Characterization of clinical and genetic spectrum of Chinese patients with cystic fibrosis

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Abstract

Background Cystic fibrosis (CF) is a rare autosomal recessive disorder caused by biallelic mutations in the CFTR gene. The clinical features and mutation spectrum of CF have been well characterized in Caucasians, while limited studies were conducted in Chinese patients.

Subjects and methods A total of 20 individuals from 19 families were diagnosed with CF in this study. We analyzed the clinical features and screened all coding exons of CFTR using a combination of Sanger sequencing and multiplex ligation-dependent probe amplification analysis.

Results The median age at onset was 9.3 years in our cohort, while the median age at diagnosis was 19 years. The respiratory system was most frequently affected in this study: all patients (100%, 19/19) presented with diffuse bronchiectasis and 61.1% (11/18) patients showed a forced expiratory volume in 1 s below 80% predicted. Six patients (6/20, 30%) exhibited allergic bronchopulmonary aspergillosis (ABPA). Only 4 (4/20, 20%) patients presented with pancreatic exocrine insufficiency (PI). Three adult male patients receiving examinations for congenital bilateral absence of vas deference (CBAVD) were all found with CBAVD. A total of 22 distinct mutations were detected in this cohort, with the variant p.G970D as the most common variant (12/38 alleles, 31.6%). Among these mutations, 5 (p.Y109D, p.I203F, p.D572E, p.R1066S and exon 2-3 deletion) were novel mutations, which expanded the mutation spectrum.

Conclusions Chinese CF patients showed different clinical features and a distinct CFTR mutation spectrum, compared with Caucasians. There is a significant diagnosis delay, suggesting the current underdiagnosis of CF in China.

Background

Cystic fibrosis (CF, OMIM # 219700) is a rare autosomal recessive disorder involving multiple organs including the respiratory tract, exocrine pancreas, male reproductive system and exocrine sweat glands. CF is caused by biallelic pathogenic mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), which is mostly expressed in epithelial cells functioning as a chloride channel protein. CF is mostly reported in Caucasians. According to the 2017 Cystic Fibrosis Foundation Patient Registry Annual Data Report (available at...
https://www.cff.org/Research/Researcher-Resources/Patient-Registry/), there were approximately 30,000 CF patients in the US, with about 1,000 newly identified individuals every year. The clinical manifestation and mutation spectrum have been well characterized in Caucasians. By comparison, only about 70 Chinese patients have been reported in literature. [1] It was considered that CF is extremely rare in Chinese population. However, more than half of these Chinese patients were reported in the recent four or five years, [2-6] suggesting that there might be a significant underestimated incidence of CF in China. With all available data from these studies, we recently made a preliminary summary of the phenotype and genotype spectra of CF in China. [1] Limited data showed that Chinese CF patients have a disposition to present atypical symptoms, mainly displaying pulmonary manifestations with fewer digestive symptoms, and showed a different CFTR mutation spectrum. Further studies are warranted to support these findings. In the present study, we collected the detailed clinical data and screened CFTR mutations in 20 additional Chinese patients, trying to describe the phenotype more accurately and expand the mutation spectrum.

Methods

Subjects

From March 2015 to August 2019, patients with suspected CF visiting Peking Union Medical College Hospital (PUMCH) were enrolled in this study. A total of 20 individuals from 19 families were diagnosed with CF according to the 2017 consensus guidelines for CF diagnosis. [7] The sweat chloride tests were conducted following the protocol described previously. [2] The diagnosis of allergic bronchopulmonary aspergillosis (ABPA) was achieved according to the consensus criteria. [8] For male patients over 18 years of age, examinations for congenital bilateral absence of vas deference (CBAVD) were recommended. The diagnosis of CBAVD was achieved based on these criteria: the presence of normal to slightly smaller testicles, non-palpable vas deferens, normal plasma levels of follicle-stimulating hormone, and reduced ejaculate volume (< 1 mL). [9] Given the inaccessibility of fecal pancreatic elastase-1 assay in China, patients with steatorrhea and positive fecal Sudan III staining were considered to have pancreatic insufficiency (PI). Informed consent was obtained from all the participants or their parents. All methods carried out in this study were
Mutation screening of CFTR

