

CYSTIC FIBROSIS MUTATION SPECTRUM IN NORTH MACEDONIA: A STEP TOWARD PERSONALIZED THERAPY

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ABSTRACT

The most prevalent “rare” disease worldwide, cystic fibrosis (CF), is an autosomal recessive multisystem disease, caused by mutations in the *CFTR* gene. The knowledge of *CFTR* mutations present in certain population is important for designing a simple, fast and cost-effective genetic testing approach, also for better management of CF patients, including the administration of novel targeted therapies. Here, we present genetic results of 158 unrelated CF patients from the National CF Registry of the Republic of North Macedonia. Initially, patients were screened for the 11 most common CF mutations. Additional CF mutations and large deletions/duplications in the *CFTR* gene were analyzed using commercial kits. If the genotype was undetermined, all *CFTR* exons were analyzed using Sanger DNA sequencing or next generation sequencing (NGS) (since 2014). The most common CF mutation, c.1521_1523del (legacy name F508del), was found with an overall incidence of 75.9%. Additionally, 26 other pathogenic variants and three large deletions were identified in the *CFTR* gene as a genetic cause of CF. Two of these, c.1070 C>T (p.Ala357Val) and c.2779_2788dup CTTGCTATGG (p.Gly930AlafsTer48), were novel. According to the distribution and prevalence of the pathogenic variants detected in our patients, a fast and cost-effective method, based on a single base extension was designed as a first-line CF genetic test with a 90.0% detection rate within our popu-

lation. Furthermore, the knowledge of *CFTR* mutation classes in our CF patients represents the first step toward personalized therapy for CF in our country.

Keywords: Cystic fibrosis (CF); *CFTR* mutation classes; Mutations.

INTRODUCTION

Cystic fibrosis (CF; MIM #219700) also known as mucoviscidosis, is a well-known disease and the most frequent autosomal recessive disease in the Caucasian population with approximately 1/2500 live births. It is caused by mutations in the CF transmembrane conductance regulator (*CFTR/ABCC7*; MIM #602421) gene [1], whose dysfunction disrupts the chloride transport in the epithelial cells of the lungs and respiratory system, sweat glands, pancreas, intestine and the vas deferens in men, resulting in various conditions such as: severe chronic pulmonary disease, salt exhaustion, pancreatic insufficiency, liver disease and infertility in men (caused by congenital bilateral aplasia of the vas deferens) [2,3].

The large spectrum of phenotypic characteristics of CF has been shown to involve not only the type of *CFTR* mutations, but also other genetic factors such as modifier genes and environmental factors. Due to the great clinical variations of CF, the diagnoses of classic CF and non classic CF have emerged. This fact has raised the need to classify the *CFTR* mutations based on molecular consequences. To the present time, more than 2000 different mutations have been reported in the Cystic Fibrosis Mutation Database (CFMD), most of them being missense, frameshift, splicing and nonsense [Cystic Fibrosis Mutation Database (<http://www.genet.sickkids.on.ca>); accessed January 2019]. Only a small number of the hundreds of *CFTR* mutations discovered to date, have been proven to

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result in classical CF, and the vast majority are associated with various clinical presentations. The classification of the *CFTR* mutations based on their consequences on the CF protein is highly important for the choice of therapy, as well as the predicted outcome.

According to their effect on the *CFTR* protein, the *CFTR* pathogenic variants can be grouped into six classes. Class I mutations (nonsense, frameshift or splice mutations) produce truncated RNA resulting with absence of *CFTR* protein at the apical membrane. Class II mutations generate defective processing and maturation of the *CFTR* protein (it does not fold correctly) and as a result, the *CFTR* protein fails to reach the apical cell membrane. After producing, the defective *CFTR* protein is destroyed by the endoplasmic reticulum-associated pathway, and the amount of *CFTR* protein present on the cell surface is significantly reduced. The most frequent *CFTR* mutation F508del belongs to this group. For class III mutations, even though the *CFTR* protein reaches the apical membrane, abnormal regulation of the chloride channel results in impaired gating. Class IV mutations evoke reduced chloride conductance, meaning that *CFTR* protein reaches apical cell membrane, but the misshaped *CFTR* pore restricts Cl⁻ flow. In the carriers of class V mutations there is a functional *CFTR* protein production, however, due to alternative splicing or reduced gene transcription the quantity of the *CFTR* protein at the cell surface is significantly decreased [4-6]. Class VI mutations are considered to decrease the stability of the functional *CFTR* protein causing accelerated protein turnover at the cell surface, resulting in unstable flow maintenance of the Cl⁻ ions.

