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ORIGINAL ARTICLE

Male Infertility

CFTR mutations causing congenital unilateral absence of the vas deferens (CUAVD) and congenital absence of the uterus (CAU) in a consanguineous family

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Cystic fibrosis (CF) is one of the most common recessive genetic diseases, with a wide spectrum of phenotypes, ranging from infertility to severe pulmonary disease. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene are considered the main genetic cause for CF. In this study, we recruited a consanguineous Iranian pedigree with four male patients diagnosed with congenital unilateral absence of the vas deferens (CUAVD), and one female patient diagnosed with congenital absence of the uterus (CAU). Testicular biopsy of one patient was performed, and hematoxylin and eosin (H and E) staining of testis sections displayed the presence of germ cell types ranging from spermatogonia to mature spermatids, indicating obstructive azoospermia. To explore the underlying genetic factor in this familial disorder, we therefore performed whole-exome sequencing (WES) on all available family members. WES data filtration and *CFTR* haplotype analysis identified compound heterozygous mutations in *CFTR* among four patients (two CUAVD patients carried p.H949Y and p.L997F, and one CUAVD and the female CAU patient carried p.H949Y and p.I148T). All these mutations were predicted to be deleterious by at least half of the prediction software programs and were confirmed by Sanger sequencing. Our study reported that *CFTR* compound heterozygous mutations in a consanguineous Iranian family cause infertility in both sexes.

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INTRODUCTION

Infertility is described as the inability to achieve pregnancy after 12 months of regular unprotected sexual intercourse, and about 15% of couples are affected worldwide.^{1–3} Reproductive failure is linked to several genetic defects, typically chromosomal anomalies and gene mutations.^{4–6} Male factors contribute to around 50% of infertility cases, while 10%–15% of infertile men suffer from azoospermia.^{7,8} Still, the major etiology of male infertility remains largely unknown.

Cystic fibrosis (CF) disorder is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which is located on chromosome 7q31, contains 27 exons, and spans about 190 kb at the genomic level.^{9–11} It encodes a transmembrane chloride (Cl⁻) channel that has anion transport and fluid reabsorption function.¹² The *CFTR* protein is a glycosylated transmembrane protein, consisting of two transmembrane domains (TMD1 and TMD2), two nucleotide

binding domains (NBD1 and NBD2), and a regulatory (R) domain that is expressed in the epithelial cells of exocrine tissues including lungs, pancreas, sweat glands, and vas deferens.^{13,14} So far, more than 2000 variants that lead to impairment in chloride transport in most of the exocrine glands, resulting in abnormally thick mucus secretion, have been identified in the *CFTR* gene (<https://www.cftr2.org/>).¹⁵ Patients with classical CF characteristics usually have bi-allelic *CFTR* variants and suffer from lung problems and pancreatic insufficiency. However, mild diseases such as congenital absence of the vas deferens (CAVD) without other typical clinical manifestations of CF may result from the variation in alternative mRNA splicing or different sensitivity levels to dysfunctional *CFTR* protein among various organs.^{16–18} The mutation spectrums of typical CF and CAVD are thus diversified.¹⁹

Approximately 95% of males with CF have primary infertility with obstructive azoospermia (OA), and *CFTR* variants are the main

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genetic cause of CAVD/CUAVD and congenital bilateral absence of the vas deferens (CBAVD).^{20,21} CBAVD is the most common subtype with 1%–2% prevalence but can go up to 25% in OA cases, while the incidence of CUAVD is 0.5%–1.0% and usually recognized during evaluations for infertility.^{22,23} According to previous reports, most CAVD patients have a compound heterozygous genotype with mutations in different alleles, and around 43% of CUAVD patients carry at least one *CFTR* variant.^{3,24,25}

Congenital absence of uterus and/or vagina (CAUV/CAU), which affects 1 in 5000 women, is associated with *CFTR* mutations. The frequency of the most common mutations of *CFTR* in patients with CAUV was 8%, twice the incidence of that in the general population (4%).^{26,27} *CFTR* variation thus rarely causes CAU, with a few sporadic cases reported so far.

