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Cystic fibrosis transmembrane conductance regulator-related male infertility: Relevance of genetic testing & counselling in Indian population

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***Background & objectives*: Due to limited information available on the frequency and spectrum of cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene mutations in congenital bilateral absence of vas deferens (CBAVD) in Indian population, it is difficult to provide accurate genetic counselling to couples. The present study was undertaken to investigate the spectrum and frequency of *CFTR* gene mutations in Indian men with CBAVD and to determine the female CF carrier status.**

***Methods*: Direct DNA sequencing of the *CFTR* gene was carried out in eighty CBAVD men, their female partners and fifty controls from the general population. Pathological significance of the identified novel *CFTR* gene variants was carried out using *in silico* tools. Appropriate genetic counselling was provided to the couples prior to intracytoplasmic sperm injection (ICSI).**

***Results*: A significant association was observed for *CFTR* gene variants in Indian CBAVD men versus controls (odds ratio: 12.1; 95% confidence interval: 4.8-30.4; *P*<0.0001). A total of 20 *CFTR* gene variants were identified in 53 CBAVD men. Eight novel missense *CFTR* gene variants (L214V, A238P, E379V, L578I, F587L, L926W, R1325K and R1453Q); two novel splice-site gene variants (c.1-30C>G and IVS1+2T>G) and ten previously reported mutations (R75Q, c.1210-12[5], F508del, A309G, R334W, I444T, R668C, R709X, A1285V and Q1352H) were detected in CBAVD men. The novel and reported *CFTR* gene mutations were L926W (2.5%, *P*=0.26), R1453Q (2.5%, *P*=0.26), F508del (8.75%, *P*=0.03) and c.1210-12[5] (42.5%, *P*=0.002). A total of 13 (16.2%) female partners were found to be a CF carrier. Nine couples had a risk of transmitting mutant CFTR allele to the offspring.**

***Interpretation & conclusions*: The heterogeneous spectrum of *CFTR* gene in Indian population suggests the necessity of screening CBAVD men and female partners for accurate genetic counselling prior to undergoing ICSI.**

Key words Azoospermia - cystic fibrosis transmembrane conductance regulator gene - congenital bilateral absence of vas deferens - genetic counselling - intracytoplasmic sperm injection - male infertility

Congenital bilateral absence of vas deferens (CBAVD), an autosomal recessive disorder, accounts for 1-2 per cent of male infertility with an estimated incidence of 1:1000 men[1]. CBAVD is considered a primary genital form of cystic fibrosis (CF), associated with abnormalities in the CF transmembrane conductance regulator (*CFTR*) gene[2]. In humans, the *CFTR* gene on chromosome 7q31.2 encodes CFTR protein that regulates the chloride ions across epithelial cell membranes[2]. Structurally, the protein comprises: two membrane-spanning domains which form the channel pores, two nucleotide-binding domains which control channel gating and one regulatory domain (R domain) which determines the phosphorylation activity[3].

More than 2000 *CFTR* gene variants have been documented in the CF mutations Databases (*http://www.genet.sickkids.on.ca/cftr/app*, *http://www.umd.be/CFTR*, *http://www.cftr2.org/*). F508del and the c.1210-12[5] are also referred as (5T) the most common severe and mild mutations, respectively, detected in CF- and CFTR-related disorders (CFTR-RDs) such as CBAVD[1]. There is a variation in the frequency of F508del, while the 5T variant is found to be present at a similar frequency in CBAVD men with different ethnicity and residing in different geographical regions[1-14]. In North Indian CBAVD men, the frequency of F508del and c.1210-12[5] was reported as 11-34 and 26-54 per cent, respectively[4,5,9]. Our previous study reported 39.4 per cent frequency of IVS9-c.1210-12[5] in CBAVD men[7]. Currently, there is no epidemiological statistics on the incidence of CBAVD phenotype in India. However, data accumulated over a decade suggest that the incidence of isolated CBAVD in Indian population might be comparable to that of Caucasians[4-9].

CBAVD men have normal spermatogenesis and can achieve biological paternity using assisted reproductive technologies (ART) such as intracytoplasmic sperm injection (ICSI)[15] with their own sperm retrieved through various sperm retrieval techniques such as microsurgical epididymal sperm aspiration, testicular sperm extraction, percutaneous epididymal sperm aspiration[16] or opt for donor insemination. Moreover, if the female partner of a CBAVD male is a CF carrier, she possesses a risk of transmitting the mutant *CFTR* allele to the progeny, resulting in a child born with a full-blown CF or CF-related disorders such as CBAVD[16,17], highlighting the significance of screening female partners. However, CF carrier frequency in the female partner of CBAVD men still remains undetermined. Routine screening for *CFTR* gene is not conducted in ART clinics in India due to inadequate knowledge of the frequency and spectrum of *CFTR* gene variants, limited awareness of the *CFTR*-related male infertility and non-availability of population-specific mutation panel.

Therefore, the present study was undertaken to identify the spectrum and frequency of *CFTR* gene variants in Indian CBAVD men and to determine their female partner CF carrier status. It was also aimed to understand the genetic risks involved, if the female partner was a CF carrier and to provide accurate counselling to the couples prior to undergoing ICSI.

Material & Methods

This study was approved by the Institutional Ethics Committee for research involving human subjects at ICMR-National Institute for Research in Reproductive Health (ICMR-NIRRH), Mumbai, India. Written informed consent was also obtained from all the study participants prior to blood sample collection.

