°C (MP 1 and MP 2) or 50°C (MP 3) for 30 s, and 72°C for 45 s, and a final extension of 10 min at

72°C. Then 2 µl of the purified PCR products were added to 9.8 µl formamide and 0.2 µl Genescan-500 Rox size standard (Applied Biosystems, Foster City, CA, USA). The fluorescent PCR products were heat denatured, chilled on ice, and separated on a 16-capillary sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). The results were processed by Genescan 3.7 software (Applied Biosystems) to obtain electropherograms for each sample. Each product was identified by its size, and fluorescence intensities were correlated to the copy number of the relevant exons. Four control DNAs were included in each experiment: two normal DNAs, that is, one from a woman and one from a man (twofold reduction of *F9* exon 5 in men compared to women); a DNA for trisomy 21 (1.5-fold increase of *DSCR1* exon 4); and a DNA carrying a previously identified *CFTR* deletion (*CFTR*dele2–3 for MP 1, *CFTR*dele4–10 for MP 2, and *CFTR*dele17a–18 or *CFTR*dele19 for MP 3). The results were first analysed visually by superimposing fluorescent profiles of tested samples and normal controls, the normalisation being performed with exon 4 *DSCR1* peaks. The peak height values were also imported into an Excel (Microsoft) spreadsheet and the copy number of each fragment was determined by calculating a dosage quotient (DQ) for each exon relative to all the other amplified exons in patients and controls (table 1).²⁰

The reproducibility of the whole procedure was assessed by several operators who tested the same samples from patients, and normal and mutant controls, on the same day (duplicates) and on different days.

Molecular characterisation of rearrangements

Several methods were used to characterise the rearrangements detected by QFM-PCR. When apparent deletions removed only one exon, mispriming at primer binding sites was first checked by amplification of the relevant exon using another set of primers bracketing the fragment amplified by QFM-PCR.

The size of rearrangements removing one or several exons was investigated by long range PCR experiments using the Expand Long Template PCR system (Roche Diagnostics, Meylan, France). The resulting fragments were sequenced to precisely define the deletion breakpoints and allow the design of primers suited to detect the deletions by conventional PCR. In the case of complete *CFTR* gene deletion, fluorescent in situ hybridisation (FISH) analysis with a *CFTR* cDNA probe

Table 1 Statistical profiles from the analysis of patient no. 7 carrying the *CFTR*dele17a–17b deletion from multiplex PCR 2 (MP 2) and 3 (MP 3)

| | Height value | | Dosage quotient | |
|------------------------|---------------|--------------|---------------------|------------------|
| | Patient no. 7 | Control male | <i>DSCR1</i> exon 4 | <i>F9</i> exon 5 |
| Reference gene | | | | |
| <i>DSCR1</i> exon 4 | 565 | 461 | – | 0.98 |
| <i>F9</i> exon 5 | 502 | 400 | 1.02 | – |
| <i>CFTR</i> exon | | | | |
| Exon 16 (MP 2) | 1156 | 1006 | 0.94 | 0.92 |
| Exon 17a (MP 3) | 827 | 1292 | 0.52 | 0.51 |
| Exon 17b (MP 3) | 360 | 690 | 0.43 | 0.42 |
| Exon 18 (MP 3) | 1052 | 827 | 1.04 | 1.01 |

The DQ values indicative of the 17a–17b deletion pattern are underlined. From our study, values of the DQ were within the range 0.75–1.31 (mean 0.95) for normal control samples, 0.38–0.64 (mean 0.48) for a heterozygous deletion control, and 1.40–1.79 (mean 1.55) for a heterozygous duplication control. They were concordant with those obtained by Yau *et al.*²⁰

As an example (indicated in bold characters), the DQ for *CFTR* exon 17a compared to *DSCR1* exon 4 is $DQ_{17a/DSCR1} = \text{Patient (CFTR 17a/DSCR1)}/\text{Control (CFTR 17a/DSCR1)} = (827/565)/(1292/461) = 0.52$.

