Molecular diagnosis of cystic fibrosis by RNA obtained from nasal epithelial cells

Carmen Prior-de Castro[1]*, Miguel Ángel Martínez Gallego[1]*, Clara Gómez-González[1], Rubén de Sancho Martín[1], Carlos Rodríguez Antolín[2], Carmen Rodríguez-Jiménez[3], Ángela del Pozo Mate[2], Ester Zamarrón de Lucas[4], Marta Ruiz de Balbuena Maiz[5], Cristina de Manuel Gómez[5], Sergio Alcolea Batres[4], María Concepción Prados Sánchez[4], Rosa J Torres[6]

[1]Department of Molecular Genetics-INGEMM, La Paz University Hospital, Madrid, Spain

[2]Department of Bioinformatics-INGEMM, La Paz University Hospital, Madrid, Spain

[3]Department of Next Generation Sequencing-INGEMM, La Paz University Hospital, Madrid, Spain

[4]Department of Pneumology, La Paz University Hospital, Madrid, Spain

[5]Department of Paediatric Pneumology, La Paz University Hospital, Madrid, Spain

[6]La Paz University Hospital Health Research Institute (FIBHULP), IdiPAZ, Madrid, Spain.Centre for Biomedical Network Research on Rare Diseases (CIBERER), ISCIII, Spain

*Both authors contributed equally

Correspondence to: Carmen Prior de Castro, PhD

Servicio de Genética

Bloque quirúrgico, planta -2

Hospital Universitario "La Paz"

Paseo de la Castellana 261

28046 Madrid, Spain

Phone: +34917277217

e-mail: carmen.prior@salud.madrid.org

Abstract

Background: The diagnosis of cystic fibrosis (CF) is established when characteristic clinical signs are coupled with biallelic *CFTR* pathogenic variants. No previously reported non-canonical splice site variants have to be considered as variants of uncertain significance unless their effect on splicing has been validated.

Methods: Two variants identified by next-generation sequencing were evaluated. The variant's effect on splicing was assayed by RNA analysis and real-time expression quantification from RNA obtained from nasal epithelial cells of a patient with the clinical suspicion of CF and two patients with milder phenotypes (CFTR-related disorders).

Results: The variant c.164+2dup causes skipping of both exon 2 (p.(Ser18_Glu54del)) and exon 2 plus 3 (p.(Ser18Argfs*16)) in CFTR mRNA. Exon 2 expression in the patient heterozygous for c.164+2dup was decreased to 7% of the exon 2 expression in controls. The synonymous variant c.1584G>A causes a partial skipping of exon 11. The exon 11 expression in two patients heterozygous for this variant was 22% and 42% of that of the controls, respectively.

Conclusion: We conclude that variant c.164+2dup affects mRNA processing and can be considered as a CF-causing variant. Moreover, the results of the functional assay showed that the p.(Glu528=) variant, usually categorized as a neutral variant based on epidemiological data, partially affects mRNA processing in our patients. This finding would allow us to reclassify the variant as aCFTR-related variant with incomplete penetrance.

RNA obtained from nasal epithelial cells is an easy and accurate tool for CFTR functional studies in patients with unclassified splice variants.

Keywords: CFTR, splicing, RNA analysis, CF-causing variant, CFTR-related variant, nasal epithelial cells

Introduction

Cystic fibrosis (CF) is the most frequent life-limiting, genetically inherited disorder in the European population. CF affects at least 100,000 individuals worldwide [1,2]. CF is a condition almost always diagnoses in childhood, and, in many countries, is diagnosed by newborn screening [3].

CF is a monogenetic disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene with an autosomal recessive mode of inheritance [4-6]. CF is a membrane electrolyte transport defect that causes multisystem disease, affecting the epithelia of the respiratory tract, exocrine pancreas, intestines, hepatobiliary system, and exocrine sweat glands [7]. The disease is clinically characterized by progressive obstructive lung disease with bronchiectasis due to viscous and thick mucus with bacterial colonization, obstruction of the pancreatic ducts causing malabsorption syndrome and malnutrition, as well as male infertility [1]. Pulmonary disease is the major cause of morbidity and mortality in CF. However, the *CFTR* gene pathogenic variant has been linked to milder phenotypes known as CFTR-related disorders (CFTR-RDs)**,** which are defined as clinical diseases that do not meet the diagnostic criteria for CF, with variable symptoms and/or reduced penetrance, and are associated with CFTR protein dysfunction. CFTR-RDs represent a spectrum ranging from single-organ manifestations to multisystem disease, such as the congenital bilateral absence of the vas deferens, acute recurrent or chronic pancreatitis, and disseminated bronchiectasis [8].

