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Short Communication

Complexity of phenotypes induced by p.Asn1303Lys-CFTR correlates with difficulty to rescue and activate this protein

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Abstract: Cystic Fibrosis is the most common recessive autosomal rare disease found in Caucasian. It is caused by mutations on the *Cystic Fibrosis Transmembrane Conductance Regulator* gene (*CFTR*) that encodes for a protein located on the apical membrane of epithelial cells. c.3909C>G (p.Asn1303Lys) is one of the most common worldwide mutations located in nucleotide binding domain 2. The effect of the p.Asn1303Lys mutation on misprocessing was studied by immunofluorescence and western blotting analysis in presence and absence of treatment. To evaluate the functionality of potentially rescued p.Asn1303Lys-CFTR, we assessed the channel activity by radioactive iodide efflux. No recovery of the activity was observed in transfected cultured cells treated with VX-809. Thus, our results suggest that multiple drugs may be needed for the treatment of c.3909C>G patients in order to correct and activate p.Asn1303Lys-CFTR as it shows folding and functional defects.

Key words: Cystic fibrosis; p.Asn1303Lys; VX-809; Functional impact.

Introduction

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disease in Caucasians caused by mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene. To date, more than 2006 CFTR genetic variations have been listed in the CFTR database (http://www.genet.sickkids.on.ca/). According to the CFTR2 global database, the c.3909C>G mutation (old nomenclature: N1303K) is present at least on one copy allele in 2.5% of world CF patients (2226 over 88664) (http://www.cftr2.org) which is relatively high. This relatively high c.3909C>G frequency tends to be higher in Mediterranean countries with a frequency of 27% in the Lebanese population (1).

The c.3909C>G mutation is class II mutation retained in the ER, incompletely glycosylated, and rapidly degraded in proteasomes. Soon after its identification, it was characterized by its severity in the pancreas and variability of pulmonary status (2). The c.3909C>G mutation induces a substitution (p.Asn1303Lys) in Nucleotide Binding Domain 2 (NBD2) that initially was predicted, unlike four other CFTR domains, to have an unessential role in a proper folding and membrane trafficking (3). However, mutating this amino acid remarkably disrupts the global CFTR conformation (4) and the gating activity (5, 6). Thus, further investigations are needed to determine the impact of this class II mutation on the processing and on the final activity of this mutant CFTR.

The presence of this mutation with other DNA alteration in cis, complex allele, was identified in several populations (7, 8, 9) may aggravate its clinical outcome (10, 11). However, the splicing impact resulting from this complex allele is not sufficient to explain the phenotype variability (9). Therefore, potential treatments applied at the protein level may induce a correction of the phenotype regardless of the genetic background.

Recently, investigations have been conducted on molecules that could rescue mutant CFTR not able to reach the cell membrane due to folding defects, including lumacaftor (VX-809), a CFTR corrector or MG132, a proteasome inhibitor. VX-809 is reported to restore CFTR activity in homozygote p.Phe508del Human Bronchial Epithelial cells (12). The corrective effect of VX-809 on this class II mutation may also be extended on other mutations showing structural abnormalities. Moreover, this drug has also shown a rescuing effect on several other mutations, one of them not located in the NBD2 (13).

Therefore, we have studied p.Asn1303Lys-CFTR protein processing and membrane trafficking in transiently transfected cell lines in presence VX-809 and MG132. We have also conducted functionality tests to determine the channel activity of any p.Asn1303Lys-CFTR that could possibly reach the membrane in presence and absence of VX-809.

Materials and Methods

Ethics statement

The study was performed according to French legislation and recommendations of the local ethics committee in accordance with the Declaration of Helsinki.

CFTR constructs

The plasmid pTCFWT used in this paper is a vector designed for the visual detection of transfected mammalian cells by the green fluorescent protein (GFP), the SV40 propoter controls synthesis of the GFP-Zeocin fusion protein while the CMV promoter controls CFTR (WT or mutated proteins). The construct pTCF N1303K (c.3909C>G) was obtained by site-directed mutagenesis using Gene tailor site-directed mutagenesis kit (Invitrogen) and specific primers (Table 1) according to manufacturer's protocol construction. In this study, pTCFWT, pTCFc.1521_1523delCTT (p.Phe508del), and pTCF N1303K plasmids were used. The inserted mutation was confirmed by direct sequencing (see primers in Table 1).

