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Molecular and Functional Basis of Cystic Fibrosis in Indian Patients: Genetic, Diagnostic and Therapeutic Implications

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ABSTRACT

Cystic fibrosis (CF, MIM#219700) is a common autosomal recessive disorder among Caucasians, which was considered as rare disease for Indian population. CF is caused due to presence of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. In this study, we established a spectrum of mutations from both classical CF as well as from infertile male patients with congenital absence of vas deferens (CAVD). In Indian classical CF patients, we reported 14 previously known and eight novel mutations, viz. *3986-3987 delC, 876-6 del4, 1792 InsA, L69H, S158N, Q493L, 1530L* and *E1329Q*. The frequency of *delta 508* was found to be 27%. Absolute linkage between *delta 508* and *KM19-GATT TUB9-M470V-T854T* haplotype predicts a relatively recent appearance of *delta 508* mutations in Indian population. The *CFTR* gene analysis in CAVD infertile males documented 13 different *CFTR* gene mutations and 1 intronic variant that led to aberrant splicing. *P.Phe 508 del* (n= 16) and *p.Arg 117 His* (n=4) were among the common severe forms of *CFTR* mutations identified. The *IVS-8-T5* allele (mild form of mutations) was formed with an allele frequency of 28.3%. Eight novel mutations were also found in the *CFTR* gene from our patient cohort. We also investigated whether genetic modifiers, viz. transforming growth factor (TGF-β) and endothelial receptor type A (EDNRA) of CF lung disease also predispose to CAVD in association with *CFTR* mutations, which were associated with the CAVD phenotype.

Functional characterization of identified 11 novel *CFTR* gene mutations disclosed that a significant reduction in channel activity for *L69H* and *S549N* mutants in *CFTR* expressing cells was observed whereas impaired *CFTR* protein maturation was noticed only in *L69H* substitute *CFTR*. *CFTR* correctors (*VX809*) rescued the defect due to *L69H* mutation, which is evidenced from detection of C band in *L69H* mutant expressing cells pre-treated with *VX809*. The chloride channel activity in *S549N* and *L69H* mutant *CFTR* was also restored in presence of *CFTR* potentiators *VX770*.

Above findings confirms heterogeneity of *CFTR* mutations in Indian classical and non-classical CF patients. They may help in developing a strategy to develop counseling and therapeutic approach for CF patients in India.

Keywords: Cystic fibrosis, genetic mutation, *CFTR*.

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Introduction

Cystic fibrosis (CF MIM#219700) is the most common lethal autosomal recessive monogenic disease in Caucasian population with an average prevalence of one in 2500 live birth with carrier frequency 1 in 25 individuals (1). Notwithstanding, CF is still thought to be very rare in Indian population (2). However, published reports indicate that CF is probably far more common in people of Indian origin than previously thought but is underdiagnosed or missed in majority of cases. Dr. Bhakoo reported first case of CF in 1968 from PGIMER, Chandigarh (3). Later, we estimated the relative frequencies of various genetic disorders, which were found, with 7.56% frequency (4). The mutations in the CF transmembrane conductance regulator gene (*CFTR* or *ABC C7; MIM # 602421*), which are responsible for both classic and non-classic presentation of the disease (CAVD, MIM # 22180) (5, 6). The *CFTR* gene was identified and cloned about two decades ago (7, 8). The *CFTR* gene is located on the long arm of chromosome 7 (region q31 – q32) encompassing 250 kb and comprising 27 exons (7-9). It encodes a trans-membrane protein of 1480- amino acids that function as a CAMP- regulated chloride channel in exocrine epithelia (10). Hydropathy plot analysis disclosed that *CFTR* protein composed of two motifs, each containing a membrane spaning domain (MSD) that is composed of six tansmembrane helices and nucleotide binding domain (NBD) that contains sequence predicted to interact with ATP (II). MSD-NBD motifs are linked by a unique domain termed as regulatory domain (R) that contains multiple phosphorylation sites and many charged amino acids. The carbonyl terminal, consisting of threonine, arginine, and leucine (TRL) which is anchored through a PDZ type – binding interaction with the cytoskeleton (11, 12) (Fig. 1).

