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ORIGINAL ARTICLE

Spectrum of *CFTR* gene sequence variants in a northern Portugal population

A. Grangeia [a,b,*], S. Alves [a], L. Gonçalves [a], I. Gregório [a], A.C. Santos [c,d], H. Barros [c,d], A. Barros [a,b], F. Carvalho [a,b,1], C. Moura [a,b,e,1]

[a] *Genetics Service, Department of Pathology, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal*
[b] *Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal*
[c] *Public Health and Forensic Sciences and Medical Education Department, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal*
[d] *EPIUnit − Instituto de Saúde Pública, Universidade do Porto, Porto, Portugal*
[e] *São João Hospital Centre, 4200-319 Porto, Portugal*

Abstract In Portugal, the spectrum of Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene variants is not known. The main objective of this work was to determine the type and frequency of *CFTR* variants in a sample from northern Portugal by the complete analysis of the *CFTR* coding sequencing performed in 512 Portuguese children. A total of 30 different *CFTR* sequence variants, already reported as cystic fibrosis (CF) or *CFTR* related disorders variants, were detected. Ninety-two children (18.0%; 95%CI: 14.7–21.6) were found to be carriers of one sequence variant and 8 (1.6%; 95%CI: 0.7–3.1) had two sequence variants. Taking into consideration only variants that may cause CF when combined with a pathogenic CF variant, the CF pathogenic variant carrier frequency was 3.3% (95%CI: 1.9–5.3). One (0.2%; 95%CI: 0.01–0.7) child presented two CF pathogenic variants.

Conclusions: The majority of *CFTR* variants detected have been associated with a less severe CF phenotype. A wide spectrum of *CFTR* variants was identified, confirming the highest *CFTR* allelic heterogeneity previously reported in Mediterranean country. Additionally, better knowledge about the *CFTR* sequence variation spectrum may contribute to more efficient genetic testing in the Portuguese population.

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Introduction

Cystic Fibrosis (CF, OMIM # 219700) is the most frequent monogenic autosomal recessive disease in the Caucasian population. The improvement in the health care of CF patients in recent years has led to an increased survival, being the median predicted survival age of 50.5 (95%CI: 47.5–53.5).[1]

The incidence of CF in Europe has become well defined in recent years due to the newborn screening programmes and the improvement in CF patient registries in different European countries facilitating accurate assessment of CF prevalence in Europe.[2,3]

On average, 1 in 25–30 individuals is a carrier of a Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) pathogenic variant and 1 in 2500–3500 newborns are affected with CF, however there are significant variations according to ethnic group and geographical location.[4,5] CF prevalence in Portugal, based on the neonatal screening initiated in October 2013, is estimated to be 1:7500 (Ana Marcão, oral presentation, 2017).

Since the identification of the CFTR gene in 1989, more than 2000 variants have been reported, with wide geographical variation.[6-9] This gene encodes a CFTR protein, which functions as a chloride channel located in the apical membrane of secretory epithelial cells.[10]

Pathogenic variants in the CFTR gene result in a variety of consequences associated with abnormal function of epithelial and exocrine glands, leading to a multisystemic disease.[11]

To date, p.Phe508del (F508del) is the most common CF-pathogenic variant, accounting for about 70% of all pathogenic variants in Caucasians.[2,4,12] Besides p.Phe508del, there are very few pathogenic variants with global frequencies higher than 1%. While in Northern and Central Europe there is a high degree of homogeneity of CFTR variants, Southern European countries have a more heterogeneous CFTR spectrum.[4,13]

Seven CF-pathogenic variants, p.Arg334Trp (R334W, 5%), p.Ala561Glu (A561E, 2.9%), p.Gly85Glu (G85E, 1.8%), p.Arg1066Cys (R1066C) and c.3272-26A>G (1.3% each), p.Gln1100Pro (Q1100P, 0.8%) and p.Pro205Ser (P205S, 0.7%), have been reported in CF Portuguese patients, presenting higher frequencies than expected and suggesting that they might be specific to the Portuguese population.[2,14] Additionally, previous studies carried out in a cohort of infertile Portuguese patients with congenital bilateral absence of the vas deferens (CBAVD) showed a very heterogeneous CFTR panel.[15]

In addition to CF, CFTR variants are also responsible for other diseases, usually monosymptomatic and classified as CFTR-related disorders (RD).[16] Many of these patients may carry a CF-pathogenic variant on one allele and a likely pathogenic variant on the other allele, or even in both alleles.

