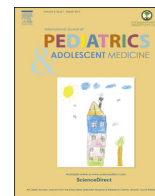


HOSTED BY



Contents lists available at ScienceDirect

International Journal of Pediatrics and Adolescent Medicine

journal homepage: <http://www.elsevier.com/locate/ijpam>

Case Report

The first case report of double homozygous of 2 different mutations in the *CFTR* gene in Saudi Arabia



Hanaa Banjar ^{a,*}, Nabil Moghrabi ^b, Tariq Alotaibi ^c, Sami Alotaibi ^c, Hisham Gamalmaz ^c

^a King Faisal Specialist Hospital and Research Centre, Saudi Arabia

^b Genetics Department, Saudi Diagnostic Laboratory, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

^c Alfaisal University, Saudi Arabia

ARTICLE INFO

Article history:

Received 10 May 2016

Received in revised form

19 September 2016

Accepted 25 September 2016

Available online 16 February 2017

Keywords:

Cystic fibrosis transregulatory gene (*CFTR*)

Mutations in the cystic fibrosis patients

Rare double homozygous in cystic fibrosis patients

ABSTRACT

The first cases of a rare double homozygosity of two different mutations in the cystic fibrosis trans-regulator gene (*CFTR*) of a cystic fibrosis patient in Saudi Arabia. Details of the family screening and a review of the literature on similar cases are discussed.

© 2016 Publishing services provided by Elsevier B.V. on behalf of King Faisal Specialist Hospital & Research Centre (General Organization), Saudi Arabia. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in Caucasians, with an incidence of ~1 in 2500–3000 for the native Caucasian population of England [1,2].

In the native Caucasian population of the United Kingdom, 56 different mutations account for 86% of CF genes ($n = 9807$) [3].

Cystic fibrosis has been reported before in detail in Saudi Arabia [4–7] and the Gulf area [8,9]. Clinical presentations mainly include chest infection, steatorrhea, and pseudo Bartter's syndrome [4–7]. *CFTR* mutations of the Saudi population have been described previously [4–7].

Our previous report showed the clinical pattern of our CF population, with poor nutrition, poor compliance with chest physiotherapy and medication, and early colonization with bacteria: fifty percent of our CF population was colonized with *Pseudomonas aeruginosa* at the time of diagnosis of 34 months, which is much

earlier than the 5–7 years reported in North America [4,5].

Kambouris et al [4] showed that the Saudi *CFTR* mutations were different from that have been described in the rest of the world and that new mutations are common in people of Saudi ethnic origin due to intermarriages that perpetuate most of these mutations.

The identification of the disease-causing mutations in a CF patient is important for many reasons. These include genetic counseling, the possibility of prenatal diagnosis (PND) for the index family, cascade carrier testing for other family members (Super et al, 1994), and studies of genotype/phenotype correlation. In time, knowledge of the actual mutation could also be important for targeting treatments aimed at the basic defect.

In this report, we present the first report of double homozygosity of two different mutations in the *CFTR* gene in Saudi Arabia.

2. Case report

Index case – A 6-year-old female patient of Saudi descent and the product of a full-term pregnancy had a birth weight of 2.2 kg and presented at 2 months of age with a history of vomiting, poor feeding, multiple episodes of diarrhea since birth, recurrent chest infections, failure to thrive, increased sweating, persistent runny nose and the subsequent development of generalized edema. Her sweat chloride measured at a local hospital was high (95 mmol/L), and she was suspected to have cystic fibrosis (CF). The patient was

* Corresponding author. King Faisal Specialist Hospital and Research Centre, Saudi Arabia. Tel.: +966 503493022.

E-mail addresses: hanaa@kfshrc.edu.sa (H. Banjar), nmoghrabi@kfshrc.edu.sa (N. Moghrabi), 19tariq88@live.com (T. Alotaibi), sami1989-09@live.com (S. Alotaibi), hisham.gamalmaz@gmail.com (H. Gamalmaz).

Peer review under responsibility of King Faisal Specialist Hospital & Research Centre (General Organization), Saudi Arabia.

given NG tube feeding with a high-calorie diet and pancreatic enzymes, MCT oil and multivitamins.