More than 2000 sequence variants have been identified in the *CFTR* gene (http/www.genet.sickkids.on.cafchrome.html) and many of them have been implicated in a variety of *CFTR* related pathologic conditions such as respiratory distress, pancreatic insufficiency, meconium ileus and congenital absence of the vas deferens (CAVD) (13). *Delta 508F* is the most common CFTR mutation

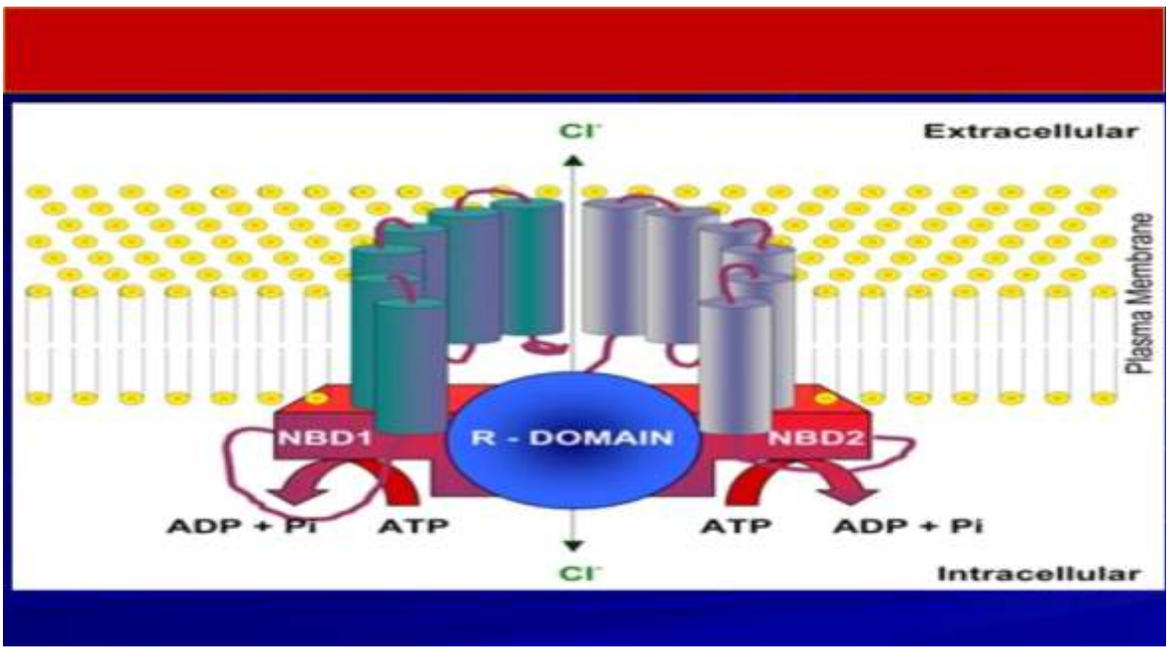

Fig.1: Hypothesized structure of CFTR showing proposed structure of CFTR protein.

worldwide, which is present upto 92% of patients with CF. However, this frequency varies among countries and ethnic groups (http://www.cftrscience.com). All these mutations are classified into six classes based on the mechanism of the disruption of CFTR (14) and the absence of the CFTR protein at the apical plasma membrane which include defective protein synthesis, impaired protein maturation leading to protein degradation, defective regulation of CFTR channel activity, altered ionic selectivity and conductance, lowered CFTR mRNA amount and decreased protein stability.

CAVD is characterized as an isolated urogenital form of CF which is associated with normal spermatogenesis, but absence of sperm in the ejaculate due to lack of vas deferens (15). More than 95% of males with CF are infertile due to obstructive azoospermia caused by absent, atrophic or fibrotic Wolffian duct structure (16). The *p. Phe 508 del, p. Arg 117H* is and *T5 allele* were identified as most common *CFTR* mutations in caucassians associated with CAVD phenotype (16, 17). Despite advances in understanding the pathophysiology of CF, there are still many inexplicable differences in its clinical association with CAVD. The role of intensifying the severity of classical CF is well established (17, 18). Transforming growth factor (TGF β), codon 10 polymorphism (rs180470) and codon 25 polymorphism (rs1800471) as well as endothelial receptor type-A (*EDNRA*) gene polymorphism have been previously established in enhancing the severity of classical CF (17, 19). Molecularly, established mutations including preferentially novel and rare mutations is urgently needed for functional characterization at cellular level, so that, therapeutic molecules could be developed to target underlying molecular defect. Additionally, the cellular and functional data on theses mutations can improve CF genetic counseling. Recent advances of targeted molecular therapies and high throughput screening have resulted in multiple drug therapies that target many important mutations in the *CFTR* protein (20).

In this manuscript, we provide the work done on CF at Postgraduate Institute of Medical Education and Research, Chandigarh with special reference to its diagnostic and molecular characterization of spectrum of mutations from both classical and non-classical forms of CF. Haplotype association have been used to trace the origin and age of different CF mutations worldwide (21). There is no information available in this regard from Indian subcontinent. Therefore, haplotype study was also carried out using associated intragenic and extragenic marker haplotypes. The study was also performed to investigate whether genetic modifiers of CF lung disease also predispose to CAVD in association with *CFTR* mutations. We also provide the latest results and current progress of *CFTR* modulators for the treatment of cystic fibrosis, focusing on potentiators of *CFTR* channel gating and *p.Phe508del CFTR* mutation. Special emphasis is placed on the molecular basis of understanding these new therapies.

