Artigos Originais

Large *CFTR* Gene Rearrangements in Infertile Patients due to Congenital Absence of the Vas Deferens

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Resumo

Aproximadamente 85% dos pacientes inférteis com ausência congénita dos canais deferentes (CAVD) possuem mutações no gene *Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)*. Apesar da análise completa do gene *CFTR*, alguns pacientes permanecem com mutações por identificar. Uma vez que grandes rearranjos genómicos não são detectáveis através dos métodos convencionais baseados na técnica da PCR, foi sugerido que este tipo de mutações poderia justificar aqueles casos não identificados. No presente estudo, pesquisou-se a presença de grandes rearranjos do gene *CFTR* em 12 pacientes com ausência congénita bilateral dos canais deferentes (CBAVD) e ausência de mutações *CFTR* após estudo completo do gene em 45 casos CAVD. Através da técnica de PCR multiplex semi quantitativo (QFMPCR) detectou-se uma grande delecção do gene *CFTR* em 1/12 (8,3%) dos pacientes. Neste paciente, anteriormente identificado como portador de um alelo IVS8-5T, a delecção dos exões 2 e 3 corresponde à deleção CFTR dele2,3(21Kb), previamente descrita com uma elevada frequência (5%) na população Eslava. Em conclusão, na CAVD, o estudo dos rearranjos do gene *CFTR* deve complementar o estudo do gene *CFTR* de modo a se poder efectuar um aconselhamento genético apropriado do paciente e do casal sobretudo com vista a um possível diagnóstico pré-implantação em caso de tratamento de infertilidade.

Abstract

Mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene are recognized in about 85% of infertile patients due to congenital absence of the vas deferens (CAVD). Despite the extensive analysis of the *CFTR* gene by several methods, some cases remain without a diagnosis. Because large genomic rearrangements of the *CFTR* gene are not detectable by conventional PCR-based methods, it has been suggested that these types of

mutations could account for those undiagnosed cases. In the present study, we have searched for large *CFTR* gene rearrangements in 12 patients with congenital bilateral absence of the vas deferens (CBAVD) and absence of *CFTR* mutations after an extensive gene screening of 45 CAVD patients. Using multiplex semi-quantitative PCR (QFM-PCR) we found a large *CFTR* exon deletion in 1/12 (8.3%) of the patients. This case, also carrying one IVS8-5T allele, was found to have a *CFTR* deletion of exons 2 and 3, which corresponds to the CFTRdele2,3(21Kb) described with a high frequency (5%) in Slavic populations. In conclusion, *CFTR* gene rearrangements should be included in *CFTR* gene analysis of CAVD patients in order to give the most appropriated genetic counseling, especially in couples seeking infertility treatment and pre-implantation genetic diagnosis.

Keywords: Cystic Fibrosis, male infertility, CBAVD, *CFTR* gene, *CFTR* gene rearrangements

Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disease found in the Caucasian population, with an incidence of 1:2500 live births and a carrier frequency of 1:25. It is caused by mutations in the cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene located at 7q31-q32, which comprises about 250Kb and encompasses 27 exons (1, 2, 3). It codes for a 1480 amino acid CFTR glycoprotein, which functions as a cAMP-regulated chloride channel that regulates salt and water transport in the cell membrane of epithelial cells (4). In its classical form, CF is characterised by chronic obstructive pulmonary disease, pancreatic insufficiency, intestinal obstruction, male infertility and elevated sweat chloride (>60 mM) (4, 5). However, the severity of the clinical phenotype depends on the nature of the underlying mutation genotype that may cause the total absence or varying levels of the CFTR protein (1, 4).

To date, over 1200 mutations and 200 sequence variations have been described in the *CFTR* gene, although distributions and estimated frequencies differ with geographic and ethnic variations (6). Of these mutations, the majority correspond to point mutations and small insertions or deletions. The most frequent disease-causing mutation that accounts for about 70% of CF chromosomes worldwide is a deletion of three base pairs in exon 10, which results in deletion of a phenylalanine at position 508 of the polypeptide (F508del).

