ELSEVIER

Journal of Cystic Fibrosis 10 (2011) 228 – 233

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Original Article

Impact of polymorphism of *Multidrug Resistance-associated Protein 1* (*ABCC1*) gene on the severity of cystic fibrosis

Andrea Mafficini [a,1,2], Myriam Ortombina [b,2,3], Isabelle Sermet-Gaudelius [c,4], Patrick Lebecque [d,5], Teresinha Leal [e,f,6], Patrizia Iansa [b,3], Gregory Reychler [d,5], Karin Dahan [f,6], Xaviers Pepermans [f,6], Gerard Lenoir [c,4], Anissa Leonard [d,5], Claudio Sorio [a,1], Baroukh Assael [b,3], Paola Melotti [b,*,3]

[a] *Department of Pathology, General Pathology Section, University of Verona School of Medicine, Strada Le Grazie, 37134 Verona, Italy*
[b] *Cystic Fibrosis Center, Azienda Ospedaliera di Verona, piazzale Stefani, 1-37126 Verona, Italy*
[c] *Necker-Enfants-Malades Hospital, 149 rue de Sèvres, 75015 Paris, France*
[d] *Pediatric Pulmonology & Cystic Fibrosis Unit, Université Catholique de Louvain, 10 avenue Hippocrate, 1200 Brussels, Belgium*
[e] *Department of Clinical Chemistry, Université Catholique de Louvain, 10 avenue Hippocrate, 1200 Brussels, Belgium*
[f] *Centre for Human Genetics; Université Catholique de Louvain, 10 avenue Hippocrate, 1200 Brussels, Belgium*

Received 9 May 2010; received in revised form 3 October 2010; accepted 18 October 2010
Available online 24 March 2011

Abstract

A 5′FR/G-260C (NCBI reference: 010393.16:g.15983174C>G) functional polymorphism of *Multidrug Resistance-associated Protein 1* (*ABCC1*) promoter has been reported which influences ABCC1 expression including inflammatory related events.

We aimed at investigating the impact of this polymorphism on the severity of CF disease.

In this multicentric study, key clinical features of 203 CF patients homozygous for the F508del mutation were recorded. Kaplan–Meier analysis showed that patients with the rare CC genotype were chronically colonized by PA around 6 years earlier (mean ± SD: 11.2 year ± 7.8, 95% CI for the mean: 5.7–16.8) than those with the GG or the CG alleles ($p <= 0.01$) and a FEV1 <60% predicted was first observed earlier in this group ($p < 0.05$). Concordant trends to better nutritional status and FEV1 were observed in the slightly older GG subgroup.

The potential role of ABCC1 promoter as a modifier gene deserves further study.
© 2011 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.

Keywords: NT_010393.16:g.15983174C>G polymorphism; Cystic fibrosis; Infection by *Pseudomonas aeruginosa*; Modifier gene; Multidrug Resistance-associated Protein 1; ABCC1; Severity of cystic fibrosis disease

1. Introduction

Cystic fibrosis (CF) is the most common fatal inherited disorder in white population. It is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, a member of the ATP Binding Cassette (*ABC*) transporter superfamily. CF is mainly characterized by bronchopulmonary disease, pancreatic insufficiency and male infertility. It presents over a wide range of disease severity and patients with identical CF genotypes can display markedly different phenotypic expression [1,2].

Among the ABC transporter superfamily, Multidrug Resistance-associated Protein 1 (*MRP-1*) and CFTR share the highest homology. MRP-CFTR regulatory interaction has been reported [3]. Studies focusing on *MRP-1* (now defined as *ABCC1*, according to the international nomenclature) have suggested that it may possibly represent a CF modifier gene [4–9]. Data obtained from the Verona Centre have demonstrated induction of ABCC1 mRNA and restoration of nasal potential difference

* Corresponding author. Tel.: +39 045 8073419; fax: +39 045 8072042.
E-mail address: paola.melotti@ospedaleuniverona.it (P. Melotti).
[1] Tel.: +39 0458027688; fax: +39 0458027127.
[2] Equal contributors.
[3] Tel.: +39 0458123741; fax: +39 0458122042.
[4] Tel.: +33 144494882; fax: +33 144381750.
[5] Tel.: +32 27641939; fax: +32 02 7648906.
[6] Tel.: +32 27646724; fax: +32 27646932.

