

Beyond The Classical Cystic Fibrosis

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ABSTRACT

Cystic Fibrosis is the most common autosomal recessive genetic disease in Caucasian population and is caused by mutations in the CFTR gene (cystic fibrosis transmembrane regulator), detected for the first time in 1989. More than 2000 mutations in the CFTR gene have been identified; many of which are so rare as to be called ‘private’ as they are only present within individual families.

Typically, CF is characterized by elevated sweat chloride levels (**SCL**), obstructive lung disease, chronic bacterial infections of lower airways and sinuses, bronchiectasis and male infertility due to obstructive azoospermia.

Often, such cases have at least one CFTR mutation with a mild functional effect, the so called “Atypical CF”, which is a result of mutations of the CFTR gene.

Moreover, an increasing number of patients is diagnosed as CFTR-related disorders (**CFTR-RD**) generally characterized by a later onset of symptoms involving a single organ, usually associated to borderline SCL and mutations causing a different degree of CFTR protein dysfunction either in coding and in non-coding CFTR gene regions.

Accurate identification of CF mutations results in more applicable programs for prevention, diagnosis, and treatment of CF.

Extending knowledge about the molecular pathology on the one hand allows better delineation of the mutations in the CFTR gene and the other to dramatically increase the predictive power of molecular testing.

Keywords: Cystic fibrosis; Cystic fibrosis conductance transmembrane regulator; Congenital bilateral absence of the vas deferens; Mutation analysis; Polymerase Chain Reaction; Screening in infertile couples

Abbreviations: **CBAVD:** Congenital bilateral absence of the vas deferens; **CF:** Cystic fibrosis; **CFTR:** Cystic fibrosis conductance transmembrane regulator; **NBS:** Newborn screening

INTRODUCTION

Cystic fibrosis (CF; OMIM#219700) is the most common autosomal recessive genetic disease for the Caucasian (white) population.

In Italy, the disease occurs in 1/2500 to 1/3000 Caucasian newborns, with a carrier incidence ranging from 1/26 to 1/30 in the general population [1,2].

CF is a complex multisystem disease related to the buildup of thick, sticky mucus that can damage many of the body's organs (epithelia of the respiratory tract, exocrine pancreas, intestine, male genital tract, hepatobiliary system, and exocrine sweat glands).

The pulmonary disease is present in 90% of patients and it is the major cause of morbidity and mortality in CF.

The CFTR gene (CFTR; OMIM*602421) provides instructions for making a glycoprotein called cystic fibrosis transmembrane conductance regulator (1,480 amino acid residues).

This protein works as a channel across the membrane of cells producing mucus, sweat, saliva, tears, and digestive enzymes [3].

The channel negatively transports charged particles called chloride ions into and out of the cells.

The transport of chloride ions helps control the movement of water in tissues, which is necessary for the production of thin, freely flowing mucus. Mucus is a slippery substance that lubricates and protects the lining of the airways, digestive system, reproductive system and other organs and tissues [4]. In particular, more than 95% of men with CF are infertile as a result of azoospermia caused by congenital bilateral agenesis of the vas deferens (**CBAVD**); it occurs in men without pulmonary or gastrointestinal manifestations of CF [5].

Typically, CF is characterised by elevated sweat chloride levels (**SCL**), obstructive lung disease, chronic bacterial infections of lower airways and sinuses, bronchiectasis and male infertility due to obstructive azoospermia.

Most patients with CF have pancreatic insufficiency (**PI**) although 10–15% have normal exocrine pancreatic function and frequently show a milder clinical picture.

Often, such cases have at least one CFTR mutation with a mild functional effect [6].

Moreover, an increasing number of patients is diagnosed as CFTR-related disorders [7] generally characterised by a later onset of symptoms involving a single organ (ie, pancreatitis, disseminated bronchiectasis, obstructive azoospermia secondary to congenital bilateral absence of vas deferens [8], usually associated to borderline SCL and mutations causing a different degree of CFTR protein dysfunction either in coding [9–11] and in non-coding CFTR gene regions [12,13].

Finally, the growing proportion of newborn screening (**NBS**) programmes revealed a large number of infants having a Cystic Fibrosis Screen Positive, Inconclusive Diagnosis (**CFSPID**) including those infants with discordance between immunoreactive trypsinogen at the NBS, SCL, CFTR genotype and clinical phenotype [14,15].

