Abstract

Background: In Sardinia the mutational spectrum of CFTR gene is well defined. A mutation detection rate of 94% can be achieved by screening for 15 CFTR mutations with a frequency higher than 0.5%. The efficiency of this molecular test suggests that Sardinians may represent a suitable population for a preconceptional screening.

Methods: Five hundred couples of Sardinia descent were screened for 38 mutations using a semi-automated reverse-dot blot and PCR-gel electrophoresis assays. This mutation panel included the 15 most frequent CF alleles in Sardinia.

Results: We identified 38 CF carriers, revealing an overall frequency of 1/25 (4%). The most common CF allele was the p.Thr338Ile (T338I) (65%), followed by the p.Phe508del (F508del) (22.5%). We also identified one couple at risk and an asymptomatic female homozygote for the p.Thr338Ile allele.

Conclusions: In spite of the low number of the couples tested, the results herein reported demonstrate the efficacy and efficiency of the preconceptional screening program and the high participation rate of the Sardinian population (99%).

© 2011 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.

Keywords: Cystic fibrosis; Preconceptional screening; Genetic counseling; Genetic testing

1. Introduction

The prerequisites suggested for a prospective approach for control of recessive genetic disease through heterozygote screening include the following: high severity of the disease, high prevalence in a defined population, the availability of simple and accurate methods for carrier identification, as well as of genetic counseling services to inform individuals and couples at risk about the disease and the reproductive choices.

Differences in disease frequency can play a role in decisions on screening for specific disorders, and countries with similar disease prevalence often reach different decisions about the appropriateness of it.

In the early 1990s, most genetic screening programs were at the pilot stage, with a few notable exceptions. For example, screening programs had been established for β-thalassemias [1] and Tay–Sachs disease [2] in certain sub-populations. Since then policy developments, advances in scientific understanding and technological developments have led to the introduction of screening tests for several other pathologies. Currently, cystic fibrosis (CF, MIM 219700) represents one of the most frequent diseases worldwide object of screening.

More than 1700 sequence variations have been identified in the CFTR gene (MIM 602421), causing both cystic fibrosis and...
other associated phenotypes, which are collectively referred to as CFTR-related disorders (CFTR-RD) (http://www.genet.sickkids.on.ca/cftr/). These mutations often have geographic or population variations in frequency. Because CF carriers are asymptomatic, they can only be identified by the detection of mutations in the CFTR gene.

The US National Institutes of Health, the American College of Obstetricians and Gynecologists, and the American College of Medical Genetics have recommended widespread CF genetic screening of couples that are either planning a pregnancy or in the early stages of pregnancy [3–5]. The different mutational spectra associated with each specific populations cause problems with the identification of CF carriers that complicate the screening process as well as pre- and post-test genetic counseling. For example, only 6 or 7 mutations account for over 95% of all CF chromosomes in Ashkenazi Jews [6], and 15–32 mutations are responsible for over 85% of all CF chromosomes in most northern Europeans. In southern European populations, however, up to 90 or more mutations need to be screened to obtain a 90% mutation detection rate in CF patients [7,8].

This extensive heterogeneity in the distribution of CFTR gene mutations in European populations makes the goal of a mutation detection rate of 92–93% very hard to achieve. In general, such screens should be designed to have high sensitivity and the ability to detect all CF-causing mutations with a frequency greater than 1% in the local population.

In Italy, the mean detection rate of the Italian CF alleles is about 86% [9–11].

We recently reported the molecular characterization of the large majority of Sardinian CF alleles (175/176) [12]. A mutation detection rate of 94% can be achieved by screening for 15 mutations with a frequency higher than 0.5% in the Sardinian population. The high diagnostic efficiency of the molecular CFTR test suggests that Sardinians may represent a suitable population for preconceptional CF screening.