Genomic DNA were extracted from peripheral blood of the patient and their parents if available following standard methods. Direct sequencing for all 27 exons of CFTR and flanking sequences was performed as previously described. [2] Sequencing traces were analyzed using the CodonCode Aligner Software (CodonCode Aligner Corporation; Centerville, MA, USA) and variants nomenclature was described according to the transcript reference NM_00492.3. Multiplex ligation-dependent probe amplification (MLPA) analysis was conducted to screen potential gross rearrangements for those with only one or no mutation identified via exon sequencing, using a commercial MLPA kit (SALSA® P091-D1 CFTR, MRC-Holland; Amsterdam, The Netherlands). Real-time quantitative PCR (qPCR) of genome DNA was performed to verify the gross deletion detected by MLPA analysis as described before, [4] with the qPCR primer pairs CFTR-E2 (F 5’-TGTAAGAGATGAAGCCTGGTATT-3’ and R 5’-AGGCGCTGTCTGTATCCTTT-3’) and CFTR-E3 (F 5’-TGGAATAGAGAGCTGGCTTCA-3’ and R 5’-ACACCTATTCACCAGATTTCTG-3’). Subsequently, the deletion breakpoints were characterized by long-range PCR and Sanger sequencing.

Genotyping of modifier genes for CF lung disease

Thorough literature review was conducted to summarize those reported non-CFTR modifier genes which play important roles in CF lung disease severity. Specific primers for the lung disease-associated loci located in or near these genes were designed by the authors (Table S1). For patient 6 – 1 and 6 – 2, DNA fragments encompassing those loci were amplified and sent for further sequencing, trying to explain the difference in the severity of lung disease between these two siblings who carried the same CFTR mutations. To construct the associated haplotypes, the loci were also sequenced for their patents.

Results

In total, 20 patients (15 females and 5 males) from 19 Chinese families diagnosed with CF were recruited into this study. The age at symptom onset ranged from newborn to 30 y with a median of 9.3 y in this cohort. The median
age at diagnosis was 19 y ranging from 4 to 44y. Patient 3 had a sister died at 7 months for unknown reason and the elder sister of patients 16 died at 10 y due to repeated pulmonary infection suggesting a suspected history of CF. Patients 6 – 1 and 6 – 2 were sisters both suffering from CF. All patient denied consanguineous marriage in their families.

Clinical manifestations of CF patients in this cohort

The respiratory tract is the most frequently affected system in this cohort. Almost all patients had diffuse bronchiectasis (100%, 19/19) and 55% (11/20) had sinusitis. Six patients (6/20, 30%) exhibited ABPA. Pseudomonas aeruginosa (PA) was the most common pathogen observed in our patients with a frequent of 16/19 (84.2%). Only 2 patients showed negative results of sputum culture. Eleven out of 18 (11/18, 61.1%) patients who accepted pulmonary function testing had a forced expiratory volume in 1 s (FEV$_1$) below 80% predicted. There were 4 (4/20, 20%) patients diagnosed with PI+, including 3 patients (No. 3, 12 and 14) diagnosed by positive Sudan III staining and 1 with fatty diarrhea. An important comorbidity of CF was CBAVD. Three adult male patients accepted CBAVD examination and all of them were found with CBAVD (3/3, 100%). The other two male patients were too young or just reluctant to accept CBAVD examination. Sweat chloride testing results were obtained from 18 patients and all of them showed elevated sweat chloride concentrations (> 60 mmol/L). The average concentration was $140.3 \pm 48.4$ mmol/L ranging from 63.0–218.4 mmol/L. Detailed information of clinical manifestations of the patients were summarized in Table 1.

