MOLECULAR CHARACTERIZATION OF FOUR FREQUENT POLYMORPHISMS IN THE *CFTR* GENE: ASSESSING ITS ROLE IN CYSTIC FIBROSIS DISEASE

A. S. EL-SEEDY

Laboratory of cellular and molecular genetics, Department of genetics, Faculty of agriculture, Aflaton St., El-Shatby, P.O.Box 21545, Alexandria University, Alexandria, Egypt

Cystic fibrosis (CF) is a common autosomal recessive genetic disorder caused by a variety of sequence alterations in the *CFTR* gene. Since the cloning of the cystic fibrosis transmembrane regulator (CFTR) gene in 1989, more than 2000 mutations were reported to the Cystic Fibrosis Consortium database (http://www.genet.sickkids.on.ca). Of more than 200 CFTR gene polymorphisms described so far, some are quite common and can be found in general population with frequencies up to 40% (Cuppens *et al*., 1993; Bombieri *et al*., 2000). The molecular pathogenesis of polymorphisms in the *CFTR* gene is not understood, although recent studies have shown that some polymorphism exhibit many features of CFTR-related disorders (CFTR-RD), suggesting the important role of these polymorphisms in *CFTR* gene expression (Cuppens *et al*., 1998).

The interactions between mutations and polymorphisms within CFTR, polymorphisms in other genes, and the environment leads not only to significant symptom variability in patients with classical CF, but possibly also to ‘‘nonclassical’’ CF (Boyle, 2003) and a growing number of monosymptomatic diseases such as obstructive azospermia, idiopathic pancreatitis, disseminated bronchiectasis, and chronic rhinosinusitis. The relative contributions of individual genetic factors and the environment to disease phenotype are currently unclear, but it is most likely that multiple CF modifier genes are involved (Davies *et al*., 2005).

Single nucleotide polymorphisms (SNPs) are being intensively studied to understand the biological basis of complex traits and diseases. The genetics of human phenotype variation could be understood by knowing the functions of SNPs (George Priya Doss *et al*., 2008). Genetic analyses indicated that functional polymorphisms in the *CFTR* gene can alter the expression of *CFTR* (Cuppens *et al*., 1998) but cannot cause CF without additional CFTR mutations (Davies *et al*., 2005).

The SNPs c.1540A>G, c.2694T>G, and c.4521G>A may have affected pre-mRNA splicing by changing regulatory sequence motifs of exonic splice enhancers, leading to lower amounts of normal transcripts (Steiner *et*

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al., 2004) and could contribute to an increased risk in the development of chronic pancreatitis (De Cid *et al*., 2010). Previous studies have suggested that the most frequent polymorphism in *CFTR* gene, the M470V in exon 10, which represents for A or G variation at position 470, plays a role in modulating CFTR protein at both transcriptional and translational levels. It was reported that the M470-CFTR was associated with a 1.7-fold of the V470 CFTR function (Cuppens *et al*., 1998). Several *CFTR* variants indicated to be more frequent in certain clinical conditions than in healthy individuals. Three of them, *5T*, M470V, and R75Q, have been shown to be relatively frequent in Serbian patients with monosymptomatic CF disorders (Nikolic *et al*., 2006).

Interestingly, the frequency of common CFTR polymorphism differ in different countries and populations, such as, the prevalence of V470 allele is strikingly low in Sub-Saharan Africans and in African-Americans, with a frequency approximately 1/5 of that in other populations (http://www.ncbi.nlm.nih.gov.gate1.inist.fr/SNP/snp_ref.cgirs= 213950). Moreover, it has been reported that poly-T, TG-repeats and M470V polymorphisms play roles in the development of CF-like diseases (Noone *et al*., 2000; Huang *et al*., 2008). The polythymidine variants in intron 8 [IVS8-poly (T)] are associated with the efficient usage of the intron 8 splicing acceptor site, and affect the transcription of exon 9 mRNA by exon skipping. The IVS8-5T results in approximately 90% of exon 9 skipping, leading to a nonfunctional CFTR, thereby is considered as a disease mutation with incomplete penetrance (Chu *et al*., 1993).

In this study, the consequence of four frequent CFTR polymorphisms separately (c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A) or in allelic complexes in cis was analyzed *in vitro* to assess the pathogenesis of these polymorphisms on clinical phenotypes.

MATERIALS AND METHODS

Construction of a green fluorescent protein (GFP)-tagged mutated CFTR.