In this study, we recruited a consanguineous Iranian family, in which male patients were suffering from CUAVD and one female patient was diagnosed with CAU. Whole-exome sequencing (WES) was performed to determine the genetic cause of CUAVD and CAU, and *CFTR* compound heterozygous mutations were identified in four patients.

MATERIALS AND METHODS

Family recruitment

An Iranian family comprising four infertile brothers and one infertile sister (IV:2, IV:7, IV:9, IV:13, and IV:5), who are offspring of a first-degree consanguineous marriage, were recruited from the affiliated Armaghan Clinic of Mashhad University of Medical Sciences, Mashhad, Iran (November, 2019). The infertile individuals were diagnosed by a panel of urologists and gynecologists, and the accessible clinical records and peripheral blood samples of all the available siblings and parents were collected. Furthermore, genomic DNA was extracted from blood using the FlexiGene DNA Kit (QIAGEN, Hilden, Germany) following the standard protocol. All the studies on patients were conducted after obtaining informed consent, and this project was approved by the ethical committee of University of Science and Technology of China (USTC; approval number: USTCEC202000003) and the Mashhad University of Medical Science (MUMS; approval number: MUMSEC130010405).

Testicular biopsy and hematoxylin and eosin (H and E) staining

Testicular biopsy is a significant component of male infertility workup to distinguish between obstructive and nonobstructive azoospermia. Open surgical biopsy is known as the gold standard for obtaining tissues and inspection of spermatogenesis.²⁸ A small tissue sample was taken from both left and right testes of patient IV:13, and the samples were perfused overnight in Bouin's fixative solution. Tissue embedding was then performed in paraffin to make blocks. Tissue sectioning was prepared by microtome, and H and E staining was subsequently carried out as described previously.²⁹ A digital Nikon DS-Ri1 camera installed on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) was used to capture the images.

WES and Sanger sequencing

For exome capturing, an AIExome Enrichment Kit V1 (iGeneTech, Beijing, China) was used. Captured libraries were constructed for all the recruited family members, as per manufacturer's instructions. Sequencing was carried out using the Novaseq 6000 platform (Illumina, San Diego, CA, USA). Raw data and WES data filtration were processed as described previously.³⁰ *CFTR* compound heterozygous mutations were confirmed by Sanger sequencing using the primers listed in **Supplementary Table 1**.

In silico analysis

The conservation of affected amino acids resulting from *CFTR* mutations was predicted using MEGA6.³¹ The pathogenicity of these mutations was assessed via seven deleterious effects prediction software programs (**Supplementary Table 2**).

RESULTS

Family description

The phenotypes of all the infertile individuals were recognized at Armaghan Clinic where they consulted for infertility treatment (**Figure 1**). Basic clinical parameters of all the CUAVD patients are summarized in **Table 1**. All the male patients including IV:13 displayed inherited lack of left vas deferens. The female with CAU (IV:5) was 36 years old with no cycle, and ultrasound test verified congenital lack of the uterus. The mother (III:2) experienced an irregular cycle and two miscarriages. Histochemical analysis indicated that IV:13 had standard testicular tissue with seminiferous tubules containing normal spermatogenesis composed of spermatogonia, spermatocytes, spermatids, and spermatozoa, consistent with a score of 9 on the Johnsen scale³² (**Figure 2**) and suggesting OA.

CFTR variants identified in the patients

Analysis of WES results showed no candidate mutations that were compatible with autosomal recessive inheritance regarding patients' parents being first cousins. Considering that *CFTR* is a major genetic factor for this type of disease, we retrieved all *CFTR* variations identified by WES.

