Should diffuse bronchiectasis still be considered a CFTR-related disorder?

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Received 5 December 2014; revised 27 February 2015; accepted 27 February 2015
Available online 18 March 2015

Abstract

Background: Although several comprehensive studies have evaluated the role of the CFTR gene in idiopathic diffuse bronchiectasis (DB), it remains controversial.

Methods: We analyzed the whole coding region of the CFTR gene, its flanking regions and the promoter in 47 DB patients and 47 controls. Available information about demographic, spirometric, radiological and microbiological data for the DB patients was collected. Unclassified CFTR variants were in vitro functionally assessed.

Results: CFTR variants were identified in 24 DB patients and in 27 controls. DB variants were reclassified based on the results of in silico predictive analyses, in vitro functional assays and data from epidemiological and literature databases. Except for the sweat test value, no clear genotype–phenotype correlation was observed.

Conclusions: DB should not be considered a classical autosomal recessive CFTR-RD. Moreover, although further investigations are necessary, we proposed a new class of “Non-Neutral Variants” whose impact on lung disease requires more studies.

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Keywords: Diffuse bronchiectasis; CFTR gene; Functional analysis; Variants classification

1. Introduction

The cystic fibrosis transmembrane conductance regulator gene (CFTR, MIM #602421) encodes a chloride channel that is expressed at the apical membrane of epithelia. More than 1900 changes have been identified in the CFTR gene. The combination of severe CFTR mutations on each allele usually leads to severe forms of cystic fibrosis (CF) (OMIM #219700). Other mutations that allow residual CFTR function (more than 10%) could result in mild or incomplete phenotypes (only one CF symptom), such as pancreatitis, congenital bilateral absence of deferens vases, or CFTR-Related Disorders (CFTR-RD). CFTR mutation implication in diffuse bronchiectasis of unknown etiology (DB) is more controversial. Increased frequency of the p.Phe508del allele (the most common CF mutation) was reported in French patients with bronchial hypersecretion [1].
in German patients with DB [2] and in French patients with DB and elevated sweat chloride concentration [3]. The presence of 

*CFTR* mutations has been correlated with abnormal nasal potential difference test results or intermediate sweat test results (40–60 mmol/L) in adults with DB [4]. These findings led to the recommendation of screening for the most frequent CF mutations in patients with DB. However, clinicians and biologists often wonder whether in-depth exploration of the *CFTR* gene is required in patients with DB but without any problem in other organs that are typically affected in CF. Indeed, the main challenge of large genetic analyses is the interpretation of unknown *CFTR* sequence variations and what kind of genetic counseling should be given to these patients and their families. This problem is heightened when rare or private variants are identified and when the molecular consequences are very mild or unknown. Moreover, *CFTR* has been extensively analyzed in many patients with DB and *CFTR* mutation heterozygosity was observed in 30 to 53% of them, while in the others no variation was detected [4–8].

Thus, determining whether DB is a real *CFTR*-RD remains relevant and challenging. The aims of this study were to determine whether the *CFTR* variations found in patients with DB may have deleterious effect on CFTR protein level and to identify potential genotype-phenotype correlations.

### 2. Materials and methods

#### 2.1. Patients

Adult patients with idiopathic DB (n = 47) were recruited at the respiratory department of the Montpellier University Hospital, France between 2007 and 2009 (CPP Ethic committee #2007.09.02 Sud Méditérranée III; DGS clinical trial number: 2007-A00216-47 Authorization N° DGS2007-0423; Authorization CNIL: 908041). All patients gave their written informed consent. The clinical characteristics and inclusion criteria are reported in Supplementary table 1 and the online Supplementary materials and methods. Controls were 47 healthy individuals whose DNA was already available in the laboratory.

#### 2.2. *CFTR* gene analysis

Genomic DNA was isolated from blood leukocytes using the Flexigene Kit (Qiagen®). Then, the coding regions (27 exons) and flanking intronic boundaries (20 to 200 bp), two deep intronic regions (IVS12 and IVS19) and 1000 bp in the promoter (upstream of transcription start site) of the *CFTR* gene were amplified by using the Single Condition Amplification/Internal Primers (SCAIP) technique as previously described [9]. Gene rearrangements were analyzed with the multiplex ligation-dependent probe amplification (MLPA) technique (Salsa MLPA Kit P091-B1 CFTR, MRC Holland, Amsterdam, Netherlands). All genotypes are reported in Supplementary table 2.