Molecular Diagnosis of Cystic Fibrosis and its Underlying Pathogenesis

The diagnosis of CF has vast implications for patients and their families. The broad spectrum of clinical disease and reports of over 2,000 different mutations have made the CF diagnosis difficult (22). The guidelines for CF diagnosis were established by the Cystic Fibrosis Foundation for diagnosis of both infants with positive newborn screening (NBS) findings and older patients presenting with an indistinct clinical picture. CF Foundation proposed the following diagnostic algorithm state: the diagnosis of CF should be based on the presence of one or more characteristic clinical features *viz*. respiratory, gastrointestinal or gastrourinary symptom, a history of CF in a sibling or a positive NBS test, plus laboratory evidence of an abnormality in the *CFTR* gene or protein (23, 24). Either biological evidence of channel dysfunction such as an abnormal sweat chloride

test or nasal potential difference and identification of CF disease causing mutation on each allele of the *CFTR* gene are acceptable evidence of a *CFTR* abnormality. Newborn screening depends on initial analysis of fetal blood for high values of immunoreactive trypsinogen (IRT) followed by genetic testing or repeat (25).

The sweat chloride test remains the initial test of choice and gold standard for CF diagnosis despite its limitations. Sweat chloride test was developed by Gibson-Cooke in 1959 (25). The test is performed *via* pilocarpine inotophoresis, which is used to stimulate sweat gland secretion. The sweat is collected and analyzed for chloride concentration. The underlying mechanism of elevated levels of sweat sodium was demonstrated by Roeve *et al* (26) (Fig. 2).

Sweat chloride concentration above 60 mmol per liter (>70 mmol/L in adolescent and adults) is suggestive of cystic fibrosis. In normal individuals and carriers of the CF gene, the mean sweat chloride concentrations are 30 mmol/L.

Cut-off value for infants is 30 mmol/L (27). A positive sweat test is diagnostic as long as it is performed on adequate amount of sweat (>100 mg) in CF. A second test for *CFTR* function such as nasal potential difference measurement or analysis of rectal mucosal biopsy is recommended if sweat test is equivocal (28).

DNA analysis in establishing CF diagnosis is most useful for those individuals with sweat chloride values in the intermediate range. Two or more disease causing *CFTR* mutations should be located on different alleles as CF is an autosomal recessive disease. CF Foundation recommends testing for the 23 CF mutation panel developed by the American College of Medical Genetics (ACMG). These mutations have been demonstrated to cause sufficient loss of *CFTR* function to confer CF disease and are therefore noted as conclusive genetic evidence for diagnosis of CF (23). Indirect non-invasive parameters available for assessing the pancreatic function test are serum immunoreactive trypsin (IRT), fecal chymotrypsin, stool immunoreactive human