Mutations in the *CFTR* gene are recognized in about 85% of infertile patients due to congenital absence of the vas deferens (CAVD) (7, 8, 9, 10) *could you please also add the reference of Costes et al., EJHG 1995.* The genetic link between CF and CAVD was suggested when an increased frequency of heterozygotes for the F508del mutation was reported in these patients (11). In 1992, the first observation of a compound heterozygo-

sity for the *CFTR* gene in a patient with congenital bilateral absence of the vas deferens (CBAVD) was described (11), and from the observation that many CBAVD men had mutations in their *CFTR* gene it was proposed that CBAVD may be a primary genital form of CF (11). Clinical symptoms of CAVD are azoospermia with low seminal fluid volume (<2.0 ml) and pH (<7.2), nonpalpable vas deferens (unilateral/bilateral), absence of the distal part of the epididymis, globus major, and different degrees of hypoplasia of the vesicula seminalis (7). In CAVD, compound heterozygosity of the *CFTR* gene is the common finding, with the majority of genotypes consisting of one severe and one mild mutation or two mild mutations. On the other hand, the classic CF phenotype is characterized by homozygosity or compound heterozygosity for two severe mutations (7). The most common *CFTR* mutations in CBAVD are F508del, R117H and the IVS8-5T allele (7, 8, 10, 12). At the boundary between exons 8 and 9, the *CFTR* gene has a splicing acceptor site, the efficiency of which varies depending on the sequence. At this splice acceptor site of intron 8 (IVS8 poly-T) there is a polymorphism of 5, 7 or 9 thymidines (13). The frequency of exon 9-skipping during splicing has been found to be inversely proportional to the number of thymidines. The IVS8-7T and IVS8-9T alleles predominantly generate normal mRNA transcripts, whereas the 5T variant results in production of mRNA lacking exon 9 and reduced levels of normal mRNA. The protein product of the *CFTR* transcript lacking exon 9 is devoid of cAMP-activated chloride conductance, and therefore the IVS8-5T allele is considered to be a mild mutation with an incomplete penetrance, resulting in CAVD and a mild form of CF (14, 15). In CAVD patients, the IVS8-5T allele is frequently associated with a severe *CFTR* mutation in the other chromosome, this genotype being the most frequent found in CAVD patients (7, 9, 10).

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Several studies have shown that commercial kits do not allow the proper diagnosis of a significant proportion of CAVD patients, since they are designed for the detection of classical CF mutations found in CF patients (16). To overcome this problem, all 27 *CFTR* exons should be studied (6, 10, 17, l8, 19). Still, some CAVD cases remain to be identified. Because large genomic *CFTR* rearrangements are not detectable by PCR-based methods, it has been suggested that such mutations could account for those unidentified cases. Recent studies have shown that 16-20% of unidentified CF alleles that remain unidentified after an extensive analysis of the *CFTR* gene by Denaturing Gradient Gel Electrophoresis (DGGE) and denaturing High Performance Liquid Chromatography (dHPLC) consist of large gene rearrangements (20, 21, 22, 23). The study of such *CFTR* gene rearrangements and their frequencies has important implications regarding the appropriate genetic counselling of CAVD patients and of their partners during infertility treatment.

In the present study, we report the results of a *CFTR* rearrangement screening in a cohort of CAVD patients, which after extensive DGGE screening had only one *CFTR* mutation identified, or had a *CFTR* genotype that could not totally explain the clinical phenotype.

Materials and Methods

Patients

All 45 male patients had neither CF typical clinical manifestations (pulmonary and/or gastrointestinal disease) nor a positive sweat test. Diagnosis of CAVD was based on physical examination (presence of globus major and absence of palpable vas deferens) and azoospermia with low seminal fluid volume, pH and fructose levels (24). Abdominal ultrasonography was performed in order to evaluate the pelvis and the upper urinary tract, patients with renal abnormalities being excluded from the study. Transrectal and scrotal ultrasonography showed abnormal seminal vesicles (hypoplasia or aplasia), absence of one or both vas deferens and a dilated epididymis head (globus major). Patients also had normal serum hormone levels, a normal karyotype and were tested negative for Y-chromosome AZF and DAZ microdelections. At the diagnostic testicle biopsy, all patients presented conserved spermatogenesis with germ cell sloughing, suggestive of tubular obstruction. CAVD patients had either microsurgical epididymal sperm aspiration or open testicular sperm extraction (TESE) depending on the presence of enough motile sperm in the dilated epididymis (25) for infertility treatment, with absence of the vas deferens and presence of epididymus globus major being confirmed during the procedure. All CAVD patients were diagnosed and treated by a single experienced urologist. In all cases, DNA analyses were performed after patient informed consent.

Screening for *CFTR* rearrangements by semi-quantitave fluorescent multiplex PCR (QFM-PCR)

The principle of QFM-PCR is based on the simultaneous amplification of short *CFTR* gene fragments followed by comparison of the fluorescent profiles with control DNA and quantification of the fluorescence of each amplicon. The amplification step of the PCR assay is stopped at the exponential phase, allowing the quantification of the number of alleles amplified.

