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ARTICLE

Assessing the residual *CFTR* gene expression in human nasal epithelium cells bearing *CFTR* splicing mutations causing cystic fibrosis

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The major purpose of the present study was to quantify correctly spliced *CFTR* transcripts in human nasal epithelial cells from cystic fibrosis (CF) patients carrying the splicing mutations c.580-1G>T (712-1G>T) and c.2657+5G>A (2789+5G>A) and to assess the applicability of this model in *CFTR* therapeutic approaches. We performed the relative quantification of *CFTR* mRNA by reverse transcription quantitative PCR (RT-qPCR) of these splicing mutations in four groups (wild type, CF-F508del controls, CF patients and CF carriers) of individuals. In addition, *in vitro* assays using minigene constructs were performed to evaluate the effect of a new CF complex allele c.[2657+5G>A; 2562T>G]. *Ex vivo* qPCR data show that the primary consequence of both mutations at the RNA level is the skipping of their neighboring exon (6 and 16, respectively). The CFTR minigenes results mimicked the *ex vivo* data, as exon 16 skipping is the main aberrant transcript, and the correctly spliced transcript level was observed in a similar proportion when the c.2657+5G>A mutation is present. In summary, we provide evidence that *ex vivo* quantitative transcripts analysis using RT-qPCR is a robust technology that could be useful for measuring the efficacy of therapeutic approaches that attempt to achieve an increase in *CFTR* gene expression.
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INTRODUCTION

The splicing mechanism is a complex process regulated by intragenic and extragenic factors resulting in a mature mRNA, which is translated to cell protein undergoing the secretory pathway. Donor and acceptor sites and branch points in intronic sequences are essential for this process.[1] In addition, enhancer and silencer sequences in exons and introns act as splicing regulatory elements that also contribute to the exon definition.[2] Splicing factors binding with these specific signals can also affect the efficiency of the splicing process.[3] It has been reported in several genetic disorders that not only splicing mutations but also point mutations can activate/inactivate these *cis* and *trans* elements thereby disrupting the synthesis or function of their encoded proteins.[4,5] Aberrant transcripts that change the open reading frame are eliminated by the cell surveillance machinery resulting in variable levels of residual gene expression. In the end, splicing efficiency affects transcript abundance and consequently the amount of functional protein, the critical point which determines a pathological effect or a predisposition to disease.[6]

Cystic fibrosis (CF; OMIM 219700) is a recessive genetic disorder due to deficient ionic transport in epithelial tissues. Most mutations in the *CFTR* gene (*ABCC7*; OMIM 602421) determine the total or partial loss of the CFTR channel function. Among these, 12% are splicing mutations (www.genet.sickkids.on.ca/cftr). Interestingly, a large and growing number of missense, synonymous and nonsense mutations have been found to disrupt the regulatory sequences involved in recognition of the exon,[7–9] and consequently, the proportion of splicing defects could be higher than that previously estimated. Changes in *CFTR* gene expression lead to phenotypic variability and low levels have been postulated as a predisposing factor to *CFTR*-related disorders.[8] Therefore, determining the amount of normal transcripts is essential to understand the complex relationship between genotype and phenotype.

The major purpose of the present study was to measure the levels of normal *CFTR* transcripts in human nasal epithelial (NE) cells from CF patients bearing the splicing mutations c.580-1G>T (712-1G>T, IVS5, class I mutation resulting in a major loss of CFTR protein) and c.2657+5G>A (2789+5G>A, IVS16, class V mutation with a partial loss of CFTR protein) and to detect differences in expression between genotypes. To this end, we have performed the relative quantification of *CFTR* mRNA of splicing mutations, in which a reduced *CFTR* expression level was unequivocally expected, by reverse transcription quantitative PCR (RT-qPCR), in order to evaluate the application of this model in *CFTR* therapeutic approaches. Furthermore, we provide new insights into a new complex allele involving the c.2562T>G SNP (exon 15) and the c.2657+5G>A mutation.

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In addition, *in vitro* assays using minigenes were performed. Finally, we evaluated the relationship between *CFTR* expression and the corresponding phenotype.

MATERIALS AND METHODS

Individuals

Fifty-three individuals previously characterized[10,11] were enrolled in this study. Samples were grouped considering four genotypes: (a) CF patients homozygous for p.Phe508del (CF-F508del), $n=7$; (b) compound heterozygous CF patients carrying one of the two different splicing mutations: the c.580-1G>T, $n=4$ [p.Phe508del (2), p.Gly542* (1), p.Ala399Asp (1)]; and c.2657+5G>A, $n=9$ [p.Phe508del (6), p.Asn1303Lys (1), c.273+1G>A (1) and p.Gly542* (1)]; (c) CF carrier relatives of these CF patients (c.[580-1G>T];[=], $n=3$; c.[2657+5G>A];[=], $n=9$); and (d) wild-type (wt) control group, $n=21$.

Human Genome Variation Society nomenclature was used (Supplementary Table S1).

The study was approved by the ethics committee. All enrolled individuals, followed up in the same CF Unit, gave their written consent. Different CF clinical parameters were evaluated (Table 1).

