Consultations in Molecular Diagnostics

WGA Allows the Molecular Characterization of a Novel Large *CFTR* Rearrangement in a Black South African Cystic Fibrosis Patient

Marie des Georges,^{*†‡} Caroline Guittard,[‡]
Carine Templin,[‡] Jean-Pierre Altieri,[‡]
Candice de Carvalho,[§] Michele Ramsay,[§]
and Mireille Claustres^{*†‡}

INSERM, U827,^{*} Montpellier, F-34000 France; Université
Montpellier,[‡] UFR Médecine, Montpellier, F-34000 France; Centre
Hospitalier Régional Universitaire Montpellier,[‡] Hôpital Arnaud
de Villeneuve, Laboratoire de Génétique, Montpellier, F-34000
France; Division of Human Genetics, National Health Laboratory
Service and School of Pathology,[§] University of Witwatersrand,
Johannesburg, South Africa

By performing extensive scanning of whole coding and flanking sequences of the cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene, we had previously identified the CF-causing mutations in black South African patients of different ethnic groups suspected with the disease. Of ten samples analyzed, there were six remaining that had either one ($n = 2$) or two ($n = 4$) unidentified *CFTR* alleles that have now been tested for large rearrangements using a semiquantitative fluorescent PCR assay. A novel deletion encompassing *CFTR* exon 2 was detected in one patient who was heterozygous for the mutation 3120+1G>A. The Caucasian deletion involving the same exon [c.54-5811_c.164+2186del8108ins182] was ruled out. The DNA had been stored for more than 12 years and only minute quantities remained. We thus used a whole-genome amplification method based on multiple displacement amplification to generate sufficient amounts of DNA to characterize the intronic breakpoints and identify the deletion at the genomic level. Mapping and sequencing the breakpoint junctions revealed a novel large deletion [c.54-1161_c.164+1603del2875]. We have designed a simple test to specifically detect the presence or absence of this large rearrangement. This study reports the first large *CFTR* rearrangement in a black South African CF patient, further defining the molecular spectrum of CF that will be useful for improving genetic

testing and counseling in this region. (J Mol Diagn 2008; 10:544–548; DOI: 10.2353/jmoldx.2008.080028)

Cystic fibrosis (CF, MIM# 219700) is still thought to be very rare in the black South African (SA) populations with no known white admixture. Between 1994 and 1997, ten DNA samples from black SA patients with clinical evidence of CF were referred to the Reference Centre of Montpellier (southern France) for extensive CF transmembrane conductance regulator (*CFTR*) gene analysis. For each sample, all coding and flanking sequences were studied by a scanning method (denaturing gradient gel electrophoresis) until late 2005, and since then by a direct automated sequencing method based on a touchdown PCR protocol that enables single amplification conditions for the 32 amplicons (SiCTA, Single Condition Touchdown Amplification) and the use of the same primers both for PCR and sequencing.¹ Four samples were found with two mutations including one homozygote for 3120+1G>A and three compound heterozygotes ([3120+1G>A]+[p.G1249E], [3120+1G>A]+[3196del54], [3120+1G>A]+[2183delAA]) and two samples were found with only one mutation (3120+1G>A and –94G>T, respectively).^{2,3} Despite an exhaustive study, a CF-causing defect was identified in only 50% of alleles. Although the vast majority of the 1556 *CFTR* gene mutations described thus far (<http://www.genet.sickkids.on.ca/cftr/app>) are point mutations or small insertions/deletions, large genomic rearrangements encompassing a single or several exons can contribute to unidentified alleles not only in Caucasian⁴ but also in non-Caucasian Asiatic populations.⁵ In this study we

Accepted for publication July 10, 2008.

Authorship: M.D.G. supervised, validated, and interpreted the technical work; C.G. performed the WGA procedure; C.G., J.-P.A., and C.T. participated in the technical and molecular studies; M.R. collected the samples; C.d.C. performed and interpreted the MLPA procedure; and M.C. conceived the report and drafted the manuscript.

