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ORIGINAL

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Gene expression profile study in CFTR mutated bronchial cell lines

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Abstract Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis conductance transmembrane regulator (CFTR). Symptoms are pancreatic insufficiency, chronic obstructive lung disease, liver disease, chronic sinusitis and infertility in male patients. The phenotypic variability may be explained only in part by the more than 1200 CFTR mutations, which are grouped into six different classes, according to their effect on the protein ranging from a severe (no synthesis or blocked processing) to mild mutation (altered conductance or reduced synthesis). However, it is now accepted that other genes (CF modifiers) influence the phenotypic spectrum of the disease. In order to identify CF modifier genes, we built a low-density home-made oligoarray containing 144 genes selected according to biochemical criteria and evaluated their expression in two CF bronchial epithelial cell lines (CuFi1 F508del/F508del; CuFi3 F508del/R553X). If we consider both cell lines, 38 genes (26.3%) show an altered expression pattern with a threshold >±1.5. Of these 38 genes, 12 are altered in CuFi1, and 26 in CuFi3. Some of these genes share the same expression pattern in both cell lines, while others have a different behaviour. These results were validated by a QRT-PCR assay (R^2_{CuFi1}=0.81 and R^2_{CuFi3}=0.91). These data could suggest that the presence of a class I allele (R553X) determines a more profound alteration of gene expression pattern than the presence of a class II allele (F508del). The identification of the genes altered by a specific CF mutation could lead to the development of a pharmacological approach specific for different CFTR genotypes.

Key words Cystic fibrosis • CFTR • Modifier genes • Microarray • Expression analysis

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Introduction

Cystic fibrosis (CF; OMIM# 219700) is a lethal inheritable disease affecting Caucasians [1]. It is caused by mutations in the gene encoding cystic fibrosis conductance transmembrane regulator (*CFTR/ABCC7*; OMIM# 602421) [2, 3], leading to defective electrolyte transport in epithelial cells [4]. The consequences are an altered mucus viscosity and recurrent episodes of obstruction, inflammation and progressive destruction of affected organs. Symptoms are pancreatic insufficiency associated with neonatal meconium ileus (~10% of patients) and chronic obstructive lung disease superimposed with recurrent opportunistic infections that progressively destroy lung tissue [5, 6]. Other complications include liver disease, chronic sinusitis, infertility in male patients and elevated sweat concentrations [7, 8]. Pulmonary complications in

CF are a direct consequence of changes in the viscosity and volume of the air-surface liquid in the airways and possible malfunctioning of the immunological defence system. The result is stasis of mucus and obstruction of the smaller airways [9]. After this process, a (chronic) infection with an uncontrolled inflammatory response leads to destruction of lung tissue and reduction in lung function. Different individuals develop a broad phenotypic spectrum caused by the organ affected and by the severity of the disease [10]. In part, the phenotypic variability may be explained by more than 1200 mutations, grouped into six different classes, each one reflecting the CFTR defective function or production [11] (http://www.genet.sickkids.on.ca/cftr). This classification allows for discrimination between mild (altered conductance or reduced synthesis) and severe (no synthesis or blocked processing) mutations [12]. In particular, class I mutations result in the total absence of protein synthesis. This class includes the nonsense mutations and those that produce a premature stop codon (anomalies of splicing and frameshift mutations). In certain cases (R553X), the mutated mRNA is unstable and does not produce the protein, causing loss of CFTR chloride channel activity in the affected epithelia [13–15]. Class II mutations alter the cellular maturation of the protein, and thus the transport of these proteins to the plasma membrane. In this way, the protein is either present in a very small quantity or absent from the plasma membrane. One mutation of this class represents the majority of CF alleles (F508del is present up to 90% in North Europe) [16].

