A polymorphism in ADRB2 gene is associated with severity of pulmonary phenotype in Cystic Fibrosis patients.

Abstract: Background: Cystic Fibrosis (CF) is a common genetic disease caused by mutations in the gene encoding for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein. It is known that modifier genes and environmental factors play a key role in determining the severity of the disease. Methods: We analyzed Single Nucleotide Polymorphisms (SNPs) in three genes, namely TNFA, TGFB1 and ADRB2, as potential modifiers of CF lung phenotype: c.−851C>T, c.−308G>A, c.−238G>A and c.+691G ins/del SNPs in TNFA, p.Leu10Pro (c.869C>T) and p.Arg25Pro (c.915G>C) SNPs in TGFB1 and p.Arg16Gly (c.46G>A), p.Gln27Glu (c.79C>G) and p.Thr164Ile (c.491C>T) in ADRB2. Results: For the c.46G>A SNP of ADRB2 the A allele (Arg16), as well as the AA genotype, were significantly more frequent in CF patients than healthy controls. When stratifying CF patients according to FEV1 (Forced Expiratory Volume in 1 second) phenotype we observed a statistically significant difference (p=0.02) in the allelic and genotype frequencies. The A allele and A/A genotype were more frequent in mild CF patients when compared to severe CF subjects and thus probably associate with a protective effect toward the development of severe pulmonary manifestation in CF patients. Conclusions: Our results are indicative of the involvement of the ADRB2 gene as modifier gene in Cystic Fibrosis pulmonary phenotype.

Keywords: Cystic Fibrosis, Modifier genes, Tumor necrosis factor-α (TNFA), Transforming growth factor-β1 (TGFB1), β2 adrenergic receptor (ADRB2), Pulmonary manifestation, Polymorphism.

1 Introduction

Cystic Fibrosis (CF) is the most common recessive genetic disease in the Caucasian population with a prevalence of 1:2500 newborns [1]. It is a multi-organ disease caused by mutations in the gene that encode the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein. CFTR is a chloride channel that is predominately localized to the lumen-facing (apical) membranes of epithelial cells, in which it participates in the regulated transport of salt and water across epithelial tissues [2].

So far more than 1800 different mutations have been described. The mutations are classified in five classes based on the molecular alteration at protein level: Class I mutations lead to defective protein products, Class II mutations result in defective protein processing, Class III mutations cause a defect in the channel regulation, Class IV mutations are defective in conductance through the channel, and Class V mutations lead to abnormal splicing. The first three classes are considered as severe mutations and the other classes as mild mutations. [3,4].

The altered protein causes dense mucous epithelial secretions and thus a clinical expression that typically involves the gastrointestinal and pulmonary apparatus, pancreas, liver, and reproductive tract. The clinical course of the disease varies widely especially at the pulmonary level [5]. This raised the possibility that other factors, such as modifier genes or environmental factors, plays a pivotal role.

Previous studies have shown strong discordance for pulmonary phenotype in patients with the same CFTR genotype and this had been observed even in siblings...
from the same families. This indicates the scarce role of environmental factors in modulating the pulmonary phenotype and suggests that modifier genes, inherited independently of CFTR, could mainly modulate pulmonary expression in a number of CF patients [6].

Tumor necrosis factor-α (TNFA) is a proinflammatory cytokine found at high levels in CF airways that contributes to severe neutrophil-predominant inflammation [7]. Transforming growth factor-β1 (TGFB1) is a cytokine with both pro-inflammatory and anti-inflammatory properties. In the airway epithelium, TGFB1 modulates fibroblast proliferation and deposition of collagen [8]. β2 adrenergic receptor (ADRB2) is a G-protein–coupled receptor, present in the airway, that stimulates adenyl cyclase activity and is known to influence airway reactivity [9]. The gene encoding for ADRB2 is highly polymorphic (at least 95 polymorphisms are reported in humans by the NCBI SNP database), and some of these polymorphisms have been studied in Cystic Fibrosis, but results were somewhat contrasting [10-12].