The GFP-tagged CFTR plasmids (wild-type (WT), and F508del-CFTR) were generously provided by B. Stanton (Dartmouth Medical School, Hanover, NH). Specific variant substitutions alone (c.1540A>G), (c.2694T>G), (c.4404C>T) and (c.4521G>A) or in different associations were introduced into the WT-CFTR plasmid using the Gene tailor site-directed mutagenesis kit (Invitrogen, Carlsbad, CA) using the designed primers and according to the manufacturer's protocol (Fig. 1 and Table 1). The sequence of each plasmid containing the desired variants in individual clones was controlled through plasmid sequencing on both strands using the ABI PRISM Big Dye Terminator™ Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Reactions were run on an ABI PRISM™ 310 automatic sequencer (Applied Biosystems).

Cell culture and transient transfection

Hela cells were grown in a DMEM medium with Glutamax-I (Life Technolo-

gies) amended with 10% fetal bovine serum (FBS, Gibco) and 100 unit/ml penicillin, 100 µg/ml streptomycin in humidified incubator at 37°C in the presence of 5% CO_2. Wild-type and mutant *CFTR* were transiently expressed in HeLa cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Examination of distribution of GFP-CFTR by confocal microscopy

Forty-eight hours after transfection, Hela cells were washed twice with ice-cold PBS and fixed in paraformaldehyde (3% in TBS) for 10 min at 4°C. Nuclei were stained with TOPRO-3 iodide (1:1000 in TBS, Molecular Probes). Cover glasses containing cells were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a reversed microscope (Olympus IX 70) equipped with a laser scanning confocal unit (model MRC-1024, BioRad), a 15 m W krypton-argon laser, and a X60 water immersion objective. GFP fluorescence was excited using the 488 nm laser line and collected using a standard fluorescein isothiocyanate filter set (522±32 nm). Fluorescence associated with TOPRO-3 iodide was excited using the 647 nm laser line and collected using a filter set (680±32 nm). Double fluorescence images were generated simultaneously. For Golgi complex staining, fixed cells were blocked by three washes in PBS/3% BSA. Cover slips were incubated for 1 h with Golgi p58 K anti-mouse antibody (Sigma, Poole, UK) at a 1:100 dilution. To detect the endoplasmic reticulum (ER), after paraformaldehyde (3% in PBS), cells were washed twice with PBS and were permeabilized with 0.5% Triton X-100 in PBS, incubated with Anti-calreticulin (Cambridge Bioscience, Cambridge, UK) for 1 h in PBS containing 3% bovine serum albumin, and washed 3 times for 5 min with PBS. Secondary antibody incubation was performed for 45 min in PBS containing 3% bovine serum albumin, after which the cells were washed as described above. Subsequently, cells were mounted in appropriate medium.

Western blot analysis

Forty-eight hours after transfection, Hela cells were harvested, and resuspended in RIPA (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl, 1% Triton X-100) buffer supplemented with protease inhibitors (Roche Diagnostics). Cell lysates were incubated on ice for 30 minutes and clarified by centrifugation at 15,000 g for 10 minutes at 4°C. Total proteins were quantified using the BCA protein assay reagent (Sigma), and bovine serum albumin as the standard. 30 µg of proteins were analysed onto a SDS-PAGE according to Laëmmli and Favre (1973). Whole cell lysates were separated on a 5% SDS-PAGE (Bio-Rad Laboratories Inc.) and transferred onto nitrocellulose membranes. The membranes were incubated with 5% non-fat dry milk in 0.1% Tween 20 in PBS at 4°C overnight. Then the membranes were washed in PBS containing 0.05% Tween 20 (PBS-T).

Primary antibody was incubated for 1 h against CFTR protein (clone M3A7, Chemicon) in PBS. M3A7 is a mouse monoclonal antibody that recognizes an epitope at the C-terminal, in the region of residues 1370 to 1380. The membranes were washed with PBS-T three times and then incubated with the horseradish peroxidase-conjugated secondary antibody (Sigma) for 1 h. They were washed in PBS-T again and the blots were developed with chemiluminescence with ECL kit (Amersham Biosciences) and visualized on x-ray film after a 1-15 min exposure. Immunoblots were quantified using Duo-Scan transparency scanner and scion image software (developed by US NIH, http://www.scioncorp.com/).

RESULTS AND DISCUSSION

Mutations and polymorphisms in CFTR gene are responsible for cystic fibrosis disease (Qiao *et al*., 2008). Genomic surveys indicate that polymorphisms affecting transcription and mRNA processing, including splicing and turnover, may account for the main share of genetic factors in human phenotypic variability. However, most of these polymorphisms remain yet to be discovered (Wang and Sadée, 2006). Previous studies have demonstrated that polymorphisms outside the *CFTR* gene (Yarden *et al*., 2005; Yarden *et al*., 2004), as well as within the gene, may affect transcription or function of the CFTR protein and modify the phenotype of some CF mutations (Huang *et al*., 2008). Polymorphisms cannot cause CF by itself, but may alter CFTR protein production and/or function. There is a variation in distribution of common polymorphisms among different populations. It was thus important to compare their frequencies in patients with the frequencies in healthy population in order to assess the possible role of these polymorphisms in the monosymptomatic CF disorders. (Nikolic *et al*., 2006).