Family history revealed two parental uncles who had sickle cell disease (HbS disease) but no family history of CF.

The patient was referred to our center for further management at the age of 11 months. On physical examination, she was pale and malnourished, with both weight (5.2 Kg) and height (45 cm) below the 5th percentile for age, a low body mass index (BMI) of 13.5, a Z score for weight of (−2.5), and a Z score for height of (−2.3) (Table 1). She also presented with post nasal drip, bilateral rhonchi and crepitation and a palpable liver. The results of sweat chloride tests performed on two separate occasions were consistently high (>105 mmol/L; normal is < 60 mmol/L). Malabsorption investigation was positive based on 72-h fat stool collection results of >60 mmol fat/day.

Respiratory culture revealed the presence of *Streptococcus pneumoniae*, and a subsequent culture showed many Beta-lactamase positive *Haemophilus influenzae* cells.

The patient had multiple pulmonary exacerbations with positive multiplex PCR findings for respiratory viruses on three occasions (for para-influenza virus, influenza virus, and C. bocavirus 1/2/3/4).

Her chest X-ray showed mild cardiomegaly with mildly prominent central pulmonary vascularity. There were no signs of pulmonary edema. There was bilateral perihilar bronchial wall thickening. Focal left lingular airspace disease was noted. There was no pneumothorax or pleural effusion.

The echocardiogram showed small patent ductus arteriosus with left-to-right shunting. There was no tricuspid or pulmonary regurgitation that could be used to estimate the pulmonary artery pressure; however, the Doppler study (IVRT) was suggestive of mildly elevated pulmonary artery systolic pressure (~35–40 mmHg). Cardiac chamber size was normal. There was good biventricular systolic function.

Bilateral mastoid X-ray, neck X-ray and sinus X-ray were performed when the patient was 4 years old and showed mild enlargement of the adenoids with narrowing of the nasopharynx and poor aeration of the paranasal sinuses. No aeration in the

mastoid air cells could be observed. The picture suggested possible recurrent sinusitis and otitis media. Abdominal US was unremarkable.

At 5 years of age, the patient presented with recurrent attacks of partial complex seizure in the form of deviation of the mouth which lasted from half a minute to 2 min, 4 times a day. Her seizures were controlled with triple anti-seizure medications: Keppra (levetiracetam), Topamax (topiramate), and Lamictal (lamotrigine).

When her clinical and laboratory presentations at diagnosis were compared with those of the rest of the CF population from the same center (Table 1), the patient was found to have an earlier presentation (at 2 months) compared with a mean age of (3.4 ± 5 years) for the whole CF population; a low body mass index (BMI) of 13.5 compared with (15.5 ± 5.8); low vitamin A, E, D, and K; a mild increase in liver enzymes; and electrolyte imbalance with hypokalemia and hyponatremia (Table 1).

Cystic fibrosis transmembrane regulator gene mutation (CFTR) analysis of the index case – DNA from the patient's lymphocytes was used to amplify the 27 exons and flanking sequences of the *CFTR* gene. The PCR products were analyzed by sequencing them in both the forward and reverse directions [4].

The sequence analysis identified two copies of a splice site consensus sequence variant, c.164+12T>C, in intron 2 (IVS2+12T>C) of the *CFTR* gene. This variant, c.164+12T>C, predicts skipping of the preceding exon during RNA splicing, leading to a nonfunctional transmembrane conductance regulator protein (Fig. 1). This mutation has been previously reported in patients with CF, but not as double homozygous state [10].

Another single nucleotide duplication of c.3889dupT was identified in the coding region in two copies of the *CFTR* gene in this individual. This duplication, c.3889dupT, predicts a sequence frameshift due to 'T' insertion at the second nucleotide position of codon 1297 for serine (c.3889_3890insT), inserting 4 amino acids and a premature termination codon, (X), in exon 24 of the regulator protein (p.S1297FfsX5) (Fig. 1). This mutation has been previously reported in patients with CF, but not as double homozygous state [11].

Table 1

A comparison of the double homozygous individual and other CF patients with homozygous *CFTR* from the same center.

