Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

Three complex alleles of *CFTR* gene identified in Lebanese, Egyptian and French population and their potential impact on splicing

Raëd Farhat[1#], Ayman EL-Seedy[2#], Marie-Claude Pasquet[3], Sandra Corbani[4], André Megarbané[5], Alain Kitzis[1,3], Véronique Ladeveze[1*]

[1]*Neurovascular Unit And Cognitive Disorders (NEUVACOD), Université de Poitiers, France*
[2]*Laboratory of cellular and Molecular Genetics, Department of Genetics, Alexandria University, Aflaton Street, El-Shatby-21545, Alexandria, Egypt*
[3]*CHU de Poitiers (France)*
[4]*University St Joseph, Unité de Génétique Médicale Lebanon, Beyrouth, Lebanon, France*
[5]*Institut Jérôme Lejeune, Paris, France*
[#]*Contributed equally to this work*

ARTICLE INFO

Original paper

Article history:
Received: December 03, 2021
Accepted: March 20, 2022
Published: April 30, 2022

Keywords:
CF, N1303K, Complex alleles, hybrid minigene, splicing

ABSTRACT

Cystic Fibrosis (CF) in Arab Mediterranean countries has a different CFTR mutational profile if compared either to Caucasians or in the Arabian Peninsula. The c.3909C>G (N1303K, p.Asn1303Lys) mutation of the Cystic Fibrosis Transmembrane Conductance Regulator gene (CFTR). This mutation represents a higher frequency in the Mediterranean countries in association with different polymorphisms or mutations in cis position constituting various complex alleles. N1303K mutation induces many phenotypes, especially pancreatic insufficiency from mild to severe and it is associated in cis with other polymorphisms. The aim of this investigation is therefore to screen complex alleles carrying N1303K mutation among Lebanese, Egyptian and French patients. All exons of the CFTR and their flanking regions were performed by PCR amplification, followed by automated direct DNA sequencing. Two complex alleles are more frequent corresponding to Wild Type and mutated haplotype. Besides that two other very rare complex alleles have been detected, one in Egyptian and French samples, and then another one in Lebanon samples. We have studied their impact on the CFTR mRNA splicing using a minigene strategy. Constructs containing wild-type and mutant CFTR cloned into the pTBNdeI hybride minigene have been expressed in HeLa, HT29 and HEK293 cells. RT-PCR analysis of mRNA using β-globin-specific primers revealed that N1303K and the polymorphisms associated with cis induce weak abnormal splicing and a modification of the quality and the quantity of CFTR protein. These different associations of identified polymorphisms with N1303K in cis could have an impact on the severity of the disease.

DOI: http://dx.doi.org/10.14715/cmb/2022.68.4.7

Introduction

Cystic Fibrosis (CF), the most common autosomal recessive genetic disease in Caucasian, is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulatory (*CFTR*) gene. To date, more than 2100 CFTR mutations and polymorphisms have been identified (CF Mutation DataBase, http://www.genet.sickkids.on.ca/cftr/). The CFTR is composed of two nucleotide-binding domains (NBD1, NBD2), two membrane-spanning domains (MSD1, MSD2) and a regulatory domain (R-domain). Multiple disease-causing mutations reside in NBD2 and the domain is critical to CFTR function since channel gating involved NBD1:NBD2 dimerization and NBD2 contains the catalytically active ATPase site in *CFTR* gene (1-2).

CF is not common in the Lebanese and Egyptian population and figures as a genetic rare disease and need further epidemiological investigations. The history of Lebanon and Egypt, characterized by flows of different ethnic groups, has enabled the introduction of new genes and a wide variety of genetic diseases. Even though few epidemiological studies were conducted to determine the CF incidence in Lebanon and Egypt, multiple factors advocate that it could be relatively high (3-4). Both countries are not like other Arab countries and the mutation spectrum in

*Corresponding author. E-mail: veronique.ladeveze@univ-poitiers.fr

the *CFTR* gene has been influenced by gene flow coming from different populations. Moreover, the high rate of consanguinity in both societies increases CF incidence and private mutations (5-6).