The diagnosis of CF is established in a proband when characteristic clinical signs are coupled with evidence of abnormal cystic fibrosis transmembrane conductance regulator (CFTR) function, including two elevated sweat chloride

values above 60 mmol/L, biallelic *CFTR* pathogenic variants, and/or a transepithelial nasal potential difference measurement characteristic of CF [1-3].

The classification of sequence variants according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines has been widely implemented and is useful for improving variant interpretation [9]. For variants in the *CFTR* gene, however, these recommendations must be applied carefully in the context of the phenotype of interest. A *CFTR* gene variant associated with a CFTR-RD could be deemed “pathogenic” for this phenotype, but not in the context of classical CF. In addition, certain *CFTR* gene variants show variable penetrance and cause CF in certain individuals but not in others.

The Clinical and Functional Translation of CFTR (CFTR2) project was developed to register variants with comprehensive clinical features when observed in at least four unrelated individuals worldwide [10]. This project classified CFTR variants into four categories: CF-causing, variants of varying clinical consequence, non CF-causing and variants of unknown significance [10]. Due to the difficulty in categorizing the variants and the broad clinical spectrum of CF, the CF external quality assessment scheme recommends following the CFTR2 classification instead of the classic ACMG classification [10].

There are currently more than 2100 variants listed in the CFTR1 locus-specific database [11], but only 485 variants (as of 29 April 2022) are recorded in the CFTR2 website with their clinical relevance. These variants are classified as CF-causing (82,7 %), variants of varying clinical consequence (10,1%), non CF-causing (4,9%) and variants of unknown significance (2,3%) [10].

In this study, we report the molecular diagnosis of a patient with a clinical suspicion of CF and a well-known CF-causing variant in one *CFTR* allele, in which a non-canonical splice site variant in the *CFTR* gene was identified by next-generation sequencing (NGS). The detected variant's effect on splicing was validated by RNA analysis to classify the clinical relevance of the change in this patient. We also performed functional RNA analyses in two patients with milder phenotypes (CFTR-RD) who were carriers of a CF-causing variant in one allele and the synonymous variant c.1584G>A p. (Glu528=) (legacy name 1716G>A), in compound heterozygosity.

Patients and Methods

Blood samples were drawn after informed consent was obtained from the patient and his parents to perform DNA and RNA analysis of his peripheral blood and nasal epithelial cells. The study was conducted according to the principles of the Helsinki Declaration.

Additional Materials and Methods are available online (Supplemental File 1)

Patients

P1 is a 38-year-old male patient diagnosed with CF at one year of age due to respiratory symptoms with a sweat chloride level of 90 mEq/L. During childhood, P1 was hospitalized 3 times for pulmonary CF exacerbations. At 18 years of age, P1 presented mainly pulmonary involvement of CF, with chronic and slightly purulent expectoration and chronic *H. influenzae* and methicillin-sensitive *Staphylococcus aureus* (MSSA) pulmonary infection but no CF exacerbations in the past year. P1 showed no gastrointestinal symptoms but

was taking pancreatic enzymes. P1 experienced 2 pulmonary *Pseudomonas* infections (eradicated) at 33 and 37 years of age and *Scedosporium aurantiacum* isolates at 30 and 33 years of age. Currently, at 38 years of age, P1 has a normal weight and is a current smoker (10 cigarettes a day). He does not experience dyspnoea but has approximately 30 mL of green expectoration a day and chronic cough. His pulmonary function test shows moderate bronchial obstruction: FVC 4820 (96%), FEV1 2280 mL (56%) and FEV1/FVC 81.5. The latest chest computed tomography (CT) revealed central and peripheral cylindrical bronchiectasis, predominantly in the upper lobes with little mucous content. P1 has chronic bronchial infection by MSSA and isolates of *Aspergillus fumigatus*. P1 has CF-related bone disease (osteopenia), and his treatment includes inhaled bronchodilators, inhaled colistin 1 MU/12 h, 500 mg of azithromycin 3 times a week, vitamins A, D, E and K, pancreatic enzymes, omeprazole and protein shakes.