Clinical symptoms and signs	Double homozygous case Values at presentation	Incidence in other CF cases (Total 229 patients)
Age at presentation	2 months	Mean of 3.4 ± 5 years
BMI at presentation	13.5	15.5 ± 5.8
Sweat cl value	107 mmol/L	Mean of 89 mmol/L
Recurrent respiratory symptoms	Present	90%
Recurrent loose bowel movements	Present	92%
Vomiting	Present	33%
Partial complex seizure	Present	5 patients (2%)
Vitamin E level < 5.5 mg/L	Value = 2.7 (low)	34.7%
Vitamin A level < 343 Mcg/L	Value = 185 (low)	51.5%
Vitamin D, 25 hydroxy (total) level < 50–100 nmol/L	Value = 19 (low)	70%
Vitamin K < 0.10 ng/mL	Value = 0.26 (normal)	7.6%
ALT > 65 U/L (normal 10–45)	Value = 49 (slightly higher than normal)	2.8%
AST > 65 U/L (normal 10–25)	Value = 49 (slightly high than normal)	1.6%
GGT > 35 U/L	Value = 5 (normal)	9.6%
Albumin <20 g/L	Value = 43 (normal)	0.8%
Electrolyte imbalance	present	30%
Z score for weight	−2.5	-1.6 ± 1.8
Z score for height	−2.3	-2.1 ± 5.9
Weight < 5th percentile for age	Present (5.2 kg at 11 months)	37%
Height < 5th percentile for age	Present (54 cm at 11 months)	25%
Homozygous <i>CFTR</i>	Present	(91%) homozygous
Compound heterozygous	Present	(9%) compound heterozygous
Respirator culture: <i>Streptococcus pneumoniae</i>	Present	11%
<i>Haemophilus influenzae</i>	Present	14%

Legend: Mg = milligram, Mcg = microgram, nmo = nanomole, u = units, g = gram, *CFTR* = cystic fibrosis transmembrane regulator gene.

Index Case

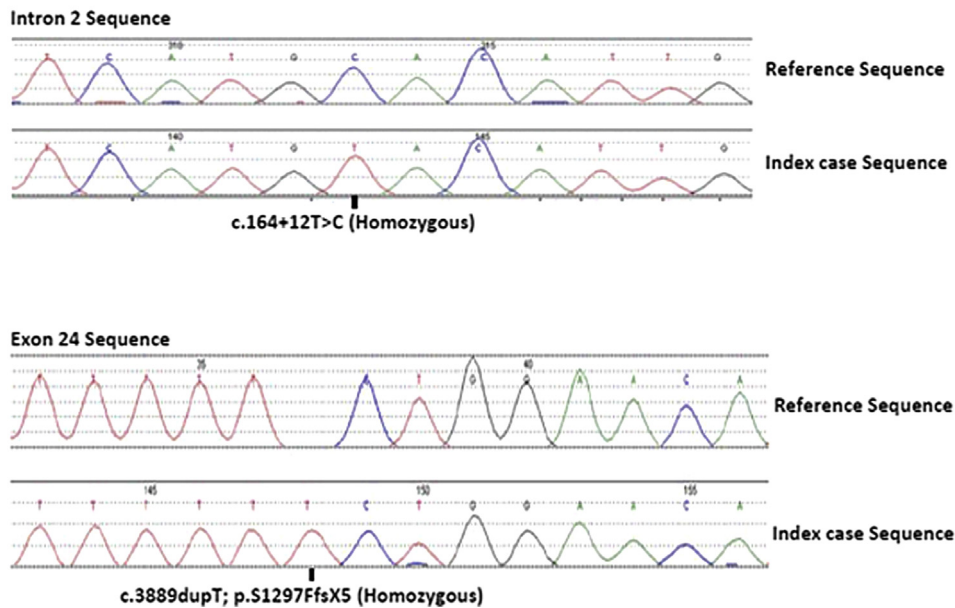


Figure 1. Index case, double homozygous c.164+12T>C/ c.3889_3890insT.

A homozygous IVS9(TG10-7T) allele polymorphism and sequence variant polymorphism, c.1408G>A; p.V470 M (rs213950) (Fig. 1), were also identified in this individual.

