Published in final edited form as:

J Med Genet. 2011 April ; 48(4): 235–241. doi:10.1136/jmg.2010.083287.

CFTR* transcription defects in pancreatic sufficient cystic fibrosis patients with only one mutation in the coding region of *CFTR

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Abstract

Background—Patients with cystic fibrosis (CF) manifest a multisystem disease due to deleterious mutations in each gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*). However, the role of dysfunctional *CFTR* is uncertain in individuals with mild forms of CF (ie, pancreatic sufficiency) and mutation in only one *CFTR* gene.

Methods—Eleven pancreatic sufficient (PS) CF patients with only one *CFTR* mutation identified after mutation screening (three patients), mutation scanning (four patients) or DNA sequencing (four patients) were studied. Bi-directional sequencing of the coding region of *CFTR* was performed in patients who had mutation screening or scanning. If a second *CFTR* mutation was

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An additional table is published online only. To view this file please visit the journal online (<http://jmg.bmj.com>).

Competing interests None.

Patient consent Obtained.

Ethics approval All studies were approved by the Johns Hopkins University and University of Minnesota IRBs and written consent was obtained from all patients or their parents.

Provenance and peer review Not commissioned; externally peer reviewed.

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not identified, *CFTR* mRNA transcripts from nasal epithelial cells were analysed to determine if any PS-CF patients harboured a second *CFTR* mutation that altered RNA expression.

Results—Sequencing of the coding regions of *CFTR* identified a second deleterious mutation in five of the seven patients who previously had mutation screening or mutation scanning. Five of the remaining six patients with only one deleterious mutation identified in the coding region of one *CFTR* gene had a pathologic reduction in the amount of RNA transcribed from their other *CFTR* gene (8.4–16% of wild type).

Conclusions—These results show that sequencing of the coding region of *CFTR* followed by analysis of *CFTR* transcription could be a useful diagnostic approach to confirm that patients with mild forms of CF harbour deleterious alterations in both *CFTR* genes.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*).¹ Typically patients develop disease in multiple organ systems leading to obstructive lung disease, pancreatic insufficiency, male infertility, and sweat electrolyte abnormalities. This constellation of symptoms is referred to as ‘classic’ or pancreatic insufficient (PI)-CF.² However, approximately 10% of CF patients manifest symptoms in only a subset of the organ systems affected in PI-CF. This phenotype is variable and frequently milder as the majority of these patients are pancreatic sufficient (PS-CF). Diagnosis of PS-CF can be difficult due to the incomplete clinical presentation and inconclusive results of sweat chloride testing. Testing of the *CFTR* gene for mutations can aid in diagnosis, particularly in adults who present late in life with an atypical phenotype.

The most common *CFTR* mutation associated with CF, $\Delta F508$ [p.Phe508del], accounts for approximately 70% of CF alleles. However, over 1800 other mutations have been identified in *CFTR* (<http://www.genet.sickkids.on.ca/cftr/>).³ Genetic testing for CF generally begins with a panel of 23 *CFTR* mutations recommended by the American College of Medical Genetics (ACMG) that identifies approximately 85% of the CF alleles in Caucasians.⁴ Most PI-CF patients carry two mutations present in the ACMG panel. Most PS-CF patients have two mutations in the coding regions of the *CFTR* gene.⁵⁶ At least one mutation permits residual *CFTR* function, allowing the patient to escape the classic CF phenotype.⁷⁸ While the 23 mutation ACMG panel includes several mutations frequently associated with PS-CF (eg, R117H [p.Arg117His], A455E [p.Arg455Glu], 2789+5G→A [c.2657+5G→A], and 3849+10kbC→T [c.3717+12191C→T]), it is not uncommon for a PS-CF patient to carry a rare *CFTR* mutation that is not present in the panel.⁹ Detection of less common mutations can be accomplished by use of extended mutation panels¹⁰ or by comprehensive analysis of the coding regions of the *CFTR* gene using scanning^{11–13} or DNA sequencing methods.¹⁴ Scanning techniques detect 85–99% of *CFTR* mutations^{15–17} while DNA sequencing has a higher sensitivity because it allows analysis of individual nucleotides.¹⁸¹⁹ However, these techniques do not identify gene rearrangements and mutations in non-coding regions that affect splicing or expression of RNA transcripts. Gene rearrangements are unlikely to be found in patients with PS-CF as they would be expected to cause absent or severe *CFTR* dysfunction. On the other hand, mutations that affect RNA processing and transcription are more likely in PS-CF since these mutations generally allow production of reduced amounts of wild-type protein.²⁰