Three fluorescent-labeled multiplex PCR assays were developed to amplify all the 27 *CFTR* exons, the promotor region and the region containing the polyadenylation signal sequence. Primers were labeled with the fluorescent phosphoramidite 6-FAM dye. Primer sequences and the size of the products generated are described elsewhere (21). The multiplex PCR-1 (MP1) amplifies the promotor, the polyadenylation signal sequence and exons 1-6a and 11. MP2 amplifies 10 exons: exons 7-10 and 12-16, and MP3 amplifies 10 exons: 6b and 17a-24. Each MP assay contained control primer pairs (internal controls) that amplified short exonic fragments from other human genes located on different chromosomes: depending on the MP used, exon 4 of *DSCR1* (chromosome 21) and/or exons 5, 7 and 8 of *F9* (X chromosome). Each experiment also included negative controls (samples with absence of deletions or duplications) and positive controls (samples with a known *CFTR* deletion). MP were performed in 25 μl reaction mixture using the Qiagen Multiplex PCR Kit (Qiagen, Courtaboeuf, France), that contained 300 ng of genomic DNA and 0.1-0.8 μM of primers. PCR cycling conditions were as follows: initial denaturation of 15 min at 95°C, followed by 21 cycles at 95°C for 30 s, 55°C (MP1, MP2) or 50°C (MP3) for 30 s, extension at 72°C for 45 s, and a final extension step of 10 min at 72°C. Primer concentrations and the number of amplification cycles were optimized to ensure that each PCR was still in the exponential phase of amplification. Purified PCR products (2 μl) were added to a mixture containing 9.8 μl formamide and 0.2 μl Genescan-500 Rox size standard (Applied Biosystems, Foster City, CA, USA), then separated on an ABI PRISM 310 DNA automated sequencer and analyzed using the Genescan 3.1 software (Applied Biosystems) to obtain the electropherograms from each

sample. Data were analyzed by superimposing fluorescent profiles of test and control DNA followed by visual comparison of the peak heights of the corresponding amplicons after being normalized against the F9 and/or DSCR1 peaks. The presence of a deletion was indicated by a drop of approximately 50% in the height of the corresponding peak, while duplication show an increase in signal intensity of approximately 50%. In order to determine the copy number of each exon amplified, the peak height values from a sample were compared against one another and against those from controls, which gives a series of dosage quotients (DQ). Peak height data from samples and controls was imported into an Excel (Microsoft) spreadsheet and DQ for pairs of exons in a sample were then calculated by dividing the ratio of the two exons peak height from the sample by the corresponding ratio obtained from the controls.

Results

Initially, 45 infertile azoospermic patients due to CAVD, 42 with bilateral (CBAVD) and 3 with unilateral (CUAVD) absence of the vas deferens were analysed for the 31 most common *CFTR* mutations, by using a commercial kit (Cystic Fibrosis Diagnostic System, Abbott, Wiesbaden, Germany) and for the poly-T variants at intron 8 (IVS8 poly-T) by fluorescent DNA amplification fragment analysis (10, 17, 18). Samples with absence or presence of only one pathogenic mutation were then subjected to denaturing gradient gel electrophoresis (DGGE) technique to screen all 27 exons of the *CFTR* gene (10). This showed a mutation detection rate of 95.5% (86/90 alleles) and left 12 CBAVD patients without a final diagnosis. These were then selected to proceed for the *CFTR* gene rearrangement screening: 2 patients with one mild *CFTR* mutation, 1 patient with one IVS8-5T allele, 7 patients whose *CFTR* mutations or IVS8-5T alleles could probably not explain the clinical phenotype (2 cases with mild/mild *CFTR* mutations, 3 cases with one mild *CFTR* mutation and a IVS8-5T allele, and 2 cases with two IVS8-5T alleles), and 2 apparently homozygous patients for a mild *CFTR* mutation but in whom the homozygous status was not possible to confirm by familial studies. Patients carrying one severe CF mutation in one allele were not selected for the *CFTR* rearrangement study, since it was not expected that another severe mutation would be detected (large deletion/insertion) in CBAVD patients without clinical manifestations of CF.

