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ORIGINAL PAPER

CFTR Exon 10 deleterious mutations in patients with congenital bilateral absence of vas deferens in a cohort of Pakistani patients

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Summary ***Congenital bilateral absence of vas deferens (CBAVD) is a urological syndrome of Wolffian ducts and is responsible for male infertility and obstructive azoospermia. This study is designed to explore the integrity of exon 10 of CFTR and its role in male infertility in a cohort of CBVAD patients in Pakistan. Genomic DNA was extracted from 17 male patients with CBAVD having clinical symptoms, and 10 healthy controls via phenol-chloroform method. Exon 10 of the CFTR gene was amplified, using PCR with specific primers and DNA screening was done by Sanger sequencing. Sequencing results were analyzed using freeware Serial Cloner, SnapGene, BioEdit and FinchTV. Furthermore, bioinformatics tools were used to analyze the mutations and their impact on the protein function and stability. We have identified 4 mutations on exon 10 of CFTR in 6 out of 17 patients. Two of the mutations were missense variants V456A, K464E, and the other two were silent mutations G437G, S431S. The identified variant V456A was present in 4 of the studied patients. Whereas, the presence of K464E in our patients further weighs on the crucial importance for its strategic location to influence the gene function at post-transcriptional and protein level. Furthermore, Polyphen-2 and SIFT analyze the mutations as harmful and deleterious. The recurrence of V456A and tactically conserved locality of K464E are evidence of their potential role in CBAVD patients and in male infertility. The data can contribute in developing genetic testing and treatment of CBAVD.***

KEY WORDS: CFTR; CBAVD; Exon 10; Mutation analysis; Missense mutations.

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INTRODUCTION

Congenital absence of vas deferens (CAVD) is a urological syndrome presumably resulting in abnormalities of the Wolffian ducts. It is also a contributing factor to obstructive azoospermia. It is categorized into three types: *unilateral* (CUAVD), *bilateral* (CBAVD), and congenital bilateral *partial* aplasia (CPAVD) (1).

CBAVD is the most prevalent subtype that follows an autosomal recessive pattern and accounts for 1-2 % of sterile but healthy men and up to 25% of obstructive azoospermia cases (2).

To date, two perspectives have been presented regarding the etiology of the CBAVD: vas deferens atresia and vas deferens agenesis. Even though the comprehensive pathological mechanism is required to be further studied and validated, these pathological mechanisms are widely known to be triggered by gene alterations (3).

Mainly CBAVD is diagnosed at adulthood during a medical examination for the exploration of infertility. Therefore, it is of prime importance to properly assess infertile men with appropriate clinical work-up, to correctly address the suitable genetic tests in an exclusive way (4). Initially, clinical diagnosis was made on the palpation of the *vas deferens* (VD), that is, on their intrascrotal portion: the diagnosis was negative if this portion was present and positive if it was absent. But in recent times, besides palpation, ultrasound imaging (transrectal and scrotal) is essential for accurate diagnosis (5). The biological alarming signal is a non-pathognomonic trio: hypospermia (semen volume < 1.5 ml), the value of seminal plasma lesser than the reference level (fructose < 13 µmol/ejaculate; *Glycerophosphocholine* (GPC) < 2 µmol/ejaculate), and acid pH (< 7.0) (6). Hence, on the basis of clinical symptoms, two clinical categories of CBAVD have been reported: CBAVD showing symptoms of cystic fibrosis referred as CF-CBAVD and CBAVD without clinical symptoms of CF referred as isolated CBAVD (iCBAVD) (7). Furthermore, apart from CF associated symptoms, other congenital genitourinary defects, primarily including dysplasia or the absence of seminal vesicles and kidney-related issues contributes towards CBAVD (8, 9).