The present study represents a part of the case–control genetic study conducted during the period 2011-2017 at ICMR-NIRRH, Mumbai. The total sample size was calculated as CBAVD (n=100), female partners of CBAVD (n=100) and fertile controls (n=50). A total of eighty unrelated sub-fertile men with a confirmed diagnosis of CBAVD were recruited from different geographic regions in India [north (n=16), northeast (n=1), west (n=48), central (n=1) and south (n=14)]. The semen parameters were evaluated as reported previously[18]. Men with semen parameters showing azoospermia, low seminal volume (<1.5 ml), absence/low fructose in semen and absence of the bilateral vas deferens on scrotal examination by the andrologists (Drs Rupin Shah and Vijay Kulkarni) were recruited as CBAVD cases. Testicular volume was assessed using Prader Orchidometer (Holtain Ltd, Crosswell, Wales, UK). Ultrasonography (USG) of the abdomen and pelvis was also performed. CBAVD men with renal abnormalities or those with congenital unilateral absence of vas deferens were excluded from the data analysis. Transrectal ultrasonography (TRUS) was carried out in CBAVD men to detect anomalies of seminal vesicles and ejaculatory ducts, wherever possible. Female partners of CBAVD men (n=80) were recruited in this study to assess carrier frequency. Proven fertile men from the general population attending the Family Welfare Clinics at ICMR-NIRRH

were recruited as controls (n=50). The controls had normal semen parameters with bilaterally palpable vas deferens. Study participants having family or personal history of CF or CF-related disorders were excluded from the study. The information related to ICSI outcomes were obtained during the follow up of the CBAVD men with the andrologist.

Cystic fibrosis transmembrane conductance regulator (CFTR) gene testing and analysis: Peripheral venous blood was collected through venipuncture in ethylenediaminetetraacetic acid (EDTA) tubes, and genomic DNA was isolated using QIAamp® DNA Blood Midi Kit (Qiagen Inc., Hilden, Germany). The essential promoter, entire coding regions and splice sites of 27 exons of the *CFTR* gene were amplified by PCR using specific primers (Supplementary Table I). Sequencing reaction and analysis were carried out as described earlier[6].

In silico prediction of pathogenicity: Bioinformatics tools such as PolyPhen-2, SIFT and Mutation Taster were used to predict the pathological significance of the identified novel *CFTR* variants. Polyphen-2 *(http://genetics.bwh.harvard.edu/pph2/*) predicted the possible impact of an amino acid substitution on the structure and function of a protein using physical and comparative considerations. Sorting Intolerant From Tolerant (SIFT) (*https://sift.bii.a-star.edu.sg*) and Mutation Taster (*http://www.mutationtaster.org*) predicted the effect of amino acid substitution on protein function based on sequence homology and the physical properties of amino acids. The pathological splicing effects of the exonic and intronic variants were predicted using Human Splicing Finder version 3.0 (*http://www.umd.be/HSF3/HSF.html*). Multiple sequence alignments using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*) were carried out for understanding the conserved nature of the novel *CFTR* variants across various species.

Statistical analysis: All data were expressed as mean ± standard deviation (SD). Differences between proportions of *CFTR* gene mutations were compared by Chi-square test using STATA software (version 8.2, Texas, USA), and $P<0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics: The age of CBAVD men was 31.97±5.18 yr (mean ± SD), range: 23-47 yr. The mean age of the female partners of CBAVD men was 27.78±4.61 yr, (range: 20-38 yr). The duration of infertility was 4.45±3.48 yr, (range: 12 to 240 months). The mean testicular size found was 20.6±4.5 ml. Based on the TRUS reports available, agenesis of seminal vesicles was found in 15 (18.8%) CBAVD men. The head of the epididymis was palpable in most of the CBAVD men. The semen parameters demonstrated azoospermia with a mean semen volume of 0.79±0.9 ml and a mean semen *p*H of 6.72±0.8.

Genotypic characteristics: Screening of complete *CFTR* gene in CBAVD men led to the identification of twenty variants comprising ten novel and ten known variants, as represented in Table I. The variants detected comprised 16 missense, two splicing, one non-sense and one frame shift mutations. Out of 80 CBAVD men, *CFTR* gene mutations were detected in 53 CBAVD men with 66.3 per cent frequency (Table II). A statistically significant association was observed for *CFTR* gene variants between Indian CBAVD men and controls (odds ratio: 12.1; 95% confidence interval: 4.8-30.4; $P<0.0001$).

Novel variants: Of the ten novel variants, seven were heterozygous missense [L214V (KU325495), A238P (KT121468), E379V (KM596832), L578I (KU325496), L926W (KM014732), R1325K (KM596834) and R1453Q (KM596833)]. A homozygous missense gene variant F587L (KJ606925) was also identified. Electropherograms of the novel *CFTR* gene variants are represented in Fig. 1. Two of the novel splice-site variants detected were c.1-30C>G (KU325497) in the essential promoter region and IVS1+2T>G (KU325498). The most frequently detected novel gene variants were L926W (2.5%) and R1453Q (2.5%). The correlation of novel *CFTR* gene variants with semen characteristics, testicular size and epididymal status is shown in Table III.

