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Molecular and Functional Analysis of the Large 5′ Promoter Region of *CFTR* Gene Revealed Pathogenic Mutations in CF and *CFTR*-Related Disorders

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Patients with cystic fibrosis (CF) manifest a multisystemic disease due to mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*); despite extensive testing of coding regions, a proportion of CF alleles remains unidentified. We studied 118 patients with CF and *CFTR*-related disorders, most with one or both unknown mutations after the scanning of CFTR coding regions, and a non-CF control group ($n = 75$) by sequencing the 6000-bp region at the 5′ of the *CFTR* gene. We identified 23 mutations, of which 9 were novel. We expressed such mutations *in vitro* using four cell systems to explore their functional effect, relating the data to the clinical expression of each patient. Some mutations reduced expression of the gene reporter firefly luciferase in various cell lines and may act as disease-causing mutations. Other mutations caused an increase in luciferase expression in some cell lines. One mutation had a different effect in different cells. For other mutations, the expression assay excluded a functional role. Gene variants in the large 5′ region may cause altered regulation of *CFTR* gene expression, acting as disease-causing mutations or modifiers of its clinical phenotype. Studies of *in vitro* expression in different cell systems may help reveal the effect of such mutations. *(J Mol Diagn 2013, 15: 331—340; http://dx.doi.org/10.1016/j.jmoldx.2013.01.001)*

Cystic fibrosis (CF) is the most frequent lethal inherited disorder among white people, with an incidence of 1:2500 newborns. It depends on alterations of the chloride channel expressed by most epithelial cells and encoded by the cystic fibrosis transmembrane regulator gene (*CFTR*).[1] The diagnosis of CF is based on symptoms, sweat chloride levels, and molecular analysis findings. However, causative mutations are identified in 90% to 95% of CF chromosomes using scanning procedures to analyze whole coding regions of *CFTR*[2] and large gene rearrangements,[3] and this percentage is lower in *CFTR*-related disorders (*CFTR*-RDs).[4] It is conceivable that some disease-causing mutations lie in gene regulatory regions.

Furthermore, a known feature of CF is the scarce genotype-phenotype correlation and the different expression of the disease in patients with CF bearing the same genotype[5] and in sib-pairs with CF.[6] A variety of modifier genes

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of the CF phenotype[7] can explain the phenotypic heterogeneity, but each acts in a small percentage of patients with CF.[8,9] These results prompted studies of noncoding regions of *CFTR,* including intronic[10] and flanking regions, potentially involved in the regulation of gene expression.[11]

The *CFTR* gene shows clear temporal and developmental regulation of its expression, but the molecular mechanisms underlying the transcriptional control in different tissues and organs are still poorly understood.[11] In fact, few regulatory elements have been detected so far. Analysis of 3.8 kb of genomic sequence upstream of exon 1 of the *CFTR* gene revealed high GC content (65%), no TATA box, multiple transcriptional start sites, and several potential Sp1 and AP-1 protein binding sites.[11] Moreover, the presence of conserved sequence tags[12] confirms that the region at the 5′ of *CFTR* may have a relevant role in the regulation of *CFTR* expression. Mutations in this region may impair the interaction between the gene and regulatory factors and act as disease-causing mutations; moreover, they may modulate expression of the gene (and thus of the disease) at different organs and tissue levels. Some examples of mutations that interfere with *CFTR* transcriptional activity[13] have been described in the more proximal 5′ region of *CFTR* in patients with CF[14] and *CFTR*-RDs,[15] but for other mutations identified in single patients (such as those reported in the Cystic Fibrosis Mutation Database, *http://www.genet.sickkids.on.ca/Home.html*, last accessed March 13, 2013.), the lack of expression studies did not permit definition of the functional role. We studied the 6000-bp region at the 5′ of *CFTR* in a large group of patients with CF and *CFTR*-RDs, most with one or both unknown mutations after scanning of the CFTR coding region, and in non-CF controls. We expressed some mutations in four cell systems to define their effect.

Materials and Methods

Participants

This study was performed on 118 unrelated Italian patients (Table 1) affected by CF (58 cases) or *CFTR*-RDs (60 cases). In detail, we studied i) 20 patients with CF homozygous for the F508del mutation, aged >18 years, and classified as having severe pulmonary and liver expression ($n = 10$) or mild pulmonary and no liver expression ($n = 10$) as previously described[7,8]; ii) 38 patients with CF with one ($n = 32$) or both ($n = 6$) undetected mutations after analysis of the most frequent *CFTR* mutations in this population,[16] *CFTR* gene scanning,[2] and the search for large gene rearrangements[3]; and iii) 60 patients with *CFTR*-RDs[4] who had normal sweat chloride levels (ie, chloride <60 mEq/L) on two test occasions, no familiarity for CF, and the absence of colonization by *Pseudomonas aeruginosa* and other pathogens typically identified in sputum from patients with CF. None of these patients met the criteria for CF diagnosis.[17] Of these 60 patients with *CFTR*-RDs, 11 had recurrent pancreatitis, 11 had disseminated bronchiectasis, and 38 had congenital bilateral absence of vasa deferentia (CBAVD) characterized by azoospermia with a low semen plasma volume (<1.5 mL) and low pH (<7.5), bilateral nonpalpable vasa deferentia, absence of the distal part of the epididymis, and hypoplasia of the seminal vesicles confirmed by ultrasound. Finally, we studied 75 unrelated non-CF controls from the same geographic area. All the participants (guardians in the case of minors) provided written informed consent to anonymously use a DNA sample and clinical data for research purposes.