#### 2.3. Epidemiology

To determine the frequency of each sequence alteration, a study was performed in 47 healthy subjects (94 chromosomes) and 50 patients with CF (100 chromosomes) by denaturing high pressure liquid chromatography (DHPLC) or high resolution melt (HRM) (Light Cycler 480 Roche). If available, the range of minor allele frequencies (MAF) found in different studies in the general population (dbSNP) or patients with CF [10,11] was presented (Table 1).

#### 2.4. *In silico* analyses

*In silico* functional analyses were performed using the following online bioinformatics tools: Human Splicing Finder [12] for the intronic and exonic variants, Consite and TFSearch for the promoter variants, and SIFT and Polyphen2 for the exonic variants.

#### 2.5. Cell lines

The cell lines Beas2B (human pulmonary epithelial cells) and Cos7 (transformed African green monkey kidney fibroblasts), from ATCC (www.atcc.org), were maintained in culture as previously described [13,14].

#### 2.6. Constructs

The detailed description of the constructs used in this study is in the online Supplementary materials and methods.

#### 2.7. Reporter assay

Beas2B cells were seeded in 96-well plates (10,000 cells/well) and transfected as previously described [14].

#### 2.8. *In vivo* splicing assay

Beas2B cells were plated in 6-well plates (250,000 cells/well) and transiently transfected with pSPL3 minigenes carrying wild type (WT) or mutant *CFTR* sequences using the Polyfect® reagent (Qiagen). Total RNAs and PCR products were processed as previously described [15].

#### 2.9. Transcript level analysis

Beas2B and Cos7 cells were plated in 6-well plates (250,000 cells/well) and transiently transfected with pcDNA3.1-CFTR (WT or mutant) (1 μg) using Polyfect®. RNAs and PCR products were processed as previously described [15]. All the oligonucleotides used for this study are available on demand.

#### 2.10. Western blotting

Western blotting was performed as previously described using Cos7 cells plated in 6-well plates (200,000 cells/well) and anti-CFTR MM13-4 (#05-581 Millipore) or anti-Lamin A/C (#05-714 Millipore) primary antibodies [14]. Relative intensities of equal areas were compared using the Quantity One® quantification analysis software (Bio-Rad).
2.11. Statistical analyses

Details of statistical analyses are available in the online Supplementary materials and methods.

2.12. Mutation nomenclature

To be consistent with the international recommendations (http://www.hgvs.org/mutnomen/), CFTR variants were named according to CFTR numbering with the A of the ATG translation start codon indicated as +1. The usual nomenclature of each sequence variation was also included (GenBank NM_000492.3) (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Sequence variation</th>
<th>Allelic frequency</th>
<th>Distribution</th>
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#### 3. Results

3.1. CFTR gene alterations

Sequence analysis of the CFTR promoter, exons and flanking regions in patients with DB (n = 47) and healthy controls (n = 47) identified a total of 43 CFTR sequence changes in 24 DB patients and 36 CFTR changes in 27 controls (Table 1, Supplementary table 2). These changes corresponded to 24 different CFTR variants in DB patients and to 22 variants in controls and were mainly localized in exons and intronic boundaries (Fig. 1A). Based on the CFTR-France database [16] and the Cystic Fibrosis Mutation database (Toronto, CA, www.genet.sickkids.on.ca/app),

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[^n]: variable, dbSNP database.
[^n1]: 100, our laboratory cohort; n2 = 27177, [10]; n3 = 778, [11].
[^CNY]: Cystic Fibrosis Mutation Database (Toronto), CFTR2 database and CFTR-France database.