Cell culture and transfection

HeLa cells were grown in DMEM medium with Glutamax-I (Life Technologies) supplemented with 10% fetal bovine serum (Gibco), 100units/mL penicillin and 100µg/mL of streptomycin in a humidified incubator at 37°C in the presence of 5% CO_2. Cells were transiently transfected by WT and mutant *CFTR* plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The GFP allowed visual detection of transfected cells. For treated cells, VX-809 (Vertex Pharmaceutical) (10 µmol/L) or MG132 (Sigma) (10 µmol/L) were added 24 hours after transfection. Cells were used after 48 hours for western blotting and functionality tests. For confocal microscopy, cells were observed after a 24h culture without drugs.

Immunolocalisation

HeLa cells grown on glass were fixed in 4% paraformaldehyde and permeabilized as described previously (5). Coverslips were incubated with human anti-CFTR MAB25031 (Invitrogen) (1/400 in 1X PBS) for 1 h at 4°C followed by the secondary antibody, conjugated to Alexa Fluor® 555 (Invitrogen) (1/800 in 1X PBS) (40 min). Images were obtained using a confocal microscope (Olympus FV100) equipped with a multiline Argon laser (457nm, 488nm, 515nm) to visualise GFP, and with a HeNe-Green laser (543nm) to visualise CFTR.

Western Blotting Analysis

HeLa cells were lysed in Laemmli buffer (Sigma) and cell lysates were clarified and quantified as before (14). Proteins were analyzed using the primary antibody against CFTR (clone M3A7; Millipore) (1/2000) in 1X TBST (Tris-buffered saline with 0.2% Tween 20) over night at 4°C. After washing three times with TBST, cells were incubated with secondary antibody conjugated to Alexa Fluor® 555 (Invitrogen) (1/100 in TBST) for 6 h. Na^+/K^+ ATPase α-1 (Clone 464.6; Millipore) (1/1000 in 1X TBST) using as a loading control. The blots were scanned with a Typhoon imager (GE Healthcare) using an excitation laser (532nm) and a 580nm band-pass filter (580 BP 30).

Functionality tests by Iodide Efflux

CFTR chloride channel activity was evaluated on transfected HeLa cells using the iodide (^{125}I) efflux assay as previously described (15, 16). The ^{125}I efflux was realized by the MultiPROBE®IIex robotic liquid handling system (Perkin Elmer Life Sciences, Courtaboeuf, France) and measured by the Packard CobraTMII gamma counter (Perkin Elmer Life Sciences, Courtaboeuf, France). Statistical analyses and graphic presentations were obtained using the GraphPad Prism version 5.0 for Windows (GraphPad Software). Sets of data were compared using analysis of variance (ANOVA) or the Student's t test. Values of $p<0.05$ were considered as statistically significant: *$p<0.05$; **$p<0.01$; ***$p<0.001$. Non-significant difference was $p>0.05$.

Results

The p.Asn1303Lys-CFTR protein is missprocessed and retained in the endoplasmic reticulum

Immunofluorescence followed by confocal microscopy imaging shows neither p.Phe508del nor p.Asn1303Lys-CFTR mutants exhibited cell surface staining (Figure 1a) but these proteins were restricted to intracellular compartments, indicating the presence of a processing defect, unlike WT-CFTR. The absence of membrane staining in mutants was also confirmed by 3D-confocal images (data not showed). A western blot analysis of WT-CFTR produced two bands (Figure 1b). The first was a narrow band of approximately 140 kDa (band B), which represented the core-glycosylated protein located in endoplasmic reticulum (ER); the second was diffuse, with an approximate molecular mass of 170 kDa (band C), and represented mature, fully glycosylated protein that had migrated through the Golgi complex to the cell membrane. For both mutated proteins (p.Asn1303Lys and p.Phe508del), only band B was observed, confirming a retention of these mutated proteins in the ER compartment. No band C was observed when cells were treated for 24h with VX-809 and MG132 respectively (Figure 1b).

Table 1. Primers used in amplification and sequencing of studied regions.

Use	Hybridation	Primers
Directed Mutagenesis to introduce the c.3909C>G mutation on the pTCF	Exon 24	5'TCTGGAACATTTAGAAAAAA[G]TTGGATCCCT3'
		5'TTTTTTCTAAATGTTCCAGAAAAAATAAATACTTT3'
Verifying the correct realization of the direct mutagenesis to introduce the c.3909C>G mutation in pTCF	Exon 23/Exon 24	5'CCTTTGGAGTGATACCACA3'
		5'ATCACAGATCTGAGCCCAA3'