The classification of the *CFTR* mutations based on the effects on *CFTR* protein production and the amount of residual *CFTR* protein function helps in establishing the treatment and the decision of which medication may be beneficial for a particular mutation. So far, there are three generally most accepted targeted approaches to enhance the function of *CFTR* protein. These include: *potentiators*, that are used for recovering the *CFTR* protein function at the apical surface of the epithelial cells, disrupted when class III or IV mutations are present; *correctors*, used for class II mutations, to raise the intracellular processing, allowing higher amounts of *CFTR* protein to reach the cell surface; and *production correctors*, which promote the read-through of premature termination codons in mRNA, boosting the production of the *CFTR* protein in class I *CFTR* mutations. Moreover, practice has shown that most of the *CFTR* mutations present multiple molecular defects and should therefore be included in more than just one class of mutations and treated with combined therapy. Furthermore, the treatment of patients with CF requires a multi disciplinary team approach [7].

This study was performed with the intention of characterizing the genotypes of all patients listed in the National Registry of Cystic Fibrosis Patients of the Republic of North Macedonia and to determine the spectrum of pathogenic variants causing CF in our country. This approach allows the implementation of a fast and cost-effective first step *CFTR* mutation screening strategy in our country that is beneficial for faster identification of the causative mutations and giving a definitive diagnosis more rapidly in newly CF suspected individuals, as well as for newborn screening protocols. Furthermore, the knowledge of *CFTR* mutation classes in CF patients in our country represents a first step toward personalized therapy for CF.

MATERIALS AND METHODS

Materials. A total of 158 patients (ages between newborn and 36 years) with positive (Cl⁻ >60.0 mEq/L) or borderline (Cl⁻ = 40.0-60.0 mEq/L) sweat test result and clinical features consistent with a diagnosis of CF: respiratory (repeated episodes of bronchopneumonia, chronic cough and sputum production, wheezing, sinus disease with nasal polyps) and non respiratory, *i.e.*, digestive (meconium ileus, failure to thrive, malnutrition, steatorrhea); dehydration with salt depletion; hepatobiliary disease or recurrent pancreatitis were referred for molecular characterization of CF, mainly from the Department of Pediatrics and the Institute for Respiratory Diseases, Skopje, Republic of North Macedonia. Of the 158 patients, 97 were of Macedonian ethnic background, 59 were of the Albanian ethnic background. Of the remaining two patients, one was of Turkish ethnicity and the other of Bosnian ethnic background. Informed written consent was obtained from all individuals included in the study. The study was approved by the Ethics Committee of the Macedonian Academy of Sciences and Arts, Skopje, Republic of North Macedonia.

Methods. DNA was isolated from peripheral blood using the standard phenol-chloroform extraction method. Initially, all patients were screened for a panel of *CFTR* mutations with SNaPshot analysis as published by Noveski *et al.* [8] (mutations screened: F508del, G542X, N1303K, 621+1G>T, G551D, R553X, R1162X, W1282X, R117H, 2184insA and 1717-1G>A). The analysis of the amplified fragments was performed on the ABI PRISM® 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). For the patients whose genotypes were not determined with the initial screening, several additional methods were applied. These include: INNO LiPA *CFTR* 19 and INNO LiPA *CFTR*+17 Tn Update detection kit for 36 mutations in the *CFTR* gene (Fujirebio Diagnostics AB, Göteborg, Sweden); multiplex ligation-dependent probe amplification (MLPA),

for detection of deletions/duplications in the *CFTR* gene (SALSA MLPA KIT P091 *CFTR*; MRC-Holland, Amsterdam, The Netherlands); next generation sequencing (NGS), with TruSight Inherited gene panel performed on MiSeq Illumina Personal Sequencer and data analyses on the Illumina Variant Studio (Illumina Inc., San Diego, CA, USA).

RESULTS

In our study, a total of 30 different mutations were detected in a cohort of 158 unrelated clinically confirmed CF patients (Table 1).

Allele Frequencies. The most frequent mutation in our patients was F508del indicating the highest prevalence of 75.9% (240/316 alleles), followed by G542X (3.5%; 11/316 alleles), N1303K (1.9%; 6/316 alleles), *CFTR* dele2,3 and G1349D (1.6%; 5/316 alleles each). Two mutations: 621+1G>T and 711+3A>G were found in four patients each (1.3%; 4/316 alleles). Five mutations