Two different compound heterozygous mutations of *CFTR* were identified in four patients. Two male CUAVD patients, IV:7 and IV:2, shared *CFTR* compound heterozygous mutations (ENST00000003084, p.H949Y and p.L997F); male CUAVD patient IV:13 and female CAU patient IV:5 carried *CFTR* compound heterozygous mutations (ENST00000003084, p.H949Y and p.I148T). Sanger sequencing of these *CFTR* mutations was performed, which further confirmed infertility phenotype co-segregated with *CFTR* compound heterozygous mutations in the pedigree (**Figure 3**).³³

The c.443T>C (rs35516286) variant gives rise to isoleucine being replaced by threonine at position 148 in the *CFTR* protein. The c.2845C>T (rs121909035) and the c.2991G>C (rs1800111) variants cause alteration of histidine to tyrosine at amino acid 949 and leucine transfer to phenylalanine at position 997 in the *CFTR* protein, respectively (**Supplementary Figure 1**). All the detected variations localized in the transmembrane domain constructed from six membrane-spanning segments, each mediating the regulation of anion (Cl⁻) transport across the apical membrane of epithelial cells.³⁴ Each of these *CFTR* compound heterozygous mutations had a rare allele frequency in the ClinVar public population database (**Supplementary Table 3**).^{35–39} *CFTR* p.H949Y (rs121909035) has already been evaluated as “pathogenic” in ClinVar, while recent studies also showed that p.L997F (rs1800111) and p.I148T (rs35516286) are pathogenic and updated in ClinVar. Further, seven deleterious effects prediction software programs were used to predict the pathogenesis of *CFTR* mutations, and they were predicted to be deleterious by more than half *in silico* programs (**Supplementary Table 2**). Further, all the identified mutations were mostly conserved in the eutherian species (**Supplementary Figure 2**).

DISCUSSION

CF is a recessive genetic disease, with most cases caused by pathogenic mutations in *CFTR*. Although CF is more frequent in the Caucasian population, it affects all ethnic groups, with particular variations (e.g., p.Phe508del) found in 90% of Caucasian patients.^{40–42} While the limited

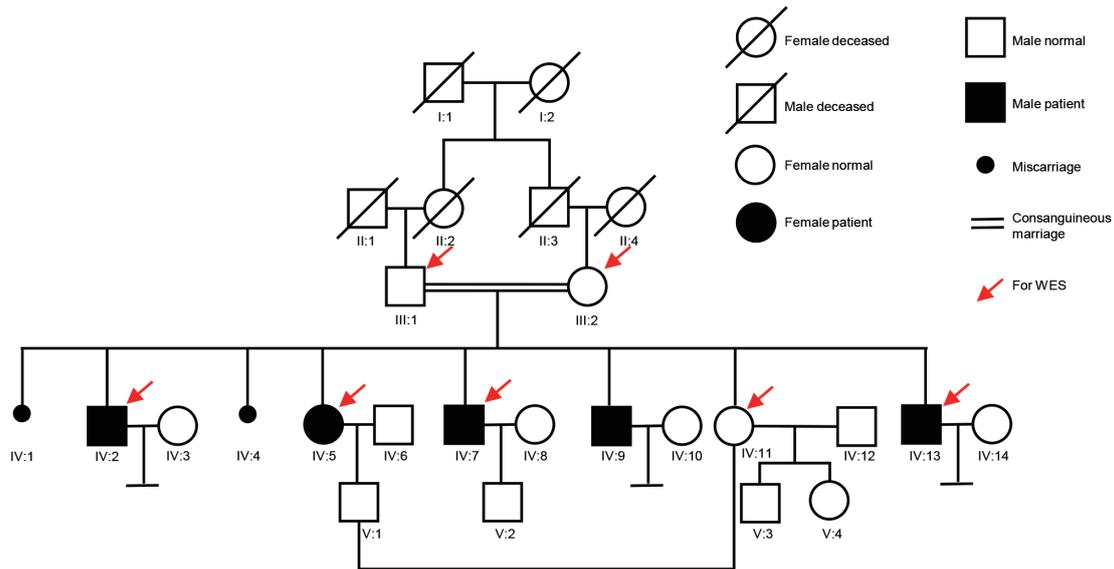


Figure 1: Pedigree structure of the family. IV:7 has an 11-year-old son but failed to conceive later. Physical examination and semen analysis showed lack of left vas deferens and azoospermia, suggesting secondary infertility. V:1 was born through a surrogate mother using IV:11 uterus for transferring and developing zygotes of IV:5 and IV:6 by the IVF method. IVF: *in-vitro* fertilization; WES: whole-exome sequencing.