Since 1989, it has become well known that CF is an ion channel disorder caused by mutations in the gene for the CF transmembrane conductance regulator (**CFTR**) [16].

The CFTR gene is located on the long (**q**) arm of chromosome 7 (7q31.2) [17,18].

More than 2000 mutations in the CFTR gene have been identified [19]; many of which are so rare as to be called ‘private’ as they are only present within individual families.

CF is characterized by wide variability of clinical expression with regard to disease severity and rate of progression. This is caused, at least in part, by (i) the large number of different mutations affecting the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, (ii) the existence of modifier genes and (iii) environmental factors, such as viral or bacterial pathogens, that influence disease phenotype [7,20,21].

Moreover, the CFTR mutation detection rate varies by test method and ethnic background.

There has been a surprising degree of difficulty encountered worldwide in establishing the diagnosis in a minority of cases and because of this, healthcare providers continue to be faced with uncertain cases and challenging diagnostic dilemmas.

Although the diagnosis of CF has traditionally relied on recognition of characteristic clinical signs and symptoms, the increased use of prenatal population screening for maternal CF carrier status, prenatal ultrasound screening (that might reveal meconium ileus, meconium peritonitis, bowel obstruction, or echogenic bowel), and NBS has resulted in the routine diagnosis of asymptomatic or minimally symptomatic infants and a consequent opportunity to foster their normal growth and development [22].

The most common mutation, called F508del, is a deletion of one amino acid at position 508 in the CFTR protein. The resulting abnormal channel breaks down shortly after it is made, so it never reaches the cell membrane to transport chloride ions.

The F508del mutation, accounts for two-thirds of all cystic fibrosis alleles worldwide.

This mutation is particularly frequent in people of northern European ancestry (70% in Anglo-Saxon countries and 50% in the Mediterranean area).

Carrier screening for cystic fibrosis involves analysis for common mutations in the CFTR gene from people with no personal history, or family history, of the disease.

This analysis shows whether a person is a carrier, at risk (one in four) of having a baby with cystic fibrosis if their partner is also a carrier.

Carrier screening has been recommended by the American College of Medical Genetics and American College of Obstetricians and Gynecologists (**ACMG/ACOG**) [23] and the Human Genetics Society of Australasia (**HGSA**) [24] and has been established in the USA, Australia, and parts of Europe [25].

Accurate identification of CF mutations results in more applicable programs for prevention, diagnosis, and treatment of CF. In a study carried out in some areas of Northern Italy, carrier screening was associated with a decrease in the incidence of CF [26].

In CF, contrary to other genetic diseases (for example beta thalassemia), the identification of carriers is possible only through molecular research of CFTR mutations.

A recent report has shown the costs of managing CF for the Italian National Health System [27].

The results reports a high cost for the chronic and evolutive aspects of the disease. As mentioned before, molecular screening of CF is highly recommended in the USA by the National Institutes of Health Consensus Development Conference Statement on genetic testing for cystic fibrosis [28].

The target of the screening could be men and women of reproductive age in the periconceptual period.

A European consensus conference in 2009 has concluded that for complete information for couples, each national health system could implement a screening program.

In Italy, the region of Veneto started a complete screening program for the general population some years ago.

The results have shown a significant reduction of the incidence of the disease. More recently, an experimental periconceptual program of CF and thalassemia screening allowed for the identification of 94% of CFTR gene mutations, with respect to 80% in the rest of Italy.

Moreover, this screening program involved a high number of couples [29].

The success of a genetic screening program is mainly the outcome of educational support informing the population about the disease, thereby offering the opportunity to make an informed decision about reproduction.

We conducted a study with the aim to establish the a priori risk of CF carrier status in the Basilicata population and to offer a reliable screening test to couples planning a pregnancy, allowing them to make informed decisions.

MATERIALS AND METHODS

To each patient after a venous blood sample was collected (in EDTA-K3) we applied the following tests.

Molecular analysis of the CFTR gene was performed following these steps:

-DNA isolation, starting from 25µl of blood, using the Promega extraction kit (DNA IQ™ System, cod. C6701; Promega Italy S.r.l., Milan, Italy).

-Polymerase chain reaction (**PCR**) and reverse hybridization.