The regulatory sequences that control *CFTR* expression are not well understood. Elements that regulate *CFTR*’s epithelial specific expression pattern are absent from the promoter, but rather appear to lie in intronic regions and outside of the gene.^{21–23} Deleterious mutations in these non-coding regions may have a mild effect and are likely to be associated with PS-

CF.²⁴ However, it is currently not feasible to sequence the 190 000 base pairs (bp) that comprise the entire *CFTR* gene to identify mutations in a diagnostic setting. Thus, we chose to use RNA based methods to identify PS-CF patients who may have a second, occult mutation in a non-coding region of *CFTR*. To assess the diagnostic utility of RNA studies, we analysed mRNA transcripts from nasal epithelial cells derived from PS-CF patients with only one *CFTR* mutation identified after *CFTR* genetic testing.

PATIENTS AND METHODS

Patient population

Eleven patients with PS-CF, including one set of siblings, were recruited from 2004 to 2006 from five CF Care Centers. Each patient had only one *CFTR* mutation identified after *CFTR* screening (n=3), scanning (n=4), or sequencing (n=4). Patients who underwent *CFTR* screening were tested for a minimum of 70 previously described *CFTR* mutations.¹⁰ *CFTR* scanning was performed using modified temporal temperature gradient electrophoresis (mTTGE).¹⁶ Patients underwent a complete history and physical examination, pulmonary function testing, and sputum culture. Chest and sinus CTs, semen analysis, and stool pancreatic elastase were obtained if not previously collected for clinical care. Nasal potential difference (NPD) measurements were made in four subjects.^{25,26} PS-CF was defined as: (1) chloride transport abnormality defined as sweat $[Cl^-] > 40$ mmol/l or abnormal NPD; (2) respiratory disease characterised by recurrent infections, chronic cough or airway obstruction; and/or (3) congenital absence of the vas deferens in males (CBAVD), in patients with no clinical evidence of pancreatic insufficiency. All studies were approved by the Johns Hopkins University and University of Minnesota institutional review boards and written consent was obtained from all patients or their parents. Verbal or written assent was obtained from children >5 years old.

CFTR DNA sequencing

To identify mutations in the coding regions of *CFTR*, the 27 exons, bordering introns, two regions containing intronic mutations known to cause abnormal splicing (3849+10kbC→T and 1811+1.6kbA→G [c.1679+1.6kbA→G]) and 360 bp of 5'-flanking DNA containing the basal promoter of *CFTR* were amplified from genomic DNA using PCR and sequenced as previously described.⁵

RNA extraction, cDNA synthesis, and *CFTR* splicing analysis

Epithelial cells were collected from the inferior turbinate of each nostril using a cytology brush.²⁷ RNA was extracted using RNeasy (Qiagen, Crawfordsville, IN, USA). cDNA was synthesised using StrataScript reverse transcriptase (Stratagene, La Jolla, CA, USA) and random primers (Invitrogen, Carlsbad, CA, USA). Splicing analysis was performed using cDNA amplified with seven sets of *CFTR* specific primers designed to produce overlapping products that were separated by size by electrophoresis. Primer sequences are available in supplementary table 1.

Quantitative *CFTR* transcript analysis

Reverse transcriptase (RT)-PCR was performed with *CFTR* specific primers fluorescently labelled with 6-FAM (carboxy-fluorescein) and products were separated by capillary electrophoresis using an ABI 3100 genetic analyser (ABI, Carlsbad, CA, USA). Sizes were estimated against an internal standard (ROX 500, 6-Carboxyl-X-Rhodamine) with GeneScan software (ABI). Analysis was performed using primers that amplified *CFTR* exons 3–6 (621+1 G→T [c.489+1G→T] and 711+3 A→G [c.579+3A→G]) or exons 10–12 (ΔF508). The amount of full length *CFTR* transcript in patients carrying ΔF508 was

calculated using methods described by Ramalho *et al*²⁸ taking into account the estimated amount of exon 9-containing transcripts based on the intron 8 T tract status of the patient (9T/7T in all patients with $\Delta F508$).²⁹ Nasal epithelial mRNA from healthy $\Delta F508$ (n=3) and 621+1 G→T (n=3) carriers was used as a control.