Address reprint requests to Pr. Mireille Claustres, M.D., Ph.D., Laboratoire de Génétique Moléculaire, Montpellier, 641 Avenue du Doyen Gaston Giraud, 34095 Montpellier Cedex 5, France. E-mail: Mireille.Claustres@inserm.fr.

Table 1. Clinical Features Associated with a Diagnosis of CF in Six Black SA Patients

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Ethnicity	Zulu	Xhosa	Tswana	Ndebele	nd	nd
Gender	Female	Male	Female	Male	Female	Male
Date of birth	1989	nd	1995	1986	1983	nd
Age at diagnosis (yrs)	7	4	2	8	12	nd
Failure to thrive	+	+	+	+	+	+
Foul smelling, fatty stools	+	+	+	+	+	+
Chronic pulmonary infections	+	+	+	+	+, asthma	+
<i>P. aeruginosa</i> colonization	—	nd	—	+	+	+
Sweat test (chloride, mmol/L)	69	+ (nd)	82	136	58, 68, 43	nd
Kwashiorkor or marasmus	+	nd	nd	No	No	No
Mutation 1	—	—	—	3120+1G>A	—94G>T	—
Mutation 2	—	—	—	CFTRdele2*	—	—

nd, no data.

*CFTRdele2 identified in this study [c.54-1161_c.164+1603del2875].

show that large *CFTR* deletions can also be present in black South African populations.

Materials and Methods

Case Description

Six black SA patients diagnosed as having clinical features suggestive of CF (Table 1) were tested for large rearrangements. This report is focused on the only case found to carry a large deletion, a male child native of Zimbabwe who had been diagnosed with CF at 8 years of age on the basis of severe chronic lung disease, pancreatic insufficiency, and a positive sweat test (136 mEq/L) (case 4 of Table 1). He presented with failure to thrive, and foul smelling and fatty stools, and suffered from recurrent respiratory tract infections, with chronic cough productive of purulent sputum that was positive for *Pseudomonas aeruginosa*. There was no history of meconium ileus. No kwashiorkor or marasmus was associated. A history of familial CF was suspected, as a younger brother presenting "chest problems" died at 1 year of age without definite confirmation of diagnosis (DNA not available). The patient was 11 years old at the time of referral to our center for DNA analysis. After extensive sequencing of all coding flanking regions of the *CFTR* gene, only one mutation was found, 3120+1G>A. We describe here our successful strategy to discover the second *CFTR* mutation responsible for the disease.

Search of CFTR Large Rearrangements

Detection by Semiquantitative Fluorescent PCR

We have further analyzed the six samples remaining with one ($n = 2$) or no ($n = 4$) mutation by using a rapid semiquantitative fluorescent multiplex PCR (SQF-PCR) assay developed in our laboratory for the detection of large rearrangements in the *CFTR* gene.⁶ The method relies on the comparison of the fluorescent profiles of multiplex fragments obtained from different DNA samples, the amplification being stopped at the exponential phase. Dosage quotient for area and height of peaks is

an indicator of the number of copies of each of the 27 exons of the *CFTR* gene.

Confirmation by Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA)⁷ is a quantitative PCR-based assay that is used to detect copy number changes and has been applied to the *CFTR* gene. This method was developed at MRC-Holland (Amsterdam, The Netherlands) and *CFTR* gene-specific probes are commercially available. MLPA was used to validate the deletion mutation in DNA from the same patient that had not been subjected to whole-genome amplification (WGA). Dosage quotients for peak areas were analyzed. A deletion or duplication of one copy of a probe target sequence will usually be apparent by a 35% to 55% reduction or increase in relative peak areas respectively.