The anatomical anomalies related to CBAVD occur at the embryonic stage. Cystic fibrosis transmembrane conductance regulator gene (CFTR) or CFTR mediated anions are essential for normal growth of the male reproductive tract (10). Mutations in the CFTR gene have a crucial impact on the vas deferens development in fetuses aged 12-18 weeks (11). These mutations lead to obstructions and denaturation of the vas deferens due to mucus accumulation, particularly pronounced during embryonic growth. Studies showed that proper fluid secretion is crucial for the mesonephric duct to develop correctly (12,

13). When fluid secretion is disrupted, it can lead to underdevelopment and deterioration of the mesonephric duct in the early stages of embryo growth (14).
Until now, over 2000 mutations in the CFTR have been detected, however, not all of them are pathogenic in nature. A relatively low number of mutations are causing CFTR associated abnormalities while the rest are not linked with any clinical syndromes. Around 370 CFTR mutations are listed in the clinical and functional translation of the CFTR project (CFTR2) (15). These pathogenic variants are classified as mild and severe mutations on the basis of their phenotypic and functional effect. Two severe mutations on both alleles of the CFTR causes *cystic fibrosis* (CF) while one severe and one mild or both mild mutations resulted in CBAVD. Moreover, all CF patients have CBAVD (16).
CFTR mutations have been classified into six classes where class I to III variants present severe manifestations and complete loss of CFTR function. However, class IV to VI variants present mild phenotypes with reduced CFTR function (2).
CFTR exhibits a great deal of heterogeneity due to the modifications in the base sequence of DNA which leads to altered protein expression. Diverse migratory patterns and settlement have led to heterogeneity in mutations worldwide. The relationship between CBAVD and CFTR is well established but it is least studied and documented in the Asian population. That was the reason of our study aiming to the analysis of the genetic integrity of promoter region and exon 10 of CFTR to identify mutations in individuals with CBAVD in a Pakistani population.

Materials and methods

Ethics statement

This study was approved by the *Ethical Review Committee of Forman Christian College* (A Chartered University) *Lahore, Pakistan* (ERC-81-2017 Dated: 11, September 2017). All the experiments were performed according to the approved guidelines.

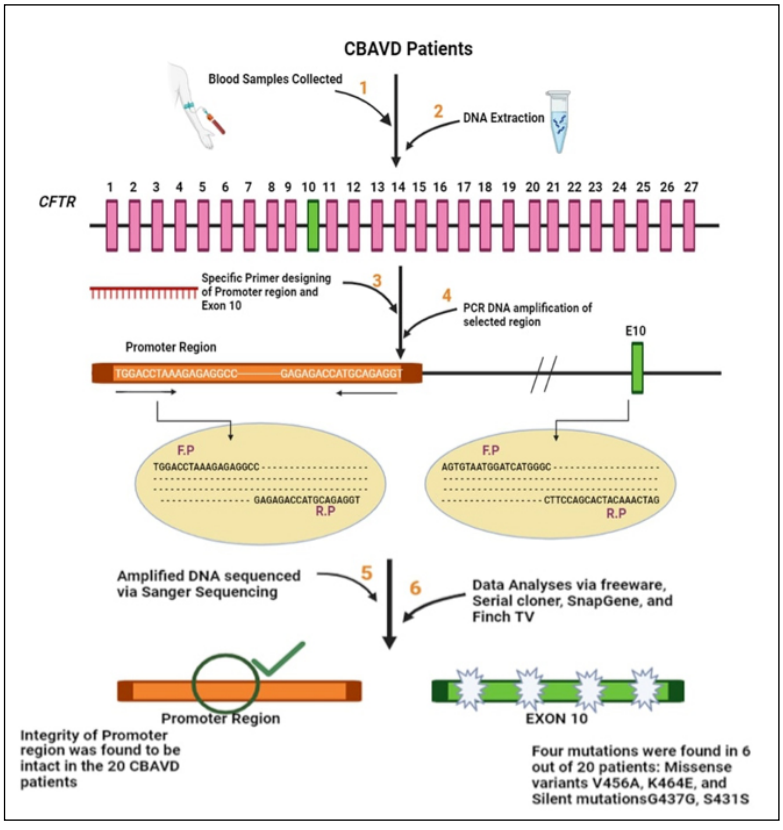
Blood sampling and DNA extraction

The term subjects has been used for both patients and healthy controls taking part in our study. Archived blood samples from 31 patients with CBAVD and azoospermia together with those of 10 healthy controls were gifted by *Fatima Memorial Hospital* (FMH), Lahore Pakistan. Blood samples were collected for research after the approval of the *Institutional Review Board of Fatima Memorial Hospital, Lahore Pakistan* (FMH-10-2018-IRB-520-F Dated: 23, October 2023) and archived for further research. Extracted DNAs from 17 patients and 10 healthy controls were selected for further processing. The authors had no access to information that could identify individual participants during or after data collection. All the patients were examined by the same physician. CBAVD was primarily diagnosed by impalpable scrotal vas on physical examination, followed by ultrasonography. Eventually, diagnosis was confirmed by cytobiochemical characteristics: decreased concentration of fructose and carnitine, azoospermia with low pH < 7 and normal hormone concentration (9). All participants had no classical symptoms of CF. Written informed consent was taken from the participants for the study. Genomic DNA was isolated from whole blood cells by using standard phenol chloroform method (17).

