Turk Thorac J 2021; 22(6): 426-431 DOI: 10.5152/TurkThoracJ.2021.20282

Orginal Article

Association Between Cystic Fibrosis Severity Markers and *CFTR* Genotypes in Turkish Children

Abdurrahman Erdem Başaran[1], Ayşen Başaran[1], Dilara Fatma Kocacik Uygun[2], Elanur Yılmaz[3], Asef Moballegh[3], Latife Öz[4], Özgül Alper[3], Ayşen Bingöl[1]

[1]Department of Pediatrics, Division of Pulmonology, Faculty of Medicine, Akdeniz University, Antalya, Turkey
[2]Department of Pediatrics, Division of Allergy Immunology, Faculty of Medicine, Akdeniz University, Antalya, Turkey
[3]Department of Medical Biology and Genetics, Akdeniz University, Faculty of Medicine, Antalya, Turkey
[4]Department of Pediatrics, Akdeniz University, Faculty of Medicine, Antalya, Turkey

Cite this article as: Basaran AE, Basaran A, Kocacik Uygun FD, et al. Association between cystic fibrosis severity markers and *CFTR* genotypes in Turkish children. *Turk Thorac J.* 2021; 22(6): 426-431.

Abstract

OBJECTIVE: To compare class I/II cystic fibrosis transmembrane conductance regulator (CFTR) mutations to class III-V mutations with regards to cystic fibrosis disease severity markers in children.

MATERIAL AND METHODS: This study was designed as a cross-sectional study in Antalya province, located on the south coast of Turkey. The study included 38 cystic fibrosis patients aged between 0.6 and 18 years. The CFTR genotype of the patients was categorized into 2 groups based on the presence or absence of class I or class II mutations in any of the alleles. Group I comprised 8 homozygous, 8 with unknown alleles, and 8 compound heterozygous patients, and group II comprised 11 homozygous and 3 compound heterozygous patients. The groups were analyzed in respect of cystic fibrosis disease severity markers, such as spirometry, ShwachmanKulczycki score, body mass index (BMI), sweat chloride concentration, chronic *Pseudomonas aeruginosa* infection, annual exacerbation frequency, and severe exacerbations requiring hospitalization during the previous year.

RESULTS: In the comparison of group I and group II patients, a significant difference was observed in pancreas insufficiency (83.3% vs. 35.7%; $P = .005$), chronic *P. aeruginosa* infection (58.3% vs. 7.1%; $P = .002$), cough severity score (1.7 ± 1.1 vs. 0.9 ± 1.5; $P = .029$), number of severe exacerbations requiring hospitalization during the previous year (0.9 ± 1 vs. 0.3 ± 0.8; $P = .03$), and sweat chloride levels (76.7 ± 15.2 vs. 61 ± 22.3; $P = .02$). All these values were higher in group I patients. The mean BMI values (15.8 ± 2.2 vs. 17.6 ± 2.8; $P = .03$) were lower in group I patients.

CONCLUSION: There seems to be a difference between class I/II CFTR mutations and class III-V mutations on the severity of the disease in cystic fibrosis patients.

KEYWORDS: Cystic fibrosis, disease severity markers, CFTR mutation classes, cystic fibrosis phenotype
Received: *November 17, 2020* ***Accepted:*** *May 4, 2021*

INTRODUCTION

Cystic fibrosis (CF; MIM 219700) is the most common life-shortening autosomal recessive hereditary disorder in Caucasians. It is characterized by chronic suppurative obstructive lung disease, multifocal biliary cirrhosis, pancreatic insufficiency, and loss of electrolytes through sweating. It is well known that CF is caused by mutations in the *CFTR* (cystic fibrosis transmembrane conductance regulator (NM_000492.3)) gene and according to their functional effect, these mutations can be grouped into 6 classes; no synthesis (class I), processing defects (class II), regulation defects (class III), decreased conductance (class IV), reduced synthesis (class V), and decreased stability (class VI).[1,2,3] While class I and II mutations affect a large proportion of the CFTR protein reaching the epithelial cell surface, the protein can be present on the cellular surface in classes III, IV, or V mutations and a certain residual function can be found.[4,5]

