Diagnostic contribution of molecular analysis of the cystic fibrosis transmembrane conductance regulator gene in patients suspected of having mild or atypical cystic fibrosis*

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Abstract

Objective: To evaluate the diagnostic contribution of molecular analysis of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in patients suspected of having mild or atypical cystic fibrosis (CF).

Methods: This was a cross-sectional study involving adolescents and adults aged ≥ 14 years. Volunteers underwent clinical, laboratory, and radiological evaluation, as well as spirometry, sputum microbiology, liver ultrasound, sweat tests, and molecular analysis of the CFTR gene. We then divided the patients into three groups by the number of mutations identified (none, one, and two or more) and compared those groups in terms of their characteristics.

Results: We evaluated 37 patients with phenotypic findings of CF, with or without sweat test confirmation. The mean age of the patients was 32.5 ± 13.6 years, and females predominated (75.7%). The molecular analysis contributed to the definitive diagnosis of CF in 3 patients (8.1%), all of whom had at least two mutations. There were 7 patients (18.9%) with only one mutation and 26 patients (70.3%) with no mutations. None of the clinical characteristics evaluated was found to be associated with the genetic diagnosis. The most common mutation was p.F508del, which was found in 5 patients. The combination of p.V232D and p.F508del was found in 2 patients. Other mutations identified were p.A559T, p.D1152H, p.T1057A, p.I148T, p.V754M, p.P1290P, p.R1066H, and p.T351S.

Conclusions: The molecular analysis of the CFTR gene coding region showed a limited contribution to the diagnostic investigation of patients suspected of having mild or atypical CF. In addition, there were no associations between the clinical characteristics and the genetic diagnosis.

Keywords: Cystic fibrosis/diagnosis; Cystic fibrosis/genetics; Cystic fibrosis transmembrane conductance regulator.

* Study carried out at the Hospital de Clínicas de Porto Alegre – HCPA, Porto Alegre Hospital de Clínicas – Universidade Federal do Rio Grande do Sul – UFRGS, Federal University of Rio Grande do Sul – Porto Alegre, Brazil.

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Financial support: This study received financial support from the Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (FIP-HPA, Research Incentive Fund of the Porto Alegre Hospital de Clínicas) and, in the form of donations, from United Medical and Roche.

Submitted: 23 October 2012. Accepted, after review: 4 March 2013.
Methods

This was a single-center cross-sectional study. We sought to evaluate the contribution of molecular analysis of the \textit{CFTR} gene to the diagnostic investigation of adolescents and adults suspected of having mild CF (diagnosis confirmed by sweat test results and mild lung disease without pancreatic insufficiency) or atypical CF (clinical phenotype consistent with CF without sweat test confirmation).

The study protocol was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (HCPA, Porto Alegre Hospital de Clínicas; Protocol no. 08-549). All of the participants gave written informed consent. Although we received donations from private companies (Roche and United Medical), those sponsors had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

The study population consisted of patients selected from among those treated at the HCPA Department of Pulmonology outpatient clinic for adolescents and adults with CF, located in the city of Porto Alegre, Brazil. We included individuals $\geq 14$ years of age with phenotypic findings of CF (digital clubbing, bronchiectasis, chronic infection with typical bacteria, and exocrine pancreatic insufficiency), with or without sweat test confirmation.

We excluded patients in whom the phenotypic findings were attributable to other diseases, such as ciliary dyskinesia and primary immunodeficiency; pregnant patients; and patients who declined to participate in the study or who declined to give written informed consent.

Volunteers were selected from among patients attending the abovementioned outpatient clinic for routine medical visits between 2009 and 2010. The selected patients were interviewed by one of the members of our study group. The interviewer used a data collection form in order to identify mutations in the \textit{CFTR} gene.\cite{6,7} Although the method is highly specific for CF, its sensitivity is low. This is due to the fact that the number of known mutations is quite large (over 1,900), although only a minority is included in commercially available panels.\cite{2,8} Despite its shortcomings, analysis of mutations in the \textit{CFTR} gene has been increasingly used as a diagnostic method for CF.