The screening of *CFTR* rearrangements led to the detection (1/12, 8.3%) of a large deletion (exons 2 and

3) in an Ukraine emigrant patient who also carried the IVS8-5T/7T genotype. No *CFTR* gene rearrangements were detected in the other 11 patients, including the two putative homozygous patients. This confirms that these two patients are true homozygous for a *CFTR* mutation previously identified by the 31-mutation panel and DGGE screening. Deletion of exons 2 and 3 was shown by a drop of approximately 50% in the height of the representative exon peaks, when compared to a control sample (Fig. 1), and appeared to be the same as that previously described with a high frequency (5%) in Slavic populations (26), which was in accordance with the ethnicity of the patient. Based on these facts, we performed a duplex PCR assay using specific primer pairs that anneal at both sides of the breakpoint junction (introns 1 and 3) and at intron 3. A 243 bp junction product was therein amplified, thus confirming the presence of exons 2 and 3 deletion (Fig. 1). This deletion spans 21.08 kb of the *CFTR* gene and includes about 25% of intron 1, exon 2, intron 2, exon 3 and approximately 45% of intron 3, and thus corresponds to the CFTR dele2,3 (21Kb) previously reported (26).

Discussion

Despite the accumulated information about mutations in the *CFTR* gene, the majority of reported mutations are single-nucleotide substitutions or small base pair deletions/insertions in exons or their flanking intronic sequences. By contrast, only a very few large deletions or insertions have been identified. The actual frequency of such *CFTR* gene rearrangements is, however, difficult to estimate given the large size of the *CFTR* gene and the technical limitations of the conventional screening methodologies. Recently, comprehensive studies examining CF patients included the analysis of *CFTR* gene rearrangements using a protocol based on semi-quantitative mutliplex fluorescent PCR assays, and reported that large *CFTR* gene rearrangements account for 1.3% of all CF mutations (20, 21, 22, 23) and for 16-20% of unidentified CF mutations where a point mutation or a short deletion/insertion has not been found.

In the present study, a *CFTR* rearrangement was detected in 1 out of 12 (8.3%) CBAVD patients with no definitive molecular diagnosis. The CFTRdele2,3(21 kb) rearrangement corresponds to a deletion of exons 2 and 3, which was previously described as the second most frequent mutation after F508del in Central and Eastern European CF patients (26). The mutation removes 21.08 kb of the *CFTR* gene and was predicted to cause a
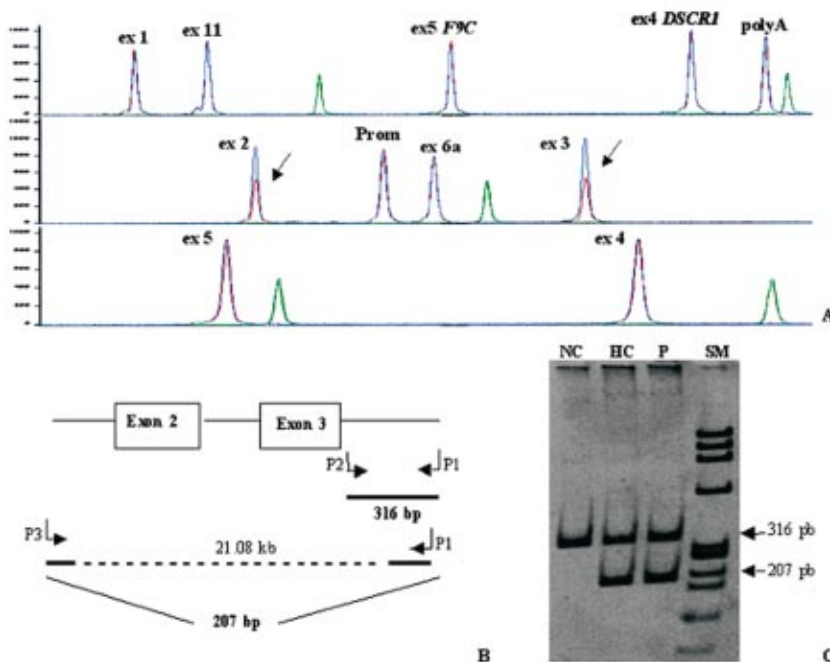
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Figure 1. (**A**) Electrophoregram from semi-quantitative fluorescent multiplex PCR showing the CFTR dele2,3 (multiplex 1) in a CBAVD patient. The y-axis corresponds to fluorescent intensities in arbitrary units. Gene fragments are indicated at the top of the corresponding peaks. Control peaks are in blue and those of the patient are in red (size markers in green: Genescan-500 Rox size standard). Profiles were superimposed and normalised against those of exon 4 of *DSCR1* and exon 5 of *F9* amplicons. Abnormal profiles are indicated by arrows (twofold decrease in peak intensity for exons 2 and 3). (**B**) Schematic representation of the duplex PCR assay for confirming the presence of the deletion, using three specific primers (P1, P2, P3) that anneal at both sides of the breakpoint junction (P3: intron 1; P1: intron 3) and at intron 3 (P2). A 243 bp juntion product was amplified when deletion of both exons (2,3) is present, whereas a 316 bp fragment was amplified in the normal allele. The deletion spans 21.08 kb of the *CFTR* gene and includes about 25% of intron 1, exon 2, intron 2, exon 3 and approximately 45% of intron 3. (**C**) Agarose gel electrophoresis, showing the size marker (SM); patient DNA (P), with a 316 bp PCR amplicon corresponding to the normal allele and a 207 bp PCR amplicon corresponding to the junction fragment of the deleted allele; heterozygote control (HC) for the deletion (exons 2,3); and normal DNA control (NC) without *CFTR* deletions.