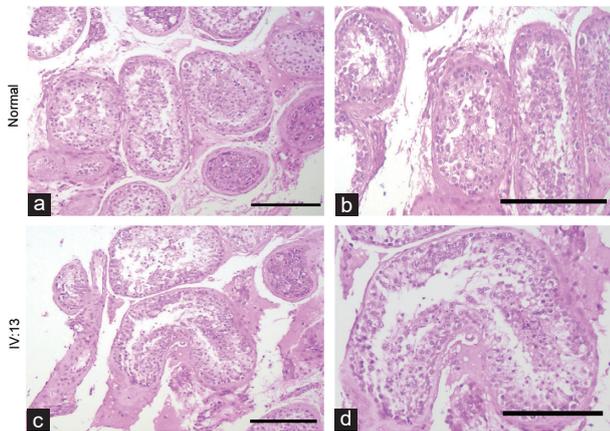


Figure 2: Normal distribution of germ cells and spermatogenesis. H and E staining of testes from normal individual (a) image captured on 20x and (b) image captured on 40x displaying normal spermatogenesis. H and E staining of the patient IV:13: (c) representative image captured on 20x and (d) representative image captured on 40x also displaying normal spermatogenesis indicating obstructive azoospermia. Scale bars = 100 μ m. H and E: hematoxylin and eosin.

knowledge on CF frequency and the spectrum of *CFTR* mutations among Iranians is probably due to misdiagnosis or lack of studies, previous research has reported that the prevalence of CF in Iran is approximately 1 in 100 000 cases, with DF508, N1303K, G542X, R347H, and W1282X being the most common mutations.^{43–45} The *CFTR* gene is located on the short arm of chromosome 7 (7q31), contains 27 exons, and spans about 190 kb,^{9,10} which encodes a transmembrane chloride (Cl^-) channel. This protein consists of two transmembrane domains, two nucleotide binding domains, and a regulatory domain that manages anion transport and fluid reabsorption function.

Almost all CF males have infertility resulting from OA, and CAVD is known as an atypical form of CF, but CAVD can occur without any typical manifestations of CF.⁴⁶ A possible reason for this event could be

the difference in alternative mRNA splicing between various organs, just as previous studies showed that mRNA splicing was more efficient in the respiratory epithelial than that in the vas deferens epithelial. Furthermore, the reproductive system is more sensitive to *CFTR* protein dysfunction than other tissues.^{10,47} Congenital absence of the vas deferens could be unilateral (CUAVD) as a result of mild *CFTR* mutation, with an incidence of 0.5%–1% in the male population and mostly double prevalence on the left compared with the right side.^{48,49} Based on Casals *et al.*'s report,⁴⁰ 38% of the CUAVD cases correlate with mutations in the *CFTR* gene.

Although women with CF have thicker cervical mucus and ovulation issues, most affected females are fertile. According to a study by Timmreck *et al.*,²⁵ CAU is a rare phenotype among women with *CFTR* mutations, and the majority of patients with CAUV/CAU do not carry *CFTR* mutations.