The identification of two CF-causing mutations confirms the diagnosis of atypical CF. The identification of one mutation is inconclusive. The absence of mutations means that the diagnosis of CF is highly unlikely, and differential diagnoses, such as ciliary dyskinesia and immunodeficiency, should be considered.\cite{9}

The objective of the present study was to evaluate the contribution of molecular analysis of the \textit{CFTR} gene coding region to the diagnostic investigation of adolescents and adults suspected of having mild or atypical CF, as well as to compare the characteristics of three groups of patients, divided according to the number of \textit{CFTR} gene mutations identified (none, one, or two or more, the last of the three confirming the diagnosis of CF).
The evaluation of chronic sinus disease was complemented by CT of the sinuses.

The Shwachman–Kulczycki score\textsuperscript{12} was used in order to assess overall disease severity. This clinical scoring system is based on four different aspects (general activity, physical examination, nutrition, and chest X-ray findings). Each is scored on a 5–25 point scale, a better performance translating to a higher score. A final score of 100 points indicates optimal clinical condition.

We reviewed the bacteriological tests performed on sputum samples collected during routine clinical visits in the last 12 months. Identification of mucoid and nonmucoid \textit{Pseudomonas aeruginosa}, methicillin-sensitive and methicillin-resistant \textit{Staphylococcus aureus}, \textit{Burkholderia cepacia}, and \textit{Haemophilus influenzae} was recorded when they were identified at least twice in sputum samples collected during routine clinical visits at intervals longer than 30 days.

Spirometry was performed in the HCPA Department of Pulmonology with a MasterScreen spirometer and the program v4.31 (Jaeger, Würzburg, Germany) in accordance with the Brazilian Thoracic Association technical acceptability criteria.\textsuperscript{13} We recorded FVC, FEV \textsubscript{1}, and the FEV \textsubscript{1}/FVC ratio. Three acceptable maneuvers were performed, the values obtained on the best test being recorded. All parameters were expressed as a percentage of the predicted values for age, height, and gender.\textsuperscript{14}

For the diagnosis of liver disease in CF, we used the ultrasound scoring system developed by Williams.\textsuperscript{15} The scoring system is based on three ultrasound features: liver parenchyma—normal (1 point), coarse (2 points), or irregular (3 points); liver edge—smooth (1 point) or nodular (3 points); and periportal fibrosis—absent (1 point), moderate (2 points), or severe (3 points). A final score of 3 is consistent with normal liver, higher scores being suggestive of progressive liver disease. A score of 8–9 is consistent with established liver cirrhosis. This ultrasound scoring system is routinely used at the HCPA. The sonographer at the HCPA interpreted and scored the findings of routine ultrasound examinations.

Molecular analysis was performed in the HCPA Department of Genetics. A sample of 5 mL of peripheral blood was collected for DNA extraction. The \textit{CFTR} gene coding region was amplified by polymerase chain reaction. Sequencing of the fragments was performed using the Big \textsuperscript{TM} Dye Terminator v3.1 Ready Reaction Cycle Sequencing (Applied Biosystems, Foster City, CA, USA) and fluorescent dye terminator sequencing on a genetic analyzer (ABI PRISM \textsuperscript{TM} 3130xl, Applied Biosystems). The analyses were performed with the DNA Sequencing Software v5.2 (Applied Biosystems). All 27 \textit{CFTR} gene exons were sequenced. The flanking regions and exon-intron organization were also included in the sequencing. The intron 8 polymorphism was also analyzed.

For the statistical analysis, the data were processed and analyzed with the aid of the IBM SPSS Statistics software package, version 18.0 (IBM Corporation, Armonk, NY, USA).

Descriptive analysis was performed, quantitative data being presented as mean ± SD or median (interquartile range). Qualitative data were expressed as n (%).