Commercial kits developed for CF screening test only a limited number of common CF-causing variants and therefore may miss sequence variants that can be responsible for less severe CFTR-RDs phenotypes. The development of molecular biology techniques such as next-generation sequencing (NGS), made possible a complete analysis of the CFTR gene in a way that is less labour-intensive and less expensive than the traditional Sanger sequencing.

In Portugal, the spectrum of CFTR sequence variants are not established. The preliminary results of the pilot study for CF newborn screening in Portugal which started in October 2013, estimates a substitutte by: 1:7500 frequency of CF (unpublished data). The recent implementation of a national CF registry centre and the different approaches used for genetic testing carried out by the different laboratories has up to now made the calculation of frequencies of CFTR variants unsafe.

The present work reports the spectrum of CFTR variants in a sample from northern Portugal.

Material and methods

Selection of individuals

This study is based on a Portuguese population-based birth cohort study – Generation XXI.[17] Participants were recruited at level III public units providing obstetrical and neonatal care in the metropolitan area of Porto, Portugal, between April 2005 and August 2006. The baseline sample comprised 8647 participants. At 4 years of age, the total cohort was invited to a re-evaluation that occurred between April 2009 and July 2011.

To fit into the general study aim, only children with parents of Portuguese nationality were included. For this specific study, 512 participants were randomly selected at the 4 years old follow-up. For each child, a sample from buccal mucosa cells was collected with a swab.

Genomic DNA was extracted from buccal mucosa cells, using a commercial kit (JETQUICK, Genomed). DNA samples were then banked in the Genetics Service of the Faculty of Medicine, University of Porto.

CFTR gene sequencing

In total 512 DNA samples were completely sequenced and analyzed. CFTR gene sequencing of the first 206 samples was performed by Sanger sequencing. For that purpose polymerase chain reaction of the 27 CFTR exons and their flanking intronic regions was performed. The amplified fragments were then sequenced and analyzed to identify DNA sequence variants.

Due to the update of the molecular techniques, the remaining 306 samples were analyzed by NGS (Ion Torrent PGM). Assay design was performed in order include the 27 CFTR exons, 50 bp into flanking introns and untranslated regions.

The annotation of CFTR sequence variants was based on the GenBank cDNA reference sequence NM_000492 and in accordance to the standard nomenclature recommended by the Human Genome Variation Society.

Clinical characterization

After molecular analysis and according to what was stated by children's parents in the specific informed consent, results of the genetic analysis were communicated to the parents.

Genetic counselling was offered to all parents and children with at least one pathogenic or likely pathogenic variant. Parents were studied to ascertain the origin of the variant and to validate compound heterozygosity in children with two pathogenic or likely pathogenic variants. Clinical evaluation of these children was performed in collaboration with Genetics Consultation of São João Hospital Centre (CHSJ). This clinical characterization included: (1) family history; (2) evaluation of clinical symptoms of CF, including stature-ponderal growth and pancreatic and pulmonary disease;

(3) quantitative sweat chloride test (positive sweat test > 60 mmol/L). Asthma diagnosis was based on the child current and past medical history, family history, physical examination and laboratory tests (prick test).

Ethics

All the procedures were explained to the participants and written informed consent forms were signed in every phase of Generation XXI, by parents or the child's legal guardian, according to the Ethical Principles for Medical Research Involving Human Subjects expressed in the Declaration of Helsinki. Also, for this particular study, a specific informed consent form was signed by the parents of the child.

The study was approved by the University of Porto Medical School/CHSJ Ethics Committee and by the Portuguese Data Protection Authority.