The c.3909C>G (N1303K, p.Asn1303Lys) mutation of *CFTR* exon 24 induces a substitution of an Asparagine (N) by a Lysine (K) in the NBD2 at the protein level. The Phoenician origin of this mutation have been previously discussed (7). Indeed, the frequency of the N1303K varies significantly between countries and ethnic groups. It has a high incidence in Europe, but it is more frequent in the south than in the north of Europe (4). N1303K has been reported to be the second most common mutation in Italy (4%). In some regions, the incidence of this mutation even is higher, for example, reaching 7.8% in the southwestern region of France (5). In the Lebanese population, N1303K is considered as the third most frequent mutation after the c.1521_1523delCTT (F508del) and c.3846G>A (W1282X, p.Trp1282X) [CF Mutation Data Base Statistics]. The described frequency varies between 9.4% (3) and 27% (8). In the Egyptian population, N1303K has a high frequency after p.Phe508del (9-10). However, in our previous study (11), no p.Phe508del was identified in Egyptian patients whereas novel mutations were detected. The N1303 residue is located in the NBD2 of the CFTR protein. In addition to that, it induces little or no CFTR at the cell surface and weak or no activity (12). It is therefore classified as class II folding defect mutation (13).

Symptoms related to N1303K mutation in homozygote patients are not clearly described but seem to be more severe at the pancreatic level than the pulmonary level (8). This mutation is classified as a severe mutation with respect to the pancreas (causing pancreatic insufficiency and diabetes mellitus) (14), it is also associated with precocious and severe lung symptoms such as newborn pneumothorax (15).

Interestingly, N1303K mutation is found in association with different polymorphisms or mutations *in cis* position in the form of complex alleles. There has been a steady increase in the number of reports of polymutant variants on the same allele, termed complex alleles (13, 17-24). In this molecular screening of Lebanese, Egyptian and French *CFTR* genes, three complex alleles are identified, two more frequently corresponding to Wild Type (WT) and mutated, the third very rare around 1% is detected in one Egyptian and one French patient. In this current study, we describe the potential impact of N1303K CFTR mutation associated with c.[744-33GATT(6);869+11C>T in the form of complex allele on splicing process using an *in vitro* hybride minigene assay.

Materials and methods

Case Description

Subjects included 5 French and 11 Lebanese CF patients that have been diagnosed as carrying the N1303K mutation among more than 1500 patients tested at the Genetics Laboratory of Centre Hospitalier Universitaire de Poitiers as a referral center for CFTR diagnosis. Furthermore, fifty Egyptian patients have been diagnosed with CF or CFTR-RD based on severe chronic lung disease and pancreatic insufficiency, but without this mutation. Sixty individuals with no history of CF have been used as a negative control. Control DNA was provided from a heterozygous spouse (French and Egyptian).

Genomic DNA extraction

DNA was isolated from collected blood samples from all diagnosed patients in EDTA (Ethylenediaminetetraacetic acid) tubes using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) as previously described. The concentration and purity of DNA samples were evaluated by the Nanodrop 2000 Spectrophotometer (ThermoFisher, USA). DNA samples were stored at 4°C until further analysis.

PCR amplification and direct sequencing

DNA polymerase chain reaction (PCR) reaction was performed using primers designed for the 27 exons of the CFTR gene and their flanking introns as previously (8). The PCR reaction was done using a 9700 GeneAmp Thermo Cycler (Perkin Elmer) as follows: initial denaturation (94°C, 2 min), followed by 30 cycles (94°C, 30 sec; 58°C, 30 sec; 72°C, 30 sec), and a final extension step (72°C, 5 min).

To perform the direct sequencing of the *CFTR* gene, PCR products were purified using the ABI PRISM Big Dye Terminator TM cycle sequencing Reading Reaction Kit (Applied Biosystem) using the following conditions: the 25 cycles: initial

denaturation (96°C, 10 sec), primers hybridization (44°C, 30 sec), and an extension step (60°C, 4 min). PCR products were purified by the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) before direct sequencing according to the manufacturer protocol. All reactions were run on an ABI PRISM™ 3100 automatic sequencer (Applied Biosystems, CA, USA).

Minigene splicing analysis

To study the impact of intronic or/ and exonic variations on aberrant splicing, the pTB*Nde*I CFTR plasmid minigene (generously provided by F. Pagani, Italy) was performed in the transfected mammalian cells. The genomic DNA region of interest (335bp intron6 - exon7 - 326bp intron7) of different patients (EGY46B, Leb-6994 and Lib-1324), was introduced into the minigene *via* a unique restriction site (*Nde*I) located in a fibronectin intron (Figure 1). The construction and validation of the hybrid minigene used in this study have been described elsewhere (14). 9Egy46B is heterozygous for the two polymorphisms but without the N1303K mutation. Leb-1324 is heterozygous for the two polymorphisms and the mutation, whereas Leb-6994 is homozygous for the two polymorphisms and the mutation (genotype c.[744-33GATT(6); 869+11T; 3909C]/ c.[744-33GATT(7); 869+11C; 3909C ; c.[744-33GATT(6); 869+11T; 3909G]/ c.[744-33GATT(7); 869+11C; 3909C] ; c.[744-33GATT(6); 869+11T; 3909G]/ c.[744-33GATT(6); 869+11T; 3909G] respectively.