P2 is a 56-year-old woman with a history of pulmonary tuberculosis in childhood treated with streptomycin for 6 months. P2 came to the clinic after 5 years with recurrent respiratory infections, chronic cough and yellow-green expectoration. P2 has 3 living children and a son who died shortly after birth due to meconium ileus. A chest CT scan showed bronchiectasis, and a sputum culture in which *pseudomonas aeruginosa* was isolated. Spirometry showed an FVC of 2120 mL (85%), an FEV1 of 160 mL (76.3%) and an FEV1/FVC ratio of 75. Sweat chloride levels were 36 mEq/L and 44 mEq/L. Based on the results and levels, P2 was diagnosed with CFTR-related disease. During the subsequent 8 years of follow-up, P2 experienced *Pseudomonas aeruginosa* colonization and moderate exacerbations annually. P2 therefore undergoes

therapy with inhaled colistin and azithromycin 3 times a week, as well as bronchodilators and daily respiratory physiotherapy.

P3 is a male patient who, at 4 years of age, was referred to our hospital's paediatric cystic fibrosis unit due to recurrent lower respiratory tract infections and a family history of bronchiectasis. A sweat chloride test was in intermediate values (47 mEq/L). P3 started presenting respiratory symptoms at 6 months of age, with recurrent lower respiratory tract infections with a good response to therapy. P3 was diagnosed with pneumonia at 1 year of age and was treated with oral antibiotics, with good clinical recovery. The diagnostic study was extended with a sweat chloride test (normal), chest CT (no abnormalities). At 8 years of age, the patient is currently in good clinical condition and has experienced no new relevant lower tract respiratory infections since the age of 6 years, showing normal spirometric values. P3 continues to be followed-up in the cystic fibrosis unit.

Results

DNA analysis results

Using the CFTR Devyser Core-Iberia Kit, P1 and P2 presented the variant c.1521_1523delCTT p.(Phe508del), while P3 presented the variant c.3846G>A p.(Trp1282Ter), all of them in heterozygosity. Both p.(Phe508del) and p.(Trp1282Ter) are CF-causing variants. The NGS study confirmed the variants detected by the CFTR Kit and the variant c.164+2dup was present in P1, while the variant c.1584G>A p.(Glu528=) (legacy name: 1716G/A) was present in P2 and P3. The variants were confirmed by Sanger sequencing. The variants p.(Phe508del) and c.164+2dup found in P1 have been confirmed to be in compound heterozygosity after studying both parents. The variants

p.(Phe508del) and p.(Glu528=) found in P2 are probably in compound heterozygosity because they are close to each other and have been observed by NGS on different alleles. Although the father could not be studied, the variants p.(Trp1282Ter) and p.(Glu528=) found in P3 can be assumed to be in compound heterozygosity because only the variant p.(Trp1282Ter) was inherited from the mother.

Variant c.164+2dup is absent from the controls and is expected to modify the splicing site according to the *in silico* bioinformatics splicing defect prediction tools AdaBoost, RandomForest and SpliceAI. Variant p.(Glu528=) has 1.7% minor allele frequency according to the Genome Aggregation Database (gnomAD) and in a Spanish CF cohort, was prevalent (22%) among patients with idiopathic chronic pancreatitis [14]. Based on epidemiologic data, however, this variant is classified as a neutral and non-CF-causing variant.

MLPA ruled out large CFTR deletions and duplications, in all patients.

RNA analysis

Given that the c.164+2dup change is located in the intron sequence between exons 2 and 3, near exon 2, of the *CFTR* gene, we designed forward and reverse primers in the 5'UTR region and exon 7 of the *CFTR* gene (table 1, supplemental file), respectively. Using RT-PCR and subsequent PCR amplification, we observed three differently sized fragments (902bp, 791bp and 682bp) of similar intensity in the agarose gel from P1 (Figure 1A). The purified longer fragments obtained from the RNA of P1 showed a normal 902bp sequence including the entirety of exons 1 and 6 of the *CFTR* gene (Figure 1A). Sequencing of the purified 791-bp fragment from the RNA of P1 revealed a *CFTR* mRNA sequence that included exon 1 to 6, with skipping of exon 2

(Figure 1B). NM_000492.4: c.54_164del p.(Ser18_Glu54del), the variant with exon 2 deletion, does not change the reading frame. Sequencing of the purified short fragment from the RNA of P1 revealed a 682bp *CFTR* mRNA sequence including exon 1 to 6, with skipping of exons 2 and 3. The variant with the NM_000492.4: c.54_273del deletion changes the CFTR reading frame with a stop codon at position 33, p.(Ser18Argfs*16) (Figure 1B). All fragments obtained from the controls' RNA showed a normal 902bp sequence.

