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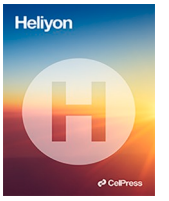

Research article

CFTR pathogenic variants spectrum in a cohort of Mexican patients with cystic fibrosis

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ABSTRACT

Background: Molecular diagnosis of cystic fibrosis (CF) is challenging in Mexico due to the population's high genetic heterogeneity. To date, 46 pathogenic variants (PVs) have been reported, yielding a detection rate of 77%. We updated the spectrum and frequency of PVs responsible for this disease in mexican patients.

Methods: We extracted genomic DNA from peripheral blood lymphocytes obtained from 297 CF patients and their parents. First, we analyzed the five most frequent PVs in the Mexican population using PCR-mediated site-directed mutagenesis. In patients with at least one identified allele, *CFTR* sequencing was performed using next-generation sequencing tools and multiplex ligation-dependent probe amplification. For variants not previously classified as pathogenic, we used a combination of *in silico* prediction, CFTR modeling, and clinical characteristics to determine a genotype–phenotype correlation.

Abbreviations: ABC, ATP-binding cassette; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; CF, cystic fibrosis; *CFTR*, cystic fibrosis transmembrane conductance regulator; CNV, copy number variant; DNA, Desoxyribonucleic acid; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; NBD1, cytosolic nucleotide-binding domain 1; NBD2, cytosolic nucleotide-binding domain 2; PV, pathogenic variant; PMS, PCR-mediated site-directed mutagenesis; TMD1, transmembrane domain 1; TMD2, transmembrane domain 2; R, regulatory domain.

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Results: We identified 95 PVs, increasing the detection rate to 87.04%. The most frequent variants were p.(PheF508del) (42.7%), followed by p.(Gly542*) (5.6%), p.(Ser945Leu) (2.9%), p.(Trp1204*) and p.(Ser549Asn) (2.5%), and CFTRdel25–26 and p.(Asn386Ilefs*3) (2.3%). The remaining variants had frequencies of <2.0%, and some were exclusive to one family. We identified 10 novel PVs localized in different exons (frequency range: 0.1–0.8%), all of which produced structural changes, deletions, or duplications in different domains of the protein, resulting in dysfunctional ion flow. The use of different *in silico* software and American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) criteria allowed us to assume that all of these PVs were pathogenic, causing a severe phenotype. *Conclusions:* In a highly heterogeneous population, combinations of different tools are needed to identify the variants responsible for CF and enable the establishment of appropriate strategies for CF diagnosis, prevention, and treatment.

1. Introduction

Cystic fibrosis (CF; OMIM #219700) is the most frequent autosomal recessive disease [1]. Its incidence in Mexico is unknown, though it is estimated to affect approximately 1/8500 live births yearly [2]. Currently, the survival of patients with CF living in high-income countries (e.g., USA, Canada, and United Kingdom) is approximately 50 years; however, in Mexico, a middle-income country, it is barely 21.37 years [3,4].

The gene responsible for CF is cystic fibrosis transmembrane conductance regulator (*CFTR*, OMIM #602421). *CFTR* is located on the long arm of chromosome 7 (7q31) and contains 27 exons, generating a 6.5-kb mRNA transcript encoding a glycoprotein of 1480 amino acids. CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily and contains two cytosolic nucleotide-binding domains (NBD1 and NBD2), two transmembrane-domains (TMD1 and TMD2), and a phosphorylation-dependent regulatory domain (R) [5,6]. CFTR acts as a chloride channel in the apical membrane of epithelial cells, playing an essential role in cell osmotic balance [7,8].

More than 2000 variants have been detected in *CFTR*, and their distribution is related to the ethnic background of the population. For example, the most frequent pathogenic variant (PV) in CF patients, p.(Phe508del), is found in almost 100% of patients in the Faroe Islands but a notably lower frequency of patients in some Latin American countries [2,7,9]. In countries with predominantly Northern European ancestry, the five most frequent PVs represent more than 86% of CF alleles. In contrast, in Mexico, where the population has a high degree of genetic admixture, 46 PVs account for only 77.7% of CF alleles [10]. Thus, the molecular diagnosis of CF is challenging in countries such as Mexico [11,12].

To improve the basis of the molecular diagnosis of CF in the Mexican population and related populations, we updated the spectrum and frequency of PVs responsible for this disease. Here, we report the identification of 95 PVs in a representative and large sample of Mexican patients with CF. Our results include 10 novel variants, their structural protein predictions, and correlations with the CF phenotype.