In order to study the role of TNFA, TGFB1 and ADRB2 as modifiers of the pulmonary phenotype in Cystic Fibrosis and confirm in a different population of CF patients previously reported association [10,13-16] we analyzed single nucleotide variations (SNPs) in these 3 genes in a group of Italian CF patients characterized by different clinical phenotypes.

2 Methods

2.1 SNPs choice:

We have chosen two polymorphisms in exon 1 of TGFB1, namely p.Leu10Pro (c.869C>T) and p.Arg25Pro (c.915G>C) that regulate levels of TGFB1 production and have been shown to modify the development and/or severity of lung disease in fibrotic lung disease, asthma and chronic obstructive pulmonary disease (COPD) [17]. For ADRB2 we studied three of the most common variants, namely p.Arg16Gly (c.46G>A) leading to enhanced down-regulation of the ADRB2, p.Gln27Glu (c.79C>G) characterized by blunted down-regulation and abnormal receptor degradation if co-expressed with Arg16, but not if coexpressed with Gly16, [18] and pThr164Ile (c.491C>T) leading to a decreased binding of agonist to the protein [10]. Finally, for TNFA we analyzed three SNPs within the promoter region, c.−851C>T, c.−308G>A and c.−238G>A and one SNP in intron 1, c.+691G ins/del, due to their known association with different modulation of TNFA production [17].

2.2 Patients and controls

We studied 70 unrelated CF Caucasian patients (32 males and 38 females; actual mean age 15.5 ± 13.3 sd) admitted to the Regional Cystic Fibrosis Centre of Trieste (Italy), whose diagnosis was made after two positive sweat tests performed according to Gibson and Cooke [19].

Informed consent was obtained from the participants or, in case of minors, from their relatives. The Burlo Garofolo Ethical Committee approved the study (Prot. CE/V – 78).

The pulmonary function of patients was evaluated on the basis of forced expiratory volume in 1 s (FEV1) expressed as a percentage of expected values corrected for sex and height. The best value of all records collected during each calendar year was taken into consideration. Based on the CF database, clinical history and FEV1 (% of predicted) at different age, and severity of lung disease was classified according to the criteria used by Drumm et al. [20]. For 45 patients the FEV1 values were available and it has been possible to classify the pulmonary phenotype as mild or severe (Table 1). In particular, the mild pulmonary phenotype group included CF patients >15 years of age (to have better long-term prediction) with

Table 1. Clinical characteristics of the 45 CF patients analyzed in this study

<table>
<thead>
<tr>
<th>Patients</th>
<th>Male (M) (n=20)</th>
<th>Female (F) (n=25)</th>
<th>Total (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age (year)</td>
<td>27.3 ± 9.72</td>
<td>21.4 ±9.46</td>
<td>24.1 ±9.9</td>
</tr>
<tr>
<td>FEV1 mild</td>
<td>19/20 (0.95)</td>
<td>21/25 (0.84)</td>
<td>40/45 (0.88)</td>
</tr>
<tr>
<td>FEV1 severe</td>
<td>1/20 (0.05)</td>
<td>4/25 (0.16)</td>
<td>5/45 (0.12)</td>
</tr>
<tr>
<td>Severe/Severe CFTR genotype</td>
<td>17/20 (0.85)</td>
<td>20/25 (0.80)</td>
<td>37/45 (0.82)</td>
</tr>
<tr>
<td>Mild /Mild CFTR genotype</td>
<td>1/20 (0.05)</td>
<td>4/25 (0.16)</td>
<td>5/45 (0.11)</td>
</tr>
<tr>
<td>Severe/unknown CFTR genotype</td>
<td>2/20 (0.10)</td>
<td>1/25 (0.04)</td>
<td>3/45 (0.07)</td>
</tr>
</tbody>
</table>
a FEV1% predicted > 97, 92, 90, 87 ... for the age of 15, 16, 17, 18 ... years, respectively. The severe pulmonary phenotype group included CF patients > 8 years of age with a FEV1% predicted < 80, 79, 78, 77 ... for the ages of 8, 9, 10, 11 ... years respectively [20]. For the remaining 25 patients it was not possible to classify their FEV1 pulmonary phenotype because of their young age, less than 8 years, or the lack of clinical data. For these reasons they were excluded from our study.