Several studies have provided evidence for a possible genetic link between CFTR polymorphisms and variation in the phenotypic expression of *CFTR* mutations, but a number of factors that might affect the interpretation of the published results need to be clarified before reliable conclusions can be reached. This study therefore was planned to investigate the effect(s) of four common polymorphisms namely c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A alone or in allelic complexes in cis in the *CFTR* gene. These different constructs was established by site-directed mutagenesis as shown in Fig. (1). In order to achieve such a purpose two different approaches were chosen and employed.

1- Subcellular localization of CFTR mutants

CFTR has been shown, by immunofluorescence analysis, to be located at the apical membrane of epithelial cells. To assess the effect of each polymorphism on membrane targeting, wild-type and mutant GFP-tagged *CFTR* were expressed in HeLa cells and their localizations were observed using immunofluorescence (Fig. 2). The wild-type CFTR was clearly pre-

sent at the cell membrane. Polymorphisms c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A showed a cell membrane protein distribution similar to that of the wild-type protein. The remaining polymorphisms P5, c.[2694T>G;4521G>A], and P6, c.[1540A>G;2694T>G; 4521G>A] mutants also showed a cell surface distribution in some cells (approximately 10% of those transfected), although the protein signal in these cells appeared to be less intense at the membrane. Furthermore, c.[1540A>G;2694T>G;4404C>T;4521G>A] (P7) failed to reach the cell membrane and appeared to be localized at distinct areas of the cytoplasm. The retention of these mutants in the cytoplasm was caused by aberrant protein processing in either the ER or the Golgi. A functionally low level of CFTR protein may arise due to alternative splicing of the CFTR transcript and this had detected in lung disease associated with the IVS8-5T allele of the *CFTR* gene (Noone *et al*., 2000).

To explore the subcellular localization of CFTR mutants in more detail, co-localization studies were performed with cellular marker proteins in HeLa cells transfected by different constructs. As shown in Figure (3b-e), there was an apparent colocalization of CFTR with the Golgi marker protein (p58k) in mutant CFTR proteins c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A. However, no colocalization between CFTR protein and p58k in other mutant CFTR was observed. Calreticulin is a soluble protein resident in the ER lumen and is used as endoplasmic reticulum (ER) marker. Immunofluroescent labelling of CFTR mutants with ER marker in HeLa cells showed that the double and triple CFTR mutants; P5, c.[2694T>G;4521G>A]; and P6, c.[1540A>G;2694T>G; 4521G>A] appeared to be localized to distinct areas of the cytoplasm, a perinuclear-endoplasmic reticulum (ER) localization mutants (Fig. 4b,c) whereas c.[1540A>G;2694T>G;4404C>T;4521G>A] is retained in the ER (Fig. 5 d). Thus, these data revealed that mutant CFTR protein accumulates at or close to the ER or ER-derived membranes in transfected HeLa cells. These results were in agreement with the energetic point of view suggested by (Riordan *et al*., 1999).

2- Processing of CFTR mutants

To study the trafficking of c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A polymorphisms in different genotypes combination expressed in HeLa cells, a western blot (wb) analysis was made. In wb experiments, a band of lower molecular mass than band B was detected by using M3A7 antibody. This band was previously shown to correspond to a polypeptide resulting from usage of an alternative translation initiation site of the CFTR mRNA. As shown in Fig. (5a), the relative amount of fully glycosylated proteins (band C) is not different between P1, c.1540A>G; P2, c.2694T>G; P3, c.4404C>T; P4, c.4521G>A polymorphims and WT-CFTR, confirming that these mutant proteins can fold and trafficking normally to the plasma membrane

in HeLa cells. However, P2, c.2694T>G and P4, c.4521G>A mutants produced significantly less band C (75.5% and 74.5% of the total CFTR, ratio C/(B+C)) than wild-type 86.5% (Fig. 5b). These results indicated a decrease of C/(B+C) in P4, c.4521G>A polymorphism in transfected cells. This decrease (around 10% compared to the WT-CFTR) is statistically highly significant ($p<0.01$) when Kruskal Wallis test (http://www. viesanimales.org) was performed. The reduction in the expression level on the apical membrane of P2 and P4 CFTR may be related to its impact on splicing process. These results were in agreement with those of Steiner *et al*. (2010), who implicated these polymorphisms in the etiology of several clinical symptoms in cystic fibrosis gene mutations due to its impact on splicing efficiency.