2. Methods

2.1. Study population

Our study included 297 patients diagnosed with CF based on positive sweat chloride test (>60 mmol/L) and clinical manifestations. Patients were referred from different health institutions around Mexico. This study was approved by the Ethic and Research Committees of the National Institute for Genomic Medicine (INMEGEN CEI 2015/10). Written informed consent and assent were obtained from patients and from the parent or legal guardian of patients <18 years old. This research was conducted in accordance with the Helsinki Declaration. To compare the frequencies of the variants, we used the public gnomAD v.2.1.1 database (https://gnomad.broadinstitute.org/gene/ENSG00000001626?dataset=gnomad_r4) and 2217 exomes from healthy unrelated adults without a history of Mendelian diseases that were previously sequenced in our laboratory [13,14].

2.2. Molecular analyses

We extracted genomic DNA from peripheral blood lymphocytes using the QIAamp DNA Blood Maxi kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The molecular screening was performed in two steps. First, we screened the five most frequent PVs in the Mexican population [p.(Ile507del), p.(Phe508del), p.(Gly542*), p.(Ser549Asn), and p.(Asn1303Lys)] using PCR-mediated site-directed mutagenesis (PSM) as described previously [10,12]. Second, in those patients with at least one unidentified allele, *CFTR* sequencing was performed using next-generation sequencing (NGS). We performed the sequencing using Multiplicom CFTR Mastr Dx (Agilent, CA, USA) in the MiSeq system (Illumina, Inc). This kit amplifies all *CFTR* exons, intron–exon boundaries, UTR regions, indels, and copy number variants (CNVs). In some cases, the multiplex ligation-dependent probe amplification (MLPA) assay was used to confirm large deletions or duplications.

2.3. Databases and in silico analysis

Short variant calling was carried out using the Genome Analysis Toolkit: UnifiedGenotyper [GATK, https://gatk.broadinstitute.org], Integrative Genomics Viewer (IGV) [https://software.broadinstitute.org/software/igv/], and MASTR Reporter software (Agilent, USA). All PVs identified in the patients were validated in their parents by PSM or direct Sanger sequencing using Big Dye Terminator (Applied Biosystems TM, Foster City, USA).

To identify potentially novel PVs, we used the cystic fibrosis mutation [http://www.genet.sickkids.on.ca], CFTR2-Clinical and Functional Translation of CFTR [http://cftr2.org], and CFTR-France [https://cftr.iurc.montp.inserm.fr/cftr/] databases [15]. To determine whether variants were pathogenic, likely pathogenic, of uncertain significance, or benign we used the InterVar [https://wintervar.wglab.org/] and Franklin [https://franklin.genoox.com/clinical-db/home] tools. For variants not previously classified as pathogenic or lacking consensus criteria, we used *in silico* prediction methods for exonic and intronic variants, including PolyPhen v.2.0 (http://genetics.bwh.harvard.edu/pph2/), Condel v.2.0 (https://bbglab.irbbarcelona.org/fannsdb/help/condel.html), FathHMM v.2.3 (http://fathmm.biocompute.org.uk/), MutPred v.2.0 (http://mutpred.mutdb.org/), CADD v.1.6 (https://cadd.gs.washington.edu/), SIFT v.1 (https://sift.bii.a-star.edu.sg/), Mutation Taster v.2021 (https://www.mutationtaster.org/), Mutation Assessor v.3 (http://mutationassessor.org/r3/), PROVEAN v.1.1 (https://www.jcvi.org/research/provean#downloads), HSF v.3.1 (http://www.umd.be/HSF/), NetGene2 v.2.42 (http://www.cbs.dtu.dk/services/NetGene2/), SpliceAI v.1 (https://spliceailookup.broadinstitute.org/), EX-SKPIP v.1 (https://ex-skip.img.cas.cz/), ASSP v.3.1 (http://wangcomputing.com/assp/), BDGP v.0.9 (https://www.fruitfly.org/seq_tools/splice.html), ESE Finder v.3.0 (https://esefinder.ahc.umn.edu/cgi-bin/tools/ESE3/esefinder.cgi), and MaxEntScan v.1 (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) [16,17]. In addition, the pathogenicity status of novel variants was assigned according to the consensus criteria of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) [18].

Finally, mutant proteins arising from novel variants in *CFTR* were modeled using the Swiss model tool (https://swissmodel.expasy.org/). The combination of CFTR modeling, pathogenicity prediction, and CF clinical characteristics allowed us to determine a genotype–phenotype correlation.