(6.5 kb) was used to confirm its presence. We also performed FISH experiments with commercially available bacterial artificial chromosomes (BACs) of the 7q31 region and designed several additional sets of QFM-PCR primers to delineate the extent of this large deletion.

To determine the haplotypes associated with the rearrangements and potentially probe their limits, intragenic microsatellites were analysed in the patients and their families using conventional PCR or semi-quantitative PCR (when segregation study was not informative): IVS1(CA), IVS6a(TTGA), IVS8(CA), IVS8(TG)mTn, IVS17b(TA), and IVS17b(CA) along with single nucleotide polymorphisms (1540A/G and 2694T/G).

RESULTS

Detection of *CFTR* rearrangements

QFM-PCR screening led to the detection of rearrangements in 11 families (table 2). Ten of them were found in the first group of 50 patients or parents of deceased patients where a mutation had been previously identified. None was detected in the second group of 12 patients who carried two unknown mutations. One further rearrangement was identified in the third group of 16 patients, in a CBAVD patient who was apparently R117H homozygous. No rearrangement was detected in the other patients of the third group, confirming that they were homozygous for a *CFTR* mutation. The results obtained in the third group thus added a CF allele to the total of unidentified alleles which were investigated for the presence of rearrangements. Overall, a rearrangement was found in 11/75 (14.7%) unidentified CF alleles. Taking into account the five CF alleles bearing known deletions that were previously identified, *CFTR* gene rearrangements accounted for 20% of 80 so called unidentified CF alleles, where a point mutation or a short deletion/insertion has not been found.

The 11 rearrangements detected consisted of eight different patterns. Most exhibited a simple deletion pattern involving one or several consecutive exons: 1; 2; 17b; 14a–17b; 17a–17b in four unrelated cases (fig 1A and table 1). Two others consisted of a complex deletion removing exons 3–10 and 14b–16, and a whole *CFTR* gene deletion (fig 1B). The former was found in *cis* with the V754M variation (exon 13), which has been described as a CF mutation (www.genet.sickkids.on.ca/cftr). The complete deletion was identified in a

patient having CBAVD, and who apparently carried two R117H–7T copies (R117H in *cis* with the IVS8–7T variant). Posterior analysis of his parents confirmed the compound heterozygosity for R117H and the deletion. The last rearrangement consisted of a duplication pattern of exons 4–8 (fig 1C).

The multiplex assay was sensitive enough to detect a number of micro-deletions/insertions within exons, which modified the fragment size, such as F508del, 394delTT, 2183AA→G, 4016insT, as well as STR variants, for example at the IVS6a(TTGA) and IVS8(TG)mTn polymorphic sites.

Molecular characterisation of the rearrangements

The 11 rearrangements consisted of eight different anomalies, of which six are new (table 3). The two already reported involve exon 1²² and exon 2 (Mekus and Tümmler, www.genet.sickkids.on.ca/cftr). We named the new rearrangements according to the nomenclature recommendations (www.hgvs.org), but the A of the ATG translation start codon was numbered +133 to be in accordance with the current *CFTR* gene numbering (GenBank NM_000492.2) and the CF mutation database, where the rearrangements were reported. The breakpoints of three new deletions involving intron 17b were determined. The 3' breakpoints were concentrated in a small AT-rich region including the IVS17b(TA) microsatellite. Hence, abnormal segregation of this microsatellite was observed in four families where such a deletion was detected. The 3' breakpoint of the partial deletion of exon 17b, 3413del355ins6, is located 9 bp after the IVS17b(TA) site, while those of *CFTR*dele14b–17b and *CFTR*dele17a–17b are located within it, leaving stretches of 13(TA) and 7(TA) repeats, respectively. The junction sequence of *CFTR*dele17a–17b was identical in the four patients carrying this anomaly, which was associated with the same extended haplotype (IVS1(CA), IVS6a(TTGA), IVS8(CA), IVS8(TG)mTn, 1540A/G, 2694T/G, IVS17b(CA); data partially shown in table 3). In all the cases characterised at the molecular level (nos. 1, 3–8), the rearrangements were inherited in a stable manner, as the breakpoints were identical in the patients and their parents.