In the statistical analysis, the patients were divided into three groups by the number of known CF-causing mutations identified (two or more, one, or none).

The three groups were compared by one-way ANOVA for continuous variables with normal distribution; by the Kruskal-Wallis test for ordinal variables or for continuous variables without normal distribution; and by the chi-square test for categorical variables, Yates’ correction or Fisher’s exact test being used when necessary.

The level of significance was set at 5%, and all tests were two-tailed.

Results

Between May of 2009 and November of 2010, we evaluated 37 patients with phenotypic findings of CF, with or without sweat test confirmation. None of the selected patients declined to participate in the study. All of the patients completed the study protocol.

Table 1 shows the general characteristics of the patients. Females predominated in the study sample, which consisted of 28 females (75.7%) and 9 males (24.3%). The mean age was 32.5 ± 13.6 years (range, 14–67 years). Most of the patients were White (only 1 patient was non-White), and 16 patients (43.2%) reported having relatives (first- and second-degree relatives) with a confirmed diagnosis of CF. Five patients (13.5%) had been diagnosed with exocrine pancreatic insufficiency. The mean BMI was 22.4 ± 3.85 kg/m\textsuperscript{2} (range, 15.3–30.0 kg/m\textsuperscript{2}). Digital clubbing was found
The mutations identified by molecular analysis are presented in Table 3. The most common mutation was p.F508del, which was found in 5 patients. The combination of p.V232D and p.F508del was found in 2 patients. In 1 patient, we identified three mutations (p.T351S and p.F508del in the first allele, and p.P1290P in the second allele). Other mutations identified were p.A559T, p.D1152H, p.T1057A, p.I148T, p.V754M, p.P1290P, and p.R1066H.

Discussion

This was a cross-sectional study evaluating patients who presented with phenotypic findings consistent with CF and who were referred to a referral outpatient clinic for the diagnosis and treatment of CF in adolescents and adults. The molecular analysis of the CFTR gene contributed to the definitive diagnosis of CF in 4 (10.8%) of the 37 patients evaluated. In 7 patients (18.9%), only one CF-causing mutation was identified, whereas, in 26 patients (70.3%), no mutations were identified. None of the clinical characteristics evaluated were found to be associated with the genetic diagnosis. The presence of exocrine pancreatic insufficiency tended to be more common in patients with a confirmed genetic diagnosis of CF, although the difference was not statistically significant. Of the patients with a genetic diagnosis of CF, only 1 had abnormal sweat test results. However, of the 7 patients in whom one mutation was identified, 3 had abnormal sweat test results, as did 10 of the 26 patients in whom no mutations were identified.

Clinical characteristics and reasons for referral included the presence of chronic lung disease and chronic sinus disease, bronchopulmonary colonization with germs suggestive of CF, digital clubbing, family history of CF, gastrointestinal abnormalities, and nutritional abnormalities. All of the patients had previously undergone sweat tests at least twice.

In the present study, of the 4 patients with a genetic diagnosis of CF, 1 can be characterized as having mild, classic CF because that patient had late manifestations of the disease and abnormal sweat test results, whereas the remaining 3 can be characterized as having atypical CF because they presented with borderline or normal sweat electrolyte values.