CFTR transcript quantification by sequencing

RT-PCR was performed using primers that amplified *CFTR* exons 13–14a and sequenced as above. The amount of *CFTR* transcript was calculated in a qualitative manner by comparing chromatogram peak heights. RNA from a healthy R764X (p.Arg764X) carrier was used as a control.

Statistical analysis

A descriptive analysis was performed with calculations of means and SDs using Microsoft Excel. A two tailed Student t test with unequal variance was used to assess statistical significance. Values of $p < 0.05$ were assumed significant.

RESULTS

Identification of a second CFTR mutation in five patients

The phenotype and genotypes of the 11 patients evaluated in this study are summarised in table 1. To determine if any mutations in the coding regions of *CFTR* had been missed during initial genetic testing, we sequenced the *CFTR* genomic DNA of the seven patients who had *CFTR* screening (patients 3–5) or scanning (patients 1, 2, 9, 10). A second *CFTR* mutation associated with PS-CF was identified in five of the seven patients, including the two siblings (figure 1 and table 1).^{30–33} Two of these mutations have been shown to alter *CFTR* function (table 2).^{34–36} However, the functional consequence of 711+3 A→G, found in siblings 4 and 5, had not been previously evaluated. Analysis of *CFTR* RNA from nasal epithelial cells of patient 4 revealed two products; one due to normal splicing (exon5+: 390 bp) and the other due to missplicing of exon 5 (exon5–: 300 bp; figure 2). The patient's other mutation, (R553X [p.Arg553X]) is associated with a low amount of *CFTR* transcript due to nonsense mediated RNA decay (NMRD) (figure 2B).^{37,38} The RNA transcripts lacking exon 5 (exon5–), were 2.7 times more abundant than the transcripts containing exon 5 (exon5+/R553X) (figure 2B). The *CFTR* RNA containing exon 5 may be transcript bearing R533X that escapes NMRD, the result of normal splicing from the 711+3 A→G allele, or a combination of both. Our results do not distinguish between these possibilities. Nevertheless, the majority of RNA transcript generated by the *CFTR* gene bearing 711+3 A→G is abnormal due to the absence of exon 5.

Reduction in CFTR RNA transcripts in five of six patients with only one identified CFTR mutation

To determine if any of the patients with only one mutation identified in the coding region of *CFTR* had a second mutation in a non-coding region that affected expression of *CFTR* RNA, we evaluated the expression from each *CFTR* gene, by comparing the level of RNA expression from the *CFTR* gene with the identified mutation to the expression of the other *CFTR* gene. Four of the six patients (patients 6–9) carried the common *CFTR* mutation, $\Delta F508$ (table 1). RT-PCR of exons 10–12 surrounding $\Delta F508$ from patient cDNA and three healthy $\Delta F508$ carriers produced two transcripts: one from the *CFTR* gene bearing $\Delta F508$ (333 bp), and one from the other *CFTR* gene that was 3 bp longer as a result of the wild type ('wt' exon 10 sequence) (figure 3A). The ratio of wild type to $\Delta F508$ transcript was >1.0 in the controls (1.22 ± 0.25 mean peak area $\pm$ SD; figure 3B). This is consistent with previous studies showing that $\Delta F508$ alleles are expressed at about 85% of wild type *CFTR*.²⁸

However, the ratio of 'wild type' (transcript from the second *CFTR* gene) to $\Delta F508$ transcript in patients 6–8 were significantly lower than controls (mean wild type/ $\Delta F508$ peak area $\pm$ SD: patient 6: 0.5 ± 0.17 , $n=4$, $p=0.0002$; patient 7: 0.85 ± 0.11 , $n=4$, $p=0.0038$; patient 8: 0.95 ± 0.18 , $n=5$, $p=0.0456$; figure 3B). The ratio of 'wild-type' to $\Delta F508$ transcript from patient 9 did not differ from the normal controls (mean wt/ $\Delta F508$ peak area=1.24, $n=1$; figure 3). The amount of each transcript was then adjusted for the lower level of expression resulting from alternate splicing of exon 9 due to the intron 8 T/TG tract length (see Methods). After these adjustments, the amount of wild-type *CFTR* transcript in each patient compared to normal individuals was estimated as: patient 6: $8.5\pm0.2.8\%$; patient 7: $14.3\pm1.8\%$; and patient 8: $16.0\pm3.1\%$.