Results

In total, 512 DNA samples were completely sequenced and analyzed. A total of 73 different *CFTR* sequence variants were identified. From these DNA variants, 30 included molecular variants that had been previously reported as CF-pathogenic, *CFTR*-RD or likely pathogenic variants (Table 1) and 43 are benign.

From the forty-three benign variants, 42 were already included in the *CFTR* mutation database[9] and one, the c.1614T>C (p.Asn538Asn) in the exon 12, was identified for the first time in one allele. The c.1614T>C substitution does not alter the codified amino acid nor any significant splicing motif; it is predicted to be a neutral variant by the PROVEAN[18] and Human Splicing Finder[19] software tools.

Taking into account the 30 *CFTR* variants already reported as CF or *CFTR*-RD pathogenic variants, a total of 123 sequence variants alleles were detected. From the 512 children studied, 92 were found to be carriers of one variant (92/512, 18.0%; 95%CI: 14.7–21.6) and 8 children had two variants (8/512, 1.6%; 95%CI: 0.7–3.1) (Table 2).

However, the majority of these *CFTR* variants have been reported in patients with a less severe *CFTR*-RD phenotype. Thus, considering only DNA sequence variants classified as CF-pathogenic (p.Phe508del, p.Arg1162X and p.Phe1257Leu) or variants that in association with a pathogenic variant may cause CF (p.Leu633Ile, p.Tyr1014Cys, p.Phe1052Val, p.Gly1069Arg and p.Met1407Thr), the CF carrier frequency decreases to 3.3% (95%CI: 1.9–5.3) (17/512) or even to 1.4% (95%CI: 0.06–2.5) (7/512), if only the 3 CF-pathogenic variants are considered.[20–23]

Out of a total of 30 *CFTR* variants, 8 children were identified with two variants (8/512, 1.6%; 95%CI: 0.7–3.1). However, when only CF-pathogenic or likely pathogenic variants were considered, only one child (p.Phe508del/p.Phe1052Val) presented two CF-causing variants (1/512, 0.2%; 95%CI: 0.01–0.7) (Table 2).

The c.1210-12T[5] was the most frequent sequence variant, detected in 35 of the 1024 alleles analyzed (3.4%; 95%CI: 2.4–4.6). It is associated with 11TGs repeats in 20 alleles, 12TGs in 11 alleles and 13TGs in 4 alleles (Table 1).

Beyond the c.1210-12T[5], the c.2002C>T (p.Arg668Cys) was identified with an allelic frequency of 1.8% (95%CI: 1.0–2.6), followed by the c.1727G>C (p.Gly576Ala) and the c.2260G>C (p.Val754Met) with allelic frequencies of 1.3% (95%CI: 0.7–2.0) and 0.8% (95%CI: 0.2–1.3), respectively (Table 1).

The CF-pathogenic variant, p.Phe508del, was identified in 5 of the 1024 alleles (0.5%; 95%CI: 0.16–1.0) (Table 1).

Segregation analysis performed in the parents of the children with two variants confirmed the compound heterozygosity.

Children with two sequence variants were evaluated for the presence of clinical signs and symptoms of CF. Currently, at 11 years of age, none of them had CF at the time of clinical evaluation, they presented normal stature-ponderal growth, had no respiratory symptoms, pancreatic insufficiency or digestive disease history. Three children had a quantitative sweat test value between 30 and 59 mmol/L, while the other child had a value below 30 mmol/L. The child identified with the p.Phe508del/p.Phe1052Val genotype has a normal stature-ponderal growth, sweat test of 48 mmol/L, pancreatic sufficiency and with the exception of allergic asthma (prick test positive for mites), no other pulmonary disease.

Discussion

In the present study, we analyzed the *CFTR* gene of 512 Portuguese children in order to estimate the spectrum of *CFTR* variants in this population.

