

Cystic fibrosis, molecular genetics for all life

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From the womb to the adult

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Abstract

Cystic fibrosis (CF) is the most frequent lethal autosomal recessive disorder among Caucasians (incidence: 1:2,500 newborn). In the last two decades CF prognosis considerably improved and many patients well survive into their adulthood. Furthermore, milder CF with a late onset was described. CF is a challenge for laboratory of molecular genetics that greatly contributes to the natural history of the disease since fetal age. Carrier screening and prenatal diagnosis, also by non-invasive analysis of maternal blood fetal DNA, are now available, and many labs offer preimplantation diagnosis. The major criticism in prenatal medicine is the lack of an effective multidisciplinary counseling that helps the couples to plan their reasoned reproductive choice. Most countries offer newborn screening that significantly reduce CF morbidity but different protocols based on blood trypsin, molecular analysis and sweat chloride cause a variable efficiency of the screening programs. Again, laboratory is crucial for CF diagnosis in symptomatic patients: sweat chloride is the diagnostic golden standard, but different methodologies and the lack of quality control in most labs reduce its effectiveness. Molecular analysis contributes to confirm diagnosis in symptomatic subjects; furthermore, it helps to predict the disease outcome on the basis of the mutation (genotype-phenotype correlation) and mutations in a myriad of genes, inherited independently by CF transmembrane conductance regulator (*CFTR*), which may modulate the clinical expression of the disease in each single patient (modifier genes). More recently, the search of the *CFTR* mutations gained a role in selecting

CF patients that may benefit from biological therapy based on correctors and potentiators that are effective in patients bearing specific mutations (personalized therapy). All such applications of molecular diagnostics confirm the “uniqueness” of each CF patient, offering to laboratory medicine the opportunity to reposition the patient in the “core” of the medical process.

Keywords

CFTR, mutations, prenatal diagnosis, genotype-phenotype correlation, modifier genes.

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Introduction

Cystic fibrosis (CF) is a chronic, systemic disease and the phenotypic expression generally includes altered sweat chloride, pancreatic insufficiency, and pulmonary inflammation and colonizations that gradually lead to respiratory insufficiency [1]. Less severe forms of CF that appear with pancreatic sufficiency, normal or borderline sweat test and single-organ involvement are included under the umbrella term of CF transmembrane conductance regulator (*CFTR*) related disorders (*CFTR*-RDs). The most known are congenital bilateral absence of vas deferens, recurrent or chronic pancreatitis and disseminated bronchiectasis [2].

In the last decades the outcome of CF patients considerably improved as it is true for their quality of life thanks to the improvement of early diagnosis by newborn screening and advances in care that include physical activity and personalized dietary correction. So, CF is no longer a pediatric disease since up to 80% of patients reach their adulthood [1], but a series of complications typical of adult CF patients appear with increasing frequency including osteoporosis, diabetes, malnutrition, severe lung disease with colonization by resistant pathogens and liver disease. In this scenario, a continuous

coordination of care among specialists, including clinical biochemists is mandatory.

CF is a recessive disease due to the reduced function of an ATP-dependent chloride channel expressed by most epithelial cells. The search of *CFTR* mutations is widely used to detect, by means of different technical approaches, the about 2,000 mutations identified in the *CFTR* disease-gene so far (www.genet.sickkids.on.ca). Molecular analysis allows the identification of about 80% alleles from CF patients if the most frequent mutations are tested [3], but the detection rate is much lower in *CFTR*-RD [4]. The detection rate for *CFTR*-RD alleles does not increase when mutations peculiar to the patient's ethnic-geographic group are included in the test [5, 6]. Large gene rearrangements, identified in about 2-3% of CF alleles are very rare in *CFTR*-RD [7]. Finally, pathogenic mutations in non-coding region of the *CFTR* gene have been described [8, 9], but they are not currently analyzed in the routine setting.

Neonatal screening: a great advantage for CF outcome

Except for 10% of patients diagnosed at born for meconium ileus (MI), in most cases CF is diagnosed for symptoms at a mean age of 3 to 4 years and up to 10% of patients are diagnosed > 18 years. Newborn screening for CF is supported by clinicians and scientific societies to early identify still asymptomatic patients and start treatments that may prevent long-term complications. Newborn screening also reduces costs of care. Experiences from all countries unequivocally demonstrated that the early diagnosis corresponds to a great improvement of quality of life (mainly in terms of nutrition and pulmonary function) and survival. In fact, in most countries newborn screening for CF is now performed on all newborns [10]. The currently available laboratory approaches for CF newborn screening include the Guthrie test, i.e., the immunoreactive trypsinogen (IRT) on dried blood performed in the third day of life, the analysis of *CFTR* mutations and sweat test. However, different protocols may be used for the screening and this causes a different efficiency of the program [10, 11]. Since the IRT gives about 2% of false positives, some centers assess again the IRT in the 20th day of life in cases positive to the first IRT, while in other centers all positive IRT cases are tested for mutations. However, molecular analysis has a detection rate of about 80% if panels of mutations are tested and reaches higher diagnostic sensitivity using gene sequencing, but this latter approach frequently

identifies novel mutations of uncertain pathogenic significance. Finally, most centers perform the sweat test on IRT positive newborn. The different combination of the three analytical approaches has a good performance in terms of diagnostic sensitivity but in several patients the discordant results between IRT, molecular analysis and sweat test causes difficulties in reporting and novel classifications are now used to define discordant patients like “*CFTR*-related metabolic syndrome”, “equivocal CF diagnosis” or “CF screening–positive inconclusive diagnosis” [12].