The development of molecular analysis techniques allowed the identification of more than 21.6% of the patients. Only 3 patients (8.1%) met diagnostic criteria for diabetes mellitus, and 13 (35.1%) met diagnostic criteria for chronic sinus disease. Regarding sputum microbiology, the most commonly isolated bacterium was P. aeruginosa (43.2%), followed by S. aureus (35.1%). H. influenzae was identified in 7 patients (18.9%), and B. cepacia was isolated in 5.4% of the patients. The mean FEV₁ was 68.31 ± 25.40% of predicted, the mean FVC was 80.0 ± 19.4% of predicted, and the mean FEV₁/FVC ratio was 71.68 ± 17.34%. Sweat electrolytes were evaluated in two samples, the mean weight of the first sample being 242.00 ± 85.99 mg and the mean weight of the second sample being 228.50 ± 95.00 mg. Chloride concentrations in the first and second samples were, respectively, 54 ± 26.14 mEq/L and 51.76 ± 23.37 mEq/L.
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1,900 mutations in the *CFTR* gene, as can be seen in the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/cftr). Genetic mutations have been classified as severe (class I-III mutations) or mild (class IV-VI mutations) on the basis of the molecular defect of the CFTR protein. In individuals with class I mutations, the CFTR protein is not synthesized; in those with class II mutations, the CFTR protein is improperly processed in the endoplasmic reticulum; in those with class III mutations, the CFTR protein is improperly regulated; in those with class IV mutations, the CFTR protein shows defective conductance; in those with class V mutations, CFTR protein production is defective; and in those with class VI mutations, there is rapid degradation of the CFTR protein. Nonclassic forms of CF have been associated with mutations that reduce but do not eliminate the function of the CFTR protein. In general, individuals

Table 1 - General characteristics of the patients suspected of having mild or atypical cystic fibrosis.

| Variables | Patients (n = 37) |
|-----------|------------------|
| Gender    |                  |
| Male      | 9 (24.3)         |
| Female    | 28 (75.7)        |
| Age, years | 32.5 ± 13.6    |
| Race      |                  |
| White     | 36 (97.3)        |
| Non-White | 1 (2.7)          |
| BMI, kg/m² | 22.4 ± 3.85    |
| Family history of CF | 16 (43.2) |
| Exocrine pancreatic insufficiency | 5 (13.5) |
| Digital clubbing | 8 (21.6) |
| Diabetes mellitus | 3 (8.1) |
| Chronic sinus disease | 13 (35.1) |
| Sputum bacteriology |           |
| *Pseudomonas aeruginosa* | 16 (43.2) |
| Mucoid *P. aeruginosa* | 7 (18.9) |
| Nonmucoid *P. aeruginosa* | 10 (27.0) |
| *Staphylococcus aureus* | 13 (35.1) |
| Methicillin-sensitive *S. aureus* | 12 (32.4) |
| Methicillin-resistant *S. aureus* | 2 (5.4) |
| *Burkholderia cepacia* | 2 (5.4) |
| *Haemophilus influenzae* | 7 (18.9) |
| FEV₁, L | 2.20 ± 0.95 |
| FEV₁, % of predicted | 68.31 ± 25.4 |
| FVC, L | 2.99 ± 0.90 |
| FVC, % of predicted | 80.0 ± 19.4 |
| FEV₁/FVC | 71.68 ± 17.34 |
| Sweat test |             |
| Sample 1 |                  |
| Sodium, mEq/L | 57.57 ± 27.34 |
| Chloride, mEq/L | 54.00 ± 26.14 |
| Sample weight, mg | 242.34 ± 85.99 |
| Sample 2 |                  |
| Sodium, mEq/L | 57.19 ± 22.12 |
| Chloride, mEq/L | 51.76 ± 23.37 |
| Sample weight, mg | 228.50 ± 95.00 |

CF: cystic fibrosis; and BMI: body mass index. Values expressed as n (%), except where otherwise indicated. Values expressed as mean ± SD.
Table 2 - Comparison of three groups of patients (those in whom two or more mutations were identified, those in whom one mutation was identified, and those in whom no mutations were identified) in terms of their characteristics.\(^a\)

