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Molecular Study of $(TG)_m(T)_n$ Polymorphisms in Iranian Males With Congenital Bilateral Absence of the Vas Deferens

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Abstract

Congenital bilateral absence of the vas deferens (CBAVD) is a frequent cause of obstructive azoospermia. Nearly 75% of men with CBAVD have at least 1 detectable common mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The different alleles at the $(TG)_m(T)_n$ polymorphic locus at the 3' end of human *CFTR* intron 8 determine the efficiency of exon 9 splicing. To study the *CFTR* gene mutations and $(TG)_m(T)_n$ polymorphisms in Iranian CBAVD patients with presumed low CF frequency and to better understand the complex regulation of exon 9 splicing among our study population, we analyzed *CFTR* mutations and $(TG)_m(T)_n$ polymorphisms in 112 Iranian CBAVD, 7 congenital unilateral absence of the vas deferens males from Iran, and 84 fertile males as controls. Moreover, we compared the rate of *CFTR* transcripts with exon 9 (9+) with reduction of the $(T)_n$ repeat in our study population. Our study showed that the *5T* mutation was present with high frequency in our patients. Longer $(TG)_m$ polymorphic tracts increase the proportion of exon 9 deletion transcripts but only when activated by the *5T* allele. The combination of the *5T* allele in 1 copy of the *CFTR* gene with a CF mutation in the other copy is the most common cause of CBAVD in the Iranian population. We also observed the highest level of

exon 9+ splicing efficiency among the tested samples with the $(TG)_{12}(T)_7$ allele, which represents the most common intron 8 splice variant allele in the general population. Our results support the idea that a putative role of the $(T)_n$ repeat is to distance the $(TG)_m$ repeat from the 3' splice site and that the different alleles at the $(T)_n$ locus affect the efficiency by which the splice acceptor consensus sequence is recognized.

Key words: CBAVD, *CFTR*, IVS8-*5T*, male infertility

In the majority of cases, congenital bilateral absence of the vas deferens (CBAVD) can be considered a genital form of cystic fibrosis (CF), presenting without the other clinical features of CF (Timmreck et al, 2003). CBAVD is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which contains 27 exons encompassing approximately 180 kb of DNA on chromosome 7q31.2. More than 1000 mutations have been described (Cystic Fibrosis Mutation Database). Approximately 20% of males with CBAVD have a single detectable *CFTR* mutation, while another 20% have 2 common *CFTR* mutations, usually 1 classic and 1 mild (Claustres et al, 2000). An intron 8 splice variant, called *5T* (IVS8-*5T*), is also frequently observed in CBAVD and seems to be specific for this condition (Chillon et al, 1995). Whether or not *CFTR* mRNA contains exon 9 depends on the variable length of a sequence of thymines in intron 8 of *CFTR*. Since the *5T* allele causes reduced levels of normal *CFTR* mRNA (Chillon et al, 1995; Cuppens et al, 1998), this DNA variant seems likely to be involved in the pathogenesis of CBAVD. The different alleles at the $(TG)_m(T)_n$ polymorphic locus at the 3' end of human *CFTR* intron 8 determine the efficiency by which exon 9 is spliced. The resulting *CFTR* transcripts lacking exon 9 lose 21% of the amino terminal end of the *NBF1* region and produce a protein that is misfolded and nonfunctional (Delaney et al, 1993). Genetic studies have found that a polymorphic polythymidine $(T)_n$ locus located within the 3' splice site of IVS8 is associated with the variable efficiency of exon 9 splicing (Chu et al, 1991). In the normal population, 3 alleles are found at this locus (5% *5T*, 84% *7T*, and 11% *9T* in populations of European descent) (Kiesewetter et al, 1993; Cuppens et al, 1994). The degree of exon skipping is inversely correlated with the length of the $(T)_n$ tract so that transcripts derived from genes that carry 5 thymidines (*5T*) at this locus have the highest levels of exon 9 skipping, whereas those with 7 or 9 thymidines (*7T* or *9T*, respectively) exhibit progressively lower levels of skipping (Chu et al, 1993). Besides the $(T)_n$ locus, additional *cis*-acting elements have been described that influence *CFTR* exon 9 alternative splicing (Niksic et al, 1999; Pagani et al, 2000; Hefferon et al, 2002). In particular, recent evidence has implicated another polymorphism, a series of 10 to 13 $(TG)_m$ located immediately upstream of the $(T)_n$ repeat. It has been reported that *T5 CFTR* genes carried by most CBAVD patients have a high number of $(TG)_m$ repeats, whereas *T5* genes carried by healthy fathers of individuals with CF (carrying a CF mutation on the other gene) have a lower number of $(TG)_m$ repeats (Cuppens et al, 1998; Groman et al, 2004). These observations suggest that the $(TG)_m$ tract can further modulate exon 9 skipping and may account for the partial penetrance of the *T5* allele.