The precise characterisation of the other rearrangements was hampered by various difficulties: (i) the size of the complete *CFTR* gene deletion, which was not detected by

Table 2 Phenotype and genotype data of patients carrying *CFTR* rearrangements

| Patient no. | Gender | Current age | Phenotype | | | | | Sweat test | Genotype | | Origin | |
|-------------|--------|----------------------------|------------------|---------------|--------------|-----------|----------|------------|--|----------|--------------------------------------|--|
| | | | Age at diagnosis | Pancr. status | Lung disease | Other | Allele 1 | | Allele 2 rearrangement involving exon(s) | Parental | Geographic | |
| 1 | M | 10 years | 1 month | PI | Severe | | 114 | F508del | 1 | Father | North eastern Italy | |
| 2 | M | 16 years | Birth | PI | Severe | | 130 | A561E | 2 | Father | Southern Italy | |
| 3 | M | 10 years | 1 year | PI | Severe | | + | R553X | 17b | Mother | France | |
| 4 | F | 13 years | 4 years | PI | Severe | NP | + | F508del | 14b–17b | Father | Eastern France | |
| 5 | F | 24 years | 1 month | PI | Severe | | 100 | F508del | 17a–17b | Mother | ND | |
| 6 | F | 21 years | Childhood | PI | Moderate | | + | F508del | 17a–17b | Father | Eastern France | |
| 7 | M | 35 years | 1 year | PI | Severe | CBAVD, NP | 103 | F508del | 17a–17b | Father | Eastern France | |
| 8* | 2 F | Deceased at 2 and 6 months | Birth | PI | Severe | | ND | F508del | 17a–17b | Father | Eastern France | |
| 9 | F | Deceased at 15 years | 5 years | PI | Severe | | 300 | 1812–1G→A | 3–10,14b–16† | Mother | Kabylie (Algeria)/ Brittany (France) | |
| 10 | M | 37 years | 37 years | PS | None | CBAVD | ND | R117H(–7T) | 1–24 | Mother | France | |
| 11 | M | Deceased at 31 years | 3 months | PI | Severe | DB | 90 | G542X | 4–8 | Mother | Eastern France | |

CBAVD, congenital bilateral absence of the vas deferens; DB, disseminated bronchiectasis; del, deletion; dup, duplication; F, female; M, male; NP, nasal polyposis; Pancr., pancreatic; PI, pancreatic insufficiency; PS, pancreatic sufficiency.

*Case 8: the deletion was identified in the father of two deceased children.

†The *CFTR*dele3–10,14b–16 deletion was identified in *cis* with the V754M variation.