Previously reported mutations: Ten previously known *CFTR* gene mutations identified in the study population are represented in Table I. Electropherograms of previously reported *CFTR* gene variants are represented in Fig. 2. The mutations/variants identified spanned the different domains of the CFTR protein and are represented in Fig. 3. The geographic distribution of *CFTR* gene variants in Indian CBAVD men is depicted in Supplementary Fig. 1. The most commonly identified *CFTR* gene mutations were F508del (8.75%, $P=0.03$), c.1210-12[5] (42.5%, $P=0.002$), R75Q (4.2%, $P= 0.17$),

Table I. *CFTR* gene known mutations and variants identified in Indian congenital bilateral absence of vas deferens (CBAVD) men

Mutations	Nucleotide change	Consequences	Exon/intron	Number of alleles
c.1-30C>G*	Splice error	-	5' UTR region	1
IVS1+2T>G*	c.53+2T>G	-	Intron 1	2
R75Q	c.224G>A	Arginine to glutamine at 75	Exon 3	3
L214V*	c.640C>G	Leucine to valine at 214	Exon 7	1
A238P*	c.844G>C	Alanine to proline at 238	Exon 7	1
A309G	c.926C>G	Alanine to glycine at 309	Exon 7	1
R334W	c.1000C>T	Arginine to tryptophan at 334	Exon 8	1
5T	c.1210-12[5]	Aberrant splicing	Intron 9	34
E379V*	c.1136A>T	Glutamic acid to valine at 379	Exon 9	1
F508del	c.1521_1523delCTT	Deletion of phenylalanine at 508	Exon 11	7
I444T	c.1331T>C	Isoleucine to threonine at 444	Exon 9	1
L578I*	c.1732C>A	Leucine to isoleucine at 578	Exon 13	1
F587L*	c.1762T>G	Phenylalanine to leucine at 587	Exon 13	2
R668C	c.2002C>T	Arginine to cysteine at 668	Exon 14	1
R709X	c.2125C>T	Arginine to pre-mature stop codon at 709	Exon 14	2
L926W*	c.2777T>G	Leucine to tryptophan at 926	Exon 17	2
A1285V	c.3854C>T	Alanine to valine at 1285	Exon 20	1
R1325K*	c.3974G>A	Arginine to lysine at 1325	Exon 25	1
Q1352H	c.4056G>C	Glutamine to histidine at 1352	Exon 25	2
R1453Q*	c.4358G>A	Arginine to glutamine at 1453	Exon 27	2

*Novel sequence variant. UTR, untranslated region

R709X (2.5%, *P*=0.26) and Q1352H (2.5%, *P*=0.26). Of the seven CBAVD men with F508del mutation, four men also harboured one of the following mutations: c.4056G>C (Q1352H), c.1517T>C (I506T), c.224G>A (R75Q) or a novel variant c.1732C>A (L578I), while only one of them harboured a c.1210-12[5] variant.

Coding single-nucleotide polymorphisms and intronic variants: Of the 26 intronic variants detected, 19 were novel (Supplementary Table II), while 7 were previously reported. Four potential regulatory coding single-nucleotide polymorphisms namely M470V (n=55), T854T (n=21), P1290P (n=4) and Q1463Q (n=30) were detected in CBAVD men.

Significant differences were observed in the frequency of *CFTR* gene variants across the isolated CBAVD and CBAVD with agenesis of seminal vesicle (*P*<0.001) Supplementary Table III.

Female partner cystic fibrosis carrier status: Of the eighty female partners of the CBAVD men screened, *CFTR* gene variant was detected in 13 (16.2%) females (Table II). Twelve females harboured a c.1210-12[5] allele, while one was a carrier of A1285V heterozygous missense *CFTR* gene variant.

In silico analysis: *In silico* analysis of the *CFTR* gene variants is represented in Supplementary Table IV. PolyPhen-2 predicted six novel *CFTR* gene sequence variants to have a score >0.5 which is the threshold for damaging mutations and therefore, it might be responsible in damaging the CFTR protein structure or function. Similarly, SIFT predicted six novel variants to be damaging, whereas Mutation Taster predicted seven variants to be disease-causing mutations. *In silico* analysis using Human Splicing Finder v.3.0 predicted novel exonic variants to affect the splicing mechanism, either by altering or creating Exonic splicing silencer and Exonic splicing enhancer sites in the *CFTR* gene (Supplementary Table IV). Multiple sequence alignments using Clustal Omega confirmed the wild-type amino acid sequence of all the novel variants except R1325K to be conserved across species such as human, mouse, rabbit, bovine, sheep, monkey, pig and horse (Supplementary Fig. 2).