Patient 10 carries 621+1 G $\rightarrow$ T (table 1). This mutation changes the canonical splice donor sequence in *CFTR* intron 4 leading to two aberrantly spliced transcripts⁴⁰⁴¹; one lacks all of exon 4 while the other lacks 93 bp of exon 4 due to the use of a cryptic splice donor. To detect these transcripts, we amplified *CFTR* exons 3 to 6 from nasal epithelial cell cDNA from patient 10, his mother (who is a healthy carrier of 621+1 G $\rightarrow$ T), and from two unrelated healthy carriers of 621+1 G $\rightarrow$ T. As expected, the 621+1 G $\rightarrow$ T carriers had three different RNA transcripts: 'wild type' (384 bp) and two products corresponding to aberrant splicing of exon 4 (figure 3C, lower trace). In the carriers, the ratio of wild type to aberrant transcript is 1.58 ± 0.78 (mean $\pm$ SD). The same three transcripts are seen in patient 10 (figure 3C, upper trace); however, the ratio of the wild-type transcript from the *CFTR* gene not bearing 621+1G $\rightarrow$ T to the two aberrant transcripts is significantly reduced (mean $\pm$ SD 0.27 ± 0.13 ; $n=4$, $p=0.04$; figure 3D). Amplification of the entire *CFTR* transcript in overlapping segments did not identify any alternatively spliced transcripts. Thus, patient 10 has a reduction in the level of RNA transcripts expressed from the *CFTR* gene without an identified mutation.

The remaining patient (patient 11) carries R764X (table 1). Because this mutation is not predicted to cause aberrant RNA splicing, we estimated transcript levels of each *CFTR* allele by sequencing of reverse transcribed RNA. Amplification of the region containing R764X (exons 13–14a) from a healthy carrier of R764X revealed minimal amounts of transcript bearing the R764X mutation, indicating that this mutation induces NMRD (red peak, third sequence trace, figure 4). However, transcript bearing R764X in patient 11 is present at a higher level than the RNA transcript from the other *CFTR* gene (red peak versus blue peak in bottom trace, figure 4). This indicates that patient 11 harbours a mutation in the second *CFTR* gene that reduces RNA transcript to a level less than that associated with NMRD.

DISCUSSION

In this study, we used DNA and RNA based methods to determine if occult mutations were responsible for the clinical features of CF in 11 patients with only one identified *CFTR* mutation. We found that the CF phenotype in 10 of these patients can be explained by either (1) deleterious mutations in the coding region of each *CFTR* gene (five patients) or (2) one deleterious *CFTR* mutation plus reduced transcript from the second *CFTR* gene (five patients). These studies support the observation that patients with clinical features of CF and one known deleterious *CFTR* mutation are likely to harbour a defect in their other *CFTR* gene⁶ and illustrate the utility of *CFTR* sequencing and RNA analysis in the diagnosis of CF in patients with PS-CF. Furthermore, prudent genetic counselling of a patient with PS-CF and a single identified *CFTR* mutation should take into account the high likelihood that the patient carries deleterious mutations in each *CFTR* gene.

Screening for common CF causing mutations using the ACMG recommended panel is an appropriate initial approach in Caucasian patients with features of CF. However, about 25%

of CF patients have only one mutation identified after screening, even with expanded mutation panels.⁴⁴² Many of these patients have a second *CFTR* mutation that can be identified by analysing the entire coding regions of the *CFTR* gene.⁵ In our study, three patients had mutations that were not present on the expanded *CFTR* mutation screening panels. Scanning methods, as opposed to DNA sequencing, have the potential to miss mutations that do not change the physical properties of DNA strands.¹⁵⁴³ In our study, we identified a second *CFTR* mutation in half of the patients who had only one mutation identified after scanning. In some patients, genomic rearrangements and mutations in non-coding sequences of *CFTR* that are not identified by standard sequencing may be present.⁴⁴⁴⁵ Consequently, a small percentage of patients with CF will still have unidentified deleterious mutations even after sequencing of the coding regions of *CFTR*.⁵¹⁴ As every method of mutation identification has limitations in sensitivity, it is important for clinicians to take the method of genetic testing into account when interpreting results, particularly when two *CFTR* mutations are not identified in a patient with biochemical and clinical features of CF.