1569-1993/$ - see front matter © 2011 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2010.10.007

parameters in CF patients treated with azithromycin [5] suggesting that functional complementation of mutant CFTR by ABCC1 could possibly contribute, at least partly, to the mechanism of action of this macrolide. This finding, however, has not been confirmed by others [10].

More recently, a functional polymorphism in *ABCC1* promoter (NBCI reference: NT_010393.16:g.15983174C>G; accession number on NIH SNP database: rs504348) that can possibly modulate the protein expression has been reported [12,13]. Accordingly, in four cell lines, the G allele of this polymorphism was associated with a significantly lower transcriptional activity of the promoter than the C allele [13].

We hypothesized that *ABCC1* polymorphism is associated with the severity of CF disease. In this study we determined the NT_010393.16*ABCC1* genotype in a large cohort of F508del homozygous CF patients and analyzed the association of C or G alleles with CF disease severity. Gene reporter assays were performed in order to establish the relevance of the NT_010393.16 variant for the *ABCC1* promoter transcriptional activity. In a subgroup of CF patients, allelic frequencies were considered in relation with ABCC1 mRNA levels and cAMP responsiveness in nasal epithelial cells.

2. Materials and methods

2.1. Genotyping

DNA was extracted from whole blood using salting out method then samples were genotyped for the NT_010393.16: g.15983174C>G (cluster report**:** rs1805010). Polymorphism of *ABCC1* promoter region of interest (−365/−211) was amplified with Polymerase Chain Reaction (PCR) in a GeneAmp PCR system 9700® (Applied Biosystem, Foster City, CA, USA) using the forward primer 5′FR (5′-CAGGATGAAATGAGGGCA-CAG) and the reverse primer 5′FR (5′-GAAGCGCCTGG-GATCTTTGG) as previously described [13]. Reactions were performed with AmpliTaq Gold ® (Applied Biosystem), in AmpliTaq Buffer 1×, 1.5 mM Mg^{2+}, 200 μM of each dNTP, 10 pmol of each primer in presence of 80 ng of DNA in a final volume of 50 μl. The temperatures applied were: 94 °C for 15′ followed by 35 cycles of 94 °C for 30″, 62 °C for 90″ and 72 °C for 60′ finally followed by 72 °C for 10′. The size of the PCR fragment was checked by electrophoresis on 2% agar-gel in the presence of Size Marker VI (Roche, Diagnostics, Mannheim, Germany). The fragment was then purified using ExoSAP-IT® (Usb-corporation, Cleveland, Ohio, USA) according to manufacturer's instructions. A dry sample containing 2 μl of purified PCR fragment and 1 μl of 4 mM primer sequencing R (5′-CCTGCGACCACTTTTCAAAT) was provided for analyses of polymorphism that was carried out using SnapShot® Multiplex system (Applied Biosystem; BMR Genomics sequencing service C.R.I.B.I.'s University of Padova). Results were analysed using GenescanView 1.2 software.