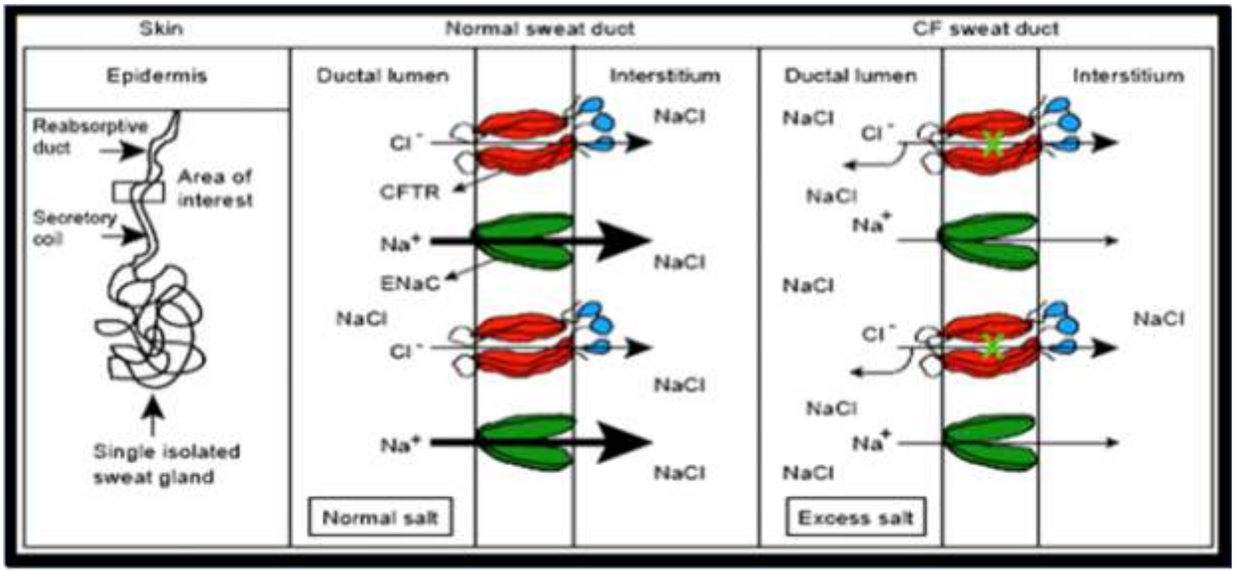

Fig.2: Mechanism understanding elevated sodium and chloride levels in the sweat of CF patients. Left panel shows cartoon of a sweat gland with sweat duct projecting out of the sweat gland through dermis into the epidermis. Middle panel shows role of CFTR and ENaC in maintaining normal salt concentration in sweat. Last panel shows dysfunctional CFTR leading to elevated salt concentration in sweat.

lipase and stool fat excretion as a gold standard (29).

Spectrum of Mutations in Classical Cystic Fibrosis

A prospective cohort study was designed to establish a spectrum of mutations in *CFTR* gene. In this study, 1005 suspected CF subtypes were subjected for sweat chloride measurement. Notably, 45 subjects were diagnosed as CF on the basis of classical clinical phenotype and elevated sweat chloride. On the basis of clinical symptoms and autopsy reports that confirmed the diagnosis of CF in three patients with normal sweat chloride were also included. Besides, 2 infants with raised IRT were included as CF subjects.

Demographics and family history of CF subjects revealed that average age at diagnosis in CF subjects was 62.8 months compared to 9.3 months at age of presentation. Male to female ratio was 35.15. Notwithstanding, females had a higher median age at diagnosis (33 months) as compared to males (18 months). History of previous sib death due to respiratory or gastrointestinal problems was observed in 9 families. Consanguinity was noticed in three and history of similar gastrointestinal or respiratory complaints was recorded in another 4 families. In 1 case, the patient's mother had a history of chronic pancreatitis and diabetes (30).

Clinical Findings

The usual presentations include failure to thrive (94%), malabsoption (82%), chronic cough (90%), recurrent or persistent pneumonia (79%) and meconium ileus (10%). Mean Schwachman-Kidczyski score was 57.34±12.07. The median age of CF patients with *P.aeruginosa* colonization was 39 months compared to 8 months with *S.aureus*. Pancreatic insufficiency was present in 37 patients (77%). Mean IRT levels in CF patients were 176.43±141.23 ug/L. Determination of fecal fat revealed steatorrhoea (7.36±4.56 g/24h) in 8 patients out of 15 tested (53.33%). Notably, all patients with liver disease (n = 6) and meconium ileus (n = 5) had pancreatic insufficiency.

Sweat Chloride

Median sweat chloride value in CF subjects was 86.25 mEq/L. All individual patient values were mean of two/three repeat sweat chloride estimation on different days. Amount of sweat chloride collected was more than 100 mg on each occasion (198±30 mg). In 2 infants, adequate sweat could not be collected. Fig. 3 shows individual sweat chloride values of CF (n

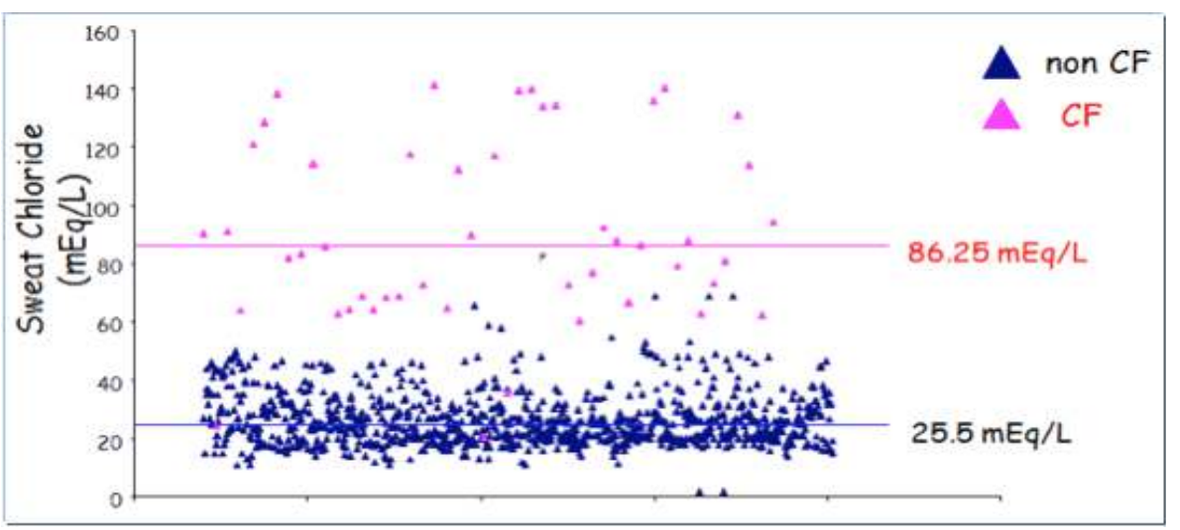

Fig.3: Comparison of sweat chloride values between CF and non-CF cases.