We studied 112 Iranian males with CBAVD using a minigene assay to determine the incidence of $(TG)_m(T)_n$ and IVS8-*5T* polymorphisms and their relationship to the *CFTR* gene mutations in these patients. These new data complete our previous published data (Radpour et al, 2006a,b).

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Samples

Blood samples were collected from 112 unrelated Iranian males with azoospermia and CBAVD visiting the Reproductive Biomedicine Research Center of Royan Institute, Iran. The diagnoses of CBAVD patients were initially suggested by impalpable scrotal vas on physical examination and transabdominal/rectal ultrasonography and subsequently confirmed by cytobiochemical characteristics: azoospermia with low semen volume (<1.5 mL), decrease in fructose (vesicular marker) levels, decrease in carnitine (epididymal marker) concentrations, and normal follicle-stimulating hormone concentrations according to World Health Organization criteria (World Health Organization, 1999). Each patient had a sperm count of zero. Also we studied 7 patients with congenital unilateral absence of the vas deferens (CUAVD) and 84 fertile males from the general population in Iran as control subjects.

CFTR Mutation Scanning

DNA samples were analyzed by a previously reported method (Radpour et al, 2006a). All 27 exons of *CFTR* were amplified by polymerase chain reaction (PCR) using the published primer pairs for sequencing (Zielenski et al, 1991) and were studied by denaturing gradient gel electrophoresis (Culard et al, 1994) or by single-strand conformation analysis (Liechti-Gallati et al, 1999). Long-range PCR was performed across *CFTR* intron 9 using primer 9i5 (Zielenski et al, 1991) located in intron 8 upstream from the $(TG)_m(T)_n$ site, together with a reverse primer located at the end of exon 10 (5'TGCTTTGATGACGCTTCTGTAT-3') and using 200 ng of genomic DNA from the patient. Nested PCR was performed to amplify the polypyrimidine sequence with previous reported primers RF9 and RR9 (Radpour et al, 2006a). The nested PCR conditions were as follows: denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 74 °C for 40 seconds, for 32 cycles. Nested PCR products were digested with *Xmn* I and visualized on a 12% nondenaturing polyacrylamide gel.

Sequencing of PCR products was carried out by VBC-Genomics (Vienna, Austria) using 50 ng (2 µL) of PCR product and 4 pM (1 µL) of nonfluorescent primers (forward and reverse separately), 4 µL of BigDye Terminator (Perkin-Elmer, Wellesley, Mass), and 3 µL of double-distilled water to adjust the volume to 10 µL. Sequencing results were compared with the sequence of the wild-type *CFTR* gene (Cystic Fibrosis Mutation Database).