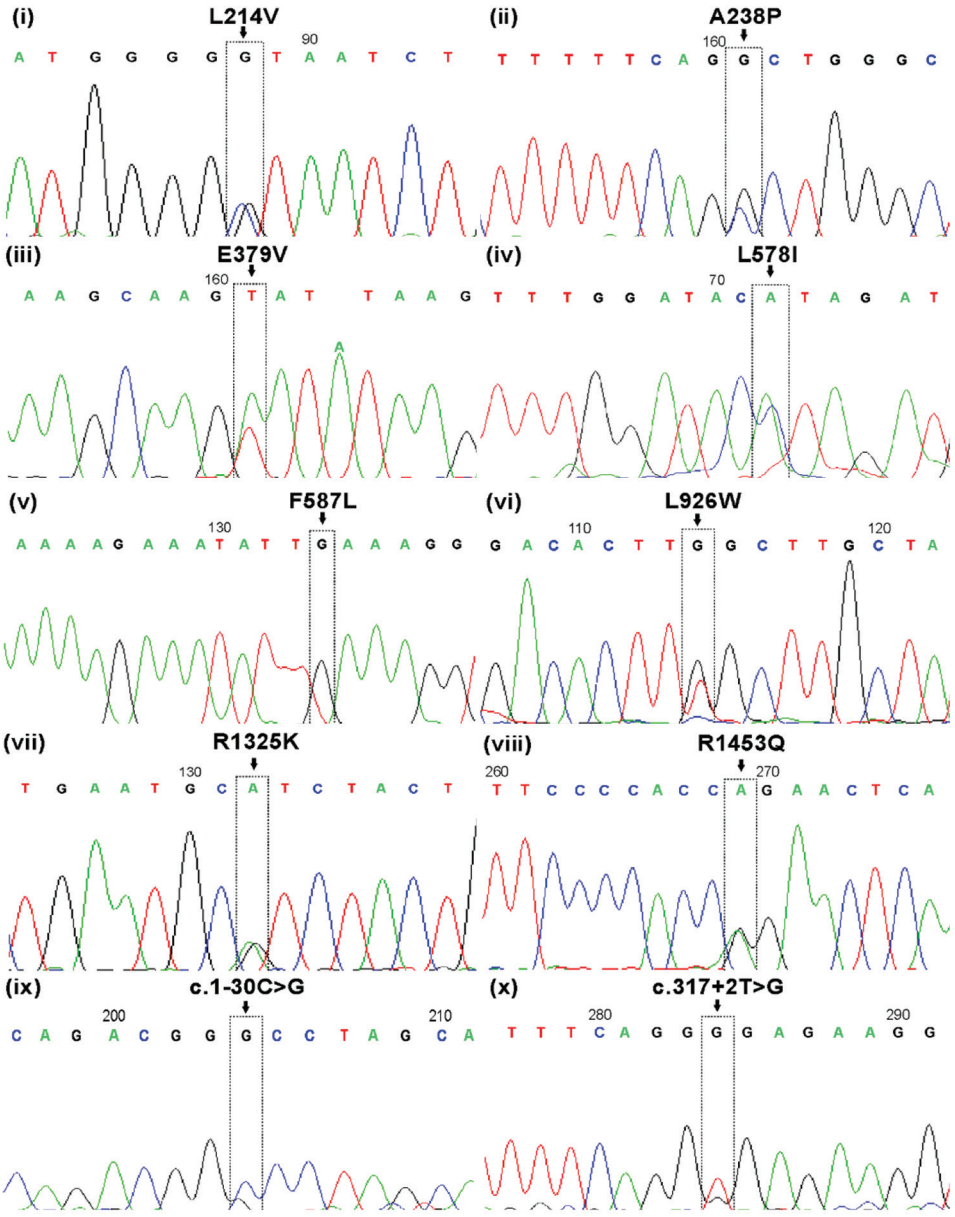

Fig. 1. Sequencing results of novel *CFTR* gene variants (L214V, A238P, E379V, L578I, F587L, L926W, R1325K, R1453Q, c.1-30C>G and c.317+2T>G) identified in Indian congenital bilateral absence of vas deferens men.

Table II. Frequency of *CFTR* gene variants in Indian population

Genotype	CBAVD men (n=80), n (%)	Female partner of CBAVD men (n=80), n (%)	Fertile men as controls (n=50), n (%)
CFTR variant detected	53/80 (66.25)*	13/80 (16.25)*	7/50 (14)
At least one severe/mild variant	41/80 (51.25)*	13/80 (16.25)	7/50 (14)
Two or more variants	12/80 (15)*	0/80 (0)	0/50 (0)

*$P \leq 0.0001$ as compared to controls. CBAVD, congenital bilateral absence of vas deferens

Table III. Semen characteristics, testicular volume and epididymal status correlation with novel CFTR gene variants

CBAVD case number and geographical location	Semen parameters				Testicular size (ml)		Epididymal status		Novel *CFTR* gene variant
	Volume (ml)	PH	Liquefaction time (min)	Fructose	Right	Left	Right	Left	
H4 (Maharashtra)	0.5	6	20	Negative	30	30	Head turgid	Head turgid	L926W
H20 (Maharashtra)	0.3	6	ND	Negative	18	18	Half head full	Half head full	c.1-30C>G
H21 (Maharashtra)	1	8	60	Negative	18	15	Head	Half head full	IVS1+2T>G, L926W
H22 (Uttar Pradesh)	0.3	6	15	Negative	24	26	Head turgid	Cystic head	R1453Q
H23 (Kerala)	1	7.8	ND	Negative	22	22	Head	Head	L578I
H28 (Uttar Pradesh)	2	7.2	ND	Negative	30	30	Head turgid	Head turgid	IVS1+2T>G
H29 (Andhra Pradesh)	1.5	7.2	40	Negative	25	25	Head	Head	R1325K
H31 (Uttar Pradesh)	0.1	6	30	Negative	20	20	Head	Head	R1453Q
H39 (Maharashtra)	1	7.2	15	Positive	15	15	One third head	One third head	F587L
H55 (Maharashtra)	0.5	6	20	Negative	20	20	Head	Head	E379V
H60 (Maharashtra)	1	7	30	Negative	15	15	Head	Head	A238P
H70 (Karnataka)	0.1	6	20	Negative	16	14	Head	Head	L214V

ND, not done; CBAVD, congenital bilateral absence of vas deferens

Risk of cystic fibrosis or CFTR-related disorders (CFTR-RD) to the offspring: Of the cohort, 53 men and 13 females who harboured a mild or a severe variant were at risk of transmitting at least one mutant variant to the offspring. Among them, nine couples were both carriers of the CF variant (Supplementary Table V). Three CBAVD men had compound heterozygous *CFTR* gene mutations L926W/c.1210-12[5] and c.1521_1523delCTT (F508del)/L578I, and their female partner harboured c.1210-12[5] or a *CFTR* gene mutation, increasing the risk by 50 per cent for the offspring who inherit two *CFTR* gene variants. Six CBAVD men had one *CFTR* gene variant and their female partners harboured c.1210-12[5], thereby increasing the risk by 25 per cent for the offspring to inherit two *CFTR* gene variants.