The results are atypical due to the presence of double homozygous variants of reported mutations in the *CFTR* gene in Trans configuration and in both alleles. These results indicate that this individual is likely affected with CF due to the presence of a reported homozygous disease-causing sequence variant of a consensus splice donor-site mutation, c.164+12T>C (IVS2+12T>C) in intron 2, and a second reported homozygous disease-causing single nucleotide insertion, c.3889_3890insT (p.S1297FfsX5), in exon 24 of the *CFTR* gene.

Family screening for the *CFTR* gene – Once the *CFTR* gene results became available, the parents were screened along with the brother, who was 10 months of age at that time and presented with cough but had normal growth and a normal sweat chloride test of 20 mmol/L (normal is <60 mmol/L). His *CFTR* screening was the same as that of his sister: double mutations in one allele only and in the CIS configuration, while the other allele was normal, as shown in Fig. 2. *CFTR* sequence analysis identified one copy of each of the splice site consensus sequence mutations, c.164+12T>C, in intron 2 (IVS2+12T>C) and the single nucleotide duplication, c.3889dupT (c.3889_3890insT), in exon 24 (p.S1297FfsX5) of the *CFTR* gene (Fig. 2).

CFTR analysis of both parents showed that they were both carriers for both mutations in the same manner as the brother in terms of the CIS configuration (Figs. 3 and 4).

Course of the disease: The patient was started on ventolin, fluticasone inhalation, amoxicillin/clavolinic acid for 2 weeks, hypertonic saline 7% on nebulization, ADEK vitamins, and Creon pancreatic enzyme replacement.

Her growth parameters gradually improved with treatment. The patient was 6 years at the time of manuscript writing, with normal growth and development and minimal chest x-ray changes.

3. Discussion

A total of 400 CF patients are followed in our CF clinic. A total of 290 patients' *CFTR*s have been identified. This is the first case report of a double homozygous mutation in our CF population.

Our patient's phenotypic picture was severe compared with that of other CF patients from the same center: she had an earlier presentation of 2 months compared with a mean age of (3.4 ± 5 years); a low body mass index (BMI) of 13.5 compared with (15.5 ± 5.8); low levels of vitamins A, E, D, and K; a mild increase in liver enzymes; electrolyte imbalance with hypokalemia and hyponatremia; and a seizure disorder (Table 1).

Both of the mutations that our patient showed were described previously by Malone et al [10] and by Cheadle et al [11]; however, the mutations were not described as double homozygous but as a single homozygous state.

Malone et al [10] described one of the mutations that was described in our patient, 621+2 (T>C), which appears to be specific to Pakistani CF families. This mutation alters the T nucleotide at the +2 position of the consensus 5' (GT) splice site of intron 4 and is predicted to affect splicing. Two unrelated patients were found to be homozygous for 296+12 (T>C). This mutation was the only abnormality found during the sequencing of all 27 exons and the intron-exon boundaries of the *CFTR* gene in patient C11. Four healthy siblings of this patient were heterozygous for the mutation. Despite its position, 12 bases from the intron-exon boundary, the mutation may affect splicing, although it does not create a new splice site.

Cheadle et al [11] reported the other detected mutation in our patient: 4016 insT, in exon 21 of the *CFTR* gene. 4016 insT is predicted to lie within the second putative nucleotide binding domain. Thus, the introduced stop codon would produce a protein with both the membrane spanning domains, the first nucleotide binding domain, the regulatory domain, and part of the second nucleotide