The impact of these mutations on the protein and the wide spectrum of CF phenotypes prompted a series of genotype-phenotype correlation studies [17]. The CFTR genotype is invariably correlated with pancreatic status – in about 85% of cases with pancreatic insufficiency and in about 15% of cases with pancreatic sufficiency. The correlations between the CFTR genotype and pulmonary, liver and gastrointestinal expression are debatable. As far as pulmonary disease, patients with presumably "severe" genotypes (F508del plus other class I, II, III mutations) had greater risks of shortened survival and *P. aeruginosa* acquisition compared with patients with presumably "mild" genotypes (class IV or V mutations in one or both alleles). Nevertheless it is now clear that CFTR genotype alone does not account for the wide diversity in CF pulmonary phenotype. Evidence is accumulating that secondary genetic factors independent from the CFTR locus significantly influence the severity of CF lung disease, the "CF modifiers" [18, 19]. These genes alter the effects of the primary disease gene and therefore result in phenotypic variability. The general classes of these potential modifier genes include inflammatory and anti-inflammatory mediators, antioxidants, mediators of airway reactivity, molecules involved in CFTR trafficking and alternative ion channels [20]. The best studied CF candidate modifiers for lung disease include mannose-binding lectin, glutathione-S-transferase, transforming growth factor-b1 (TGF-b1), tumour necrosis factor-a (TNF-a), b2-adrenegic receptor, HLA class II antigens, a1-AT and nitric oxide synthase (NOS)-1 [21, 22].

The primary goal of this study is to verify the expression patterns of CF putative modifier genes in CF cells with different CFTR genotypes, through a low-density oligoarray composed of 144 genes.

Candidate genes have been chosen based on the literature data, and because they encode for protein interacting with CFTR (degradation pathway, chaperones, channels, kinases and phosphatases, and CFTR interacting proteins), genes that take part in some processes in which CFTR is involved (inflammation), genes localised in the 19q13.3 region delimited between APOC2 and D19S112 markers, and genes mapping in *cis* with CFTR on human chromosome 7q32 (miscellaneous).

To this aim, we analysed two CF bronchial epithelial cell lines deriving from two patients with a different CFTR genotype: CuFi1 (F508del/F508del) homozygous for two class II mutations, and CuFi3 (F508del/R553X), a heterozygous compound with one class I allele and one class II allele.

Data analyses allowed to characterise a total of 38 (26.3%) differentially expressed genes gave results for both cell lines: 12 in CuFi1 (8 up- and 4 downregulated) and 26 in CuFi3 (25 up- and 1 downregulated).

Materials and methods

Human airway epithelial cell line culture model

CuFi1, CuFi3 and NuLi1, derived from CF and normal human airway epithelial cells, were obtained from J. Zabner (University of Iowa). Cells were cultured in collagen-coated plastic dishes (type VI, human placental) and serum-free bronchial epithelial cell growth medium with supplements (BEGM, Cambrex) [23]. The passage number of each cell type was recorded and cells were analysed at passage 15.

Microarray design and printing

We developed a "low-density" oligoarray (CFchip) containing a large number of genes involved with intracellular processes in cells where CFTR is normally expressed and genes occurring in cells that respond to abnormalities caused by CFTR defect (i.e., inflammatory, ion flux), to identify genes and pathways interacting with or compensating for CFTR functions. The oligonucleotide set (MWG Biotech, Ebersberg, Germany) was printed in triplicate onto UltraGAPS glass slides (Corning, Schiphol-rijk, The Netherlands) using TheRoboArrayer (Microgrid Compact Plus, BioRobotics). Printed slides were dried overnight and cross-linked with UV light at 600 mJ using a Strata-linker 2400

(Stratagene, Glenville, VA) and stored in a desiccator at room temperature. Before hybridisation, each slide was incubated in a prehybridisation buffer (5xSSC, 0.1% SDS, and 0.1 mg/ml BSA) at 42°C for 45–60 min.