2.2. Cell lines

The following airway epithelial cell lines were used: 2CFSMEo, a CF tracheobronchial gland epithelial cell line kindly provided by D. Gruenert, University of California, CA, USA [14]; IB3-1, a CF respiratory epithelial cell line and C38, its corresponding rescued isogenic cell line expressing a plasmid encoded copy of functional CFTR; both were kind gifts of Pamela Zeitlin, Johns Hopkins University, USA [15]. 2CFSMEo cells were grown in Eagle's MEM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% foetal bovine serum (FBS, Cambrex Bio Science) and 1% L-glutamine (Cambrex Bio Science). IB3-1 and C38 were cultured in LHC-8 media (Biosource, Camarillo, CA, USA) supplemented with 5% FBS. Cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. In vitro analysis of promoter SNP NT_010393.16

The promoter SNP NT_010393.16-reporter constructs for this study were kindly provided by Dr. Caroline Lee from National Cancer Centre of Singapore [12]. A 635 bp promoter region of *ABCC1* gene including the SNP region was inserted upstream of the β-galactosidase reporter gene in an expression vector which also contains the enhanced green fluorescent protein (EGFP) reporter gene driven by cytomegalovirus promoter for normalization against differences in transfection efficiency. Promoter–reporter constructs were transiently transfected using Fugene Hd (Roche).

EGFP fluorescence was measured in cellular lysates with a 485 nm excitation filter and a 530 emission filter with a Packard fluoroCOUNTER (PerkinElmer Inc., Waltham, Massachusetts, USA). β-galactosidase activity was quantified by means of a colorimetric assay (β-galactosidase reporter gene activity detection Kit, Sigma-Aldrich, St. Louis, MO, USA) with ONPG (O-Nitrophenyl-β-D-galactopyranosid) as substrate. The reaction was detected 4 h after incubation at 37 °C in a Packard SpectraCOUNTER (PerkinElmer Inc.) microplate reader at 405 nm. Values were normalized against blank and the corresponding cell lines were transfected with an empty vector. β-galactosidase expression was normalized against EGFP activity.

2.4. Nasal brushing, mRNA quantification and anion conductance assays

The procedures were performed as previously described [9,16]. A halide-sensitive videoimaging assay with fluorescent dye 6-methoxy-N-3′-sulfopropylquinolinium (SPQ, Molecular Probe, Cedex, France) was used to measure chloride transport at the cellular level. After loading with 10 mM SPQ, cells were continuously perfused with isotonic NaI solution, followed by $NaNO_3$ solution to which cAMP agonists were added. The maximum change in fluorescence rate after addition of cAMP agonists was defined as cAMP-dependent anion conductance ($\Delta FcAMP/\Delta t$) and the CFTR-mediated cAMP-stimulated anion conductive pathway was considered present when $\Delta FcAMP/\Delta t > 0.1$. At least 5 nasal ciliated cells (NCC) were studied in each cell preparation experiment. The percentage of cells displaying cAMP response (%cAMP) in each individual experiment represented the SPQ assay endpoint.

2.5. Study population

209 CF patients, all homozygous for the F508del mutation, were recruited from three European centres (Verona: 138, Brussels: 51, and Paris: 20). They were aged from 6 to 40 years and all had reliable spirometry. Functional in vivo studies were performed from nasal brushing samples collected from 20 CF patients from the Paris centre. Genotype for the NT_010393.16 polymorphism of *ABCC1* was determined in 130 adult non-CF Italian subjects and in all 209 CF patients. Healthy subjects were negative for the most common mutations of *CFTR* gene except for 4 heterozygous subjects (healthy carriers), consistently with epidemiological data about carrier frequency.

Informed signed consent for the use of DNA and other samples was obtained following approval by local Ethical Committees.

2.6. Clinical data of CF patients

Clinical data were of 203 patients collected thanks to electronic databases. Main characteristics are shown in Table 1. Anthropometric parameters and FEV1 were normalized using Freeman's [17] and Knudson's [18] equations respectively.

Diabetes was defined by the need for insulin therapy. Chronic *Pseudomonas aeruginosa* (PA) colonization was reported using the European consensus definition [19]. In order to take into account the accelerated FEV1 decline with age in CF patients, the last value of FEV1 was also expressed as FEV1 percentile using CF-specific reference equations [20]. Outcome variables were then stratified in quartiles. Upper and lower quartiles from each centre were then pooled for further statistical analysis.