Maturation patterns of mutant CFTR P5, c.[2694T>G; 4521G>A] ; P6, c.[1540A>G; 2694T>G; 4521G>A] and P7, c.[1540A>G; 2694T>G; 4404C>T; 4521G>A] indicated that band density of the 150-kDa was present while that of 170-kDa was absent in all of these constructs (Fig. 5c). This suggests that these mutated constructs are present as the immature and core-glycosylated form. The absence of glycosylation is consistent with the hypothesis that these polymorphisms are trapped in the ER and are not transferred to the Golgi apparatus where the glycosylation takes place (Riordan *et al*., 1999). Therefore, these polymorphisms caused a misprocessing defect and decreased the mature CFTR form. These results are consistent with subcellular localization data as p.Phe508del-CFTR were not detectable in the plasma membrane. This postulate is also consistent with the immunofluorescence observations, which indicated that P5, P6 and P7 mutant CFTR constructs are located in ER or ER-derived membranes in transfected Hela cells (Fig. 5d). Such alterations may be clinically insignificant in individuals without additional CFTR mutations, but may have influence on disease phenotype in patients with other relevant mutations (Davies *et al*., 2005). Hence, Quantitative approaches rather than conventional genomic analysis are required to interpret the role of cSNPs (Steiner *et al*., 2004). Furthermore, these polymorphisms could contribute to an increased risk in the development of chronic pancreatitis (De Cid *et al*., 2010). However, these findings support the hypothesis that differences in CF phenotype could be related to the effect of the genotype on CFTR protein production and function. Further studies could help verifying these results in an attempt to characterize the possible influence of *CFTR* gene polymorphisms in cystic fibrosis disease.

CONCLUSION

The results of the present *in vitro* study suggested that c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A which are frequently observed individually in the *CFTR* gene, cause normal protein maturation, whereas the occurrence of the double c.[2694T> G;4521G>A], triple c.[1540A>G;2694T >G;4521G>A] and

quadruple c.[1540A>G; 2694T>G;4404C> T;4521G>A] polymorphisms had impacts on maturation of CFTR protein. These pieces of information should be useful in understanding the pathogenesis of CF. These findings reveal that reported polymorphisms may impair the quantity and quality of CFTR protein and could contribute to CF disease development. In addition, cSNPs may be responsible for variations in the phenotypic expression of CFTR mutations. Confocal microscopy was used to determine the subcellular localization of wild-type and mutant CFTR in transfected Hela cells. F508del-CFTR was localized preferentially to the endoplasmic reticulum (ER) compartment. In contrast, mutant CFTR showed a markedly different subcellular localization pattern in the transfected cells. It did not localize to the ER. Instead, the pattern of its subcellular distribution was consistent with an aggregated form in the cytoplasm. However, the findings support the hypothesis that differences in CF phenotype could be related to the effect of the genotype on CFTR protein production and function. It is then suggested that in addition to modifier genes, SNPs may also contribute to the differences observed in the symptoms of various CF patients.

SUMMARY

To date, more than 200 sequence polymorphisms have been identified in the *CFTR* gene thus far and only a minority has been characterized at the cellular localization and the protein levels. In previous studies, molecular genetic analysis of the entire coding region of the *CFTR* gene in patients identified four common polymorphisms: c.1540A>G is located in Exon 10 of *CFTR*; c.2694T>G in Exon 14a, whereas c.4404C>T and c.4521G>A are located in Exon 24. The aim of this study was therefore to examine the possible effects of these polymorphisms on subcellular localization and *CFTR* processing in different constructs to disclose their impact on the clinical phenotype. The subcellular localization using confocal microscopy of coding single-nucleotide polymorphisms c.1540A>G, c.2694T>G, c.4404C>T and c.4521G>A have shown correct membrane localization and normal effect on maturation of CFTR protein. Although maturation patterns were not affected, total amounts of mature CFTR protein were reduced for c.2694T>G and c.4521G>A polymorphisms. The double c.[2694T>G; 4521G>A], triple c.[1540A>G;2694T> G;4521G>A] and quadruple c.[1540A>G; 2694T>G;4404C>T; 4521G>A] polymorphisms have shown to be exclusively cytoplasmic consistent with an endoplasmic reticulum localization. Western blot analysis of CFTR protein indicated that the double and triple mutants had effects on the maturation of CFTR protein. So, more severe effect on CFTR protein was present when these polymorphisms were combined in complex alleles in cis, supporting the influence of these frequent polymorphisms on the clinical features of CF patients. These findings suggest that SNPs may be responsible for variation in

the phenotypic expression of *CFTR* mutations.

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Table (1): Sequences of site-directed mutagenesis primers. Two primers were used for each mutagenesis reaction. The altered bases, which encode the mutated amino acids are underlined and in bold shape.