In the current study, we presented a consanguineous Iranian family suffering from infertility in both sexes, and for which, so far, there is no report of CF pedigree showing only infertility phenotype in both sexes. Further clinical study showed CUAVD in the male patients and CAU in the female patient. To investigate further, we examined gonadal hormone levels in serum, seminal volume and pH, and testicular size, which revealed normal results. Subsequently, spermatogenesis histochemical evaluation showed a standard sperm production procedure. This acquired information indicated OA. Herein, we performed WES of all the available family members' gDNA to determine the role of genetic factors in this familial disorder. We found that male CUAVD patients IV:7 and IV:2 shared *CFTR* compound heterozygous mutations (ENST00000003084, p.H949Y and p.L997F), while male CUAVD patient IV:13 and female CAU patient IV:5 carried *CFTR* compound heterozygous mutations (ENST00000003084, p.H949Y and p.I148T). The identified mutations are located in transmembrane domains that mediate the regulation of anion (Cl^-) transport across the apical membrane of epithelial cells. p.H949Y was found in all infertile patients, except IV:9, whose gDNA sample was not available. We assumed he would show a compound heterozygous

Table 1: Clinical parameters of congenital unilateral absence of the vas deferens patients

Clinical parameter	IV:2	IV:9	IV:13	IV:7	Reference value (men), range
Age (year)	41	34	26	35	NI
Height (cm)	165	180	178	178	NI
Weight (kg)	90	87	78	70	NI
BMI (kg m ⁻²)	33.1	26.9	24.6	22.1	NI
Physical examination	Lack of left vas deferens	NI			
Karyotype analysis	Normal	Normal	Normal	Normal	NI
Sperm concentration (×10 ⁶ ml ⁻¹)	0	0	0	0	>15
Semen volume (ml)	2.0	2.5	2.1	2.9	>1.5
pH	7.5	7.9	7.4	8.0	7.2–8.0
FSH (IU ml ⁻¹)	6.3	10.0	7.5	11.7	1.3–19.3
LH (IU l ⁻¹)	8.1	6.4	7.9	5.0	1.9–9.0
Testosterone (ng dl ⁻¹)	358	730	571	607	200–800

NI: not included; FSH: follicle-stimulating hormone; LH: luteinizing hormone; BMI: body mass index