Molecular Characterization of a Novel CFTR Large Deletion Using DNA Recovered by WGA

Several methods were used to identify the breakpoints in introns 1 (24,105 bp) and 2 (4670 bp) of the deletion detected by SQF-PCR. First, we analyzed polymorphic sites known in these regions with the aim to detect loss of heterozygosity, using standard fluorescent PCR-based techniques on 5 ng of genomic DNA. The next step was to use a strategy based on multiple PCRs to obtain a fragment spanning the intronic junctions. However the patient DNA had been stored at -80°C since 1993 and the amount available (less than 150 nanograms) was not compatible with the study. Consequently, we used a WGA kit (GenomiPhi, Amersham Biosciences, Sydney, Australia) to generate DNA. WGA was accomplished during 16 hours at 30°C using $1\ \mu\text{l}$ (5 ng) of the stock DNA according to the manufacturer's protocol. The amplified DNA was purified (QIAquick Purification kit from Qiagen) and quantified by spectrophotometry. About $4\ \mu\text{g}$ of genomic DNA (amplification efficiency $\times 800$) was produced from the GenomiPhi procedure, allowing a series of cross-PCR-amplifications in introns 1 and 2 to obtain a

Figure 1. Characterization of a novel large deletion of the *CFTR* gene involving exon 2 [c.54-1161_c.164+1603del2875] in a CF black SA patient. **A:** This 2875 bp-deletion occurred between the nucleotide 54-1161 of *CFTR* intron 1 (IVS1 nt 22,944) and the nucleotide 164 + 1603 of intron 2 (IVS2 nt 1603) according to the recommended international nomenclature with the A of the ATG start codon numbered as +1 (<http://www.hgvs.org/mutnomen>). It is also termed [186-1161_c.296+1603del2875] with the A of the ATG translation start codon numbered as +133 in accordance with the GenBank reference sequence for the *CFTR* gene on chromosome 7 (NM_000492.2) and the CF mutation database (<http://www.genet.sickkids.on.ca/CFTR/app>). **B:** The breakpoints junction (vertical bar) was determined by direct sequencing of a WGA-amplified junction fragment (primers F21-IVS1F and IVS2R) and confirmed by direct sequencing of patient DNA (primers CF23036F and IVS2R). **C:** Specifically-designed junction fragment test (duplex PCR using primers CF23036F and IVS2R and primers for control exon 10), giving a 960-bp specific product in the presence of deletion c.54-1161_c.164+1603del2875 on 2% agarose gel. Lane 1, control DNA heterozygote for the Caucasian deletion of exon 2 [c.54-5811_c.164 + 2186del8108ins182]; lane 2, normal control DNA; and, lane 3, patient DNA carrying deletion [c.54-1161_c.164+ 1603del2875] involving exon 2.

fragment spanning the breakpoints junction. Nine different combinations of primer pairs were tested until successful specific PCR amplification of the deleted allele occurs with primers F21-IVS1F [5'-attggcaaatgccgtatgctc-3'] and IVS2R [5'-gttaagccagataattctgc-3']. This 2-kb PCR product containing the junctional breakpoint region was then sequenced to identify the deletion.

The presence and identity of the deletion was confirmed by sequencing directly the genomic DNA of the patient using flanking primers CF23036F [5'-gaccaaggaagtataaagaag-3'] and IVS2R. It was also confirmed by a simple duplex PCR assay designed to specifically detect this mutation. Primers CF23036F and IVS2R amplify a 960-bp fragment only when the deletion is present, whereas primers generating a 491-bp product containing exon 10⁸ ensure an internal amplification control (primers amplifying exon 2 could not be used due to cross-amplifications).

Nomenclature of Mutations

For convenience to readers accustomed to the nomenclature of the Cystic Fibrosis Mutation Database (<http://www.genet.sickkids.on.ca/cftr/app>), we used the usual names of *CFTR* mutations. According to the international nomenclature guidelines recommended in the Human Genome Variation Society web page (<http://www.hgvs.org/mutnomen>), 3120+1G>A is c.2988+1G>A, 3196del54 is

c.3064del54, 2183delAA is c.2051delAA, and -94G>T is -226G>T.