Table 1
Clinical manifestations and CFTR mutations for CF patients from this study.

| No. | sex | Age (y) | Weight/Height (kg/m) | Symptoms | Pancreatic insufficiency | Sputum culture | Pulmonary function test (FEV1% pred; FEV1/FVC) | Comorbidity | CFTR Variant 1 | CFTR Variant 2 |
|-----|-----|---------|----------------------|----------|-------------------------|--------------|-----------------------------------------------|-------------|---------------|---------------|
|    |     |         |                      |          |                         |              |                                               |             |               |               |
| 1   | F   | 22      | 10                   | 48/1.6   | none                    | 113.7        | chronic gastritis                             | none        | c.2909G > A | p.G970D       |
| 2   | F   | 11      | newborn              | 39/1.5   | none                    | 164.7        | ABPA                                          | none        | c.3068T > G | p.I1023X*     |
| 3   | F   | 14      | 12                   | 43/1.6   | sister died at 7 months | 183          | diarrhe a                                     | none        | c.2909G > A | p.G970D       |
| 4   | F   | 18      | 8                    | 52/1.6   | diarrhe a               | 199          | ABPA                                          | none        | c.3068T > G | p.I1023X*     |
| 5   | M   | 22      | 2                    | 65/1.3   | none                    | 151          | non                                          | none        | c.2909G > A | p.G970D       |
Germline CFTR variants detected in this study

All patients were screened for CFTR mutations by direct sequencing and MLPA analysis. Biallelic CFTR variants were detected in all the patients, except for patients 9 and 15, for which only one mutation was detected (Table 1). In total, 22 distinct variants were detected, including 10 missenses, 5 nonsenses, 3 indels, 3 splicing mutations and 1 gross deletion (Fig. 1). Among them, 5 mutations (c.325T > G, p.Y109D; c.607A > T, p.I203F; c.1716C > A, p.D572E; c.3196C > A, p.R1066S and exon 2–3 deletion) turned out to be novel mutations. In the 19
unrelated probands, the variant c.2909G > A (p.G970D) was the most common variant detected, with an allele frequency of 31.6% (12/38 alleles). The variants c.293A > G (p.Q98R) and c.1766 + 5G > T were also observed more than once. The most frequent mutation p.F508del in Caucasian CF patients was not observed in our cohort. Direct sequencing of all CFTR exons failed to identify any pathogenic mutations in patient 9. Subsequently, MLPA analysis of CFTR was performed and revealed a heterozygous gross deletion involving exons 2–3 (△E2-3) inherited from her mother (Fig. 2a). The maternal deletion of CFTR exons 2–3 was confirmed by qPCR (Fig. 2b). Subsequent breakpoint characterization showed that there was a deletion of 13.4 Kb encompassing exons 2 and 3 of CFTR (Fig. 2c). The complete removal of exons 2-3 resulted in a frameshift and pre-mature termination codon (p.S18Rfs*16). The second mutation from the paternal chromosome was not detected.

Different phenotype and modifier gene genotype in sibling 6 – 1 and 6 – 2

Even bearing the same homozygous p.G970D mutation (Fig. 3a), patients 6 – 1 and 6 – 2, as siblings, have very different lung disease severity and lung function. For patient 6 – 1, the symptoms began at age of 15 year. She suffered from more severe lung disease with diffuse bronchiectasis in bilateral lungs and continuous deterioration of pulmonary function (FEV1 = 38.4% Pred, FEV1/FVC = 60.32%). Patient 6 – 1 also experienced severe weight loss. By comparison, her elder sister, patient 6 – 2, was diagnosed with CF by genetic test at her 30’s. Patient 6 – 2 had a normal daily life and were in good nutrition. Chest computed tomography (CT) showed that she had focal bronchiectasis involving the right upper lobe (Fig. 3b), however, the result of pulmonary function test turned out to be quite normal (FEV1 = 91.6% Pred, FEV1/FVC = 73.32%). In addition, she had a negative result of sputum culture but a high level of sweat chloride.