Analysis of nasal epithelial *CFTR* RNA can help identify mutations that are not detected by sequencing. Using this approach, we were able to show that five of the six remaining PS-CF patients studied had a statistically significant reduction in RNA transcription from the *CFTR* gene without a coding region mutation compared to the *CFTR* gene with an identified mutation. The amount of normal *CFTR* RNA expressed in these patients ranges from approximately 8.4–16%. Variable transcript levels and skipping of exons 9 and 12 have been observed in healthy individuals.^{46–48} Because the primers used to quantify the amount of $\Delta F508$ were located in exon 12 it is possible that we did not account for *CFTR* transcripts lacking these exons in our calculations. Using RT-PCR of overlapping *CFTR* exons we determined that patients 6–8 did not express any *CFTR* transcripts missing exon 12. However, we did not assess exon 12 splicing in patient 9. Thus, it is possible that our estimates of *CFTR* transcript level may be inaccurate if this patient missplices exon 12.

The amount of *CFTR* expression needed to maintain a normal pulmonary phenotype has been inferred from a number of studies. Several investigators have suggested that expression of full length *CFTR* RNA above 20% of normal is necessary to escape a CF phenotype.²⁰²⁸⁴⁹⁵⁰ Therefore, we concluded that the reduced level of RNA transcript from the *CFTR* gene without an identified mutation in the five patients was clinically significant. The significant but not complete reduction in *CFTR* RNA transcript likely explains the PS phenotype observed in each patient, as shown for splicing mutations such as 3849 +10kbC→T.²⁰ The precise molecular cause of the reduction in *CFTR* transcript in these patients is currently not known and is the focus of future studies.

In conclusion, these results demonstrate that identification of only one *CFTR* mutation in patients with a clinical diagnosis of PS-CF does not exclude *CFTR* as the cause of their disease. When appropriate, methods such as DNA sequencing and RNA analysis can provide molecular evidence that *CFTR* is dysfunctional. These studies highlight the potential of using RNA analysis to identify patients who may benefit from sequencing of the entire *CFTR* gene.⁵¹ More broadly, these findings illustrate the utility of RNA analysis in the molecular diagnosis of CF and suggest that RNA analysis might be useful in other recessive disorders, particularly those where mutations in regulatory elements are known to contribute to disease.

Acknowledgments

Special thanks to Gail Sharpless for technical assistance; Lois Brass for performing NPD measurements; Robert Castille, MD, James Yankaskas, MD, and Bradley Chipps, MD for referring patients to this study; and Nadine Caci

and Brooke Noren for data collection. Most of all, we would like to thank the patients and families for their willingness to participate in this study.

Funding This work was supported by grants from the National Institutes of Health (NIDDK R37 99003 to GRC) and the Cystic Fibrosis Foundation.

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Legend: *2-comprehensive scan; 3-mutation screen
 **2-comprehensive scan; 4-*CFTR* sequencing

Figure 1.
 Flow chart illustrating the results of studies performed on patients with one *CFTR* mutation identified after scanning, screening, or sequencing.

Figure 2. Reverse transcriptase (RT)-PCR analysis of patient 4 carrying R553X and 711+3 A to G. (A) Diagram of RNA splicing of *CFTR* exons 3–6 and the predicted effect of 711+3 A→G on *CFTR* splicing. (B) Electropherogram showing products from RT-PCR of *CFTR* exons 3–6 from nasal epithelium cDNA from patient 4. The area under each curve is proportional to the amount of RNA transcripts present in the nasal epithelial cells. The 300 bp product is missing the 90 bp *CFTR* exon 5.

Figure 3. RT-PCR results from patients 6–10 with one mutation in the *CFTR* coding region. (A) Electropherograms illustrating a product of 333 bp amplified from the *CFTR* gene bearing ΔF508 and the 336 bp product amplified from a ‘wild type’ gene. The area under each curve is proportional to the amount of RNA transcripts present in the nasal epithelial cells. (B) Graph depicting the ratio of the wild type product to the ΔF508 product for three healthy ΔF508 carriers and four patients from this study (patients 6–9). The mean is shown and whiskers indicate 1 SD for each patient/control. The bold whisker depicts the mean±SD of the wild type/ΔF508 ratio for the three controls. (C) Electropherograms showing the products of RT-PCR of *CFTR* exons 3–6a from nasal epithelial cDNA from patient 10 and a healthy carrier of 621+1 G→T. As predicted 621+1 G→T produces two different alternatively spliced transcripts. The area under each curve is proportional to the amount of RNA transcripts present in the nasal epithelial cells. (D) Graph depicting ratio of the wild type product to the sum of the mutant products for patient 10 and 3 healthy 621+1 G>T carriers. The mean is shown and whiskers indicate 1 SD for each patient/control. Patient 10 has reduced expression compared to each control. The asterisk indicates results from the mother of patient 10 who carries 621+1 G→T.