RNA extraction, labelling, hybridisation

Total RNA was isolated by the TRIZOL standard protocol (Invitrogen). A small aliquot of RNA was then used for quantification and quality control using, respectively, a spectrophotometer (Nanodrop) and agarose gel electrophoresis. Twenty-five micrograms of total RNA were labelled using the CyscribeFirst Strand Labeling kit (Amersham Biosciences, Uppsala, Sweden). Unincorporated nucleotides were removed from cDNA by using the QIAquick Purification Kit (Qiagen). The labelled cDNA was eluted in 30 µl of PB buffer, and labelling efficiencies were determined by spectrometry (Nanodrop). cDNA differentially labelled were mixed and precipitated; each pellet was dissolved in 15 µl of hybridisation buffer (50% formamide, 5xSSC, 0.1% SDS and 0.1 mg/ml of a nucleic-acid blocker such as sonicated salmon sperm DNA) and after a denaturation of 5 min at 95°C, it was put onto the surface of printed slides and incubated at 42°C overnight. After hybridisation, the slides were washed at 42°C in buffer 1 (2xSSC, 0.1% SDS) for 5 min, in buffer 2 (0.2xSSC) for 2 min and twice in buffer 3 (0.05 SSC) for 2 min at room temperature. Finally, the slides were dried by centrifugation for 5 min at 1500 rpm. Then slides were analysed with laser scanner GenePix 4000B (Axon).

Image analysis and processing

The acquired images were analysed with Genepix Pro 5.0 software (Axon Instruments, Union City). Oligonucleotide spots were automatically segmented, local backgrounds (B635 and B532) were subtracted, and total intensities (F635 and F532) as well as the fluorescence ratios of the two dyes were calculated for each spot. The spots were flagged when they exhibited poor hybridisation signals or when they were saturated (F635 or F532 median=65535). We removed systematic bias in the data by applying the dye-swap normalisation [24], which makes use of the reverse labelling in the two microarray replicates. This normalisation procedure is well suited to treat low-density microarray data, where the majority of the spotted genes are expected to change their expression level. Identical results were obtained by subtracting or not the background from the spot foreground signals, as the average background level was very low.

Genes with signal to background ratio below two were flagged, together with genes strongly disagreeing by fold change: if two out of three replicates were overexpressed, while the third had a fold change below the cut off –2, the gene was flagged; and similarly for underexpressed genes. To establish the significance of observed regulation for each gene, we used *t*-tests with Welsh's correction, and then controlled for the multiplicity of testing. We took into account the multiplicity by controlling the false discovery rate (FDR) [25] in order to avoid a large number of false rejections. FDR control was given by the method proposed in Farcomeni [26]. FDR control at level 0.05 implies that the expected number of genes erroneously declared significant divided by the number of significant genes is below 5%. Finally, only genes with a satisfactory effect (absolute value of the fold change at least 1.5) were considered.

Validation of relative gene expression by real-time RT-PCR

Total RNA of the three cell lines (CuFi1, CuFi3, NuLi1) on which we performed microarray analyses was reverse-transcribed to cDNA according to the protocol of High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Incubation conditions were the following: 10 min to 25°C and 2 h to 37°C. We performed real-time quantitative PCR (QRT-PCR) using the Taqman system (Applied Biosystems, Foster City, CA, USA). The expression levels of a total of 5 genes and an internal reference (*GAPDH*) were measured by multiplex PCR using Assay-on-Demand™ gene expression products (Applied Biosystems, Foster City, CA, USA) labelled with 6 carboxyfluorescein (FAM) or VIC (Applied Biosystems). The analysed genes are the following: Hs00168906 (*ENaC*), Hs00232219 (*SMAD3*), Hs00168299 (*GRM8*), Hs00189032 (*CalR*) and Hs00191186 (*NHERF2*). The simultaneous measurement of each gene-*FAM* and *GAPDH-VIC* permitted normalisation of the amount of cDNA added per sample. We performed PCRs using the Taqman Universal PCR Master Mix and the ABI PRISM 7000 Sequence Detection System. A comparative threshold cycle (C_T) was used to determine gene expression relative to a calibrator (control subjects). Hence, steady-state mRNA levels were expressed as *n*-fold difference relative to the calibrator. For each sample, our gene's C_T value was normalised using the formula $DC=C_{tgene}-C_{tGAPDH}$. To determine relative expression levels, the following formula was used: $\Delta\Delta C_T=\Delta C_{Tsample}-\Delta C_{Tcalibrator}$ and the value used to plot relative gene expression was calculated using the expression $2^{-\Delta\Delta C_T}$.

