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Original Research

Cystic Fibrosis assessment in infertile couples: genetic analysis trough the Next Generation Sequencing technique

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Abstract

Background: Cystic Fibrosis (CF) is a genetic disease which is responsible for different systemic conditions. In particular, CF could be responsible for infertility, especially in the male partner due to congenital bilateral absence of vas deferens (CBAVD). Moreover, in Assisted Reproductive Techniques CF screening is performed in order to detect possible risks for the newborn. For this reason, CF testing is one of the main genetic screening performed in infertile couples. **Methods**: In this scenario, we present a prospective observational study in CF testing with Next Generation Sequencing (NGS) technique on 360 subjects referring to an *In-Vitro* Fertilization center. **Results**: 360 subjects were screened for CFTR. Of them, 19 subjects presented CF causing variants, 44 subjects presented CFTR-RD associated, 22 subjects had variants of uncertain significance and 19 subjects with no clinical consequences. **Conclusion**: Results clarify proportions of the main CF mutations. Actually, there are no more advanced techniques rather than Next Generation Sequencing (NGS) technique, although it is not yet widely used as a test for the identification of the CF carrier.

Keywords: Cystic Fibrosis; Infertility; Next Generation Sequencing; *CFTR*; IVF

1. Introduction

Cystic Fibrosis (CF) is an autosomal recessive genetic disease with a frequency of 1:2500 in the Caucasian population; in Italy the incidence is 1:4500 [1,2]. This condition is caused by mutations in the gene that codes for the protein called *CFTR* (Cystic Fibrosis Transmembrane Conductance Regulator) expressed and functioning in the apical portion of the membrane of epithelial cells and which regulates the secretion of chlorine, sodium and bicarbonate ions in epithelial tissues [3]. The disease involves numerous organs and systems: the respiratory system, from the upper airways to the lung tissue, the pancreas in the production of digestive enzymes, the liver, the intestine and the reproductive system, especially the vas deferens in males. The gene that codes for the CFTR protein is located on chromosome 7 and is composed of 27 exons. About 2000 variants in the *CFTR* gene have been identified, although fewer are responsible for the disease. To date, circa than 400 appear to be the cause of the disease [4,5]. Moreover, thanks to the new generation techniques, the number of variants described in the worldwide databases is progressively increasing.

CFTR variants are grouped into classes that reflect their functional consequences; those that lead to loss of cell surface gene expression or loss of function are generally "severe" mutations associated with a phenotype with lung disease and pancreatic insufficiency. The final clinical phenotype of CF patients depends on the combination of the genetic variants [6].

In fact, there are forms of classical disease with heavy clinical manifestations but also "mild" forms of digestive symptom, like pancreatitis and even *CFTR*-related disorder in which, for example, the only sign of disease is bilateral agenesis of the vas deferens with consequent sterility in the healthy male (CBAVD). The most frequent severe mutation is F508del. The relative frequency of *CFTR* variants is highly variable in relation to the geographic area. Molecular analysis of the *CFTR* gene is of fundamental importance in order to identify CF carriers. For this reason, it is the subject of continuous studies aimed at improving the sensitivity and accuracy of genetic tests. All those individuals at greater risk of being carriers (blood relatives of the affected) and the partners of individuals who are already CF carriers

are subjected to molecular analysis of the *CFTR* gene. In Medically Assisted Procreation, the CF testing is performed for couples in some conditions. In particular, when individuals present signs or symptoms CF correlated (like CBAVD positive male partner) CF is tested to help diagnosis and provide genetic counselling [7]. For example, signs of obstructive azoospermia in spermiogram (when azoospermia, sperm with acidic pH (<7.2), low volume of semen, and very low level of fructose are detected) and other situations such as Pre-implantation Genetic Diagnosis for couple with CF risk with or without male infertility require *CFTR* gene analysis in Medically Assisted Procreation (MAP) context [8,9]. Secondly, CF is tested also in asymptomatic individuals with the purpose of genetic counselling approach in elective population in order to reduce the risk of CF occurrence in the offspring. Population eligible for CF screening could include siblings, CF carrier partner, and people undergoing Assisted Reproductive Techniques (ART) treatment as well. Various types of molecular biology tests have been developed in order to identify the variants responsible for Cystic Fibrosis and those most commonly used have a detection rate of about 85% (in technical terms, detection rate, or diagnostic efficiency) [10]. In clinical practice, we can distinguish between variants that cause CF disease, variants that result in a *CFTR*-related disorder, variants with no known clinical consequence, and variants of unproven or uncertain clinical relevance. The first two groups can overlap since, for example, CF variants can hamper pancreatic sufficiency. These terms are heavily important in the counselling with the infertile couple. The clinical relevance and the consequences of the gene findings should be explained both for the individual, the future newborn, and for his/her family. Attention should be given to the information provided, which should be clear and concise, as too much information may confuse the couple [11].