Figure 4.

Quantification of *CFTR* RNA transcripts in cells from patient 11. Electropherograms from DNA sequencing of genomic DNA and cDNA. The R764X mutation is caused by the replacement of a C nucleotide at 2422 by a T nucleotide (compare upper and second tracings). cDNA was generated from nasal epithelial cell RNA obtained from a healthy carrier of the R764X mutation and from patient 11. The C: T ratio indicates the amount of DNA or cDNA derived from the wild type *CFTR* gene in the healthy carrier or the *CFTR* gene without a coding region mutation in the patient (C) to the amount of DNA or cDNA derived from the *CFTR* gene with the R764X mutation (T).

Table 1

CFTR genotypes and clinical characteristics of patients with one *CFTR* mutation after *CFTR* screening or scanning

ID	Sweat [Cl ⁻]*	FEV ₁ [†]	<i>Pseudomonas</i> infection	NPD	CBAVD	<i>CFTR</i> genotype upon entry to study (method)	<i>CFTR</i> genotype after sequencing
1	52	57	Yes	ND	ND	ΔF508/unknown, IVS8 5T/9T (c.1210-12T[5]/c.1210-12T[9]) (comprehensive scan)	ΔF508/IVS8-TG12-5T
2	98	79	No	CF	Yes	ΔF508/unknown (comprehensive scan)	ΔF508/S492F [p.Ser492Phe]
3	89	62	No	ND	NA	ΔF508/unknown (screened – 86 mutations)	ΔF508/P205S [p.Pro205Ser]
4 [‡]	65	58	Yes	ND	NA	R553X/unknown (screened – 86 mutations)	R553X/711+3 A→G
5 [‡]	66	82	Yes	ND	Yes	R553X/unknown (screened – 70 mutations)	R553X/711+3 A→G
6	72	71	Yes	CF	No	ΔF508/unknown [¶]	ΔF508/unknown ^{‡‡}
7	59	106	Yes	Abnormal [§]	NA	ΔF508/unknown [¶]	ΔF508/unknown ^{‡‡}
8	37	85	No	CF	NA	ΔF508/unknown [¶]	ΔF508/unknown ^{‡‡}
9	40	112	No	ND	ND	ΔF508/unknown (comprehensive scan)	ΔF508/unknown
10	66	ND	Yes	ND	ND	621+1G→T/unknown (comprehensive scan)	621+1G→T/ unknown ^{‡‡}
11	58	ND	No	ND	ND	NA ^{**}	R764X/unknown ^{‡‡}

* Sweat [Cl⁻] concentration is expressed as mmol/l. Normal <40 mmol/l; borderline 40–60 mmol/l; CF >60 mmol/l.

[†] Forced expiratory volume in 1 s (FEV₁) is expressed as per cent predicted.

[‡] Patients 4 and 5 are siblings.

[§] Baseline in the CF range with normal responses to amiloride, low [Cl⁻] and isoproterenol.

[¶] Patients are described in Groman et al 2002.⁵

^{**} No *CFTR* screening was performed.

^{‡‡} Reduction in RNA from 'unknown' allele.

CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; ND, not done; NPD, nasal potential difference.

Table 2

Functional consequences of *CFTR* mutations identified after *CFTR* sequencing

ID	Second <i>CFTR</i> mutation identified after sequencing	Amino acid conservation *	Functional consequence	References
1	TG12-5T	NA	Missplicing—loss of exon 9 from <i>CFTR</i> transcript	Cuppens <i>et al</i> 1998 ³⁵ Groman <i>et al</i> 2004 ³⁶
2	S492F	Conserved in mammalian orthologues except platypus	None described	Ferec <i>et al</i> ³¹ Wine <i>et al</i> 2001 ³⁹
3	P205S	Conserved in mammalian orthologues	Defective biosynthesis, decreased amount of fully glycosylated CFTR	Sheppard <i>et al</i> 1996 ³⁴
4, 5	711+3 A→G	NA	Missplicing—loss of exon 5 from <i>CFTR</i> transcript	This study (figure 2)

* Sequences were obtained from GenBank. Mammalian orthologues included macaque, mouse, rat, dog, cat, cow, platypus, opossum, guinea pig, and rabbit. Amino acid alignment was generated using ClustalW.