= 48) and non-CF (n = 956) subjects. Median sweat chloride value of 86.25 mEq/L in CF was significantly elevated as compared to 25.5 meq/L in non-CF individuals. In India, there are only few centers where sweat testing is performed.

Identification and Molecular Characterization of Mutations in the *CFTR* Gene from Classical CF

The spectrum of mutations in *CFTR* gene from classical CF at PGI is listed in Table 1. In this study, 100 unrelated CF chromosomes were first analyzed for most common *delta F508* mutation, which was found with 27% of CF chromosomes *R117H, R553X, N1303K* and *G551D* were identified by ARMS on a total of five chromosomes.

By restriction digestion, 3849+10 KbC-T was found in 3 CF subjects with normal sweat chloride. Using SSCP and subsequently DNA sequencing, mutations were molecularly characterized in 31 of the remaining 65 CF chromosomes which include nine missense mutations, viz. *L69H, S158N, Q493L, Y517C, V520F, I530L, S549N, E1329Q* and *Y1381H*, one insertion mutation (1792 insA), three splice site mutations (*876-6del4, 1525-1G-A, 3120+1G-A*), two deletion mutation (*1161 delC, 3986 delC*), and one nonsense mutation (*L218X*). *S549N* and *1525-1G-A* were the second most common mutations observed in our population (5% each) Among them, 8 novel mutations, viz. *L69H, Q493L, S158N, I530L, E1329Q, 1792 InsA* and *3986-3987 delC* were identified in one patient each. Notably, none of novel mutation was found in 50 control subjects.

It is noteworthy here that output productions score for 3 novel mutation (*S158N, I530L and E1329Q*) was found 0.05, representing deleterious effects using molecular modeling and Bioinformatics CMMB. The most common CF mutation worldwide is the deletion of a phenylalanine residue at position 508. A review of all genotyped South Asian patients showed that delta 508 is identified in 19% to 44% CF alleles (31-33), which is lower than the reported frequency of 66% worldwide CF population. In our study, delta F 508 represents only 27% of the analyzed and 41% of the identified CF allele (30). The striking characteristic of the mutation spectrum is absence of some mutations common in the Mediterranean and European population (34). S549N and 1525-1G-A were the second most common mutations, followed by 3849+10 kbC-T. This corroborates the study of Shastri *et al* (35) which has recommended testing Indian CF patient for *delta F508, 1161 delC, 3849+10 kbC-T* and *S549N*. Absolute linkage between *delta F508* and *KM.19-GATT -TUB9-M470V-T854T* haplotype (*2-2-1-1-1*) predicts a relatively recent appearance of delta F508 in Indian CF patients (28). Low frequency of delta F508 mutation and detection of eight novel and thirteen rare mutation imitate a heterogeneous spectrum of mutation in Indian CF patients. Notwithstanding, CF mutation frequencies and haplotypes vary widely among different populations and haplotype heterogeneity has been found to be greater in population with lower *delta F508* frequencies (36). We documented NBD1 and NBD2 as the hotspots identified in *CFTR* protein in CF patients at our institute (Table 1).

Identification and Molecular Characterization of Mutations in the *CFTR* Gene from Non -Classical CF (CAVD)

Demographics and Clinical Variables

At our center, we used the following algorithm: male CAVD patients comprising of bilateral absence of vas deferens, unilateral absence of vas deferens, absence of epididymis, and absence of seminal vesicle as well as renal agenesis. The median age of the CAVD patients at the time of enrollment was 28 years and most of the patient consulted an urologist only after the evaluation of their female partner by a gynecologist. Semen value (1.6±0.61) was much less in comparison to normal range (2-4 mL),

Table 1: Frequency of CFTR mutaitons identified in Indian CF Patients. Total Chromosomes-100; known mutations - 66%, unknown mutaions - 34%

