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Identification of a novel duplication CFTRdup2 and functional impact of large rearrangements identified in the *CFTR* gene

Magali Taulan [a,b,*], Victoria Viart [a,b], Corinne Theze [a,b], Caroline Guittard [b,c], Jean-Pierre Altieri [b,c], Carine Templin [b,c], Laurent Mely [d], Mireille Claustres [a,b,c], Marie des Georges [b,c]

[a] Université Montpellier1, UFR de Médecine, Montpellier, France
[b] INSERM U827, Laboratoire de Génétique de Maladies Rares, Montpellier, France
[c] CHU Montpellier, Hôpital Arnaud de Villeneuve, Laboratoire de Génétique Moléculaire, Montpellier F-34000, France
[d] Centre de Ressources et Compétences pour la Mucoviscidose, Hôpital Renée Sabran, Giens, France

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ABSTRACT

In European populations, large rearrangements contribute to approximately 2% of CF mutations. Here, we reported a novel duplication, the CFTRdup2, identified in a patient heterozygous for Phe508del and suffering from a mild CF. Using a combination of functional tests, we studied the impact of duplication/deletion on CFTR expression. We showed that the copy number variations of exon 2, in addition to abolishing the rate of the mature CFTR protein, affect the *CFTR* mRNA levels. These data illustrate the importance to perform functional analysis to better understand the molecular basis responsible for cystic fibrosis. Determining the impact of deletions or duplications is relevant for a more comprehensive diagnosis and prognosis of patients.
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1. Introduction

CF (cystic fibrosis) is a life-consuming disorder, characterized by progressive lung disease, pancreatic insufficiency and elevated sweat electrolyte concentration. CF is caused by malfunction in the CFTR (cystic fibrosis transmembrane conductance regulator) protein, a Cl^- channel expressed at the apical membrane of epithelial cells. The CFTR protein consists of 1480 amino acid residues and comprises a cytoplasmic N-terminal part (~80 residues), two membrane-spanning domains formed by six transmembrane segments, two nucleotide-binding domains (NBDs) where interaction with ATP takes place, and a regulatory R domain containing multiple consensus phosphorylation sites (Riordan et al., 1989). CF or CFTR-related disorders are presented when both alleles of the *CFTR* gene are mutated. Although the vast majority of CFTR mutations are either point mutations or short deletions/insertions, large genomic rearrangements contribute to approximately 2% of CF mutations, with duplications being less frequent than deletions (www.genet.sickkids.on.ca/cftr/app). Here, we report a new duplication spanning exon 2, identified in one patient presenting a mild form of CF. To our knowledge, there has been no previous report demonstrating the effect of large rearrangements. Although the consequence of frameshift deletion such as the CFTRdelEx2,3 is evident (Dork et al., 2000), the molecular mechanism explaining the impact of other deletion/duplication in frame is less clear. Then, in addition to delineating the exact breakpoint junctions of the duplication, we used functional approaches to study the impact of naturally occurring large rearrangements, CFTR-dup2 and two previously reported deletions, CFTR-del2 and CFTR-del19 (Ferec et al., 2006; Taulan et al., 2007, 2009).

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide-binding domain; SQF-PCR, semiquantitative fluorescent PCR; qPCR, quantitative PCR; ER, endoplasmic reticulum; WT, wild-type.

* Corresponding author at: IURC, 641 Avenue du Doyen Gaston Giraud, 34093 Montpellier Cedex 5, France. Tel.: +33 4 11 75 98 64; fax: +33 4 11 75 98 82.
E-mail address: magali.taulan@inserm.fr (M. Taulan).

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2. Materials and methods

2.1. CF patient and identification of gross rearrangement by semiquantitative fluorescent PCR (SQF-PCR)

This report focused on one 20-years-old patient, diagnosed through neonatal screening, with a mild form of CF (positive sweat test (90 mEq/L), pancreatic sufficient (PS) and mild pulmonary symptoms FVC: 93%; FEV: 65%). The patient carries the Phe508del mutation inherited from her father; extensive sequencing failed to detect a maternal mutation. Informed consent to *CFTR* studies has been previously obtained from parents at time of referral. A SQF-PCR assay was used to detect large rearrangements in the *CFTR* gene as previously reported (primers on demand) (Taulan et al., 2009). The method relies on the comparison of the fluorescent profiles of multiplex PCR fragments obtained from different samples, the amplification being stopped at the exponential phase. The 27 exons were grouped into three multiplex PCRs with one primer of each pair 5′-labeled with the 6-FAM fluorochrome. All amplicons multiplexed in the same PCR have

different length. The PCR cycling conditions were selected using QIAGEN Multiplex PCR Kit (Qiagen, Courtaboeuf, France) and amplified DNA fragments were separated on an ABI 310 or 3130xl sequencer at 60 °C. The data were analyzed using Genescan and Genotyper (ABI 310) or GeneMapper (ABI 3130xl) softwares.

2.2. Confirmation and determination of the breakpoint junctions by qPCR assays

To identify the break-end junctions, a set of amplification using Light Cycler technology (Roche Diagnosis) was realized (Taulan et al., 2009). We performed the relative quantification of several sequences from intron 1 to intron 2 and their copy number was assessed. Exact delineation of the duplication was assessed by sequencing the patient genomic DNA using primers flanking exon 2 (primers in Table 1).

