Title:

The CFTR gene mild variants Poly-T, TG-repeats and M470V detection in Indian men with congenital bilateral absence of vas deferens

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Abbreviations

ART = assisted reproductive techniques

CBAVD=congenital bilateral absence of vas deferens

CF=cystic fibrosis

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CFTR=cystic fibrosis transmembrane conductance regulator gene

CFTR-RD= cystic fibrosis transmembrane conductance regulator gene related disorder

ICSI = Intracytoplasmic sperm injection

USG = Ultrasonography

Short Title: Poly-T, TG-repeats and M470V in Indian CBAVD men.

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SUMMARY

The aim of the study was to detect the frequency of the *CFTR* gene variants poly-T, TG-repeats and c.1408A>G p.Met470Val (M470V) in Indian men with congenital bilateral absence of the vas deferens (CBAVD). Men diagnosed with CBAVD (n=76), their female partners (n=76) and healthy men from general population (n=50) were recruited. Genomic DNA was isolated and the polymorphic regions of IVS9-c.1210-12T [5] and M470V were amplified using specific primers followed by Sanger's DNA sequencing. A statistically significant increase in the frequency of heterozygous IVS9- c.1210-12T [5] (39.4%) was observed in CBAVD men as compared to controls (14%). The allelic distribution of c.1210-12T [5], c.1210-12T [7] and c.1210-12T [9] in CBAVD men was 21%, 64.4% and 13 % and that in healthy controls were 7%, 73% and 20% respectively. Longest TG-repeat c.1210-34TG [13] was found in association with c.1210-12T [5] with an allelic frequency of 5.9% in CBAVD men. We found a significant association of c.1210-34TG [12] /c.1210-34TG [13] - c.1210-12[5] -V470 allele in CBAVD men. Twelve female partners harbored a heterozygous c.1210-12T [5] allele. The study emphasizes the need to screen both partners for the polymorphisms M470V, poly T, TG tract repeats in addition to population specific known *CFTR* gene mutations.

INTRODUCTION

Congenital bilateral absence of the vas deferens (CBAVD) is one of the phenotype observed in 95% male Cystic fibrosis (CF) patients and in about 2% otherwise healthy men (Grangeia et al., 2004; Bombieri et al., 2011). CF and CFTR related disorders (CFTR-RDs) are caused due to defects in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The gene is located on chromosome 7q31.2, containing 27 exons with more than 2000 polymorphisms reported (<http://www.genet.sickkids.on.ca/cftr/app>). Genotype and phenotype studies have categorized mutations as severe mutations (with no functional CFTR protein) that belong to class I-III and mild mutations (with adequate CFTR activity for performing sustained function) belonging to classes IV-V (Claustres et al., 2000). Men with CBAVD,

harbor at least one severe CFTR gene mutation in one allele and one mild CFTR gene mutation in the other, or two mild CFTR gene mutations in both alleles (Grangeia et al., 2004). Apart from c.1521_1523delCTT, p.Phe508del, the most commonly tested variations in CBAVD are c.1210-12T [5] and M470V.

Repeated thymidine's (poly T) near the splice acceptor site in intron 9 (IVS9Tn) of the *CFTR* gene is responsible for the splicing efficacy of exon 10 (Chu et al., 1993). Three main poly-T variants are reported worldwide: c.1210-12T [5], c.1210-12T [7] and c.1210-12T [9]. Approximately 1–2% of European CBAVD patients have a homozygous 5T genotype (Chillón et al., 1995; Claustres et al., 2000). The TG repeats (TGm) located upstream to the poly T tract in intron 9, and the polymorphism c.1408G>A, p. (Met470Val) (M470V) rs213950 in exon 11 (HGVS) have been implicated in CBAVD (de Meeus et al., 1998; Groman et al., 2004). It has been demonstrated that a polymorphism at Val 470 results in CFTR protein with reduced activity compared to the Met 470 allele (Cuppens et al., 1998). Strong linkage disequilibrium is evident among c.1210-12T [5] and the Val allele of M470V polymorphism in CBAVD but not in general population (de Meeus et al., 1998). Studies in population from North India reported 25-56% frequency of IVS-9- c.1210-12T [5] (Sharma et al., 2009; Sachdeva et al., 2011). The ethnicity of the Indian population is quite heterogeneous with different geographical distribution. Hence studies including men from different geographical locations are required.