(V456F, G126D, E822X, R347P and 1811+1G>C) were present with frequencies of 0.95% (3/316 alleles) each. Eight mutations (R117C, Y161D, 457TAT>G, R1158X, S466X (TAG), 2789+5G>A, 2184insA and 3849G>A) were found only in a heterozygous state with frequency of 0.6% (2/316) separately. A spectrum of eight mutations (E92X, A357V, E379X, E585X, R1066C, c.2779_2788dup CTTGCTATGG, 3850-1G>A and 711+1G>T) were found only once with a frequency of 0.3% (1/316) individually. The A357V (c.1070C>T; p.Ala357Val) and c.2779_2788dup CTTGCTATGG (p.Gly930AlafsTer48) are novel mutations, found for the first time in our cohort. Both patients also had F508del, but testing parental samples was available only for the carrier of A357V, where the *trans* position of the variants was confirmed. The A357V (c.1070C>T; p.Ala357Val) mutation was determined in a male infant with dehydration, vomiting, anorexia and weight loss, first reported by Fustik *et al.* [9], where the clinical symptoms of the patient were described in more

Table 1. The frequency of pathogenic *CFTR* mutations in different ethnic cohorts in our study (316 alleles).

Mutations	HGVS (reference sequence: LRG 663t1)	Exon/Intron	Mutation Type	Class of Mutation	Macedonian n = 194 (%)	Albanian n = 118 (%)	Other ^a n = 4(%)	All Alleles n = 316 (%)
CFTRdele2,3 (21 kb)	c.54-5940_273+10250del	2,3	deletion	I	4 (2.1)	1 (0.9)	–	5 (1.6)
E92X	c.274G>T	4	nonsense	I	–	1 (0.9)	–	1 (0.3)
457TAT>G	c.325_327delTATinsG	4	deletion/insertion	II ^b	2 (1.0)	–	–	2 (0.6)
R117C	c.349C>T	4	missense	IV	2 (1.0)	–	–	2 (0.6)
G126D	c.349C>T	4	missense	III ^b	1 (0.5)	2 (1.7)	–	3 (0.9)
Y161D	c.481T>G	4	missense	III ^b	2 (1.0)	–	–	2 (0.6)
621+1G>T	c.490+G>T	4	mRNA splicing defect	I	2 (1.0)	1 (0.9)	1 (25.0)	4 (1.3)
CFTRdele4,5,6,7,8	c.(273+1_274-1)_1116+1_1117-1)del	4, 5, 6, 7, 8	deletion	I ^b	1 (0.5)	–	–	1 (0.3)
711+1G>T	c.579+1G>T	5	mRNA splicing defect	III	–	1 (0.9)	–	1 (0.3)
711+3A>G	c.579+3A>G	5	mRNA splicing defect	V	1 (0.5)	3 (2.5)	–	4 (1.3)
R347P	c.1040G>C	8	missense	IV	1 (0.5)	2 (1.7)	–	3 (0.9)
A357V	c.1070C>t	8	missense	IV ^b	1 (0.5)	–	–	1 (0.3)
E379X	c.1135G>T	9	nonsense	I ^b	1 (0.5)	–	–	1 (0.3)
V456F	c.1366G>T	10	missense	IV ^b	3 (1.5)	–	–	3 (0.9)
S466X(TAG)	c.1397C>T	11	nonsense	I	2 (1.0)	–	–	2 (0.6)
F508del	c.1521_1523del	11	deletion	II	143 (73.7)	94 (79.7)	3 (75.0)	240 (75.9)
CFTRdele11	c.(1392+1_1393-1)_1584+1_1585-1)del	11	deletion	I ^b	–	1 (0.9)	–	1 (0.3)
G542X	c.1624G>T	12	nonsense	I	8 (4.1)	3 (2.5)	–	11 (3.5)
1811+1G>C	c.1679G>C	12	mRNA splicing defect	V ^b	3 (1.5)	–	–	3 (0.9)
E585X	c.1753G>T	13	nonsense	I	1 (0.5)	–	–	1 (0.3)
E822X	c.2464G>T	14	nonsense	I	1 (0.5)	2 (1.7)	–	3 (0.9)
2184insA	c.2052_2053insA	14	insertion	I	2 (1.0)	–	–	2 (0.6)
2789+5G>A	c.2657+5G>A	16	mRNA splicing defect	V	–	2 (1.7)	–	2 (0.6)
p.Gly930AlafsTer48	c.2779_2788dupCTTGCTATGG	17	insertion	I ^b	1 (0.5)	–	–	1 (0.3)
R1066C	c.3196C>T	20	missense	II	–	1 (0.9)	–	1 (0.3)
R1158X	c.3472C>T	22	nonsense	I	2 (1.0)	–	–	2 (0.6)
3849G>A	c.3717G>A	22	mRNA splicing defect	V ^b	2 (1.0)	–	–	2 (0.6)
3850-1G>A	c.3718-1G>A	22	mRNA splicing defect	V ^b	1 (0.5)	–	–	1 (0.3)
N1303K	c.3909C>G	24	missense	II	5 (2.6)	1 (0.9)	–	6 (1.9)
G1349D	c.4046G>A	25	missense	III	2 (0.5)	3 (2.5)	–	5 (1.6)

^a Other (Bosnian, Turks).