Primer name	Nucleotide sequence
10A- 10B	(F) 5'GCAAGACTTCACTTCTAATGGTGATTATGGG 3' (R) 5'CATTAGAAGTGAAGTCTTGCCTGCTCCAGT 3'
14A- 14B	(F) 5'ACATACCTTCGATATATTACGGTCCACAAGAG 3' (R) 5' GTAATATATCGAAGGTATGTGTTCCATGTAGT 3'
24A- 24B	(F) 5' GAGAACAAAGTGCGGCAGTATGATTCCATC 3' (R) 5' TACTGCCGCACTTTGTTCTCTTCTATGACC 3'
24C- 24D	(F) 5' AAGTGCAAGTCTAAGCCCCAAATTGCTGCTC 3' (R) 5'TGGGGCTTAGACTTGCACTTGCTTGAGTTC 3'

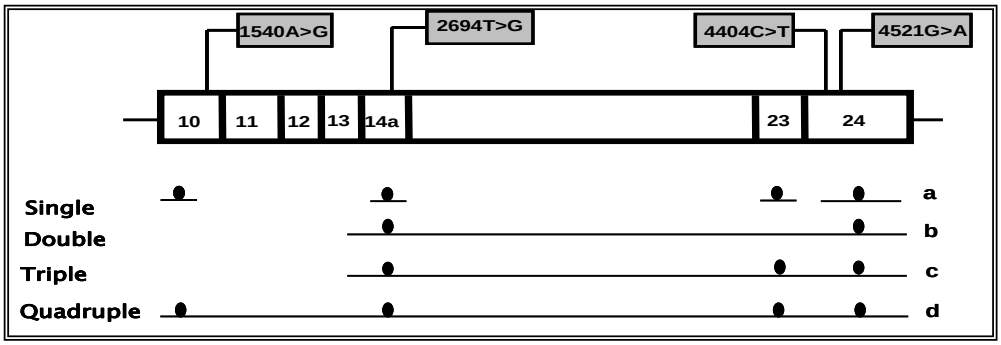

Fig. (1): Schematic presentation of the partial *CFTR* gene illustrating the location of four CFTR polymorphisms as indicated in gray boxes and different mutant CFTR constructs characterized in the present study:

a. Single mutant construct for each studied polymorphism c.1540A>G (P1), c.2694T>G (P2), c.4404C>T (P3), and c.4521G>A (P4).
b. Double mutant construct contain two polymorphisms c.[2694T>G;4521G>A] (P5).
c. Triple mutant construct contain three polymorphisms c.[1540A>G;2694T>G;4521G>A] (P6).
d. Quadruple mutant construct contain four polymorphisms c.[1540A>G;2694T>G;4404C>T;4521G>A] (P7).