Characterization of nasal epithelial cells

For details, Supplementary Information is available at the *Eur J Hum Genet.* website.

RNA isolation and cDNA synthesis

For details, see Masvidal *et al*[12] and Supplementary Information.

Specific criteria were applied for inclusion of the RNA samples in order to reduce inter-sample variability: (a) RNA concentration ≥ 50 ng/μl; (b) 260/280 nm ≥ 1.90; and (c) RNA integrity number ≥ 5.

Each RNA sample (500 ng) was reverse transcribed (RT) twice using the High Capacity cDNA Reverse Transcription kit and random primers (Applied Biosystem, Foster City, CA, USA).

Analysis of splicing mutations

See *in silico* and qualitative analysis in Supplementary Information.

qPCR

CFTR gene expression analysis was performed with Taqman assay-on-demand products on an ABI 7300 real-time PCR system (Applied Biosystem). Available Taqman assays for *GUSB* (Hs_99999908_m1) and *ATP2B4* (Hs_00608066_m1) previously validated as reference genes[12] were used. Specific custom Taqman assays were synthesized for the two *CFTR* splicing mutations (Supplementary Table S2). Only *CFTR* correctly spliced transcripts (CST) can be amplified using these assays (Supplementary Figure S2). Triplicate reactions (20 μl each) from the two independent RT reactions were carried out for each sample/gene. Control samples such as 'no-RT' and 'no-RNA' were included to test for PCR and gDNA contamination, respectively. Furthermore, a calibrator sample was included in every run to correct for inter-run variability.

The raw *Cq* values were determined by using the SDS software v1.3.1 (Applied Biosystem), and normalization of data was performed by using the qBase^{Plus} software (Biogazelle, Ghent, Belgium).[13] Mean values from both RT-qPCR for each sample and median expression for each group were considered for statistical analysis. Intra-group expression variability was assessed by the median absolute deviation (MAD).

Statistical analysis

Qualitative and quantitative clinical variables were analyzed using the Fisher's exact test and the two-tailed Student's *t*-test, respectively. Differences in expression between groups were assessed by Mann–Whitney test. All tests were performed using the SPSS 12.0 software (IBM, North Castle, NY, USA). Differences were considered statistically significant when $P<0.05$.

Minigenes construction and RNA analysis

The CFTR minigenes were produced using a 'sticky feet PCR' strategy to introduce *CFTR* introns (IVS14, IVS15 and IVS16) consecutively into the pCDNA5/FTR/CFTR mammalian expression vector carrying the complete wt-CFTR cDNA. Site-directed mutagenesis was used to introduce the mutations c.2657+5G>A and c.2562T>G into the minigenes[14] (see Supplementary Information).

Protein analysis

CFTR detection was performed by western blot[15] (see Supplementary Information).

Table 1 Phenotypic characteristics of the cystic fibrosis patients

Clinical features	*c.2657+5G>A/CF* (n=*11*)	P	*F508del/F508del* (n=*45*)	*c.580-1G>T/CF* (n=*3*)
Sex, (male/female)	5/6	NS	28/17	2/1
Current age, years (mean ± SD)	36.8 ± 7.0	0.0001	23.8 ± 5.3	22.6 ± 3.5
Age at diagnosis, years (mean ± SD)	21.0 ± 8.1	0.0001	3.5 ± 4.5	5.6 ± 1.1
Sweat test, mEq/l (mean ± SD)	103.4 ± 23.3	NS	112.5 ± 20.3	102.3 ± 4.0
FEV1% predicted (mean ± SD)	82.4 ± 31.4	0.004	47.4 ± 23.1	32.8 ± 5.2
FVC % predicted (mean ± SD)	84.2 ± 32.1	0.045	61.4 ± 23.9	53.2 ± 4.7
Oxigen saturation, % (mean ± SD)	96.1 ± 1.5	0.020	93.9 ± 4.6	91.6
BMI (mean ± SD)	22.3 ± 4.3	0.037	19.1 ± 2.7	20.6
Trypsine, μg/l (mean ± SD)	343.8 ± 169.8	0.0001	15.6 ± 20.7	24.5 ± 48.8
Pancreatic insufficiency, *n* (%)	3 (27)	0.0001	43 (93)	3(100)
Pancreatitis, *n* (%)	2 (18)	0.035	0 (0)	0 (0)
Diabetes mellitus, *n* (%)	0	NS	9 (20)	2 (66)
Lung symptoms at diagnosis (%)	82	0.0001	60	—
Bronchiectasis, *n* (%)	11 (100)	NS	45 (100)	3(100)
Bilateral bronchiectasis, *n* (%)	8 (77)	NS	12 (27)	0
Diffuse bronchiectasis, *n* (%)	3 (23)	NS	33 (73)	3(100)
Bronchial colonization, *n* (%)	10 (91)	NS	44 (98)	3(100)
Pseudomonas colonization, *n* (%)	7 (64)	NS	38 (84)	2(66)
Hemoptysis, *n* (%)	11 (100)	0.001	21 (47)	—
Sinusitis, *n* (%)	11 (100)	0.024	30 (66)	3(100)
Admission to hospital (mean ± SD)	1.3 ± 2.7	NS	2.0 ± 4.6	—

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; BMI, body mass index; NS, not statistically significant.