| Variables                          | No. of mutations | p*  |
|------------------------------------|------------------|-----|
|                                    | ≥ 2 (n = 4) | 1 (n = 7) | 0 (n = 26) |
| **Gender**                         |               |       |       |
| Male                               | 1 (25.0)     | 0 (0.0) | 8 (30.8) | 0.242 |
| Female                             | 3 (75.0)     | 7 (100.0) | 18 (69.2) |
| **Age, years**                     | 30.80 ± 10.90 | 30.00 ± 9.95 | 33.00 ± 14.43 | 0.851 |
| **Race**                           |               |       |       |
| White                              | 4 (100.0)     | 7 (100.0) | 25 (96.2) | 0.805 |
| Non-White                          | 0 (0.0)       | 0 (0.0) | 1 (3.8) |
| **BMI, kg/m\(^2\)**                | 22.9 ± 3.7    | 23.3 ± 2.3 | 22.2 ± 4.3 | 0.801 |
| **Family history of CF**           | 3 (75.0)     | 2 (28.6) | 11 (42.3) | 0.322 |
| **Exocrine pancreatic insufficiency** | 2 (50.0) | 0 (0.0) | 3 (11.5) | 0.570 |
| **Digital clubbing**               | 0 (0.0)       | 0 (0.0) | 8 (30.8) | 0.115 |
| **OGTT**                           |               |       |       |
| Glucose intolerance                | 0 (0.0)       | 2 (28.6) | 4 (15.4) | 0.624 |
| Diabetes                           | 0 (0.0)       | 1 (14.3) | 2 (7.7) |
| **Chronic sinus disease**          | 2 (50.0)     | 4 (57.1) | 7 (26.9) | 0.266 |
| **Sputum bacteriology**            |               |       |       |
| Pseudomonas aeruginosa             | 1 (25.0)     | 5 (71.4) | 10 (38.5) | 0.218 |
| Mucoid P. aeruginosa               | 1 (25.0)     | 3 (42.9) | 3 (11.5) | 0.162 |
| Non-mucoid P. aeruginosa           | 0 (0.0)       | 3 (42.9) | 7 (26.9) | 0.306 |
| Staphylococcus aureus              | 3 (75.0)     | 3 (42.9) | 7 (26.9) | 0.154 |
| Methicillin-sensitive S. aureus    | 2 (50.0)     | 3 (42.9) | 7 (26.9) | 0.530 |
| Methicillin-resistant S. aureus    | 1 (25.0)     | 1 (14.3) | 0 (0.0) | 0.062 |
| Burkholderia cepacia               | 0 (0.0)       | 0 (0.0) | 2 (7.7) | 0.639 |
| Haemophilus influenzae             | 0 (0.0)       | 1 (14.3) | 6 (23.1) |
| **Shwachman-Kulczycki score**      | 80 (32.5)     | 75 (20.0) | 70 (31.3) | 0.748 |
| Liver score\(^c\)                  | 3 (0)         | 3 (0)    | 3 (0)    | 0.399 |
| Presence of bronchiectasis         | 2 (50.0)     | 6 (85.7) | 19 (73.1) | 0.439 |
| **FEV\(_1\), L**                  | 3.01 ± 0.71   | 2.44 ± 0.70 | 2.20 ± 1.13 | 0.317 |
| **FEV\(_1\), % of predicted**     | 89.7 ± 26.0   | 72.2 ± 14.7 | 68.2 ± 29.0 | 0.338 |
| **FVC, L**                         | 3.7 ± 0.4     | 3.2 ± 0.5 | 2.9 ± 1.2 | 0.359 |
| **FVC, % of predicted**            | 95.5 ± 16.1   | 81.7 ± 6.2 | 78.5 ± 23.2 | 0.320 |
| **FEV\(_1\)/FVC**                 | 80.3 ± 12.5   | 74.8 ± 14.1 | 73.7 ± 19.0 | 0.785 |
| **Sweat test**                     |               |       |       |
| Sample 1                           |               |       |       |
| Sodium, mEq/L                      | 56.8 ± 20.3   | 59.14 ± 27.3 | 56.5 ± 28.0 | 0.973 |
| Chloride, mEq/L                    | 53.8 ± 15.7   | 61.3 ± 23.10 | 51.34 ± 27.7 | 0.674 |
| Sample 2                           |               |       |       |
| Sodium, mEq/L                      | 53.3 ± 10.3   | 56.4 ± 27.8 | 58.1 ± 22.2 | 0.941 |
| Chloride, mEq/L                    | 49.7 ± 10.50  | 52.2 ± 26.0 | 52.2 ± 24.4 | 0.985 |
| **Sweat test result**              |               |       |       |
| Normal                             | 1 (25.0)     | 1 (14.3) | 12 (46.2) | 0.306 |
| Borderline                         | 2 (50.0)     | 3 (42.9) | 4 (15.4) |
| Abnormal                           | 1 (25.0)     | 3 (42.9) | 10 (38.5) |