Using modern reproductive technologies such as intracytoplasmic sperm injection (ICSI), CBAVD men can achieve fatherhood (Okada et al., 1999). If both parents are heterozygous carriers of a *CFTR* gene mutation their offspring might develop clinical manifestations of CF and/or CF related disorders such as CBAVD (Okada et al., 1999). Hence, all couples should be informed of this possibility and the significance of genetic testing for *CFTR* gene mutations prior to ICSI (Okada et al., 1999; Zielenski, 2000). Information on the female partner carrier status of the *CFTR* gene mutations in the Indian population is limited. Therefore, the aim of the present study was to detect the frequency of the *CFTR* “mild” mutation IVS9- c.1210-12T[5] in a cohort of CBAVD Indian men ; their female partners and it's potential association to the frequent variant p. Met 470Val (M470V).

MATERIALS AND METHODS

Ethical Approval

The study was conducted at the National Institute for Research in Reproductive Health; (NIRRH) Mumbai, India during 2011-2016. This study was approved by the Institutional Ethics Committee of the National Institute for Research in Reproductive Health (NIRRH)

Mumbai, India. Written informed consent was obtained from all the study participants before collection of blood samples.

Recruitment of study participants

The study participants were recruited from different geographical locations in India. Men diagnosed with congenital bilateral absence of the vas deferens (n=76) and their female partners (n=76) were recruited. Peripheral venous blood (5ml) was collected for genotyping studies. The diagnosis of CBAVD was based on azoospermia, low semen volume (<1.5 ml), absence/low fructose in seminal fluid and absence of vas deferens on scrotal examination by the experienced andrologists. Medical history and clinical examination was carried out to rule out pulmonary and gastrointestinal manifestations of CF. All the participants had no family history of CF. Ultrasound (USG) of the abdomen and pelvis was performed to rule out any associated renal anomalies. **Previous studies reported lower incidence of *CFTR* gene mutations in CBAVD patients having renal anomalies suggesting that it could likely be a distinct disorder with a different genetic origin from isolated CBAVD (Bombieri et al., 2011; Schwarzer and Schwarz, 2012). Therefore, CBAVD patients with renal abnormalities were excluded from our study.** A total of fifty healthy proven fertile men from general population were recruited as controls whose semen parameters were normal as per WHO guidelines (WHO, 2010). Scrotal examination of the controls revealed a bilaterally palpable vas deferens.

CFTR gene mutation analysis

Genomic DNA was isolated from peripheral blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes using QIAamp® DNA Blood Midi Kit (Qiagen Inc., Hilden, Germany). The complete intron 9 and exon 11 of the *CFTR* gene were amplified using genomic DNA from CBAVD men, their female partners and controls by polymerase chain reaction (PCR) using specific primers (Gajbhiye et al., 2016). PCR cycling condition of 35 cycles for intron 9 were as follows: 95°C for 3 minutes, 56°C for 1 minute and 72°C for 1 minute. The conditions for exon 11 were as follows: 95°C for 3 minutes, 58°C for 1 minute and 72°C for 1 minute. Direct DNA sequencing reaction was carried out using either commercial or institutional DNA sequencing facility. The sequencing results were compared with the wild type *CFTR* gene sequence published in the Gen Bank (NM_000492.3).

Statistical Analysis

The statistical analysis of the data was performed using the chi-square or Fisher exact test using the Open Source Epidemiologic Statistics for Public Health software version 3.01. A $p \leq 0.05$ was considered to be statistically significant.

RESULTS

Frequency of the poly T variants

Three main IVS9 poly-T variants: c.1210-12T [5], c.1210-12T [7] and c.1210-12T [9] identified in the study are represented as Table 1. Thirty CBAVD men (39.4 %) revealed the c.1210-12T [5] variant, of which two individuals (2.6 %) had a homozygous c.1210-12T [5] / c.1210-12T [5] variant and twenty eight (37 %) harbored a heterozygous variant. Screening of their female partners revealed a heterozygous c.1210-12T [5] variant in 15.7% (12 /76). A statistically significant increase was observed in the frequency of the c.1210-12T [5] allele in CBAVD men compared to controls (Table 1). There was no statistical difference in the allelic frequency of c.1210-12T [7] and c.1210-12T [9] variants among CBAVD men, their female partner and the controls (Table 1). One CBAVD male showed a rare 3T allele.