Outcome of intracytoplasmic sperm injection (ICSI): Among the cohort of CBAVD men who were mild or severe variant (n=53), 21 underwent at least one cycle of ICSI (40%). Among these couples, four successful pregnancies with live births including one twin pregnancy were recorded. One couple opted for adoption after ICSI failures. In nine couples wherein both partners were carriers of CFTR mutations/variants, six underwent ICSI, with two successful pregnancies resulting in live birth.

Discussion

In the present study, a heterogeneous spectrum of *CFTR* gene variants is reported with 66.3 per cent frequency in Indian men with CBAVD, which is lower than that in the Caucasian population[1]. CBAVD men were classified as patients with: (*i*) one variant (51.25%), (*ii*) two variants (15%) and (*iii*) no mutations (33.7%). Two *CFTR* gene mutations detected in 15 per cent of the CBAVD men, were comparable to those found in other studies[13,19,20]. The most common mutation, F508del, was observed at a lower allelic frequency (8.75%) as compared to that of the Caucasian population[1] but was higher than that of Chinese[2], Japanese[21] and Taiwanese[22] populations and was comparable with that of north Indian CBAVD men[4,5,8]. On the contrary, IVS-9 c.1210-12[5] variant was reported at a higher frequency (42.5%) compared to that of studies in literature[4,5,9]. Failure to detect *CFTR* gene variants could be either due to the presence of large rearrangements such as exon deletion, insertion and duplications[23,24] or because of genetic aetiology other than *CFTR* gene. Recent studies suggest that gene *ADGRG2* at a frequency of 11-15 per cent in CBAVD cases is negative for *CFTR* gene mutations[25].

Out of the ten novel sequence variants, eight were located in highly conserved regions, which could functionally alter CFTR protein organization. Two novel splice-site variants (c.1-30C>G and c.317+2T>G) were predicted to induce splicing error and thus damage the translated CFTR protein. R75Q mutation detected in 4.2 per cent is a known variant for chronic obstructive pulmonary disease (COPD)[26]. R709X detected in 3 per cent of CBAVD men is predicted to result in a truncated

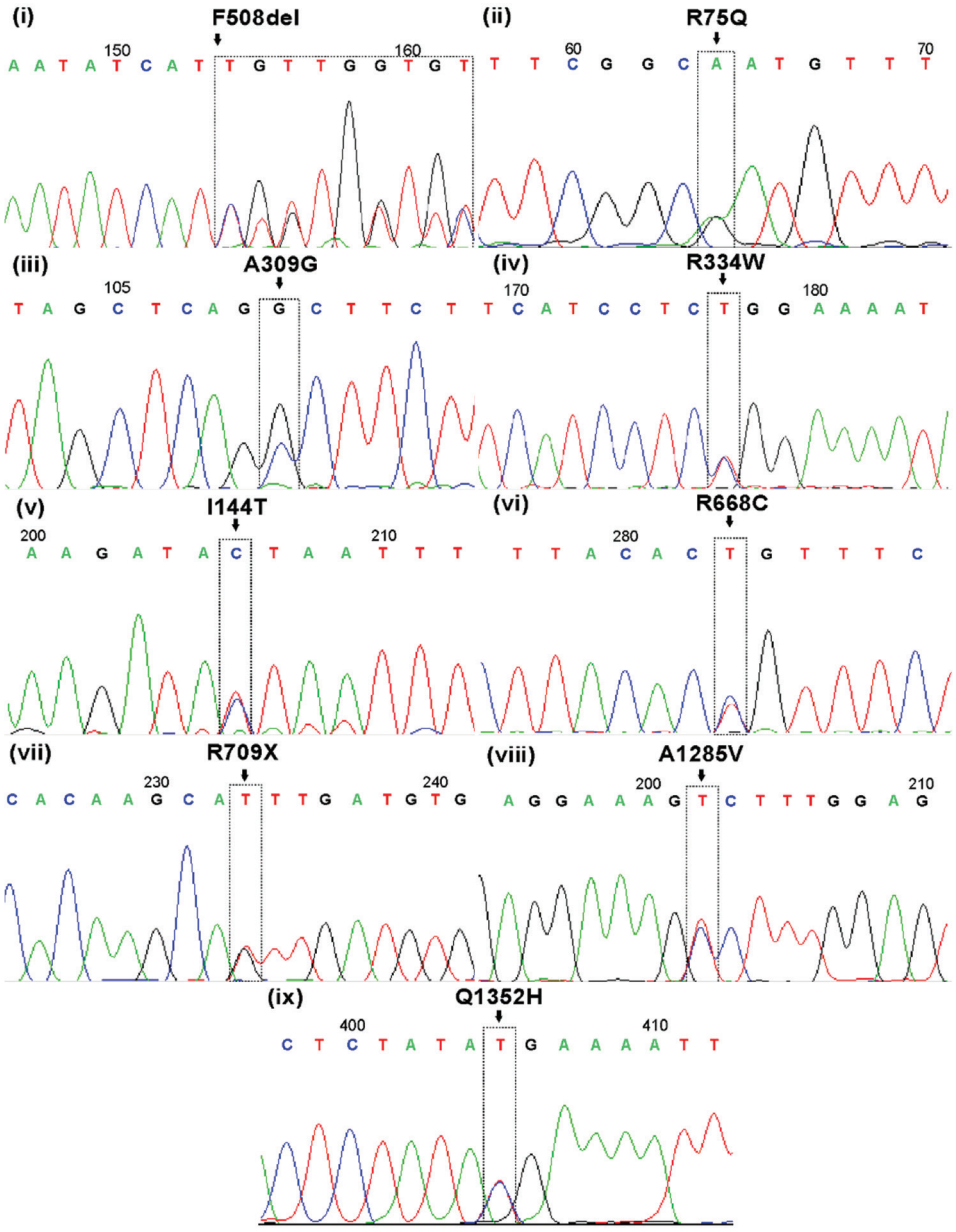

Fig. 2. Sequencing results of previously reported *CFTR* gene mutations (F508del, R75Q, A309Q, R334W, I144T, R668C, R709X, A1285V and Q1352H) identified in Indian congenital bilateral absence of vas deferens men.