3. Results

Our study enrolled 297 unrelated Mexican patients with a clinical diagnosis of CF. Using diverse molecular strategies, we identified 95 PVs distributed along the *CFTR* gene (Fig. 1). During the first step of our strategy, using PMS, 47.5% of the alleles were identified as one of the five most frequent variants. Next, *CFTR* sequencing using NGS or MLPA enabled us to increase the number of identified

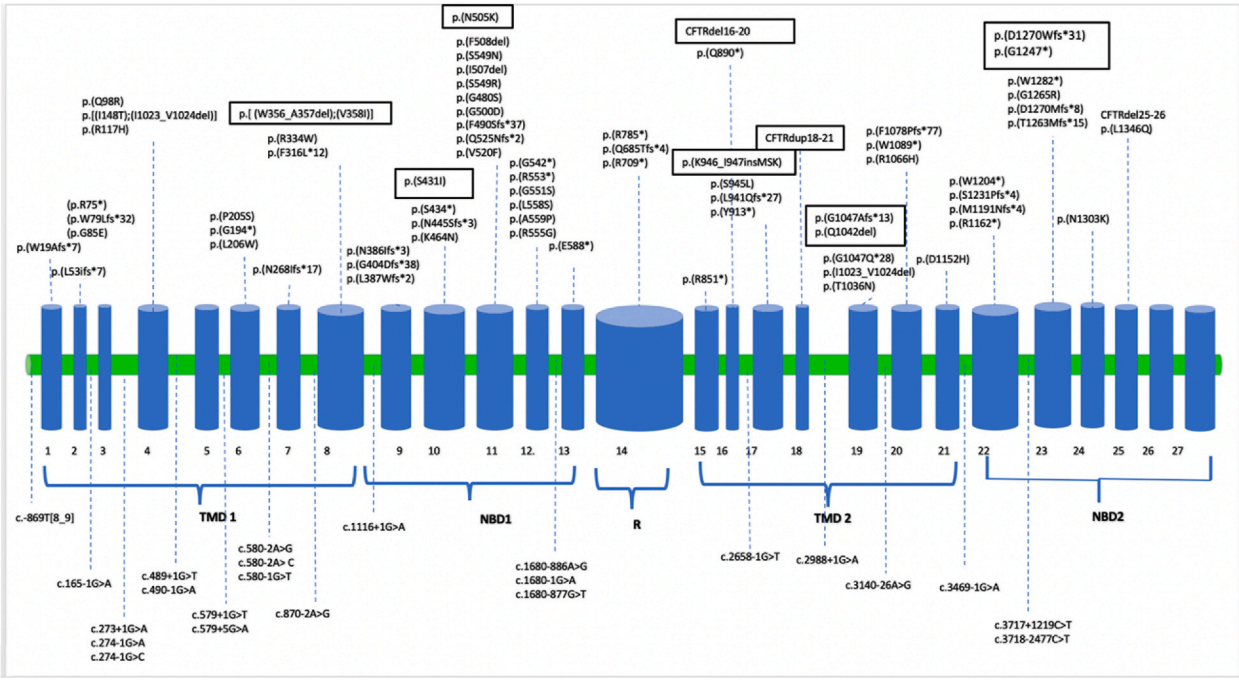

Fig. 1. Ninety-five pathogenic variants (PV) found in the CFTR gene in Mexican patients with cystic fibrosis. The PV enclosed in a rectangle are the novel variants. The cylinders represent the exons of the gene. The brackets indicate the region of the gene that transcribes to different domains of the protein: two cytosolic nucleotide-binding domains (NBD1 and NBD2), two transmembrane-domains (TMD1 and TMD2), and regulatory domain (R). The protein nomenclature is one letter and is in accordance with the human genome variation society (HGVs) guidelines.

alleles to 87.04%. Among these, 74% had two identified alleles and 26% had one.

As expected, the most frequent PVs were p.(Phe508del) (42.7%), followed by p.(Gly542*) (5.6%), p.(Ser945Leu) (2.9%), p.(Trp1204*), p.(Ser549Asn) (2.5%), CFTRdel25-26 (2.3%), and p.(Asn386Ilefs*3) (2.3%). The remaining variants had frequencies <2% and some were exclusive to one family (Table S1, Fig. 2). When the frequencies of these 95 PVs were compared with those reported in the public gnomAD v.2.1.1 database and the 2217 exomes from healthy unrelated adults, we found 56 and 10 variants in the gnomAD and exomes from healthy Mexicans, respectively. The frequencies of these variants were significantly higher in our CF patients in relation to those reported in both databases ($P \leq 0.010$, Table S1). Only three variants did not show significant differences [p.(R117H) 0.2 vs. 0.143, $p = 0.3821$; c.3140-26A > G, 0.4 vs. 0.068, $p = 0.1225$; p.(D1152H), 0.2 vs. 0.068, $p = 0.1225$ (Table S1)].