In this study we report the results of a pilot preconceptional carrier screening of CF in Sardinian couples, with no family history of CF. The aim was to establish the a priori risk of CF carrier status in the Sardinian population and to offer a reliable screening test to couples planning a pregnancy allowing them to make informed decisions. Furthermore, Sardinian population, intensively involved and sensitized in the carrier screening of β-thalassemia, carried out successfully since the late 1970s, has facilitated the organization of this survey.

2. Patients and methods

2.1. Patients

A total of 505 couples were enrolled from a group of people who had voluntarily requested hematological screening for β-thalassemia at the Genetic Screening and Counseling Service, Thalassemic Regional Hospital, Cagliari.

All couples were of Sardinian descent, either planning a pregnancy or in the early stage of pregnancy (3–10 weeks), without family history of CF or CFTR-related disorders. The individuals ranged in age from 18 to 54 years.

Five hundred couples, for a total of 1000 individuals, agreed to participate in the molecular screening.

Prior to the collection of blood samples, the couples were informed about the availability of the pilot study and then asked to fill out an informed consent. The informed consent, approved by the Local Ethic Committee of the Hospital, provided information about the clinical aspects of CF, carrier screening tests, the risk of having an affected child if both partners were found to be carriers, and the consequences of a negative test result.

Participants were invited to fill in a self-administered questionnaire in order to assess impact and understanding of the CF screening among them. The questionnaire included multiple choice questions on their knowledge of CF and their reproductive intentions within the next 2 years.

2.2. Methods

DNA was extracted from 400 μl of peripheral blood using Biomek NX (Beckman Coulter, California, USA) according to manufacturer’s protocol.

The samples were screened for the 15 most common Sardinian CF mutations [12], allowing an allele detection rate of 94%. The Cystic Fibrosis Nuclear Laser kit (Nuclear Laser Medicine, Italy), which screens by a reverse dot-blot assay 36 CF Italian mutations at once, included 13 of the 15 common Sardinian mutations. Hybridization was performed using Profiblot T48 (Tecan, Switzerland) according to the manufacturer’s protocol.

The remaining two Sardinian mutations, the c.54-5811_164+2186del18108ins182 (exon 2 deletion) and the p.Asn287-LysfsX19 (991del5) were detected by PAGE analysis [12].

Four patients with anomalous hybridization signal required an extensive molecular analysis at both DNA and RNA levels. Sequencing analysis of exon 10 and its flanking regions was performed using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, California, USA) and the sequencing reactions were performed on an ABI PRISM 3130XL (Applied Biosystems).

RNA was extracted from nasal epithelial cells collected using cyto-brush from patients and a non-CF control subject. cDNA synthesis was performed using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s instructions. The cDNA spanning exons 8–12 was amplified, and the resulting PCR products were resolved on 2% agarose gel, and sequenced as described above.

If one of the partners in a couple was diagnosed as a carrier, the other, testing negative for the 94% of mutations identified in this screen, was further analyzed by a multiple ligation probe amplification assay (MRC-Holland, the Netherlands), to exclude the possibility of large CFTR gene rearrangements.

2.3. Mutation nomenclature

The gene variants at the protein level were named as recommended in the Human Genome Variation Society (HGVS) web page (http://www.hgvs.org/mutnomen/). For
variation described at the nucleotide level, the A of the ATG translation start codon was numbered as +133 in accordance with the current CFTR gene numbering based on cDNA sequence (GenBank NM_000049.2) and on the CF mutation database. These variations were also given in parentheses following the approved nomenclature format (A of the ATG translation start codon as +1).

2.4. Statistical analysis

Data are reported as counts and percentages. Comparison between qualitative variables was performed by using Chi-square test.

3. Results

This study involved 500 couples of Sardinia descent, who had no family history of CF and were either planning a pregnancy or in the early stages of pregnancy (3–10 weeks of gestation). They were enrolled from a group of patients requesting voluntary screening for β-thalassemia. The majority were from the south of Sardinia (77.8%), with the remainder from the middle (20.1%), and north (2.1%) part of the island.

