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V232D mutation in patients with cystic fibrosis

Not so rare, not so mild

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Abstract

The frequency of some *Cystic Fibrosis (CF) Transmembrane Conductance Regulator gene (CFTR)* mutations varies between populations. Genetic testing during newborn screening (NBS) for CF can identify less common mutations with low clinical expression in childhood and previously considered mild but not fully characterized, such as the mutation p.Val232Asp (c.695T > A). The aim of this study was to describe CF patients with the V232D mutation. We identify CF children with the V232D mutation detected by NBS and compare them with CF adults with this mutation whose diagnosis was prompted by clinical symptoms in the same period. We studied clinical, biochemical, spirometric, and prognostic features in both populations. NBS program tested 276,523 children during a period of 14 years (2003–2017) and identified 54 cases of CF. Six children (11%) had the V232D mutation. Over the same period, 5 adults (age 37.6 ± 16.29 years old) with symptoms of CF and this mutation were also diagnosed. Follow-up duration was mean 10.1 years for adults and mean 6.5 years for children. In the adult group, lung function was impaired at diagnosis in all patients (Forced Expiratory Volume$_1$—FEV1—67.12% ± 13.09) and worsened in children tested during evolution (FEV1$_{first}$: 113%; FEV1$_{last}$: 64%). Pancreatic insufficiency was present in adult group, with recurrent pancreatitis in 1 present. Although with less clinical expression in children, V232D is associated with pulmonary and pancreatic involvement during adulthood and CF cannot be considered mild. This mutation is present in 11% of all patients diagnosed with CF in our region. Its inclusion in some NBS programs should be taken into account in order to improve the prognosis of affected children.

Abbreviations: BMI = body mass index, CF = cystic fibrosis, CFTR = cystic fibrosis transmembrane conductance regulator gene, CFTR1 = Cystic Fibrosis Mutation Database, CFTR2 = Clinical and Functional Translation of CFTR Database, FEV1 = forced expiratory volume at first second, IRT = immunoreactive trypsinogen, NBS = newborn screening, TM = transmembrane segment.

Keywords: CFTR, genetic testing, lung function, newborn screening, pancreatic insufficiency

1. Introduction

The cystic fibrosis (CF) transmembrane conductance regulator gene (*CFTR*; MIM no. 602421) consists of 27 exons, spanning approximately 250 kb on 7q31.2,[1] and encodes an anion transporter protein in the epithelium. This protein forms a chloride channel pore that plays a role in chlorine and bicarbonate transport[2] and has secondary effects on sodium transport. Its dysfunction leads to increased salt concentration in sweat and thickened secretions in various organ systems. To date, more than 1700 mutations and polymorphisms have been identified throughout the *CFTR* gene. The list of mutations is continuously updated in the CF Mutation Database (CFTR1)[3] and the Clinical and Functional Translation of CFTR database (CFTR2).[4] The most common *CFTR* mutation is F508del, which accounts for approximately 75% of all *CFTR* alleles in patients with CF. Its prevalence decreases as on moves from northwest to southeast Europe.[5] The remaining 25% of *CFTR* alleles are heterogeneous. In the CFTR1 database,[3] missense mutations account for 42% of all alleles, frameshift mutations for 15%, splicing mutations for 12%, nonsense mutations for around 10%, inframe insertions/deletions for 2%, large insertions/deletions for 3%, promoter mutations for 0.5%, and sequence variations not predicted to be disease-causing for 15%.

The frequency and distribution of *CFTR* mutations vary among populations. Very few mutations have a worldwide frequency above 0.1%,[5] but it is important to identify the correlation with symptoms or clinical expression.