Notably, we found 10 novel variants localized in different exons, with a frequency range of 0.1–0.8% (Table 1). To interpret the pathogenicity of the novel variants, we used *in silico* prediction methods. We also employed the ACMG/AMP criteria [18].

These variants included two large rearrangements: a deletion of five exons (CFTRdel16–20) in one patient (p.[CFTRdel16–20]; [Trp19Alafs*7]) and a four-exon duplication (CFTRdup18–21) in four patients, two in a homozygous state and two *in trans* with p.(Phe508del) and p.(Ser945Leu). In the cases of patients homozygous for the variants, their families denied consanguinity. Protein modeling revealed that both large rearrangements generated a deletion or duplication of the TMN2 and NBD2 domains, altering the channel structure (Fig. 3a).

The novel identified missense variants [c.1515T > G:p.(Asn505Lys) and c.1292G > T:p.(Ser431Ile)] induced structural changes in the transmembrane domains TMN1 and NBD1, thereby affecting protein stability (Fig. 3b). Patients harboring these variants were compound heterozygotes with p.(Phe508del).

Moreover, we identified three novel variants generating stop codons: p.(Gly1047Alafs*13) in three patients, p.(Gly1247*) in one patient, and p.(Asp1270Trpfs*31) in one patient. Although p.(Asp1270Trpfs*31) was found in the homozygous state, the others were identified *in trans* with p.(Phe508del), p.(Ser945Leu), c.-869[8_9]T, and p.(Ile507del). The prediction tools showed that these variants generated a partial loss of the TMN2 domain and a total loss of the NBD2 and R, leading to protein instability and affecting ATP hydrolysis (Fig. 3c).

Furthermore, we found two insertion–deletion variants. The variant p.(Lys946_ile947insMetSerLys) was found *in trans* with p.(Phe508del) and generates structural changes in TMN2, affecting its anchorage to the membrane. The variant p.(Gln1042del) was found *in trans* with p.(Gln685Thrfs*4) and affected the catalytic activity of NBD2, compromising ATP hydrolysis (Fig. 3d).

For the first time, we identified a complex allele (p.[Trp356_Ala357del; p.Val358Ile]) in a compound heterozygous patient with p.(Phe508del). This complex allele caused conformational changes in loop 6 of the TMN1, which plays a crucial role in the protein's stability and anchoring, leading to ion flow dysfunction (Fig. 3e). The frequencies of these variants were significantly higher in affected individuals (CF patients) than in 2217 healthy unrelated adults and populations included in the gnomAD database (Table S2). Thus, all novel variants were classified as strongly pathogenic.

Finally, the genotype–phenotype correlation of the patients carrying these variants allowed us to assume that all generated a severe phenotype from birth, with elevated sweat chloride levels, pancreatic insufficiency (PI), severe pulmonary abnormalities, malnutrition, and vitamin deficiency. It was not possible to determine the genotype–phenotype correlation in the case harboring the complex allele p.[Trp356_Ala357del; p.Val358Ile] because we did not have clinical data.

4. Discussion and conclusions

CF is an autosomal recessive disease characterized by alteration of chloride and bicarbonate transport in epithelial cells, resulting in a multiorgan disease, mainly characterized by chronic lung disease, PI, and elevated sweat chloride levels. CF is caused by the presence