Cell Culture, Minigene Expression, and RT-PCR Analyses

Three different cell lines namely; HeLa, HT29, and HEK293 were used. Cells were maintained in DMEM medium with Glutamax-I (Life Technologies) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 100µg/ml of streptomycin in a humidified incubator at 37°C in the presence of 5% CO2. Cells were transiently transfected by WT and mutant *CFTR* minigenes constructed with Lipofectamine 2000 (Invitrogen) kit. Three independent transfections were performed for each cell line for RNA extraction and expression analysis.

Total RNA was extract from cell lysates using the RNeasy Mini Kit (Qiagen, Germany) and was then dissolved in 50 µL of sterile water. cDNA synthesis and RT-PCR analyses were performed as previously described (11) using cDNA primers designed in Table 1. PCR products were detected by 1.5% agarose gel electrophoresis and 10% polyacrylamide gel as well.

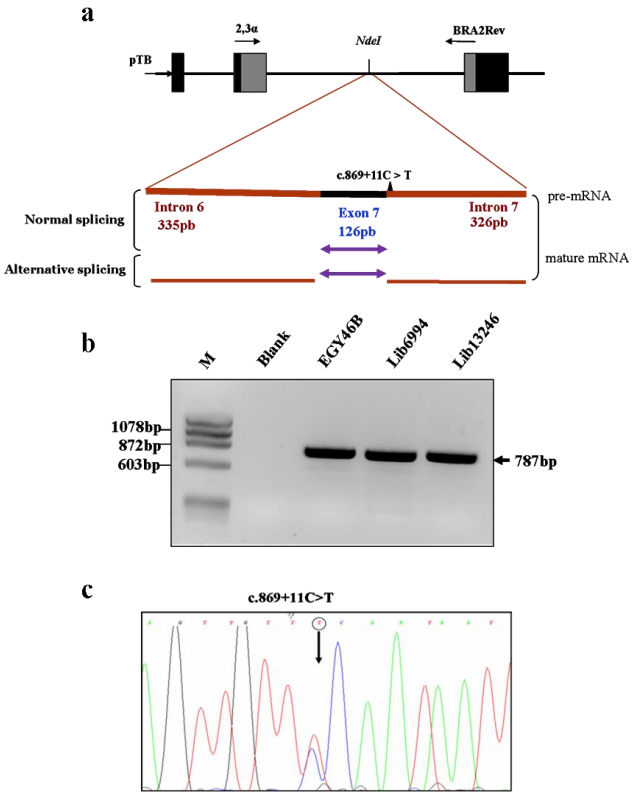

Figure 1. Construction of pTB minigene showing c.869+11C>T polymorphism associated with N1303K. (a) Schematic diagram of a linear CFTR covering intron6-exon7- intron7 which use to bind to pTB minigene demonstrating the position of c.869+11C>T polymorphism. (b) PCR amplification of the desired insert from the patients DNA. Lane 1. DNA marker 4x174 cut with *Haelll*. Lane 2. Negative control. Lanes 3-5. The amplification product of c.869+11C>T polymorphism. (c) Nucleotide alterations of c.869+11C>T detected by direct sequencing of exon7

Table 1. Oligonucleotides used in RT-PCR, and sequencing of the inserted fragment.

Name	Sequence
pTB2160Dir	5′-TATTCAGATATTTATGTCTAGG-3′
pTB2270Rev	5′-CCCATGTGAGATATCTAGG-3
2,3α*	5′-CAACTTCAAGCTCCTAAGCCACTGC-3′
BraRev*	5′-AGGGTCACCAGGAAGTTGGTTAAATCA-3′

* indicate the sequence of RT-PCR oligonucleotides

Results and discussion

The present study aims to describe CFTR complex alleles associated with N1303K in Lebanese, Egyptian and French population and their potential impact on splicing using appropriate methods. In Egyptian patients studied here, no N1303K is detected. In French samples, only five N1303K patients are observed, all are heterozygous in our cohort. In Lebanon one patient is homozygous, the others (10) are heterozygous for N1303K (Table 2). Moreover, the haplotype of GATT repetition noted in this table as GATT (n) corresponds to c.[744-33GATT(6)] or c.[744-33GATT(7)]. The polymorphism +11C>T corresponds as c.869+11C>T. +11T>T indicates a homozygous pattern for this polymorphism. TG (n) and T(n) correspond to c.[12010-35TG (m);-12T(n)] polymorphism located at the site of the donor of intron 9 (at the end of 3') (Table 2).