PCR conditions and identification of variants

For screening purposes, the *promoter region* (PR-CFTR) and *exon 10* (EX10-CFTR) were amplified by polymerase chain reaction (PCR) using specific sets of primers (Table 1 - ***Supplementary Materials***) (Figure 1).
PCR conditions for the amplification of selected regions were: initial denaturation at 94°C for 5 minutes followed by 35 cycles of 1 min at 94°C, annealing for 48s at 61.6°C for EX10-CFTR and at 58.8°C for PR-CFTR, elongation carried out at 72°C for 1 minute and final elongation at 72°C for 5 min. PCR products of the 17 patients and 10 control samples were purified using *MinElute PCR Purification Kit*, (*Qiagen, Valencia, CA*) and then sequenced by *Macrogen* (*Inc. Company, South Korea*).
DNA sequencing was performed using forward primers of both exon 10 and promoter region labeled as EX10-CFTR-FWD and PR-CFTR-FWD respectively; afterwards, sequencing results were analyzed and confirmed using *Serial Cloner*, *SnapGene*, *BioEdit* and *FinchTV*.

Figure 1.



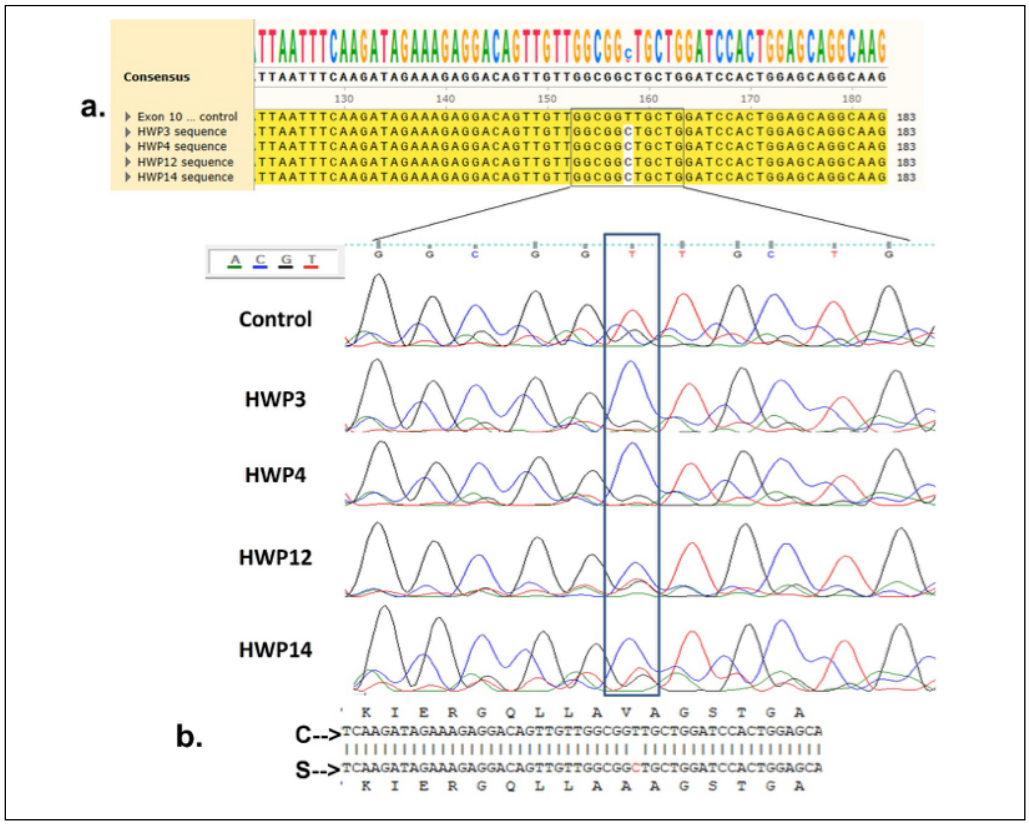


Figure 1.
(a) Sequence Alignment of Exon10-control, HWP3, HWP4, HWP12 and HWP14: Rectangular box indicates the region expanded to visualize DNA sequence quality. (b) Comparison of control and sample DNA sequence with their corresponding in-frame amino acid sequences. C and S represent control and sample sequence