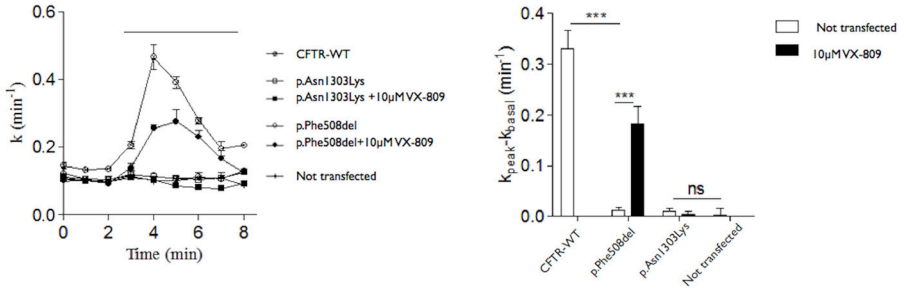

Figure 1. Impact of the p.Asn1303Lys mutation on protein localisation and processing (**a**) Subcellular localization of WT and mutant CFTR proteins in transfected HeLa cells as assessed by Immunofluorescence followed by confocal microscopy imaging. The green color results of the auto-fluorescence of the GFP and the red color results of CFTR protein recognized by the Alexa Fluor 555® (Invitrogen). The mutants p.Phe508del and p.Asn1303Lys are mislocalized compared with the WT transfected cells. A mean number of 15 cells were examined in three independent experiments for each CFTR protein analyzed. ER, endoplasmic reticulum; Nc, Nucleus; Mb, Membrane. Scale Bar, 10 µm. (**b**) Processing of WT and mutant CFTR proteins as assessed by the glycosylation status of the CFTR protein at steady state on Western Blotting. HeLa cells were transiently transfected with WT, or mutants (p.Phe508del and p.Asn1303Lys). CFTR protein was detected by M3A7 (Millipore). Na^+/K^+ ATPase α-1 is the control. Black arrows on the right indicate the positions of core-glycosylated (band B) and fully glycosylated (band C) forms of CFTR. Ladder: Precision Plus Protein Kaleidoscope Standards (Biorad). 1) WT CFTR, 2) WT CFTR+MG132, 3) p.Phe508del CFTR, 4) p.Phe508del CFTR+MG132, 5) p.Phe508del CFTR+VX-809, 6) p.Asn1303Lys CFTR, 7) p.Asn1303Lys CFTR+MG132, 8) p.Asn1303Lys CFTR+VX-809.

Depletion of CFTR channel activity of the p.Asn1303Lys protein

We assessed the effect of Asn1303Lys substitution on channel activity by iodide efflux. We observed no activity of the mutated protein stimulated by 10 µM forskolin and 30 µM genistein. The absence of activity was maintained, in these same conditions, even after the treatment of the transfected cells by 10 µM VX-809 for 24h. This is unlike p.Phe508del-CFTR that expressed a significant activity after folding correction with VX-809.

Discussion

The variable severity regarding the lung phenotype in c.3909C>G homozygous and heterozygous patients (2) was previously investigated at the splicing level. The polymorphisms c.744-33GATT[6] and c.869+11C>T associated in several populations to the c.3909C>G mutation (7, 8, 9) seem to have no notable influence on the normally spliced mRNA *in cellulo* (9). Therefore, investigations were extended in order to better understand the effect of the p.Asn1303Lys (c.3909C>G) on CFTR processing and activity levels.

The p.Asn1303Lys is the only NBD2 mutation known to disrupt CFTR processing (17) despite the capacity of the cotranslationaly (18) folded protein, lacking of this domain, to escape the ER quality control system (3). Indeed, as the protease resistance of the four other folded domains is preserved and the protein maturation is moderately affected after NBD2 truncation, this domain is considered nonessential for the processing but contributes to CFTR stabilization (4). However, these facts were not observed in full-length protein carrying the p.Asn1303Lys, as this NBD2 mutation has impaired the channel membrane trafficking and induced MSD1 and MSD2 conformational changes (4). In this study, p.Asn1303Lys-CFTR processing analyses are consistent with these observations as no glycosylated form was detected (Figure 1). The absence of band C in cell extracts transiently transfected with c.3909C>G mutant was previously reported (19), but is in conflict with other observations (17, 20) showing some band C. To better assess the residual activity of the possibly maturate form of p.Asn1303Lys-CFTR, our channel activity tests have provided evidence showing an absence of residual functionality even when cells were treated with VX-809 (Figure 2). Concordant with our functionality test results, Awatade *et al.* (2015) in a previous study, conducted on Human Bronchial Epithelial cells mounted in micro-Using chambers, also showed that CFTR activity was not significantly restored after VX-809 treatment (13). Thus, authors hypothesized that