The procedure includes two steps:

PCR amplification using biotinylated primers and hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines.

Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

The amplification and the reverse hybridization on a strip were obtained with the use of commercial kits produced by Nuclear Laser Medicine S.r.l., Settala (**MI**), Italy (cod. AC023/AC025 and AC089): genetic tests aimed at checking 60 mutations in the CFTR genes (F508del, I507del, F508C, I502T, 1706del17, 1677delTA, G542X, 1717-1G>A, R553X, Q552X, G551D, S549R(A>C), 2183AA>G, N1303K, 4016insT, 3849+10kb C>T, G85E, 711+5G>A, 711+1G>T, 2789+5G>A, W1282X, G1244E, 621+1G>T, R117H, 852del22, T338I, R347P, 1259insA, R1066H, L1065P, L1077P, D1152H, 4382delA, R1162X, R1158X, S912X, I148T, 3199del6, D110H, H139R, G178R, 991del5, R334W, R347H, R352Q, 1717-8G>A, D579G, 1874insT, E585X, 1898+1G>A; 1898+3A>G, 1898+5G>T, 2184insA, 2522insC, 3120+1G>A, R1066C, 3272-26A>G, 3659delC, G1349D, 4015delA, 14b-17b del; Ex2del; Ex1del; 22,23,24del; 22,23del; 2,3del; 17a, 17b, 18del. *PoliT: 5T, 7T, 9T.*)

The test has sensitivity and a specificity of more than 99%. With a direct analysis of 60 mutations of the CTFR gene, with reverse dot blot, it is possible to detect 90% of the most common CF alleles in Southern Italy (the regions of Campania, Puglia, Basilicata and Molise).

- The patients who tested negative or with a single mutation detected by reverse dot blot and with a clinical suspicion of atypical cystic fibrosis were analyzed with a complete scanning of the codificant region, through amplification and direct sequencing of 27 exons of the CFTR gene. In patients negative for reverse dot blot and in whom there were no clinical signs of cystic fibrosis, sequencing of the CFTR gene was not necessary. DNA sequencing is not essential, since the detection of innocent polymorphisms is not important to control the disease.

RESULTS

In the 964 infertile couples examined, 132 subjects (69 women and 63 men) were heterozygous for one of the mutations of CFTR, with a carrier occurrence of 6.85%.

The carrier occurrence in infertile couples is significantly higher than the hypothetical value of the general population (4%). The statistical analysis was performed according to the 'hypothesis test for a proportion'. Our data indicated an expectation of 6.85% with a sample of 1,928 subjects.

The Z-test revealed a value of 6.38% with a p value <0.0001. Nevertheless, mutation I148T, at first classified as a mutation, now is considered an 'innocent' polymorphism, able to be pathogenic only if present in the same gene (on the same chromosome) with another polymorphism (3199del6).

Hence it is necessary, in presence of polymorphism I148T, to detect the presence of polymorphism 3199del6.

The polymorphism 3199del6 was not detected in all patients with the I148T variant.

Among the infertile couples, three men, two of whom were brothers, were azoospermic with CBAVD.

The molecular analysis of the CFTR gene revealed that the two brothers, aged 26 and 29 years old respectively, were both 'compound heterozygotes G542X/D1152H', while the third one, aged 37 years old, and was 'compound heterozygotes G542X/E831X'.

With regard to the 76 patients with cystic fibrosis and a positive sweat test, all have two CFTR-mutated genes.

This study shows that, in the Basilicata region (Italy), it was observed that the CFTR phenotype is caused by a small number of mutations.

The knowledge of the frequency of mutations prevalent in CFTR genes in a specific geographic region, can allow the development of kits for their detection.

Extending knowledge about the molecular pathology, on the one hand, allows better delineation of the mutations in the CFTR gene, and on the other, dramatically increases the predictive power of molecular testing.

ATYPICAL CYSTIC FIBROSIS: CASE REPORT

Besides the classical form of severe disease, other clinical forms of CF have been identified and called “atypical” forms. These one show monosymptomatic phenotype (recurrent pancreatitis, congenital bilateral agenesis of the vas deferens, asthma, bronchiectasis) and benign prognosis than the classic form of CF [30].