In total, 73 different variants were identified in the *CFTR* gene. From these variants, 42 have already been reported as benign and 1, c.1614T>C (p.Asn538Asn), was detected for the first in this study.[9] This variant was also classified as benign, based on the analysis of the protein change and bioinformatics programmes.[18,19]

The remaining 30 variants included CF-pathogenic or likely pathogenic variants. The classification of these variants is difficult to establish due to in part to the lack of genotype-phenotype correlation and the variability of disease expression in patients with a mild CF phenotype or *CFTR*-RDs.[24]

Based on previous studies and CF database, only 3 out of the 30 variants identified could be classified as CF-pathogenic, p.Phe508del, p.Arg1162X and p.Phe1257Leu.[21,22] On the other hand the p.Leu633Ile, p.Tyr1014Cys, p.Phe1052Val, p.Gly1069Arg and p.Met1407Thr variants, have varying consequences and may cause CF when combined with another CF-pathogenic variant.[20,23] Other sequence variants here detected do not cause CF, but have been associated with a less severe

Table 1 *CFTR* sequence variants classified as pathogenic or likely pathogenic, found in the analyzed cohort of 512 Portuguese children.

Exon/Intron	cDNA change	Protein change	No. alleles	Allelic freq. (%)
ex 2	c.91C>T	p.Arg31Cys (R31C)	1	0.1
ex 3	c.224G>A	p.Arg75Gln (R75Q)	2	0.2
ex 5	c.509G>A	p.Arg170His (R170H)	1	0.1
ex 6	c.601G>A	p.Val201Met (V201M)	1	0.1
ex 8	c.890G>A	p.Arg297Gln (R297Q)	1	0.1
ex 8	c.1052C>G	p.Thr351Ser (T351S)	2	0.2
ex9	c.1163C>T	p.Thr388Met (T388M)	1	0.1
intron 9	c.1210-12[5T] (IVS8-5T)		35	3.4
	c.1210−12[5T11TG] (IVS8-5T11TG)		(20)	(2.0)
	c.1210−12[5T12TG] (IVS8-5T12TG)		(11)	(1.0)
	c.1210−12[5T13TG] (IVS8-5T13TG)		(4)	(0.4)
ex 10	c.1327G>T	p.Asp443Tyr (D443Y)	1	0.1
ex 11	c.1521_1523delCTT	p.Phe508del[a] (F508del)	5	0.5
ex 12	c.1616T>C	p.Ile539Thr (I539T)	1	0.1
ex 13	c.1684G>A	p.Val562Ile (V562I)	3	0.3
ex 13	c.1727G>C	p.Gly576Ala (G576A)	13	1.3
ex 14	c.1897C>A	p.Leu633Ile[b] (L633I)	1	0.1
ex 14	c.2002C>T	p.Arg668Cys (R668C)	18	1.8
ex 14	c.2173G>A	p.Glu725Lys (E725K)	1	0.1
ex 14	c.2260G>C	p.Val754Met (V754M)	8	0.8
ex 15	c.2502T>G	p.Phe834Leu (F834L)	1	0.1
ex 17	c.2735C>T	p.Ser912Leu (S912L)	1	0.1
ex 17	c.2855T>C	p.Met952Thr (M952T)	1	0.1
ex 17	c.2900T>C	p.Leu967Ser (L967S)	3	0.3
ex 19	c.2991G>C	p.Leu997Phe (L997F)	5	0.5
ex 19	c.3041A>G	p.Tyr1014Cys[b] (Y1014C)	2	0.2
ex 20	c.3154T>G	p.Phe1052Val[b] (F12052V)	4	0.4
ex 20	c.3205G>A	p.Gly1069Arg[b] (G1069A)	2	0.2
ex 20	c.3322G>C	p.Val1108Leu (V1108L)	1	0.1
ex 22	c.3484C>T	p.Arg1162X[a] (R1162X)	1	0.1
ex 22	c.3705T>G	p.Ser1235Arg (S1235R)	5	0.5
ex 23	c.3771T>G	p.Phe1257Leu[a] (F1257L)	1	0.1
ex 26	c.4220T>C	p.Met1407Thr[b] (M1407T)	1	0.1
			123	

[a] CF pathogenic variant.
[b] Variant that may cause CF when in compound heterozygosity with a CF pathogenic variant. The old terminology is within brackets ().

phenotype or with *CFTR*-RDs. In these patients, although the clinical symptoms are not expected to be severe enough to meet the criteria of CF, they may present a monosymptomatic disease that could appear later in life.