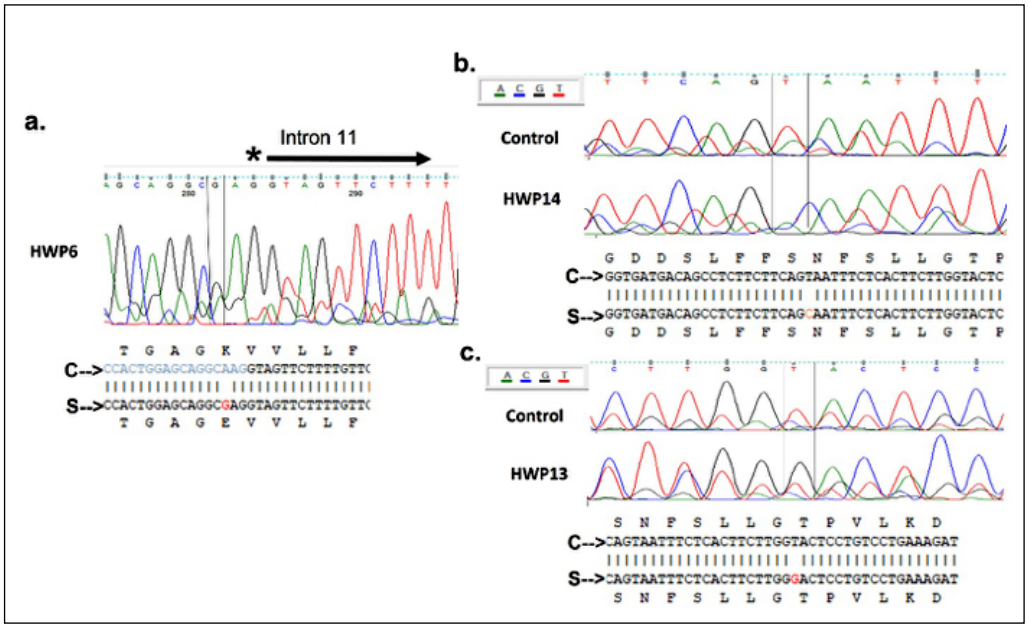


Figure 2.
(a) Sequence analysis of HWP6, (*) indicate the last base pair of exon 10 and protein sequence comparison with control. (b & c) DNA and protein sequence comparison of missense mutations. C and S indicate control and sample sequence respectively.

For mutation confirmation, sequencing data with quality chromatogram peaks were selected (Figures 2, 3).

In-silico analysis of CFTR protein with respect to missense mutations (V456A and K464E)

Analysis of conserved amino acid residues

The evolutionary conserved amino acid sequences of CFTR protein were checked by *ConSurf*. By using *multiple sequence alignment* (MSA) of homologous protein sequences as primary input, *Consurf* calculates a conservation score (1-9) for each amino acid position in the protein sequence where 7-9 score indicates conserved amino acid (18).

Probing of structural and functional effects of mutations

HOPE (*Have (y) Our Protein Explained*) (*https://www3.cmbi.umcn.nl/hope/*) was used for analysis of the potential impact of amino acid substitutions on protein function and stability. Amino acid sequence of the protein along with information about the mutation was provided as input and

HOPE provided insights into the potential consequences of mutations via integration of various computational approaches (19).

Analysis of missense mutations on protein stability and functional outcomes

To understand the potential out-turn of mutations on protein stability and functions, five different in silico tools were used. Polyphen-2 (20) and SIFT (21) were used to find out the harmful effect of variants whereas MuPRO (22, 23), CUPSAT (24) and mCSM (25) were used to anticipate the effects of mutagenesis on protein stability (Table 2).