TG repeat frequency

The frequency of TG repeats (c.1210-34TG [10], c.1210-34TG [11], c.1210-34TG [12], and c.1210-34TG [13]) is represented as Table 2. The longest TG repeat c.1210-34TG [13] was observed in 7 out of 76 CBAVD men with two individuals showing a homozygous variant. Only one female showed a c.1210-34TG [13] allele. The frequency of c.1210-34TG [12] allele was found to be decreased significantly in CBAVD men (26.9%) than in controls and female partners. A statistically significant difference was observed between the CBAVD men and controls when values for c.1210-34TG [12] and c.1210-34TG [13] were combined. On the other hand, the c.1210-34TG [11] was significantly increased in both CBAVD men and their female partners as compared to controls ($p \leq 0.05$). We also observed c.1210-34TG [10] variant which was statistically not significant.

Comparison of the different linkage TG-T haplotypes

Table 3 shows the different linkage TG-T haplotype in CBAVD men, their female partners and controls. The c.1210-34TG[11];1210-12T[5]] haplotype did not vary significantly among the three groups. The comparison of the c.[1210-34TG[12];1210-12T[5]] haplotype revealed a significant two fold increase in CBAVD men compared to females and controls. The c. [1210-34TG [12]; 1210-12T [5]] and c. [1210-34TG [13]; 1210-12T [5]] variants accounted for 30 of 152 alleles in CBAVD men, and were significantly increased in CBAVD men than the controls. There was also a statistical difference between the three groups for the c.1210-34TG [11]; 1210-12T [7]] and c. [1210-34TG [12]; 1210-12T [7]] haplotypes.

Distribution of the c.1408A>G p.Met470Val allele and its association with IVS9- c.1210-12T [5]

Overall a significant difference was observed in the M470 and V470 allele in the CBAVD men as compared to controls (Table 4). However, there was no difference in the genotypic and allelic distribution of M/M and V/V among the CBAVD men. We further analyzed the association of c.1210-12T[5] variant and M470V in CBAVD men, their female partners and controls (Table 5) Statistical analysis showed that the c.1210-34TG[12]/ c.1210-34TG[13]-c.1210-12T[5] -V470 allele was significantly associated with CBAVD .

DISCUSSION

The present study reports the TG repeat and poly thymidine tract (TG) m T (n) in intron 9, their association with M470V allele in Indian men with CBAVD and their female partners. The c.1210-12T [5] variant has been known to be associated with different CFTR related disorders such as chronic pancreatitis, bronchiectasis, rhinosinusitis, aspergillosis, as well as CBAVD (Ferec and Cutting 2012). A varied frequency of c.1210-12[5] (IVS9-5T) has been reported across different populations (Xu et al., 2014). Notably, we found the c.1210-12T[5] allele to be significantly higher in the cohort of CBAVD men (39.4%) as compared to the healthy men. Our observations fall in the range 28 -54% reported in other Indian studies (Sharma et al., 2009a; Sachdeva et al., 2011). It was higher than Italian (8.3%) (Pradal et al., 1998) , Iranian (10%) (Radpour et al., 2007), Argentinian (11%) (Levy et al .,2004), German (12.3%) (Dork et al., 2000), American, (17.9%) Wang et al., 2002), Taiwanese (19.4%) (Wu CC et al., 2005), French (16.3%) (Claustres et al., 2000), Portuguese (27.4%) (Grangeia et al., 2004), and Egyptian (28.6%) CBAVD (Fathy et al., 2016). The c.1210-12T [5] allele was lower than the studies reporting (43.7%) in Egyptian (Lissens et al., 1999) and Chinese population (46.5%) (Li et al., 2012).

Reports in literature indicated that incidence of harboring a c.1210-12T [5] and a *CFTR* mutation is higher in infertile men than the females (Morea et al., 2005), wherein the c.1210-12T[5] variant has a milder clinical significance (Sun et al 2006). In the present study, the allelic frequency of c.1210-12T [5] detected in CBAVD men is higher than the females. This may be biased as the females recruited in the study were tested as part of the screening for carrier status. In nine of the twelve females with c.1210-12T [5] allele, their husband also had a c.1210-12T [5] signifying that the carrier frequency may be high among Indians (Kapoor et al., 2006; Sharma et al., 2008, 2009b).