truncated CFTR protein lacking all functional domains (26). The clinical evaluation of homozygotes for this 21--kb-deletion showed that it represents a severe CF allele, since all the homozygous patients had pancreatic insufficiency, early onset of diagnosis and pulmonary disease (26). The present CBAVD patient with the CFTRdele2,3(21 kb) rearrangement also carried the IVS8- 5T/7T genotype. Since no familial studies were possible to perform, allele segregation could not be determined. In a previous study, a compound hetero-zygote patient presenting with mild pancreatitis (CFTR--associated disease) was identified with the CFTRdele 2,3(21 kb)/R117H and the IVS8-7T/7T genotype (27). If the deletion of exons 2-3 is supposed to occur on a IVS8-7T chromosome background, then the IVS8-5T allele of the present patient could be considered to occur in *trans* (on the other chromosome). Thus, the combination of a CFTRdele2,3(21kb) with a IVS8-5T allele on the other chromosome is a sufficient finding to predict a reduced

CFTR channel function and to fully explain the CBAVD phenotype. Furthermore, the presence of the IVS8-5T allele is considered to be a mild mutation with incom-plete penetrance, not associated with classical CF mani-festations. Two CBAVD patients carrying the genotypes R117H/CFTRdele2,3(21 kb) and IVS8-5T/CFTRdele2,3 (21 kb) have also been previously reported (28), thus supporting our hypothesis. Another recent study also detected a large *CFTR* deletion of exons 22, 23 and 24 in 1/48 (2%) CBAVD patients tested, who also carried the IVS8-5T allele (29).

The determination of *CFTR* gene copy numbers allows the discrimination between true homozygotes for a *CFTR* mutation and of compound heterozygotes for a *CFTR* mutation and a large *CFTR* deletion. Exon dele-tions cannot be detected in compound heterozygotes using conventional PCR-based screening techniques, because the amplification of a non-deleted allele masks the lack of a PCR product corresponding to the deletion

site. In the present series, *CFTR* rearrangements were also studied in two homozygous patients for a mild *CFTR* mutation but in whom the homozygous status was not possible to confirm by familial studies. We here confirm that these patients are true homozygotes, as no *CFTR* rearrangement could be detected. The complete deletion of the *CFTR* gene in one allele of a CBAVD patient, apparently homozygous for a R117H mutation, was also identified in a previous study (21). Overall, of 45 CAVD patients, the *CFTR* rearrangement study enabled to increase the mutation detection rate from 95.5% (86/90 alleles) to 96.7% (87/90 alleles). The remaining unsolved cases could be explained by mutations that escaped detection by DGGE screening, to splicing abnormalities located within introns, or to mutations located in other disease causing genes that may account for the CAVD phenotype.

In conclusion, the results presented here and elsewhere (6, 10, 17, 18, 21, 29) highlight the need to achieve a complete *CFTR* gene analysis, which is of most importance in CAVD patients wishing to have an infertility treatment, as the genetic tests and counselling of both couple members are needed for preimplantation genetic diagnosis and CF prevention in the offspring (30). Because commercial kits for the detection of *CFTR* mutations have been shown to be inadequate to detect the majority of mutations in CAVD patients, and due to the intrinsic technical limitations of PCR-based methods, the proposed strategy for the molecular diagnosis of CAVD patients should include the following serial steps: genotyping for 30-35 most common CF gene mutations by using available commercial kits; if negative, one should then proceed to the complete screening of all 27 exons by one of the following detection techniques: DGGE, single SSCP, dHPLC and DNA sequencing; if still negative, the screening of *CFTR* large exonic deletions and duplications should be performed.

Acknowledgements

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