To correlate the expression value obtained from microarray analysis and QRT-PCR we used the $\log_2$ transformation of the median value of each gene for both cell lines.

Results

To identify genomic response to different CFTR mutations, total RNA was isolated from two different human epithelial bronchial-derived cell lines, CuFi1 (F508del/F508del) and CuFi3 (F508del/R553X), and from a healthy subject, NuLi1 at the same passage (P15). We performed gene expression analyses through microarrays containing 144-human oligonucleotide set for genes that interact with or compensate for the mutated CFTR to maintain physiological functions. They have been subdivided into 7 different classes, according to their molecular function or to the biological process in which they are active (inflammation, protein degradation, chaperones, protein kinase and phosphatase, channels, CFTR interacting proteins, miscellaneous).

Table 1 Genes differentially expressed with a threshold expression level >±1.5. The table lists the genes that are found over- or underexpressed in CuFi1 and CuFi3 after expression profiling experiments with a threshold expression level >±1.5. The expression data correspond to the mean value of the two different experiments for each cell line

		Accession no.	Fold-change
CuFi1	*Upregulated*		
AP1M2	Adaptor-related protein complex 1, mu 2 subunit	NM_005498	2.85
HSPA5	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa)	NM_005347	2.66
AP3B1	Adaptor-related protein complex 3, beta 1 subunit	NM_003664	2.58
ENaC	Sodium channel, nonvoltage-gated 1 alpha	NM_001038	2
CANX	Calnexin	NM_001746	1.82
S100A11	s100 calcium-binding protein a11 (calgizzarin)	NM_005620	1.7
STX1A	Syntaxin 1a	NM_004603	1.55
CALR	Calreticulin precursor	NM_004343	1.52
	Downregulated		
MADH3	mad, mothers against decapentaplegic homologue 3	NM_005902	–3.46
GRM8	Glutamate receptor, metabotropic 8 precursor	NM_000845	–1.97
NDUFA5	nadh dehydrogenase (ubiquinone) 1 alpha subcomplex, 5	NM_005000	–1.6
ACTA1	alpha 1 actin precursor	NM_001100	–1.52
CuFi3	*Upregulated*		
HSPB1	Heat shock 27kda protein 1; hspb1	NM_001540	3.25
ENAC	Sodium channel, nonvoltage-gated 1 alpha	NM_001038	2.61
GOPC	pist; pist	NM_020399	2.32
S100A8	s100 calcium-binding protein a8; s100a8	NM_002964	2.19
STAT1	Signal transducer and activator of transcription 1 isoform beta; stat1	NM_139266	2.08
S100A11	s100 calcium binding protein a11 (calgizzarin); s100a11	NM_005620	2.07
SMAD3	mad, mothers against decapentaplegic homologue 3	NM_005902	2.04
GRM8	Glutamate receptor, metabotropic 8 precursor	NM_000845	2.02
UFD1L	Ubiquitin fusion degradation 1-like; ufd1l	NM_005659	1.86
PRKAA2	Protein kinase, amp-activated, alpha 2 catalytic subunit; prkaa2	NM_006252	1.8
SLC4A4	Solute carrier family 4, sodium bicarbonate cotransporter, member 4; slc4a4	NM_003759	1.69
KRAS2	c-k-ras2 protein isoform b; kras2	NM_033360	1.68
KCNJ1	Potassium inwardly rectifying channel j1 isoform a; kcnj1	NM_000220	1.67
MUC5AC	Gastric mucin; muc5ac	AF043909	1.67
SARA1	sar1a gene homologue 1; sara1	NM_020150	1.59
S100A9	s100 calcium-binding protein a9; s100a9	NM_002965	1.59
PPP2R4	Regulatory subunit pr 53 of protein phosphatase 2a isoform b; ppp2r4	NM_021131	1.58
PRKCG	Protein kinase c, gamma; prkcg	NM_002739	1.57
CLCN3	Chloride channel 3; clcn3	NM_001829	1.57
PPP3CA	Protein phosphatase 3 (formerly 2b), catalytic subunit, alpha isoform (calcineurin a alpha); ppp3ca	NM_000944	1.55
SNAP25	Synaptosomal-associated protein 25 isoform snap25a; snap25	NM_003081	1.54
PSMC1	Proteasome 26s atpase subunit 1; psmc1	NM_002802	1.52
SGK	Serum/glucocorticoid regulated kinase; sgk	NM_005627	1.52
PSMD11	Proteasome 26s non-atpase subunit 11; psmd11	NM_002815	1.5
UBE2E1	Ubiquitin-conjugating enzyme e2e 1 (ubc4/5 homologue, yeast); ube2e1	NM_003341	1.5
	Downregulated		
HSPA8	Heat shock 70kda protein 8 isoform 2; hspa8	NM_153201	–1.66