The aim of our work was to improve the detection rate of *CFTR* gene variants in the infertile population through the genetic study of couples seeking medically assisted procreation, through the use of the Next Generation Sequencing (NGS) technique. The main advantage is the ability to produce a large volume of data at lower costs, more rapidly, and to increase diagnostic efficiency.

2. Material and methods

This is a prospective observational study assessed on infertile couples entering the *In Vitro* Fertilization (IVF) Center of the University of Palermo. Every patient involved in the study signed an informed consent for participating to the study. The CF analysis is part of the routine diagnosis tests assessing the infertility and every patient signs an informed consent form also for the usage of data for academic purposes. Ethical review and approval were waived for this study, due to the design of the study, according to our ethical committee guidelines. Genetic analysis was performed on 360 patients who had to undertake a path of medically assisted procreation. Peripheral blood in ethylene diamine tetra acetic acid-containing (EDTA) collection tubes was used to extract genomic DNA (standard phenol-chloroform method and Salting-out extraction method). Subsequently, the NGS technique was used on the Ion Torrent PGM platform.

The *CFTR* panel with 102 amplicons was used for library preparation. Library preparation and Ion Torrent sequencing were performed according to standard procedures detailed in the manufacture's guideline and previous publication [12,13]. The Ion Ampliseq Library Kit Plus, Ion PGM™ HI-Q View OT2 and Ion PGM™ HI-Q View Sequencing Kit v2 chemistry were used (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). All sequences were aligned to the human reference genome sequence and were analyzed with the Ion Torrent Software Suite (Vs. 3.6) (Thermo Fisher Scientific, Waltham, MA, USA) using the plug-in variant caller (Vs. 3.6.43647). To confirm the variants identified in NGS, we utilized the Sanger sequencing analysis.

The DNA was amplified using the polymerase chain reaction (PCR): PCR mix (50 μL) contained 200 ng of genomic DNA, 1.5 μL of 10 pmol primers, 5 μL of buffer 10x, 1.5 μL of $MgCI^2$ 50 mmol, 1 μL of 40 mmol dNTP and 2.5 U of Taq polymerase enzyme (Invitrogen Corp., Carlsbad, CA, USA). PCR products were analyzed on SYBR-Safe 3% agarose gel and displayed to the ultraviolet lamp. PCR products were sequenced bidirectional directly using BigDye terminator 3.1 cycle sequencing kit and run-on ABI PRISM 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Primers used for PCR and sequencing were designed in our laboratory (available on demand).

To identify deletions causing CF (*CFTR* dele2ins182, *CFTR* dele1; *CFTR* dele 22, 23, 24; *CFTR* dele 22, 23; *CFTR* dele 2,3, *CFTR* dele 17a, 17b, 18; *CFTR* dele 14b–17b), we used the FC DEL kit (Nuclear Laser Medicine SRL) based on multiplex PCR and reverse hybridization on the strip [14,15].

To analyze the combination between the poly-T tract and the TG tract (usually TG11, TG12, or TG13) (difficult to detect by NGS methods) we used Sanger sequencing.

Specific databases were used to understand the causative role of the identified variants (CFTR2, http://cftr2.org; http://www.genet.sickkids.on.ca; http://cftr.iurc.montp.inserm.fr/cftr/) and the main bioinformatics tools for prediction, or in silico, which calculate the risk that the variant is pathogenic by carrying out various assessments (polyphen, sift, mutation tasting, splice site finder, CADD, Varsome).

3. Results

Next Generation Sequencing was used for the genetic analysis of couples who had to undergo medically assisted procreation (MAP) protocols.

We carried out, through NGS, the genetic analysis of *CFTR* gene on 360 subjects of Caucasian origin who had to undergo MAP. From this analysis, through the use of reference databases such as *CFTR2* (https://www.cftr2.org) and tools for prediction, we identified several variants of which some pathogenic and others of uncertain significance (VUS) classified using the specific databases (See **Supplementary Material**). This approach allows us to identify 11% of couples at risk out of 125 couples who attended our laboratory. Of these we considered 6 (4.8%) couples at risk for CF: in one couple there were two CF causing variants, in three couples the combination of the variants gave a genotype with variable consequences, while in two couples the combination of the variants gave a genotype with unknown consequences. 8 (6.4%) couples at risk for CFTR-RD had one carrier of a variant that causes the disease or a variant with variable consequences and the other carrier had a CFTR-RD variant.

Rare and unknown *CFTR* variants have been identified. In most cases it was possible to clarify the pathogenetic nature of some variants by consulting both the reference databases and the prediction tools, in other rare cases the nature of the nucleotide variants remained uncertain (See **Supplementary Material**).

We have screened 360 subjects of which 19 subjects presented CF causing variants, 44 subjects presented CFTR-RD associated, 22 subjects had variants of uncertain significance and 19 subjects with no clinical consequences. No subjects had the TG13-5T allele. Among the CF causing variants we found 12 (3.3%) subjects with F508del (c.1521_1523delCTT), 2 (0.5%) with 2789 + 5G >A (2657 + 5G >A), 1 (0.25%) with R347P (c.1040G >C), 1 (0.25%) with 4382delA (c.4251delA), 1 (0.25%) with G542X (c.1624G >C), 1 (0.25%) with P5L (c.14C >T) and 1 (0.25%) with Y38X (c.114C >G).