DB, diffuse bronchiectasis; CF, cystic fibrosis or severe; M, mild; UV, unclassified variant; NNV, no neutral variant; P, polymorphism; NA: not applicable; ND: not determined.
these variants were classified in four categories: severe mutations (CF; only detected in patients with DB), mild mutations (M), unclassified variants (UV) and rare polymorphisms (P) (Fig. 1B shows their occurrence in the two populations).

The 12 CFTR exonic variants found in patients with DB led to amino acid loss/changes in various domains of the CFTR protein (Fig. 1C). Three alterations were well-known CF mutations: p.Phe508del was found in two patients, whereas p.He507del and p.Ala46Asp were each detected in only one patient. The mild mutation p.Leu997Phe was found in two patients and one control, whereas p.Arg117His was associated with the c.[1210-12 T] allele in one patient. The c.[1210-34TG[12];1210-12 T[5]] was detected in one patient with DB (Supplementary table 2 and Fig. 1B). The UVs p.Gly576Ala and p.Arg668Cys were found in six patients with DB and two controls. They were usually reported to segregate as complex allele p.Gly576Ala,p.Arg668Cys[8,17]; however no familial segregation was available for the present study. Furthermore, p.Arg668Cys was found alone in one healthy control. Thus, these two UVs were evaluated together and separately in in vitro functional analyses.

Analysis of the CFTR promoter allowed to detect three variants in patients with DB (c.−1043dupT, c.−966 T > G, c.−812 T > G) and three in controls (c.−966 T > G, c.−730A > G, c.−274C > A). Six intronic variants were identified for the first time: c.489 + 23C > G and c.4242 + 71G > T in two patients with DB and c.580-90C > T, c.3873 + 69A > C, c.4137-116G > T, c.4137-142A > T in the control group (Supplementary table 2).

Rare polymorphisms, as defined by CFTR databases, were identified in 17 controls and in 14 DB patients (in green in Supplementary table 2). We also noted the association of several common polymorphisms. Specifically, homozygotes for the haplotype c.[1210-34TG[11];1210-12 T[7]]p.Met470Val were slightly more frequent in the DB group (29.8%) than in controls (23.4%). The frequent polymorphisms c.1767 + 152 T > A and c.2562 T > G (p.Thr854Thr) were very often associated (in 18/18 patients with DB and 22/23 controls). Similarly, the frequent variants c.2909-92G > A and c.3469-69C > A as well as c.4137-139G > A and c.4389G > A (p.Gln1463Gln) were repeatedly associated (19/19 patients with DB and 14/14 controls and 16/16 patients and 11/16 controls, respectively). Although it was not possible to determine whether these changes were on the same alleles, they often segregate together in CF families.

3.2. Epidemiological data

Epidemiological analyses were performed to determine the allelic frequencies of the UVs and polymorphisms identified in the DB cohort. To this aim, the control group (n = 94 chromosomes) and a CF cohort already available in our laboratory (n = 100 chromosomes), were analyzed using the Sanger Sequencing or HRM technique (Table 1). The results of this analysis and comparison, when possible, with the MAF range for the general population (dbSNP database) or the CF population (our laboratory cohort or previously published control cohorts [10,11]) indicated that the variants p.Arg75Gln, p.Gly576Ala and p.Arg668Cys were twice more present in the DB cohort than in the general population. Conversely, the variants c.−966 T > G and p.Glu528Glu were three times more frequent in the control group than in patients with DB. The c.3139 + 42 T > C intronic variant was identified in both DB and CF groups at the same frequency and was twice more frequent in the control group.

3.3. Functional analysis

To estimate the putative effect of the identified CFTR variants on CFTR transcripts/splicing variants and CFTR protein synthesis, in silico and in vitro functional analyses were then carried out. In silico analysis predicted that among the variants exclusively found in the control group only p.Ser1235Arg could induce functional alterations. However, a previous study excluded a major effect of this mutation on CFTR [18]. Therefore, variants only found in leucine binding domain; R-domain, regulator domain; N-ter, N-terminal extremity; C-ter, C-terminal extremity.