Results

In this series of six samples from black SA CF patients tested for large rearrangements by SQF-PCR, a novel deletion encompassing exon 2 was detected in one sample previously found to be heterozygote for 3120+1G>A. The presence of the common Caucasian deletion involving the same exon [*CFTRdele2* or c.54-5811_c.164+2186del8108ins182] was ruled out since the specifically designed amplification junction test⁶ was negative in the black SA sample (data not shown). As the sample displayed heterozygosity for three microsatellites IVS1(GA)n, IVS1(CA)n, and IVS1(TG)n, the search for breakpoints was restricted to a genomic region of about 10 kb instead of 28.7 kb (Figure 1A). Using a whole genome amplification method based on multiple displacement amplification (MDA), we could generate sufficient amounts of genomic DNA to perform the PCR-based experiments necessary for the molecular characterization of the deletion. A combination of cross-amplifications through introns 1 and 2 resulted in the location of the junction fragment whose sequencing revealed a simple deletion of 2875 bp [c.54-1161_c.164+1603del2875] encompassing exon 2 (Figure 1B), which occurred between

two short direct repeats [aaatg], one being lost in the rearranged segment (data not shown), which is compatible with a mechanism of occurrence involving a classical model of replication slippage. A computer-assisted sequence analysis of the sequences flanking the breakpoints junction using several web-available programs did not reveal any motif (Alu, LINE, or SINE) known to promote genomic gross deletions through homologous recombination. The presence of the deletion can be rapidly tested on a simple agarose gel by using a PCR assay that specifically amplifies a 960-bp junction fragment in the presence of the deletion (Figure 1C).

MLPA mutation detection using the PO91 kit (MRC-Holland) on non-WGA DNA confirmed a heterozygous deletion of *CFTR* exon 2 in this patient. This provided an independent validation of the efficacy of the SQF-PCR assay.

Discussion

Since our first report of CF-causing mutations in three black SA patients,² further evidence of the presence of CF in several black ethnic groups has been illustrated despite difficulties in diagnosing the disease at clinical and laboratory levels.^{9,10} Commercial panels for detecting the most common CF point mutations in the white populations are not appropriate and extensive scanning or sequencing of all coding and flanking sequences has to be performed to find mutations responsible for the disease. In the black populations of South Africa, the most common CF-causing mutation is 3120+1G>A (46% of alleles so far identified). Mutation p.F508del, if present, seems very rare in Africans since it has not yet been detected among about twenty samples analyzed so far in SA or in France, whereas it accounts for 43% of alleles in the "Coloured" (individuals of mixed Khoisan, Malay, European and African ancestry) CF populations (9 and unpublished data). Due to the recent development of appropriate techniques, the mutational spectrum of *CFTR* mutations causing CF has now been expanded to gross genomic rearrangements, which can account, in some Caucasian populations, for up to 20% of alleles negative for point mutations.⁴ Using a SQF-PCR assay and confirmed by MLPA, we demonstrate in this study the presence of a large rearrangement in one SA black patient heterozygous for 3120+1G>A. This novel simple deletion of 2875 bp [c.54-1161_c.164+1603del2875] is different from the complex indel mutation also involving exon 2 ([c.54-5811_c.164+2186del8108ins182], also named *CFTRdele2*, characterized by a gross deletion of 8108 bp and an insertion of 182 bp), which has been found in several Caucasian CF alleles, particularly in Mediterranean populations.¹¹ The four samples that did not have detectable point mutations despite exhaustive investigations remained negative for large rearrangements, as was also the case of the sample carrying the sequence change in the *CFTR* promoter region (−94G>T). Although it is possible that CF mutations as yet unidentified may lie in introns or in regulatory regions, which are not investigated by the available strategies, misdiagnosis of CF cannot be ruled out.

The breakpoints' characterization of the large deletion detected in this study would not have been possible without a whole genome amplification of patient DNA. The MDA method¹² is a non-PCR-based technique that uses random exonuclease-resistant hexamers and bacteriophage Phi29 DNA polymerase for double-stranded DNA displacement and primer extension. Its robustness, accuracy and reliability for genetic screening of clinical samples has been recently demonstrated.¹³ MDA presents several advantages over other WGA techniques including i) consistent DNA amplification using minute amounts of starting DNA, ii) a proofreading activity of Phi29 DNA polymerase with a very low error rate, iii) an average length of MDA products of about 10 kb. MDA may thus be useful for genetic diagnosis on extremely reduced quantities of starting DNA, including prenatal or preimplantation genetic diagnosis.¹⁴

In conclusion, we have described the first large *CFTR* rearrangement responsible for CF in a South African black patient. This information contributes to defining the molecular spectrum of CF in South Africa and will be useful for improving genetic testing and counseling in the country.