Results

DNA sequencing and analysis

After a quality control with rigorous selection criteria, sequencing data of 8 patients revealed that 4 mutations in 6 individuals accounted for 35% of total 17 CBAVD patients in our experiment. All these mutations were present on exon 10 while we didn't find any significant mutation in the promoter region of CFTR in the above cohort. Of these four identified mutations, two were missense mutations (V456A, K464E) whereas the other two were silent mutations (G437G, S431S).

V456A is an important missense variant identified in four patients (HWP3, HWP4, HWP12 and HWP14) of our study cohort (Figure 1a). Nucleotide sequences of these patients contain base substitution at position 158 of exon 10 where T is replaced by C resulting in change in amino acid valine to alanine (Figure 1b). Previously, V456A was reported in the South Asian population as a CF causing variant. Furthermore, its association with male infertility of CBAVD individuals has been established. However, other identified mutations were novel and found only in one patient each.

Another important point mutation K464E has been identified in one patient (HWP6). We observed base substitution A to G mutation at position 181 of exon 10 which changed amino acid lysine (K) to glutamic acid (E) (Figure 2a). It is important to note that the total length of exon 10 is 183 base pairs and the integrity of DNA sequence near the splice site is critical for RNA splicing machinery to function normally. Therefore, in addition to its impact due to change in amino acid, K464E can interrupt spliceosome activity at post-transcriptional level leading to excessive loss of function. V456A and K464E are present on the NBD1 domain of CFTR (26) which is crucial for ATP hydrolysis for normal channel function (27).

Interestingly, in addition to V456A, patient HWP14 contains a silent mutation S431S (Figure 2b). This additional mutation does not affect the nature of the amino acid. Another silent mutation G437G has been detected at position 102 of exon 10 in HWP13 (Figure 2c). These two silent mutations are irrelevant in the perspective of protein function but can influence *single nucleotide polymorphism* (SNP).

Frequency and mutation spectrum of CFTR is variable and known to be confined within different ethnic groups. In this study, we have found genetic mutations on critically functional locations of CFTR in CBAVD patients (Table S2 - ***Supplementary Materials***). The recurrence of V456A mutation in four CBAVD patients highlights its potential role in disease and diagnosis. Mutation K464E is crucial due to its location and is capable of affecting CFTR function drastically.

Structural visualization of CFTR protein and in silico analysis of identified mutations in exon 10

We visualize the structure of the CFTR protein (PDB ID: 6O1V), using PyMol software (*https://pymol.org/2/*) to explore critical regions and mutations within exon 10 (Figure S4 - ***Supplementary Materials***). The orthoscopic view of CFTR provides an overall structure, while the functional site in exon 10 is highlighted in cyan (Figure S4 - ***Supplementary Materials***). Within this selected region of exon 10, we pinpoint three specific mutations—V456A, K464E, and G437G—represented as sticks. Each mutation is labeled with its corresponding amino acid change, providing insight into the spatial arrangement of these vital regions within the protein (Figure S4, D-F - ***Supplementary Materials***).

ConSurf identified V456 (score: 7) and K464 (score: 9) as conserved and highly conserved residues respectively (Figure S5 - ***Supplementary Materials***). Both Polyphen-2 and SIFT analyze the mutations as harmful and deleterious. In case of Polyphen-2, score 0-0.5 means benign effect and above 0.5 is considered damaging whereas for SIFT score 0-0.5 is considered non tolerant and close to 1.00 is allowed (Table 1). Mutational effects of MuPRO, CUPSAT and mCSM suggested the overall mutations resulted in decreased stability, these tools predict ΔΔG (kcal/mol) where negative value indicative of destabilizing mutation (Table 2).

HOPE software revealed structural alterations caused by

Table 1.
In silico checking for the pathogenicity of mutants.

S. No.	Mutation	Polyphen-2		SIFT	
		Score	Effect	Score	Effect
1	V456A	0.989	Probably damaging	0.01	Intolerant
2	K464E	0.973	Probably damaging	0.00	Intolerant

Table 2.
Computational analysis of protein stability in mutants.