CFTR Minigene Analysis of Exon 9 Deletion in Patients Carrying the 5T Allele

Total RNA was isolated from whole blood and cultured epididymal cells using an RNA extraction kit (PrepMate; Bioneer, Daejon, South Korea) according to the manufacturer's instructions. The concentration and purity of the RNA samples were determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a spectrophotometer. The desired A_{260}/A_{280} ratio of pure RNA was between 1.8 and 2.0. The integrity of the RNA samples was further confirmed by electrophoresis on 1% agarose gels. cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas, Hanover, Md) and a poly(dT) primer according to its instructions. Exon 9 amplification was performed with designed primers (forward, 5'-CTTCTAATGGTGATGA CAGCCTC-3'; reverse, 5'-ACTACCTTGCCTGCTCCAGT G-3') (Radpour et al, 2006a). Hot start PCR with AmpliTaq Gold DNA polymerase (Roche Molecular Diagnostics, Pleasanton, Calif) was used for all amplifications. The reaction mixtures were preheated at 95 °C for 10 minutes before thermal cycling. The routine PCR program was 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 45 seconds at 72 °C. The amount of RNA in each sample was standardized by PCR amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Zielenski et al, 1991; Radpour et al, 2006a). GAPDH was amplified for 25 cycles at an annealing

temperature of 55 °C using primers published previously. PCR products were resolved on a 2% NuSieve agarose gel containing 10 µg/mL ethidium bromide.

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The analysis of the entire coding sequences of the *CFTR* gene allowed us to identify 19 different mutations in Iranian CBAVD patients (Figure 1). These mutations have been described previously in Iranian patients with CBAVD (Radpour et al, 2006a,b). Of these, 5 cases were homozygous or compound heterozygous (+/+), 67 were positive for only 1 mutation (+/−), and 49 were negative for both mutations (−/−) (IVS8-5*T* was not involved) (Table 1). The result of our study reflects the high allelic heterogeneity of *CFTR* gene mutations, although 2 mutations, IVS8-*5T* and F508del, were found to be more common in Iranian CBAVD patients. IVS8-*5T* was observed with $(TG)_{12}$ or $(TG)_{13}$ haplotypes on 61 chromosomes, thus confirming the association of this splice site variant with CBAVD in Iranian patients. Screening for the IVS8-*5T* and F508del together led to the identification of more than one third of alleles. All patients with completely resolved mutation genotypes carried a missense or splicing mutation on at least 1 allele; however, in 3 cases, we could only find 1 nonsense mutation (Table 1). Also in 84 fertile males (as control subjects), we could not isolate *CFTR* mutations or IVS8-*5T* variants.

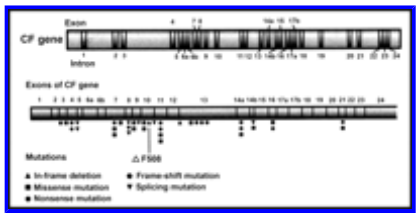

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Figure 1. Cystic fibrosis transmembrane conductance regulator gene mutation spectrum of Iranian congenital bilateral absence of the vas deferens patients. This picture shows the panel of 19 different mutations with their frequencies in our study population.

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Table 1. CFTR *gene mutations in 112 CBAVD patients and 7 CBAVD patients**

Study of the polypyrimidine tract in front of exon 9 revealed a high frequency of the *5T* allele in CBAVD males without renal anomalies. Eleven males were homozygous for *5T* while 39 males were heterozygotes with a *7T* or *9T* on the other allele. In contrast, none of the normal males were found to carry a *5T* allele. To further explore the role of the $(T)_n$ polymorphic locus in the alternative splicing of *CFTR* exon 9 and its implication in the physiopathology of CBAVD, we prepared minigenes containing exons 8, 9, and 10 of the *CFTR* gene and part of the flanking introns, with different $(TG)_m(T)_n$ alleles at the 3'-end of intron 8, and 7 different variants at the intron 8 $(TG)_m$ and $(T)_n$