CF is a multiorgan disease with great phenotypic as well as genetic heterogeneity, and many studies have attempted to reveal the relationship between disease genotype and phenotype.[6,7] In previous studies, it has been shown that the class IV-V mutations are associated with a milder phenotype.[8] The presence of residual function in at least 1 allele confers less severe lung disease and improved nutritional status.[9] An association between genotype and phenotype has been determined in terms of lung function, pancreatic function, nutritional status, age at diagnosis, and *Pseudomonas aeruginosa* infection.[10,11]

The aim of this study was to compare class I/II CFTR mutations to class III-V mutations with regards to CF disease severity markers, such as spirometry, Shwachman-Kulczycki (SK) score, body mass index (BMI), sweat chloride concentration, chronic *P. aeruginosa* infection, annual exacerbation frequency, and severe exacerbations requiring hospitalization during the previous year in children.

Corresponding author: Abdurrahman Erdem Başaran, e-mail: erdembasaran15@hotmail.com

MATERIAL AND METHODS

This study was designed as a cross-sectional study in Antalya province, located on the south coast of Turkey. The study included CF patients who regularly attended the department of pediatric pulmonology, Akdeniz University hospital, between 2018 and 2020. This clinical genetic study was approved by the Ethical Review Committee for the Protection of Human Subjects in Research, Akdeniz University, Turkey (decision number: 736, decision date: September 23, 2020). The patients who fulfilled the following diagnostic criteria of CF were included in the study: (a) two sweat test results > 60 mmol/L chloride or 1 sweat test result > 60 mmol/L chloride and the presence of 2 identified diseases causing CF mutations, (b) sweat test result < 60 mmol/L and typical clinical presentation of CF and 2 identified diseases causing CF mutations.[12] Patients were excluded from the study if CFTR mutations were not detected in both alleles.

The chloride level in the sweat test was measured with the CFΔ Collection System® sweat test analysis system (UCF 2010 Iontophoresis Unit and UCF 2011 Sweat Analysis Unit), which is used to analyze both the conductivity and chloride concentration of sweat via conductivity measurement by a colorimetric endpoint software method.[13] The SK score was calculated by 2 pediatric pulmonologists specialized in CF. To obtain the SK score, the 4 domains of physical examination, general activity, radiological findings, and nutrition were scored from 5 to 25, according to the degree of disease, and the total of these scores was defined as the SK score. For cooperative children aged > 5 years, spirometry (ZAN 100 Spiromed, Germany) was performed while the patient was at baseline health without pulmonary exacerbation. BMI was calculated as (weight in kg)/(height in m)^2 and BMI z-score was calculated using the references published by Neyzi.[14] Chronic *P. aeruginosa* infection was defined as ≥ 50% of sputum or cough swab or nasopharyngeal aspirate samples being positive in the preceding 1 year.[15]

Pulmonary exacerbations were defined as respiratory infectious episodes requiring antibiotic therapy.[16] In each patient, exacerbation frequency was calculated as the number that occurred in the preceding 1 year and was determined based on a review of clinical charts and patient history. The patient cough severity score (CSS) were obtained by scoring daytime cough symptoms from 0 to 5 (0 = no cough; 1 = 1 or 2 short coughing episodes in a day; 2 = 2 short coughing episodes in a day; 3 = frequent coughing but does not affect school or other activities; 4 = frequent cough affecting school or other activities; 5 = severe cough inhibiting most activities).[17] Pancreatic insufficiency (PI) was defined as a fecal pancreatic elastase-1 value of < 100 µg/g stool and a condition requiring pancreatic enzymes.[18]

MAIN POINTS

- The genotype-phenotype relationship of cystic fibrosis (CF) disease is not well known.
- CF genotype classification is very important in terms of public health, predicting the course of the disease and taking precautions, and genetic counseling.
- There seems to be a difference between class I/II CFTR mutations and class III-V mutations on the severity of the disease in CF patients.