Table 3 Molecular characterisation of the *CFTR* rearrangements

| Patient no. | Rearrangement | Simplified name | Exon(s) involved | Linked haplotype IVS1(CA)-IVS8(CA)-IVS17b(TA)-IVS17b(CA) | Motif sequence at the breakpoints |
|-------------|---|-----------------------------|--------------------------------|--|-----------------------------------|
| 1 | c.136_c.185+69del119bpins299bp* | 136del119ins299 | Part of 1 (codons 2–18) | 23–16–29–13 | Inverted <u>CCATG</u> |
| 2 | c.186-?_c.296+?del† | <i>CFTR</i> dele2 | 2 | 24–16–30–14 | ND |
| 3 | c.3413_c.3499+268del355bpins TGTTAA | 3413del355ins6 | Part of 17b (codons 1094–1122) | 23–16–del–13 | Direct <u>CTGT</u> and AT rich |
| 4 | c.2752–674_c.3499+198del9855bp | <i>CFTR</i> dele14b–17b | 14b–17b | 23–16–13–13‡ | Direct <u>TCGG</u> and AT rich |
| 5–8 | c.3121–977_c.3499+248del2515bp | <i>CFTR</i> dele17a–17b | 17a–17b | 23–16–7–13‡,§ | Symmetric <u>ATG</u> and AT rich |
| 9 | [c.297-?_c.1716+?del; c.2752-?_c.3120+?del] | <i>CFTR</i> dele3–10,14b–16 | 3–10 and 14b–16 | 22 or 23–16–7–17¶ | ND |
| 10 | <i>CFTR</i> dele1–24 | <i>CFTR</i> dele1–24 | 1–24 | del–del–del–del | ND |
| 11 | c.406-?_c.1341+?dup | <i>CFTR</i> dup4–8 | 4–8 | 27–17–7–17** | ND |

The nomenclature recommendations were followed (www.hgvs.org) but the A of the ATG translation start codon was numbered as +133, according to the current *CFTR* gene numbering (GenBank NM_000492.2) and the CF mutation database. The new rearrangements are indicated in bold.

*The same indel was described in a CF patient with paternal isodisomy²² and further found in a French CF patient.¹⁴

†The same *CFTR*dele2 as that described by Mekus and Tümmler (www.genet.sickkids.on.ca/cfr) was identified using specific primers provided by T Dörk.

‡Abnormal segregation of the IVS17b(TA) microsatellite was observed using the flanking primers,⁴² as the 3' breakpoint is located within this site. The number of (TA) repeats, indicated in italics, has thus been determined by sequencing.

§The linked haplotype was demonstrated in case nos. 5 and 6 and hypothesised in case nos. 7 and 8.

¶The precise IVS1(CA) allele could not be determined, as the father's DNA was not available.

**The linked haplotype was hypothesised, considering the most frequent haplotype IVS8(CA)23-IVS17b(TA)33-IVS17b(CA)13 linked to G542X.⁴³

conventional karyotyping but confirmed by FISH analysis (data not shown); (ii) the lack of DNA or cDNA to study the complex *CFTR*dele3–10,14b–16 deletion, which removes at least 54 kb, and the *CFTR*dup4–8 duplication, which is predicted to extend over a minimum of 11 kb.

DISCUSSION

CFTR rearrangements account for 20% of unidentified CF alleles

In the population studied, *CFTR* rearrangements were identified in 11 unrelated families. All these rearrangements were characterised in families where a CF mutation had been previously identified. Taking into account the number of alleles bearing one of three known deletions (*CFTR*dele2–3, *CFTR*dele17a–18, and *CFTR*dele19) in our population, large *CFTR* gene rearrangements represented 20% of CF alleles where a point mutation or a short deletion/insertion has not been found, and 1.3% of overall CF anomalies. These figures indicate a slightly higher proportion of *CFTR* rearrangements in our larger and probably more heterogeneous population than in that studied by Audrezet *et al.*¹⁴ Screening for *CFTR* gene rearrangements should especially benefit populations where the rate of point mutations is particularly low compared to that studied here. The remaining unsolved cases could be explained by mutations that escaped DGGE or DHPLC screening, or splicing anomalies located within introns. Alternatively, it is possible that a number of patients who were diagnosed with CF but who had no identified mutation after extensive gene screening have been indeed misdiagnosed, or that mutations in a gene other than *CFTR* could account for their CF-like disease.²³

Audrezet *et al.* documented heterogeneity in five large deletions,¹⁴ in terms of location, extent, and mutational mechanism. We further document this heterogeneity, as we identified eight different rearrangements, of which six are new. They include deletions or indels involving one exon (1, 2, 17b) or several contiguous exons (14b–17b; 17a–17b), a complex deletion (*CFTR*dele3–10,14b–16), the first described deletion of the whole gene, and the first described *CFTR* duplication (*CFTR*dup4–8). Although the functional consequences of the identified rearrangements have not been evaluated, we can postulate that they preclude *CFTR*

expression and are thus null mutations, in keeping with the observed phenotypes. Likewise, given the classical CF phenotype of patient no. 11, who carries a duplication of exons 4–8, we hypothesise that the duplicated region is located inside the *CFTR* gene and interferes with the transcription or translation process, thus resulting in a null mutation.