Table 2. Genotypes of Lebanon CF patients carrying the N1303K mutation and related polymorphisms.

Patients	GATT (n)	TG (n)	T(n)	N1303K	Other variations
Lib-6994 *	GATT 6/6 +11T>T	10/10TG	9/9T	K1303K[¥]	
Lib-7274 [ψ]	GATT 7/6 +11C>T	10/10TG	9/7T	N1303K	c.4389G>A
Lib-7423	GATT 7/6 +11T>T	10/10TG	9/9T	N1303K	
Lib-8149	GATT 7/6 +11C>T	10/11TG	9/7T	N1303K	M470V
Lib-8361	GATT 7/6 +11C>T	10/11TG	9/7T	N1303K	M470V
Lib-10193	GATT 7/6 +11T>T	10/10TG	9/9T	N1303K	F5078del
Lib-10362	GATT 7/6 +11T>T	10/11TG	9/7T	N1303K	S549R
Lib-11769	GATT 7/6 +11T>T	10/10TG	9/9T	N1303K	F508del
Lib11791	GATT 7/6 +11C>T	10/11TG	9/7T	N1303K	M470V
Lib-13246*	GATT 7/6 +11C>T	10/11TG	9/7T	N1303K	M470V
Lib-15485	GATT 7/6 +11T>T	10/10TG	9/9T	N1303K	F508del

*These two patients were used to establish inserts for minigene splicing analysis. [¥] Lib-6994 is homozygous for the mutation N1303K and the two polymorphisms studied. [ψ] c.4389G>A (NN) polymorphism was detected in exon27 of Lib-7274 patient.

Table 3 summarizes the four haplotypes detected and their frequency in our study in Egyptian, Lebanese and French patients. WT Haplotype c.[744-33GATT(7); 869+11C;3909C] and mutated Haplotype c.[744-33GATT(6);869+11T;3909G] are more frequently detected. The haplotype K1303 (c.3909G) is always detected with 6 GATT and the haplotype N1303 (c.3909C) with 7GATT except in one Egyptian patient and one French patient with 6 GATT. Only four complex alleles have been found but two of them are very rare, one only in an Egyptian patient (EGY46B) and in one French patient, the other only in two Lebanon patients. The very rare haplotype c.[744-33GATT(6); 869+11T; 3909C] combines the two polymorphisms without the mutation. The estimated frequency in Table 3 does not allow for statistics given the small sampling of the Lebanese but allows for highlighting very rare alleles. However, it is clear that the most frequent allele with N1303 is 7GATT and c.869+11C whereas the most frequent with K1303 is 6GATT and c.869+11T.

Table 3. Haplotypes were identified in Lebanese (L), Egyptian (E) and French (F) subjects in this study

Genotype	IVS6GATT	Intron 7	number of alleles frequency			Total - frequency
			L	E	F	
N1303	GATT (7)	c.869+11C	6 0.273	199 0.995	154 0.9625	359 - 0.94
N1303	GATT (7)	c.869+11T	4 0.182	0 0	0 0	4 – 0.01
N1303	GATT (6)	c.869+11T	0 0	1 0.005	1 0.0065	2 – 0.005
K1303	GATT (6)	c.869+11T	12 0.545	0 0	5 0.031	17 – 0.045

Furthermore, the sequencing of all CFTR 27 exons and their surrounding introns confirmed the presence of N1303K (homozygous or heterozygous state) and revealed the presence of different mutations or polymorphisms. For each allele, the number of TG and T (intron 9) has been determined respectively 10 or 11 and 9 or 7, inducing a very weak alternative splicing (23). Indeed, the c.[12010-35TG(m);-12T(n)]polymorphism located at the site of the donor of intron 9 (at the end of 3') has an important effect on exon 9 skipping if this site contains a high number of TG and a low number of T tract.