A total of 96 unrelated Caucasian healthy blood donors (46 males and 50 females; actual mean age 30.5 ± 10 sd), coming from the Transfusion Center of Children Hospital Burlo Garofolo, were recruited as controls. No spirometry data were available for the controls. Informed consent was obtained from the participants.

Genomic DNAs were extracted from peripheral whole blood using the EZ1 DNA extraction kit and EZ1 robotic device (Quiagen, Milan, Italy) following manufacturer’s instructions.

CFTR genotyping was performed using the INNO-LiPA CF kit (Innogenetics, Ghent, Belgium). If mutations were undetectable by using the INNO-LiPA CF kit, we screened the whole CFTR gene by DHPLC (Transgenomic, Omaha, NE, USA) and direct sequencing using the ABI 3130 Genetic Analyzer (Applied Biosystems).

2.3 ADRB2 SNPs genotyping

ADRB2 SNPs were genotyped by using Taqman SNPs genotyping assay (Applied Biosystems, Foster City, CA, USA): C_2084764_20 for the c.46G>A (p.Arg16Gly, rs1042713) SNP; C_2084765_20 for the c.79C>G (p.Gln27Glu, rs1042714) SNP; C_8950503_20 for the c.491C>T (p.Thr164Ile, rs1800888) SNP following manufacturers’ protocols. Real-time PCR were run using a Real-time PCR platform (ABI 7900 HT) and analyzed with Sequence Detection System 2.3 software (Applied Biosystems).

2.4 TNFA SNPs genotyping

A fragment containing the c.308G>A (rs1800629) and c.238G>A (rs361525) polymorphisms was amplified with PCR using the primers: Forward 5’–CCCTGAGGTGTCTGGTTTTC–3’; Reverse 5’–CTCAGTATGGTGAGAGTAATG – 3’. Fragments were then sequenced using the BigDye Terminator 3.1. Sequencing kit (Applied Biosystems, Foster City, California, USA).

The c.-851C>T (rs1799724) SNP was genotyped using allele-specific fluorescent probes TaqMan® SNP genotyping assay C_11918223_10 (Applied Biosystems, Foster City, CA, USA) using a Real-time PCR platform (ABI 7900 HT) and analyzed with Sequence Detection System 2.3 software (Applied Biosystems).

2.5 TGFB1 SNPs genotyping

A fragment containing the c.869C>T (rs1800470) and c.915G>C (rs1800471) polymorphisms was amplified with PCR using the primers: Forward 5’–CGAGGGAGCCAGCTTTGG–3’; Reverse 5’–CGCAGCTGGACAGGATCT-3’. Fragments were then sequenced using the BigDye Terminator 3.1. Sequencing kit (Applied Biosystems, Foster City, California, USA).

2.6 Statistical analysis

Hardy–Weinberg equilibrium was assessed by a χ² test with 1 degree of freedom. Allele and genotype frequencies were calculated by direct gene counting. Haplotypes were computed using Arlequin software (version 3.1) [21]. Differences in alleles, genotypes and haplotypes distribution were analyzed using the exact Fisher test. P-value < 0.05 were considered to be significant. Odds ratio (OR) and 95% confidence intervals (CI) were also calculated. Statistical analysis were performed using the open source R package (available at the www.r-project.org site).

3 Results

Clinical features of the 45 patients analyzed in this study are shown in table 1. 37 patients had two severe CFTR mutations: 15 F508del/F508del; 2 homozygous for other severe mutations; 16 compound heterozygotes F508del/other severe mutation; 4 compound heterozygotes for two different other severe mutations. 5 patients had a mild CFTR genotype: 4 compound heterozygotes F508del/mild mutation; 1 compound heterozygotes for two different mild mutations. The last 3 patients had one F508del allele whereas the second allele remained unknown.