Genomic DNA was isolated from blood samples of each individual[19] and molecular genetic analysis of the *CFTR* gene was performed with next-generation sequencing analysis. In order to investigate the possible disease causing-variants, the Ion AmpliSeq CFTR Panel v2 was used (all coding exons, intron-exon boundaries, UTRs; 8.49kb) (Thermo Fisher Scientific, MA, USA). The raw data were aligned to the hg19 reference genome. Variant calling and mapping were performed using Torrent Suite Software v. 5.10.5.0 (Thermo Fisher Scientific, MA, USA). The data (SNPs, InDels) were annotated and filtered with The Ingenuity Variant Analysis (Qiagen, CA, USA). Variants in the non-coding regions and synonymous changes were filtered out. Variants listed in population databases such as 1000G, dbSNP, and ExAC browser were limited <1% for minor allele frequency (MAF). Following the examination of the remaining variants, Sanger sequencing analyses were performed for the suspected variants both in the gDNA samples obtained from patients and their parents. Genotypic data were grouped according to the functional classification, first suggested by Tsui[20] and later modified by Welsh[1] and Zielenski.[21] Patients with mutant *CFTR* genotype were separated into 2 groups based on the presence or absence of class I or class II mutations in any of the alleles. The overall data from clinical charts, SK scores, and spirometry results of 38 patients aged between 0.6 and 18 years were analyzed based on the groups.

Statistical Analysis

Data obtained in the study were analyzed statistically using The Statistical Package for Social Sciences version 23.0 software (IBM Corp.; Armonk, NY, USA). Descriptive statistics were presented as frequency, percentage, mean, standard deviation, and minimum and maximum values. The normality assumption was assessed with the Shapiro–Wilk test. In the analysis of differences between the 2 groups, the independent samples *t*-test was used if the data fit the normal distribution, and the Mann–Whitney *U*-test was used if the data were not normally distributed. A value of $P < .05$ was considered statistically significant.

RESULTS

In this study, 38 patients, comprising 14 females and 24 males with a mean age of 8 ± 5.3 years, were evaluated. The descriptive characteristics of the cases are all shown in Table 1. PseudoBartter syndrome was detected in 18 (47.4%), pancreatic insufficiency in 25 (65.8%), chronic *P. aeruginosa* infection in 15 (39.5%), chronic *Staphyloccocus aureus* infection in 5 (13.2%), bronchopulmonary aspergillosis in 3 (7.9%), nasal polyps in 2 (5.3%), and a positive history of meconium ileus in 4 (10.5%) patients. Parental consanguinity was determined in 10 (26.3%) patients, and 16 (42.1%) patients had siblings with CF.

In 26 (68.4%) patients who underwent pulmonary function test, the mean forced expiratory volume in 1 second (FEV1) was 78.5 ± 26.6%, mean forced vital capacity

Table 1. Demographic and Clinical Characteristics of the Patients

Participant Characteristics	*N* = 38
Age (years), mean (std. dev.)	8.0 (5.3)
Gender, male, *N* (%)	24 (63.2)
Age at diagnosis (months), median (range)	7.5 (1, 156)
BMI (kg/m^2), mean (std. dev.)	16.5 (2.6)
BMI z score, mean (std. dev.)	–0.8 (1.7)
Sweat chloride concentration (mEq/L), mean (std. dev.)	71 (19.5)
Number of patients performing spirometry test (%)	26 (68.4)
FVC (% predicted), mean (std. dev.)	71.1 (23.5)
FEV1 (% predicted), mean (std. dev.)	78.5 (26.6)
MEF 25-75 (% predicted), mean (std. dev.)	95.8 (40.5)
Scwahman-Kulczycki score, mean (std. dev.)	81.2 (17)
Cough severity score, mean (std. dev.)	1.4 (1.3)
Annual exacerbation frequency, mean (std. dev.)	1.2 (2)
Number of severe exacerbations requiring hospitalization during the previous year, mean (std. dev.)	0.7 (1)

BMI, body mass index; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; MEF 25-75, maximum mid-expiratory flow rate.