To have more information, the construction of minigene containing the region of interest was realized from patient DNA (EGY46B, Leb6994 and Leb13246) to study splicing. EGY46B is carried the rare haplotype c.[744-33GATT(6); 869+11T; 3909C], Leb6994 is homozygous c.[744-33GATT(6);869+11C;3909T] and Leb13246 is heterozygous (Table 3). Figure 2 shows that normal or alternative splicing is observed in presence of these genotypes. Figure 2 shows the splicing pattern of examined complex alleles from different patients. Figure 2A shows a part of the CFTR gene with the region of interest (both polymorphisms studied are associated with N1303K mutation). Figures 2B 2C reveal the alternative splicing detected by analyzing agarose electrophoresis gel and acrylamide gel

respectively. Figures 2D 2E show normal splicing sequence and skipping of exon 7 respectively.

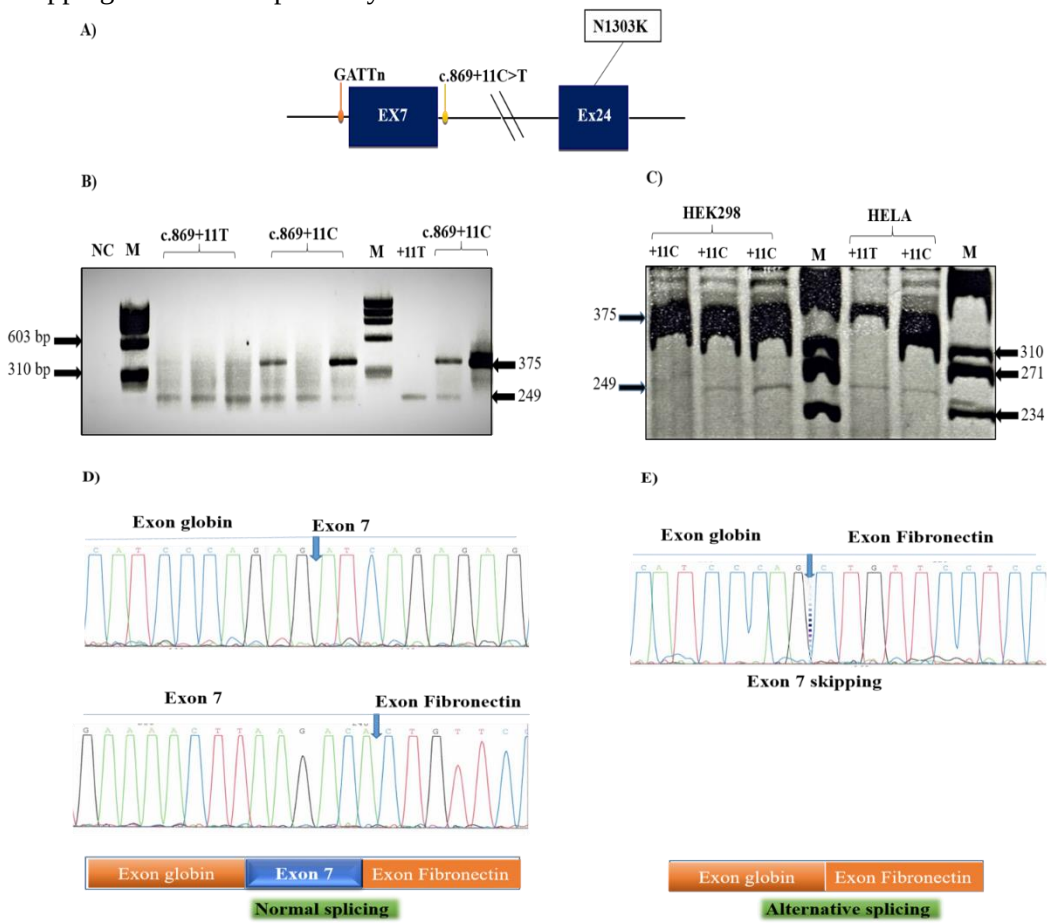

Figure 2. Splicing patterns of examined complex alleles from different patients using minigene splicing assay protocol. **(A)** the region of interest with N1303K mutation and both polymorphisms. **(B)** RT-PCR analysis by agarose electrophoresis gel. Lane 1. Negative control (NC) in which no reverse transcriptase was added. Lane 2. M, DNA marker 4x174 cut with *Hae*lll .Lanes 3-5. Amplification product from c.869+11T>T CFTR cDNA, Lanes 6-8. Amplification product from c.869+11C>C CFTR cDNA from HeLa (lines3-8), HT29 (lanes 10-11) and Hek293 (lane 12) cells . (C) Polyacrylamide gel electrophoresis shows two fragments obtained by RT-PCR: 375 bp of exon 7 with normal splicing and 249 bp with exon 7 skipping. (D and E) DNA sequence of RT-PCR product showing sequences of normal and alternative splicing (skipping of exon7) respectively.