2.7. Statistical analysis

For in vitro functional studies, the correlation between *ABCC1* genotype and ABCC1 mRNA expression or percent nasal potential both under basal conditions and after cAMP stimulation was assessed using the *t* test with Welch's correction where variances were found to be significantly different.

Clinical data were compared using chi-squared test for categorical variables and unpaired *t* test or Mann–Whitney test for continuous variables, as appropriate. Chi-square was also used to compare allelic distribution between upper and lower quartiles. Kaplan–Meier plot was obtained for ages at chronic colonization by PA and at first FEV1 value <60% predicted, under stable conditions.

Table 1
Characteristics of 203 CF patients homozygous for the F508del mutation recruited from 3 different European centres.

	Paris (Necker)	Brussels (St Luc)	Verona
Subjects n	14	51	138
Male/female	8/6	29/22	60/78
Age years	17.43±3.32	21.33±9.65	24±9.44
FEV1 (% predicted)	73±18	79±25	58±29
FEV1 CF (specific percentile)	56±28	68±24	45±31
Weight Z score	−1.95±1.64	−0.98±1.16	−1.01±1.40
Height Z score	−0.85±1.75	−0.9±1.18	−0.69±1.22
BMI Z score	−1.45±1.19	−0.61±1.03	−0.82±1.28
cc by PA n (%)	6 (42.9%)	16 (31.4%)	88 (63.8%)
Diabetes n (%)	0	10 (19.6%)	37 (26.8%)

Data are presented as mean±SD unless otherwise stated. FEV1: forced expiratory volume in one second; and cc by PA: chronic colonization by *Pseudomonas aeruginosa.*

3. Results

3.1. ABCC1 SNPNT_010393.16 genotyping

Among 130 healthy control subjects, the observed frequencies for CC, CG and GG polymorphic genotypes were 4%, 39% and 57% respectively. The distribution was similar to that observed in 209 CF patients (5.9%, 38.9% and 55.2%) and in keeping with that reported by Wang and colleagues in a European non-CF population [13].

3.2. Functional in vitro analysis

IB3-1 and C38 cell lines were efficiently transfected, as detected by EGFP fluorescence, but β-galactosidase activity was undetectable. In 2CFSMEo-cells, repeated assays showed no statistically significant difference of regulation by the major allele G-containing promoter when compared with the C-allele.

3.3. Functional in vivo/ex vivo analysis

mRNA quantification and anion conductance assays in the subgroup of 20 patients from the Paris centre did not show any significant difference between GG and GC genotypes mRNA levels ($p=0.1152$;%BASAL $p=0.1911$; after cAMP $p=0.4964$).

3.4. Clinical status of CF patients and genotype for the NT_010393.16 polymorphism of ABCC1

No significant difference could be demonstrated between anthropometric data, FEV1, diabetes prevalence and the genotype for the NT_010393.16 polymorphism of *ABCC1*. However, there was a slight but concordant trend for the GG group to have higher height Z score, weight Z score, BMI Z score and better FEV1 (Table 2).

In the study group, 110 CF patients (54%) were chronically colonized by PA. Rates of chronic colonization by PA were comparable in the three groups. Age at chronic colonization by PA was available for 103 out 110 CF patients. Kaplan–Meier analysis showed that patients harbouring the CC genotype were chronically colonized by PA around 6 years earlier (mean±SD: 11.2 year±7.8, 95% CI for the mean: 5.7–16.8) than those with the GG or the CG alleles ($p<=0.01$, Fig. 1).

When comparing upper and lower quartiles for normalized FEV1, no genotype for the NT_010393.16 polymorphisms of *ABCC1* (CC, CG or GG) was found to be overrepresented in any subgroup (Table 3).

Table 2
Clinical data of 203 CF patients homozygous for the F508 del mutation according to the genotype for the NT_010393.16:g.15983174C>G polymorphism of *ABCC1*.