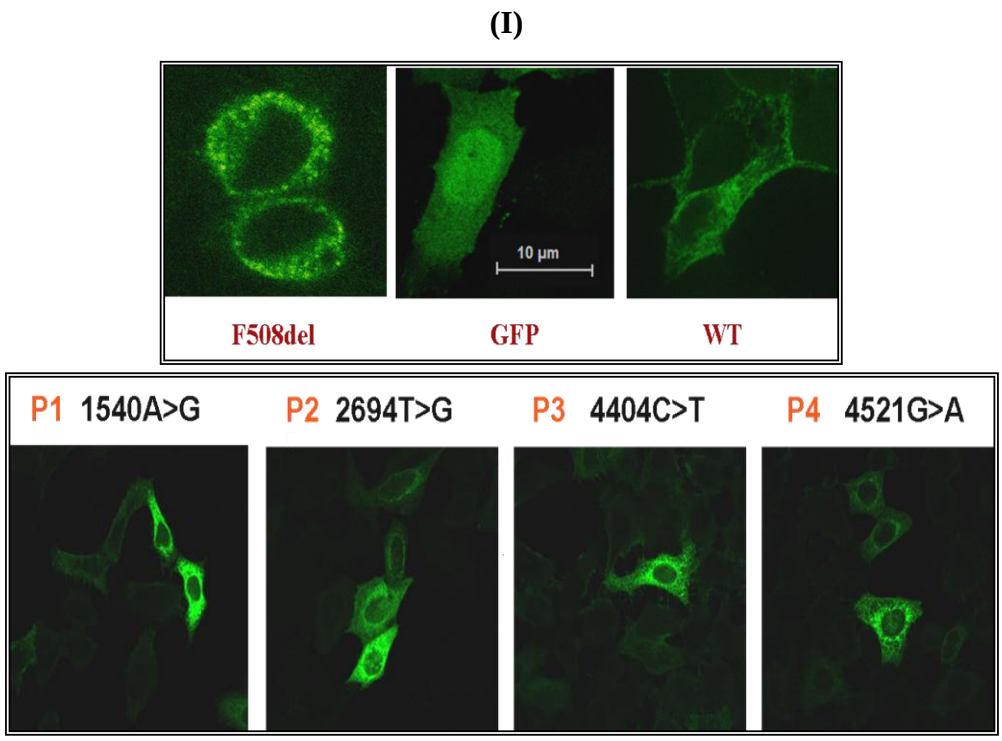

Fig. (2): Subcellular localization of wild-type and mutant CFTR protein in expressing HeLa cells as assessed by confocal laser scanning microscopy. CFTR was stained green. (I) p.Phe508del; GFP; Wild-type CFTR. Single polymorphisms: c.1540A>G (P1); c.2694T>G (P2), c.4404C>T (P3); c.4521G>A (P4) were targeted at least to the plasma membrane. (II) GFP (A); Wild-type (B); p.Phe508del (C); complex polymorphisms: P5 (D), P6 (E), and P7 (F) were not targeted to the plasma membrane. They either mislocalized or diffused in the cytoplasm compared with the wild-type transfected cells. A mean number of 15 cells were examined in three independent experiments for each CFTR protein analyzed. Scale Bars, 10μm.

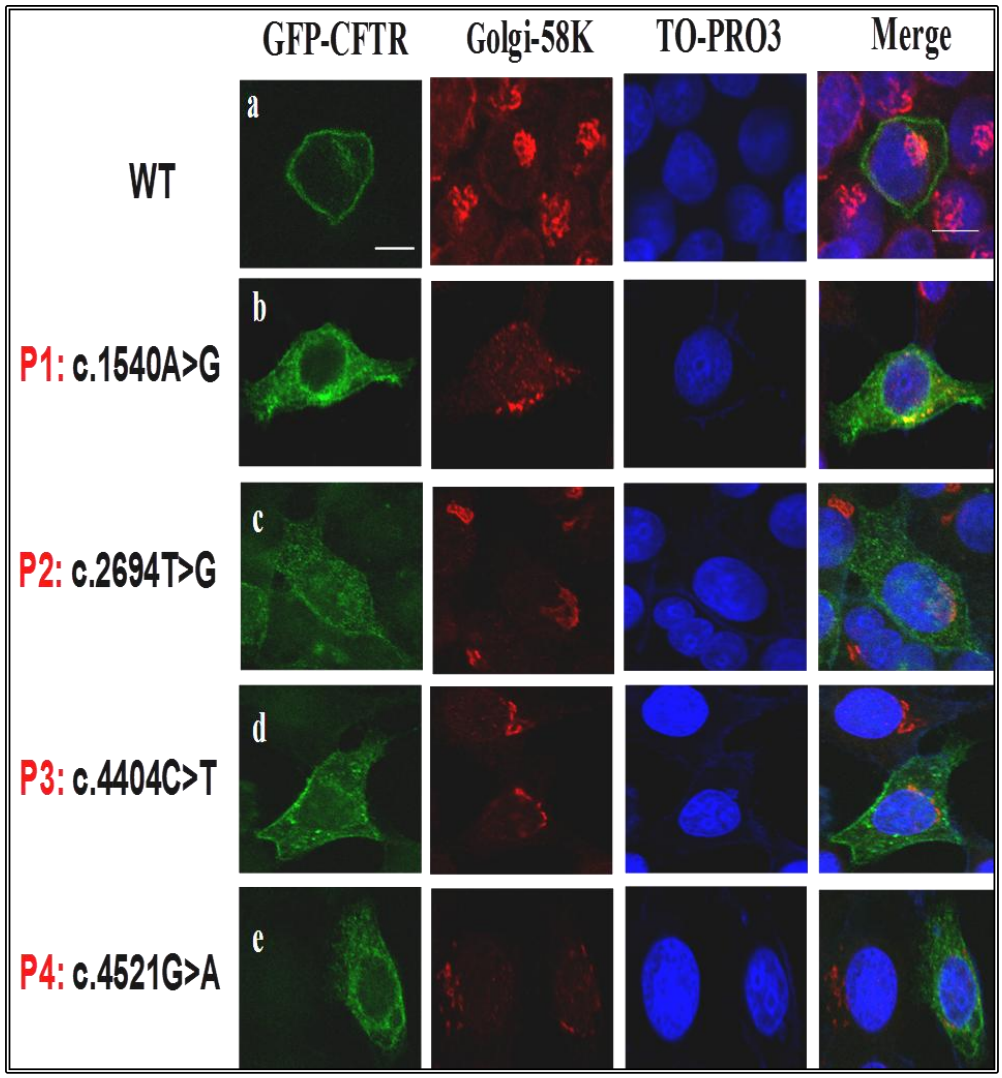

Fig. (3): Subcellular localization of wild-type and mutant CFTR. HeLa cells were transiently transfected with wild-type CFTR (a) or mutant (b-e) GFP-tagged CFTR. Cells were stained for Golgi body with the Golgi 58K antibody (Golgi marker; red) followed by incubation with incubation with Alex-Fluor 555 goat anti-mouse secondary antibody. Cells were labelled with anti-CFTR (green) and. TO-PRO was used to counter stain the nuclei (blue). Yellow displays co-localization of CFTR protein with organelle-specific marker. Mutant proteins: P1, c.1540A>G (b); P3, c.2694T>G(c); P4, c.4404C>T (d); P4, c.4521G>A (e) shown strong membrane fluorescence similar to the wild-type CFTR.