Given that the c.1584 G>A change is located in the last nucleotide of the exon 11 sequence of the *CFTR* gene, which has previously been found to produce exon 11 skipping, we designed forward and reverse primers in exons 8 and 14 of the *CFTR* gene (table 1, supplemental file), respectively. Using RT-PCR and subsequent PCR amplification, we observed two differently sized fragments (984bp and 792bp) in the agarose gel of P2 and P3 (Figure 2A). In both patients, the longer 984bp band was more intense. The purified longer fragments obtained from P2 and P3 showed a 984bp sequence heterozygous for the p.(Phe508del) variant and c.1584 G>A change respectively (Figure 2A). Sequencing of the purified shorter fragments from P2 and P3 patients revealed a 792bp *CFTR* mRNA sequence with skipping of exon 11 (Figure 2B):NM_000492.4: c.1393_1584del p.(Thr465_Glu528del) deletion that does not change the reading frame. Using RT-PCR and subsequent PCR amplification, we obtained several DNA fragments from the controls' RNA, with the longer band of approximately 984bp being the most intense and mRNA sequences with skipping of exon 10, exon 13 or both exons. None of the purified shorter fragments from the controls revealed a *CFTR* mRNA sequence with skipping of exon 11.

CFTR mRNA expression quantification

We found a exon 2 expression decreased to 29% and 30% of control levels in P2 and P3, respectively, suggesting a general decrease in *CFTR* transcript expression due to the p.(Phe508del) and p.(Trp1282Ter) variants, respectively. However, the exon 2 expression in P1 was only 7% that of the controls (Figure 3A).

P1 and P2 presented the variant c.1521_1523del p.(Phe508del) in exon 11, which has been related to a general decrease in *CFTR* transcript expression. Exon 11 expression was decreased by 22% and 38% for P2 and P1, respectively, compared with that of the controls. Exon 11 expression was 42% of control in P3 in absence of any mutation in exon 11 (Figure 3A).

The predicted mRNA *CFTR* without exon 11 transcript expression was 1500% and 3800% that of the controls in P2 and P3, respectively (Figure 3B). The controls presented a minimal amount of mRNA *CFTR* without exon 11 transcription of approximately 0.3% of the complete transcript expression with exon 11 transcription. However, the mRNA *CFTR* without exon 11 transcript expression from P2 and P3 was 20% and 22%, respectively, of the complete transcript expression.

Table 2 shows a summary of the results for P1, P2 and P3.

Discussion

The variant c.164+2dup is a thymine duplication in the second nucleotide of the intron; the affected nucleotide is therefore actually the third intronic nucleotide. According to ACMG/AMP guidelines [9], this position is not canonical. Non-canonical splice site variants are difficult to interpret and should be considered as variants of uncertain significance unless their effect on

splicing has been validated. This study demonstrated that the variant c.164+2dup causes both exon 2 and exon 2 + exon 3 skipping. Single exon 2 deletion, c.54_164del p.(Ser18_Glu54del), does not change the reading frame; however, the skipping of exons 2 and 3, c.54_273del deletion, changes the CFTR reading frame with a stop codon at 33 position, p.(Ser18Argfs*16). We also found that this variant causes significantly decreased expression of complete (with exon 2) *CFTR* mRNA in nasal epithelium.

Although the ClinVar database (National Center for Biotechnology Information) recently reported the variant c.164+2dup as likely pathogenic, there is no evidence or publications for this non-canonical splice site variant. Given that we have confirmed that this variant affects normal RNA splicing, we can classify this variant as CF-causing. We reached a molecular diagnosis of CF in P1, who was heterozygous for two CF-causing variants: c.164+2dup and p.(Phe508del) and guiding the family's genetic counselling.

For comparison, we also analysed the effect of the c.1584G>A variant, present in two separate patients with suspected CFTR-RDs, in the splicing of the *CFTR* gene. In addition to being carriers of a CF-causing variant, P2 and P3 also carried the synonymous variant c.1584G>A , a relatively frequent variant in the general population with an allele frequency of 1.7% (gnomAD). It is more prevalent in patients with chronic pancreatitis [12] and is classified as a neutral and non-CF causing variant. The peculiarity of this synonymous variant is its location, the last nucleotide of exon 11 of the *CFTR* gene is mutated. Most of the variants affecting pre-mRNA splicing occur at the 5' splicing site, where the first two intronic nucleotides and the last nucleotide of the exon are highly conserved, which can lead to decreased splicing strength at the boundary