Early detection of CF by newborn screening (NBS) enables the establishment of preventive measures and treatment before the onset of irreversible changes in the respiratory tract or other complications. NBS protocols vary across countries and the cost-effectiveness has been debated.[6] Protocols generally combined a

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first-tier strategy consisting of immunoreactive trypsinogen (IRT) testing with cutoffs at the 96th and 99.5th percentiles and a second-tier strategy consisting of a second IRT, pancreatic-associated protein testing or *CFTR* mutation analysis. *CFTR* mutation panels have also been proposed as third-tier test. Genetic testing during NBS can identify less common mutations that may have little clinical relevance in childhood but can cause lung and pancreatic problems later. The inclusion of these mutations in NBS programs could improve the prognosis of the disease in affected children.[7,8]

The broad phenotypic spectrum of CF is not explained by obvious genotype-phenotype correlations[5], and multiple additive effects may be responsible for *CFTR*-related disorders.[9] However, some mutations have been considered related to mild CF, but its phenotype has not been fully described and the frequency can be high in some regions. One such example is the p.Val232Asp (c.695T > A) mutation, not included in CFTR2 database despite being listed in CFTR1 and considered so far to cause *mild* CF.

The aim of this study is to characterize the clinical, biochemical, spirometric, and prognosis features of CF patients with the V232D mutation identified by NBS or genetic testing in adulthood.

2. Methods

2.1. Study population

The study population comprised children and adults with CF carrying the V232D mutation. The children were identified by NBS and the adults were diagnosed following genetic testing prompted by clinical symptoms of CF and abnormal sweat chloride test. The period of the study was from January of 2003 to December of 2017.

The following data were evaluated at diagnosis: sex, age at diagnosis, geographic origin, family history of CF, *CFTR* mutations in the other allele, sweat chloride levels, pulmonary colonization, and lung and pancreatic function. Follow-up test included anthropometric measurements, spirometry (percent of predicted forced expiratory volume in the first second—FEV1%), and biochemical tests including measurement of fat-soluble vitamin levels. Follow-up duration was mean 10.1 years for adults and mean 6.5 years for children.

The study was approved by the local ethics committee and informed consent was obtained from all patients and/or from their parents.

2.2. Analytical and anthropometric tests

NBS for CF was performed using dried blood samples collected for routine NBS. The first tier in the protocol was IRT with a standard cutoff of 70 ng/mL (this cutoff is calculated every day according to batch results). All patients with high IRT levels confirmed in a second batch underwent *CFTR* genetic analysis.

DNA was isolated using standard protocols from blood samples obtained from all patients and their parents in the case of children. Analysis of *CFTR* mutations was performed using mass spectrometry (Sequenom Inc, San Diego, CA) following an in-house assay using the Sequenom MassARRAY iPLEX system.[10] For carrier detection of V232D CFTR mutation, the exon 6a of the CFTR gene was amplified by polymerase chain reaction using the forward primer 5′-CTATGCATAGAGCAGTCCTG, and the reverse primer 5′-TTAGTGTGCTCAGAACCACG, with the following program: 2 minutes at 95 °C, 35 cycles of 60 seconds at 94 °C, 60 seconds at 55 °C, 60 seconds at 72 °C, and finally 7 minutes at 72 °C. Sequencing reactions were run using the same primers and the Big Dye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA) and the products analyzed on an ABI 3730XL capillary sequencer.

Nutritional status was assessed with the body mass index (BMI), calculated as weight (kg)/height squared (m^2), and Shukla Index, calculated as (actual weight [kg]/actual height [m])/(50th percentile weight/50th percentile height) × 100 using the World Health Organization growth standards as a reference.[11]

For the sweat chloride test, sweat was induced by pilocarpine iontophoresis and chloride concentration was measured on the Sweat Chloride Analyzer (Advanced Instruments, Inc., Norwood, MA) as previously described.[12] Lung function was evaluated by spirometry in adults and older children according to reference procedures.[13] Pancreatic function was investigated using the fecal pancreatic elastase enzyme-linked immunosorbent assay kit (Bioserv Diagnostics, Rostock, Germany), with normal value set at >200 μg elastase/g stool. Vitamin levels were determined by high-pressure liquid chromatography (Bio Rad, Hercules, CA). Normal values for children: vitamin D 20 to 100 ng/mL; vitamin A 0.2 to 0.49 mg/L; vitamin E 3.00 to 15.00 μg/mL. Normal values for adults: vitamin D 20 to 100 ng/mL; vitamin A 0.3 to 0.8 mg/L; vitamin E 5.00 to 18.98 μg/mL.