Furthermore, different cut-off are adopted for IRT with a variable diagnostic sensitivity and specificity of the screening; in particular some programs privilege a high diagnostic sensitivity to detect also a percentage of *CFTR*-RD or mild CF, but the benefit to early identify these forms is discussed. In addition, the screening is not adopted in all countries/regions and this may cause difficulties to the pediatricians in countries where a high immigration from less developed countries is present. Finally, only few laboratories participate to adequate quality control programs [13].

Carrier screening: still few evidences support its effectiveness

Given the high incidence of asymptomatic CF carriers (i.e., 1:25), recommendations for carrier screening or population screening have been proposed by several scientific societies and colleges of physicians [14, 15]. The carrier screening is aimed to identify high-risk couples before the birth of a CF child in the general population. The efficacy of CF screening program depends on the possibility to identify the CF carrier status of each partner, which helps to determine the risk for the fetus. The screening of couples can follow two approaches: the female partner is screened first, and if she is revealed as CF carrier, then the male partner is tested; otherwise both partners are screened concurrently. The main limit of the population screening is the low diagnostic sensitivity of molecular analysis (that is the lone approach to reveal asymptomatic carriers). As previously discussed, panel of mutations have a detection rate of about 80% of CF alleles, while gene sequencing (that may reach a sensitivity of 95%) is too expansive. Thus, in all couples negative to the test the residual risk (i.e., the probability that the members still carry a copy of a *CFTR* mutation each despite negative testing) should be clarified to the couple through an adequate multidisciplinary

counseling [15]. Similarly, the aim of screening, the voluntary nature of screening, medical and genetic issues surrounding CF and its prevalence of CF, the interpretation of the test results should be clarified to each couple during the counseling. However, given the high number of couples to be included in a population screening program it is difficult to offer an effective counseling to all couples. For these reasons, even if some preliminary experiences in limited geographical area demonstrated that the carrier screening contributed to reduce the incidence of CF, such program is not still adopted in any country. Otherwise, a number of heterozygote couples can be identified following the birth of an affected child or through the offer of *CFTR* mutation analysis to relatives of patients, i.e., cascade screening [16].

Prenatal diagnosis: an opportunity for a reasoned reproductive strategy in high-risk couples

The increase in couples at high-risk for CF increased the demand for prenatal diagnosis (PD). In fact, PD helps high-risk CF couples to make informed decisions regarding reproductive strategies and to avoid unnecessary terminations and irreversible pre-conception strategies that a high number of couples with an affected child adopted before the availability of PD [17]. For example, before the availability of PD for CF in our region, only 11 of 150 (7.3%) couples that had a CF child initiated another pregnancy and in eight cases they opted for voluntary interruption. Similar results were reported by others [17]. After 1993, 149 of the 250 couples with a CF child monitored in our regional center planned a further pregnancy and asked for 181 PDs (which resulted in the birth of 139 non-CF children).

PD can be performed on DNA from fetal cells obtained by amniocentesis (second trimester) or by chorionic villi (CV) sampling in the first trimester [18]. Non-invasive approaches have been described based on the analysis of fetal DNA in maternal blood [19]; these procedures are still poorly standardized. Preimplantation diagnosis is also available [20], but in some countries the legislation does not permit such approach.

From the analytical point of view PD requires a series of steps that include [17]: i) pre-test multidisciplinary counseling [18]; ii) the knowledge of the mutations of the family (usually the affect proband); iii) the sampling and the check of the absence of maternal contamination of fetal tissue (by microscope); iv) the analysis of a set of short tandem repeats (STR) to confirm the purity of fetal

DNA and to confirm paternity (since fetal tissue is usually tested only for the mutations identified in the family); v) the analysis of the mutations in the fetal sample (better if using two independent techniques). If the mutations of the family are not known, a set of intragenic STR can be tested for linkage diagnosis; vi) reporting and counseling.

The pancreatic status influences most CF symptoms. It may be corrected by enzyme replacement

About 90% of patients with classic CF have pancreatic insufficiency. Molecular analysis permits to predict the pancreatic status, since there are several mutations (classified as mild) that are associated to CF with pancreatic sufficiency (PS), while other mutations (classified as severe) are associated to insufficiency (PI). This classification is now under revision, because some mutations usually considered mild may cause PI. For example, in a recent multicentric study we demonstrated that the *D1152H* mutation (considered a mild mutation) may cause PI in about 5% of CF patients [21]. It is mandatory to well define the pancreatic status in CF patients because the early enzyme supplementation permits to reduce malnutrition, and consequently improve growth, pulmonary function and survival. The biochemical golden standard to define the pancreatic status is the secretin-pancreozymin test that is invasive (i.e., duodenal intubation and EV injection of stimulants) and cumbersome. In alternative, most centers offer the quantitative fecal fat test that has an acceptable diagnostic efficiency provided that the patient perform a 72-h fecal collection and a care dietary record (particularly difficult in infancy). In the last decade, fecal elastase gained a relevant diagnostic role thanks to the poor invasivity and reduced costs and to a diagnostic sensitivity and specificity of about 95% [22, 23]. The main limit is the scarce diagnostic sensitivity for the mild PI. However, considered that the pancreatic status in CF patients may change, an acceptable workflow may be the analysis of fecal elastase every year.