The breakpoints of three new deletions were determined. Interestingly, *CFTR*dele17a–17b was found in four unrelated patients originating from Eastern France and is probably associated with the same haplotype, which suggests a founding effect. It would be interesting to screen for this particular deletion in patients from neighbouring countries. In this deletion and in those involving exon 17b and exons 14b–17b, an AT-rich environment, together with a short repeat at the breakpoints, may have favoured non-homologous recombination by slipped mispairing (table 3), as has been described for other *CFTR* deletions^{11 12 14 19 24} and deletions of other disease causing genes.²⁵ The sequences bordering the breakpoints were screened for the presence of motifs known to be associated with site-specific recombination, mutation, cleavage, and gene rearrangements²⁶; no such element was observed. Query to RepeatMasker2 (<http://www.repeatmasker.org/>) revealed no sequence homology that could have favoured homologous unequal recombination. In particular, the 5' breakpoint of *CFTR*dele14b–17b is located within an *Alu* sequence, but no homologous sequence was found around the 3' breakpoint, not even a partial core sequence.²⁶ A mechanism of non-homologous recombination could also be invoked to explain the occurrence of the *CFTR*dup4–8, as has been demonstrated for duplications in other disease causing genes^{27–29}; and the complex *CFTR*dele3–10,14b–16 anomaly, although homologous unequal recombination remains a possible hypothesis given the presence of a number of interspersed repeat elements in the introns involved in the rearrangements. Another complex deletion removing exons 4–7 and 11–18 (*CFTR*50kdel) has been reported,³⁰ but its breakpoints remain unknown so far. Interestingly, intron 10 is involved in both complex rearrangements. Unequal homologous recombination is more likely to have occurred for the complete *CFTR* gene deletion which removes at least 3 Mb.³¹

Considering the *CFTR* deletions already described and those we report here, it clearly appears that some *CFTR* sequences may be prone to rearrangements. Strikingly, the small intron 17b (2.8 kb) is involved in the 3' breakpoint of five deletions (*CFTR*dele14b–17b, *CFTR*dele16–17b, *CFTR*dele17a–17b, 3413del355ins6, and the deletion removing the entire exon 17b described by Magnani *et al*²⁴), the IVS17b(TA) site appearing as a critical hotspot involved in the three described here. The *CFTR* introns 1, 3, and 18 are involved in the breakpoints of four, six, and five other deletions, respectively. However, these introns are large and it is not documented whether identical sequences are involved in the rearrangements.

Implications for genetic counselling

Identification of a CF rearrangement definitely confirmed the CF diagnosis in all cases but that of the CBAVD patient, although, at present, a moderate form of CF cannot be ruled out. Some of the patients were referred to our laboratory several years ago and, even if the diagnosis had been clearly established on the basis of clinical and biological findings,¹⁵ the presence of only one CF mutation after exhaustive screening of the *CFTR* gene coding regions could have thrown doubt upon the diagnosis. Moreover, in the context of a hereditary disease, identification of each parental CF anomaly allows the psychological burden to be shared in the family. Indeed, family studies showed that all the rearrangements were inherited from a parent (father in six cases, mother in the five others including that of the duplication). In other respects, it greatly facilitates genetic counselling and cascade screening in relatives and makes feasible prenatal diagnosis by direct analysis of the causative mutations. Precise identification of the breakpoints enables the design of primers for conventional PCR which may be easier and cheaper to use in these situations (in particular, the PCR primers and conditions for the detection of the common *CFTR*dele17a–17b are available upon request). The search for *CFTR* gene rearrangements should also be considered in patients presenting with a *CFTR* related disease and who carry a mild mutation, since they may have a severe CF mutation in *trans*.