To explain the different manifestations between the sisters, several reported CF lung disease-associated SNP located in or near 8 genes were selected by literature review (Table S1). Most of the genotypes of these SNPs were the same for the siblings. Interestingly, patient 6 – 1, who had more severe lung manifestations, carried the risk genotype codon 10 CC in TGFB1 (rs1800470), [10] while patient 6 – 2 had a CT genotype at the same locus. Moreover, the haplotype composed of the – 509 C, codon 10 T and intron 5 C allele (C-T-C haplotype), which was reported to be highly associated with increased lung function, [11] was observed in patient 6 – 2 but not in patient 6 – 1 (Fig. 3c). In addition, there were three other previously reported lung function-associated SNPs
showing different genotypes for the siblings. Patient 6 – 1 carried the risk alleles for all of these SNPs (A allele for rs3103933 in MUC4/MUC20, [12] C allele for rs9268905 [13] and rs9391781 [12] in HLA II), but patient 6 – 2 did not (Table S1).

**Discussion**

Understanding the phenotype and genotype features in a specific population helps to make correct diagnosis for CF patients. Numerous CF studies conducted in Caucasians has successfully characterized the clinical features and CFTR mutation spectrum of Caucasian CF patients. While, limited knowledge of the spectrum of CF phenotype and genotype in Chinese is available. Recently, a systematic review preliminarily summarized the clinical and genetic characteristics of 71 reported Chinese CF patients. [1] The authors also reported a significant under-recognition and diagnosis delay of CF in China, with the median age at onset of 1 y and median age at diagnosis of 10 y. [1] The present study collected 20 CF patients, of which the median age at onset and diagnosis were 9.3 y and 19 y, respectively. Most of the patients were firstly diagnosed with CF by sweat chloride tests or genetic screening. The reason for the much larger age at onset and diagnosis in this cohort might be due to selection bias, because all these patients were recruited from the Department of Pulmonary and Critical Care Medicine, a clinic mainly for adults in PUMCH, while, children with suspected CF often visit pediatricians. Despite that, a similar significant diagnosis delay of about 10 years was also observed in this study. Out of 20 patients, 15 (15/20, 75%) had experienced misdiagnosis, including bronchiectasis, diffusive pan-bronchiolitis, pulmonary tuberculosis and pneumonia.

As to the clinical manifestation, respiratory symptom was most commonly observed in our cohort, consistent with the results of previous studies performed in CF patients of Chinese origin. [1, 3, 5] Interestingly, about 30% patients in this study presented with ABPA, which was far more than that reported in Caucasians (7–9%). [8] Similarly, several recent studies [2, 3, 5] also reported higher rate of ABPA in Chinese patients, ranging from 37.5–57.1%. Furthermore, our recent review demonstrated that out of all 71 reported Chinese CF patients, 15 had developed ABPA, reaching a high rate of 21.1%. [1] Therefore, from the data in these independent studies, we would conclude that Chinese CF patients might be more prone to developing ABPA than Caucasians.

In addition, a lower frequency (4/20, 20%) of PI was found in this cohort, diagnosed by positive Sudan III staining and/or fatty diarrhea. In contrast, the same frequency was about 85% in Caucasians as reported in the 2017
Cystic Fibrosis Foundation Patient Registry Annual Data Report (available at https://www.cff.org/Research/Researcher-Resources/Patient-Registry/). Similar results can be found in several recent studies conducted in Chinese population. [1-3, 5] PI and PS status in CF are predisposed by the genotype at the CFTR locus. [14, 15] The most common mutation p.F508del detected in Caucasians and other mutations severely impairing CFTR function were reported to be associated with PI. [14] Thus, the substantial different CFTR mutation spectrum from Caucasians (see below) might be responsible, at least partially, for the lower frequency of PI observed in Chinese.

CBAVD is a one of the comorbidities commonly seen in Caucasian male CF patients. [16] As reported previously, [1] only one case was adult male who suffered from CBAVD. In this study, 3 of the 5 male patients received semen tests and/or urological ultrasound examination, and all turned out to have CBAVD. Thus, all the 4 Chinese male patients received CBAVD examination, including 3 reported in this cohort, showed manifestation of CBAVD. In other words, it seems like that CBAVD is also frequently presented in male CF patients of Chinese origin. Further studies with larger sample size or long-term follow-up data may make us more confident to draw the conclusion.