(FVC) was 71.1 ± 23.5%, and mean maximum mid-expiratory flow rate (Mef 25-75) was 95.8 ± 40.5%. The mean SK score of 37 cases was 81.2 ± 17.

All CF patients were fully genotyped by next-generation sequencing analysis. The functional classifications of the patients based on *CFTR* genotypes are given in Table 2. The patients were separated into 2 groups based on the presence or absence of class I or II mutation in any alleles. Group I comprised 8 homozygous, 8 with unknown alleles, and 8 compound heterozygous patients, and group II comprised 11 homozygous and 3 compound heterozygous patients. A significant difference was observed between the groups in respect of pancreas insufficiency (83.3% vs. 35.7%; *P* = .005), chronic *P. aeruginosa* infection (58.3% vs. 7.1%; *P* = .002), cough severity score (1.7 ± 1.1 vs. 0.9 ± 1.5; *P* = .029), number of severe exacerbations requiring hospitalization during the previous year (0.9 ± 1 vs. 0.3 ±0 .8; *P* = .03), and sweat chloride levels (76.7 ± 15.2 vs. 61 ± 22.3; *P* = .02). All these values were higher in group I patients. The mean BMI values (15.8 ± 2.2 vs. 17.6 ± 2.8; *P* = .03) were lower in group I (Table 3).

In the comparisons of the patients in group I and group II, there was no significant difference between the groups in terms of the history of PseudoBartter syndrome, history of siblings with CF, chronic *S. aureus* infection, bronchopulmonary aspergillosis, nasal polyp, meconium ileus, pulmonary function test, and SK score.

DISCUSSION

The genotype-phenotype relationship of CF disease is not well known. There is a limited number of clinical genetic studies. The aim of this study was to investigate the relationship between the CFTR genotypes and their potential on phenotype as disease markers. The study results demonstrated a correlation between having class I or class II mutations in any of the alleles and CF disease severity markers. It was found that patients with class I or class II mutations in any alleles had higher pancreas insufficiency, chronic *P. aeruginosa*

Table 2. Functional Classifications of the Patients According to CFTR Genotype

	Functional Classes	Genotype	Number of Subjects
Group I	I-*Unknown*	p.E92X/*Unknown*	1
		p.W1310X/*Unknown*	1
		p.S1455X/*Unknown*	2
		p.S466X/*Unknown*	1
	I-I	c.1677del TA/c.1677del TA	1
		c.406-1G>A/c.2184insA	1
		p.G542X/p.G542X	2
		c.2755del T/c.2755del T	1
	I-IV	p.S466X/p.R1070Q	1
		p.E217G/c.2183AA>G	1
	II-*Unknown*	delta F508/*Unknown*	2
		p.N1303K/*Unknown*	1
	II-II	delta F 508/delta F 508	3
		delta F 508/p.G85E	1
		delta F508/p.N1303K	2
		p.G85E/p.G85E	1
		p.S549R/delta F508	1
	II-IV	p.E92K/p.S955L	1
Group II	III-III	p.C866Y/p.C866Y	1
	IV-IV	p.L997F/p.L997F	3
		p.R297Q/p.F1052V	1
		p.Q1044G/p.Q1044G	1
		p.D1152H/p.D1152H	1
	IV-V	p.R334W/c.1766+2T>C	2
	V-V	c.2789+5G>A/ c.2789+5G>A	5

Table 3. Demographic and Clinical Characteristics of the Groups Based on CFTR Genotypes