RESULTS

Assessment of the *CFTR* mutation effect on splicing

For details, see Supplementary Information.

Quantitative analysis of *CFTR* CSTs

Independent expression analysis of *CFTR* was carried out for the two splicing mutations in four different groups. The same controls, the wt ($n=21$) and the CF-F508del ($n=7$) groups, were analyzed for both mutations. In addition, expression analysis for the c.580-1G>T included CF carriers ($n=3$) and CF patients ($n=4$) and CF carriers and patients ($n=9$, each one) for the c.2657+5G>A. In total, the *CFTR* expression analysis was performed in 35 (c.580-1G>T) and 46 (c.2657+5G>A) individuals.

CFTR gene expression was calculated relative to the expression of *GUSB* and *ATP2B4* genes.[12] Previous to the analysis, several quality-control parameters were taken into account. First, the inter-run and intra-run coefficients of variation (CVs) were calculated for all three genes analyzed. Intra-run CV ranged between 0.005 and 0.008 and inter-run CV between 0.014 and 0.026. PCR efficiency was also determined. Both *CFTR* assays showed an efficiency of 1.822; *GUSB* and *ATP2B4* efficiencies were 1.888 and 1.817, respectively. The use of *GUSB* and *ATP2B4* as suitable reference genes was determined by means of the gene expression stability value of multiple candidate genes (M) and the CV from normalized data.[16] The observed values were in agreement with the reference values M<0.5, CV<0.200.

The c.580-1G>T mutation transcript analysis showed a marked decrease in the number of CSTs in those groups bearing the mutation, the expression values being (median±MAD, arbitrary units): wt controls, 1.120±0.187; CF-F508del, 0.935±0.170; CF carriers, 0.735±0.030 and CF patients, 0.445±0.015 (Figure 1a). A similar expression pattern was observed for the c.2657+5G>A analysis in which CSTs accounted for 1.000±0.350, wt controls; 0.620±0.320, CF-F508del; 0.380±0.105, CF carriers; and 0.240±0.090 CF patients (Figure 1b). Statistical analysis of expression data for both mutations showed significant differences between the wt group and those groups with the splicing mutations ($P=0.032$ for c.580-1G>T CF carriers; $P=0.002$ for c.580-1G>T CF patients; and $P<0.001$ for c.2657+5G>A CF carriers and patients). A minor but significant difference was also found between the control groups, wt and CF-F508del, for the c.580-1G>T mutation ($P=0.041$; Figure 1a).

It should be noted that *CFTR* gene expression level displayed a wide range between samples in the same group, irrespective of whether they were patients, carriers or controls, this finding being attributable to inter-individual variability.

When the *CFTR* gene expression in CF carriers and patients bearing the splicing mutations were compared with that in wt controls, the percentage of expression reduction was 34 and 60%, respectively, for c.580-1G>T mutation and 62 and 76%, respectively, for c.2657+5G>A mutation (Figure 1c). Thus, a more pronounced reduction of *CFTR* expression, in relative values, was observed in those samples bearing the c.2657+5G>A mutation. Taking into account that the assays had similar PCR efficiencies, there have to be other reasons underlying these unexpected data.

Effect of the SNP c.2562T>G in the transcript level of samples bearing the c.2657+5G>A mutation

Contrary to what was expected before this study, we have found that the individuals with class V c.2657+5G>A mutation have lower levels of *CFTR* CST compared with individuals bearing the class I c.580-1G>A mutation. To explain this result, we have considered the