The frequency of N1303K mutation varies significantly between countries and ethnic groups; this mutation is relatively more frequent in the countries around the Mediterranean and almost null in other populations and produces different phenotypes. In the Egyptian population, N1303K has a high frequency of around 6% (11). In the Lebanese population, the described frequency varies between 9.4% and 27% in different published investigations (3, 22).

WT Haplotype c.[744-33GATT(7); 869+11C;3909C] and Mutated Haplotype c.[744-33GATT(6);869+11C>T;3909C>T] induce both minor exon 7 skipping (results presented here and in (10-11). The consequences of mutated haplotype induce 2 classes of mutation Class V and Class II (22). Both haplotypes are identified in Lebanese and French patients. Furthermore, a rare haplotype was detected in one Egyptian patient and in one French patient: c.[744-33GATT(6); 869+11T; 3909C]. The IVS6GATT repeat polymorphism (also name rs34543279) is a mostly dimorphic tetranucleotide tandem repeat located in intron 6 (25-26). It has only two common alleles with six and seven repeats, as described, and both are present in all populations studied. The GATT (7) is the most frequent and considered WT (26). No other alleles were detected in Lebanese, Egyptian and French populations tested whereas other rare alleles were described as GATT(4), (5), and (8) were found in one Adygea and two Basque chromosomes (29). It is important to note

that GATT (6) is only present in 1.4% of normal chromosomes in actual European (30). This percentage is around (a little higher than) the one obtained in this study (0.9%), even though no normal Lebanese chromosomes were detected with this polymorphism. The c.869+11C>T (also named rs1800503) was identified in 1991 by Cuppens et al.(31) in the CFTR data base, it occurs in the 3′ flanking regions of exon 7 and is considered as Neutral (32).

Besides, these studies corroborate that the exon7 (coding 42 amino acids) could be skipped and the two polymorphisms could induce this weak alternative splicing (minus 42 amino acids). We have shown previously (13) that N1303K has no impact on splicing. If N1303K has grave consequences on the pancreas, the impact on the pulmonary state is unpredictable. To explain this unpredictable severity on the pulmonary level in both homozygous and heterozygous states, the weak alternative splicing induced by polymorphisms could be implicated but is not sufficient. However, the association of other polymorphisms in cis could have an impact on the severity of the disease. This research will serve as a platform for epidemiological and clinical studies to improve patient care in the Arab world and worldwide (33,34)

Conclusions

In this study comparing Lebanon, Egyptian and French patients versus negative control provided by the heterozygous spouse (French and Egyptian), and four complex alleles were detected. Focusing on three sites of polymorphisms or mutations, two major haplotypes present always the same association in cis, except in one Egyptian patient and one French patient with the haplotype N1303, and in two Lebanon patients with the haplotype K1303 respectively. Both very rare polymorphisms were observed. The impact of the mutated haplotype induces a weak abnormal or alternative splicing and so a modification of the quality and the quantity of CFTR protein. More *in vitro* and *in vivo* (nasal epithelial patient-derived cells) experiments should be performed. However, we still do not have epithelial cells from patients carrying this genotype to confirm our *in vitro* results.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