	Group I	Group II	*P*
Age (years)*	7.9 ± 4.9	8.3 ± 5.9	*.81*
Age at diagnosis (months)*	24.1 ± 40.6	24.5 ± 39.7	.64
Cough severity score*	**1.7 ± 1.1**	**0.9 ± 1.5**	**.029***
Number of severe exacerbations requiring hospitalization during the previous year*	**0.9 ± 1**	**0.3 ± 0.8**	**.032***
Annual exacerbation frequency*	1.4 ± 2.3	0.9 ± 1.5	.60
Scwahman-Kulczycki score*	77.9 ± 17.1	87.3 ± 15.7	.07
Sweat chloride concentration (mEq/L)*	**76.7 ± 15.2**	**61 ± 22.3**	**.02***
BMI (kg/m^2)*	**15.8 ± 2.2**	**17.6 ± 2.8**	**.03***
BMI z score*	−1.1 ± 1.5	−0.3 ± 1.8	.15
FVC% predicted*	70.5 ± 24.2	72.3 ± 23.6	.85
FEV1% predicted*	77.5 ± 27	80.1 ± 27.4	.81

*Values presented as mean ± SD.
BMI, body mass index; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second.

infection, cough severity score, number of severe exacerbations requiring hospitalization during the previous year, and sweat chloride levels. These patients also had a lower BMI.

Interestingly, the cases included in this study had a high rate (42.1%) of siblings with CF. It has been known for many years that there are differences in the clinical course of siblings with CF.[22] The most notable differences relate to the severity of lung involvement in patients. After great variability was observed in respiratory disease outcomes in patients with homozygous Phe508del, attention turned to the identification of genetic modifiers.[23] In a recent study[24] evaluating 3 sibling cases using whole-exome sequencing, it was stated that many variants could cause clinically positive or negative results for the patient, and genetic modifiers and pharmacogenomics could also contribute to this situation.

It is well known that the main advantage of classifying *CFTR* mutations is to identify the mutations according to their functional defects and to categorize molecular treatments for each class. Since mutation classes are poorly correlated with clinical features, they cannot be used as categorical predictors, but some useful generalizations have been identified. In general, *CFTR* gene with class I, II, and III mutations has less function, and patients may show more severe disease symptoms. These severe types of mutations have been associated with meconium ileus,[25] pancreatic insufficiency,[26,27] CF liver disease,[9] and CF-associated diabetes.[28] The class I-III allele is associated with pancreatic insufficiency if it matches the other class I-III allele, but there is a pancreatic function sufficiency if one class IV or V allele is present. In the current study, pancreatic insufficiency was observed more frequently in cases with class I (*p.E92X*, *p.W1310X*, *p.S1455X*, *p.S466X*, *c.1677delTA*, *c.406-1G>A*, *c.2184insA*, *p.G542X*, *c.2755del T*, *c.2183AA>G*) or class II (Phe508del, *p.N1303K*, *p.G85E*, *p.S549R*, *p.E92K*) mutations in any allele similar to the findings in the literature,[25] but the same effect on meconium ileus was not seen.

In literature, class IV and V mutations have been found to be associated with a milder phenotype, and its presence in at least 1 allele has been associated with less severe lung disease and better nutritional status.[9] In the present study, lower BMI was determined in group I. No statistically significant difference was found in pulmonary function parameters associated with severe lung disease, but chronic *P. aerugonisa* infection was seen to be frequent.

In CF patients, pulmonary phenotypes can be defined by the pulmonary functional tests (e.g., FEV1), bacteriological marker types (pulmonary exacerbations requiring IV antibiotic, chronic *P. aeruginosa* infection), measurement of structural changes (with high-resolution computed tomography, HRCT scores), and survival time. Previous studies have shown that patients with class I, II, and III mutations have an extreme rate of decline in FEV1 values than patients who carry 1 class IV or V mutation.[29] On the other hand, other studies have shown that it is difficult to predict the reliable severity of pulmonary disease based only on *CFTR* genotypes.[10] In the current study, higher values were detected in patients who had class I or II mutations in any alleles which are clinically associated with the severity of lung disease, such as the number of severe exacerbations requiring hospitalization with a value of 0.9 during the preceding 12 months, cough severity score with 1.7 and chronic *P. aeruginosa* infection (58.3%). However, no significant difference was determined between the groups in respect of pulmonary function tests.