and non-functional CFTR protein[11]. In addition, *in vitro* functional assays are warranted to provide significant insight regarding the pathogenicity of the identified variants. Although CBAVD men can achieve biological paternity via ICSI, they are at a higher risk of transmitting lethal mutations to offspring[7,27]. A few studies have attempted to screen for CF female carrier status in the Indian population. Being said that, due to the increasing burden of mutations in the *CFTR* gene, it has becomes difficult to provide precise molecular diagnosis and accurate genetic counselling to CBAVD patients. Considering the functional significance of the identified known and novel *CFTR* gene variants, genetic counselling was provided by assessing female CF carrier status. In the present study a heterozygous A1285V variant was identified in one female whose male partner had a heterozygous F508del mutation. Interestingly, A1285V variant has been reported as a novel mutation in Indian CBAVD male[9]. Since both the partners were carriers of *CFTR* gene mutation, there was a 25 per cent risk of having a child with classic CF or CFTR-RD. In addition to this, eight females who

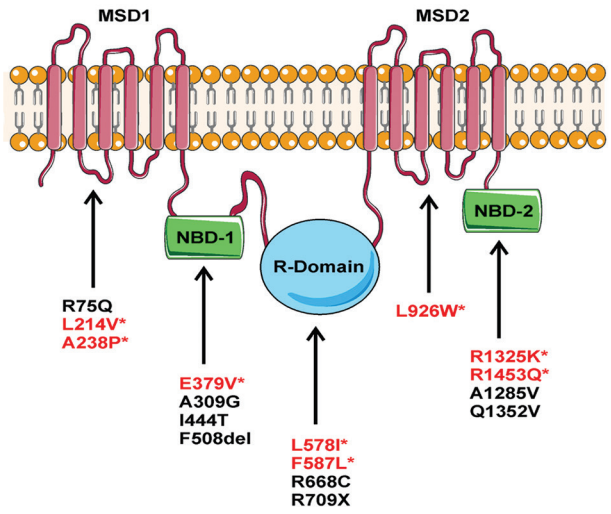

Fig. 3. Schematic diagram representing putative domain-type structure of the CFTR protein and identified *CFTR* gene variants. Novel *CFTR* gene variants are indicated in red and *. Previously known *CFTR* gene mutations are indicated in black. MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R domain, regulatory domain.

were carriers of heterozygous c.1210-12[5] variant had their male partners with a mild or severe *CFTR* gene variant. Therefore, genetic counselling for couples in which the male partner has CBAVD is important to estimate the risks and possible genotype–phenotype correlations prior to undergoing ICSI.

In conclusion, we found the spectrum and frequency of *CFTR* gene variants in the Indian population to be different from that of Caucasians. Detection of female partners as CF carriers may be useful for providing genetic counselling to the couples before enrolling them for ICSI. Complete *CFTR* gene screening is expensive and therefore a population-specific panel should be designed based on the known and novel variants identified in the Indian population. Further studies are underway to identify the genetic basis of *CFTR*-negative CBAVD subgroup.

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***Conflicts of Interest*:** None.

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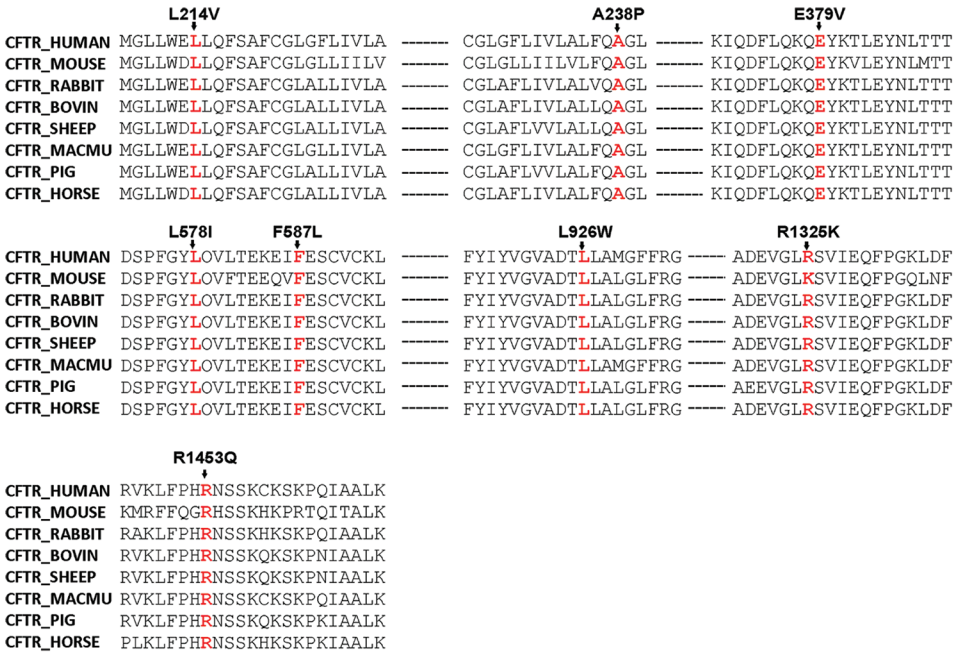

Supplementary Fig. 1. Multiple alignments (*https://www.ebi.ac.uk/Tools/msa/clustalo/*) of conserved CFTR amino acid sequences from multiple species (human, mouse, rabbit, bovine, sheep, monkey, pig and horse) and eight novel CFTR gene variants (L214V, A238P, E379V, L578I, F587L, L926W, R1325K and R1453Q) identified in Indian congenital bilateral absence of vas deferens males. The conserved amino acids are highlighted in red across different species.