S. No.	Mutation	mCSM		CUPSAT		MuPRO	
		Overall stability	Predicted ΔΔG (kcal/mol)	Overall stability	Predicted ΔΔG (kcal/mol)	Overall Stability	Predicted ΔΔG (kcal/mol)
		Score	Effect	Score	Effect		
1	V456A	Destabilizing	-2.0	Destabilizing	-1.2	Destabilizing	-1.8
2	K464E	Destabilizing	-0.7	Destabilizing	-2.1	Stabilizing	0.3

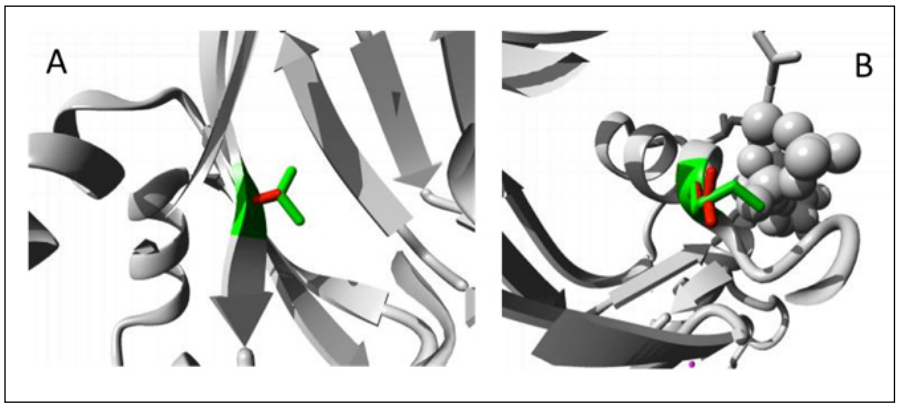


Figure 3.
Structural change V456A (A) and K464E (B) instanced by project HOPE. The protein is colored grey, the side chains of both the wild-type and the mutant residues are indicated as green and red respectively, ligand represented as grey ball structure (B).

the V456A (Figure 3A) and K464E (Figre 3B) mutations. These visualizations illustrate how these mutations impact local protein structure, providing insights into their functional consequences.

Discussion

Prior research has documented *Cystic fibrosis* (CF) as a major autosomal recessive disease in ethnic groups. Moreover, there are up to 2000 CFTR mutations that have been reported, with variable frequencies depending on ethnic and geographic backgrounds. It is imperative to highlight the various variables of CFTR mutations that are prevalent in a given population to help focus on the diagnostic test. It's a prerequisite for setting up efficient molecular diagnostics and for furthering the genetic treatment to help its prevention (1).

Since the last two decades, growing evidence has revealed a multifaceted function of CFTR in controlling a number of physiological processes associated with male infertility. Besides its familiar role of regulating electrolytes and fluid concentration of the male reproductive duct, recent investigations have indicated its participation in previously uncertain processes, such as sperm capacitation and spermatogenesis (26, 27), unfolding further potential reasons associated with male infertility, and strengthening the relation of CFTR mutations with CBVAD.

As new forms of infertility in men are identified and linked with CFTR defects and polymorphisms, it wouldn't be entirely illogical to consider CFTR as the molecular marker of male infertility. Based on strong correlation between the quality of sperm and CFTR mutations, a screening of CFTR gene for mutations in obstructive, azoospermia and non-obstructive individuals is suggested before they opt for intracytoplasmic sperm injection due to strong link-up between sperm quality and CFTR mutations (18).

Common mutation panels used for mutation analysis of males with obstructive azoospermia and CBAVD are unable to identify CFTR mutation variants in a given population. Initially, the mutation panel was designed to detect the most frequent CF causing mutations in the affected individuals of North European Caucasians (19). On the contrary, the genetic spectrum of South Asian people reported increased prevalence of novel mutation such as F508 which comprises 40-50% of cases as compared to 66% reported CF cases worldwide. Moreover, in South Asians frequency of mutation detection is lower than the Caucasians i.e., 50% and 77% respectively. Also, in several cases of CF, mutations remained undetected (23).