S.No	Mutation	Nucleotide change	Consequence	Exon/Intron	Domain	Frequency
1	Delta F508	Deletion of 3bp (CTT or TTT) between 1652 and 1655	Deletion of Phe at 508	Exon 10	NBD1	27
2	1161 delC	Deletion of C at 1161	Frameshift	Exon 7	TM6 of MSD1	2
3	3986-3987 delC*	Deletion of C at 3986	Frameshift	Exon 20	NBD2	2
4	1792ins A*	Insertion of A at 1792	Frameshift	Exon 11	NBD1	2
5	R117H	G to A at 482	Argininie to Histidine at 117	Exon 4	Extracytoplasmic loop llindking Tm1-TM2	2
6	LC 9H*	T To A at 338	Leucine to Histidine at 70	Exon 3	NH2 terminal	1
7	S158N*	G To A at 605	Serine to Asparagine	Exon 4	Intracytoplamic loop llinking TM2-TM3	1
8	Q493L*	A to T at 1609	Glutamine to Leucine at 493	Exon 10	NBD1	1
9	Y517C	A To G at 1682	Tyrosien to Cystine at 517	Exon 10	NBD1	1
10	V520F	G To T at 1690	Valine to phenylalanine	Exon 10	NBD1	1
11	G551D*	A To C at 1 720	Isoleucine to leucine at 530	Exon 11	NBD1	2
12	S549N	G To A at 1778	Serine to Asparagine at 549	Exon 11	NBD1	5
13	G551D	G to A at 1784	Glycine to Asparatate at 551	Exon 11	NBD2	1
14	E1329Q*	G to C at 4117	Glutamate to Glutamine at 1329	Exon 22	NBD2	1
15	Y1381H	T To C at 4273	Tyrosine to Histidine at 1381	Exon 23	NBD2	2
16	N1303K	C To G at 4041	Asparagine to Lysine at 1303	Exon 21	Extracytoplasmic loop llindking Tm3-TM4	1
17	L218X	T To A at 785	Leucine to stop	Exon 6a	NBD1	1
18	R553X	C to T at 1789	Arginine to stop	Exon 11	-	1
19	876-6del4*	Deletion of 4 bp TACA from 876 -4	Splice site mutation	Intron 6a	-	2
20	1525-1G-A	G to A at 1525 -1	Splice site mutation	Intron 9	-	5
21	3120+1G-A	G To A at 3120 -1	mRNA splicing defect	Intron 16	-	2
22	3849+10kbC-T	C-T at 3849	Exon 9 skipping	Intron 19	-	3

* denotes novel mutation; TM- transmembrane region; MSD - Membrane spanning domain; NBD - Nucleotide binding domain; Sharma et al (2008) Ann Human Genetics.

Table 2: Spectrum of CFTR gene mutation in Indian CAVD males (n=110)

Mutations	Nucleotide Change	Consequences	Exon/ Intron	No. of alleles
T5	Reduction of oligo T tract to 5T, c.1210-12T(5)	Aberrant splicing	Intron 8	59
F508del	c.1521_1523del CTT	Deletion of phenylalanine at 508	Exon 11	26
p.Gly480Ser	c.1438 G>A	Glycine to Serine at 480	Exon 11	1
p.Arg518Lys [a]	c.1553 G>A	Arginine to Lysine at 518	Exon 11	1
p.Arg117His	c. 350 G>A	Arginine to Histidine at 117	Exon 4	7
p.Gly126Cys [a]	c.376 G>T	Glycine to Cystine at 126	Exon 4	1
p.Ala141Gly [a]	c. 422 C>G	Alanine to Glycine at 141	Exon 4	1
p.His139Gln [a]	c.417 C>G	Histadine to Glutamine at 139	Exon 4	1
p.Ser118Pro [a]	c.352 T>C	Serine to Proline at 118	Exon 4	1
p.Arg170Cys	c.508 C>T	Arginine to Cystine at 170	Exon 5	1
p.Glu585Gln [a]	c.1753G>C	Glutamate to Glutamine at 585	Exon 13	1
p.Met281Arg [a]	c.842 T>G	Methionine to Arginine at 281	Exon 7	1
p.Arg933Thr [a]	c.2798 G>C	Arginine to Theronine at 933	Exon 17	1
p.Ser549Asn	c.1646 G>A	Serine to Asparagine at 549	Exon 12	1
p.Leu69His	c.338T > A	Leucine to histidine at 69	Exon 3	1
p.Phe87Ile	c.391T > A	Phenylalanine to isoleucine	Exon 3	1
p.Gly126Ser [a]	c.508G >A	Glycine to serine at 126	Exon 4	1
p.Phe157Cys [a]	c.602T > G	Phenylalanine to cystine at 157	Exon 4	1
p.Glu543Ala [a]	c.1760A> C	Glutamate to alanine at 543	Exon 11	1
p.Tyr852Phe [a]	c.2687A > T	Tyrosine to phenylalanine at 852	Exon 14a	1
3120 þ 1 G-A	G >A 3120 þ 1	Aberrant splicing	Intron 16	1
p.Pro1021Ser	c.3193C>T	Proline to serine at 1021	Exon 17a	1
p.Asp1270Glu [a]	c.3942T >A	Aspartate to glutamate at 1270	Exon 20	1

[a] Novel mutations

Sharma *et al.,* 2009 (Human Reproduction) & Sharma *et al.,* 2014 (Molecular Human Reproduction)

however, the mean sweat chloride was in intermediate range (40-60 mEq/L). The mean serum hormone level of FSH, LH and testosterone were in normal range in CAVD patients.