^b Functional class previously unreported (presumed according to mutation type).

detail. The observation of this patient for 2 to 5 years revealed a mild CF phenotype. Additionally, two large deletions (*CFTR*dele11 and *CFTR*dele4-8) were detected in a heterozygous state in one patient each (0.3%, 1/316). Respectively, the most frequent functional class of mutation was class II (78.8%; 249/316 alleles) with four mutations, due to the high presence of F508del allele in our cohort. Following class II, the second most frequent mutation class is class I (11.1%, 35/316 alleles) with 13 different mutations. The third most frequent mutation class is class V (3.8%, 12/316 alleles) with five different mutations, followed by class III (3.5%, 11/316 alleles) presenting with four different mutations and class IV (2.8%, 9/316 alleles) with four different mutations. Mutations belonging to class VI were not present in our patients. The mutation classes are presented in Table 1.

Genotype Frequencies. As expected from the allele frequencies, the most common genotype was F508del/F508del (57.6%; 91/158) followed by the F508del/non F508del genotype (36.7%; 58/158) and non F508del/non F508del (5.7%; 9/158). Following the F508del/non F508del genotype, most frequent was F508del/G542X (12.1%; 7/58). From the non F508del/non F508del genotypes, four included the G542X mutation in combination with other mutations and the other five genotypes (621+1G>T/1811+1G>C, E585X/G126D, 2184insA/*CFTR*dele2,3, R347P/R347P and 2789+5G>A/2789+5G>A) were found in one patient each. According to the mutation classes, the most frequent genotype class was II/II (63.3%, 100/158), followed by I/II (17.7%; 28/158), II/III (5.7%; 9/158), II/V (5.1%; 8/158) and II/IV genotype (3.8%; 6/158). The distribution of *CFTR* genotypes in our CF patients is presented in Table 2.

DISCUSSION

The molecular characterization of CF in the Republic of North Macedonia was initiated in 1989, shortly after the identification of the *CFTR* gene. Until 1990, a total of 19 unrelated families from the southern part of Yugoslavia were analyzed at the Research Center for Genetic Engineering and Biotechnology “Georgi D. Efremov”, Macedonian Academy of Sciences and Arts, Skopje, Republic of North Macedonia, showing a very low distribution of the F508del mutation (39.5%) [10]. In the following few years, more CF families were examined, but the frequency of the F508del mutation remained low (47.9%) [11]. In 2001, a reevaluation of the clinical features of the CF patients from the CF registry was performed and resulted with increasing the overall distribution of the F508del mutation to 62.4%, close to distributions observed in other Southeast European populations and neighboring regions.

Table 2. The number of various *CFTR* genotypes and corresponding classes found in our cohort (158 patients).

Genotype Class	Genotypes	Patients n = 158
I/I	G542X/E822X	1
	GF42X/R1158X	1
	2184insA/ <i>CFTR</i> dele2,3	1
I/II	F508del/G542X	7
	F508del/ <i>CFTR</i> dele2,3	4
	F508del/621+1G>T	3
	F508del/E822X	2
	F508del/S466X(TAG)	2
	F508del/A357V	1
	F508del/E92X	1
	F508del/ <i>CFTR</i> dele11	1
	F508del/R1158X	1
	F508del/E379X	1
	F508del/ <i>CFTR</i> dele4-8	1
	F508del/p.Gly930AlafsTer48	1
	F508del/2184insA	1
	G542X/Y61D	1
	E585X/G126D	1
I/IV	621+1G>T/1811+1G>C	1
	G542X/3850-1G>A	1
II/II	F508del/F508del	91
	F508del/N1303K	6
	F508del/457TAT>G	2
	F508del/R1066C	1
II/III	F508del/G1349D	5
	F508del/G126D	2
	F508del/711+1G>T	1
	F508del/Y161D	1
II/IV	F508del/V456F	3
	F508del/R117C	2
	F508del/R347P	1
II/V	F508del/711+3A>G	4
	F508del/3849G>A	2
	F508del/1811+3G>A	2
IV/IV	R347P/R347P	1
V/V	2789+5G>A/2789+5G>A	1