Recently, the studies on the genetics of CF have followed different directions, showing that there are a number of proteins that interact with the CFTR increasing up to 6 times the activity, like the SLC26 carrier family. Mutations in these genes may result in a defective activation of CFTR [9].

The identification of individuals with atypical cystic fibrosis can sometimes present particular difficulties of interpretation.

For this reason, if there is a concrete clinical suspicion of CF, it is always advisable, to perform the biochemical study by the sweat test, followed by sequencing of the CFTR gene.

The diagnostic pathways in Assisted Reproductive Technology (**ART**) Centres are aimed at finding the causes of infertility in couples and to implement procedures to solving the problem.

A couple came to our attention to do these exams: Kario typing and CFTR molecular screening such as preliminary exams for assisted reproduction cycle.

Pelvic ultrasonography, performed to the lady, showed the presence of a retroflexed uterus, with a normal profile, echostructure and dimensions. Her endometrium had a normal echographic aspect. Both her right and left ovary were normal with respect to dimension and form, without any liquid effusion.

A hysterosalpingogram confirmed the normal uterus-tubal anatomy. Serum anti-ovarian and anti-adrenal antibodies were absent.

On third day of the menstrual cycle the patient had normal levels of gonadotropins (LH: 6.4 IU/L and FSH: 7.3 IU/L). Her thyroidstimulating hormone, free tri-iodothyronine and free thyroxin hormone levels were normal, while the levels of anti-thyroid peroxidase antibodies and anti-thyroglobulin antibodies were very normal.

The husband had regular pubertal development, absence of cryptorchidism, absence of sexually transmitted diseases or signs of genital inflammation and normal endocrine function.

By history the gentleman reported to suffer from chronic sinusitis and who had previously undergone two operations for nasal polyposis.

Specialised diagnostic tests showed that the man did not suffer of bronchitis or bronchopneumonia. The biochemical pancreatic and hepatic parameters were within normal limits (normal values of steatocrito and fecal elastase). Urological examination showed bilateral absence of the vas deferens. Two semen collections also revealed azoospermia.

For these reasons, were performed the following tests:

Analysis of the karyotype of both spouses. Karyotype was obtained from T lymphocytes extracted from peripheral blood using the common culture technique. The obtained chromosomes were banded with Q-banding methods using quinacrine.

Sweat test (Gibson and Cooke). Made only to the husband for the presence of azoospermia and bilateral agenesis of the vas deferens.

Molecular analysis of the CFTR gene was performed following these steps:

- DNA isolation: starting from 25 µl of blood venous collected in EDTA-K3.
- Polymerase Chain Reaction (**PCR**) and reverse hybridization: The test has sensitivity and a specificity of more than 99%. With a direct analysis of 60 mutations of the CFTR gene (commercial kits produced by Nuclear Laser Medicine S.r.l., Settala (**MI**), Italy).

The patients who tested negative or with a single mutation detected by reverse dot blot and with a clinical suspicion of atypical cystic fibrosis were analyzed with a complete scanning of the codificant region, through amplification and direct sequencing of 27 exons of the CFTR gene.

Both spouses had a normal karyotype. The sweat test resulted positive (>60 mmol/L) for the husband. From a sweat collection of 198 mg, we found Na⁺ concentration of 99 mmol/l and Cl⁻ concentration of 118 mmol/l. The molecular analysis by reverse hybridization confirmed that the husband appeared heterozygous for G542X mutation (c.1624G>T; p.Gly542X). Since the man had clinical signs compatible with a framework of atypical cystic fibrosis, it was necessary the sequencing of the complete CFTR gene.

The sequencing of the gene revealed a second mutation, exactly the E831X (c.2491G>T; p.Glu831X). For that reason the man resulted to be compound heterozygote (G542X/E831X) for the CFTR gene. The woman was not carrier of one of the 60 mutations studied by reverse dot blotting. To prevent that the lady could be a carrier of a mutation not included in the 60 studied, we also proceeded for her to the sequencing of complete CFTR gene that confirmed the absence of mutations.

In this way we have explained to the spouses that the possibility of conceiving a son or daughter affected from CF is zero; and that all the children will be carriers of the mutation G542X or mutation E831X.

The genetic structure found in the husband, shows a very good chance to find sperm in the testicles. Consequently spouses may try to become biological parents, undergoing a cycle of assisted reproduction (**IVF**).