Molecular Analysis of *CFTR* Coding Regions

Genomic DNA was extracted from whole blood samples collected on EDTA (Nucleon BACC3; Amersham Biosciences, Little Chalfont, UK). To detect *CFTR* gene mutations, we analyzed a panel of the most frequent mutations in Italy and then scanned all 27 exons and the intronic boundaries by denaturing gradient gel electrophoresis and gene sequencing.[2] Large *CFTR* rearrangements were tested by scanning all the *CFTR* exons using a commercial kit based on quantitative PCR followed by capillary electrophoresis (MLPA SALSA kit; MRC-Holland, Amsterdam, The Netherlands).

Molecular Analysis of the 5′ *CFTR* Region

Molecular analysis of the 6000-bp region at the 5′ of the gene was performed by direct sequencing. DNA was amplified as seven fragments of various lengths (400 to 900 bp) by PCR

Table 1 Main Features of the 118 Study Patients

Diagnosis	*CFTR* genotype	Sweat test results	Clinical expression	No. of cases
CF	F508del/F508del	Positive	PI, severe L, severe P	10
CF	F508del/F508del	Positive	PI, no L, mild P	10
CF	Mutation/U	Positive	Variable	30
CF	Mutation/mutation*	Positive	Severe	1
CF	Mutation/TG12-T5-470V	Borderline	Mild	1
CF	U/U	Positive	Variable	6
CBAVD	Mutation/U	Negative	CBAVD alone	32
CBAVD	U/U	Negative	CBAVD alone	6
Recurrent pancreatitis	Mutation/U	Negative	PS, no L, no (or mild) P	11
Disseminated bronchiectasis	Mutation/U	Negative	PS, no L, no (or mild) P	11

*The patient originally had the genotype 2789 + 5G>A/unknown; during the present study, we revealed the second mutation (ie, Y849X).
CF, cystic fibrosis; CBAVD, congenital bilateral absence of vasa deferents; L, liver expression; P, pulmonary expression.

Table 2 Sequences of Oligonucleotides

Primer name	Primer sequence
CFTR promoter sequencing primers	
Fragment from −263 to +97 forward	5′-CCGGTAATTACGCAAAGCAT-3′
Fragment from −263 to +97 reverse	5′-CTGGGCTCAAGCTCCTAATG-3′
Fragment from −1276 to −333 forward	5′-TTTGGGTGACCACAAGTCAA-3′
Fragment from −1276 to −333 reverse	5′-AACGCTGGAGGACAGAAGAA-3′
Fragment from −2219 to −1256 forward	5′-TTTCTGCTTTCCTGTTTCATTG-3′
Fragment from −2219 to −1256 reverse	5′-TTGACTTGTGGTCACCCAAA-3′
Fragment from −3138 to −2197 forward	5′-TGTAAGAAGCACCCAGCACA-3′
Fragment from −3138 to −2197 reverse	5′-CAATGAAACAGGAAAGCAGAAA-3′
Fragment from −4078 to −3118 forward	5′-GCTAAGTGTGGTGCCAGGAT-3′
Fragment from −4078 to −3118 reverse	5′-TGTCTGGGTCTTCTTACA-3′
Fragment from −5049 to −4058 forward	5′-GCAAAGGGACATTTTCACCA-3′
Fragment from −5049 to −4058 reverse	5′-ATCCTGGCACCACACTTAGC-3′
Fragment from −6000 to −5029 forward	5′-GTGACTTCATGTCCCGTCCT-3′
Fragment from −6000 to −5029 reverse	5′-TGGTGAAAATGTCCCTTTGC-3′
Expression vector construct primers	
Construct 1 forward	5′-TTTCTGCTTTCCTGTTTCATTG-3′
Construct 1 reverse	5′-GACCCGAGTTCGAGGATTAC-3′
Construct 2 forward	5′-GTGACTTCATGTCCCGTCCT-3′
Construct 2 reverse	5′-ACCACTTTTACAGGGAAACG-3′
Construct 3 forward	5′-TGTTAGTGCCCATGTGCAAT-3′
Construct 3 reverse	5′-AACTGAACACCAGTGGGTTT-3′
Site-directed mutagenesis primers	
−542pGL3B forward	5′-ATACGAAAGGCACACTTTCCTTCCCTTTTC-3′
−542pGL3B reverse	5′-GGGAAGGAAAGTGTGCCTTTCGTATATCAA-3′
−680pGL3B forward	5′-TTGGAGTTCACGCACCTAAACCTGAAACTA-3′
−680pGL3B reverse	5′-TCAGGTTTAGGTGCGTGAACTCCAAGGGTG-3′
−1176pGL3B forward	5′-TACTTTCCTTTGAGTTTTTCAATTCAAACACAATGTATGCTTGC-3′
−1176pGL3B reverse	5′-CATACATTGTGTTTGAATTGAAAAACTCAAAGGAAAGTAAAAAT-3′
c.-1773_-1772delATpGL3B forward	5′-GAGTTCAATCACGTCTGGGAAAAGTCAATAG-3′
c.-1773_-1772delATpGL3B reverse	5′-CTTTTCCCAGACGTGATTGAACTCACCACAT-3′
−2068pGL3B forward	5′-ACACAGTGATAGGAATAATGGTTTAGAACT-3′
−2068pGL3B reverse	5′-TAAACCATTATTCCTATCACTGTGTAATAC-3′
−3500pGL3B forward	5′-CACTGTTGAATAGCTGTGGCTGTTCTTACC-3′
−3500pGL3B reverse	5′-AACAGCCACAGCTATTCAACAGTGGGCCAC-3′
−5183pGL3B forward	5′-TAAGACTTCCTTAATAAGAAACTACCTTTA-3′
−5183pGL3B reverse	5′-GGTAGTTTCTTATTAAGGAAGTCTTAAAGA-3′
−5782pGL3B forward	5′-TATTGCCTTTTCTCAGATATCAGGTTATGAGAATAATA-3′
−5782pGL3B reverse	5′-ATTCTCATAACCTGATATCTGAGAAAAGGCAATATGTA-3′