Determination of *CFTR* copy number allows discrimination between true homozygotes for identified CF mutations and compound heterozygotes for a CF mutation and a deletion removing at least the relevant exon. In cases of apparent homozygosity for a *CFTR* mutation, ruling out a sequence variation at the primer binding sites can be performed first by sequencing the corresponding exon using a set of external primers.³² Then, determination of copy number, particularly useful when segregation analysis is difficult or not possible in the family, can be rapidly performed. Such a discrimination is crucial when cascade screening and prenatal diagnosis are requested. Geneticists must also be aware of correlations between genotype and phenotype. In the particular case of patient no. 10, we considered that a R117H–7T homozygous genotype could not explain the CBAVD phenotype and suspected rather the presence of a severe CF anomaly in *trans* of R117H. Identification of a gene rearrangement in this patient had notable implications for genetic counselling for himself and his partner, as the couple had been referred for advice about assisted reproduction, and for his family. Indeed, once studied, the patient and his parents forwarded the genetic information to other family members. In other respects, the proven homozygous genotype for mild *CFTR* mutations found in CBAVD or DB patients of the third group, such as R74W-D1270N³³ or L997F,^{6 34–36} is not considered as deleterious enough to account for their disease. Other *CFTR* mutations may have escaped detection or, alternatively, mutations in other disease causing genes may account for

the phenotype, possibly acting in concert with *CFTR* mutations.

Further screening for rare mutations or rearrangements should also be considered when patients carry missense mutations whose deleterious effect is questionable with regard to the biochemical properties of the amino acids and the conservation of the *CFTR* protein sequence among species, and in comparison to other ABC proteins. We considered a possible complex allele in patient no. 9, on a V754M background, as the corresponding part of the R domain is not well conserved among species, residue V754 being a valine in primates but a methionine in rabbit and mouse species. In addition, residue V754 is not located in the refined functional R domain.³⁷ V754M was described as a CF mutation, as it was found in a patient having classical CF (www.genet.sickkids.on.ca/cftr). The identification of the complex *CFTR*dele3–10,14b–16 in *cis* with V754M thus leads to the reconsideration of V754M as probably not disease causing, which will reassure individuals studied for carrier screening who are V754M heterozygotes but do not carry any other mutation/deletion. This observation highlights the need to achieve a complete *CFTR* gene analysis, including screening for rearrangements, when novel or rare missense mutations are found, since their deleterious effect cannot be easily proven.

A revised strategy for *CFTR* molecular studies

Among the gene quantification methods used to identify unknown gene deletions and duplications in hereditary