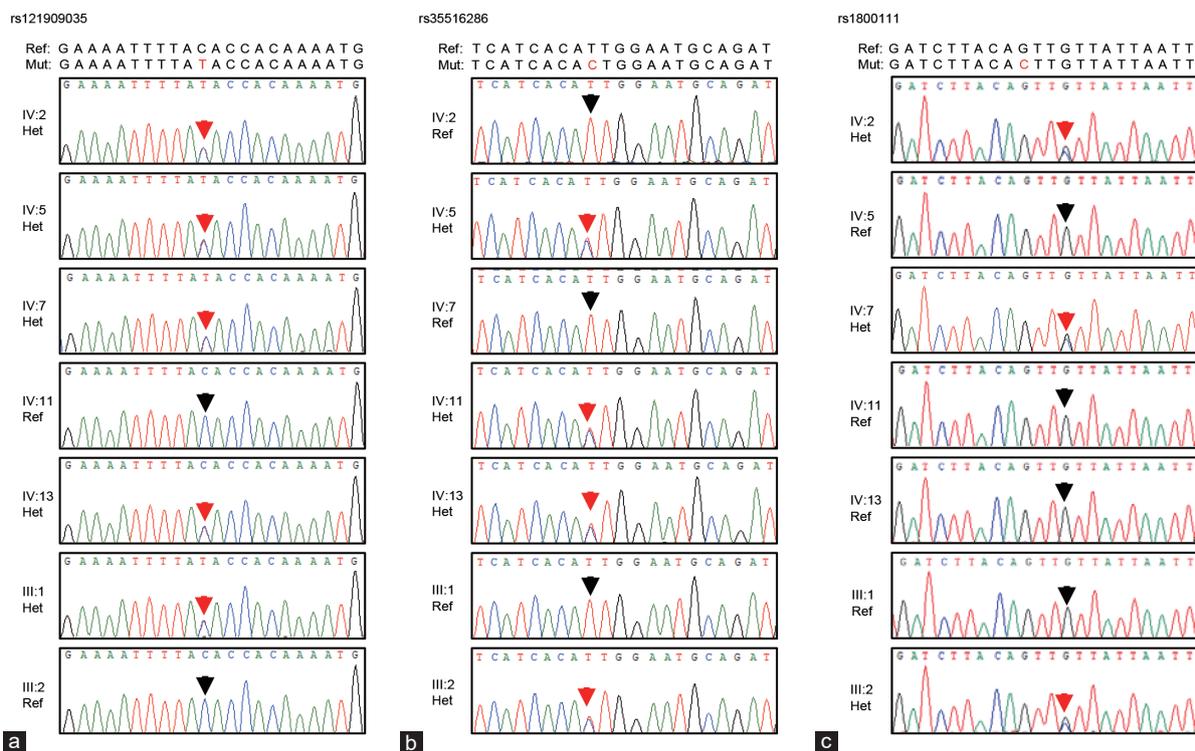


Figure 3: Validation of *CFTR* variants among family members by Sanger sequencing. (a) Chromatogram of *CFTR* variations showing the father and four patients (III:1, IV:2, IV:5, IV:7, and IV:13) were heterozygous (indicated by red arrows) for rs121909035 mutation, while the mother and normal sister (III:2 and IV:11) carried normal alleles (black arrows). (b) In rs35516286 variant, the father and two patients (III:1, IV:2, and IV:7) were normal, and the other participants carried heterozygous variation. (c) In the case of rs1800111, the mother and two patients (III:2, IV:2, and IV:7) carried heterozygous allele, while the father and the other offspring (III:1, IV:5, IV:11, and IV:13) were normal. Ref: reference; A: adenine; C: cytosine; G: guanine; T: thymine; *CFTR*: cystic fibrosis transmembrane conductance regulator; mut: mutant; het: heterozygous. IV:9 patient's DNA is not available, we assumed he would be in compound heterozygous state.

state. This mutation is recorded in ClinVar as “pathogenic,” and although mutations in another *CFTR* allele were different in these patients, our results provide solid evidence that the pathogenicity of *CFTR* p.H949Y is in an autosomal recessive pattern. All these *CFTR* mutations (p.I148T, p.H949Y, and p.L997F) were predicted to be disease causative by more than half of the deleterious prediction software programs. Previous studies have reported some bi-allelic variants of *CFTR* in some unrelated patients. Our study, in which *CFTR* compound heterozygous mutations were co-segregated with

infertility phenotype in a large family, provides the first-known familial evidence that only recessive variants (homozygous or compound heterozygous) cause CUAVD/CAU.

CONCLUSION

In conclusion, we identified *CFTR* mutations in a consanguineous Iranian pedigree comprising several infertile offspring in both sexes. *CFTR* p.H949Y was found in both CUAVD and CAU patients, indicating that it played a critical role in the synergistic pathogenesis

of complex heterozygous mutations; the other two mutations attained pathogenic effect. Our results provide important genetic evidence that only recessive variants in *CFTR* can cause CUAVD in men and CAU in women.