3. Results

A total of 276,523 newborns were included in NBS program from 2003 to 2017. During this period, 54 patients with positive genetic study were identified (Fig. 1). Six patients presented with high IRT levels and 2 *CFTR* mutations in both alleles, one of which was V232D. One patient moved to another country, so 5 patients were included in the study (Table 1). Over the same period, the V232D mutation was detected in 5 adult patients with varying clinical symptoms of CF and elevated sweat chloride levels (Table 2). All the patients were Caucasian. We divided the patients into 2 groups: adults diagnosed on the basis of typical symptoms (classic CF) and children diagnosed by NBS (asymptomatic CF at diagnosis).

3.1. Adults with clinical symptoms

The 5 adult patients with CF and the V232D mutation were diagnosed at a mean age of 37.6 ± 16.29 years old (median 33.0, range 26–66) (Table 2). CF had been suspected because of pulmonary disease in 4 cases. The fifth patient was tested because she had a sibling with CF. A family history of CF was common: 2 of the patients were siblings (patients 1 and 2) and a third had a cousin with CF (patient 5). Mutation analysis of the other allele revealed 4 different mutations. The F508del mutation was detected in just 1 patient (patient 5). Mean ± SD sweat chloride concentrations were 87 ± 21.94 mEq/L (median 81.0, range 63–110).

The 4 symptomatic patients had bronchiectasis at the time of diagnosis and were colonized by several opportunistic agents. Lung function was also impaired in the asymptomatic patient (patient 4). Mean FEV1% at diagnosis was 67.12% ± 13.09 (median 71.4, range 44–77, normal parameter for adults ≥80%). Patient 4 had undergone a left upper lobectomy for a lung abscess and patient 3 had undergone radiofrequency turbinate reduction. The patient with the worst lung function is currently being evaluated for a lung transplant (patient 3).

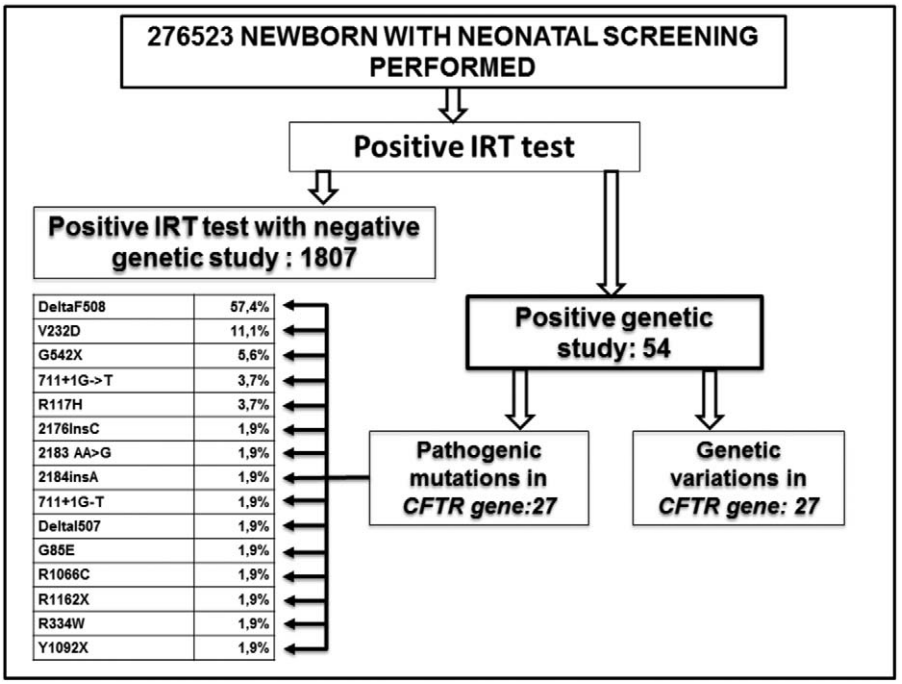

Figure 1. Cystic fibrosis mutations detected by newborn screening in Galicia, Spain (2003–2017).

Table 1

Characteristics of children diagnosed by newborn screening.