Table 2 shows ADRB2 polymorphisms’ allele and genotype frequencies in the 45 CF patients and in 96 healthy controls. Allele and genotype frequencies were in Hardy–Weinberg equilibrium in both patients and controls.
Modifier genes in Cystic Fibrosis

Allele and genotype frequencies of c.46G>A (p.Arg16Gly) SNP are significantly different between CF patients and healthy controls. The A allele (Arg16) is more frequent in CF patients (48% - 43/90) than healthy controls (32% - 61/192). The A/A genotype is more frequent in CF patients (24%) than healthy controls (11%). These allele and genotype differences are statistically significant (p=0.009 and  p=0.04 respectively).

When stratifying CF patients according to FEV1-(mild or severe) phenotype a statistically significant difference (p=0.02) is observed: the A allele and the A/A genotype are more frequent in FEV1-mild CF patients (52% and 27% respectively) when compared to FEV1-severe CF subjects (10% and 0% respectively). Thus, the A allele associates with a protective effect toward severe pulmonary manifestations (OR = 0.10, 95% CI = 0.004 – 0.66). To further elucidate the effect of the c.46G>A SNP, we compared the genotype distribution between FEV1-mild patients and FEV1-severe patients using a dominant and a recessive model. The dominant genetic model compares individuals with one or two c.46G>A A alleles (AA and GA subjects), with the group carrying none of that allele (GG subjects). The recessive model compares the homozygous genotype AA with the combined heterozygous or homozygous genotypes for the G allele (GG and GA). We observe that the association between the A allele and FEV1-mild phenotype is compatible with a dominant model (AA+AG vs. GG, p= 0.019; OR=0.078, 95% CI= 0.001 – 0.91). This means that the c.46G>A A allele confers a protective effect toward severe pulmonary expression when the SNP is present in homozygosis and heterozygosis. Since patients’ CFTR genotype (mild or severe) may possibly influence the severity of the disease,
being a possible bias of our results, we then divided the CF patients according to their CFTR-(mild or severe) genotype and performed the genetic association analysis between c.46G>A SNP and FEV1 genotype only in patients characterized by a CFTR-severe genotype. A statistically significant difference (p=0.02) in allele and genotype frequencies was observed: the A allele and the A/A genotype are more frequent in CFTR-severe/FEV1-severe CF patients (53% and 25% respectively) than CFTR-severe/FEV1-mild CF patients (10% and 0% respectively). Thus, the A allele associates with a protective effect toward severe pulmonary manifestations (OR = 0.10, 95% CI = 0.004 – 0.66) and the association was compatible with a dominant model (AA+AG vs. GG, p= 0.013; OR=0.064, 95% CI= 0.001 – 0.79).

The statistical significance was maintained when adjusting the analysis for sex (data not shown).

Allele and genotype frequencies of the c.491C>T (p.Thr164Ile) SNP are not significantly different between CF patients and healthy controls. The T allele (Ile164) is very rare in the samples we analyzed (3%) and has been found only in heterozygous form. When dividing the CF patients according to FEV1-(mild or severe) phenotype a statistically significant difference (p=0.022) in allele frequencies was observed: the C allele (Thr164) is more frequent in FEV1-mild CF patients (100%) than FEV1-severe CF patients (80%) and is thus probably associated with a protective effect toward severe pulmonary expression. This difference remains significant even when stratifying the patients similarly for what done for c.46G>A SNP and comparing CFTR-severe genotype/FEV1-severe phenotype patients with CFTR-severe genotype/FEV1 mild phenotype patients (p = 0.03).

No significant differences were found in TNFA (supplementary table E-1) and TGFB1 (supplementary table E-2) SNPs when comparing CF patient and healthy controls, CF patients with a FEV1-severe and FEV1-mild phenotype and patients with CFTR-severe genotype/FEV1 severe phenotype and CFTR-severe genotype/FEV1-mild phenotype.