References

1. Vergani P, Lockless SW, Nairn AC, Gadsby DC. CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. Nature. 2005 24; 433(7028):876-80. doi: 10.1038/nature03313. PMID: 15729345; PMCID: PMC2756053.
2. Riordan JR. CFTR function and prospects for therapy. Annu Rev Biochem. 2008; 77:701-26. doi: 10.1146/annurev.biochem.75.103004.142532. PMID: 18304008.
3. Desgeorges M, Megarbane A, Guittard C. et al., Cystic fibrosis in Lebanon: distribution of CFTR mutations among Arab communities. Human Genetics 1997; 100(2): 279–283
4. El Falaki Mona M., El Attar Mona M., El Basha Nossa, El Refai Nihal, Shahin Walaa, Mohsen Hanan, Ali Sara, Mostafa Amr M., Hamed Dina H. Cystic Fibrosis in Egyptian Children: Achievements and Future Directions. Pediatric Sciences Journal 2021, 1(1); 1-6. DOI:0.21608/cupsj.2020.52045.100
5. Farra, C. Menassa R. Awwad J et al., Mutational spectrum of cystic fibrosis in the Lebanese population. Journal of Cystic Fibrosis 2010; 9 (6): 406–410.
6. El-Falaki M. M, Shahin W. A., El-Basha N. R, Ali A. A., Mehaney D. A., El-Attar M. M., Profile of cystic fibrosis in a sin M. M. El-Falaki, W. A. Shahin, N. R. El-Basha, A. A. Ali, D. A. Mehaney, M. M. El-Attar, Profile of cystic fibrosis in a single referral center in Egypt. J. Adv. Res. 5, 563–568 (2014).
7. Farhat R, Pasquet M-C, Corbani S, Megarbané A , Kitzis A and Ladevèze V. Is Phoenicia the Origin of the N1303K CFTR Mutation? Int. J. Rare Dis. Disord. 2019; 2(1): 6-10.
8. Osborne L, Santis G, Schwarz M et al. Incidence and expression of the N1303Kmutation of the cystic fibrosis (*CFTR*) gene. Human Genetics 1992; 89 (6): 653–658.
9. Federici S, Iron A, Reboul MP, Desgeorges M, Claustres M, Bremont F, et al. CFTR gene analyis in 207 patients with cystic fibrosis in southwest France: high frequency of N1303K and 1811+1.6bA>G mutations. Arch. Pediatr. 2001; 8: 150–157. PMID: 11232455

10. Stewart C, Pepper MS, Cystic Fibrosis on the African Continent. Genetics in Medicine. 2016; 18(7): 653-662.
11. Shahin WA, Mehaney DA, El Falaki MM, Mutation spectrum of Egyptian children with cystic fibrosis. Springerplus, 2016; 5(1): 686. doi: 10.1186/s40064-016-2338-7
12. El Seedy A, Pasquet MC, Shafiek H, Morsi T, Kitzis A Ladevèze V, Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene mutations in North Egyptian population: implications for the genetic diagnosis in Egypt. Cell Mol. Bio. 2016; 62(13): 21-28. doi: 10.14715/cmb/2016.62.13.5.
13. Farhat R., Puissesseau, G. El-Seedy A Pasquet , M-C, Adolphe C, Corbani S, A Mégarbané A, A Kitzis, V Ladeveze (2015). N1303K (c.3909C>G) mutation and splicing: Implication of its c.[744-33GATT(6); 869+11C>T] complex allele in CFTR exon 7 aberrant splicing. BioMed Research International. Volume 2015, Article ID 138103, 2. doi: 10.1155/2015/138103
14. Dekkers JF, Gogorza Gondra RA, Kruisselbrink E, Vonk AM, Janssens HM, de Winter-de Groot KM, et al., Optimal correction of distinct CFTR folding mutants in rectal cystic fibrosis organoids. The European respiratory journal. 2016; 48(2): 451–458.
15. Cotellessa M, Mazzella M, Bruno C, Buzzanca C, Minicucci L, Gandino M, et al. N1303K mutation and diabetes mellitus in cystic fibrosis. Arch Dis Child 1996; 75 (6): 546. doi: 10.1136/adc.75.6.546.
16. Ataseven F, Ozer S, Yilmaz R, Senayli A. Bilateral spontaneous pneumothorax in a newborn with N1303K mutation of cystic fibrosis (CFTR) gene. Tuberk Toraks 2010; 58: 181–183.
17. Savov A, Angelicheva D, Balassopoulou A, Jordanova A, Noussia-Arvanitakis S, Kalaydjieva L. Double mutant alleles: are they rare? Hum Mol Genet 1995; 4:1169-1171.
18. Fanen P, Clain J, Labarthe R, Hulin P, Girodon E, Pagesy P, Goossens M, Edelman A. Structure-function analysis of a double-mutant cystic fibrosis transmembrane conductance regulator protein occurring in disorders related to cystic fibrosis. FEBS Lett. 1999; 452: 371-374.
19. Romey MC, Pallares-Ruiz N, Mange A, Mettling C, Peytavi R, Demaille J, Clusters M. A. naturally occurring sequence variation that creates a YY1 element is associated with increased cystic fibrosis transmembrane conductance regulator gene expression. J Biol Chem. 2000; 275: 3561-3567.
20. Clain J, Fritsch J, Lehmann-Che J, Bali M, Arous N, Goossens M, Edelman A, Fanen P. 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: 9045-9049.
21. Niel F, Legendre M, Bienvenu T, Bieth E, Lalau G, Sermet I, Bondeux D, Boukari R, Derelle J, Levy P, Ruszniewski P, Martin J, Costa C, Goossens M, Girodon E. a new large CFTR rearrangement illustrates the importance of seraching for complex alleles. Hum Mutat. 2006; 27(7): 716-717. doi: 10.1002/humu.9431.
22. Claustres M, Altiéri JP, Guittard C, Templin C, Chevalier-Porst F, Des Georges M. Are p.I148T, p.R74W and p.D1270N cystic fibrosis causing mutations? BMC Med. Genet. 2004; 2: 5-19.
23. Krasnov KV, Tzetis M, Cheng J, Guggino WB, Cutting GR. Functional studies of rare missense mutations in CFTR facilitate interpretation of genotype-phenotype relationships. Hum Mutat. 2008; 29(11): 1364-1372.
24. El-Seedy A, Girodon E, Norez C, Pajaud J, Pasquet MC, de Becdelièvre A, Bienvenu T, des Georges M, Cabet F, Lalau G, Bieth E, Blayau M, Becq F, Kitzis A, Fanen P, Ladeveze V., CFTR mutation combinations producing frequent complex alleles with different clinical and functional outcomes. Hum. Mutat. 2012; 33(11):1557-1565. doi: 10.1002/humu.22129
25. Chillón M, Casals T, Giménez J, Ramos MD, Palacio A, et al. Analysis of the CFTR gene confirms the high genetic heterogeneity of the Spanish population: 43 mutations account for only 78% of CF chromosomes. Hum. Genet. 1994; 93: 447-451.
26. Farhat R, El-Seedy A, Norez C, Talbot H, Pasquet MC, Adolphe C, Kitzis A, Ladevèze V. complexity of phenotypes induced by p.Asn1303Lys-CFTR correlates with difficulty to rescue and activate this protein. Cell Mol Biol. 2017; 63(11):106-110. doi: 10.14715/cmb/
27. Chehab EF, Johnson J, Louie E, Goossens M, Kawasaki E,Erlich H. A dimorphic 4-bp repeat in the cystic fibrosis gene is in absolute linkage disequilibrium with the DF508 mutation: implications for prenatal diagnosis and mutation origin. Am J Hum Genet 1991; 48:223-226.
28. Gasparini P, Dognini M, Bonizzato A, Pignatti PF, Morral N, Estivill X. A Tetranucleotide repeat polymorphism in the cystic fibrosis gene. Hum. Genet. 1991; 86: 625.