Spectrum of Mutations Identified in CAVD Patients

Overall, 220 CRTR alleles were screened for mutation. We identified twenty three mutations (Table 2) (37-39). Two most common mutations, viz. *p. Phe 508 del* and *p. Arg 117H* is were found on 26 and 7 allele, respectively. Both of these mutations were found in the heterozygous conditions in all the patients. Another most common mild form of *CFTR* mutation, viz. *IVS8-T5 allele* observed on 59 alleles in infertile CAVD males. In 13% of CAVD, the mild mutation was present in both the alleles whereas in 15% of cases, *IV58-T5* was detected in a compound heterozygous form with other mutation present in coding region of the *CFTR*. Other mutations were very rare. Among them, fourteen mutations were novel. All these novel mutations were subjected for determination of prediction score using SIFT (*http://blocks.fh crc.org/sift/SIFT.html*) and confirmed by polyphen-2 (*http//genetics.bwh. harvard.edu/php*). Threshold for pathological mutations was 0.05. Among them, *Gly126Cys, Ser118Pro, Met281Arg, Arg933Thr, Lys69His, Glu543Ala* and *Asp1270Glu* were damaging mutation, which can perturb the protein structure (37-39).

Genetic analysis of the *CFTR* gene led to identification of the mutations in 81% of the Indian CAVD males and this detection was very similar to that of Caucasians, however, the spectrum of mutation in this study was different from that Caucasians (40-41). Notably, the allele frequency of *p. Phe508del* mutation in our population is similar to that of France (4) and Spain (5). The *5T allele* in our population was found with an allele frequency of 28% is very similar to that of Canada, Brazil (42, 43).

Association of Cystic Fibrosis Genetic Modifiers with Congenital Bilateral Absence of the Vas Deferens

To investigate whether genetic modifiers of CF lung disease also predispose to CAVD in association with *CFTR* mutations, we tested the hypothesis that polymorphisms of TGF-β1 and *EDNRA* polymorphism might play a role in penetrance of CAVD. These polymorphisms have been reported to modify CF lung disease, and might also contribute as genetic modifiers of CAVD. Sixty CAVD subjects and fifty controls were investigated for candidate polymorphism *TGF-β1* or *EDNRA* associated with a more serve lung phenotype among CF disease. Importantly, CC genotype (SNP r5335) of *EDNRA* and TT genotype (SNP r51982073) TGF- β1 were associated with CAVD to occur more frequently among CF individuals (37). TGF- β1 is the best described modifier of CF associated with pulmonary phenotype. In reference to our study, the human vas deferens, epididymis and seminal vesicles develop from the Wolffian duct. Notably, TGF- β1 and related signaling pathways play an important role in normal Wolffian duct development and differentiation (44, 45). On the other hand, other modifier of CF lung disease (EDNRA) is also associated with CAVD.

EDNRA has been implicated in normal formation of mammalian nervous system, the anorectum, and craniofacial structures such as mandible (46-48). *EDNRA* plays an important role during development of the vas deferens which could be due to loss of the vas in the setting of *CFTR* insufficiency. This is the first study in reference to the Indian population where we identify possible involvement of these pathways in an atypical CF- related condition, namely CAVD. Recently, it has been documented TGF- β1 receptor and P 38 MAPK signaling reduce *CFTR* activity and probably enhance the effect associated with heterogeneous *CFTR* mutations and may result in male infertility (49).

Functional Characterization and Pharmacological Correction of Novel and Rare Mutations