4 Discussion

The β2-adrenergic receptors (ADRB2) plays an important role in lung fluid regulation. Stimulation of the ADRB2 causes an increase in cAMP and protein kinase A (PKA) leading to an increase in the number of ENaCs (Epithelial Sodium Channels) on the apical portion of type-II alveolar cells, and the probability of an open ENaC [22]. In addition, ADRB2 stimulation leads to smooth muscle relaxation of the pulmonary lymphatics, which also play a key role in lung fluid balance, particularly in preventing excessive interstitial fluid accumulation [23].

In this study we found an association between c.46G>A ADRB2 SNP and protective effect toward severe pulmonary phenotype. Previous studies [24,25] suggested that subjects homozygous for Arg16 have an attenuated receptor function than subjects homozygous for Gly16. This might suggest that the genetic variation of ADRB2 at amino acid 16 influence lung fluid clearance. In Cystic Fibrosis, a decreased Cl permeability and increased sodium absorption account for the abnormal respiratory tract fluid. This causes the production of extremely thick and dehydrated mucus. Our findings show that the Arg16 homozygous genotype is associated with mild phenotype in CF patients. We may thus hypothesize that the presence of a Arg16 allele leads to an increased mucus hydration and a better mucus clearance. Our data are in line with previous results obtained by Büscher et al. [10] indicating that c.46G>A SNP influence the severity and progression of lung disease in CF patients.

In Büscher’ study [10], also the presence of p.Thr164Ile ADRB2 T allele (although very rare, <5%) was associated with markedly reduced pulmonary function in patients. Also in our samples T allele (Ile164) is very rare in both patients and controls, 2% and 3% respectively, and has been found only in heterozygous form; interestingly the C/T genotype was present only in CF patient with FEV1-severe phenotype.

Our findings, in agreement with Büscher’ study [10], seem to exclude a role of c.79C>G ADRB2 SNP in the modulation of Cystic Fibrosis pulmonary phenotype.

No significant differences were found in our study when analyzing TNFA SNPs (c.−851C>T, c.−308G>A, c.−238G>A and c.+691G ins/del). Thus our results seem to exclude a role for these SNPs in the modulation of Cystic Fibrosis pulmonary phenotype. This is in line, for the c.308G>A and c.238G>A SNPs, with a previous study of Yarden at al. [15]; conversely, in their study Yarden et al. [15] reported that the presence of the c.+691G del allele was likely to be associated with better pulmonary function and c.−851C homozygotes with worse pulmonary function. This findings is discordant with our results.

For TGFB1 c.869C>T and c.915G>C SNPs we didn’t evidence any statistically significant difference. Previously, also Brezova et al. [26] did not find association between TGFB1 polymorphisms and lung function in CF patients. Other studies have suggested that the T allele of c.869C>T TGFB1 SNP correlated with better lung function [20,27].
5 Conclusions

We are aware that the limited number of samples analyzed is a weak point our study, and this may have affected the power of our study to identify possible associations; for this reasons we believe that the involvement of ADRB2, TNFA and TGBF1 genes in CF should deserve further investigation.

Unfortunately the reduced number of sample is the drawback of including in the study only a well characterized cohort of patients enrolled in a limited center such as our Regional Cystic Fibrosis Centre of Trieste (Italy). However, it is important to notice that, despite the small number of samples, we have observed statistically significant differences in ADRB2 frequencies between patients. The c.46G>A SNP resulted to be associated with a protective effect toward severe pulmonary expression; this finding was also reinforced by the observation that the association was maintained also when patients were stratified for CFTR-severe genotype, thus avoiding the possible bias due to the presence of different CFTR genotypes.

If it’s true that the p values we reported are just slightly within the statistical significant threshold and applying the Bonferroni correction the statistical significance will probably be lost, nonetheless we think that finding these differences even in such a small number of samples, should be indicative of the involvement of the ADRB2 gene as modifier gene in Cystic Fibrosis pulmonary phenotype, and further study aimed to disclose the involvement of this gene in the disease, are desirable.

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References