Acknowledgments

We thank the hospital of Montpellier and VLM (Vaincre la Mucoviscidose, France) and the South African NHLS and NRF for continuous support.

References

1. Bareil C, Guittard C, Altieri JP, Templin C, Claustres M, des Georges M: Comprehensive and rapid genotyping of mutations and haplotypes in CBAVD and other CFTR-related disorders. *J Mol Diagn* 2007, 9:582–588
2. Carles S, Desgeorges M, Goldman A, Thiant R, Guittard C, Kitazos CA, de Ravel TJ, Westwood AT, Claustres M, Ramsay M: First report of CFTR mutations in black cystic fibrosis patients of southern African origin. *J Med Genet* 1996, 33:802–804
3. Romey MC, Guittard C, Carles S, Demaille, Ramsay M, Claustres M: First putative sequence alterations in the minimal CFTR promoter region. *J Med Genet* 1999, 36:263–264
4. Ferec C, Casals T, Chuzhanova N, Macek M Jr., Bienvenu T, Holubova A, King C, McDevitt T, Castellani C, Farrell PM, Sheridan M, Pantaleo SJ, Loumi O, Messaoud T, Cuppens H, Torricelli F, Cutting GR, Williamson R, Ramos MJ, Pignatti PF, Raguénès O, Cooper DN, Audrézet MP, Chen JM: Gross genomic rearrangements involving deletions in the CFTR gene: characterization of six new events from a large cohort of hitherto unidentified cystic fibrosis chromosomes and meta-analysis of the underlying mechanisms. *Eur J Hum Genet* 2006, 14:567–576
5. Girardet A, Guittard C, Altieri J-P, Templin C, Stremmer N, Beroud C, des Georges M, Claustres M: Negative genetic neonatal screening for cystic fibrosis caused by compound heterozygosity for two large CFTR rearrangements. *Clin Genet* 2007, 72:1–4
6. Taulan M, Girardet A, Guittard C, Altieri JP, Templin C, Beroud C, des Georges M, Claustres M: Large genomic rearrangements in the CFTR gene contribute to CBAVD. *BMC Med Genet* 2007, 8:22–27
7. Schouten J, McElgunn C, Waaijer R, Zwiijnenburg D, Diepvens F, Pals G: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucl Acids Res* 2002, 30:e57
8. Zielenski J, Rozmahel R, Bozon D, Kerem B, Grzelczak Z, Riordan JR, Rommens J, Tsui LC: Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* 1991, 10:214–228

9. Goldman A, Labrum R, Claustres M, Desgeorges M, Guittard C, Wallace A, Ramsay M: The molecular basis of cystic fibrosis in South Africa. *Clin Genet* 2001, 59:37–41
10. Westwood T, Henderson B, Ramsay M: Diagnosing cystic fibrosis in South Africa. *S Afr Med J* 2006, 96:304–305
11. Faa V, Bettoli PP, Demurtas M, Zanda M, Ferri V, Cao A, Rosatelli MC: A new insertion/deletion of the cystic fibrosis transmembrane conductance regulator gene accounts for 3.4% of cystic fibrosis mutations in Sardinia: implications for population screening. *J Mol Diagn* 2006, 8:499–503
12. Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS: Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci USA* 2002, 99:5261–5266
13. Maragh S, Jakupciak JP, Wagner PD, Rom WN, Sidransky D, Srivastava S, O'Connell CD: Multiple strand displacement amplification of mitochondrial DNA from clinical samples. *BMC Med Genet* 2008, 9:7–15
14. Spits C, Le Caignec C, De Rycke M, Van Haute L, Van Steirteghem A, Liebaers I, Sermon K: Whole-genome multiple displacement amplification from single cells. *Nat Protoc* 2006, 1:1965–1970