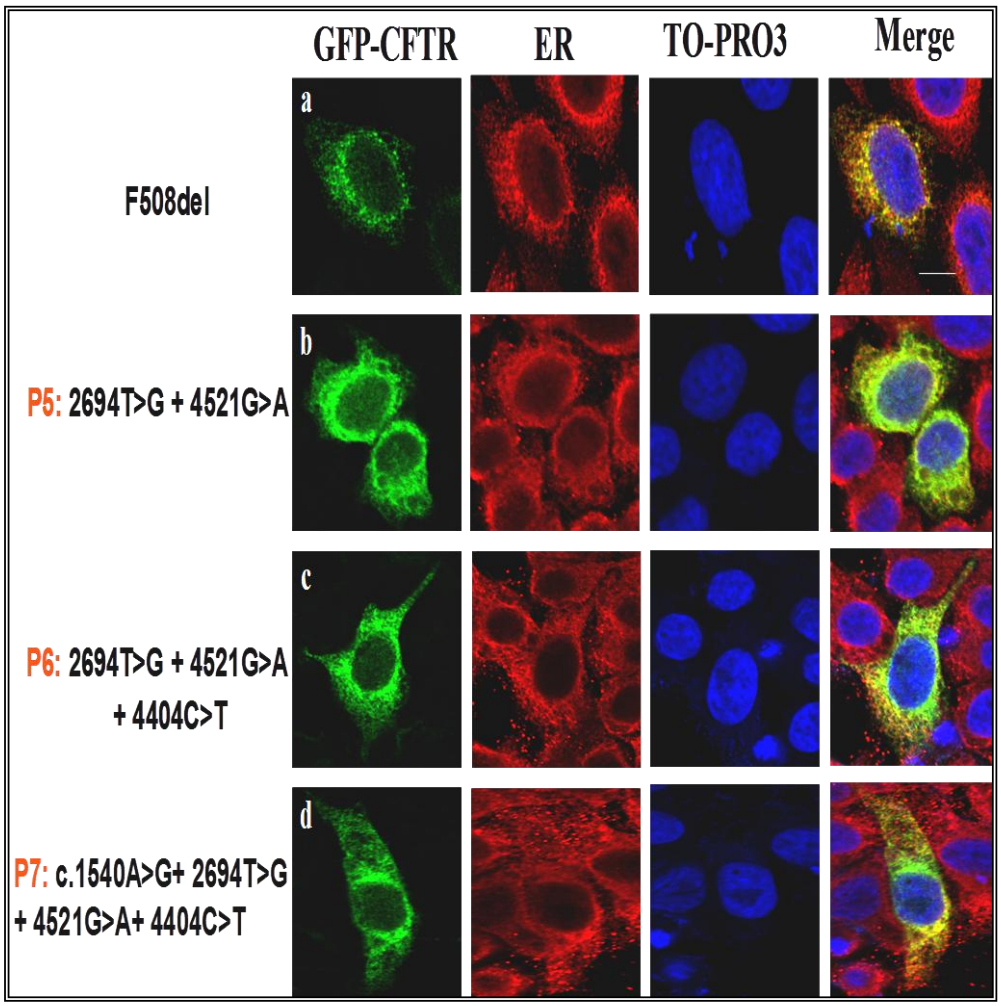

Fig. (4): Subcellular localization of F508del and mutant CFTR. HeLa cells were transiently transfected with F508del or mutant (b-d) GFP-tagged CFTR. Cells were stained for ER with the calreticulin antibody followed by incubation with Alex-Fluor 555 donkey anti-rabbit secondary antibody. Cell nuclei were stained blue with TO-PRO-3as indicated above each column. Yellow displays co-locolization of CFTR protein with organelle-specific marker. Mutant proteins: P5, c.[2694T>G;4521G>A] (b); P6, c.[1540A>G;2694T>G; 4521G>A] (c); P7, c.[1540A>G; 2694T>G; 4404C>T; 4521G>A] (d) were retained in the cytoplasm consistent with an endoplasmic reticulum localization compared with the F508del transfected cells.