We performed microarray experiments in duplicate reverted *RNA* labelled, and relative mRNA abundance was represented by the expression ratio of Cy3-labelled test sample to Cy5-labelled reference sample signals. The expression data of all the genes studied are listed in the supplementary file (Supplementary Table 1).

After data normalisation, statistical differences were identified. Of the 144 analysed genes, we observed differential expression levels in all the groups.

A total of 38 (26.3%) differentially expressed genes, with a threshold expression level >±1.5, resulted considering both cell lines; 12 in CuFi1 (8 up- and 4 downregulated) and 26 in CuFi3 (25 up- and 1 downregulated) (Table 1). In particular, differential expression of genes involved in inflammation (5 in CuFi3 *vs.* 2 in CuFi1) and in degradation pathways (4 in CuFi3 *vs*. 2 in CuFi1) is represented in the two cell lines; on the contrary, other biochemical classes are specifically altered in one of the

two cell lines. Channels, protein kinases and phosphatases, and CFTR interacting proteins are mainly altered in CuFi3; in fact 6 genes encoding channels are differentially expressed in CuFi3 and 1 in CuFi1; 5 of the kinases and phosphatases in CuFi3 and nothing in CuFi1; 3 of the CFTR interacting proteins in CuFi3 and 1 in CuFi1. So, data analysis with a threshold expression level >±1.5 could reveal that the presence of a class I allele determines a more significant misregulation of gene expression than class II allele. In fact, CuFi3 has a more altered gene expression pattern compared with CuFi1, with 26 genes that show a differential expression compared to the 12 of CuFi1.

To increase the reliability of the data, we perform analysis with a threshold expression level >±2. We identified a total of 15 genes (9% of the genes represented in the CF chip), 7 in CuFi1 (5 up- and 2 downregulated) and 8 in CuFi3 (8 up- and none downregulated). This allowed us to characterise some genes with the same threshold expression >2 in both cell lines, like *ENaC*, and some genes with different expression, like *GRM8* and *SMAD3*, both overexpressed in CuFi3 and underexpressed in CuFi1 (Fig. 1).

To confirm CFchip results, a total of five genes were selected (*ENaC*, *SMAD3*, *GRM8*, *CalR*, *NHERF2*) and analysed for differential expression by QRT-PCR. In Table 2 we compared the expression values obtained with the QRT-PCR and oligoarray assays; a Pearson correlation analysis demonstrates a statistically significant positive correlation (CuFi3: R^2=0.91, P<0.001; CuFi1: R^2=0.81, P<0.001) (Fig. 2a,b).

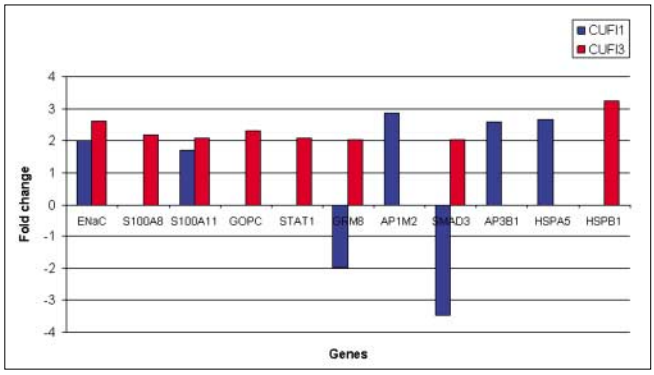

Fig. 1 Fold change of altered genes with a threshold expression level >±2 in CuFi1 and CuFi3 cell lines. A total of 15 genes with a threshold expression level >±2 (9% of the genes represented in the CF array), 7 in CuFi1 (5 up and 2 down) and 8 in CuFi3 (8 up and 0 down) are identified