Three decades have passed away since the cystic fibrosis gene was firstly identified. [17] Extensive efforts have been directed towards the research of CFTR and this disease. Over 2000 mutations have been detected in CF patients across the world as recorded in the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca). The mutation spectrum has been well established in Caucasians. A distinct CFTR mutation spectrum in Chinese has been suggested in previous studies. [1, 2] In the present study, we detected 22 distinct mutations, with p.G970D as the most common mutation counting for 31.6% (12/38 alleles) of CFTR alleles. Mutations detected more than once also included two other variants, p.Q98R and c.1766 + 5G > T. The three mutations were all among the five most common mutations observed in Chinese. [1] In contrast, the most frequent mutation in Caucasians, p.F508del, was not observed; only one mutation, p.R553X detected in patient 11, was in the screening panel recommended by the American College of Medical Genetics, which consists of the 23 most common mutations in Caucasians. [18] Thus, the present study further confirmed the ethnic-specific mutation spectrum in patients of Chinese origin.

Five novel mutations were identified in this study. The variants p.Y109D and p.R1066S both were novel missense
amino acid changes at the same positions as reported pathogenic missense mutations, p.Y109N and p.R1066C, respectively. This is considered moderate evidence supporting their pathogenicity but cannot be assumed to be pathogenic. [19] The potential pathogenicity of the rest 2 novel variants, p.I203F and p.D572E, was unknown. Further functional study for these missense mutations are warranted to determine their effects on CFTR function. The deletion of CFTR exons 2–3 found in patient 9 was the fourth gross rearrangement reported in CF patients of Chinese origin. △E2-3 is very like to be a pathogenic mutation, because it was predicted to result in a pre-mature termination codon and no functional CFTR protein. A similar deletion of 21 Kb also removing exons 2–3 was found to be a frequent and severe CF mutation in Central and Eastern Europe, [20] supporting the pathogenicity. It is noteworthy that the other three gross deletion were also detected by our group. [2–4] There might be an underestimated detection rate of rearrangement in CFTR in Chinese patients, for which overlook and the inaccessibility of MLPA analysis should be responsible. Further emphasis should be placed on the necessity of MLPA analysis in routine CFTR mutation screening in Chinese patients, especially for those with only one or no mutations identified via direct sequencing of CFTR exons.

In our cohort, substantial differences in pulmonary function and radiological findings in the lung (Table 1 and Fig. 3b) were observed in the siblings 6 – 1 and 6 – 2, who carried the same homozygous p.G970D. It has been well known that the severity of CF lung disease varies dramatically even in patients with identical CFTR genotypes, in which genetic factors play an important role. [21–23] A review collected recent efforts aiming at identifying non-CFTR modifier genes for lung disease severity in cystic fibrosis. [21] Genotyping of reported modifier loci showed that patient 6 – 1 carried several risk alleles in the TGFB1, MUC4/MUC20 and HLA II genes but patient 6 – 2 did not; the rest loci turned out to be the same for the siblings (Table S1). The TGFB1 codon 10 CC genotype (rs1800470) detected in patient 6 – 1 was reported to be related to severe lung function with an odds ratio of about 2.2. [10] This genotype has been associated with elevated TFGβ1 expression level, which could be responsible for the severe lung function due to increased inflammation. [24] In the same gene, the protective C-T-C haplotype [11] was seen in patient 6 – 1 with very mild pulmonary manifestations. TGFB1 has multiple functions including regulating immune responses and inflammation process, and it has been associated with other lung diseases like chronic obstructive pulmonary disease and asthma. [25, 26] The MUC4/MUC20 genes encode proteins located on ciliated airway mucosal surface, which are involved in mucus secretion and
mucociliary clearance.[27] As to the risk alleles in HLA II, several recent studies reported the association of SNPs in HLA II or HLA class II pathway with severe lung disease in CF patients, using genome-wide association study or gene expression approaches [12, 13, 28]. However, what we should notice is that studies about these reported modifier genes often yielded conflicting results, due to small sample size and the lack of replications. For example, the purported modifier gene TGFB1 mentioned above failed to reach the genome-wide significance in another GWAS study. [13] Furthermore, regardless of the reliability of the associations, these genes can only explain limited proportion of the variability of lung disease. Finally, environmental factors like secondhand smoke exposure may also contributed to the variability of pulmonary phenotype observed in the patients.