Fig. 1. CFTR variations found in patients with DB and healthy controls. A. Number of different CFTR variations and their localization in the CFTR locus (reference sequence GenBank NM.000492.3) in patients with DB (light gray) and controls (dark gray). B. Number of severe (CF) or mild mutations, unclassified variants or rare polymorphisms identified in patients with DB (light gray) or controls (dark gray) (some variants were identified several times and a patient could harbor 1 to 3 variants). C. Schematic showing the localization in the CFTR protein of the exonic variants identified in patients with DB. TMD, transmembrane domain; NBD, nucleotide binding domain; R-domain, regulator domain.
control group were not functionally tested. Moreover, two alterations found in patients with DB were also not studied because one was the subject of another study in our laboratory (c.[1210-34TG[11];1210-12 T[5]]) unpublished data) and the other had a well-characterized pathogenic effect (p.Ile507del).

Two known CF mutations were used as controls: p.Ala46Asp and p.Gly551Asp.

A reporter gene assay was used to assess the effect of the variants identified in the promoter region. The c.-966 T > G variant did not affect the promoter transcriptional activity, in agreement with the epidemiological data (frequency up to 0.562 in the dbSNP database). Conversely, the c.-1043dupT and c.-812 T > G alleles significantly reduced luciferase activity compared to the construct with the wild type (WT) promoter (Fig. 2A; upper panel). In silico analysis of these two polymorphic sequences predicted that the nucleotide change c.-812 T > G and the T duplication at position c.-1043 create a binding motif for E2F and FOX transcription factors, respectively. EMSA assays showed that incubation of nuclear protein extracts with the labeled mutant DNA probes yielded specific DNA–protein complexes that were abolished by an excess of unlabeled competitors (E2F or FOX DNA probes), but not of an unrelated oligonucleotide (NS) (Fig. 2A; lower panel).

The effect on CFTR pre-mRNA splicing of variants found in patients with DB was assessed using minigenes (Fig. 2B). Splicing was altered in the minigenes containing the exonic variants p.Gly576Ala, p.Arg668Cys and, to a lesser extent, p.Thr966Thr. For p.Gly576Ala, complete exon 13 skipping was observed, as previously described [19], although the bioinformatics analysis predicted no dramatic change. Indeed, the in silico analysis predicted that only the p.Arg668Cys missense variants could lead to a splicing defect. In agreement, when using the exon 14-WT and the c.2002C > T (p.Arg668Cys) minigenes, three transcript populations were identified, corresponding to complete exon 14 inclusion (upper band) or skipping (lower band), or inclusion of exon 14 lacking the first 248 nucleotides (middle band), as already described [20]. Compared to the exon 14-WT minigene, the expression of the transcript lacking the first 248 nucleotides was increased due to enhanced recognition of an alternative 3′ splice site located 248 nucleotides downstream of the native splice site (+23.64% of variation with the MaxEnt matrix) as a result of the strengthening of the poly-pyrimidine tract just before the canonical acceptor site. This splicing alteration causes a frameshift that might induce a stop codon 50 nucleotides downstream of the new junction. Moreover, for the variants p.Gly576Ala and p.Arg668Cys, the quantity of normal CFTR transcript was reduced by 57 and 37%, respectively, compared to the WT gene (Fig. 2C).

With the c.2898G > A (p.Thr966Thr) minigene, a lower migrating fragment was observed that corresponded to total skipping of exon 16 (Fig. 2B). The c.2620-26A > G variant was expected to create an alternative splice site, thus causing reduction of full-length mRNA synthesis. However, no splicing defect was detected with the in vitro splicing assay. Similarly, the other intronic and exonic variants did not affect the splicing process (Supplementary fig. 1).

Then, the effect of the exonic variants on CFTR mRNA production was tested. Transfection of full length CFTR containing the mutation p.Arg75Gln, p.Gly576Ala/p.Arg668Cys (alone and together), p.Val754Met or p.Thr966Thr induced a 30–50% decrease in CFTR mRNA level, compared to WT CFTR (Fig. 2C). The strongest effect was obtained upon transfection of the p.Phe508del (CF) and p.Glu528Glu (P) variants.