Instead the individuals with CFTR-RD 20 (5.5%) were carriers of TG12-5T, 6 (1.6%) subjects with L997F (c.2991 G >C), 4 (1.1%) with R1162L (c.3485G >T) and 4 subjects had complex allele (G576A; R668C), 2 (0.5%) with R668C (c.2002C >T), 2 (0.5%) with F1052V (c.3154T >G), 2 (0.5%) with P750L (c.2249C >T), 1 (0.2%) with R74Q (c.221G >A), 1 (0.2%) with V201M (c.601G >A), 1 (0.2%) with R297Q (c.890G >A) and 1 (0.2%) with L967S (c.2900T >C). The other variants of uncertain significance and with no clinical consequences, according to the prediction tools, were not reported.

4. Discussion

The aim of our work was to improve the detection rate of *CFTR* variants in the infertile population through the use of the NGS technique. Furthermore, the combined use of NGS technology and commercial kits for the study of deletions has increased the detection rate up to 97%. This approach, used by our laboratory, made it possible to identify rare and sometimes even unknown mutations. In fact, thanks to the latest generation technology it was possible to identify in a heterozygous patient, the Y38X variant not described in *CFTR2* and *CFTR*-France, not included in the commercial kits which is a null (non sense) variant that can be considered as “disease causing”. In addition, the use of the combined test, compared to the use of other genetic tests, applied to subjects belonging to the infertile population, has made it possible to reduce the residual risk of both being a CF carrier and having a child affected by the disease. Therefore, a subject who tests negative for CF causing-variants with a third level test that has a diagnostic efficiency of 97%, has a risk of being a CF carrier of about 1 in 968 (0.10%). The couple comprised of a CF carrier and a subject with a residual risk of being a carrier of 1 in 968, has a risk of having a child with Cystic Fibrosis estimated to be around 1 in 3871 while in a couple where both tested negative the risk becomes of about 1 in 3,745,515. To date, there are no more advanced diagnostic techniques that allow greater precision and exclude the risk of having a child with CF. Additionally, no commercial method allows us to identify all the mutations of the *CFTR* gene. The NGS technique is widely used over the world by genetic laboratories for CF and *CFTR*-RD diagnosis. Bioinformatical tools are now available at a lower cost in most countries. Nevertheless, this technique is currently little used in healthy people (except in CF partners for those extensive CFTR analysis is recommended) because of the high frequency of rare variants in this gene, the high number of those with unknown significance, and sometimes, the lack of experts in their interpretation.

This represents a limitation when carrying out genetic counselling as it is extremely difficult to communicate to the patient the doubt about the variant found. Moreover, in some cases, when genetic counselling is addressed to couples who should undergo MAP, calculating the risk of having a child affected by the classic form of the disease becomes problematic, both when only one of the two partners shows the presence of a VUS, and when both have two VUS and the effect of the combination of these variants on the clinical phenotype cannot be predicted. In these cases, genetic consultation is mandatory but a wider approach which keeps into consideration the psychological well-being of the couple is strongly needed [16,17]. The Cystic Fibrosis diagnosis has a plethora of clinical signs and symptoms, of which two main consequences can be considered in this review as: the infertility impairment [8,9,18] and the newborn quality of life [10]. On this scenario there are different techniques which allow to detect the CF presence in embryos before implantation [19]. Regarding infertility, although *In Vitro* Fertilization (IVF)-Intra-Cytoplasmic Sperm Injection (ICSI) outcomes can be hampered, new molecules are studied which can help the restore both of the ovarian function and the sperm quality. Myoinositol, for its biochemical properties is a new target therapy which is gaining wide popularity [20–24].

5. Conclusions

In conclusion, our study added data in literature about the usefulness of NGS for CF diagnosis and detection. Probably, although medicine is trying to achieve better results in terms of CF therapy, a critical approach about the real importance of new *CFTR* variants detected is strongly required.

6. Limitations

Our study provide data from an infertile population accessing only one IVF Center. Moreover, we lack data about the CBVAD proportion of our population.

Author contributions

ED, GG, AE and VA—extraction and drafting of the manuscript; ED, GG, GC, AP and ASL—design and revision; ED, SB, SS, FL, AM and IV—analysis of data; AV, MN, GB—manuscript editing and revision. All the authors conform the Journal and the International Committee of Medical Journal Editors (ICMJE) criteria for authorship, contributed to the intellectual content of the study and gave approval for the final version of the article.

Ethics approval and consent to participate

Every patient involved in the study signed an informed consent. Due to the observational format of the study the ethical committee approval is not required.

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Conflict of interest

The authors declare no conflict of interest. GB, AV, ASL, GC and GG are the Guest Editor of this journal. MN, ASL, AV are the Editorial Board of this journal. We declare that they had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to MD.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.ceog4905105.

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