The striking characteristic of the identified mutations at our center were the presence of severe rare mutations but the absence of the most common mutations identified in the Mediterranean and European population, which clearly represented the heterogeneous spectrum of *CFTR* mutations at our Institute. These rare mutations are called *orphan mutation* because of their very low incidence. Moreover, rare familiar mutations cannot provide sufficient information on the phenotype due to these mutations, viz. *L69H, F87I, S118P, G126S, H139Q, F157C, F494L, E543A, S549N, Y852F* and *D1270E* from both classical CF patients and CAVD patients (40). It is noteworthy here that the cellular and function data on these mutations can improve CF genetic counseling. For functional characterization and therapeutic implications for these CF rare mutations, baby hamster kidney cells are an adherent cell line used for this study (50). Functional findings revealed that L69H was found as a novel class II CF mutation. The trafficking to the plasma membrane of L69H-*CFTR* is abnormal as corroborated by western blot analysis. Further confocal microscopic imaging showed the abundance of L69H mutated *CFTR* protein in the endoplasmic reticulum and absence on the plasma membrane. In view of above finding, the processing pathway of this variant is similar to that of F508 del-*CFTR* mutation which is the most common mutation worldwide. Misfolded F508 del-*CFTR* is retained by the ER and undergoes ubiquitination, thereby accelerating its proteome degradation and reducing F508 del-*CFTR* trafficking in the plasma membrane (51, 52). The model of 3D structure of *CFTR* which is predicted using the Sav1866 experimental 3D structure. L69H is studied in a short cytosolic α-helix in the N-terminus of *CFTR* preceding the first transmembrane helix TMI of MSD that is called "elbow helix", which is conserved feature among the ABC exporter family. Possibly, L69H mutation may perturb the network formed hydrophobic side chains of the cytosolic extension of MSD1TMI helices. MSDI-NBDI linker (1368) might thus play an important role for MSD1 folding and destabilization of this domain at the membrane. Another important S549N located in the LSGGQ signature motif of NBD1 and may alter the hydrolysis of ATP to regulate channel activity (53) as a result S549N classified as class III CF mutation (54). Normal maturation of S459 N-*CFTR* protein was observed in our study as reported by others (54). Further, the study was conducted to evaluate the effect of some potentiator *viz* Miglustat, Isolab and VX-809 to increase the activity of defective *CFTR* protein due to presence of these mutations. Ivacaftor (VX-770) is an investigational, orally bioavailable agent which was shown to augment the chloride transport activity of G551D-*CFTR* protein in vitro (54). In our study, the pharmacological corrector VX809 was found to activate L69H in BHK-21 cells in similar to that of delta F508 (50). The activation of L69H and delta F508 were both significantly corrected in terms of maturation and translocation of *CFTR* protein to cell membrane when cells were treated with VX809 potentiator. Whereas, S549N mutation classify as type III mutation was also corrected as its translocation to plasma membrane (50). It is noteworthy here that VX809 molecule is FDA approved, so that this potentiator can be used as a therapeutic molecule to rescue from type II and III mutations associated with Indian CF patients.

Current Progress in Treatment of Basic Defects in CF

CFTR modulator therapies have been directed towards specific disease causing mutations and the molecules pathways that underlie these causes. *CFTR* mutations grouped into conservative classes have led to the development of specific approaches towards treating the molecular effect in CF (Table 3) (55-58). Non-sense mutations in *CFTR* have been shown to be rescued with application of a compound ataluren, derived from

Table 3: CFTR based therapies completed or in progress towards treating the basic defect in CF

CFTR mutation	Mutation Frequency (%)	Therapeutic approach	Status
G551D/other	4	Ivacaftor	FDA approved
Non-G551D gating/other	1	Ivacaftor	Phase II/III
R11 7H/other	5	Ivacaftor	Phase III
ΔF508/ ΔF508	49	Lumacaftor+ivacaftor VX-661+ivacaftor	phase III planned Phase II
PTC/other	10	Ataluren	Phase III

aminoglycoside antibiotic that can induce read-through of premature termination codons. This investigational drug is in phase III clinical trial (55). Two new molecules have been developed for correction, VX809 and potentiator, VX770, of the delta F508 mutations. Some of class III mutants respond to VX770 by increasing the chlorides transport. *CFTR* potentiator Ivacaflor (Kalydeco, VX-770, vertex pharmaceuticals, Boston, MA, USA) FDA and European regulatory authorities approved drug to treat CF patient with a class III G551D mutation (56-58). Recent advances of targeted molecular therapies and high throughput screening have resulted in multiple drug therapies that target many important mutations in the CFTR protein (59).

In summary, these findings represent an important milestone in the development of treatments designed to improve *CFTR* protein function as a means of addressing the underlying cause of cystic fibrosis.

Future Directions

In Indian scenario, the diagnosis of CF is important since sweat chloride measurement using pilocarpine iontophoresis is limited to only few centers despite a large population in this country. Sweat chloride measurement technique facility is must at every district level to pick up the CF population. The most reliable is the sweat induction by pilocarpine iontophoresis, followed by sweat chloride collection on a gauze or filter paper. Sweat chloride concentration in the CF patient is in intermediate range. In these cases, gene sequencing is essential to provide positive CF diagnosis as well as, mutations specific therapies which have become available recently (59, 60).

Other mechanisms of corrector therapy are being developed. Recently, the direct and indirect modulation of the nitric oxide (NO) pathway has been investigated as a possible corrector mechanism in delta F508-*CFTR* (58, 59). If future drug combinations are sufficiently robust to correct *CFTR* function in individuals with only one copy Phe508 del, this modulator will ideally provide clinical benefit to over 90% of all patients with CF. Thus, other approaches such as drugs that read through premature stop mutation and gene replacement or editing must continue (61).

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