To date, the severe type of *CFTR* mutations has been clearly found to be associated with pancreatic insufficiency, higher sweat chloride levels, and increased risk of *P. aeruginosa* infection. In the current series, there was seen to be a higher level (76.7 mEq/L) of sweat chloride in patients with class I or II mutations in any of the alleles. Thus, this finding is highly correlated with the findings in the literature.[30]

While CFTR genetic analysis panels are expected to detect > 95% mutations, they fail to achieve this in populations with low Phe508del and/or high genetic heterogeneity. Even with the advent of next-generation sequencing, full

sequencing of large disease-associated genes and intronic regions is not economically cost-effective. The combined use of all techniques cannot guarantee the detection of both mutated alleles in all patients with CF, and 1-5% of CF alleles remain undetermined. Evidence has been accumulated that a group of intragenic rearrangements (i.e. large deletions and, to a lesser extent, insertions) account for about 1-3% of all CFTR mutations.[31] In Turkey, the rate of mutation detection is low as there is low Phe508del mutation and high genetic heterogeneity. According to the 2018 data of the Turkey cystic fibrosis registry system, CFTR mutation analysis from 1351 patients with CF revealed that at least 2 mutations were identified in 59.9%, only 1 mutation was detected in 13.5%, and no mutations were identified in 17.3% patients. Phe508del was the most common mutation with 27.15% allele frequency.[32] In another study from Turkey, only 52.5% of disease-causing mutations were detected in patients with CF, and 47.5% of CF alleles were unidentified, which reflected the high molecular heterogeneity of the Turkish population.[33] A review of worldwide *CFTR* mutations analysis[34] has shown that the rate of mutation detection varies from a minimum of 33.3% in Venezuela to a maximum of 100% in Belgium. In the same study, the rate of mutation detection in Turkey was reported as 65%. According to the 2016 US Cystic Fibrosis Foundation Patient Registry,[35] the class of mutations in any alleles in 18% of patients is unknown, so the current study population is important in terms of reporting clinical features in CF patients who do not harbor mutations in both alleles. In the current study cases, Phe508del mutation was present in 12 of 76 (15.8%) alleles, and no genetic mutations were detected in 8 of 38 patients, giving a rate of 21% for unknown alleles. Although CF is a monogenic disease caused by *CFTR* mutations, there is substantial clinical variability among patients with identical *CFTR* genotypes suggesting the presence of additional modifier genes. To date, it is known that CF-related disease may develop based on multiple combining effects, such as complex alleles, mutations in genes (SCNN1A for instance) that mimic CF phenotypes, modifier genes, and epigenetic factors.[36]

In conclusion, CF genotype classification is very important in terms of public health, predicting the course of the disease and taking precautions, and genetic counseling. There seems to be a difference between class I/II CFTR mutations and class III-V mutations on the severity of the disease in CF patients.

Ethics Committee Approval: This study was approved by Ethics committee of Akdeniz University, (Approval No: 736).

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer Review: Externally peer-reviewed.

Author Contributions: Design - A.E.B., A.B., Ö.A.; Data Collection and/or Processing - D.F.U., L.Ö., A.M., E.Y.; Data Collection and/or Processing - A.B., L.Ö., E.Y.; Analysis and/or Interpretation - A.E.B., A.B., A.B.,Ö.A.- Literature Search - A.E.B., A.B., D.F.U.; Writing Manuscript - A.E.B, A.B.; Critical Review - A.B., Ö.A.

Acknowledgments: The authors would like to thank Caroline Walker for further editing of English.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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