Brother

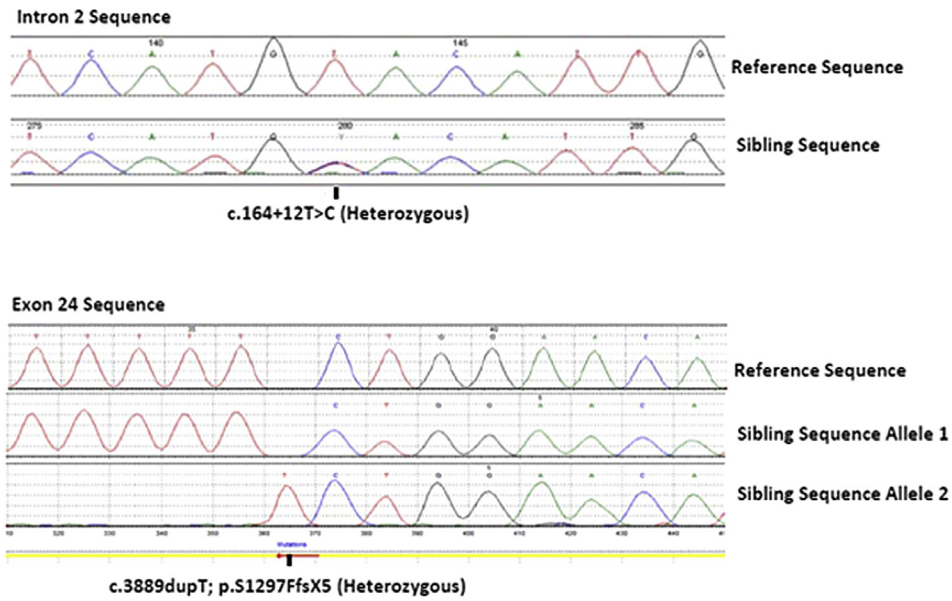


Figure 2. Brother with double mutation c.164+12T>C/ c.3889_3890insT in the same allele in CIS position.

binding domain. This truncated protein may be integrated into the membrane (with perhaps some retention of function), or it may be degraded. Protein studies should help to resolve this issue.

This mutation causes the insertion of a single nucleotide in a run of six identical nucleotides. This type of insertion is thought to arise from the misincorporation of an extra base by the DNA polymerase

because of slipped mispairing at the replication fork [12]. Other examples of this type of insertion have been described in Lesch-Nyhan syndrome, osteoporosis, and oculocutaneous albinism, as reviewed by Cooper [13].

Few reports have described the presence of double mutations in each allele.

Mother

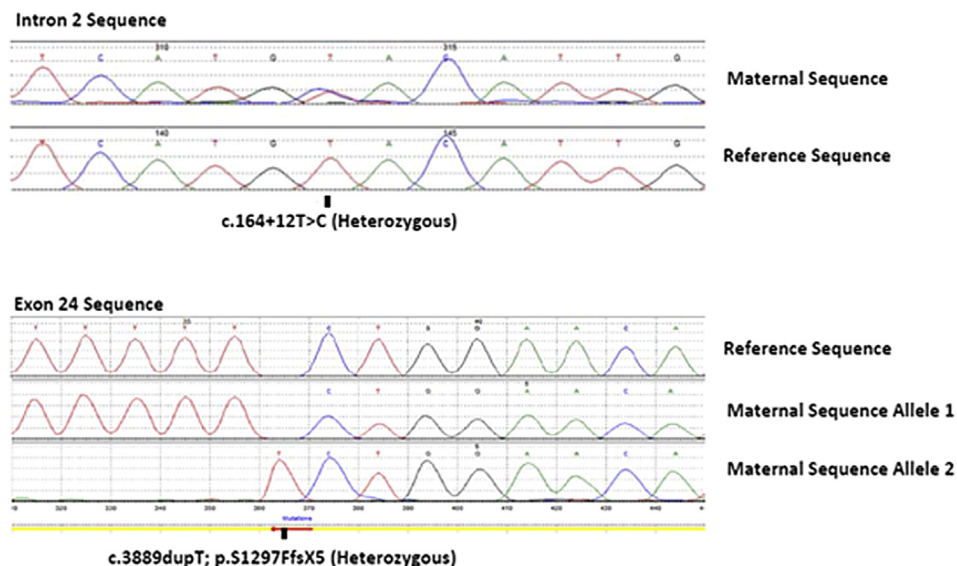


Figure 3. Mother with double mutation c.164+12T>C/ c.3889_3890insT in the same allele in CIS position.