The fate of transcription and translation of the *CFTR* gene is related to the number of TG repeats in intron 9, (Cuppens et al., 1998). We found c.1210-12T [5] in cis with different TG

repeats c.1210-34TG [10], c.1210-34TG [11], c.1210-34TG [12] and c.1210-34TG [13] as reported in literature (Groman et al., 2004). The c.1210-34TG [12]- c.1210-34TG[13] in co-existence with c.1210-12T[5] haplotype observed in CBAVD men in the present study corroborates with reports in individuals with CBAVD (Groman et al., 2004) , asthma (Tzetzis et al., 2001) and chronic pancreatitis (Cuppens et al., 1998). One CBAVD male showed c.1210-34TG [10] - c.1210-12T [5] which has been reported earlier (Morea et al., 2005). It was interesting that the different TG repeats associated with the c.1210-12T [5] variant observed by us showed low allelic frequency (19.7%) than reported in Indian (58.75%) and Chinese (96.91%) men with CBAVD (Sharma et al., 2009, Ni et al., 2012). This could be attributed to the study population recruited from diverse geographical locations. A rare c.1210-12T [3] allele was detected in one CBAVD male which has earlier been suggested to be a fully penetrant mutation, in contrast to c.1210-12[5] allele (Disset et al., 2005). To the best of our knowledge, this is the first study to report a c.1210-12T [3] allele in Indian men. The penetrance of the c.1210-12[5] is also under the regulation of another common polymorphism c.1408G>A, p. (Met470Val) (M470V) rs213950 in exon 11 of the *CFTR* gene (Cuppens et al., 1998). The V470 allele in presence of c.1210-12[5] is related to CBAVD and male infertility (Claustres, 2005) while in absence is linked with higher fertility rates (Kosova et al., 2010). In the present study, a statistically significantly difference was observed in CBAVD men than the controls. However, no difference was seen in the Met470 and Val470 allele among the CBAVD men which corroborates with other studies (Ni et al., 2012). Linkage disequilibrium has been shown to exist between the c.1210-12[5] allele and the Val470 allele in CBAVD but not in general population (de Meeus et al., 1998). Interestingly, we also found c.1210-34TG [12] /c.1210-34TG [13] - c.1210-12[5] –V470 allele to have a significant association with CBAVD which corroborates with reports in literature (Sun et al., 2006, Ni et al., 2012, Li et al., 2012).

Females with c.1210-12[5] and Val 470 have been reported to be symptomatic with respiratory diseases (Sun et al., 2006). In this study, only two females were identified to have a Val 470 in association with c.1210-12[5]. The c.1210-12T [5] , a mild *CFTR* variant, which in combination with long TG repeats and the V470 allele /a severe *CFTR* gene mutation on the other allele, could possibly cause CF or CFTR- RDs (Bombieri et al., 2011). Thus, it has been put forth that an allele by itself might not be responsible for harmful effects; but the alignment of specific alleles at different loci could cause an aberrant *CFTR* transcription and translation (Cuppens et al., 1998; Claustres, 2005). Hence accurate genetic counseling must be provided to the couple prior to ICSI.

In conclusion, the cohort of Indian CBAVD men showed the haplotypes carrying the V470 allele in exon 11 with a high frequency of c.1210-12T [5] alleles at the poly T locus and the c.1210-34TG [12] and c.1210-34TG [13] alleles at the TG (m) locus in intron 9. The c.1210-12T [5] variant among females is low. The couples were provided appropriate genetic counseling for the risk of having a child with CF or CF related disorders such as CBAVD, pancreatitis or bronchitis. The study suggests screening for the polymorphism M470V, poly T, TG tract repeats in addition to the known *CFTR* mutations in both the couples prior to ICSI.