2.3. Expression plasmids constructions

To evaluate both *CFTR* mRNA and protein contents, expression vectors containing either the wild-type or the mutated *CFTR* cDNA (pcDNA3.1-WT and pcDNA3.1-CFTR-dup2) were constructed. To compare the impact of copy number mutants, constructions containing the deletions of exon 2 (pcDNA3.1-CFTR-del2) and exon 19 (pcDNA3.1-CFTR-del19) were also created. Full-length human *CFTR* cDNA was excised from the pTG5985 vector and ligated into pcDNA3.1(−) expression vector as previously described (Lopez et al., 2011). To generate deletion mutants, a PCR-fusion method was used to produce specific deletion of exons 2 or 19. Two specific fragments, on both sides of the deleted sequence, were amplified in separate PCRs (PCR1a and PCR1b) using primers (noted A/B or C/D, Table 1), which are complementary to each other and have 5′ sequences corresponding to the fusion flanking sequence of the opposite fragment. The separates PCR products are used as template for an ultimate PCR (PCR2) using the outermost primers (A and D, Table 1); the PCR products, deleted from exons 2 or 19, were ligated to the pcDNA3.1 plasmid recreating the human *CFTR* cDNA without exons 2 or 19 (CFTR-del2, CFTR-del19). To generate the duplication mutant, a similar approach was used except modifications. Two specific fragments, each including exon 2, are amplified in separate PCRs (PCR1a and PCR1b) with two copies of exon 2. Each PCR product was ligated to the pcDNA3.1 plasmid followed by a final ligation recreating the human *CFTR* cDNA with two copies of the exon 2 (CFTR-dup2). As a control, the construct CFTR-Phe508del, containing the deletion of three nucleotides, was generated by directed mutagenesis (Quickchange II™ site-directed mutagenesis kit, Stratagene). All the constructs were verified by direct sequencing.

Table 1
Primers used for the experimental approaches.

Direct sequencing	
DUP2F	5′-TACATTGTTTAGTTGAAGAGAGAAATTCAT
DUP2R	5′-GCTAAAATAAAGAGAGGAGGAACAGATATT
Construction of the copy number mutants	
PCR1a	
Exon 2 deletion	A: 5′-CCAAGCTGGCTAGCGTTTAA
	B: 5′-CCATTCTCTGAAAAAAAGTTTGGA
Exon 2 duplication	A: 5′-CCAAGCTGGCTAGCGTTTAA
	B: 5′-CTTTCCAATTTTTCAGATAGATTGTCAG
Exon 19 deletion	A: 5′-CAACACGTTGAAAGCAGGTG
	B: 5′-AGGCCCACCAAGCTATCC
PCR1b	
Exon 2 deletion	C: 5′-CTTTTTTTCAGAGAATGGGATAGAGA
	D: 5′-TATTTTATCTAGAACACGGCTTGACA
Exon 2 duplication	C: 5′-CTGGACCAGACCAATTTTGAGG
	D: 5′-TATTTTATCTAGAACACGGCTTGACA
Exon 19 deletion	C: 5′-GATAGCTTGGTGGGCCTCTT
	D: 5′-AAGACACACCATCGATCTGGA

2.4. Cell culture and transient transfections

Beas2B, a human bronchial epithelial cell line with a weak CFTR expression level and Cos-7, a monkey kidney fibroblast cell line that does not express CFTR, were transiently transfected as previously described (Lopez et al., 2011). For the *CFTR* mRNA level analyses, cells were seeded in 6-wells and transfected using Polyfect reagent (Qiagen) with 1 μg of the indicated pcDNA3.1 expression vectors. Forty eight hours after transfection, cells were lysed for RNA extraction. For stability assays, 24 h after transfection, cells were incubated with actinomycin D (5 μg/ml), which inhibits mRNA synthesis by blocking the transcription process, and RNAs were extracted at indicated times. Similar transfections were performed for protein extracts, resuspended directly in 1× Laemmli sample buffer.

2.5. Reverse-transcriptase PCR and RT-qPCR

Total RNAs were extracted using the RNeasy Plus Mini kit (Qiagen). RT was performed as previously described (Lopez et al., 2011), and cDNA were amplified using LightCycler® 480 Real-Time PCR System (Roche). The level of *CFTR* mRNA was normalized to the expression of endogenous *b-Actin* mRNA, used as an internal control. All PCR reactions were performed at least in triplicate in three independent experiments (separates cDNAs synthesis from different biological samples).

2.6. Immunoblot analyses

Total whole-cell proteins from transfected Beas2B and Cos-7 cells were analyzed by western blot. The PVDF membranes (Immobilon) were incubated overnight with 1:400 diluted anti-CFTR primary antibody (L12B4, recognizes a 386 to 412) in 5% skim milk. The protein levels of the Lamin AC housekeeping gene were assayed for internal control of protein loading.

2.7. Statistical analyses

Data are expressed as the mean ± SE. Paired comparisons were made using Student's *t*-test using InStat (GraphPad Software, version 3.0). Data were considered statistically significant when $p < 0.05$.

2.8. Nomenclature of mutations

For convenience to readers, we used usual mutation nomenclature reported to International Consortium Mutation Database (http://www.genet.sickkids.on.ca/CFTR/app). However, for the exon 2 duplication, the international nomenclature recommended in the Human Genome Variation Society web page (http://www.hgvs.org/mutnomen/) was also indicated [in italic and in brackets].

3. Results and discussion

3.1. Identification of the exon 2 duplication in one patient with Phe508del mutation on the other chromosome

Using SQF-PCR, we detected a duplication of exon 2 in the DNA of both the patient's and the patient's mother (Fig. 1A). The familial segregation confirmed that the exon 2 duplication is inherited from the patient's mother. We next used a previously developing and practical technique to determine the endpoints using quantitative PCR technology (Taulan et al., 2009). With reductions in gene copy number, from two to one, more readily identifiable, we successfully revealed this duplication using our previously developed and practiced quantitative PCR technology (Taulan et al., 2009), followed by direct DNA sequencing to precisely map the duplication breakpoint junctions

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