This study wants to underline that the identification of individuals with atypical cystic fibrosis can sometimes present particular difficulties of interpretation.

On that ground, if there is a strong clinical suspicion, it is always advisable the biochemical study by performing the sweat test, followed by sequencing of the CFTR gene.

P.LEU636PRO MUTATION IS ASSOCIATED WITH CBAVD: CASE REPORT

CBAVD is found in 1–2% of infertile men, whereas CBAVD is present in approximately 99% of males with CF.

Both CF-causing mutations and CFTR mutations other than the common mutations have been identified in CBAVD patients, and these mutations showed extreme heterogeneity in spectrum and frequency. In CBAVD, the most common mutations are p.Phe508del (allele frequency 17%), IVS8-5 T (allele frequency 25%) in cis with longer IVS8-TG repeats (IVS8-TG12 or TG13) and p.Arg117His (allele frequency 3%) in cis with IVS8-7 T or IVS8-5 T.1 These three alleles account for all but a small fraction of CFTR mutations observed in CBAVD, and the remaining mutations were detected in just a few cases. In general, among CBAVD patients, 78% had at least one CFTR mutation, 46% having two and 28% only one [31-34].

We describe the case of a 36-year-old Caucasian man who was evaluated because of infertility and familiarity for CF (a daughter of his sister was diagnosed with CF by newborn screening with p.Phe508del homozygosity). The patient provided written informed consent with guarantees of confidentiality. The patient's clinical history had infertility as the only remarkable symptom.

Infertility was a result of obstructive azoospermia caused by CBAVD, as diagnosed by an andrologist, in the absence of other anomalies of the urogenital tract. Sweat testing resulted in Cl⁻ levels of 47 and 49 mEq/L in two separate tests (normal value <40; pathological value >60). Genetic analysis (reverse dot blot followed by DGGE and sequencing) showed the presence of the p.Phe508del mutation in heterozygosity with p.Leu636Pro mutation. The IVS8-Tn alleles were 7 T/9 T. To assess the compound heterozygosity, we analyzed the genotype of the sister of the patient, an obligate carrier, who in fact had only the p.Phe508del mutation. Chest radiograph and spirometry were normal (FVC 6.21 L [112% of predicted], FEV1 5.04 L [118%], FEF25–75 7.02 L/s [148%]). The cultures of induced sputum were negative. Stool analysis showed normal values of fecal elastase. Assessment results justified a diagnosis of CFTR-RD with the phenotype of isolated CBAVD and we advocated regular annual follow up visits for physical examination and follow up testing at the CF center.

Genetic counseling was also recommended for the couple, who were attempting assisted reproduction technology [35].

To the best of our knowledge, this is the first report on the phenotype associated with the rare CFTR mutation p.Leu636Pro in compound heterozygosity with a CF-causing mutation in an adult. Indeed, the mutation p.Leu636Pro, a missense mutation, whose effect on CFTR function has never been studied, was previously reported only in a female adolescent with diffuse bronchiectasis and no other stigmata of CF [19].

On the basis of the clinical features (isolated CBAVD with borderline sweat chloride level and no evidence of lung disease), we postulate that p.Leu636Pro is a mild CFTR mutation associated with CFTR-RD. No functional tests for CFTR analysis were carried out because of patient refusal, so we made in silico analysis of p.Leu636Pro mutation effect on CFTR protein by the PANTHER software [34].

The PANTHER score was -2.63 , corresponding to a 41% probability that the variant will cause a deleterious effect on protein function.

These data, along with the presence of the severe p.Phe508del mutation, could postulate a reduction of CFTR activity associated with the phenotype of CBAVD with sweat chloride levels intermediate between those of patients with pancreatic sufficiency and carriers [36].

Understanding of the CFTR gene has permitted relationships between genotype and phenotype to be made, and the knowledge of the natural history of patients with rare genotypes and late diagnosis might be very useful to physicians (i.e. urologist, andrologist, pulmonologist) attempting to guide patients, when young adults are identified with the genetic potential for CF or CFTR-RD. Asymptomatic or oligosymptomatic individuals identified with a genetic background for CF, even if mild, should be regularly monitored at the CF centers for disease progression, and for better knowledge of resulting phenotypes in order to improve the information discussed at the time of clinical and genetic counseling [32].

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