using the Veriti thermal cycler (Applied Biosystems, Foster City, CA) and the HotStarTaq DNA polymerase kit (Qiagen Inc., Valencia, CA); the sequences of the PCR primers are listed in Table 2. The PCR was standardized according to the manufacturer's instructions. The assay was performed in a 30-μL reaction mixture containing 2 μL of genomic DNA (120 ng), 8 μmol/L of each primer, 3 μL of the 10X buffer, 10 mmol/L dNTPs, HotStarTaq DNA polymerase (0.75 U per reaction), and autoclaved water. The cycling conditions were as follows: HotStarTaq DNA polymerase activation at 95°C for 15 minutes, 50 cycles at 94°C for 30 seconds, 54° to 58°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. All amplification products were checked by electrophoresis in agarose gel (20 g/L) with the Tris-borate-EDTA buffer and then were sequenced.

Nomenclature of Mutations of *CFTR*

The recommendations of the Human Genome Variation Society[18] were followed in naming the mutations, using + 1 as the A of the initiation ATG codon in the reference sequence NM_000492.3. Table 3 reports the official nomenclature and the current legacy name for each mutation.

Real-Time PCR of *CFTR* Transcripts in Some Cell Lines

Total RNA was isolated from frozen pellets of the HepG2, HeLa, PanC-1, and A549 cell lines using TRIzol reagent (Invitrogen, Carlsband, CA) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop,

Table 3 Gene Variants Identified in the Region of 6000 bp at the 5′ of *CFTR*

Variant (cDNA name)	Variant (legacy name)	No. (%) of alleles		
		Control subjects (150 alleles)	Patients with CF (116 alleles)	Patients with *CFTR*-RDs (120 alleles)
c.-274C>A	-142C>A	5 (3.3)	0	0
c.-275G>A*	-143G>A	2 (1.3)	0	1 (0.8)
c.-461A>G	-329A/G	3 (2.0)	0	1 (0.6)
c.-593A>G	-461A->G	2 (1.3)	2 (1.7)	2 (1.7)
c.-674T>C*	-542T>C	0	0	3 (2.5)
c.-737G>A		5 (3.3)	0	0
c.-751A>G		2 (1.3)	2 (1.7)	2 (1.7)
c.-812T>G	-680T>G	2 (1.3)	0	2 (1.7)
c.-869T[8_9]	-790T9/8	4 (2.7)	1 (0.9)	0
c.-887C>T	-816C->T	4 (2.7)	1 (0.9)	0
c.-966T>G	-834T>G	9 (6.0)	2 (1.7)	1 (0.8)
c.-1043dupT	-911dupT	6 (4.0)	0	0
c.-1043delT	-911delT	1 (0.6)	0	1 (0.8)
c.-1308A>G	-1176A>G	0	3 (2.6)	0
c.-1773_-1772delAT*	-1641-1640delAT	0	2 (1.7)	0
c.-2200G>A*	-2068G>A	2 (1.3)	1 (0.9)	0
c.-3136T>G*	-3004T>G	4 (2.7)	0	0
c.-3632G>T*	-3500G>T	2 (1.3)	1 (0.9)	0
c.-3966T>C	-3834T>C	40 (26.7)	83 (71.6)	24 (20.0)
c.-5315G>A*	-5183G>A	1 (0.6)	0	1 (0.8)
c.-5671C>T	-5539C>T	41 (27.3)	88 (75.9)	29 (24.2)
c.-5914A>G*	-5782A>G	0	0	1 (0.8)
c.-5947T>G*	-5815T>G	4 (2.7)	0	0

*Novel variants.

Wilmington, DE); reverse transcription was performed on 1 μg of total RNA resuspended in diethyl-pyrocarbonate–treated Nanopure water using a QuantiTect reverse transcription kit (Qiagen Inc.) and the protocol supplied by the manufacturer. To check levels of *CFTR* transcripts in the HepG2, HeLa, PanC-1, and A549 cell lines, relative quantification of real-time PCR was performed in duplicate using the LightCycler 480 Probes Master containing *CFTR* primers and a TaqMan *CFTR* probe (*CFTR* and *H. sapiens,* ID. Assay 102716; Roche Diagnostics GmbH, Mannheim, Germany). Amplification was performed using the LightCycler 480 systems for real-time PCR (Roche) with a two-step PCR protocol (preincubation of 10 minutes at 95°C followed by 45 cycles of amplification at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1 second). mRNA quantification results were normalized using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene (ID. Assay 101128; Roche) as an endogenous control. In all the studies, each sample was run in triplicate, and the C_T method ($\Delta\Delta C_T$ method) was used to examine the relative quantification of the samples. Fold expression changes were calculated using the following equation: $2 - \Delta\Delta C_T$.