29. Mateu, E, Calafell F, Lao O, Bonne´-Tamir B, Kidd JR, Pakstis A, Kidd KK, Bertranpetit J. Worldwide Genetic Analysis of the CFTR Region Am. J. Hum. Genet. 2001; 68:103–117.
30. Morral N, Dörk T, Llevadot R, Dziadek V, Mercier B, et al. Haplotype analysis of 94 cystic fibrosis mutations with seven polymorphic CFTR DNA markers. Hum. Mutat. 1996; 8: 149-159.
31. Cuppens H, Teng H, Raeymaekers P, De Boeck C, Casslman JJ. CFTR haplotype backgrounds on normal and mutant CFTR genes. Human Molecular Genetics. 1994; 3 (4) 607-614.
32. Trujillano D, Weiss ME, Köster J, Papachristos EB, Werber M, Kandaswamy KK, Marais A, Eichler S, Creed J, Baysal E, Jaber IY, Mehaney DA, Farra C, Rolfs A. Validation of semiconductore next-generation sequencing assay for the clinical genetic screening of CFTR. Mol Genet Genomic Med. 2015; 3(5): 396-403. doi: 10.1002/mgg3.149.
33. Muhammad I, Sale P, Salisu M, Muhammad T, Abubakar B, Maidala A, Nuwanyada E. Molecular analysis of Bio-makers of Chloroquine resistance in Plasmodium falciparum Isolate from Gombe Local Government Area, Gombe State, Nigeria. Cell Mol Biomed Rep 2022; 2(1): 42-55. doi: 10.55705/cmbr.2022.335753.1033
34. AL-Sadeq D, Abunada T, Dalloul R, Fahad S, Taleb S, Aljassim K, Al Hamed F, Zayed H Spectrum of mutations of cystic fibrosis in the 22 Arab countries: A systematic review. Respirology 2019; 24: 127–137.