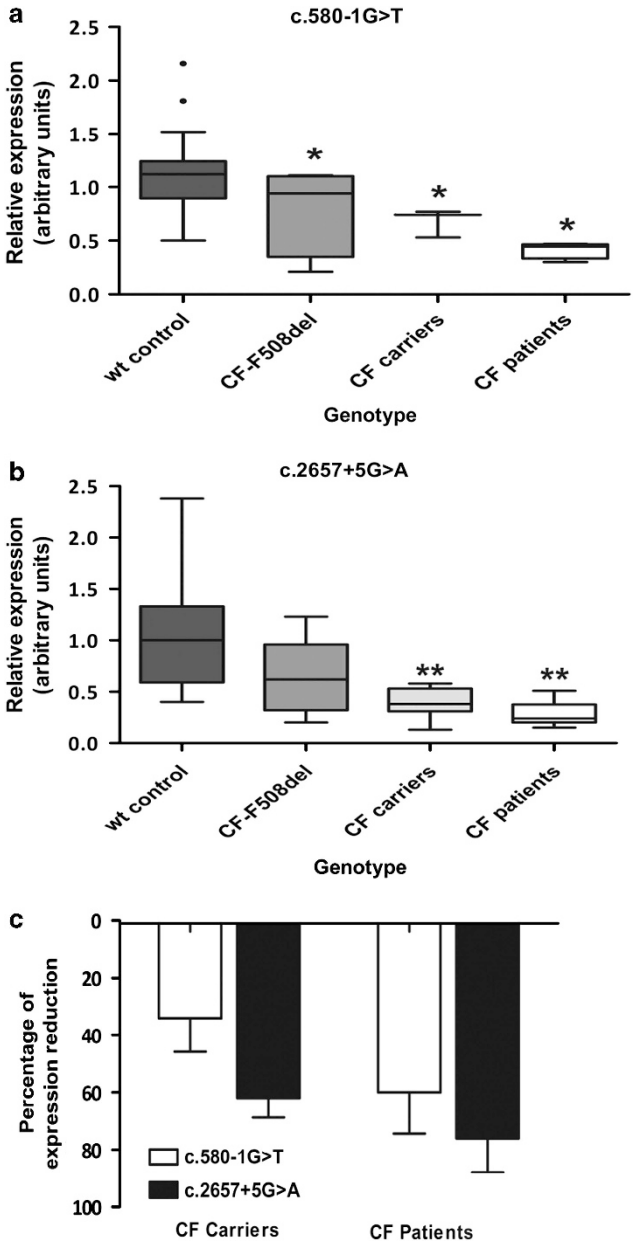

Figure 1 Quantitative *CFTR* expression analysis. Box plot analysis of *CFTR* expression results for c.580-1G>T (**a**) and c.2657+5G>A (**b**) obtained in the different groups of this study. (**c**) Percentage of *CFTR* expression reduction in CF carriers and patients compared with wt controls for the c.580-1G>T mutation (white) and the c.2657+5G>A mutation (black). Significant differences are indicated by asterisks (*$P<0.05$; **$P<0.001$).

putative role of other sequence variants in exon processing. Different *CFTR* synonymous and non-synonymous SNPs are present in patients and controls in similar frequencies (data not shown), suggesting that their effect, if any, must be similar in all the groups analyzed. Surprisingly, a common SNP located in exon 15, c.2562T>G, showed allelic frequencies with statistically significant differences between those groups with the c.2657+5G>A mutation and the two control groups ($P=0.012$). Thus, 26% alleles of wt controls and 14% alleles in the CF-F508del group showed the c.2562T>G SNP. Meanwhile, groups with the c.2657+5G>A mutation had c.2562T>G frequencies that reached 50% in CF

patients and 61% in CF carriers, suggesting linkage disequilibrium between these two *CFTR* variants.

The c.2562T>G SNP had been previously reported to influence the splicing efficiency process[8] in agreement with the *in silico* analysis, showing the loss of binding sites for splice enhancers (SC35 and SRp40). To evaluate a potential synergic effect on splicing of the c.2657+5G>A and c.2562T>G variants when occurring in *cis*, we have performed further complementary studies by cDNA qualitative analysis and the construction of minigenes.

Several cDNA samples were qualitatively analyzed (Figure 2a). The cDNA sample from the CF carrier c.[2657+5G>A; 2562T>G];[2562T>G] showed three transcripts, the CST (796 bp) and two aberrantly spliced transcripts (ASTs) (758 bp and 667 bp), from which sequencing analysis shows evidence for the skipping of exon 16 (r.2620_2657del) and exon 15 (r.2491_2619del), respectively. The wt control c.[2562T>G];[2562T>G] showed two transcripts, the CST and the one with the skipping of exon 15, whereas, the wt controls without the SNP c.2562T>G showed only the CST. It must be noted that ASTs were observed in a low proportion in these samples, so we cannot rule out the presence of other transcripts with even lower proportions. To show whether an additional AST lacking exons 15 and 16 was produced, a specific PCR was carried out using a CF patient c.[2657+5G>A;2562T>G];[1521_1523delCTT] and one wt control c.[2562T>G];[2562T>G] (Figure 2b). A 326 bp band was only observed in the CF sample, corresponding to an AST lacking exons 15 and 16 (r.2491_2657del). This result suggests the synergic effect of variants, c.2562T>G and c.2657+5G>A, both present in *cis* in our c.2657+5G>A patient cohort. Overall, patients with the complex CF allele c.[2657+5G>A; 2562T>G] have a lower level of