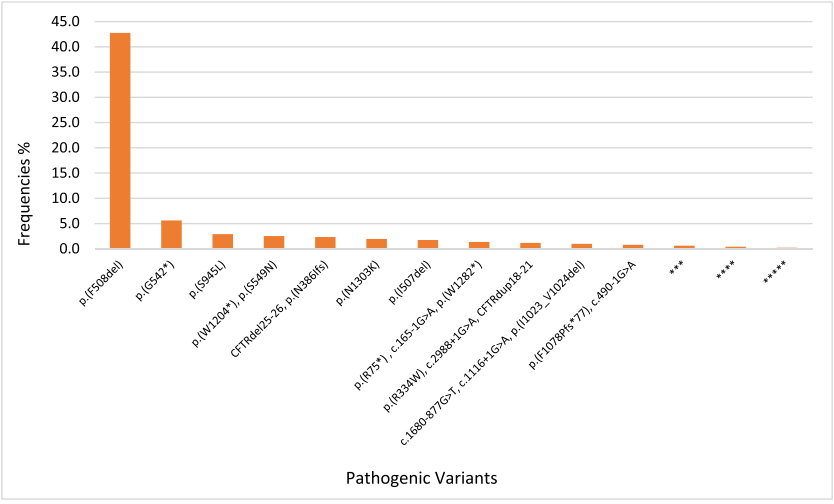

Fig. 2. Frequencies of Ninety-five pathogenic variants (VP) in Mexican patients with cystic fibrosis. The VPs of the asterisks are listed in Table S1.

Table 1
Molecular characteristics of the ten novel variants and their frequency.

ID		Location (hg19)	Variant type	Frequency (# alleles)
DNA	Protein[a]			
CFTRdel16-20	–	117242880	Large rearrangements	0.2 (1)
CFTRdup18-21	–	117246728		1.2 (6)
c.1515T >G	p.(N505K)	17199640	Missense	0.6 (3)
c.1292G >T	p.(S431I)	17188777		0.2 (1)
c.3139delG	p.(G1047Afs*13)	117250722	Stop codons	0.6 (3)
c.3739G >T	p.(G1247*)	117282513		0.2 (1)
c.3807_3808delCG	p.(D1270Wfs*31)	117282580		0.4 (2)
c.2832_2840dupGTCGAAAAT	p.(K946_I947insMSK)	117243758	Insertions–deletions	0.2 (1)
c.3125_3127delAAC	p.(Q1042del)	117250705		0.2 (1)
c.[1066_1071delTGGGCT; 1072G >A]	p.[W356_A357del; p.V358I]	117180349 117180356	Complex allele	0.2 (1)

[a] Protein nomenclature (one letter).