In addition to PI, CF patients may experience recurrent pancreatitis that may evolve to chronic pancreatitis enhancing the risk of pancreatic cancer. Usually, the risk for pancreatitis is higher in CF patients with PS and serum lipase is the golden standard to identify the acute attack [24], while serum amylase (still performed by most laboratories lacks of diagnostic specificity). Recently, it is

emerging that the risk of pancreatitis in CF patients may depend on a genetic predisposition involving mutations in genes inherited independently of the *CFTR* [25]. Thus, in a future scenario such genes will be tested to define the individual risk of CF patients to experience pancreatitis.

Modifier genes: a myriad of genes may influence the CF clinical course, but their analysis is still far from a routine context

The clinical expression of CF and its complications are strongly heterogeneous: meconium ileus at birth affect about 15% of newborns; a severe CF liver disease appears in 10-20% of cases; the *CFTR* related diabetes appears in about one third of patients; pancreatitis appears in 20% of cases (mostly in patients with pancreatic sufficiency). Similarly, the pulmonary expression of the disease spans from cases with a severe insufficiency in the first decade (with multiple pulmonary colonizations) to cases that reach their adulthood with an excellent pulmonary function. Once identified the disease-gene in 1989, the clinical discordance of the disease was related to the effect of the *CFTR* mutations. In fact, most *CFTR* mutations are grouped into classes according to the effect they exert on protein synthesis, trafficking or activity [26]. Although life-expectancy, the pancreatic status and the severity of the disease differ depending on the class of mutations [27], there is a wide clinical heterogeneity in CF patients carrying the same *CFTR* genotype [28], and even in siblings and twins with CF [29, 30] suggesting a role of other genetic or environmental factors. In the last decade various studies explored the putative role of modifier genes predisposing to a severe CF pulmonary or liver phenotype, meconium ileus and diabetes [31-33] using two approaches [34]: i) wide genome association studies; ii) the approach of candidate gene. The North American CF Gene Modifier Consortium studied more than 3,400 CF patients with the gene wide association approach; the European CF Twin and Sibling Study focused on twins and siblings with different clinical expression; dozens of other authors analyzed specific genes (candidate gene approach). This mass of studies concluded that a myriad of genes may influence the clinical expression of CF but each in a small percentage of patients. Thus, the analysis of all such genes is still far from a routine diagnostic context. On the other hand, environmental factors, such as the quality of health care, compliance to therapy [35], lifestyle, and the socio-demographic,

cultural, and family context [36] may play a pivotal role in the outcome of CF, and may impact on the genotype-phenotype correlation.

Personalized therapies in CF patients

The molecular mechanism through which some *CFTR* mutations cause CF is known in detail. For example, class 2 mutations impair the maturation of the protein and its transport from the endoplasmic reticulum to the plasma membrane. The *F508del* (i.e., the most frequent CF mutation) causes both a trafficking and a gating defect. Class 3 mutations like the *G551D* strongly reduce the time of the *CFTR* channel in the open state (gating defect).

With parallel to the knowledge of the pathogenic mechanism of *CFTR* mutations, in the last decade drugs that may specifically correct these alterations were studied [37]. At the state of the art, two main groups of molecular drugs are under study, i.e., the correctors and potentiators [38]. Correctors act as pharmacological chaperones by interacting with the *CFTR* protein bearing mutations that cause a misfolding, and facilitate the folding and cellular trafficking of the protein. Potentiators increase the chloride gating activity of the *CFTR* channel with a wide range of mechanisms that include the R-domain phosphorylation, the restoring of *CFTR* defective cAMP regulation and the ATPase activity. Kalydeco™ (also known as Ivacaftor or VX-770), is a potentiator developed by Vertex Pharmaceuticals, recently approved by the US FDA and by the European Medicines Agency (EMA) for the treatment of CF patients carrying at least one *CFTR* allele with the *G551D* mutation. The same drug may also be effective on other class 3 mutations like *G1244E*, *G1349D*, *G178R*, *G551S*, *S1251N*, *S1255P*, *S549N* or *S549R*. While, the corrector VX-809 significantly improves *F508del* *CFTR* trafficking *in vitro*, and is now under study in clinical trials. A series of studies are now focusing on other molecules that may correct or potentiate the *CFTR* protein activity and the scenario of the next few years could be a treatment specifically targeted on the basis of the *CFTR* mutation. In this scenario, molecular analysis will gain the further role of therapy guidance.

Declaration of interest

The Authors declare that there is no conflict of interest.

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