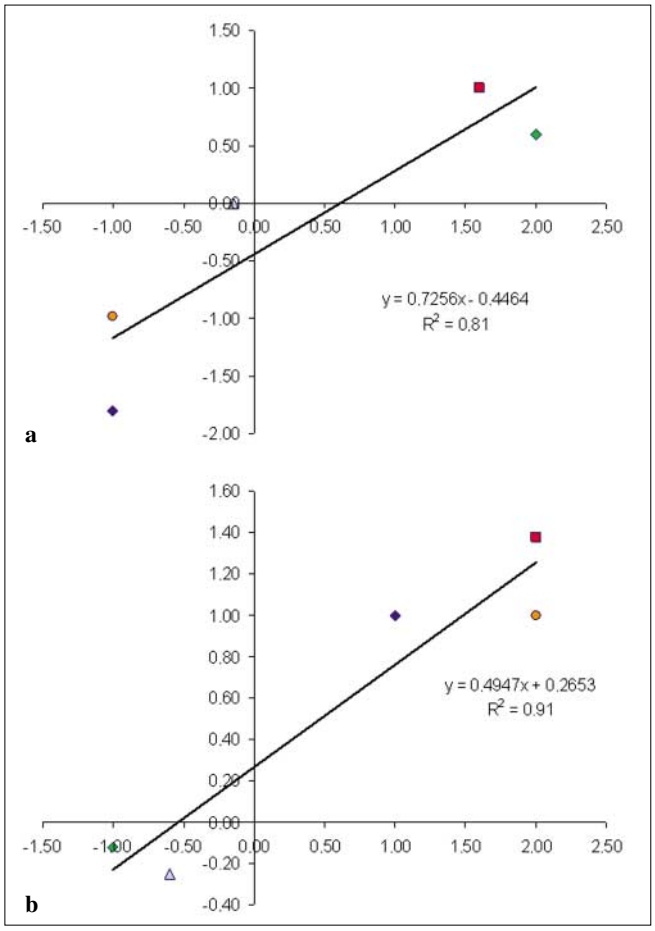

Fig. 2a,b Correlation analysis between the expression values of QRT-PCR and microarray. A Pearson correlation analysis demonstrates a statistically significant positive correlation in CuFI1 (**a**, R^2=0.81, P<0.001) and in CuFI3 (**b**, R^2=0.91, P<0.001) between the expression values obtained with the QRT-PCR and cDNA microarray assays (*ENaC*, red; *SMAD3*, blue; *GRM8*, yellow; *NHERF2*, grey; *CalR*, green)

Table 2 Correlation analysis between the expression values of QRT-PCR and microarray. This table shows the 5 genes we analysed for differential expression by QRT-PCR to confirm CF chip results. For each gene the values of QRT-PCR (log_2RT) and of cDNA microarray (log_2microarray) are shown in the two cell lines

Gene name	CuFi1 log_2RT	CuFi1 log_2microarray	CuFi3 log_2RT	CuFi3 log_2microarray
ENaC	1.60	1.00	2.00	1.38
SMAD3	–1.00	–1.80	1.00	1.00
GRM8	–1.00	–0.98	2.00	1.00
NHERF2	–0.15	0.00	–0.60	–0.25
Calreticulin	2.00	0.60	–1.00	–0.12