Supplementary Fig. 2. Geographic distribution of CFTR gene variants in congenital bilateral absence of vas deferens men in India identified in this study. The red arrow indicates the identified CFTR variants from the region. * indicates novel CFTR variants.

Supplementary Table I. Primers used for polymerase chain reaction amplification and sequencing of the *CFTR* gene

Amplification site	PCR primer	Amplicon length (bp)	Annealing temperature (ºC/35 cycles)
Promoter + Exon 1	Forward: 5' - GTGGAGAAAGCCGCTAGAGCAAAT - 3' Reverse: 5'- GCAAGTAGATGTGGCTCTCTA - 3'	360	58
Exon 2	Forward: 5' - GTGCATAATTTTCCATATGCC - 3' Reverse: 5'- TTAGCCACCATACTTGGCTC- 3'	341	58
Exon 3	Forward: 5' - CATGCAACTTATTGGTCCCAC - 3' Reverse: 5'- TTCACCAGATTTCGTAGTCTTTTC - 3'	232	58
Exon 4	Forward: 5' - TCTTGTGTTGAAATTCTCAGGG - 3' Reverse: 5'- AAAACTACAACAGAGGCAGTTTACAG - 3'	525	58
Exon 5	Forward: 5' - GAACCTGAGAAGATAGTAAGCTAGATG - 3' Reverse: 5'- GAAAACTCCGCCTTTCCAG - 3'	321	56
Exon 6	Forward: 5' - GGGTGGAAGATACAATGACACC - 3' Reverse: 5'- TCCTGGTTTTACTAAAGTGGGC - 3'	287	56
Exon 7	Forward: 5' - TGCCCATCTGTTGAATAAAAG - 3' Reverse: 5'- CAAACATCAAATATGAGGTGGAAG- 3'	340	57
Exon 8	Forward: 5' - CTTCCATTCCAAGATCCCTG - 3' Reverse: 5'- TGAACATTCCTAGTATTAGCTGGC- 3'	476	57
Exon 9	Forward: 5' - TGCTTGGCAAATTAACTTTAGAAC - 3 Reverse: 5'- GCACCTGGCCATTCCTCTAC - 3'	440	58
Exon 10	Forward: 5' - GGCCATGTGCTTTTCAAACT - 3' Reverse: 5'- CGCCAACAACTGTCCTCTTT - 3'	270	56
Exon 11	Forward: 5' - CAAGTGAATCCTGAGCGTGA - 3' Reverse: 5'- CCGATTGAATATGGAGCCAA - 3'	336	58
Exon 12	Forward: 5' - GGAAGATGTGCCTTTCAAATTC - 3' Reverse: 5'- CAGCAAATGCTTGCTAGACC - 3'	204	58
Exon 13	Forward: 5' - GACCAGGAAATAGAGAGGAAATG - 3' Reverse: 5'- TGCAATCTATGATGGGACAG - 3'	213	56
Exon 14	Forward: 5' - AAATGCTAAAATACGAGACATATTGC - 3' Reverse: 5'- GGATGCTGTTGTCTTTCGGT - 3'	678	58
Exon 15	Forward: 5' - ACAATGGTGGCATGAAACTG - 3' Reverse: 5'- TGAGCTTTCGAATCTCTTAACC - 3'	547	57
Exon 16	Forward: 5' - AATTTAGATGTGGGCATGGG - 3' Reverse: 5'- GGATTACAATACATACAAACATAGTGG - 3'	201	58
Exon 17	Forward: 5' - GGCTGCCAAATAACGATTTC - 3' Reverse: 5'- GATGGTGGATCAGCAGTTTC - 3'	329	58
Exon 18	Forward: 5' - GAGAAATTGGTCGTTACTTGAATC - 3' Reverse: 5'- CTAAATGTGGGATTGCCTCAG - 3'	566	58
Exon 19	Forward: 5' - AAATGTTTACTCACCAACATGTTTTC - 3' Reverse: 5'- TTTACAAGATGAGTATCGCACATTC - 3'	225	62
Intron 19	Forward: 5' - CATTCAGTGGGTATAAGC AGC - 3'	327	58
Exon 20	Forward: 5' - TCTATTCAAAGAATGGCACCAG - 3' Reverse: 5'- CAATGGAAATTCAAAGAAATCAC- 3'	549	56
Exon 21	Forward: 5' - CATGAGGTTCATTTACGTCTTTTG - 3' Reverse: 5'- CACAGTGACCCTCAATTTATCTG - 3'	276	57
Exon 22	Forward: 5' - TTGTGAAATTGTCTGCCATTC - 3' Reverse: 5'- CACAGTCTAACAAAGCAAGCAG - 3'	339	57

Contd...