	Sex	Age at diagnosis (mo)	Follow-up time (y)	Mutation	IRT (mg/mL)	Sweat chloride (mEq/L)	Main colonizing agent	First FEV1%	Last FEV1%	Elastase (μg/g)	Vitamin D (ng/mL)	Vitamin A (mg/L)	Vitamin E (μg/mL)	BMI (kg/m^2) at diagnosis (*Z* score)	Last BMI (kg/m^2) (*Z* score)	SNI at diagnosis	Last SNI
1	F	6	6.7	c.1521_1523delCTT/ c.695T > A	68	80		116		>500	40.5	0.35	17.0	16.4 (0.37)	15.3 (0.22)	102.6	100.2
2	F	2	5.9	c.1624G > T/c.695T > A	101	98	*Streptococcus pyogenes* + *Candida albicans*			>500	27.0	0.27	10.8	17.7 (1.22)	19.3 (1.91)	125.0	128.2
3	M	23	5.0	c.1519_1521delATC/ c.695T > A	92	92		114		385	107.9	0.18	11.9	16.6 (0.51)	16.2 (0.53)	100.1	103.9
4	M	2	12.0	c.579 + 1G > T/c.695T > A	76	100	*Staphylococcus aureus*	113	64	>500	57.0	0.23	11.2	16.7 (0.52)	18.4 (0.48)	110.6	92.5
5	F	3	3.0	c.579 + 1G > T /c.695T > A	101	99				>500	40.0	0.30	10.7	14.1 (−1.99)	18.1 (1.03)	105.2	107.4

BMI = body mass index, F = female, FEV1 = forced expiratory volume at first second, IRT = immunoreactive trypsinogen, M = male, SNI = Shukla Nutritional Index (actual weight [kg]/actual height [m])/(weight P50/height P50) × 100.
*Normal values: FEV1 ≥ 80%; elastase >200 μg/g; fecal fat <2.00 g/24 h; vitamin D 20–100 ng/mL; vitamin A 0.2–0.49 mg/L; vitamin E 3.00–15.00 μg/mL.

Table 2

Characteristics of adult patients.

	Sex	Age at diagnosis (y)	Follow-up time (y)	Reason for genetic analyses	Mutation	Sweat chloride (mEq/L)	Main colonizing agent	First FEV1%	Last FEV1%	Elastase (μg/g)	Vitamin D (ng/mL)	Vitamin A (mg/L)	Vitamin E (μg/mL)	BMI (kg/m^2) at diagnosis (*Z* score)	Last BMI (kg/m^2) (*Z* score)	Observations
1	F	26	7.8	Respiratory	c.711 + 1G > T/c.695T > A	71	*Staphylococcus aureus*	71.4	78.8	500	23	0.42	13.2	19.0 (−1.02)	19.0 (−1.02)	Bronchiectasis + pancreatitis
2	F	33	6.9	Sibling with CF	c.711 + 1G > T/c.695T > A	81	*Staphylococcus aureus*	73.3	59.1	500	37	0.36	12.1	18.6 (−1.23)	19.6 (−0.76)	
3	F	35	22.0	Respiratory	c.1624G > T/c.695T > A	110	*Pseudomonas* sp. + *Streptococcus pneumoniae*	44.2	36.0	78	28	0.40	16.6	27.8 (−1.23)	27.4 (1.18)	Bronchiectasis + pancreatitis + radiofrequency turbinate + severe respiratory failure
4	M	66	4.3	Respiratory	c.4143C > A/c.695T > A	63	*Staphylococcus aureus*	77.0	86.0	225	9	0.45	19.3	28.2 (−1.26)	29.0 (1.39)	Bronchiectasis + left upper lobectomy
5	F	28	9.6	Respiratory	c.1521_1523delCTT/ c.695T > A	110	*Staphylococcus aureus*	69.7	77.1	500	11	0.29	13.4	21.8 (0.01)	21.8 (0.01)	Bronchiectasis

BMI = body mass index, CF = cystic fibrosis, F = female, FEV1 = forced expiratory volume at first second, M = male.
*Normal values: FEV1 ≥ 80%; elastase >200 μg/g; fecal fat <7.00 g/24 h; vitamin D 20–100 ng/mL; vitamin A 0.3–0.8 mg/L; vitamin E 5.00–18.98 μg/mL.