polymorphic locus were obtained from genomic DNA. The number of $(TG)_m$ was varied and included 4 different polymorphisms: $(TG)_{10}$, $(TG)_{11}$, $(TG)_{12}$, and $(TG)_{13}$ (data was similar between CBAVD and CUAVD patients); we could not find IVS8-$(TG)_{13}$ polymorphisms in normal fertile males. Forty-four individuals were homozygous for the $(TG)_m$ polymorphism and 68 individuals were heterozygous CBAVD patients, whereas 2 individuals were homozygous and 5 individuals were heterozygous CUAVD patients (Table 1). We found that the IVS8-*5T* allele in CBAVD patients is exclusively associated in *cis* with long IVS8-$(TG)_{12-13}$ tracts, which have been shown to increase the extent of exon 9 skipping induced by *5T* variants (Figure 2). Deletion analysis of exon 9 showed the rate of exon skipping in *CFTR* mRNA (Table 2). Exon 9 skipping was strongly correlated with *5T*/*5T* genotype, the rate of normal *CFTR* mRNA increased by having IVS8-*9T* $(TG)_{10-11}$ and IVS8-*7T* $(TG)_{10-12}$ (Figure 3).

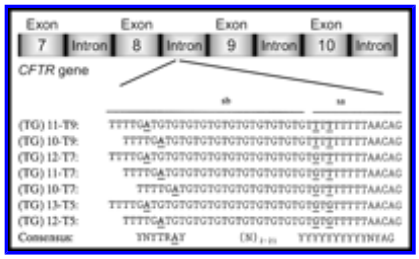

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Figure 2. DNA variants in intron 8 of the Iranian cystic fibrosis transmembrane conductance regulator gene: the alleles at $(TG)_m$ modulate the partial penetrance of the *5T* allele. The sequence at the 5' and 3' splice sites in intron 8 share most of the canonic features of the splicing site, including the 5'-GT donor site, 3'-AG acceptor site and pyrimidine-rich region at the splice acceptor site (Cuppens et al, 1998). Seven different haplotypes of $(TG)_m(T)_n$ have been found in the Iranian population, with $(TG)_{12}(T)_7$ being the most common combination. Longer alleles at $(TG)_m$ associated with shorter alleles at $(T)_n$ cause exon 9 skipping.

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Table 2. *Minigene analysis of exon 9 of CFTR transcripts in Iranian CBAVD patients**

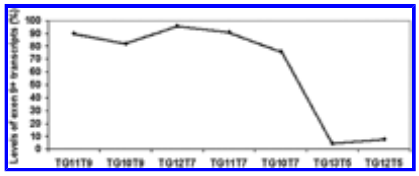

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Figure 3. Comparison of the splicing efficiency across the 7 different $(TG)_m(T)_n$ polymorphisms. The graph displays the levels of normally spliced RNA transcripts.

Taken together, we report the following findings: 1) the *5T* allele in intron 8 of *CFTR* has clinical effects related to male infertility; 2) in 28.57% of cases, the CBAVD phenotype results from the combined action of the *5T* allele and a CF mutation on the other chromosome; and 3) the highest level of exon 9+ splicing efficiency among the tested samples was observed with the $(TG)_{12}(T)_7$ allele, and

a decrease in $(T)_n$ at the polymorphic locus in a $(TG)_{13}$ or $(TG)_{12}$ background determines a reduction in exon 9+ transcripts that emphasizes the role of the *T5* allele in CBAVD/CUAVD.

