CLINICAL SCIENCES

NOVEL *CFTR* MISSENSE MUTATIONS IN BRAZILIAN PATIENTS WITH CONGENITAL ABSENCE OF VAS DEFERENS: COUNSELING ISSUES.

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PURPOSE: Screening for mutations in the entire Cystic Fibrosis gene (*CFTR)* of Brazilian infertile men with congenital absence of vas deferens, in order to prevent transmission of *CFTR* mutations to offspring with the use of assisted reproductive technologies.
METHOD: Specific polymerase chain reaction (PCR) primers were designed to each of the 27 exons and splicing sites of interest followed by single strand conformational polymorphism and Heteroduplex Analysis (SSCP-HA) in precast 12.5% polyacrylamide gels at 7ºC and 20ºC. Fragments with abnormal SSCP migration pattern were sequenced.
RESULTS: Two novel missense mutations (S753R and G149W) were found in three patients (two brothers) together with the IVS8-5T allele in hetrozygosis.
CONCLUSION: The available screenings for CF mutations do not include the atypical mutations associated to absence of vas deferens and thus, when these tests fail to find mutations, there is still a genetic risk of affected children with the help of assisted reproduction. We recommend the screening of the whole *CFTR* gene for these infertile couples, as part of the work-up before assisted reproduction.

KEYWORDS: Vas deferens. CFTR. Male infertility. Azoospermia. Prevention.

INTRODUCTION

Cystic fibrosis (CF) is the most prevalent life-shortening autosomal recessive disorder in Caucasian patients of European descent and is associated to mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene.[1] Dysfunction of CFTR Cl^- channel in the genetic disease CF disrupts transepithelial ions transport and mucociliary clearance in a variety of organs lined by epithelia resulting in a wide-ranging of misleading clinical manifestations that may include: pulmonary disease, pancreatic failure, meconium ileus, elevated levels of salt in sweat and male infertility due to congenital bilateral absence of vas deferens (CBAVD).

Some patients may have all the classical manifestations of CF from infancy and have a relatively poor prognosis, while others have much milder or even atypical disease manifestations, with single organ involvement, and according to the World Health Organization[2] should also be considered as CF patients. The CBAVD men without other CF symptoms fall into this category.

Azoospermic CBAVD patients usually have normal spermatogenesis. The male gamete can be retrieved by Microsurgical Epididymal Sperm Aspiration (MESA) or Testicular Sperm Extraction (TESE) and used in assisted reproductive techniques such as intracytoplasmic sperm injection (ICSI) allowing biological paternity to CBAVD patients.[3,4]

Today, screening for a panel of *CFTR* mutations is offered to these men prior to ICSI, and includes only the most common mutations found in CF patients of European and North American origin. The atypical CBAVD phenotype, however, is caused by milder mutations, most of them very

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rare or even not yet described, and thus not included in the panel of CF mutations usually screened.

These facts lead us to undertake a complete analysis of the entire *CFTR* gene in Brazilian patients with congenital absence of the vas deferens (CAVD) prior to ICSI, and two novel mutations were identified in the patients studied. The high cost of the test, the anxiety of the couple to be submitted to assisted reproduction and the unawareness of the genetic risks involved are especial features of the genetic counseling of these cases.

Methods: patients and procedures

The study was approved by our Institutional Ethics Committee for Research Protocols.

After a brief explanation about the test and informed consent signature, 4 mL of whole blood samples were drawn from patients into EDTA vacuum tubes (Becton Dickinson). DNA extraction was performed according to a previously described salting-out protocol.[5] The mutation analysis included PCR amplification for each of the 27 exons of the *CFTR* gene, with primers designed with the PrimerOut software (primers sequences and conditions are available on request). PCR fragments were submitted to SSCP-HA (Single Strand Conformational Polymorphism and Heteroduplex Analysis) in precast 12.5% polyacrylamide gels (GeneExel GE, Amersham Biosciences, UK). Two SSCP-HA gels were ran for each *CFTR* exon, one at 7^{O}C and the other at 20^{O}C. Fragments with abnormal SSCP migration pattern were selected to be sequenced on an AlfExpress (Pharmacia Biotech, Sweden). The products of new PCR reactions, purified with the Concert Rapid PCR Purification System (Gibco BRL) were used for sequencing with both forward and reverse primers using the Cy™5 Thermo Sequenase™ Dye Terminator Kit (Amersham Pharmacia Biotech).

For the analysis of the splice-junction site located in the boundary of intron8/exon9 (IVS8) to access the number of timines (5T, 7T or 9T), specific reverse primers were designed for direct sequencing on AlfExpress, using the same Dye Terminator Kit.

Table 1 shows the results found.

Table 1 – Novel mutations and polymorphisms leading to CFTR gene dysfunction in CBAVD patients.