Discussion

We performed a low-density microarray analysis on two bronchial epithelial cell lines, CuFi1 (F508del/F508del) and CuFi3 (F508del/R553X), compared with a cell line from a healthy subject (NuLi1). We analysed the expression pattern of 144 genes considered putative CF modifiers. The advantage of using a low-density oligoarray is major specificity towards candidate genes; in fact genes have been chosen based on literature data, and because their products physically interact with CFTR or take part in some process in which CFTR is involved. Microarray experiments have been used to evaluate gene expression in CF. Norkina et al. [27] and Kaur et al. [28] have studied the gene expression in the CF mouse small intestine and exocrine pancreas. They found increased expression of several genes, many of which are components of the innate immune system, demonstrating that the CF mouse intestine exhibits an innate immune response. Eidelman et al. [29] used microarray in IB-3 cell line to study the TNF-a/NFkB pathway. Xu et al. [30] performed high-density microarray analysis of lung mRNAs on whole lung tissue from mice lacking CFTR. They demonstrated that the absence of CFTR starts a genomic response that causes changes in gene expression also in the absence of detectable infection or inflammation.

In our experiment, expression analysis of the different cell lines has shown results very similar to the situation of CF knock-out mice and IB-3 cell line. It confirms that, in absence of inflammation or infection, the CF bronchial cell has an innate immune response both in absence of CFTR and in the presence of two CF alleles. Moreover it also confirms the genomic response of genes actively involved or elements of the degradation pathway, channels, kinases and phosphatases, and CFTR interacting proteins.

Data analysis with a threshold expression level >±1.5 revealed that CuFi3 shows a more dysregulated gene expression pattern compared with CuFi1. Furthermore, some subgroups of genes are mainly altered in CuFi3, like those encoding for channel, kinases and phosphatases and CFTR interacting proteins; all these genes are rarely differentially expressed in CuFi1.

Changes in inflammatory response

Changes in inflammatory response were represented in both cell lines. Interestingly, we found *SMAD3*, a gene of the TGF-b1 signal, with an opposite expression level >±2 in both cell lines; in fact, in CuFi1, according to Kelley et al. [31], it is the most underrepresented gene (–3.46), while it is over-represented (2.04) in CuFi3. Probably, different SMAD3 protein expression selectively alters TGF-b1-mediated signalling in CF epithelium, potentially contributing to different inflammatory responses.

Similarly, the signal transduction and activator of transcription-1 (STAT1) [32] gene encoding a protein activated by phosphorylation on tyrosine in response to different ligands is not differentially expressed in CuFi1, while it is overexpressed in CuFi3 (2.08).

Among the genes that have a similar pattern of overexpression in both cell lines, we found a family of calcium-binding proteins termed the calgranulins (*S100A8, S100A11*), expressed primarily by alveolar macrophages, whose expression is enhanced by various cytokines like TNF-a, interleukin-b and interferon-g. This family of genes share potent chemoattractant activities, stimulating inflammatory cell trafficking. Increased expression of *S100A8* (2.19 in CuFi3) was previously demonstrated in alveolar macrophages from *Cftr* mutant mice, and authors suggest that increased *S100A8* may contribute to the enhanced inflammatory responses seen in the absence of CFTR. Here, we observed overexpression of *S100A8* gene in the presence of CFTR mutated alleles in epithelial cells. Furthermore, we documented the overexpression of *S100A11* (1.7 in CuFi1 and 2.07 in CuFi3), in agreement with Renaud et al. [33]. Interestingly, the level of expression of these genes is considerably more elevated in CuFi3, in agreement with the presence of a class I mutation.

These data from CF bronchial epithelial cell lines are similar to data from *Cftr* knock-out mice with no histological inflammation, and give strength to other observations of an innate immune inflammatory response in CF cells.

Changes in protein degradation pathways and molecular chaperones

Regulation of the CFTR expression is very complex both at the transcriptional level and at the (post)-translational level, where the equilibrium between protein maturation and degradation is determined by ubiquitination, degradation and the action of different chaperones [34]. Overexpression of ubiquitination and degradation pathway genes was represented in the two cell lines. CuFi3, which produces a truncated form of CFTR protein, showed an increased expression of *PSMC1* (1.52), *PSMD11* (1.5), *UFD1L* (1.86) and *UBE2E1*(1.5). Interestingly, UFD1L is a component of the N-end rule pathway of degradation and, recently, perturbations of this pathway were described in patients with Johanson-Blizzard syndrome, a recessive disorder characterised by pancreatic insufficiency and developmental alterations [35]. Instead, CuFi1, which produces a small amount of protein, showed an overexpression of the chaperones that try to fold CFTR into a protease-resistant form able to leave the ER and reach the