AUTHOR CONTRIBUTIONS

MDG, RK, and HZ performed the experiments and wrote the manuscript. MDG, MM, MJ, MK and AS collected the samples and analyzed the data. AW, HZ, MZ, UH, and QHS analyzed and modified the manuscript. QHS and HZ conceived and supervised the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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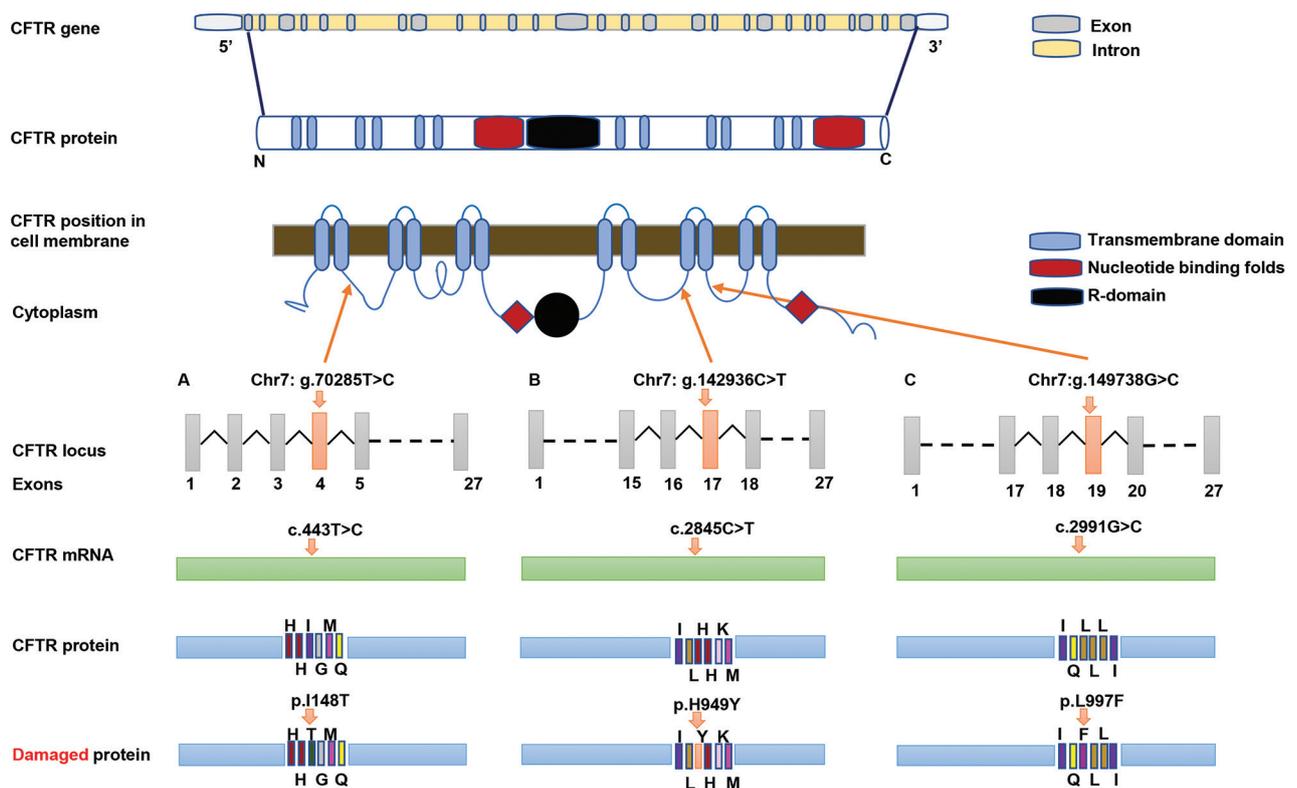