Reporter Gene Construct and Site-Directed Mutagenesis

To facilitate the promoter cloning, we divided the region of 6000 bp into three fragments that were cloned progressively. Construct 1 extends from positions −2219 to 97 (referred to as the major transcription initiation site), construct 2 extends from nucleotides −6000 to −5030, and construct 3 extends from nucleotides −5727 to −1256. Segments were amplified using specific primers (Table 2), and these primers were flanked at the 5′ end by a synthetic restriction enzyme site; the reaction was performed using a PfuUltra high-fidelity DNA polymerase (Stratagene, an Agilent Technologies company, La Jolla, CA). Construct 1 had at the 5′ end an XhoI restriction site and at the 3′ end an XbaI restriction site; construct 2 had at the 5′ end a KpnI restriction site and at the 3′ end an XbaI restriction site; and the last fragment had at both ends an XbaI restriction site. The integrity and accuracy of the wild-type (WT) cloned promoter regions in the constructs were checked by DNA sequencing. After this step, they were inserted in sequence in a temporary expression vector (pGEM-T vector; Promega Corp., Madison, WI) using the T4 DNA ligase kit (Promega Corp.). Then, the entire WT 6-kb construct was excised from the pGEM-T vector by a restriction reaction with KpnI and XhoI endonucleases and then was ligated into a pGL3-Basic vector (Promega Corp.); this vector contains a modified firefly luciferase coding sequence upstream of the SV40 late poly(A) signal and no eukaryotic promoter or enhancer sequences. Mutagenesis of the WT–pGL3-Basic 6-kb construct was achieved by using a QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions; all the primers used are listed in Table 2. Subsequently, we checked for each genetic variant by direct sequencing. Plasmids were

sequenced after site-directed mutagenesis to confirm the changes and to rule out additional nonspecific changes.

Cell Culture

Cell lines were purchased from ATCC (Manassas, VA). Human hepatocellular carcinoma cells (HepG2, ATCC number HB-8065), human cervical carcinoma cells (HeLa, ATCC number HB-8065), human lung carcinoma cells (A549, ATCC number CCL-185), and epithelioid carcinoma pancreatic duct cells (PanC-1, ATCC number CRL-1469) were maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) without the addition of antibiotics; cell cultures were maintained in a 5% (v/v) CO_2—humidified atmosphere at 37°C.

Transient Transfection and Gene Expression Assays

For HeLa and A549 cells, transient transfection was performed using Attractene reagent (Qiagen Inc.), whereas PanC-1 and HepG2 cells were transfected using Lipofectamine 2000 CD and Lipofectamine LTX with Plus reagent (Invitrogen), respectively, according to the manufacturer's specifications. All the cells were plated in a 24-well culture dish 24 hours before the experiments. HeLa cells were seeded at a density of approximately 8×10^4 per well; A549 at 5×10^4 per well; PanC-1 at 2×10^4 per well; and HepG2 at 1.2×10^5 per well. When cells reached an appropriate confluence (approximately 50% to 60% for HepG2 and PanC-1 cells and 70% to 80% for HeLa and A549 cells), they were cotransfected with 700 ng per well of plasmid reporter and 50 ng per well of internal control pRL-CMV containing *Renilla* luciferase (Promega Corp.) driven by a cytomegalovirus promoter to normalize transfection efficiency. Because pGL3-Basic vector lacks any eukaryotic promoter or enhancer sequences, it was used as a negative control. Cells were grown for an additional 24 hours, washed in phosphate-buffered saline, lysed in 100 μL of passive lysis buffer (Promega Corp.), collected, and centrifuged for 10 minutes at 12,000 rpm to remove cell debris. Ten microliters of cleared lysate was assayed sequentially for firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay system (Promega Corp.) according to the manufacturer's recommendations. Luminescence measurements were performed using a VICTOR^3-1420 multilabel counter (PerkinElmer, Waltham, MA) with a 5-second measurement period; firefly luciferase activity was normalized to *Renilla* luciferase activity. Experiments were made at least in duplicate, and each set of transfections was repeated four times for each cell line with independently purified plasmid DNA preparations and reaction mix.

Statistical Analysis

Comparisons of promoter activity between WT and variant *CFTR* promoter constructs from luciferase assays were conducted by using the unpaired *t*-test; data were considered statistically significant at $P < 0.001$; data are expressed as means ± SE of four different assays.

Results

Identification of *CFTR* Regulatory Variants

Molecular analysis of the 6000-bp region at the 5′ of the *CFTR* gene was performed in all patients with CF and *CFTR*-RDs and in control subjects. We identified 23 mutations, 9 of which are novel (Figure 1). A summary of these gene variants appears in Table 3. Two mutations have a high frequency: c.-3966T>C, with an allele frequency ranging from 20.0% in patients with *CFTR*-RDs to 71.6% in patients with CF, and c.-5671C>T, with an allele frequency ranging from 24.2% in patients with *CFTR*-RDs to 75.9% in patients with CF. The high frequency of such mutations in

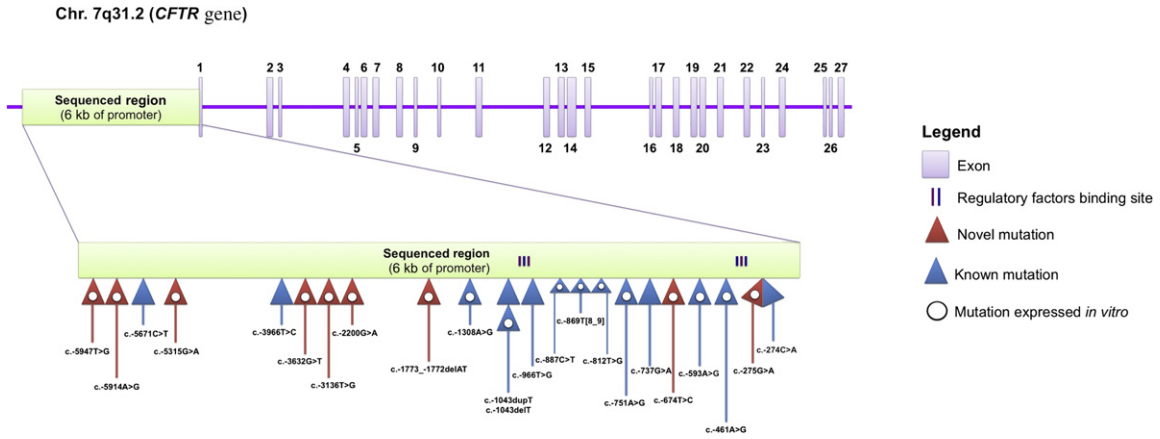

Figure 1 Regulatory variants identified in *CFTR* promoter. Diagram of the chromosome 7q31.2 region containing *CFTR* and magnification of the sequenced region with annotation of the 23 mutations identified in the present study.