Finally, the effects on CFTR protein expression level and maturation was assessed based on the detection of immature (core-glycosylated, B band, ~150-kD) and mature (additional glycosylation in the Golgi, C band ~170–190 kDa) CFTR forms by western blotting (Fig. 2D, upper and middle panels). In cells transfected with the p.Phe508del mutant, CFTR was detected mainly as immature form due to ER retention, as previously reported [21]. Similar results were obtained for the p.Ala46Asp variant. Quantification of the blots indicated that the level of mature CFTR protein was decreased by 17%–26% in cells expressing the p.Arg75Gln, p.Arg117His, p.Gly576Ala, p.Arg668Cys (alone and together), p.Leu997Phe or p.Thr966Thr variant, and by 48% and 39% in cells expressing p.Glu528Glu and p.Val754Met, respectively (Fig. 2D, lower panel). Conversely, p.Gly551Asp (used here as control) and p.Tyr1424Tyr CFTR were normally processed.

3.4. CFTR variations and functional data

The molecular consequences of the CFTR variations ranged from neutral (polymorphism) to severe (CF mutation). The in vitro functional analyses confirmed the impact of known CF pathogenic mutations; however, for the variants with an intermediate impact, defining a threshold remained challenging. The functional data confirmed the deleterious effect of mild mutations, such as the p.[Gly576Ala;p.Arg668Cys] complex allele and the p.Leu997Phe missense mutation [22]. On the other hand, the functional data suggest that the exonic variants p.Arg75Gln, p.Glu528Glu, p.Val754Met and p.Thr966Thr and the promoter variants c.-1043dupT and c.-812 T > G, which are usually categorized as neutral variants based on epidemiological data, could be considered as in vitro non-neutral variants (NNV). Therefore, based on the functional assay results, the CFTR variants identified in this study were re-classified by adding the NNV category (corresponding to a not CF-causing mutation but with a damaging molecular impact) to the classical P, M, UV and CF categories (Table 1, last column). This new classification was then used to classify variants according to their molecular consequences in DDB and control group (Supplementary fig. 2) and to study genotype–phenotype correlations.

3.5. CFTR variants and clinical data of patients with DB

To identify genotype–phenotype correlations, patients with DB were divided in two groups (group 1: patients with one or two CF/M mutations or UVs, such as c.[1210-34TG[12];1210-12 T[5]] or c.[1210-34TG[11];1210-12 T[5]]; n = 13; group 2: patients with at least one NNV, P or no variation, n = 34) and their demographic data, sweat test results, DB status (age at diagnosis,
FVC and FEV₁ volume and percentage, chronic bacterial or fungal infections) and nutritional status (BMI) were compared by univariate analyses (Supplementary table 3). This analysis highlighted that group 1 was more likely to have sweat test values higher than 40 mmol/L (p-value = 0.04302) and to have higher sweat test values than group 2 (p-value = 0.04546). This confirmed that the c.[1210-34TG[12];1210-12 T[5]] allele could be considered as a mild mutation (also based on other publications and epidemiological studies). Moreover, in group 1, chronic *Aspergillus fumigatus* colonization was less frequent than in group...
2 (p-value = 0.0046). This analysis was then repeated by including the three patients with at least one NNV in group 1 (group 1, n1 = 16 versus group 2, n2 = 31, Supplementary table 4). Overall, results did not significantly change, but for an increased tendency of group 1 to have lower FVC (p-value = 0.0507) or FEV values (p-value = 0.06077) compared to group 2. Note that penalized methods were not used for multiple testing procedures to avoid a too conservative selection.

Finally, we studied the link between common variants associations (describe in the last section of 3.1) and DB clinical data. No correlation was found.

4. Discussion

To determine whether DB is a real CFTR-RD, we assessed whether the CFTR variations found in patients with DB may have deleterious effect on CFTR protein level and evaluated the existence of potential genotype–phenotype correlations. The findings should guide clinicians and biologists who wonder whether an exhaustive exploration of the CFTR gene in these diseases is required.