between the exon and the intron [13]. According to AdaBoost and RandomForest [14], there is a high probability (0.98 and 0.99, respectively) that a splicing error will occur, while for SpliceAI [15], the probability is low (0.035), and partial skipping of exon 11 due to this variant has been previously reported in patients homozygous for this variant [16,17]. Once the cDNA study was conducted, different mRNA products were found in both P2 and P3. The majority product corresponded to the normal transcript, but the minority product had the deletion of exon 11. Our study quantified exon 11 expression and found that P2 and P3 presented 22% and 42% of normal expression, respectively after normalising the expression with a constitutive gene (ACTB). P2 and P3 expressed the transcript without exon 11 1500% and 3800% more than the controls, respectively, suggesting causality of exon 11 skipping due to the variant.

The p.(Glu528=) variant is usually categorized as a neutral variant based on epidemiological data. Based on the functional assay results on the effect of the variant on mRNA processing in our patients, however, we could reclassify the variant as non-CF-causing but with a damaging molecular impact. We classified the variant as CFTR-related with incomplete penetrance, given that P2 and P3 developed mild symptoms and recurrent respiratory infections, which are CFTR-related diseases. It is noteworthy that the p.(Glu528=) variant (exon 11 skipping) did not change the reading frame, producing an incomplete CFTR protein in the NBF1 domain, as occurs with the 5T variant (exon 10 skipping) [18]. The 5T variant (c.1210–12T[5]) of the *CFTR* gene alters the skipping of exon 10 mainly when it is associated with TG12 or TG13 repeats (c.1210–34TG[*12*] or c.1210–34TG[*13*]). The higher the number of TG repeats, the lower

the efficiency of splicing. According to the CFTR2 database, 5T;TG12 and 5T;TG13 are variants with varying clinical consequences because patients with these variants combined with another CF-causing variant on the other *CFTR* allele might present the whole clinical spectrum of CF phenotypes, or the absence of disease. However, the 5T variant is associated with CFTR-RD, predominated by the congenital bilateral absence of the vas deferens. Patients carrying (TG)12(T)5 or, more importantly, (TG)13(T)5, in *trans* with a CF-causing variant, might develop other symptoms suggestive of a mild form of CF and therefore need a clinical evaluation and long-term follow-up. When 5T is found in a patient with typical CF or in the context of neonatal screening, whatever the (TG) length, other variants in *cis* (forming complex alleles) should be sought [19]. In a large Italian cohort of patients [20] with the F508del/5T;TG12 *CFTR* genotype, the patients presented with a heterogeneous clinical expression. The authors showed that a significant number of the patients initially diagnosed with an inconclusive diagnosis or CFTR-RD might progress to CF over time. These results strongly suggested that long-term follow-up is needed because there are currently no markers for identifying patients at a higher risk of more severe outcomes. Bampi et al. [16] suggested that aberrantly spliced CFTR transcripts in c.1584G>A might accumulate over time, explaining the late disease onset in certain patients, and that the exclusion of the possibility that splicing variant c.1584G>A has the potential to result in a CFTR-RD warrants further study. It is possible that unknown genetic factors might modify the penetrance of this variant.

This study aimed to highlight the importance of correctly classifying variants in the *CFTR* gene, as well as the performance of functional studies to

assist in determining their clinical impact. RNA obtaining from nasal epithelial cells is an easy and accurate tool for performing CFTR functional studies in patients with unclassified splice variants.

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Author contributions

Carmen Prior-de Castro: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & edit.

Miguel Ángel Martínez Gallego: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft.

Clara Gómez-González: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & edit

Rubén de Sancho Martín: Investigation

Ester Zamarrón de Lucas, María Concepción Prados Sánchez, Marta Ruiz de Balbuena Maiz, Cristina de Manuel Gómez, Sergio Alcolea Batres: Resources, Writing – original draft

Carlos Rodríguez Antolín, Carmen Rodríguez-Jiménez, Ángela del Pozo Mate: Investigation

Rosa J Torres: Conceptualization, Methodology, Writing – review & edit, Investigation, Formal analysis, Funding acquisition, Resources, Supervision

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. CFTR mRNA amplification and sequencing: Forward and reverse primers were designed in the 5'UTR region and exon 7 of the *CFTR* gene, and in exon 8 and exon 14 of the *CFTR* gene, respectively**.** For Q-PCR, adequate intron-spanning PCR primers were designed for *CFTR* exon 2, *CFTR* exon 11, and for the ACTB gene. Adequate PCR primers were also designed for a predicted mRNA *CFTR* without exon 11 transcript with a forward primer in the predicted exon 10 and 12 junction.