In this study, we present the results of the comprehensive genotyping of 158 CF patients, including the distribution of mutation classes in our country. The F508del mutation still remains the most common mutation in our cohort, being present in 75.9% of the analyzed alleles (143/194 Macedonian alleles; 73.7%, 94/118 Albanian alleles; 79.7% and 3/4 other alleles 75.0%). This is similar to the frequency reported in Serbia and Montenegro (72.28%) [12] and correlates with most of the European populations. Among the other countries in the Balkan region, a lower frequency of F508del was reported (Albania 69.9%, Greece 53.4%, Bulgaria 62%) [12-14]. The second most frequent mutation was G542X found in 3.5% of our patients, similar to the

frequencies reported in our neighboring countries (Bulgaria 4.2%, Greece 3.9%, Serbia and Montenegro 2.57%). This mutation is most commonly found in the Mediterranean regions of Europe and Africa. However, several mutations that were found more than once in our group of patients, have not been mentioned in the studies of the neighboring countries: G1349D, V456F, G126D and R117C.

Accordingly, the genotype F508del/F508del is the most frequently found in all ethnic cohorts analyzed in our group of CF patients with a distribution of 57.6%. The F508del/G542X and F508del/N1303K genotypes, which are most frequent after F508del/F508del, were found in four patients from the Macedonian cohort and in three patients from the Albanian cohort; and in five patients from the Macedonian cohort and in one patient from the Albanian cohort, respectively. Two mutations that were found in a homozygous state (R347P/R347P and 2789+5 G>A/2789+5G>A) were exclusive to two Albanian patients, while the F508del/621+1G>T genotype was the only F508del/non F508del genotype represented in all of the three different cohorts.

To date, molecular characterization of the CF patients leads toward personalized therapy as the best proposed therapy for class I mutations, which cause premature termination codons (PTC), include the aminoglycosides that are active against PTCs. Also, other formulas, such as ELX-02, are in ongoing clinical trials evaluating the effect on class I mutations. Unfortunately, so far no single drug is able to accomplish the complete rescue of the *CFTR*-F508del protein that is carried by 94.3% (149/158) of our patients. Recently, the combined use of *potentiator* and *corrector* molecules, verified to be beneficial for the homozygous F508del patients, have been approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) [15]. Likewise, since 2012, the use of a small molecule acting as *potentiator*, called ivacaftor, upgraded the treatment of patients with CF, stabilizing the open gate state and by that increasing the chloride transport by the mutant type *CFTR* protein up to 50.0%. This treatment corresponds to class III mutations (G1349D) but is also beneficial for several class IV (R117C) and class V mutations (711+3A>G and 2789+5 G>A) that are present in 7.6% (12/158) of our patients' genotypes. The comprehensive molecular characterization of CF in our country identified the patients that would benefit from a novel targeted therapy. At the present time, the treatment would be most beneficial for 16 patients who have mutations belonging to class III, plus the mentioned mutations from other classes (R117C, 711+3A>G and 2789+5G>A). The knowledge of *CFTR* mutation classes in our CF patients represents a first step toward personalized therapy for CF in our country.

Moreover, determining the spectrum of mutations in the registered CF patients is leading toward adjusting the first line screening protocol for the most common mutations in our population. Comparison between various commercial kits for detection of CF mutations was also performed. We concluded that the available kits (INNO LiPA *CFTR* (19 and 17+Tn) cover 86.0% of the mutations found in our patients. Elucigene kits: CF30v2, CFEU2v1, CF DE and CF ITALIA (same as the CF UK panel) cover: 84.0, 87.0, 85.0 and 0.63%, respectively, of the mutations detected in our cohorts and ViennaLab kits: CF, CF TUR and CF GER cover: 86.0, 85.0 and 86.0%, respectively, of our patients' mutations. This raised the need to design method with another panel of mutations, more appropriate and accurate for our population. The previously introduced SNaPshot method published by Noveski *et al.* [8], was modified with introduction of the recurrent mutations determined in our cohort of CF patients to include 11 different *CFTR* mutations (F508del, G542X, N1303K, 621+1 G>T, 2184insA, V456F, G126D, G1349D, E822X, 711+3 A>G, R117C). With this approach, we are covering *ca.* 90.0% of the mutations found in our population. This makes the modified screening method more cost-effective and with a better detection rate in our population compared with the available commercial kits. To date, CF mutation detection is not a first line method in newborn screening of CF in our country. This kind of well-adjusted protocol facilitates faster genotyping of suspected CF individuals, giving the definite diagnosis and possibility to adjust treatment and to prolong the life expectancy of the CF individual. Furthermore, using this approach we would also have an effective first-line screening of the newborns.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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