	CC	GC	GG	CC versus GG p-value
n	12	79	112	
Age (years±SD)	21.5±6.9	22.46±9.5	23.3±9.5	0.24
Height — Z score	−0.84±1.45	−0.79±1.28	−0.72±1.21	0.76
Weight — Z score	−1.35±2.13	−1.21±1.37	−0.93±1.28	0.32
BMI — Z score	−0.97±1.88	−0.91±1.20	−0.70±1.16	0.47
FEV1 — % predicted	61.7±37.8	61.1±29.4	67.3±27	0.52
cc by PA — n (%)	10 (83.3%)	39 (49.4%)	61 (54.5%)	0.11
Age at cc by PA — years	11.2±7.8	16.8±7.8	17±9.1	0.0644
Diabetes — n (%)	3 (25%)	17 (21.5%)	28 (25%)	0.73

Data are presented as mean±SD unless otherwise stated. FEV1: forced expiratory volume in one second; BMI: body mass index; and cc by PA: chronic colonization by *Pseudomonas aeruginosa*.

Table 3
Comparison of upper (UQ) and lower (LQ) quartiles according to the Kulich's CF specific FEV1 reference values.

	UQ	LQ	p-value
n	48	50	
Male/female	27/21	19/31	0.11
Age — years	21.21±7.66	24.1±8.47	0.08
FEV1 — % predicted	95±14	33±18	<0.0001
FEV1 — CF specific percentile	88±8	13±15	<0.0001
Weight — Z score	−1.06±1.57	−1.72±1.37	0.0286
Height — Z score	−1.15±1.16	−0.61±1.38	0.0378
BMI — Z score	−0.44±1.23	−1.67±1.14	<0.0001
cc by PA — n (%)	18 (37.5%)	36 (72%)	0.0012
Diabetes — n (%)	4 (8.3%)	22 (44%)	0.0002
ABCC1 genotype n (%)			
CC	5 (10.4%)	4 (8%)	0.95
CG	18 (37.5%)	24 (48%)	0.40
GG	25 (52.1%)	22 (44%)	0.55

Data are presented as mean±SD unless otherwise stated. FEV1: forced expiratory volume in one second; BMI: body mass index; and cc by PA: chronic colonization by *Pseudomonas aeruginosa*.

Kaplan–Meier analysis revealed that age at first FEV1 value below 60% predicted under stable conditions was significantly lower in patients with CC genotype in comparison with CG or GG ($p<0.05$) (Fig. 2).

4. Discussion

To the best of our knowledge this is the first study looking at the potential role of *ABCC1* single nucleotide polymorphisms in CF patients.

Clinical data from the present study show that in patients homozygous for the F508 del mutation, the rare CC phenotype of the NT_010393.16:g.15983174C>G polymorphism of *ABCC1* promoter region is associated with a significantly younger age at which a FEV1 <60% predicted is first observed under stable conditions and a younger age at chronic colonization by PA. Age at first PA infection and age at chronic colonization by PA have both been used in other studies of modifier genes in CF [21,22] but the latter is more meaningful as chronic colonization by PA has been clearly associated with an accelerated rate of FEV1, poorer survival and increased costs of treatment [23–26]. In addition, concordant trends to better nutritional status and FEV1 were observed in the slightly older GG subgroup.

Beside or together with environmental factors (including therapies), modifier genes are believed to contribute to the variable clinical severity of CF in patients sharing the same *CFTR* genotype. However, studies looking at CF modifier genes have often yielded conflicting results. Even though the reasons for this are not fully understood, it is clear that many small studies are underpowered [27–29].

In order to circumvent common pitfalls, our multicenter study focused on a single CFTR genotype and investigated a large population size of more than 200 patients. Participating centres have long been used reliable CF-specific electronic databases.