Compound heterozygotes patients for one of these variants with one CF-pathogenic variant may have a clinical spectrum ranging from CBAVD to CF with sufficient pancreatic function. So, considering only the CF-pathogenic variants, p.Phe508del, p.Arg1162X and p.Phe1257Leu, the CF carrier frequency is 1.4% (7/512) and none of the children were found to simultaneously carry two CF-pathogenic variants. However, taking also into account the likely pathogenic variants, the carrier frequency increases to 17/512 (3.3%) and the prevalence of CF to 1/512 (0.2%). Although the carrier frequency is in accordance to what is expected and to what has been reported in other countries, 3–4%, the CF prevalence observed was higher 1/512 (0.2%)[2,5] (Ana Maracão, oral presentation, 2017). This higher prevalence

could be explained by the fact that not all patients with likely pathogenic variants have CF and therefore this prevalence could have been overestimated. It is important to highlight that patients with a likely pathogenic variant, even when in compound heterozygosity with a CF-pathogenic variant may have a phenotype that ranges from CF to a *CFTR*-RD.

After clinical evaluation, the child with the p.Phe508del/p.Phe1052Val genotype does not fulfil the criteria for CF diagnosis. However, since the likely pathogenic p.Phe1052Val variant has varying consequences and the clinical manifestations of CF can vary over the course of a person's lifetime, this patient would be evaluated periodically.

The other children with two *CFTR* variants, although not expected to have CF, were also evaluated for the presence of signs or symptoms related to a less severe CF phenotype or to a *CFTR*-RD. None of them had CF.

Table 2 *CFTR* gene sequence variants and clinical data in children with two pathogenic or likely pathogenic variants.

Patient code	Protein/cDNA change		Sweat test (mmol/L)	Clinical data		
	Allele 1	Allele 2		Respiratory symptoms	Gastrointestinal disease	Others
1 – Male	p.Arg297Gln (R297G)	p.Glu725Lys (E725K)	Not available	–	Pancreatic sufficiency	Autism spectrum disorder
2 – Female	p.Phe508del[a] (F508del)	p.Ser912Leu (S912L)	32	–	Pancreatic sufficiency	Tonsilittis recurrents
3 – Male	p.Phe508del[a] (F508del)	p.Leu967Ser (L967S)	40	–	Pancreatic sufficiency	Tonsilittis recurrents
4 – Male	p.Phe508del[a] (F508del)	p.Phe1052Val[b] (F1052V)	48	Allergic asthma	Pancreatic sufficiency	–
5 – Male	p.Gly576Ala – p.Arg668Cys (G576A-R668C)	p.Leu997Phe (L997F)	Not available	–	Pancreatic sufficiency	–
6 – Male	p.Met952Thr (M952T)	c.1210–12[5T11TG] (IVS8-5T11TG)	Not available	–	Pancreatic sufficiency	–
7 – Female	p.Leu967Ser (L967S)	c.1210–12[5T12TG] (IVS8-5T12TG)	26	Chronic inflammatory disease of the airways	Pancreatic sufficiency	–
8 – Male	p.Ser1235Arg (S1235R)	c.1210–12[5T12TG] (IVS8-5T12TG)	Not available	Unknown	Unknown	Unknown

[a] CF pathogenic variant.
[b] Variant that may cause CF when in compound heterozygosity with a CF pathogenic variant. The old terminology is within brackets ().