Two patients (4 and 5) had positive fecal fat values, and recurrent pancreatitis was present in 1 patient despite pancreatic insufficiency (elastase level <200 μg/g stool). All patients had adequate vitamin E levels (mean 14.9 ± 2.6 μg/mL, median 13.4, range 12.1–19.3); and variable vitamin D (mean 21.6 ± 10.5 ng/mL, median 23, range 9–37) and vitamin A levels (mean 0.38 ± 0.06 mg/L, median 0.40, range 0.29–0.45). There was no evidence of malnutrition. The BMI *Z*-score was above normal in 2 patients (3 and 4) and within the normal range in other 3.

3.2. Children diagnosed by NBS

Three of the 5 children with CF detected by NBS were girls and 2 were boys. Their mean age at genetic diagnosis was 7.2 ± 8.9 months (median 3.0, range 2.0–23.0). Their mean current age is 7.1 ± 2.9 years (median 6.9, range 3.2–12.2). Two of the children were siblings (patients 4 and 5). There was no other relevant family history. IRT levels were high in all cases during NBS (mean 87.6 ± 14.9 ng/mL, median 92, range 68–101). The mutation analysis of the other allele of the *CFTR* gene showed genetic variability, with detection of F508del, 711 + 1G-T, G542X, and I507del. All the patients had elevated sweat chloride levels (mean 93.8 ± 8.32 mEq/L, median 98, range 80–100).

Because of children's age, we were only able to obtain spirometry in 3 patients. The results of FEV1 in younger children were into normal values (116% patient 1 and 114% patient 3). The older child (patient 4) showed worsening of lung function, with first FEV1 113% and last test with FEV1 64%, during a period of time of 4.2 years (normal parameter at this age ≥80%). This patient is also currently colonized by *Staphylococcus aureus*. A second patient is colonized by *Streptococcus pyogenes* and *Candida albicans* (patient 2).

All the children had normal vitamin A levels (mean 0.26 ± 0.07 mg/L, median 0.27, range 0.18–0.35). Vitamin E levels were within the upper limit of normal, with a mean value of 12.3 ± 2.6 μg/mL (median 11.2, range 10.7–17). Vitamin D levels were near 30 ng/mL in all patients (mean 54.5 ± 31.7, median 40.5, range 27–107). Pancreatic function was normal in all children and consequently no patient had positive values of fecal fat. The current Shukla Index score is similar to that at diagnosis (106.4% vs 108.7%). Four of the patients are within normal limits and 1 has mild obesity (patient 2) (index 120% at diagnosis and 128% at present).

4. Discussion

This study analyzed clinical, biochemical, spirometric, and prognostic features of CF in a group of patients heterozygous for the V232D mutation detected by NBS or genetic testing prompted by clinical symptoms. Our findings shed light on factors that could potentially influence the inclusion of this mutation in NBS programs, such as clinical outcomes during adulthood.

NBS programs across Spain include screening for CF. In our region, NBS for CF started in 2003 and until 2017 had identified 25 cases of CF (1:11,061), 16 cases of inconclusive CF (1:17,283), and 13 cases of *CFTR*-related diseases, with a wide variability of mutations. V232D mutation was detected in 6 of 54 positive results for CF. This corresponds to 11% of all patients diagnosed with CF by NBS between 2003 and 2017. Overall, the V232D mutation is carried by 10 patients, which based on the total number of patients with CF in our region (n = 105), corresponds to a prevalence rate of 9.5%.

According to a study published by Alonso et al[14] in 2006 in the *CFTR* gene in patients of Spanish ancestry, this mutation presented with a frequency of 0.71% (n = 1954). A national monograph on CF published in 2005[15] described a prevalence of 0.51% for V232D in 780 Spanish families with CF. The frequency observed in our series suggests a higher prevalence of this mutation in northwest Spain. Familial aggregation factor (a pair of siblings in each group with exactly the same mutations) and a geographical location (all the adult patients were from 2 local areas) may of course have an important role in the observed frequency.

The V232D mutation has been previously described in the Brazilian population. In a study published in 2000, molecular analyses of 160 Brazilian CF patients with CF detected the mutation in 1 patient (0.62%), who also had the R334W mutation.[16] This association was not detected in our series. A more recent study of 37 patients with suspected CF in Brazil reported 2 cases of CF with the V232D mutation (5.4%).[17] Both patients had F508del in the other allele.