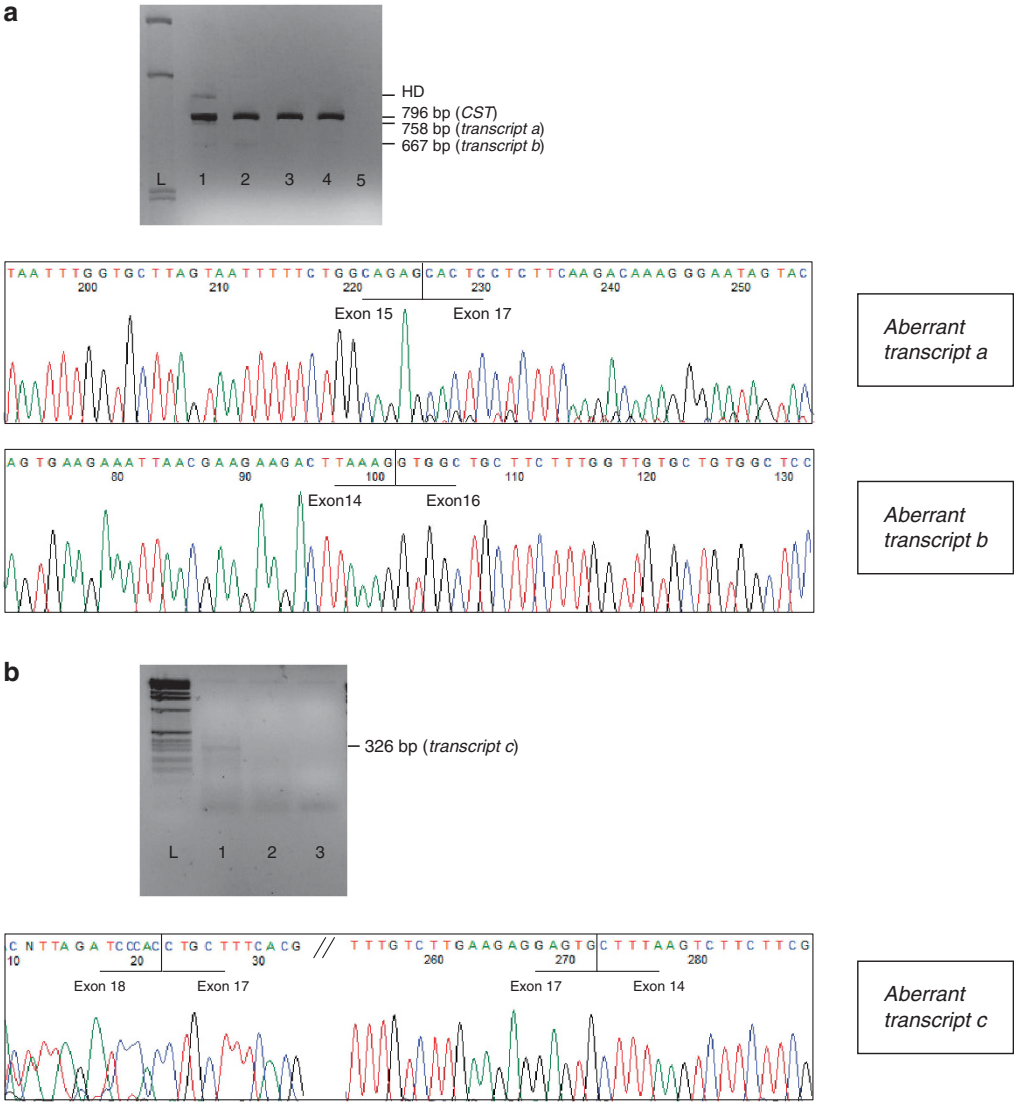

Figure 2 Qualitative analysis of the synergic effect of c.2657+5G>A mutation and c.2562T>G SNP on exon skipping. cDNA samples from NE cells were amplified and PCR products separated on 2% agarose gel. (**a**) Long PCR showed two aberrant transcripts: *transcript a* lacking exon 16 and *transcript b* lacking exon 15. L, molecular weight ladder; lane 1, CF carrier c.[2657+5G>A;2562T>G];[2562T>G]; lane 2, wt sample c.[2562T>G];[2562T>G]; lanes 3 and 4, wt samples without the SNP c.2562T>G; lane 5, NTC (negative PCR control); HD, heteroduplex band. (**b**) Specific PCR to show the additional production of aberrant *transcript c* lacking exons 15 and 16. L, molecular weight ladder; lane 1, CF sample c.[2657+5G>A; 2562T>G];[1521_1523delCTT]; lane 2, wt sample c.[2562T>G];[2562T>G]; lane 3, NTC (negative PCR control).

CSTs than expected likely due to an additive proportion of different ASTs alternatively losing exon 15 or exon 16 or both.

Minigenes analysis

Additionally, the impact of the c.2562T>G and c.2657+5G>A *CFTR* variants was evaluated by *in vitro* assays using CFTR triple-minigenes. RT-PCR analysis of HEK cells transiently expressing the wt CFTR triple-minigene show correct splicing of IVS14, IVS15 and IVS16, as the fragment obtained is of the same length as the transcripts from HEK cells expressing the CFTR cDNA minigene (Figure 3a, lanes 3 and 2, respectively). The transcripts from cells expressing the CFTR triple-minigene carrying the c.2562T>G SNP show the same splicing pattern (lane 4) as the wt *CFTR* minigene (lane 2), that is, no ASTs are observed. Whereas the CFTR triple-minigene carrying the c.2657+5G>A mutation (lane 5) showed three PCR fragments corresponding to CSTs, ASTs lacking exon 16, and ASTs lacking both exon 15 and 16 (smaller faint band). The double mutant (c.2562T>G and c.2657+5G>A; lane 6) shows the same alternative splicing pattern as the c.2657+5G>A single mutant. These results were confirmed by sequencing.

To determine whether other low-abundant transcripts are present but were not detected due to low sensitivity of the agarose gel, we performed a fluorescent RT-PCR semiquantitative analysis. The results confirmed the above data (see Supplementary Information). In addition, it revealed that the single- and double-mutant minigenes containing the c.2657+5G>A only produce 29%±2.3 and 23%±0.4 of CST, respectively (compared with the total *CFTR* transcripts of each minigene) (Figure 3b). The majority of the transcripts expressed by the single and the double mutants correspond to transcripts lacking exon 16 (63%±1.4 and 73%±1.6, respectively). The smaller percentage of transcripts, (8%±3.0 and 4%±1.2, respectively) correspond to transcripts lacking both exons 15 and 16.