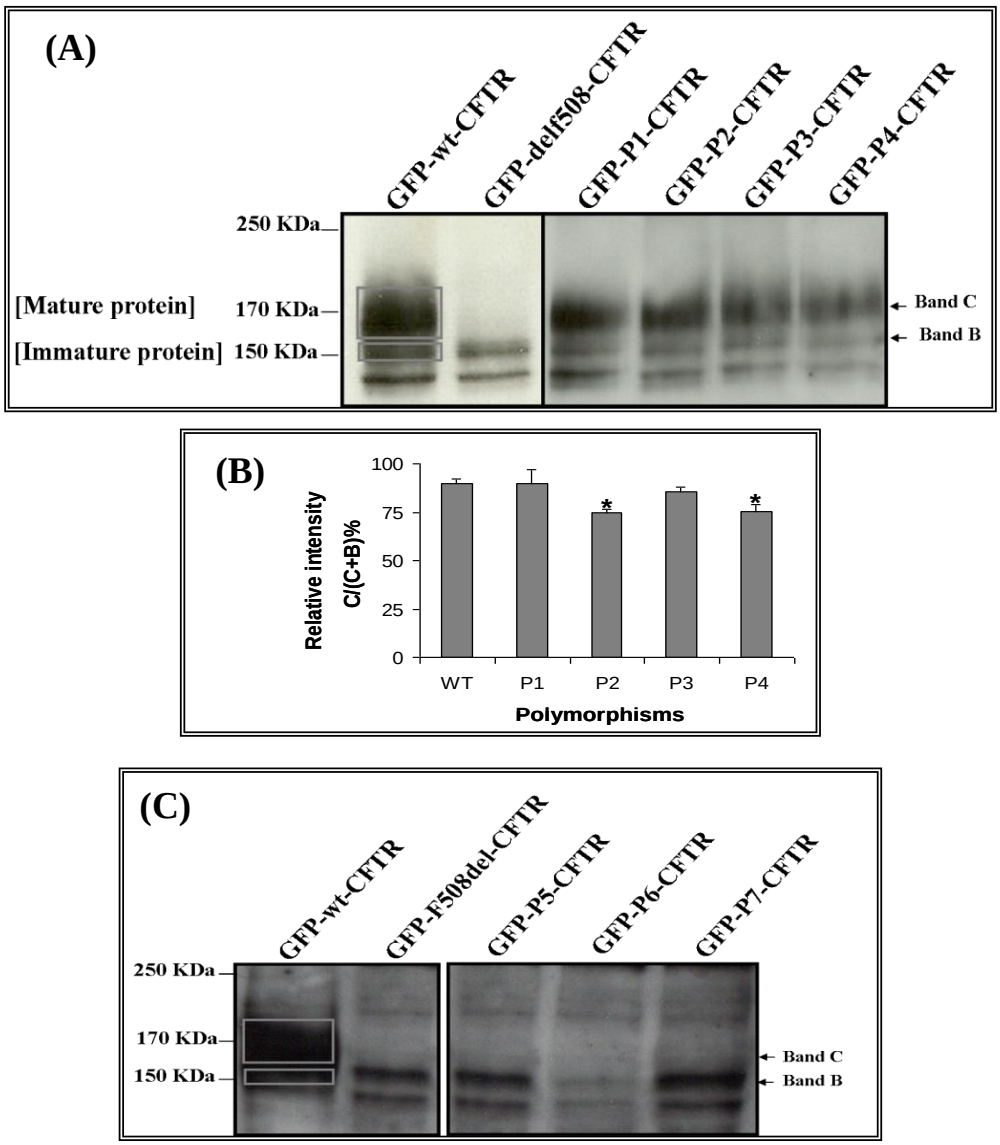

Fig. (5): A: Western blot analyses of cell extracts from Hela cells transiently expressing the wild-type and mutated forms of CFTR protein. CFTR was detected by M3A7, a mouse monoclonal antibody that recognizes an epitope at the C-terminal of CFTR in the region of residues 1370–1380. Arrows on the right indicate the positions of core-glycosylated (band B) and fully glycosylated (band C) forms of CFTR. Single polymorphisms c.1540A>G (P1), c.2694T>G (P2), c.4404C>T (P3), and c.4521G>A (P4). B: Histogram of percentage of C/B+C ratio of CFTR obtained by three Western blots. The histogram numbers correspond to CFTR proteins described in part A of this figure. Data are the means ± SD of at least three independent transfection experiments. C: Western blot analysis of the CFTR expression of complex polymorphisms: c.[2694T>G;4521G>A] (P5), c.[1540A>G;2694T>G; 4521G>A] (P6), and c.[1540A>G; 2694T>G; 4404C>T; 4521G>A] (P7).