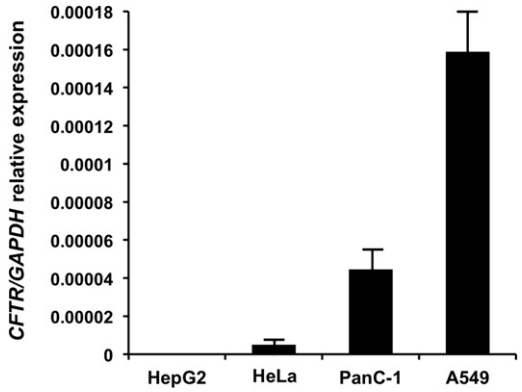

Figure 2 Relative quantification of *CFTR* mRNA expression in the HepG2, HeLa, PanC-1, and A549 cell lines. The values of *CFTR* mRNA are reported as means ± SD ratios to GAPDH housekeeping mRNA.

patients depends on the linkage disequilibrium of both the mutations with the F508del, which is the most frequent mutation in this series of patients. c.-274C>A, c.-737G>A, c.-966T>G, and c.-1043dupT have allelic frequencies of 3.3%, 3.3%, 6.0%, and 4.0%, respectively, in control subjects. The other 17 mutations have allelic frequencies <3.0% and were expressed *in vitro*.

In Vitro Expression of Mutations and Their Functional Effect on Gene Expression

To study the functional effect of *CFTR* mutations, we used four cell systems with different baseline levels of CFTR expression because such cells would produce different (levels of) *CFTR* interactors. We tested *CFTR* expression by real time-PCR and reported the result as a ratio to GAPDH mRNA. As shown in Figure 2, no basal *CFTR* expression was found in HepG2 cells, HeLa cells expressed low basal levels of *CFTR* mRNA, PanC-1 cells expressed an intermediate basal level of *CFTR* mRNA, and A549 cells expressed the highest basal levels of *CFTR* mRNA.

Thus, we expressed the 17 *CFTR* mutations in the four cell systems to determine their functional impact in modulating *CFTR* promoter activity compared with the WT sequence. Table 4 shows the results of the mutations expressed *in vitro* and tested with Dual-Luciferase Reporter Assay. Most mutations (ie, c.-275G>A, c.-461A>G, c.-593A>G, c.751A>G, c.-887C>T, c.-1043delT, c.-3136T>G, c.-5315G>A, and c.-5947T>G) do not influence expression of the reporter system in any of the four cell systems. Four mutations (ie, c.-674T>C, c.869T[8_9], c.-1308A>G, and c.-1773_-1772delAT) cause a significant reduction in reporter gene expression in one or more cell systems. The c.-812T>G mutation and the c.-2200G>A and c.-5914A>G mutations cause a significant increase in gene expression in HepG2 and HeLa cells, respectively. Finally, the c.-3632G>T mutation causes a significant increase in gene expression in PanC-1 cells and a significant reduction in HepG2 cells.

Genotype-Phenotype Correlation

Thus, eight mutations significantly modified the levels of gene expression in some cell lines (Figure 3). We related such variants to the clinical expression of the patients (Table 5). The novel c.-674T>C mutation was identified in three patients with CBAVD (cases 1 to 3) with normal sweat chloride levels and no other signs or symptoms of CF. The mutation was homozygous in case 1, who was also heterozygous for N1303K; however, the two other patients had the

Table 4 Luciferase Expression (% Compared with the WT) of Mutated *CFTR* Promoter in Various Cell Types

Mutation	A549	PanC-1	HeLa	HepG2
c.-275G>A*	106.9 (18.3)	99.5 (21.1)	106.1 (19.1)	NT
c.-461A>G	96.9 (11.1)	97.5 (11.1)	NT	103.9 (12.1)
c.-593A>G	98.6 (13.4)	82.6 (7.4)	NT	NT
c.-674T>C*	57.1 (9.4)†	89.4 (14.1)	79.9 (24.7)	33.2 (5.7)†
c.-751A>G	98.5 (7.2)	120.7 (12.7)	NT	96.2 (7.1)
c.-812T>G	91.4 (12.6)	83.6 (13.7)	81.6 (13.1)	145.7 (14.4)†
c.-869T[8_9]	54.5 (11.6)†	53.8 (10.8)†	NT	95.6 (8.9)
c.-887C>T	88.5 (15.0)	112.3 (14.6)	NT	97.4 (18.3)
c.-1043delT	106.3 (11.6)	93.6 (11.0)	92.2 (14.1)	95.9 (14.7)
c.-1308A>G	86.5 (10.6)	33.6 (11.0)†	106.2 (20.7)	95.4 (24.6)
c.-1773_-1772delAT*	97.3 (9.1)	55.9 (4.5)†	135.8 (23.1)	28.9 (4.2)†
c.-2200G>A*	112.8 (10.3)	105.8 (17.7)	328.0 (29.3)†	113.2 (15.5)
c.-3136T>G*	97.6 (11.7)	96.3 (20.1)	116.5 (19.3)	115.0 (21.3)
c.-3632G>T*	97.6 (7.4)	149.7 (10.9)†	124.0 (16.9)	64.1 (14.8)†
c.-5315G>A*	102.8 (18.1)	59.7 (18.0)	100.0 (11.2)	107.1 (18.1)
c.-5914A>G*	118.5 (12.2)	140.8 (15.7)	209.8 (5.7)†	100.1 (21.4)
c.-5947T>G*	102.6 (12.4)	89.4 (11.1)	97.6 (18.9)	98.4 (13.6)

*Novel variants.
†Values significantly different ($P < 0.001$) compared with those obtained with the WT.
NT, not tested.