Primer	Primer sequence	localization	NM_000492.4	Size
***CFTR* mRNA amplification**				
CFTR 1-FOR	GGTCTTTGGCATTAGGAGCTTG	5'UTR region	7-28	902
CFTR 7-BACK	CTTCTTCCCAGCAGTATGCCT	exon 7	908-888	
CFTR 8-FOR	TTTCCCTGGGCTGTACAAACAT	exon 8	1130-1151	984
CFTR14-BACK	TTCTGTCCAGGAGACAGGAGC	exon 14	2113-2093	
Q-PCR				
CFTRex1-F	AGAGGTCGCCTCTGGAAAAG	exon 1	75-94	95
CFTRex2-B	TTCCAGGCGCTGTCTGTATC	exon 2	169-150	
CFTR11-F	CGTCATCAAAGCATGCCAACT	exon 11	1627-1647	232
CFTR11-B	AGCCATCAGTTTACAGACACA	exon 14	1858-1838	
CFTRw11-F	**AGCAGGCAAG**GACATCTCCAA	exon 10 and 12 junction		213
CFTR11-B	AGCCATCAGTTTACAGACACA	exon 14	1858-1838	
			NM_001101.5	
ACTB-F	GAGCGGGAAATCGTGCGTGACATT	exon 4	697-720	225
ACTB-B	GAAGGTAGTTTCGTGGATGCC	exon 5	901-921	

Table 2: Summary of the results for patients P1, P2 and P3 at the genomic and mRNA levels

	CF-causing variant	Splice variant	Result of the splice variant
P1	c.1521_1523delCTT p.(Phe508del) (legacy name: F508del)	c.164+2dup	Exon 2 and exons 2 plus 3 skipping. Exon 2 expression 7% of control
P2	c.1521_1523delCTT p.(Phe508del) (legacy name: F508del)	c.1584G>A p.(Glu528=) (legacy name: 1716G/A)	Exon 11 skipping Exon 11 expression was 22% mRNA *CFTR* without exon 11: 20% of the complete transcript expression
P3	c.3846G>A p.(Trp1282Ter) (legacy name: W1282X)	c.1584G>A p.(Glu528=) (legacy name: 1716G/A)	Exon 11 skipping Exon 11 expression was 42% mRNA *CFTR* without exon 11: 22% of the complete transcript expression.

FIGURE LEGENDS.

Figure 1. RNA analysis from patient P1. Given that the c.164+2dup change is located in the intron sequence between exons 2 and 3, near to exon 2, of the *CFTR* gene, we designed forward and reverse primers in the 5'UTR region and exon 7 of the *CFTR* gene, respectively. **Figure 1A:** Agarose gel from patient P1 with three differently sized fragments (902 bp, 791 bp and 682 bp) of similar intensity. The sequence of the purified longer fragments obtained from the RNA of patient P1 showed a 902-bp normal sequence including the entirety of exons 1 and 6 of the *CFTR* gene. **Figure 1B:** Sequencing of the purified 791-bp and 682-bp fragment from the RNA of patient P1. Sequencing of the purified 791-bp fragment revealed a *CFTR* mRNA sequence including exon 1 and exons 3 to 6, with skipping of exon 2. Sequencing of the purified short fragment from the patient's RNA revealed a 682-bp *CFTR* mRNA sequence including exon 1 and exons 4 to 6, with skipping of exons 2 and 3.

Figure 2. RNA analysis from patients P2 and P3. Figure 2A: Agarose gel showing two differently sized fragments (984 bp and 792 bp) in patients P2 and P3. In both patients, the longer band of 984 bp was more intense. The purified longer fragments obtained from patient P2 showed a 984-bp sequence heterozygous for the p.(Phe508del) variant. The purified longer fragments obtained from patient P3 showed a 984-bp normal sequence except for the c.1584 G>A change. **Figure 2B:** Sequencing of the purified shorter fragments from patients P2 and P3 revealed a 792-bp *CFTR* mRNA sequence with skipping of exon 11.

Figure 3. Figure 3A: Quantification of *CFTR* exon 2 and exon 11 expression in patients P1, P2 and P3 and in three controls by real-time

PCR. CFTR exon 2 expression was expressed as a percentage of the control.

Figure 3B: Quantification of a predicted mRNA *CFTR* without exon 11 transcript in patients P2 and P3 and in three controls. CFTR expression was expressed as a percentage of the control.