Regarding the frequency and clinical relevance of CF mutations, the V232D mutation has been described in more than 0.1% of *CFTR* mutations in the Spanish CF population and the Hispanic CF population in the USA.[5] According to the Exome Aggregation Consortium (http://exac.broadinstitute.org/).[18] However, this variant is only present in one of 33,364 European reference samples (1.499e−05 population frequency). Despite the scarcity of information on V232D in CF databases (it is not listed in CFTR2, for example), our findings show that this mutation must be considered a CF causing mutation and included in some NBS programs.

The V232D mutation involves a change from a neutral residue (Valine) to a polar residue (aspartic acid) that affects the fourth transmembrane segment (TM4) of the CFTR protein. It has been hypothesized that the aspartate residue forms a non-native hydrogen bond with Gln207 in TM3 to disrupt interactions between TM3 and TM4.[19] However, a more recent study[19] suggested that V232D might inhibit maturation of the protein through a mechanism of misfolding, that is, it appears that the V232D mutation introduces a hydrophilic residue into a hydrophobic pocket trapping the protein. This partially folded intermediate can be efficiently rescued with correctors.[20]

Although V232D may be specific to certain geographic areas and population groups, it cannot be considered "mild." All the adult patients in our series had impaired respiratory function and colonization by resistant microorganisms and such conditions cannot be considered typical of a mild mutation.[5,6,21,22] Between 10% and 50% of patients with idiopathic bronchiectasis have a *CFTR* mutation,[23] and in our series, CF studies were performed because of differential diagnosis of bronchiectasis in 4 of 5 adult patients.[24,25]

Lung disease in CF becomes more evident with age, but clinical and tests data suggest that it starts in early life. Two of the adults in our series (patients 3 and 4) had "nasal problems"[26] in childhood and the only child old enough to undergo various *spirometries* had impaired respiratory function before 5 years old. The detection of the V232D mutation by NBS in our population enables CF to be diagnosed before the onset of clinical manifestations and lung damage. Other studies of uncommon CF mutations[27] that go undetected by conventional screening have shown a similar clinical pattern (nasal problems in early life, severe bronchiectasis and colonization by resistant microorganisms). Studies specifically analyzing the V232D mutation,

however, have not described the pulmonary status of patients.[16,17]

Pancreatic involvement in CF can present in different forms, and it is more common with certain genetic patterns.[28–30] In a study of Brazilian population,[16] the V232D mutation was largely associated with pancreatic sufficiency, but the few other studies that have analyzed this mutation unfortunately did not describe pancreatic function. In our series, all the children had adequate pancreatic function, and one of the adults had pancreatic insufficiency. Recurrent acute pancreatitis, however, was observed in 2 adults, including the patient with pancreatic insufficiency. Acute pancreatitis has been reported in CF patients with pancreatic insufficiency,[31] although recurrent pancreatitis is more common in CF patients with adequate function.[32] Our results are consistent with reports in the literature that 20% of pancreatic-sufficient patients have chronic or recurrent acute pancreatitis which can progress to pancreatic insufficiency as a result of inflammatory injury.[33] The normal pancreatic function observed in almost all the patients in our series could explain why we detected no cases of malnutrition or vitamin deficiency.

Our study has some limitations. First, we have described a series of cases from a specific geographic region. Considering, however, the high frequency of this mutation in our community and the scarce data available on the V232D mutation, our description is relevant. Second, because of the children's age, we were only able to study lung function in 3 of the 5 patients diagnosed by NBS. Nevertheless, inclusion of the V232D mutation in the NBS protocol enabled early diagnosis and treatment before the onset of clinical manifestations and worsening of lung function.

In summary, we have described a series of adult and pediatric CF patients carrying the V232D mutation in which all the adults and 1 child had lung impairment. The high prevalence of this mutation in our population and the phenotype of pulmonary disease are important considerations. Description of genetic CF patterns and phenotypes in specific geographic areas can help to improve NBS programs and early diagnosis with the prognostic repercussion that this can imply.

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