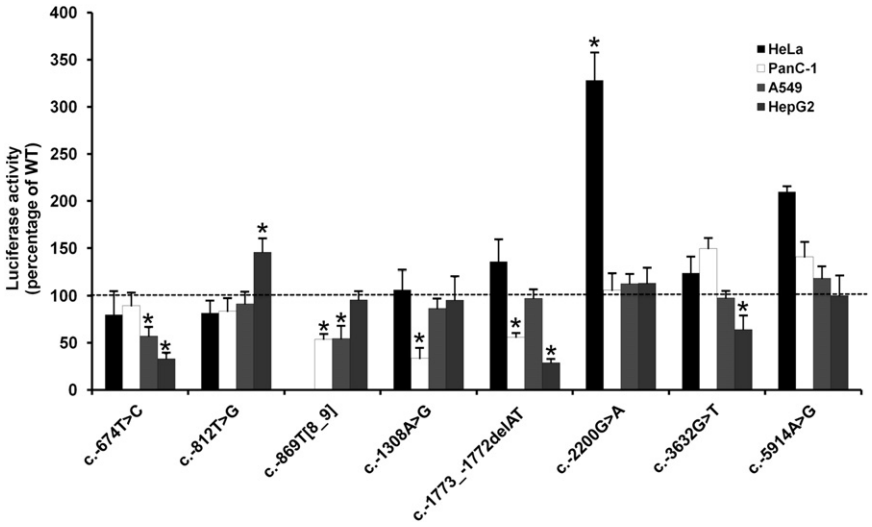

Figure 3 Relative promoter activity of mutations identified in the study. Plasmid constructs expressing firefly luciferase under control of WT or variant *CFTR* promoters were individually cotransfected with a *Renilla* luciferase reporter construct (pRL-CMV) in the HeLa, PanC-1, A549, and HepG2 cell lines. Firefly luciferase expression was measured and normalized to that of *Renilla* luciferase; then, *Renilla*-normalized promoter activities for mutated constructs were compared with those of the WT construct (indicated by the **dashed horizontal line**) to provide relative *CFTR* promoter activity of the variant. The assay was conducted at least in duplicate, and each set of transfections was repeated four times with independently purified plasmid DNA preparations and reaction mix. Data are given as means ± SD. *$P < 0.001$ compared with WT.

c.-674T>C mutation *in trans* with the F508del mutation. The c.-674T>C mutation significantly reduces luciferase expression in A549 cells (57.1%, $P < 0.001$ compared with the WT construct) and in HepG2 cells (33.2%, $P < 0.001$).

The c.-869T[8_9] mutation was identified in a patient with CF (case 4) *in trans* with the F508del. The patient (current age, 41 years) has pancreatic insufficiency (PI) and mild pulmonary expression.

The c.-1308A>G mutation was heterozygous in three patients with CF (cases 5 to 7) with mild CF and slightly altered sweat chloride levels. In case 5, it was *in cis* with the TG12-T5-470V allele; the patient had the F508del mutation on the other allele. The patient (current age, 30 years) has CF with PI, mild pulmonary expression (allergic bronchopulmonary aspergillosis and bronchiectasis), nasal polyposis, and chronic sinusitis. Case 6 (current age, 32 years) had the c.-1308A>G mutation and the N1303K mutation on the other allele. He has mild CF with PI and mild pulmonary expression with colonization by *P. aeruginosa*. Case 7 had c.-1308A>G *in trans* with G542X; he has PI and mild pulmonary expression. The c.-1308A>G mutation significantly reduces luciferase expression (ie, 33.6%, $P < 0.001$ compared with the WT construct) in only PanC-1 cells.

The novel c.-1773_-1772delAT mutation was identified in two patients with CF (cases 8 and 9). It was *in cis* with the 2789 + 5G>A mutation in case 8 (current age, 20 years), who had the 2789 + 5G>A/Y849X genotype. This patient has severe CF with PI and severe pulmonary and liver expression (ie, cirrhosis with portal hypertension). In the second case, the c.-1773_-1772delAT was observed in a patient with CF who has the F508del mutation on the other allele. The patient (case 9) had PI with mild pulmonary expression and no liver involvement. The c.-1773_-1772delAT mutation significantly reduced luciferase expression in PanC-1 cells (55.9%, $P < 0.001$ compared with the WT construct) and in HepG2 cells (28.7%, $P < 0.001$); however, it did not significantly modify the expression in HeLa and A549 cells.