Efforts were made to normalize data and to use clear definitions and standardized reference values [17–20]. Clinical

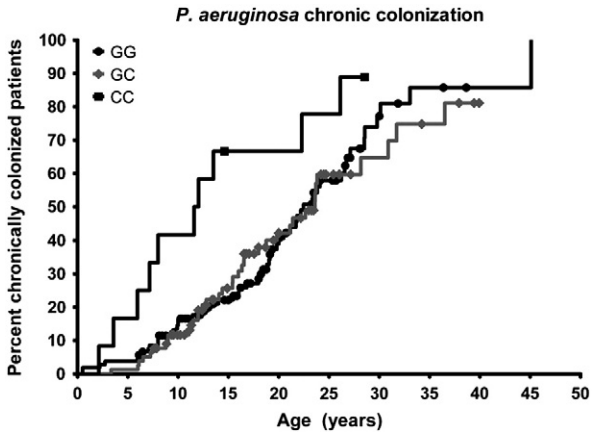

Fig. 1. Age at chronic *P. aeruginosa* infection according to ABCC1 genotype. Kaplan–Meier plots relative to age at chronic infection by *P. aeruginosa* in patients with CC, GG and GC alleles (n=103) show different trends for CC versus GG and GC (P=0.0065 and P=0.0056 respectively).

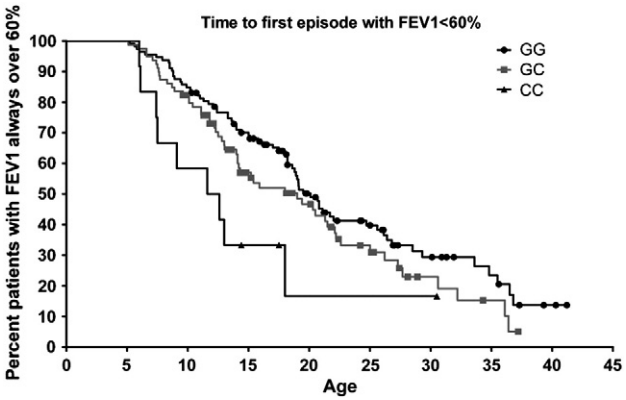

Fig. 2. Lung function decline according to ABCC1 genotypes. Kaplan–Meier plots relative to age at first episode with FEV1 <60% of predicted value in patients with CC, GG and GC alleles (n=153) are shown. The overall difference between the three curves is also significant (P=0.024), with a significant trend towards worse prognosis from GG to CC (P=0.015).

data were analyzed in two ways: not only by looking at relevant parameters in the whole population and stratifying patient subgroups according to the genotype for the NT_010393.16 polymorphism of *ABCC1*, but also by looking at possible overrepresentation of a given genotype in upper and lower quartiles according to FEV1 percentiles using CF-specific reference equations for age, height and gender [20]. The latter approach was initially performed at each centre reducing possible influences of local policies. In addition, two laboratory studies were conducted in parallel that could contribute to interpret clinical results.

It should be stressed however that given the (expected) wide range of normalized FEV1 values, the study remained underpowered to detect a 10% FEV1 difference which would be accepted as significant. The low frequency of NT_010393.16 polymorphism further affects the power of the statistical analyses.

In the present study, laboratory investigations do not provide additional support to the hypothesis of an impact of polymorphism on promoter transcriptional activity, mRNA levels, basal and cAMP-induced anion transport. However, based on our experimental model (airway tract cell lines) we cannot formally exclude that an upstream element associated in cis with the C allele (not included in our gene reporter construct) could inhibit the transcriptional activity. In four epithelial cell lines not deriving from the respiratory tract transfected with constructs containing either C or G alleles at the position of ABCC1 SNP, Wang et al. demonstrated a lower ABCC1 activity in G-containing promoter cells [13]. This was not observed in the present study but a tissue specific regulatory effect on the ABCC1 mRNA levels and function of the NT_010393.16 ABCC1 in the lung, which is not detectable in the primary human nasal cells, could be hypothesized. It is also important to stress that only 2 out of 20 patients who underwent nasal brushings for in vivo functional analysis displayed the CC genotype.