Case		1	2a	2b
Age		30y	32y	27y
mutation		S753R	G149W	G149W
IVS8-Tn		5T/9T	5T/7T	5T/7T
IVS8-TGm		10TG/12TG	10TG/11TG	10TG/11TG
polymorphisms		M470V	M470V	M470V
			2694T>G	2694T>G
Sweat chloride*		26nmol/L	24nmol/L	45nmol/L
Seminal Vesicles	right	hypoplastic	normal	normal
	left	hypoplastic	agenesis	agenesis

*ref: normal<30nmol/L; borderline 30-60nmol/L; high >60nmol/L

Case 1

The patient, a 30 year-old Caucasian man of Spanish descent, was referred to the Center for Human Reproduction of the São Paulo University Medical College Hospital by a pulmonologist due to chronic respiratory infections, sinusitis and rhinitis since early childhood. Physical examination showed bilateral absence of vas deferens (CBAVD) and a cyst on the head of the right epididymus. Seminal analysis revealed azoospermia and low volume of ejaculate (<1mL) secondary to bilateral hypoplastic seminal vesicles identified by transrectal ultrasound. The abdominal ultrasound showed normal topic kidneys and the sweat test showed normal chloride concentration.

The new mutation found in the patient is an AGC to AGG change in DNA leading to a serine-to-arginine substitution at position 753 (S753R) in the CFTR protein; S753 is a consensus phosphorylation site in the regulatory domain of CFTR protein.[6] No other classical *CFTR* mutation was found, but a 5T allele was identified in intron 8 (IVS8-5T) together with an IVS8-9T in the other allele. In exon 10 a M470V polymorphism was found in heterozygosis, corresponding to a methionine to valine substitution at protein position 470.

Case 2

Two brothers of the typical mixed ethnicity found in a considerable percentage of Brazilians were referred from a University Hospital to be screened for *CFTR* mutations. The patients came with diagnosis of azoospermia and CBAVD. Ultrasound showed normal topic kidneys and left seminal vesicle agenesis in both brothers. The sweat chloride concentration was normal (24nmol/L) for the older brother and borderline (45nmol/L) for the younger. No gastrointestinal or pulmonary phenotype was present in both brothers.

A glycine-to-tryptophan substitution at protein position 149 (G149W) was detected in both brothers. We requested the parents to be tested and the same mutation was found in the mother. A third brother, with unilateral duplication of vas deferens diagnosed at the time of his vasectomy, was also tested but no mutation was found.

Both brothers had no other *CFTR* classical mutation but were heterozygotes to the IVS8-5T allele and to other polymorphisms: the M470V in exon 10 and the 2694T>G in exon 14a, the later of unknown physiological consequences.

DISCUSSION

Infertility is an important health problem, affecting 10% of all men in reproductive age around the world. Among the causes of infertility, 10% are due to CBAVD and are associated to mutations in the *CFTR* gene.[7] A large body of evidence proved that CBAVD represents an atypical form of CF[8], and are also associated to mutations in the *CFTR* gene[9] or better called *CFTR* dysfunction.[2] It is therefore suggested that men who have CBAVD should be considered for CF screening prior to ICSI procedure, despite a negative family history of CF. Because of the high carrier frequency of about 1 in 25 for *CFTR* mutations in the general population,[10] when CBAVD is a clinical feature and the couple is considering MESA or TESE with ICSI, genetic counseling is recommended and screening is proposed to the female only if the male proves to be a carrier. If both partners are CF carriers, the reproductive technology should include pre-implantation genetic diagnosis (PGD) and transfer of mutation-free embryos. Because ICSI might bypass the normal reproductive constraints of infertile men, counseling of these couples requesting IVF with ICSI is vitally important in order to help them understand the elevated risk for CF and/or infertility in their offspring, and to help them to cope with the diagnosis.

To determine which tests actually might prove beneficial to patients, both individually and collectively, cost-benefit ratios, in addition to clinical and preventive implications, should be weighed as part of patient counseling. The currently recommended screening panels of mutations in the *CFTR* gene do not detect all disease-associated mutations and are even less effective in detecting the less frequent mutations associated to the CBAVD phenotype, specially in heterogeneous populations like Brazilian. Only an extensive *CFTR* gene screening can detect rare mutations that are not found with conventional screenings and commercial tests, and can thus improve the diagnosis and care of CF and CAVD and prevention of new cases with the use of reproductive technologies.

In our study, we performed a screening of all the exons and splicing of sites of interest of the *CFTR* gene in eighteen CBAVD patients and two novel mutations were detected: a serine-to-arginine at an alternative phosphorylation site in the regulatory domain (S753R), and a glycine-to-tryptophan substitution at position 149 (G149W) in the second intracellular domain of the CFTR protein.

Structural and functional studies of the CFTR channel carrying the serine substitution at position 753 found in case 1 are not yet available and so its impact on the CFTR channel function is unknown. Considering the clinical presentation of the patient, S753R might not be a polymorphism once CBAVD was present together with a clear pulmonary phenotype, although not together with sweat-chloride elevation. Besides, S753 is one of the ten serine residues submitted to phosphorylation (one in the NBD1 S-422 and nine in the R domain S-660, -670, -700, -712, -737, -753, -768, -795, -813) to exert the primary control of activation of Cl- conductance.[27]

The second novel mutation found was a G149W. A different mutation (G149R) at the same position was previously reported in a CBAVD patient[11] but was not found in normal individuals nor in CF patients tested, placing the G149R in the category of Class V mutation usually associated to the CBAVD phenotype.[11] The G to W substitution described here is probably even milder than G to R, once glycine and tryptophan are both not polar amino acids. The presence of the same mutation in both brothers and the borderline sweat-chloride level found in one of them, favor G149W as a novel CBAVD Class V CFTR mutation.