In our study, screening of CFTR promoter region and exon 10 was achieved by the DNA sequence method in CBAVD patients. We have identified one or two mutations in 35% of our sample patients. Discovering the presence of V456A in 4 of 17 patients further adds evidence for its pathogenic nature and supports its inclusion in the genetic diagnostic of CBAVD, especially in the South Asian population. V456A was initially described as polymorphism (28), but with further investigation the mutation was labeled as a mild disease-causing mutation particularly in adults with Bronchiectasis, while it also paired up with more prominent mutations (F508) (29). Previously, it has been linked with CBAVD as well (28). Regardless, it is a rare mutation occurring only in 2.4% of 78 south Asian patients (30). *Danziger et al.*, and clinical evidence from *Uppaluri et al.*, suggested that V456A holds importance as a disease-causing mutation and is not merely a mild polymorphism (31).

Exon 10 of the CFTR gene codes a portion of the nucleotide-binding domain (NBD I and II) in CFTR protein. NBD plays a crucial role in the regulation of CFTR function. It is involved in ATP (adenosine triphosphate) binding and hydrolysis, which is necessary for the opening and closing of the chloride channel formed by CFTR. ATP binding to NBD triggers conformational changes that enable the channel to transport chloride ions across the cell membrane. Mutations in exon 10 can disrupt the structure and function of NBD (I or II); impairing ATP binding, hydrolysis, and overall CFTR channel activity. The impaired ATP binding reduces the ability of CFTR to properly respond to cellular signals and regulate chloride ion transport, contributing to the dysfunction observed in cystic fibrosis. Mutations can also affect ATP hydrolysis, which is necessary for channel gating. The specific impact of mutations in exon 10 can vary depending on the nature and location of the mutation within the exon (1).

Based on literature, the overlapping function of NBD1 and NBD2 domain and the relation of exon 10 in the proper functioning of NBD domain (32, 33) hints towards a relation between the NBD domains of CFTR protein and the mutations observed in our study group; K464E, a missense variant which is formed as a result of base substitution of A by G at nucleotide position 181 of exon 10 of CFTR.

It can be assumed that this mutation could potentially affect the NBD domains, impacting its ability to bind and hydrolyze ATP, as proved, and described by the literature

for other exon 10 mutations. The K464E mutation also implicates to be defective for translational protein insertions on the membrane gated channels and ribosome binding (34), whereas the V456A mutation found in four of our CBAVD patients (HWP3, HWP4, HWP12 and HWP14) has been previously linked with its adverse effects on the *Nucleotide-binding Domain 1* (NBD1) of the CFTR protein. The V456A mutation refers to the substitution of the amino acid *valine* (V) with *alanine* (A) at position 456 within NBD1.

HOPE server illustrated that mutation driven physicochemical changes in protein might be deleterious. In case of V456A, mutated residue is smaller in contrast to wild type and mutation is located within a domain (ABC transporter 1 as annotated in UniProt) important for binding of other molecules and in contact with residues in a domain that is important for the activity of the protein. The mutation might affect this interaction and thereby disturb signal transfer from the binding domain to the activity domain (Figure 3A).

Regarding K464E, the size difference of mutated residue (smaller) disturbs the interaction with Mg2+ and might result in destabilization of the domain as divalent cations enhance ATP binding (35). K464 interacts with ligand ATP which might be disturbed by mutation and there is also change in charge of mutated residue (negative) as compared to wild type (positive) where changes in size and charge can cause loss of interaction with ligand (Figure 3B).

Mutations within NBD1, such as the V456A mutation, can disrupt these processes, leading to a dysfunctional CFTR protein and ultimately contributing to the development of cystic fibrosis. Therefore, its presence in CBAVD patients emphasize the significance of the mutation in the prevalence of the disease. The specific effects of the V456A and K464E mutations on the CFTR protein domain may vary, and further research may be required to fully understand its impact on the progression of CBAVD disease.

The new silent mutations S431S and G437G found in two of the patients do not seem to disrupt the coding sequencing as the resultant protein remains the same but further analysis is needed for a more concrete ruling as their influence as single nucleotide polymorphism cannot be ignored based on our study, which involves small number of CBAVD patients and lacks the inclusion of multiple ethnicities in the region. On the basis of these promising results, further research needs to be planned on a larger sample size. Furthermore, whole genome sequencing and protein analysis are required to be performed for more sensitive and consistent results. Hence, there is an extreme need for further investigations in genetics and epigenetics to provide deep understanding of male infertility, especially gene-environmental interaction, not just to provide detailed information about its etiology but also to help in proper genetic counseling. Also, further studies will be beneficial to develop preventive measures and therapies.

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