ABCC1 not only plays a role in protecting cells against drugs, toxins and heavy metals but also contributes to antioxidative defense system and inflammation which is believed to play a major role in the pathophysiology of CF lung disease.

In mice lacking the multidrug resistance-associated protein, Wijnholds et al. reported a decreased response to inflammatory stimulus [11]. Interestingly, Schultz et al. noted that in mice, loss of the *abcc1* gene results in increased resistance to *Strepococcus pneumoniae* induced pneumonia [30]. In triple knock-out mice lacking the gene for *abcc1*, van der Deen et al. observed a reduced inflammatory response to cigarette smoke and lower levels of several cytokines and chemokines in the lungs, independent of smoke exposure [31]. More recent data confirmed an association between *ABCC1* single nucleotide polymorphisms and FEV1 in two independent COPD cohorts, consistent with a detrimental effect of higher *ABCC1* expression [12].

Our finding of a possible deleterious effect of the CC genotype in CF patients is in keeping with these studies.

We do believe that this issue deserves further work. Would another team be interested in pursuing the investigation in much larger study populations, we would be pleased to offer them our complete set of well defined clinical and genetic data in order to increase the power of the study.

Acknowledgements

We thank Federica Quiri for the excellent technical assistance for cell cultures and Emily Pintani for the assistance about clinical database.

This study was supported by the French Mucoviscidose and by the ABC Foundation.

References

[1] Lester LA, Kraut J, Lloyd-Still J, Karrison T, Mott C, Billstrand C, et al. Delta F508 genotype does not predict disease severity in an ethnically diverse cystic fibrosis population. Pediatrics 1994;93:114–8.

[2] Grasemann H. Disease modifier genes in cystic fibrosis. Eur Respir Mon 2006;35:50–6 Printed in UK. Copyright ERS Journals Ltd 2006.

[3] Abraham EH, Shrivastav B, Salikhova AY, Sterling KM, Johnston N, Guidotti G, et al. Erythrocyte membrane ATP binding cassette (ABC) proteins: MRP1 and CFTR as well as CD39 (ecto-apyrase) involved in RBC ATP transport and elevated blood plasma ATP of cystic fibrosis. Blood Cells Mol Dis 2001;27:165–80.

[4] Altschuler EL. Azithromycin, the multidrug-resistant protein, and cystic fibrosis. Lancet 1998;351:1286.

[5] Pradal U, Delmarco A. Long term azithromycin in cystic fibrosis: another possible mechanism of action? J Chemother 2005;17:384–400.

[6] Cigana C, Nicolis E, Pasetto M, Assael BM, Melotti P. Effects of the macrolide azithromycin on the expression of ATP-Binding cassette transporters in cystic fibrosis airway epithelial cells. J Chemother 2007;19:643–9.

[7] Lallemand JY, Stoven V, Anneraud JP, et al. Induction by antitumoral drugs of proteins that functionally complement CFTR: a novel therapy for cystic fibrosis? Lancet 1997;350:711–2.

[8] Leslie EM, Deeley RG, Cole SP. Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology 2001;167:3–23.

[9] Hurbain I, Sermet-Gaudelus I, Valle B, et al. Evaluation of MRP1-5 gene expression in cystic fibrosis patients homozygous for the ΔF508 mutation. Pediatr Res 2003;54:627–34.

[10] Barker PM, Gillie DJ, Schechter MS, Rubin BK. Effect of macrolides on in vivo ion transport across cystic fibrosis nasal epithelium. Am J Respir Crit Care Med 2005;171:868–71.

[11] Wijnholds J, Evers R, van Leusden MR, et al. Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. Nat Med 1997;11:1275–9.

[12] Siedlinski M, Boezen HM, Boer JM, Smit HA, Postma DS. ABCC1 polymorphisms contribute to level and decline of lung function in two population-based cohorts. Pharmacogenet Genomics 2009;19:675–84.