The novel c.-812T>G mutation was observed in two unrelated patients with CBAVD (cases 10 and 11) with normal sweat chloride levels and no other signs or symptoms of CF. Both patients had the F508del/U genotype and we were unable to define whether the novel variant was *in cis* or *in trans* with the F508del mutation. The novel c.-812T>G mutation causes increased expression in HepG2 cells and no changes in the other cell lines. The c.-2200G>A mutation was identified in a patient with CF (case 12; current age, 20 years) with pancreatic sufficiency (PS) and mild pulmonary expression. The patient had the F508del/TG12-T5-470V genotype, and the c.-2200G>A variant was *in cis* with the complex allele. On the same allele, 1525-1delG was also present. The c.-2200G>A variant significantly increased luciferase expression in HeLa cells; however, it did not modify gene expression in the three other types of cells. The novel c.-3632G>T mutation was observed in two control subjects and in a patient with CF (case 13) *in trans* with the F508del. The patient (current age, 26 years) has CF with PS, mild pulmonary expression with no colonization, and no liver involvement. The variant caused higher luciferase expression in PanC-1 cells (149.7%, $P < 0.001$ compared with the WT construct) and a reduction of expression in HepG2 cells (54.1%, $P < 0.001$) and no effect in the other two types of cells. Finally, the c.-5914A>G mutation was identified in a patient with CBAVD (case 14) with both unidentified *CFTR* mutations. The c.-5914A>G mutation caused a significant increase in gene expression in HeLa cells; however, it did not affect the other cells.

We then studied the 6000-bp 5′ region of *CFTR* in 20 patients with CF homozygous for the F508del mutation (of which 10 had severe pulmonary and liver expression and 10 mild pulmonary and no liver expression) to assess whether promoter mutations would be differently present in such patients, potentially acting as modifier factors of the CF phenotype. We recorded only c.-3966T>C and c.-5671C>T in all the chromosomes. No other mutations were observed.

Table 5 Features of Patients Bearing Variants Studied *in Vitro*

Case no.	Diagnosis	Clinical expression	Sweat chloride (mEq/L)	*CFTR* genotype	FEV 1%	Current age (years)
1	CBAVD	CBAVD alone	38	[N1303K;c.-674T>C]/c.-674T>C	90	32
2	CBAVD	CBAVD alone	36	F508del/c.-674T>C	106	28
3	CBAVD	CBAVD alone	36	F508del/c.-674T>C	104	30
4	CF	PI, mild P, no L	70	F508del/c.-869T[8_9]	78	41
5	CF	PI, mild P, nasal polyposis, chronic sinusitis, no L	76	F508del/[TG12-T5-470V; c.-1308A>G]	80	30
6	CF	PI, mild P, colonization by *P. aeruginosa*, no L	68	N1303K/c.-1308A>G	84	32
7	CF	PI, mild P, no L	71	G542X/c.-1308A>G	80	17
8	CF	PI, severe P, severe L	115	[2789+5G>A;c.-1773_-1772delAT]Y849X*	44	20
9	CF	PI, mild P, no L	90	F508del/c.-1773_-1772delAT	76	28
10	CBAVD	CBAVD alone	40	F508del/c.-812T>G	96	31
11	CBAVD	CBAVD alone	23	F508del/c.-812T>G	102	41
12	CF	PS, mild P, no L	20	F508del/[1525-1delG;TG12-T5-470V;c.-2200G>A]†	84	20
13	CF	PS, mild P, no L	70	F508del/c.-3632G>T	88	26
14	CBAVD	CBAVD alone	22	c.-5914A>G/U	98	

*The patient originally had the genotype 2789+5G>A/unknown; during the present study, we revealed the second mutation (ie, Y849X).

†During the study, we revealed the presence of the 1525-1delG mutation *in cis* with the complex allele TG12-T5-470V and with the c.-2200G>A mutation.

CF, cystic fibrosis; CBVAD, congenital bilateral absence of vasa deferents; FEV 1%, forced expiratory volume in 1 second; L, liver expression; P, pulmonary expression; PI, pancreatic insufficiency; PS, pancreatic sufficiency; U, unidentified mutation.

Discussion

This is the first systematic study of the 6000-bp region at the 5′ of *CFTR* in a large cohort of patients affected by CF and *CFTR*-RDs and in control subjects. We found 23 mutations, of which 9 were novel. To assess their effect on gene expression, we expressed 17 of these mutations *in vitro*. We selected four different cell systems, ie, HeLa (cervix), PanC-1 (pancreas), A549 (pulmonary), and HepG2 (liver) because three of these cells originate from tissues typically involved in CF and because the baseline expression of *CFTR* is different in these four systems, suggesting that different regulatory factors (or different amounts of regulatory factors) of *CFTR* expression may be present in the four cell lines. In fact, baseline *CFTR* expression ranged from no expression in HepG2 cells according to previous data[19] and very low expression in HeLa cells[20] to intermediate expression in PanC-1 cells[21] and, finally, high expression in A549 cells, not in agreement with previous studies.[22]

Of the 17 mutations tested, 8 significantly modified the expression of gene reporter, with significant differences among the four cell lines. This suggests that such mutations impair the interaction of *CFTR* with regulatory factors that are produced at different amounts by the different types of cells. Thus, unlike mutations of *CFTR* coding regions (whose effects involve CFTR activity in all cells), the mutations in the promoter region may have a different effect on different tissues, thus influencing the clinical expression of CF in the single patient. The correlation of the effect of such mutations with the clinical expression of patients confirms this view

Table 6 Summary of the Features Associated with the Most Important Mutations Identified in the Study

Mutation	Allele frequency	Clinical presentation	Luciferase expression (cell lines)
c.-674T>C	2.5% in *CFTR*-RDs	CBAVD	Reduction in A549 and HepG2
c.-869T[8_9]	0.9% in CF	CF: PI, mild P, no L	Reduction in A549 and PanC-1
c.-1308A>G	2.6% in CF	CF: 5 cases with PI, mild P, nasal polyposis, chronic sinusitis, no L; 6 cases with PI, mild P, colonization by *P. aeruginosa*, no L; 7 cases with PI, mild P, no L	Reduction in PanC-1
c.-1773_-1772delAT	1.7% in CF	CF: 8 cases with PI, severe P, severe L; 9 cases with PI, mild P, no L	Reduction in PanC-1 and HepG2
c.-812T>G	1.7% in *CFTR*-RDs	CBAVD	Increase in HepG2
c.-2200G>A	0.9% in CF	CF: PS, mild P, no L	Increase in HeLa
c.-3632G>T	0.9% in CF	CF: PS, mild P, no L	Increase in PanC-1, reduction in HepG2
c.-5914A>G	0.8% in *CFTR*-RDs	CBAVD	Increase in HeLa

CF, cystic fibrosis; CBVAD, congenital bilateral absence of vasa deferents; L, liver expression; P, pulmonary expression; PI, pancreatic insufficiency; PS, pancreatic sufficiency.