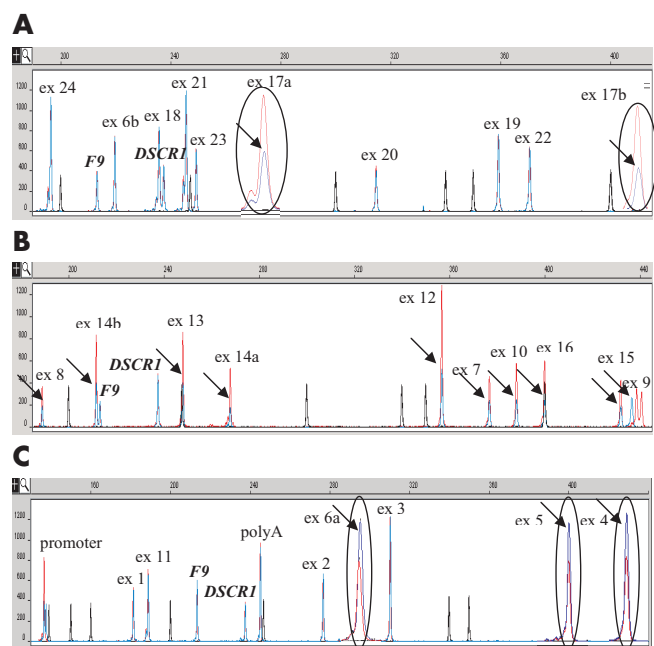


Figure 1 Electropherograms from semi-quantitative fluorescent multiplex PCR experiments. The x axis displays the computed length of the PCR products in base pairs as determined by using an internal lane standard, which is indicated in black. The y axis shows fluorescent intensities in arbitrary units. Gene fragments are indicated at the top of the corresponding peaks. The electropherograms of the controls are in red and those of the patients are in blue. The profiles were superimposed and normalised using the exon 4 *DSCR1* amplicon. The abnormal profiles have been highlighted by arrows and extended (windows). (A) *CFTR*dele17a–17b visualised from MP 3 in patient no. 7 (twofold decrease in peak intensities for exons 17a and 17b). (B) *CFTR*dele1–24, visualised from MP 2 in patient no. 10 (twofold decrease in peak intensity for all *CFTR* exons). For *CFTR* exon 9, the presence of a double peak in the control is attributable to the (TG)_mTn polymorphism. (C) *CFTR*dup4–8, visualised from MP 1 in patient no. 11's mother (1.5-fold increase in peak intensity for exons 4–6a). She carries in *trans* the –912dupT polymorphism in the promoter region (double peak).

disorders, the QFM-PCR approach is attractive because of the simplicity of its implementation in the clinical setting, its capacity to simultaneously analyse multiple gene loci, and its cost effectiveness as compared to the real time quantitative approach. It has been successfully applied in an increasing number of diseases following various protocols^{13 20 38–40} and, very recently, to the diagnosis of CF.¹⁴ The protocol described in the present study enables the rapid detection within a few hours of unknown *CFTR* gene deletions and duplications. Choosing intronic primers, except for the large exon 13, allowed us to design only three multiplexes comprising amplicons of a broader size range (138–440 bp). A great advantage of the protocol lies in the choice of reference genes amplified in each multiplex: (i) *DSCR1* serves as a double-copy control, as described elsewhere³⁸; a control DNA for trisomy 21 was used as a triple-copy control to compare with the *CFTR* exons 4–8 duplicated pattern observed in patient no. 11; in other respects, combining information on trisomy 21 could be advantageous in prenatal diagnosis of CF; (ii) *F9* serves as a single- or double-copy control depending on patient gender, thus allowing a further check of samples. Moreover, the use of genes located outside the *CFTR* locus validated the complete *CFTR* gene deletion pattern observed in patient no. 10.

Besides rearrangements detected by gene dosage, the technique described here is sensitive enough to detect length variations as small as 1 bp insertions/deletions, such as F508del, 394delTT, 1078delT, and 2184insA, as well as STR variants such those at the IVS8(TG)mTn polymorphic site. Micro-deletions/insertions and large rearrangements thus account for 24% of the reported CF mutations (www.genet.sickkids.on.ca/cftr), while their cumulative frequency is above 70% of CF alleles in most Caucasian populations. After screening using a commercial kit for approximately 30 frequent mutations which account for 82% of French CF alleles,⁴¹ the use of our QFM-PCR system could contribute to identifying a further 4.5% of CF alleles, and possibly more in other populations. The simplicity and rapidity of such a system, which can be routinely applied in the clinical laboratory, makes it the method of choice for second line screening when frequent mutations are not found. Finally, the procedure can be readily applied to the molecular diagnosis of many other hereditary diseases.

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ELECTRONIC-DATABASE INFORMATION



The URLs mentioned in this study are: www.genet.sickkids.on.ca/cftr, www.hgvs.org, and <http://www.repeatmasker.org/>.

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