Golgi: *Calnexin* (1.82), *Calreticulin* (1.52) and *HSP70* (2.66). This may suggests that homozygosity for F508del alleles might cause an adaptation of the CF cells, which try to increase the amount of CFTR that can go into the Golgi. Finally, we described different expression levels in CuFi1 of subunits *AP1M2* (2.85) and *AP3B1* (2.58). These are members of the clathrin complex involved in receptor-mediated and fluid phase endocytosis from the plasma membrane to early endosomes, and transport from the trans-Golgi network (TGN) to endosomes [36, 37].

Channels

CFTR is a regulator of a variety of ion channels and transporters in cells. We detected overexpression of the epithelial Na^+ channel (*ENaC*) in both cell lines (2.62 in CuFi3 and 2 in CuFi1). Interestingly, studies on epithelial cells have demonstrated that activation of CFTR inhibits *ENaC* [38, 39]. Moreover, it has been demonstrated that the mechanism for inhibition of EnaC is rather non-specific and is mediated by Cl^-. Otherwise, ENaC is inhibited by nitric oxide (NO) in alveolar monolayers. Serum- and glucocorticoid-inducible kinase (SGK1) interacts with NOS and may be important in regulating NO production in alveolar epithelium, and might indirectly regulate the expression of ENaC [40]. As we found an overexpression of *SGK1* (1.52) in CuFi3, we suggest that the regulation of *ENaC* is conditioned by different pathways. The results of this interaction may have an important role in the development of the CF phenotype because alternative channels in the airway may be able to partially compensate for the CF defect.

CFTR interacting proteins

CFTR is regulated by interactions with various other proteins [41, 42]. The amino terminal tail physically associates with components of the SNARE machinery, which mediates membrane fusion in eukaryotic cells [43]. The last 3 amino acids of the carboxy terminal tail of CFTR bind to PDZ domain-containing proteins [44]. PDZ interactions may serve as a scaffolding function to promote CFTR channel clustering or interactions with other membrane proteins that may serve a regulatory function. In our experiments, we found overexpression of the *GOPC* gene (2.32), encoding a PDZ domain containing Golgi protein, in CuFi3. Overexpression of *GOPC* reduces CFTR chloride currents and modulates the surface expression of CFTR [45, 46]. As for other interacting proteins, we found overexpression of *Syntaxin1A* in CuFi1 (1.55). This gene encodes for a transmembrane SNARE protein present in the cell membrane of neurons and airway epithelial cells that inhibits the CFTR chloride channel [47]. Syntaxin 1A also interacts physically with ENac, reducing channel currents and ion channel insertion into the plasma membrane [48]. So, its overexpression has to be considered important for both the channels.

The N-terminal domain of CFTR is bound by two other t-SNARE proteins, SNAP23 and SNAP25, that form heterodimers with Syntaxin1A [49]. They work cooperatively and all reduce the cAMP-mediated chloride current. *SNAP25* was overexpressed in CuFi3 (1.54). Both the precise method of action of these proteins and the meaning of an overexpression in the CF cells remain to be elucidated.

Conclusions

This work describes the first microarray approach to evaluating the effect of genotypes on CF modifiers. Also, if gene expression profiles are affected variably between cell models, and the genotypes of the two cell lines are very similar, these data could suggest that gene expression profile is influenced by the severity of the mutation. In this case, the presence of allele R553X in CuFi3 induces a generally more profound alteration of gene expression pattern, because the cell tries to produce CFTR from an RNA that does not produce the protein. Instead, F508del in CuFi1 produces a small amount of protein, and shows an overexpression of the chaperones that try to fold CFTR into a protease-resistant form able to leave the ER and reach the Golgi.

It is hoped that this information will be useful to select targets for potential CF drugs and for the identification of novel transcriptional biomarkers of the diseases.

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