Father

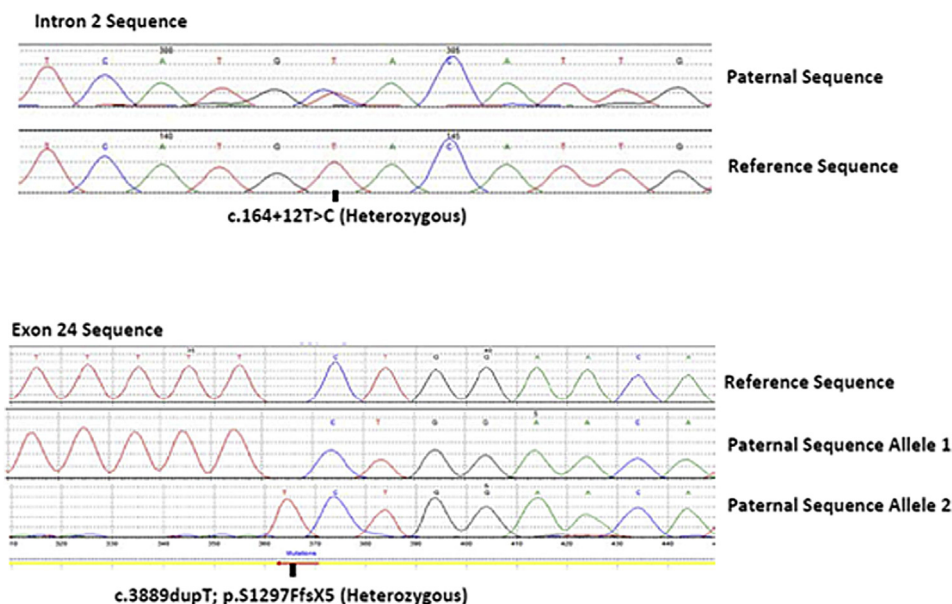


Figure 4. Father with double mutation c.164+12T>C/ c.3889_3890insT in the same allele in CIS position.

Miolo [14] reported double heterozygosity for the F508del and 852del22 mutations in *CFTR* in a 42-year-old with infertility. The patient did not have any clinical manifestations of CF and was incidentally tested for unexplained infertility. The first hypothesis was that both the F508del and 852del22 mutations were located in the cis combination. However, the molecular analyses performed on the patient's father and sister supported the hypothesis that he was a compound heterozygote for the 852del22 and F508del mutations, contrary to his normal phenotype. Intriguingly, direct sequencing of the exon 6A showed the absence of the 852del22 mutation and characterized the heteroduplex formation as an A to T transversion at nucleotide position 875p11.

Abramowicz et al [15] reported a sibship of two brothers and an ongoing pregnancy, in all of whom two mutated *CFTR* alleles were identified after fetal bowel hyperechogenicity (FBH) ascertainment. The older brother was asymptomatic, while the younger had some respiratory history and a slightly abnormal sweat test. The N1303K mutation was found in the fetus and the father, and the maternal *CFTR* allele was found to carry three missense mutations, D443Y (1459G>T, exon 9), G576A (1859G>C, exon 12), and R668C (2134C>T, exon 13), which have each previously been reported in males with CBAVD. During family counseling, a history of two episodes of lower respiratory tract infection was reported in the youngest child, whose weight, height, and physical examination were within normal limits. The routine DNA analysis showed the presence of the paternal N1303K mutation in both the younger and the older brother. On further analysis, the complex mutated maternal allele was found in the younger boy. Because of this finding, he underwent a sweat test, which showed a slightly increased chloride concentration (35 mEq/l, with a normal upper limit of 28 mEq/l in this age group). More surprisingly, compound heterozygosity was also found in the strictly asymptomatic older brother. The family was counseled for atypical CF with a mild clinical course, at least in childhood, as observed in the two brothers, with the possibility of a probably mild CF-related lung

disease later in life in any of the children. A normal 3150-g baby girl was delivered at term. The parents declined sweat testing for their oldest son.

Polizzi [16] reported five patients with a novel complex *CFTR* allele with two mutations, H939R and H949 L, inherited in cis in the same exon of *CFTR* gene and one different mutation per patient inherited in trans in a wide population of 289 Caucasian CF subjects from south Italy. The genotype–phenotype relationship in patients bearing this complex allele was investigated. The two associated mutations were related to classical severe CF phenotypes.