We further analyzed the *CFTR* expression from these minigenes at the protein level by immunoblotting (Figure 4). The results showed a reduction of 53%±15.6 and 65%±4.7 for single (lane 5) and double (lane 6) mutants, respectively (comparing with wt triple minigene; lane 3). In addition, a very faint band with a smaller molecular mass (~100 kDa) was detected on both mutant minigenes (lane 5 and 6). From the RNA studies, we have shown that both mutant minigenes produce two smaller transcripts, one skipping exon 16 and another skipping both exons 15 and 16 (Figure 3). These transcripts would lead to truncated forms of CFTR with only 881 amino acids and 838 amino acids, respectively, and a molecular mass of 100 and 95 kDa, respectively. This prediction leads us to assume that the smaller faint protein band corresponds to these truncated forms.

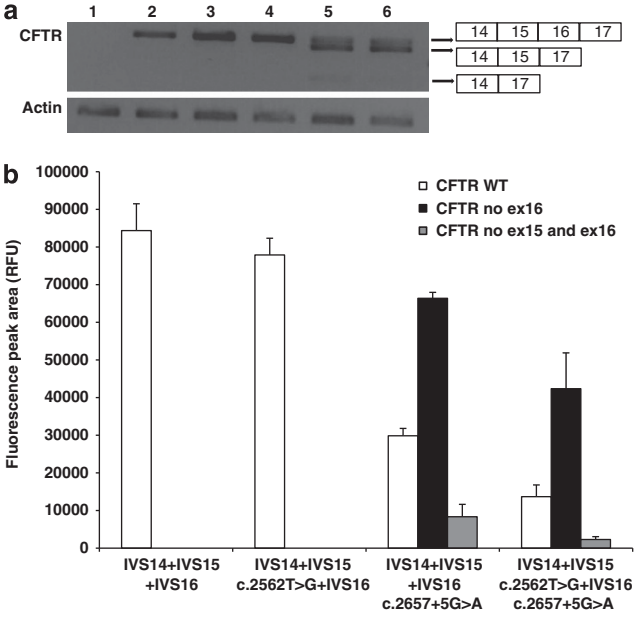

Figure 3 RT-PCR analysis of minigene transcripts expressed in HEK cells. (**a**) HEK cells transfected with the different minigenes were analyzed by RT-PCR using the D2R and G15L primers (see Methods section). Lane 1 corresponds to non-transfected cells. Lane 2: CFTR minigene containing wt (full-length CFTR cDNA); Lane 3: 'triple' CFTR minigene consisting of full-length CFTR cDNA and introns IVS14, IVS15 and IVS16; Lane 4: 'triple' CFTR minigene with the alteration c.2562T>G in exon 15; Lane 5: 'triple' CFTR minigene with the mutation c.2657+5G>A in exon 16; Lane 6: 'triple' CFTR minigene containing both alterations in exons 15 and 16. Bottom panel shows actin levels for each sample. (**b**) Graph showing the quantification from the fluorescently labeled RT-PCR for each minigene construct. Data are expressed as mean±SEM of three independent experiments. Percentages calculated for the c.2657+5G>A single and double mutant minigenes considering 100% as total amount of CFTR transcripts for each of the minigenes were: 29%±2.3 of CFTR wt (CST), 63%±1.4 CFTR no ex16 and 8%±3.0 CFTR no ex15 and ex16 (single) and 23%±0.4 of CFTR wt (CST), 73%±1.6 CFTR no ex16 and 4%±1.2 CFTR no ex15 and ex16 (double). RFU, relative fluorescent units.