The three patients had no other CFTR mutation but all were also heterozygotes for the IVS8-5T allele and for the M470V polymorphism; the two brothers were also heterozygotes for a 2694T>G polymorphism in exon 14a.

The 5T variant in intron 8 of the *CFTR* gene is the most frequent mutation associated to the CBAVD phenotype[12] and can be the sole cause of disease, such as CBAVD.[12,13] The 5T allele leads to a higher proportion of transcripts lacking exon 9 than the two other alleles, 7T and 9T. However, this 5T variant has incomplete penetrance and variable expressivity, suggesting that some other regulatory factors may modulate the splicing of exon 9. [14]

The association of the IVS8-5T allele in the 3 patients, once again places the IVS8-5T allele as a CBAVD causing genotype variation as extensively reported in the literature. In Italy more than 20,000 control subjects and over 1,800 in the infertile situation were tested; 94% of the 5T alleles were found in the infertile group of men affected by CBAVD, and one 5T allele together with a *CFTR* mutation are three times more frequent in infertile men. [15]

For national policies of CF prevention such as the one adopted in Italy[15], it is acceptable to include only the most frequent mutations present in the population, which allows a 90% detection rate. Nevertheless, when a patient presents with infertility, a thorough mutation search has to be undertaken because even in homogeneous populations, the mutations associated to CBAVD are rare.

The carrier rate and mutation frequencies vary widely in different populations so that screening tests with high detection rates for *CFTR* mutations have to consider the population ethnicity. In heterogeneous populations such as the Brazilian, complete genetic information is currently lacking to build up solid population-based *CFTR* screen-

ing programs that could enable adequate carrier detection of either typical or atypical CF patients and their family members. Identification of new mutations is clinically relevant not only to birth defects prevention using reproductive technologies, but also to a better molecular understanding of the involvement of the *CFTR* gene in the urogenital phenotype of these men. Additionally, the approach will help to develop new strategies to improve and extent the number of mutations screened.

Counseling a CBAVD patient with a *CFTR* mutation and an IVS8-5T allele is a difficult task because it is not possible to determine whether this genotype would lead to the same phenotype in the child, or if the phenotype would be as severe. The fact that the children born with the help of ICSI may be completely healthy, even though carrying the same CBAVD genotype of the father should not discourage the indication of *CFTR* screening. Even for those couples in which both are carriers, the biological paternity can be obtained by ICSI but, in these cases, preimplantation genetic diagnosis ought to be performed, with blastomere biopsy of each embryo produced and transfer of healthy embryos only.

Assisted Reproduction underscores the importance of mutational analysis of the *CFTR* gene when an infertile couple is seeking for IVF, but we strongly recommend the screening of the whole *CFTR* gene for all infertile couples, man and wife, as part of the workup before assisted reproduction with IVF-ICSI.

RESUMO

Pieri PC, Missaglia MT, Roque JA, Moreira-Filho CA, Hallak J. Mutações novas no gene CFTR de pacientes brasileiros portadores de agenesia dos vasos deferentes: dificuldades no aconselhamento. Clinics. 2007;62(4):385-90.

OBJETIVO: Pesquisar mutações em toda a extensão do gene que causa a Fibrose Cística (CFTR) de homens brasileiros inférteis por agenesia congênita dos vasos deferentes, com a finalidade de prevenir a transmissão de mutações em CFTR à prole com o uso das tecnologias de reprodução assistida.

MÉTODOS: Foram desenhados oligonucleotídeos específicos para realização de reação de polimerização em cadeia (PCR) para cada um dos 27 exons e sítios de processamento de interesse no gene CFTR. O PCR foi seguido pela técnica de SSCP-HA (polimorfismos de conformação no DNA de fita simples e na formação de heteroduplexes) em géis pré-fabricados de poliacrilamida a 12,5% em duas temperaturas, 7^{o}C e 20^{o}C. Os fragmentos com padrão alterado na migração do SSCP foram submetidos a seqüenciamento automatizado.

RESULTADOS: Foram identificadas duas mutações novas com alteração de aminoácidos (S753R and G149W) em 3 pacientes (dois irmãos) juntamente com o alelo IVS8-5T em heterozigose.

CONCLUSÕES: O rastreamento básico de mutações típicas da Fibrose Cística não inclui as mutações atípicas associadas à ausência dos deferentes. Desta forma, quando esses testes resultam normais, ainda assim existe um risco genético de crianças afetadas serem geradas com auxílio das Assisted Reproduction Technologies. Por este motivo, recomenda-se que a pesquisa de mutações em todo o gene *CFTR* seja o exame a ser oferecido para todos os casais inférteis em que o homem seja portador de agenesia dos vasos deferentes, antes da realização de reprodução assistida.

UNITERMOS: Vasos deferentes. CFTR. Infertilidade masculina. Azoospermia. Prevenção.

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