Amplification site	PCR primer	Amplicon length (bp)	Annealing temperature (ºC/35 cycles)
Exon 23	Forward: 5' - CTGAATTATGTTTATGGCATGG - 3' Reverse: 5'- TTGCAGAGTAATATGAATTTCTTGAG - 3'	317	56
Exon 24	Forward: 5' - TGATGGTAAGTACATGGGTGTTTC - 3' Reverse: 5'- TCAGCCATTTGTGTTGGTATG - 3'	283	62
Exon 25	Forward: 5' - TCAAATGGTGGCAGGTAGTG - 3' Reverse: 5'- TGATTCTGTTCCCACTGTGC - 3'	362	58
Exon 26	Forward: 5' - CTACCCCATGGTTGAAAAGC - 3' Reverse: 5'- TGAGTAAAGCTGGATGGCTG - 3'	421	58
Exon 27	Forward: 5' - GTCTGACCTGCCTTCTGTCC - 3' Reverse: 5'- ATTCCATGAGCAAATGTCCC - 3'	291	58

PCR, polymerase chain reaction

Supplementary Table II. Splice-site analysis of novel intronic *CFTR* gene variants in congenital bilateral absence of vas deferens (CBAVD) men

Novel intronic variants	Intron	Interpretation
c.185+84G>A	1	Alteration of an intronic ESS site Creation of an intronic ESE site
c.405+24A>T	3	No significant splicing motif alteration detected
c.621+91G>A	4	Alteration of an intronic ESS site
c.875+12G>A	6	No significant splicing motif alteration detected
c.1248+80A>C	8	Creation of an intronic ESE site
c.1249-106A>T	9	Alteration of an exonic ESE site
c.1259-167A>C	9	No significant splicing motif alteration detected
c.1898+45A>T	13	No significant splicing motif alteration detected
c.1898+25delT	13	No significant splicing motif alteration detected
c.2751+855delTA	15	Alteration of an intronic ESS site Creation of an intronic ESE site
c.2751+106T>A	15	Alteration of an intronic cryptic acceptor site Creation of an intronic ESE site
c.2751+174A>C	15	Creation of an intronic ESE site
c.2751+86T>C	15	No significant splicing motif alteration detected
c.2751-75T>G	15	Activation of an intronic cryptic acceptor site
c.3120+529insC	19	No significant splicing motif alteration detected
c.3120+228T>C	19	No significant splicing motif alteration detected
c.4095+61T>A	24	Alteration of an intronic ESS site Creation of an intronic ESE site
c.4268+30G>A	25	No significant splicing motif alteration detected
c.4269-93A>T	26	Alteration of an exonic ESE site

ESS, exonic splicing silencer; ESE, exonic splicing enhancer

Supplementary Table III. Comparison of *CFTR* gene variants between isolated CBAVD and CBAVD with agenesis of the seminal vesicle

Mutations/novel variants	Isolated CBAVD (n=130 alleles)	CBAVD with agenesis of seminal vesicle (n=30 alleles)
c.1-30C>G*	1	0
IVS1+2T>G*	2	0
R75Q	3	0
L214V*	1	0
A238P*	1	0
A309G	1	0
R334W	1	0
5T [c.1210-12T[5]]	30	4
E379V*	1	0
F508del	7	0
I444T	1	0
L578I*	1	0
F587L*	2	0
R668C	1	0
R709X	2	0
L926W*	2	0
A1285V	1	0
R1325K*	1	0
Q1352H	1	1
R1453Q*	2	0

*Novel sequence variant. CBAVD, congenital bilateral absence of vas deferens

Supplementary Table IV. Pathological prediction of novel *CFTR* gene variants identified in CBAVD men

Novel variants	Nucleotide change (HGVS nomenclature)	Polyphen-2	SIFT	Mutation Taster	Human splicing finder (v. 3.0) interpretation
L214V	c.640C>G	Probably damaging	Damaging	Disease causing	Possible creation of new donor site in exon 7
A238P	c.844G>C	Probably damaging	Damaging	Disease causing	Possible creation of a new exonic ESS site in exon 7
E379V	c.1136A>T	Probably damaging	Damaging	Disease causing	Possible creation of new donor site in exon 9
L578I	c.1732C>A	Probably damaging	Damaging	Disease causing	Possible creation of a new exonic ESS site in exon 13
F587L	c.1762T>G	Probably damaging	Damaging	Disease causing	Possible creation of a new exonic ESS site in exon 13
L926W	c.2777T>G	Probably damaging	Damaging	Disease causing	Could affect splicing due to broken ESE site in exon 17
R1325K	c.3974G>A	Benign	Tolerated	Polymorphism	Could affect splicing due to broken ESE site in exon 25
R1453Q	c.4358G>A	Benign	Tolerated	Disease causing	Possible creation of new acceptor site in exon 27

The PolyPhen-2 score ranges from 0.0 (tolerated) to 1.0 (deleterious). Variants with scores of 0.0 are predicted to be benign. Values closer to 1.0 are more confidently predicted to be deleterious. PolyPhen-2 and SIFT scores use the same range, 0.0 to 1.0, but with opposite meanings. A variant with a SIFT score of 1.0 is predicted to be benign. ESS, exonic splicing enhancer; ESE, exonic splicing silencer; HGVS, Human Genome Variation Society.

Supplementary Table V. Comparison of CBAVD men genotype with female partner

Couple number	CBAVD genotype	Female genotype
1	c.[1210-34TG[12];1210-12T[5]]	c.[1210-34TG[12];1210-12T[5]]
2	c.[1210-34TG[12];1210-12T[5]]/L926W	c.[1210-34TG[12];1210-12T[5]]
3	R709X/-	c.[1210-34TG[12];1210-12T[5]]
4	c.[1210-34TG[12];1210-12T[5]]	c.[1210-34TG[12];1210-12T[5]]
5	c.[1210-34TG[12];1210-12T[5]]/L926W	c.[1210-34TG[13];1210-12T[5]]
6	R1453Q/-	c.[1210-34TG[13];1210-12T[5]]
7	F508del/L578I	A1285V/-
8	R668C/-	c.[1210-34TG[12];1210-12T[5]]
9	F508del/-	c.[1210-34TG[12];1210-12T[5]]