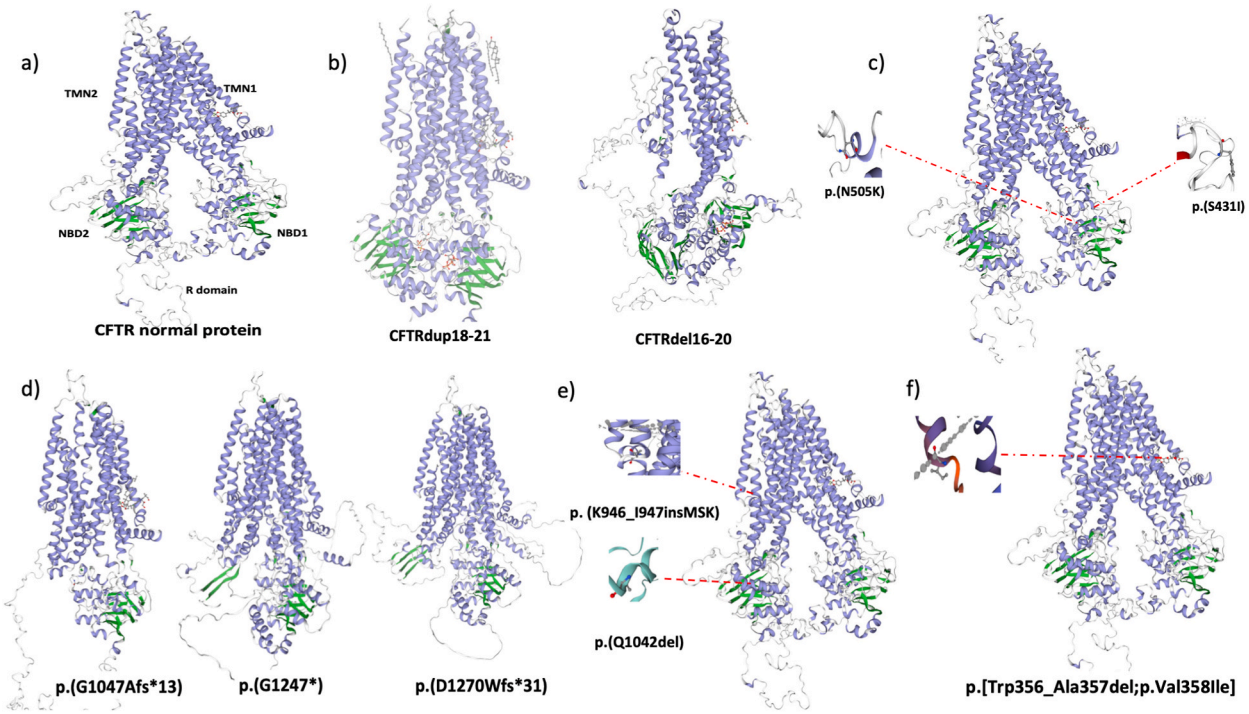

Fig. 3. 3D structural models of the CFTR proteins in patients carrying of novel variants. A) Normal protein b) Large rearrangements, c) Missense variants, d) Novel variants generating stop codons, e) Variants of type insertions-deletions, and f) Complex allele. Gray color represents affected segments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of PVs in *CFTR* [19]. To date, more than 2000 different variants have been identified, and their distribution is related to the ethnic background of the population. CFTR modulators have been introduced in the populations in which patients' PVs can be characterized, making them accessible to 50% of the world's population with CF, and even more so in those populations in which certain PVs, such as p.(Phe508del), are more frequent. The introduction of CFTR modulators represents a change from the standard prophylactic and reparative symptomatic treatment of CF [20].

The molecular diagnosis of CF was established in Mexico three decades ago however, the Mexican population includes a broad spectrum of causal variants of CF, being one of the most genetically heterogeneous populations in terms of the presence of PVs in *CFTR* [10]. This is because the Mexican population has a complex genetic structure due to admixture between European, Indigenous, and African populations [21]. As the molecular diagnosis of the disease in Mexico is very complex, and because Mexico is a middle-income country, we applied a combination of methodologies. As a first step, we screened for the five most frequent mutations by PSM [10,12], a low-cost tool that allowed us to identify more than 40% of the alleles. This step was followed by PV screening using NGS and MLPA. Our comprehensive analysis significantly increased the identification of CF-causing PVs in the Mexican population to 95 PVs, increasing the detection rate to 87.04% of CF alleles, though 13% of alleles remained unidentified.

In this research, we identified 10 novel variants. The use of *in silico* tools, together with the ACMG/AMP criteria, proved pivotal in predicting how these novel variants could impact the protein structure and classifying them all as pathogenic. Various reports have shown that these algorithms have an accuracy of 65–92% and can facilitate a deeper understanding of the correlation between a genotype and the clinical context of the patients [22]. A strength of our present study was the combined use of several tools, such as NGS, *in silico* analysis, and ACMG/AMP criteria, as well as the correlation of genotype with the patient's clinical manifestations, which made it possible to document the pathogenicity of the *CFTR* variants in a highly admixed population. In the future, one of the limitations of this study could be the complexity of detecting *CFTR* variants in a highly mixed population, as the vast majority of our country is middle-income and not all patients have access to NGS. Another limitation, it was a cross-sectional study, so we did not have follow-up of all patients.

In conclusion, the use of NGS tools is necessary to identify all of the PVs responsible for CF in a highly heterogeneous population, such as the Mexican population. The method used in our present study makes it possible to establish appropriate strategies for disease diagnosis, prevention, and treatment.

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Data availability statement

The list of pathogenic variants has not been deposited in a public database however all the variants are described in Table S1 included in the manuscript.

CRediT authorship contribution statement

Angélica Martínez-Hernández: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Elvia C. Mendoza-Caamal:** Validation, Methodology, Formal analysis, Data curation. **Namibia G. Mendiola-Vidal:** Visualization, Software, Resources, Formal analysis. **Francisco Barajas-Olmos:** Writing – original draft, Visualization, Software, Resources, Methodology, Data curation. **José Rafael Villafan-Bernal:** Software, Resources, Formal analysis, Data curation. **Juan Luis Jiménez-Ruiz:** Validation, Resources, Methodology, Investigation, Data curation. **Tulia Monge-Cazares:** Validation, Resources, Methodology, Investigation, Data curation. **Humberto García-Ortiz:** Writing – original draft, Validation, Software, Resources, Methodology, Data curation. **Cecilia Contreras- Cubas:** Writing – original draft, Visualization, Software, Resources, Methodology, Data curation. **Federico Centeno-Cruz:** Writing – original draft, Visualization, Software, Resources, Methodology, Data curation. **Carmen Alaez-Verson:** Validation, Methodology, Formal analysis, Data curation. **Soraya Ortega-Torres:** Validation, Data curation. **Adriana del C. Luna-Castañeda:** Supervision, Resources, Methodology, Investigation. **Vicente Baca:** Supervision, Resources, Methodology, Investigation. **José Luis Lezana:** Supervision, Resources, Methodology, Investigation. **Lorena Orozco:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28984.

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