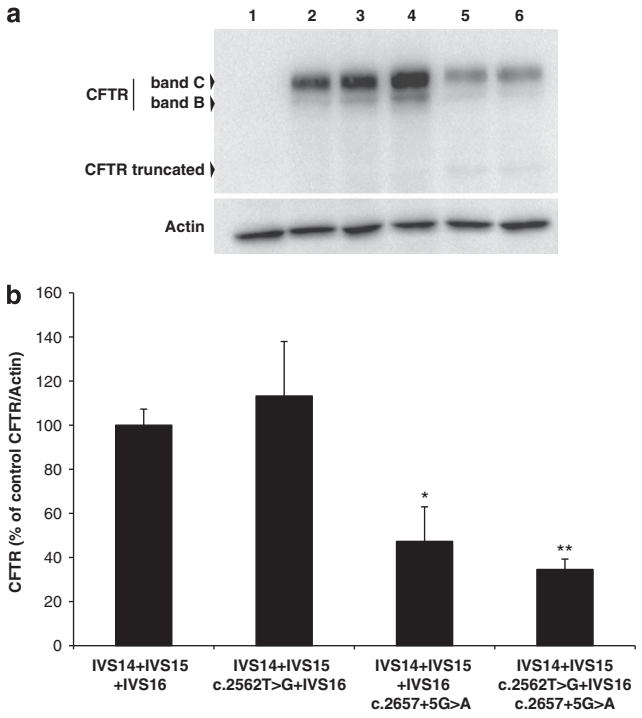

Figure 4 Protein analysis of minigene products expressed in HEK cells. (**a**) Representative western blot analysis of protein expressed in HEK cells transfected with the different 'triple' minigenes: wt (lane 3); with c.2562T>G in exon 15 (lane 4); with c.2657+5G>A in exon 16 (lane 5); with both variants, c.2562T>G in exon 15 and c.2657+5G>A in exon 16 (lane 6). Controls correspond to non-transfected cells (lane 1) and wt-full-length CFTR cDNA (lane 2). Bottom panel shows actin as loading control for each sample. (**b**) CFTR protein quantification (band C) shown as percentage of expression related to the control (wt triple-CFTR minigene). Data are the mean of three different experiments and shown as mean±SEM. Significant differences are indicated by asterisks (*$P<0.05$; **$P<0.001$).

Genotype–phenotype correlation

Different clinical parameters were evaluated to determine the relationship between the amount of *CFTR* transcripts and the clinical outcome in CF patients. To provide a more robust data, other patients of the same CF Unit were included. Thus, statistical analysis was performed on 11 CF patients bearing the c.2657 + 5G > A mutation (CF-splicing group) and 45 F508del homozygous patients (CF-F508del control group) (Table 1). The small number of CF patients bearing the c.580-1G > T mutation prevented statistical analysis from being performed. However, their clinical parameters looked quite similar to the corresponding features of the CF-F508del group (Table 1).

The CF-splicing c.2657 + 5G > A patients were mainly characterized by mild clinical CF manifestations in childhood; diagnosis was carried out in adulthood due to the presence of lung disease secondary to sinusitis, bilateral bronchiectasis and chronic bronchial infections by *Pseudomonas aeruginosa*. On the other hand, pancreatitis and pancreatic insufficiency were unusual. When comparing with the CF-F508del group, the CF-splicing group were older at the time of diagnosis as well as when the NE samples were collected ($P < 0.001$), they showed a higher body mass index ($P < 0.037$) and a better lung function (FEV1, $P < 0.0001$; FVC, $P < 0.01$; O_2 saturation $P < 0.02$), and less bronchiectasis and also lower PI incidence ($P < 0.0001$). However, CF-splicing patients showed a higher incidence of recurrent hemoptysis ($P < 0.001$). Hemoptysis was massive in 4 out of 11 (36%) CF-splicing patients requesting arterial embolization ($n = 3$) and surgery ($n = 1$). Recurrent pancreatitis was also present in 18% of the CF-splicing group. Overall, these data suggest a less severe course of the disease, at least at its beginning, in the CF-splicing patients. Hence, although c.2657 + 5G > A patients have a greater life expectancy, this class V mutation causes a severe chronic lung disease in older patients supporting our results from the expression analysis. In fact, one of these patients needed a lung transplant and another died at the age of 50 years. No significant differences between the two groups were observed concerning the number of times that patients needed hospitalization.

DISCUSSION

The *CFTR* gene has been found to be expressed at low levels in epithelial tissues[17] and also to show tissue specificity.[18,19] Consequently, previous *CFTR* gene expression studies needed complex analysis methods and were mainly focused on the splicing efficiency of exon 9.[20,21] Currently, these initial adverse conditions surrounding the *CFTR* expression analysis can be overcome using more sensitive tools, such as the RT-qPCR technology.

The pathological effect of many *CFTR* splicing variants is difficult to predict, particularly those in non-obvious regulatory elements or those that do not change the amino acid sequence. Here, the main aims were to assess the levels of *CFTR* CST in human NE cells from CF patients bearing splicing mutations, using the RT-qPCR technology, as a model to elucidate whether this technology could provide, in any mutation affecting gene transcript level, reliable data for *CFTR* transcript quantification that could be applied for further clinical purposes. Thus the study, including different genotypes, is focused on the analysis of class I and V CF splicing mutations to ensure that a reduction in CSTs will be observed. Taking into consideration that there is a correlation between the amount of CSTs and the abundance of functional protein,[22] the assays were designed to specifically quantify only the CSTs. In this way, we have prevented erroneous estimations as a consequence of different PCR efficiencies related to the size of the different spliced transcripts. Furthermore, complementary studies such as qualitative analysis of differently spliced transcripts and minigene expression analysis were performed.

The *CFTR* gene expression analysis has highlighted some interesting findings. First, we have observed a wide variability in *CFTR* expression among individuals from each group analyzed. A high variability in expression had already been reported in donor respiratory epithelium samples.[23] In addition, the variable NMD efficiency could contribute to this variability in CF patients with mutations generating a PTC.[24] The present and previous studies suggest the necessity of analyzing groups as large as possible for gene expression studies.