Our study shows that 24 patients with DB and 27 healthy controls harbored at least one CFTR variation (CF M, UV or rare polymorphisms) among the 47 individuals included in each cohort. This frequency (48.9%) is higher than in other comprehensive studies evaluating the role of CFTR gene alterations in patients with DB (Supplementary table 5), because they did not include rare polymorphisms or UV variants. Conversely, only seven patients with DB had one CF and/or M mutation (classified according to the CFTR databases). This lower frequency compared to previous studies could be explained by the exclusion of potential atypical CF patients (our patients had no nasal polyposis, rheumatologic, digestive or genital symptoms).

Many of the identified CFTR alterations have been previously described as UVs or rare polymorphisms because of the elevated frequency in the general population or the absence of functional analysis. To determine whether DB is a real CFTR-RD, the major challenge is the functional interpretation of these alterations, especially when rare or private.

The functional data suggest that the exonic variants p.Arg75Gln, p.Glu528Glu, p.Val754Met and p.Thr966Thr and the promoter variants c.-1043dupT and c.-812 T > G, which are usually categorized as neutral variants based on epidemiological data, might be considered NNV. Based on the proposed new classification of variants identified in this work (Table 1), 16 patients with DB and 14 controls (ns; p = 0.82) have at least one variant with no neutral in vitro effect. Concerning the DB population, this is not different from other studies except when they did not sequence all CFTR exonic regions [23] (p = 0.0257; Supplementary table 5). For the control group, this number seems to be higher than in the two other studies, but the difference is not significant [5] p = 0.21; [7] p = 0.14). In the next years, the real challenge will be to correctly and more precisely define an in vitro threshold to allow the classification of UV and NNV alterations. The use of a combination of functional biomarkers (for instance, ex vivo CFTR function, rectal transepithelial chloride and in vivo nasal potential difference measurement) will certainly be necessary [24].

Only 25% of all CFTR synthesized is processed to mature protein [21] and the presence of 10% of epithelial cells expressing wild type CFTR is sufficient to normalize ion transport in CF bronchial epithelial cells in vitro [25]. Moreover, we did not find any significant correlation between clinical presentation and genetic data, except for an elevated sweat test (which is a biochemical indicator). This is consistent with a previous study [4] and confirms that the sweat test is a useful biomarker of CFTR activity [26]. However, patients with CFTR mutations that were considered in this study as mild or NNV variants (more than 50% of mature CFTR protein produced) showed a slightly higher susceptibility towards lower respiratory capacity (Supplementary table 4). Other studies including larger cohort of DB patients, are needed to determine if these in vitro non-neutral variants may have impact on lung diseases, in association with common CFTR polymorphisms or variants in other genes.

It has recently proposed that a reduction of CFTR activity, due to environmental factors, could lead to a CF-like acquired disease. Indeed, increased sweat chloride concentration and reduced intestinal CFTR currents [27] and protein stability [28] have been reported in cigarette smokers with or without chronic obstructive pulmonary disease (COPD) [29]. CFTR protein function defects are probably a key factor in airway respiratory diseases; but CFTR gene alterations are probably not the main etiology. For instance, mutations in the SLC26A9 gene may prevent the SLC26A9-CFTR interaction and thus CFTR functional activation in CF-like lung disease [30]. Although the role of CFTR in lung diseases, other than CF, is conflicting, genome-wide association studies of COPD [31] and asthma [32] have not identified the CFTR gene as a susceptibility gene.

In conclusion, based on the genotype–phenotype correlation data in the DB cohort (Supplementary table 3) and the fact that the CFTR2 database considers that two CF/mild mutations should be found in trans in CFTR-RD, we think that DB should not be considered a classical autosomal recessive CFTR-RD. Indeed, the isolated DB phenotype cannot be exclusively explained by the CFTR genotypes. Nevertheless, complete screening of the CFTR gene could be useful to highlight variants of lung disease susceptibility or to classify patients with DB for future targeted therapy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2015.02.012.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

We thank Jean-Pierre Altieri, Caroline Guittard and Laure Raymond for their technical assistance. We also thank Caroline Raynal for her comments. This work was supported by grants from the CHU Montpellier (internal grants, 2007-A00216-47) and the French association Vaincre La Mucoviscidose.
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