There were some limitations in our research. First, all patients were recruited from a single center, Department of Pulmonary and Critical Care Medicine, PUMCH. Some of the patients came to our center due to suspected ABPA. So, the higher rate of ABPA in Chinese CF patients found in the present study should be carefully used because of potential selection bias. Second, due to cultural reasons, patients often refuse semen examination, making it difficult to screen CBAVD in Chinese CF patients. Studies with more patients accepting CBAVD screening are warranted to confirm the high frequency of CBAVD observed in this study.

Conclusion
In summary, the present study showed some different features of the clinical manifestations in Chinese CF patients compared with Caucasians, including more ABPA presence and a lower frequency of PI. The additional patients with CBAVD reported in this study indicates a probable same high frequency of CBAVD in male CF patients of Chinese origin, which need further studies to confirm. We also observed a distinct CFTR mutation spectrum in Chinese, with p.G970D as the most frequent mutation. Five novel mutations were reported, which expanded the mutation spectrum. There is still significant diagnosis delay and under-recognition of CF in China. Better characterization of the phenotype and genotype spectra and increasing physician’s awareness of CF will help to improve the current situation in China.

Abbreviations
ABPA: Allergic Bronchopulmonary Aspergillosis
CBAVD: Congenital Bilateral Absence of Vas Deference
CF: Cystic Fibrosis
CFTR: Cystic fibrosis transmembrane conductance
CT: Computed Tomography
FEV₁: Forced expiratory volume in 1 second
FVC: Forced vital capacity
MLPA: Multiplex ligation-dependent probe amplification
PI: Pancreatic Insufficiency
PUMCH: Peking Union Medical College Hospital
qPCR: Real-time quantitative PCR

Declarations

Ethics approval and consent to participate
The study was approved by the Institutional Review Board committee at Peking Union Medical College Hospital (PUMCH) and all participants signed written informed consent.

Consent for publication
Not applicable.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional file.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
YL, XT, K-FX, and XZ designed the study. KL, YL, MX, X-YZ, QZ, JS performed the molecular genetic testing, mutation analysis and minigene assays. XT, WX, KC and K-FX collected patients’ samples and clinical information. KL, YL and XT drafted the manuscript. All authors read and approved the final manuscript.
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Figures

Figure 1

CFTR mutations detected in this CF cohort. Different mutation types are shown in indicated colors in the upper panel; the gross deletion of exons 2-3 is indicated with a green solid line in the lower panel. The novel mutations identified in the current study are highlighted in green.
Figure 2

CFTR exons 2-3 deletion found in patient 9. a Exons 2-3 deletion in patient 9 and her mother was detected by MLPA. The X-axis shows the genomic positions of the probes and the Y-axis represents the signal ratio compared with control. The red arrow represents the heterozygous deletion. b Quantitative real-time PCR confirmed the CFTR exons 2-3 deletion in the patient, which was inherited from her mother. Experiments were performed in triplicates. c Sanger sequencing revealed a deletion of about 13.4 Kb encompassing CFTR exons 2-3. The breakpoints are shown using red line.
Different lung manifestations and potential modifier loci in patients 6-1 and 6-2. a As siblings, patients 6-1 and 6-2 are both homozygous for p.G970D, and the parent both are heterozygous carriers. b Chest CT showed that patients 6-1 presented with diffuse bronchiectasis in bilateral lungs, while patient 6-2 only had focal bronchiectasis in the right upper lobe. c Genotypes of the three lung disease severity-associated SNPs (rs1800469, ‘-509’, rs1982073, ‘codon 10’, and rs8179181, ‘intron 5’) in the TGFB1 gene. Patient 6-1 carried the risk genotype CC at codon 10 indicated in red, and patient 6-2 carried the protective C-T-C haplotype shown in green.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
Table S1.docx