Contrary to what was expected, we have found a lower amount of CSTs in individuals with the class V mutation than in individuals with class I mutation, thus pointing out an apparent discrepancy between the genotype–phenotype relationship for these splicing mutations. We have found, at least, two factors contributing to these controversial data. First, concerning the genotype–phenotype relationship, different clinical features in class V patients (age at diagnosis, current age, etc.) indicated a milder phenotype compared with CF-F508del patients in agreement with previous reports[25,26] and also with c.580-1G > T patients, although in this case statistical analysis was unavailable. Nevertheless, we have also observed some severe clinical complications in class V patients probably attributable to the aging process; we should keep in mind that the c.2657 + 5G > A CF patients analyzed in this study are in the upper range of the life expectancy among CF patients. It had been previously reported that splicing efficiency decreases with age,[27] thus it is not unexpected that the more advanced age of class V patients contributes to the low transcript levels observed and their subsequent severe lung disease. To provide some light on the genotype–phenotype relationship, it would be interesting to perform a replicate study, including younger CF patients, for instance, from neonatal screening programs. Second, we have widely analyzed the DNA region surrounding the mutation c.2657 + 5G > A to determine whether other genetic factors could contribute to the reduction in amounts of CSTs. We have identified a common c.2562T > G SNP in exon 15, probably in linkage disequilibrium with the c.2657 + 5G > A mutation. Our data support that this common SNP leads to the skipping of exon 15, meanwhile the c.2657 + 5G > A mutation is associated to the skipping of exon 16. Thus the c.2562T > G SNP further reduces the levels of *CFTR* CSTs in patients also carrying the c.2657 + 5G > A, due to skipping of each one and both exons. This finding undoubtedly makes our results on *CFTR* gene expression more robust, as well as supplies further evidence that a synonymous coding SNP regulates the splicing efficiency. In fact, the controversial neutrality of synonymous SNPs had been reviewed recently.[28] Despite the c.2562T > G SNP and the c.2657 + 5G > A mutation being largely known, this is the first study suggesting a linkage disequilibrium between them and demonstrating the relevant consequences at the RNA level of this c.[2562T > G; 2657 + 5G > A] complex allele.

Minigene constructs have been largely used as a model to determine the effect of mutant alleles. However, their results have been corroborated by *ex vivo* analysis very few times due to unavailability of suitable samples. Here, we have performed CFTR minigene constructs to compare how close the results for *CFTR* transcript levels are in both *ex vivo* and *in vitro* approaches. The CFTR minigenes results mimicked the *ex vivo* data, as exon 16 skipping is the main AST and the CST level was observed in a similar proportion, when the c.2657 + 5G > A mutation is present. However, we were unable to detect the AST that lack exon 15 resulting from the c.2562T > G mutant or the c.[2562T > G; 2657 + 5G > A] double

mutant, as was observed in the *ex vivo* analysis. In contrast, the *ex vivo* analysis was performed with some samples homozygous for the c.2562T>G SNP as well as primers specifically designed to detect the skipping of exons 15–16, probably forcing the detection of the ASTs that were very poorly represented.

Using the minigene strategy, we could analyze the impact of the *CFTR* variants at the RNA and protein level, which is not very easily feasible in *ex vivo* as CFTR protein detection requires a large amount of tissue. Analysis of the protein is of crucial importance to evaluate the impact of mutations on the amount of correctly processed CFTR.

The discrepancies observed between *ex vivo* and *in vitro* analysis in *CFTR* transcripts can be due to different factors. First, differences in *CFTR* expression can be due to the different cell types used in the two approaches; the human epithelial cells and the HEK cell line, as it is well known that the efficiency of alternative splicing is different among human tissues/cell types, probably determining the typical organ involvement in CF.[20,29] Second, we compare *ex vivo* 'physiological conditions' in a limited individual series with the *in vitro* model in which the *CFTR* is highly overexpressed. It is also worth noting that we cannot use the versatility of minigene constructs when using the human physiological nasal cells, because in our study there is no CF patient/carrier bearing the splicing mutation without the SNP, suggesting linkage disequilibrium between both variants. In summary, different factors can explain these apparent controversial data without reducing the value of either approach, the minigenes model has been widely used to evaluate the mutant genes behavior, and also there is extensive evidence of the high sensitivity of RT-qPCR analysis in human tissues. In all, we believe that *ex vivo* analysis of human samples, despite its own limitations, provides the most accurate approach to determine what is happening in physiological conditions. However, when tissues samples are not available, the minigene approach is a good strategy to determine the effect of splicing mutations. The minigene strategy provides the advantage of allowing individual discrimination for each mutation effect (only one allele is present) and also protein analysis.

In conclusion, more attention should be given to the analysis of splicing mutations as many factors can influence the transcript processing and thus result in a less functional protein. Quantification of functional *CFTR* transcripts is important as the expression of full-length *CFTR* RNA >10% of the levels found in normal individuals has been suggested to prevent the CF phenotype.[30] In this context, we postulate that RT-qPCR is a robust and reliable technology to offer the sensitivity necessary to evaluate in *ex vivo* studies the efficacy of those therapeutic approaches that attempt to achieve an increase in the *CFTR* gene expression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

This study was conceived and designed by TC. Samples and clinical data were selected by AA and JdG. NE samples processing and qPCR experiments was performed by LM. MDR performed qualitative gene expression experiments. Gene expression analysis was supervised by SL. Minigene study design was performed by AR. Minigene experiments was performed by SI and supervised by AR and MA. The manuscript was written by MA, SL and TC. All aspects of the study were supervised by SL and TC.

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