The questionnaire was returned by 700 out of 1000 participants (70%). Among this group, 42% had not previously heard about CF. The remaining individuals who knew about the disease received their information from advertising promotions/mass media (60.9%), general practitioner/gynecologists (8.6%), and other sources (30.5%) (Table 1).

The main reason given by couples for participating in the screening program was that they perceived as serious the risk associated with being a carrier of cystic fibrosis and with having a child with CF. Seventy per cent of couples (350/500) completed the questionnaire, demonstrating a positive impact to the screening program. Among these, 200 (57.1%) were planning to have a child in the next 2 years.

All the couples were screened for 38 mutations (Table 2), using a reverse dot-blot assay for 36 and a PCR-gel electrophoresis assay for the other 2 (c.54-5811_164+

<table>
<thead>
<tr>
<th>Table 2</th>
<th>List and frequency (%) of CF mutations included in the screening test.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation nomenclature</td>
<td>Alleles (%)</td>
</tr>
<tr>
<td>T338I (p.Thr338Ile)</td>
<td>26 (65.0)</td>
</tr>
<tr>
<td>F508del (p.Phe508del)</td>
<td>9 (22.5)</td>
</tr>
<tr>
<td>N1303K (p.Asn1303Lys)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>2183A→G (c.2051_2052delAAinsG)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>621+1G→T (c.489+1G→T)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>exon 2 del (c.54-5811_164+2187del8108ins182)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>R347P (p.Arg347Pro)</td>
<td>1 (2.5)</td>
</tr>
</tbody>
</table>

The most common CF allele was the p.Thr338Ile mutation described at the nucleotide level, the A of the ATG translation start codon was numbered as +133 in accordance with the current CFTR gene numbering based on cDNA sequence (GenBank NM_000049.2) and on the CF mutation database. These variations were also given in parentheses following the approved nomenclature format (A of the ATG translation start codon as +1).

2.4. Statistical analysis

Data are reported as counts and percentages. Comparison between qualitative variables was performed by using Chi-square test.

3. Results

This study involved 500 couples of Sardinia descent, who had no family history of CF and were either planning a pregnancy or in the early stages of pregnancy (3–10 weeks of gestation). They were enrolled from a group of patients requesting voluntary screening for β-thalassemia. The majority were from the south of Sardinia (77.8%), with the remainder from the middle (20.1%), and north (2.1%) part of the island.

The questionnaire was returned by 700 out of 1000 participants (70%). Among this group, 42% had not previously heard about CF. The remaining individuals who knew about the disease received their information from advertising promotions/mass media (60.9%), general practitioner/gynecologists (8.6%), and other sources (30.5%) (Table 1).

The main reason given by couples for participating in the screening program was that they perceived as serious the risk associated with being a carrier of cystic fibrosis and with having a child with CF. Seventy per cent of couples (350/500) completed the questionnaire, demonstrating a positive impact to the screening program. Among these, 200 (57.1%) were planning to have a child in the next 2 years.

All the couples were screened for 38 mutations (Table 2), using a reverse dot-blot assay for 36 and a PCR-gel electrophoresis assay for the other 2 (c.54-5811_164+2187del8108ins182 and the p.Asn287LysfsX19) mutations included in the CF panel were not detected in the population tested. Sardinian mutations leading a detection rate of 94% are in bold.

**Table 1**

Knowledge questionnaire: overall results.

| Knowledge questionnaire completed | 462 (66.0) |
| Knowledge questionnaire not completed | 238 (34.0) |
| Total | 700 |
| Knowledge about CF |  |
| No | 194 (42.0) |
| Yes | 268 (34.0) |

By mass media | 163 (60.9) |
By general practitioner/gynecologists | 23 (8.6) |
By school/university | 21 (7.8) |
By occupation (high school teachers, physicians, nurses, etc.) | 17 (6.3) |
By friends/relatives previously screened | 17 (6.3) |
By acquaintance with a CF patient | 12 (4.5) |
Others | 15 (5.6)
An asymptomatic 43 year old female homozygous for the p. Thr338Ile (T338I) allele was also identified (Table 3) whose parents, both T338I heterozygotes, were first cousins. The genotype was confirmed by sequencing analysis of exon 7 of CFTR gene. She was referred to the Regional Reference Centre for Cystic Fibrosis for clinical evaluation. The sweat chloride concentration was 93.7 mmol/L. Clinical lung and pancreatic involvement was absent and the woman reported severe dehydration in summer resulting from abnormal loss of sweat electrolytes. During the genetic counseling session, she did not show concern about her condition.