(a summary of these data is available in Table 6). In fact, the c.-674T>C mutation causes a significant reduction in luciferase expression in A549 and HepG2 cells; it was identified in three patients with CBAVD who had a severe mutation on the other allele; thus, it is conceivable that the novel mutation has a pathogenic role, acting as a mild mutation responsible for CBAVD. The c.-869T[8_9] mutation was identified in a patient with CF *in trans* with the F508del. The patient (current age, 41 years) has mild CF, confirming the pathogenic role of the c.-869T[8_9] mutation, which causes a strong reduction in luciferase expression in A549 and PanC-1 cells.

The c.-1308A>G mutation significantly reduces luciferase expression in only PanC-1 cells, and it was identified in three patients with CF. All show CF with mild pulmonary expression, and all of them have PI with severe malabsorption. In the first case, the c.-1308A>G was *in cis* with TG12-T5-470V, which usually acts as a mild mutation and gives rise to *CFTR*-RDs or to mild CF with PS. We suggest that the novel c.-1308A>G mutation may act as a negative modifier factor in this patient, causing impaired *CFTR* expression at the pancreatic level, causing PI and altered sweat chloride levels. Also in the other two cases, c.-1308A>G identified *in trans* with a severe mutation may act as a disease-causing mutation.

The c.-1773_-1772delAT mutation significantly reduces luciferase expression in PanC-1 and HepG2 cells. It was found in two patients with CF. The first patient had the severe Y849X mutation[23] *in trans* with the 2789 + 5G>A mutation. The c.-1773_-1772delAT mutation was *in cis* with the latter and may act as a negative modifier factor, further reducing the low expression of *CFTR* due to 2789 + 5G>A. In fact, 2789 + 5G>A is a splicing mutation associated with some residual production of the CFTR protein.[17] It is a mild mutation[24]; in fact, it was identified (*in trans* with the F508del) in two long aged (65 and 67 years old) siblings with CF with very mild CF,[25] although our patient showed a severe CF phenotype with PI. Furthermore, the patient had severe liver expression despite the fact that he was negative for mutations in the *A1AT* (data not shown),[9] *TGF-β*, and *MDR3*[8] genes and homozygous for the *MBL* haplotype with the highest protein activity,[8] thus excluding the possibility that the severe liver phenotype could depend on mutations in such modifier genes. Similarly, the second patient bearing the c.-1773_-1772delAT (*in trans* with the F508del) had a typical CF phenotype with altered sweat chloride levels and PI.

Three variants caused an increase in luciferase expression in only one type of cell (ie, the novel c.-812T>G mutation in HepG2 cells, the novel c.-2200G>A mutation in HeLa cells, and the c.-5914A>G mutation again in HeLa cells). It is difficult to define the role of such variants in the pathogenesis of the disease. The c.-812T>G mutation, identified in two patients with CBAVD, in both cases *in trans* with the F508del, could be present *in cis* with an unidentified mutation responsible for the disease. The c.-2200G>A mutation was found in a patient with a *CFTR*-RD with the F508del/TG12-T5-470V genotype *in cis* with this latter complex allele and with the novel 1525-1delG variant.[4] This adult patient showed a *CFTR*-RD with mild pulmonary expression (bronchiectasis and colonization by *P. aeruginosa*). Interestingly, he did not have CBAVD, which is typically present in CF and in patients with the F508del/TG12-T5-470V genotype. We suggest that the novel c.-2200G>A mutation might allow some expression of *CFTR* during embryogenesis, counteracting the effect of *TG12-T5-470V* and of the 1525-1delG alleles, thus permitting the development of vasa deferentia. On the other hand, a mutation that enhances *CFTR* expression and mitigates the effect of the S549R mutation has been described.[26] c.-5914A>G was identified in an adult patient with a *CFTR*-RD with both undetected mutations who had only bronchiectasis and a borderline sweat chloride level. Thus, it is difficult to speculate on its effect. Similarly, it is difficult to establish the role of c.-3632G>T identified in a patient with CF with the F508del on the other allele because c.-3632G>T has a different effect on PanC-1 cells (enhanced expression) and on HepG2 cells (reduced expression).

Finally, based on patients with CF with the F508del genotype, no differences between the two groups with different clinical expression were evidenced in the 6000-bp region at the 5′ of the gene. These data are preliminary because they have been obtained on a small number of patients (although carefully classified on the basis of the phenotype) all homozygous for the F508del mutation to reduce the influence of different *CFTR* genotypes on clinical expression.

These data add to existing studies that reported gene variants in the 5′ region that impairs *CFTR* gene expression, even if all these studies analyzed only the proximal region of 500 to 1000 bp.[13,27] We conclude that the whole portion of the *CFTR* promoter of 6000 bp at the 5′ of *CFTR* is strongly involved in the regulation of gene expression and that mutations in this region may either reduce or increase gene expression at different tissue levels, having a role in the pathogenesis of the disease or in modulation of its phenotype.

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