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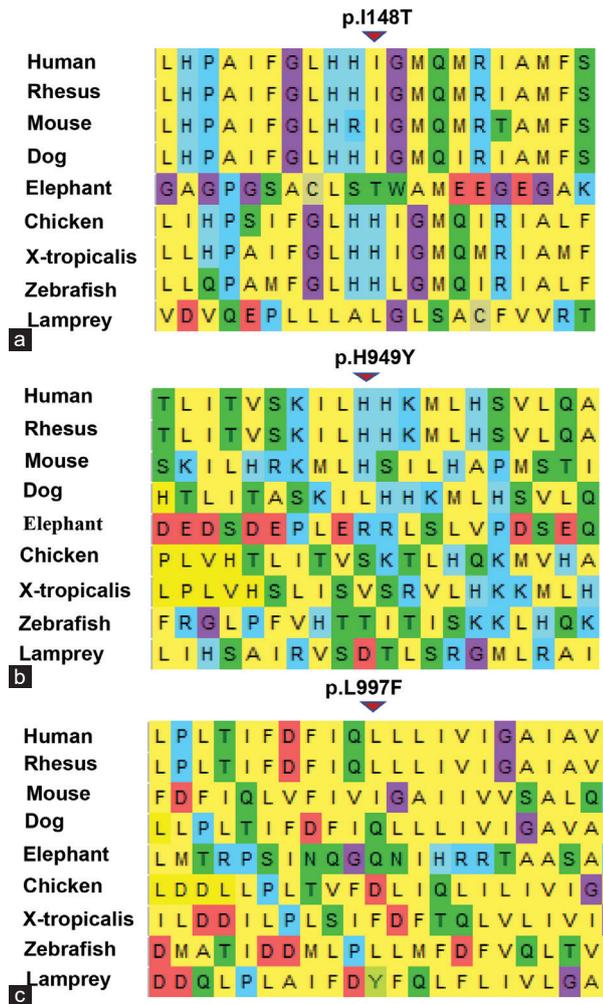
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Supplementary Figure 1: Identification and prediction effect of the CFTR variants. **(A)** The rs35516286 mutation take place in exon 4 which T altered to C at gDNA position 70 285 nucleotide (nt). In transcript mRNA, the mutated site is present at 443 nt. The CFTR protein comprises of transmembrane domain, nucleotide binding domain, and R- domain. In mutant protein, I replace with T at amino acid 148 which lies in transmembrane domain. **(B)** The rs121909035 variation arise in exon 17 leads to alteration of C to T at gDNA position 142 936 bp, and 2845 nt in cDNA resulting in replacement of H with Y at amino acid 949 in protein. This mutation placed in transmembrane domain. **(C)** The rs1800111 variant occurs in exon 19 and in this variant G replaced with C at position 149 738 nt in gDNA and 2991 nt in cDNA. Following this mutation, L exchange with F at amino acid 997 which lies in transmembrane domain. Pink arrow heads indicate mutation sites and the altered amino acid.



Supplementary Figure 2: The conservation of affected amino acids. Sequence alignment using MEGA6 predicted the conservation of isoleucine (a), histidine (b), and leucine (c) amino acids.

Supplementary Table 1: Primers used for Sanger sequencing

<i>CFTR variants</i>	<i>Forward primer</i>	<i>Reverse primer</i>	<i>Size (bp)</i>
rs121909035	5'-AGTAAGTAACTTTGGCTGCC-3'	5'-CAGTCAAATGGAGTTCAAC-3'	431
rs1800111	5'-ACCTAATGCTAGATGACGAG-3'	5'-TTACAAGATGAGTATCGCAC-3'	428
rs35516286	5'-TTGTAGGAAGTCACCAAAGC-3'	5'-GAGCATTAAATTATCCTGCC-3'	377

CFTR: cystic fibrosis transmembrane conductance regulator

Supplementary Table 2: Prediction pathogenicity of mutations by several software

<i>Prediction method</i>	<i>p.I148T</i>	<i>p.H949Y</i>	<i>p.L997F</i>
SIFT	Tolerated	Deleterious	Tolerated
Polyphen2	Benign	Probably damaging	Probably damaging
MutationTaster_pred	Disease causing	Disease causing automatic	Disease causing
MutationAssessor_pred	Low	High	Medium
Fathmm-MKL	Deleterious	Deleterious	Deleterious
GERP++_RS	5.73	4.97	2.87
SiPhy_29way_logOdds	16.314	16.24	2.683
Number of software predict deleterious	4	7	4
Ration	0.57	1	0.57

SIFT: Sorting Intolerant From Tolerant; MKL: Multiple Kernel Learning; GERP: Genomic Evolutionary Rate Profiling; RS: Reference SNP Cluster ID

Supplementary Table 3: Allele frequency in public population database

<i>Allele frequencies in public population database</i>	<i>p.I148T</i>	<i>p.H949Y</i>	<i>p.L997F</i>
avsnp147 ID	rs35516286	rs121909035	rs1800111
1000G	0.0014	0	0.0018
Esp6500	0.0005	0	0.0015
Exac03	0.0019	0	0.0021
GnomAD	0.0018	0	0.0023
ClinVar interpretation	VCV000053949: CF, CBAVD from CFTR mutation	Pathogenic	VCV000007229.22: pathogenic

CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; CBAVD: congenital bilateral aplasia of vas deferens