A total of 38 CF carriers were identified, resulting in an overall frequency of 1/25 (4%).

A medical report and details of the genetic test was reported to the participants during the post-test genetic counseling session. The residual risk for having an affected child was calculated for all the couples (Table 3). In addition, all subjects who were diagnosed as carriers were invited to inform their relatives about the opportunity to be tested for CF (cascade screening).

### 3.1. Difficulty to detect the p.Gln525LeufsX37 (1706del17) allele

An extensive molecular analysis either at DNA and RNA level was performed on four patients because they completely lacked a hybridization signal for the p.Gln525LeufsX37 mutation in exon 10, such that neither the wild type nor the mutated alleles were detected. To determine the reason for the assay failure, exon 10 and its flanking regions were sequenced. These four individuals resulted homozygous for both the wild-type p.Gln525LeufsX37 allele and the mutated c.1584G>A (1716G>A) allele. The latter is a synonymous variation which prevents the p.Gln525LeufsX37 probes from annealing to the DNA. In heterozygote state, this variation has been reported to cause exon 10 skipping and be associated either with idio-pathic chronic pancreatitis or CBAVD [13,14]. To define the effect of this mutation in the homozygote state on the CFTR transcript, RNA from two of these individuals was analyzed. PCR spanning exons 8–12 of the cDNA resulted in two PCR products of 650 and 458 bp respectively. Sequence analysis showed that the 650 bp fragment contained five correctly spliced exons, while the 458 bp fragment lacked exon 10 completely. A control individual not having this mutation completed the normal 650 bp fragment, as expected. To date, this variation is considered to have no clinical consequences [15]. In our study, the c.1584G>A variant has a frequency of 0.4%.

### 4. Discussion

The success of a genetic screening program is mainly a product of the educational support that fully informs the population about the disease, thereby offering them the opportunity of making an informed decision about reproduction. The thalassemia screening program that has been performed in Sardinia since the late 1970s is an example of a successful genetic screening program [1].

Following this model of success, we report a pilot preconceptional carrier screening of CF in Sardinian couples, with no family history of CF. The aim was to establish the a priori risk of CF carrier status in the Sardinian population and to offer a reliable screening test to couples planning a pregnancy who wanted to know their risk of conceiving a CF child. This CF molecular screening test was offered to 505 couples enrolled from among the individuals voluntarily requesting screening for β-thalassemia, and 500 (99%) accepted. Given that 38 CF carriers were identified out of 1000 subjects with no previous history of CF, the overall carrier frequency observed in the general Sardinian population was 1/25 (4%).

Our study showed that the most frequent mutation in Sardinia is the p.Thr338Ile (T338I) (65%), a mild mutation previously described by our group in Sardinian CF patients either in homozygous state (11 patients) or in compound heterozygous state with other severe mutations (18 patients) [16,17]. All the patients are pancreatic sufficient with a sweat definitely positive.