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As in men with CF, the CBAVD patients in our study have no urinary tract malformations or renal agenesis, and this is probably explained by the fact that defects in the genital ducts due to *CFTR* dysfunction occur after the splitting of the Wolffian duct into its reproductive and ureteral parts at 7 weeks of gestation. In an intensive study of 112 Iranian males with CBAVD, the entire *CFTR* coding/flanking sequences were scanned. Also we compared the rate of decrease in exon 9+ transcripts with reduction of the $(T)_n$ repeat in our studied population. Twenty-eight of the studied patients were F508del carriers. A high frequency of the IVS8-*5T* variant was found in these patients: 61 of 224 alleles (27.23%). Approximately 1% of individuals with CBAVD have 2 *5T* alleles (Chillon et al, 1995), but our studied population showed a higher frequency (9.82%). As stated earlier, R117H is considered a mild mutation unless it is found in *cis* with the *5T* variant. The frequency of IVS8-*5T* in the Iranian patients was similar to data released in Portugal (27.4%) (Grangeia et al, 2005) and Taiwan (29.2%) (Wu et al, 2004) but higher than Turkish patients (19.6%) (Dayangac et al, 2004). Previous studies suggested that when IVS8-*5T* is combined with additional activity-reducing variations, such as the higher number of IVS8 (TG)m, it shows higher disease penetrance (Cuppens et al, 1998). The longer $(TG)_m$ tract increases the proportion of exon 9− transcripts but only when activated by the *5T* allele, with $(TG)_{13}(T)_5 > (TG)_{12}(T)_5$ (Figure 3).

Much attention has been devoted to understanding the mechanisms regulating the alternative splicing of *CFTR* exon 9 (Chiba-Falek et al, 1999). Even though recent studies have shown that *CFTR* exon 9 inclusion is modulated by multiple and well-characterized exonic and intronic *cis*-acting elements (Nissim-Rafinia et al, 2000; Pagani et al, 2000; Buratti and Baralle, 2001; Hefferon et al, 2002), the extent to which exon 9− transcripts are found is predominantly determined by the alleles at the $(TG)_m$ and $(T)_n$ loci. A high number of TG repeats and a low number of T repeats favor the exclusion of exon 9 in the mRNA, and Cuppens et al (1998) had initially suggested that increased exon 9 skipping from transcripts bearing more TG repeats increases the penetrance of the *5T* allele, causing disease. Recently it has been demonstrated that the TG repeat number is a predictor of benign vs pathogenic *5T* alleles (Groman et al, 2004). In this study, we demonstrated that a short $(T)_n$ induces a higher rate of exon skipping among our population. Using a minigene assay designed to include all the *cis*-acting elements reported so far in the vicinity of exon 9 and variable combinations at the $(TG)_m(T)_n$ polymorphic locus, we determined the levels of exon 9+ and exon 9− transcripts. We demonstrated that the residual amount of normally spliced *CFTR* transcripts in the presence of a short $(T)_n$ repeat does not depend on the general splicing efficiency. Indeed, samples with comparable levels of exon 9+ transcripts in the presence of a *T7* allele may have significantly different levels of exon 9+ transcripts when the $(T)_n$ repeat is reduced. We also observed the highest level of exon 9+ splicing efficiency among the tested samples with the $(TG)_{12}(T)_7$ allele, which represents the most common IVS8 allele in the general population.

In all studies performed so far, a high frequency of *CFTR* mutations or the IVS8-*5T* variant has been found in patients without urinary tract dysfunctions. However, a substantial fraction of patients have only 1 mutation, 1 IVS8-*5T* variant, or no identifiable abnormalities in the *CFTR* gene. The

reduced levels of normal *CFTR* mRNA due to the deletion of exon 9 depend on the presence of the *5T* allele sequence in intron 8 (Table 2). This nonfunctional *CFTR* mRNA accounts for up to 92% of the total mRNA when both *CFTR* genes have the *5T* allele (Anzai et al, 2003; Grangeia et al, 2005). We have found that a significant proportion of men with CBAVD have the *5T* allele compared with men in the general population, which suggests that this allele functions as a disease mutation in Iranian men with CBAVD.

In summary, our results showed that longer $(TG)_m$ tracts increased the proportion of exon 9– transcripts but only when activated by the *5T* allele and supported the hypothesis that a putative role of the $(T)_n$ repeat is to distance the $(TG)_m$ repeat from the 3' splice site and that the different alleles at the $(T)_n$ locus affect the efficiency by which the splice acceptor consensus sequence is recognized.

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Footnotes

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