The four patients carrying the complex allele [H939R; H949 L] associated in trans with the severe mutations G542X, 1259insA, G1349D and F508del presented the classic CF phenotype. In contrast, the patient who carried the same complex allele with the R248T mutation showed a *CFTR*-RD. This is likely because R248T is a mild mutation (thought to affect *CFTR* mRNA splicing based on the Cystic Fibrosis Mutation Database), and subjects carrying this mutation might have residual function of the *CFTR* protein. It seems that the complex allele [H939R; H949 L] greatly reduces the residual function of *CFTR* and, when the other allele also has a severe mutation that produces a very low residual function, the combined effect is an overall great reduction of *CFTR* functionality; in contrast, when the other allele carries a mild mutation, the overall effect is a cumulative greater *CFTR* functionality.

Clain et al [17] reported that two mild cystic fibrosis-associated mutations resulted in severe cystic fibrosis when combined in cis and reveal a residue that is important for *CFTR* processing and function. Clinical data suggested that R347H and D979A, two mild CF-associated mutations, can produce severe CF similar to that of DF508 homozygotes when combined in cis. The authors determined the contribution of each mutant to the double mutant phenotype. D979A reduces the amount of *CFTR* protein at the cell membrane, whereas R347H generates a defective Cl channel. The mutant R347H-D979A combines both defects for a dramatic decrease in the Cl current. The magnitude of the Cl current in vitro

paralleled the severity of the disease with D979A (congenital bilateral absence of the vas deferens), R347H (mild CF), and R347H-D979A (severe CF). This study highlights the importance of structure–function analysis of naturally occurring mutants for deciphering complex genotypes and identifying residues that are important for CFTR processing and/or chloride channel activity. These results also have important implications for CF as they show that two mutations in cis can act in concert to dramatically alter CFTR function. This may contribute to the wide phenotypic variability of CF disease and points to the need to screen for all mutations.

Savov et al [18] reported the presence of two different mutations carried by the same CF allele in four out of 44 Bulgarian CF patients during a systematic search of the entire coding sequence of the *CFTR* gene. Two of the double mutant alleles include one nonsense and one missense mutation. Two nucleotide substitutions in exon I of the *CFTR* gene were detected in a severely affected CF patient who also carried the common CF mutation N1303K. A C→T transition at nucleotide position 136 generates a termination codon at amino acid position 2 (Q2X), and an A→G transition at position 139 results in the substitution of tryptophan for arginine at amino acid position 3 (R3W). Subsequent family studies demonstrated that both Q2X and R3W were inherited from the father.

One double mutant carried two missense mutations, whose contribution to the CF phenotype was difficult to evaluate. The authors suggested that double mutant alleles may be more common than expected and could account for some of the problems in phenotype–genotype correlations. Such alleles may have important implications for molecular diagnosis and genetic counseling.

The diagnostic implications of triple mutants are obvious. Failure to identify the presence of a second mutation in the same allele may result in errors in prenatal diagnosis or carrier detection if recombination between the two mutations occurs. Although the frequency of such cases will be low, their importance cannot be underestimated. Additional mutations in the same gene, and the interactions between them, may also turn out to play a relatively important role in modifying the phenotypic expression in Mendelian disorders.

In summary, double mutations are probably more common than reports indicate. The clinical picture usually reflects the severity of both detected mutations. Proper and extended genetic counseling is needed for the same family and their relatives to prevent similar mutations.

4. Ethical considerations

The confidentiality is covered in item number 3.7 of the RCF proposal. In detail, this point is under the RCF policy which is implemented on every registry that comes under RCF's responsibility (see the attached RCF policy w.r.t data security and confidentiality).

For the web-access of patient's data, the software will have unique identifiers for every user to access the application along with encryption techniques to safeguard the passwords.

Electronic data will be stored on the centralized web server which already has several layers of security in place through ITA

and KACST.

A search will be done to determine whether a case is a new registration or the case is already in the registry. Based on the result, the case will be registered or information will be updated. The treatment details of the register will be completed when the patient has completed his/her treatment. The follow-up part of the register will be completed at specified times in the patient's treatment. Outcomes will be assessed at specified times in the patient's treatment.

Conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

References

- [1] Pugh RJ, Pickup JD. Cystic fibrosis in Leeds region: incidence and life expectancy. *Arch Dis Child* 1967;42:544.
- [2] Hall BD, Simpkins MJ. Inheritance of fibrocystic disease in Wessex. *J Med Genet* 1968;5:262.
- [3] Schwarz MJ, Malone GM, Haworth A, Cheadle JP, Meredith AL, Gardner A, et al. Cystic fibrosis mutation analysis: report from 22 UK regional genetics laboratories. *Hum Mutat* 1995;6:326–33.
- [4] Kambouris M, Banjar H, Mogarri I, Nazer H, Al-Hamed M, Meyer BF. Identification of novel cystic fibrosis mutations in Arabs with CF: their impact on the CFTR mutation detection rate in Arab population. *Eur J Ped* 2000;159(5):303–9.
- [5] Banjar H. Overview of cystic fibrosis: patients aged 1–12 years in a tertiary care center in Saudi Arabia. *Middle East Pediatr Dec*. 1999;4(2):44–9.
- [6] Banjar H. Morbidity and mortality data of cystic fibrosis patients. *Saudi Med J* 2003;24(7):730–5.
- [7] Banjar H. Cystic fibrosis: presentation with other diseases, the experience in Saudi Arabia. *J Cyst Fibros* 2003;2:155–9.
- [8] Dawson KP, Frossand PM. Cystic fibrosis in the United Arab Emirates: an unrecognized condition. *Trop Dr* 1995;25(3):110–1.
- [9] Abdul Wahab A, Dawod ST, Al Thani G. Cystic fibrosis in a large kindred family in Qatar. *Ann Trop Ped* 2000;20:203–7.
- [10] Malone G, Haworth A, Schwarz MJ, Cuppens H, Super M. Detection of five novel mutations of the cystic fibrosis transmembrane regulator (CFTR) gene in Pakistani patients with cystic fibrosis: Y569D, Q98X, 296+12(T>C), 1161delC and 621+2(T>C). *Hum Mutat* 1998;11(2):152–7.
- [11] Cheadle JP, Al-Jader LN, Meredith AL. Two novel frame-shift mutations: 977 insA in exon 6B, and 4016 insT in exon 21, of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Hum Mol Genet* 1993;2(3):317–9.
- [12] Kunkel TA, Alexander S. The base substitution fidelity of eucaryotic DNA polymerases. *Biol Chem* 1986;261(1):160–6.
- [13] Cooper DN, Krawczak M. Mechanisms of insertional mutagenesis in human genes causing genetic disease. *Hum Genet* 1991;87:409–15.
- [14] Miolo G, Crovatto M, Manno M, Pivetta B, Tessitori G, Picci L. Heterozygous variant at nucleotide position 875+11A>T in exon 6A cystic fibrosis transmembrane conductance regulator gene induces 852del22 mutation false-positivity by line probe assay. *Fertil Steril* 2011;95(3):1121.
- [15] Abramowicz MJ, Dessars B, Sevens C, Goossens M, Girodon-Boulandet E. Fetal bowel hyperchogenicity may indicate mild atypical cystic fibrosis: a case associated with a complex CFTR allele. *J Med Genet* 2000;37(8):E15.
- [16] Polizzi A, Tesse R, Santostasi T, Diana A, Manca A, Logrillo VP, et al. Genotype-phenotype correlation in cystic fibrosis patients bearing [H939R; H949 L] allele. *Genet Mol Biol* 2011;34(3):416–20.
- [17] Clain J, Fritsch J, Lehmann-Che J, Bali M, Arous N, Goossens M, et al. Two mild cystic fibrosis-associated mutations result in severe cystic fibrosis when combined in cis and reveal a residue important for cystic fibrosis transmembrane conductance regulator processing and function. *J Biol Chem* 2001;276(12):9045–9.
- [18] Savov A, Angelicheva D, Balassopoulou A, Jordanova A, Noussia-Arvanitakis S, Kalaydjieva L. Double mutant alleles: are they rare? *Hum Mol Genet* 1995;4(7):1169–71.