[13] Wang Z, Wang B, Tang K, Lee EJ, Chong SS, Lee CG. A functional polymorphism within the MRP1 gene locus identified through its genomic signature of positive selection. Hum Mol Genet 2005;14:2075–87.

[14] Cozens AL, Yezzi MJ, Chin L, Simon EM, Finkbeiner WE, Wagner JA, et al. Characterization of immortal cystic fibrosis tracheobronchial gland epithelial cells. Proc Natl Acad Sci U S A 1992;89:5171–5.

[15] Zeitlin PL, Lu L, Rhim J, Cutting G, Stetten G, Kieffer KA, et al. A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. Am J Respir Cell Mol Mol Biol 1991;4:313–9.

[16] Verkman AS. Development and biological applications of chloride-sensitive fluorescent indicators. Am J Physiol 1990;259:375–88.

[17] Freeman JV, Cole TJ, Chinn S, Jones PR, White EM, Preece MA. Cross sectional stature and weight reference curves for the UK, 1990. Arch Dis Child 1995;73:17–24.

[18] Knudson R, Lebowitz M, Holberg CJ, Burrows B. Changes in the normal maximal expiratory flow–volume curve with growth and aging. Am Rev Respir Dis 1983;127:725–34.

[19] Döring G, Conway SP, Heijerman HG, Hodson ME, Høiby N, Smyth A, et al. Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a European consensus. Eur Respir J 2000;16:749–67.

[20] Kulich M, Rosenfeld M, Campbell J, Kronmal R, Gibson RL, Goss CH, et al. Disease-specific reference equations for lung function in patients with cystic fibrosis. Am J Respir Crit Care Med 2005;172:885–91.

[21] Garred P, Pressler T, Madsen HO, et al. Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J Clin Invest 1999;104:431–7.

[22] Dorfman R, Sandford A, Taylor C, et al. Complex two-gene modulation of lung disease severity in children with cystic fibrosis. J Clin Invest 2008;118:1040–9.

[23] Kerem E, Corey M, Gold R, Levison H. Pulmonary function and clinical course in patients with cystic fibrosis after pulmonary colonization with *Pseudomonas aeruginosa*. J Pediatr 1990;116:714–9.

[24] Konstan MW, Morgan WJ, Butler SM, et al. For the Scientific Advisory Group and the Investigators and Coordinators of the Epidemiologic Study of Cystic Fibrosis. Risk factors for rate of decline in FEV1 in children and adolescents with cystic fibrosis. J Pediatr 2007;151:134–9.

[25] Cystic Fibrosis Foundation. Patient registry 1996 annual data report. Maryland: Bethesda; August 1997.

[26] Baumann U, Stocklossa C, Greiner W, von der Schulenburg JM, von der Hardt H. Cost of the care and clinical condition in paediatric cystic fibrosis patients. J Cyst Fibros 2003;2:84–90.

[27] Slieker MG, Sanders EA, Rijkers GT, Ruven HJ, van der Ent CK. Disease modifying genes in cystic fibrosis. J Cyst Fibros 2005;4:7–13.

[28] Davies J, Griesenbach U, Alton E. Modifier genes in cystic fibrosis (CFTR state of the art series). Pediatr Pulmonol 2005;39:383–91.

[29] Boyle MP. Strategies for identifying modifier genes in cystic fibrosis. Proc Am Thorac Soc 2007;4:52–7.

[30] Schultz MJ, Wijnholds J, Peppelenbosch MP, et al. Mice lacking the multidrug resistance protein 1 are resistant to *Streptococcus pneumoniae*-induced pneumonia. J Immunol 2001;166:4059–64.

[31] van der Deen M, Timmens W, Timmer-Boscha H, et al. Reduced inflammtory response in cigarette smoke exposed MrpI/MdrIa/MdrIb deficiënt mice. Respir Res 2007;8:49.