In both groups age at diagnosis ranged from 2 months to 40 years. Patients homozygous for the T338I mutation are often asymptomatic otherwise showing a very mild phenotype. Clinical manifestation of patient compound heterozygotes for T338I and other severe mutation are slightly more severe ranging from isolated azoospermia, with or without CBAVD, to mild CF phenotype characterized by a mild to moderate pulmonary involvement. However in both homozygotes and compound heterozygotes patients, the increased sweat electrolyte concentration may lead, especially in warm-arid climates and in infancy, to sporadic episodes of severe hypotonic dehydration associated with hyponatremia, hyponatremia, metabolic alkalosis, that often represent the moment in which the diagnosis is made. Moreover seven patients belonging from both groups (3 patients T338I/T338I, 2 patients T338I/F508del, 1 patient T338I/2183AA>G, 1 patient T338I/R1066H), affected by azoospermia with no other CF symptoms, were occasionally identified when they requested CF genetic test for assisted reproduction technologies (ART) (Rosatelli, personal communication).

There is statistically significant difference between the frequency of the p.Thr338Ile (T338I) allele (65%), highlighted in the present study, and the findings of our previous study on Sardinian CF patients (19.3% (p = 0.0001) [12]. Conversely, the difference between the frequency of the p.Phe508del (F508del) mutation in CF Sardinian patients (50%) and in the

### Table 3

Overall results of preconceptional CF screening in general Sardinian population.

<table>
<thead>
<tr>
<th>n (%)</th>
<th>Risk to have a CF child*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrolled couples</td>
<td>500</td>
</tr>
<tr>
<td>Couples at risk</td>
<td>1(0.2)</td>
</tr>
<tr>
<td>One spouse CF carrier</td>
<td>36 (7.2)</td>
</tr>
<tr>
<td>One spouse homozygote for a CF mutation</td>
<td>1'(0.2)</td>
</tr>
<tr>
<td>Negative couples (sensitivity of 94%)</td>
<td>462 (92.4)</td>
</tr>
</tbody>
</table>

* Population risk: 1/2500.

b p.Phe508del/c.489+1G>T (F508del/del) or c.489+1G>T).

c Genotype: p.Thr338Ile (T338I) homozygote.
general Sardinian population (22.5%, this study) is not statistically significant (p=0.375) (Fig. 1) [12]. As alluded above, this discrepancy is likely due to the mild phenotype of patient p.Thr338Ile (T338I) homozygotes or compound heterozygotes which can escape a diagnosis.

Due to the high frequency of a mild mutation resulting in mild or asymptomatic phenotypes, careful management and genetic counseling are of utmost importance. In the counseling session, patients as well as couples at risk should be exhaustively informed about the asymptomatic or mild phenotype of the T338I allele. On the other hand they have to be informed particularly about the risk of acute episodes of metabolic alkalosis in infancy.

Our test, using an RDB assay comprising 36 different mutations in combination with PAGE analysis, has been shown to be a robust screening test for the evaluation of CF carrier status and the molecular diagnosis of affected individuals. Most children affected by CF are born to parents who were not aware that they could have a CF child; preconceptional carrier screening remain thereby central to inform the couples about reproductive choices.

The results of this pilot screening are encouraging as demonstrate the efficacy and efficiency of the preconceptional screening program and the high participation rate (99%) of the Sardinian population.

Conflict of interest statement

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence their work.

Acknowledgments

This work was supported by the Italian Cystic Fibrosis Research Foundation (grant FFC# 5/2008) with the contribution of “C.M.A.E. Club Milanese con Dompè Farmaceutici; Net Center Padova; Sky Italia s.r.l”.

The authors would like to thank Dr Francesca Sessini, Dr Angelo Ideo, and Prof Paolo Moi from the Genetic Screening and Counseling Service, Thalassemic Regional Hospital, Cagliari for genetic counseling; Antonella Tibbio, Francesca Culeddu, and Gianna, from the Genetic Screening and Counseling Service, Thalassemic Regional Hospital, Cagliari for collecting the samples; and Maria Demurtas from the Molecular Genetic Laboratory, Thalassemic Regional Hospital, Cagliari for technical support.

References


Fig. 1. Mutational spectra of CFTR gene in CF patients vs individuals screened in Sardinia. Frequency of F508del mutation in CF patients vs general population p=0.375. Frequency of T338I mutation in CF patients vs general population p=0.0001.