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Declaration

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Table 1: Allelic frequency (%) of poly-T variants

poly-T variants	CBAVD men (n=76)	Healthy men (controls) (n=50)	Female partners of CBAVD men (n=76)
c.1210-12T[5]	32/152(21)*	7/100 (7)	12/152 (8.3)
c.1210-12T[7]	98/152 (64.4)	68/100(68)	112/152 (73.6)
c.1210-12T[9]	20/152(13.1)	20/100 (20)	27/152 (17.7)

* $p \leq 0.05$ with respect to controls

Table 2: Allelic frequency (%) of TG-repeats

TG-repeats	CBAVD men (n=76)	Healthy men (controls) (n=50)	Female partners of CBAVD men (n=76)
c.1210-34TG[10]	19/152 (12.5)	16/100 (16)	12/152(7.8)
c.1210-34TG[11]	82/152 (53.9)*	30/100 (30)	75/152 (49.3)*
c.1210-34TG[12]	41/152* (26.9)	49/100(49)	64/152 (42.1)
c.1210-34TG[13]	9/152(7.2)#	0/100 (0)	1/152 (0.6)

* $p \leq 0.05$ with respect to controls,

$p \leq 0.0000001$ for c.1210-34TG [12] and c.1210-34TG [13] with respect to controls

Table 3: The allelic frequency of poly-T and TG-repeats haplotype

Linkage Haplotypes	CBAVD men (n=76)	Healthy men (Controls) (n=50)	Female partners of CBAVD men (n=76)	p
c.1210-34TG[10];1210-12T[5]]	1/152(0.65%)	-	-	
c.1210-34TG[10]-];1210-12T[7]]	7/152(4.6%)	8/100 (8%)	7/152 (4.6%)	0.4308
c.1210-34TG[10]-];1210-12T[9]]	10/152 (6.6%)	9/100 (9%)	4/152(2.6%)	0.08575
c.1210-34TG[11];1210-12T[5]]	1/152 (0.65%)	0/100 (0%)	2/152(1.3%)	NA
c.1210-34TG[11];1210-12T[7]]	74/152 (48.6%)	17/100 (17%)	65/152 (42.7%)	0.000001
c.1210-34TG[11];1210-12T[9]]	7/152(4.6%)	8/100 (8%)	8/152 (5.2%)	0.5019
c.[1210-34TG[12];1210-12T[5]]	21/152 (13.8%)	7/100 (6%)	9/152 (5.9%)	0.04000
c.[1210-34TG[12]; 1210-12T[7]]	21/152(13.8%)*	41/100 (41%)	40/152 (40%)	0.000006
c.[1210-34TG[12]; c.1210-12T[9]]	-	2/100 (2%)	-	NA
c.[1210-34TG[13]; c.1210-12T[5]]	9/152 (5.9%) *	-	1/152 (0.65%)	NA

*p ≤0.01 for combined c.[1210-34TG[12];1210-12T[5]] c.[1210-34TG[13]; c.1210-12T[5]] with respect to controls. NA- statistically not applicable.

Table 4: Distribution and allelic frequency of M470V genotype

Genotype distribution	CBAVD men (n=76)	Healthy men (controls) (n=50)	Female partners of CBAVD men (n=76)
V/V	25/76 (32.8%)*	4/50 (8%)	9/76 (11.8%)
M/V	27/76(35.5%)	14/50 (28%)	27/76 (35.5%)
M/M	24/76 (31.5%)*	32/50 (64%)	40/76 (52.6%)
Allelic frequencies			
V/V (V470)	77/152(50.6%)*	22/100(18%)	45/152(29.6%)
M/M (M470)	75/152 (49.3%)*	78/100 (78%)	107/152(70.3%)

*p≤ 0.05 versus controls

V/V–Homozygous V470, M/V- heterozygous M470V, M/M- homozygous M470

Table 5: Association of TG12-TG13-5T and M470V

(IVS9- c.1210-12T [5])	M470V genotype		
	V/V	M/V	M/M
CBAVD men (n=30)	15/30(50%)*	8/30 (26.6%)	7/30(23.3%)
Female Partners of CBAVD men (n=12)	2/12 (16.6%)	6/12 (50%)	4/12 (33.3%)
Healthy men (controls) (n=6)	-	3/6 (50%)	3/6 (50%)

* $p \leq 0.05$ as compared to controls

V/V- Homozygous V470, M/V- heterozygous M470V, MM- homozygous M470 allele

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