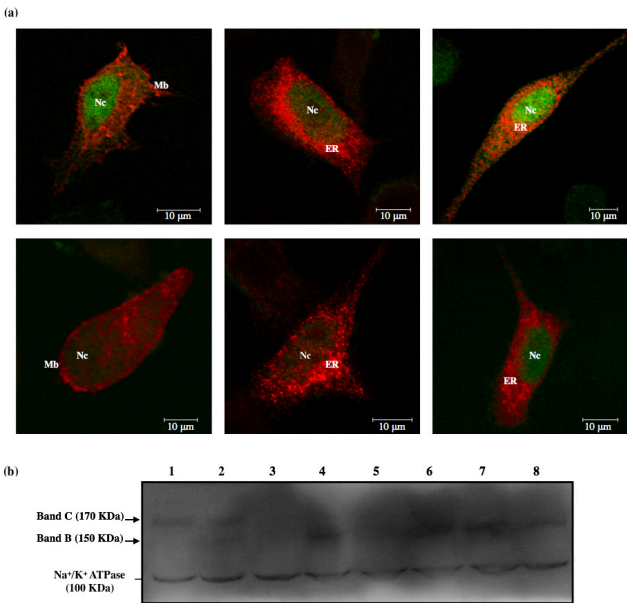

Figure 2. Impact of the p.Asn1303Lys CFTR mutation on protein activity. a) Time-dependent stimulation of iodide effluxes in cells transiently transfected by the WT (CFTR-WT), c.1521_1523delCTT (p.Phe508del), and c.3909C>G (p.Asn1303Lys) pTCF plasmids. Channel activity was measured in absence then in presence of 10µM forskolin and 30µM genistein. The horizontal bar indicates the application of these drugs. Channel activity was also measured in cells treated with 10µM VX-809 for 24h in the same conditions. b) Histogram showing the corresponding activity of each transfection in presence and absence of 10µM VX-809. ***p<0.001; ns: non significant.

VX-809 may not be able to correct the structural conformation changes induced by the p.Asn1303Lys mutation. However, it is also possible that VX-809 is able to correct p.Asn1303Lys but this mutation alters the channel functionality. Our western blot analyses did not show a maturate form of p.Asn1303Lys when cells are treated by VX-809 (nor with MG132). These results are not consistent with previously reported maturation studies that showed the band C form from p.Asn1303Lys-CFTR was significantly increased when cells were treated with VX-809 (21) or MG132 (20). These results support a corrective role of these molecules on p.Asn1303Lys-CFTR processing, but it stays non-functional at the membrane, unlike the p.Phe508del-CFTR that shows activity after correction (12). One must note that the divergences in western blot results could be explained by the variable sensibility of the technique depending on the type of transfection (stable or transient) and on the antibody. This also could explain the absence of mature form in treated p.Phe508del-CFTR in our results. However, it is important to note that our results are consistent with (22) showing no residual or correct-induced activated by VX-809 for p.Asn1303Lys-CFTR mutation in rectal cystic fibrosis organoids.

The absence of activity in VX-809 treated p.Asn1303Lys-CFTR channel could be a result of the inability of this drug to recognize the conformational changes induced by p.Asn1303Lys-CFTR mutation. Moreover, this amino acid has a crucial position in NBD2. Indeed, it stabilizes the connection of the ATP to the alpha-phosphate linker by establishing hydrogen bonds (5) and forms with two coevolving NBD2 amino acids (p.Phe1296 and p.Arg1358) a triad that undergoes a rearrangement upon ATP binding (23, 24). A hydrogen bond is established between p.Asn1303 and p.Phe1296 when ATP is bound to the protein (24), whereas, a hydrogen bond links p.Asn1303 to p.Arg1358 in the ADP-bound form (6). This switching highlights the fundamental function of the p.Asn1303 amino acid in the NBD2 "induced-fit conformation". Consequently, a decreased rate of channel opening and closing was detected when mutated p.Asn1303-CFTR are measured in low temperature patch-clamp assay (5) or in *Xenopus laevis* oocytes membranes (6). Similar kinetic perturbations emerged in other mutants as p.Asn1303His, p.Asn1303Ile, p.Asn1303Ala and p.Asn1303Lys-CFTR (5).

In conclusion, the structural defect induced by p.Asn1303Lys may be different from the one created by p.Phe508del-CFTR protein that is corrected by VX-809. Furthermore, we added new evidences to better assess the impact of c.3909C>G mutation on CFTR functionality and confirmed the absence of residual activity even when p.Asn1303Lys-CFTR is treated by VX-809. Thus VX-809 is inefficient at rescuing p.Asn1303Lys-CFTR protein and this mutation needs combined correctors in order to increase the CFTR maturation and its delivery to the plasma membrane that could help to restore its function. These observations support the fact that c.3909C>G may be a class III or IV mutation and needs multiple drug treatment in order to correct and activate this CFTR mutant.

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