Nevertheless, and according to the new Consensus Guidelines for Diagnosis of CF,[25] these three children with a quantitative chloride sweat test value between 30 and 59 mmol/L, should perform another quantitative sweat test in a different occasion and if the results are within the same range they may have CF. In these cases, evaluation of CFTR dysfunction could be assessed by nasal potential difference or intestinal current measurement in order to confirm the diagnosis of CF. However, in this cohort of children with no CF clinical symptoms, it was not yet possible to do these tests. The child with the p.Leu967Ser/c.1210–12T[5]TG[12] genotype also presented asthmatic bronchitis, which may be correlated with the *CFTR* variants. The child with the p.Ser1235Arg/c.1210–12T[5]TG[12] is currently living outside Portugal and therefore could not be further analyzed. Although the c.1210–12T[5]TG[12] in compound heterozygosity with a pathogenic or likely pathogenic variant is unlikely to cause disease, the c.1210–12T[5]TG[12] may result in a mild form of CF or a *CFTR*-RD.[13,26]

The p.Glu725Lys and p.Ser912Leu, detected in two children with other two variants have been reported in combination with the c.1766+5G>A and p.Gly1244Val, respectively, on the same chromosome.[27,28] However, in these children, the second variant in *cis*, c.1766+5G>A and p.Gly1244Val, were not detected. Since the p.Glu725Lys and p.Ser912Leu had been thought to be benign variants, even in compound heterozygosity with p.Arg297Gln and p.Phe508del, these children are not expected to meet criteria for CF diagnosis. However, the presence of mild symptoms or *CFTR*-RDs were evaluated in these children.

In fact, although no CF symptom was detected at present, 6 out of the 8 children with two *CFTR* sequence variations are boys and may have CBAVD. Additionally, apart from the clinical characterization, genetic counselling was also offered to these families.

These children were randomly selected from the northern Portuguese population and so it would be expected that the spectrum of *CFTR* variants detected during this work would represent the *CFTR* spectrum of the north of Portugal. The high percentage of likely pathogenic variants may reflect a higher percentage of patients with milder forms of CF or *CFTR*-RDs. These patients are usually diagnosed only in adolescence or adulthood and some of them are misdiagnosed because the clinical manifestations differ from those present in classical CF. Due to the difficulty in establishing the pathogenicity of some of the *CFTR* variants detected during the gene screening, the level of CFTR dysfunction could be confirmed by electrophysiological tests such as nasal potential difference measurement. Additionally, the majority of these variants are not included in the commercial kits for CF screening. In fact, only the p.Phe508del and the c.1210-12T[5], detected in the present study, would have been identified if commercial screening kits had been used. This highlights the limited power of detection of *CFTR* variants by commercial kits in heterogeneous populations such as the Portuguese population, compared to others in which a high detection level permits a quick genetic testing. As a result, the complete analysis of the *CFTR* gene should be recommended for patients with clinical symptoms of classic CF with only one or no pathogenic variant detectable with

commercial test kits and also for patients with clinical signs of moderate CF or *CFTR*-RDs.

Conclusions

Our data confirm previous studies that have shown that the spectrum of *CFTR* variants varies according to the geographic and/or ethnic origin of each population, the highest *CFTR* allelic heterogeneity being detected in Mediterranean countries. More knowledge about the *CFTR* variants spectrum contributes to a more efficient genetic testing system in the Portuguese population.

Additionally, it is important to compare these results with data obtained from the Neonatal CF Screening Programme, in order to facilitate the early diagnosis of symptom-free newborns carrying likely pathogenic variants.

Author contributions

Grangeia A., BSc, PhD performed the molecular genetics work, data analysis, data interpretation, writing the manuscript and study conception. Alves S., Gonçalves L. and Gregório I., BSc, performed the molecular genetics work and data analysis. Santos A.C. BSc, PhD and Barros H, MD, PhD, were responsible for study design, supervision of fieldwork and reviewed the final version of the manuscript. Barros A, MD, PhD. reviewed the manuscript. Carvalho F., BSc, PhD, supervised the molecular genetics work, was responsible for study conception and reviewed the final manuscript. Moura C., MD, PhD, was responsible for the clinical data, for study conception and reviewed the final manuscript. Carvalho F. and Moura C. contributed equally to this work as senior molecular genetics and senior clinician, respectively and share senior authorship.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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