\(^a\)Values expressed as n (%), except where otherwise indicated. \(^b\)Values expressed as mean ± SD. \(^c\)Values expressed as median (interquartile range). \(^d\)One-way ANOVA for continuous variables with normal distribution or Kruskal-Wallis test (ordinal variables or continuous variables without normal distribution); chi-square test (categorical variables), Yates' correction or Fisher's exact test being used when necessary.
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The low sensitivity of the method is generally due to the large number of known mutations and to the fact that commercially available panels include only a minority of those mutations. It should be taken into consideration that our study sample consisted of patients selected from among those referred to our facility (a referral center for CF) because of the difficulty in establishing a diagnosis.

One group of authors reported 6 cases of CF diagnosed in adulthood, with suppurative lung disease, preserved pancreatic function, and only one mutation identified. The authors concluded that the diagnostic spectrum of patients investigated in adulthood is different from that of those diagnosed in childhood.

One study sought to determine whether changes in the CFTR protein were responsible for the full spectrum of variant CF phenotypes. The authors analyzed the CFTR gene in 74 patients with nonclassic CF. Of those patients, 29 had two mutations in the CFTR gene, 15 had one mutation, and 30 had no mutations. As occurred in our study, there were no differences among patients with two, one, or no mutations in terms of their clinical characteristics and sweat chloride concentrations. The authors concluded that factors other than mutations in the CFTR gene can produce phenotypes clinically indistinguishable from nonclassic CF caused by CFTR protein dysfunction.

In 2004, one group of authors published a study evaluating the clinical characteristics and diagnostic parameters of patients diagnosed with CF in adulthood. Of 1,051 individuals with...
CF, 73 (7%) were diagnosed with the disease in adulthood. Of the 46 patients diagnosed with CF after 1990, 30 (65%) were diagnosed by sweat testing, 15 (33%) were diagnosed by mutation analysis, and 31 (67%) were diagnosed by a combination of both methods. Nasal potential difference measurements alone confirmed the diagnosis in the remaining 15 patients (33%). The authors concluded that patients with CF presenting in adulthood generally present with pancreatic insufficiency, inconclusive sweat test results, and a high prevalence of mutations that are not seen in childhood.

The present study has some limitations. The major limitation of the present study is the small sample size (in particular, the small number of cases with a confirmed diagnosis), which precludes the generalization of the results of the comparison among the three groups in terms of their clinical characteristics and limits the external validity of the study. Another limitation is that we did not perform nasal potential difference measurements, because the method was not available for use at our hospital during the study period.

The clinical implication of the present study is that it demonstrates the difficulty that specialists face in diagnosing CF in adolescents and adults clinically suspected of having the disease. Diagnostic investigation requires diagnostic tests other than the sweat test. The sweat test is not specific enough to rule out CF in such patients.

In conclusion, in this population of patients who had findings consistent with CF and who were referred to a referral program for adolescents and adults with CF for evaluation, the molecular analysis of the CFTR gene coding region contributed to the definitive diagnosis of CF in 3 patients (8.1%) and allowed the identification of mutations in 1 patient with mild CF, previously confirmed by sweat testing. Patients with abnormal sweat electrolytes and with no identified mutations probably have alterations that are currently unknown. None of the clinical characteristics evaluated were found to be associated with the genetic diagnosis.

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