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Functional characteristics of L1156F-CFTR associated with alcoholic chronic pancreatitis in Japanese

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ABSTRACT

Although cystic fibrosis is rare in Japanese, measurement of sweat Cl^- has suggested mild dysfunction of cystic fibrosis transmembrane conductance regulator (CFTR) in some patients with chronic pancreatitis. In the present study, we have investigated the association of CFTR variants and chronic pancreatitis in Japanese and the functional characteristics of a Japanese-, and pancreatitis-specific CFTR variant, L1156F. Seventy patients with alcoholic chronic pancreatitis, 18 patients with idiopathic chronic pancreatitis, and 180 normal subjects participated. All exons and their boundaries and promoter region of the *CFTR* gene were sequenced. HEK 293 cells were transfected with 3 CFTR variants (M470V, L1156F, and M470V+L1156F) and the protein expression was examined. *Xenopus laevis* oocytes were injected with the CFTR variants and HCO_3^-/Cl^- transport activity was examined. CFPAC-1 cells were transfected with the CFTR variants and Cl^--HCO_3^- exchange activity was examined. Six variants (E217G, I556V, M470V, L1156F, Q1352H, and R1453W) were identified in the coding region of the *CFTR* gene. Cystic fibrosis-causing mutations were not found. The allele frequencies of L1156F and Q1352H in alcoholic chronic pancreatitis (5.0 and 7.9 %) were significantly ($p<0.01$) higher than those in normal subjects (0.6 and 1.9 %). L1156F was linked with a world-wide CFTR variant M470V. Combination of M470V and L1156F significantly reduced CFTR expression to ~60%, impaired CFTR-mediated HCO_3^-/Cl^- transport activity to 50-60%, and impaired CFTR-coupled Cl^--HCO_3^- exchange activity to 20-30%. The data suggest that the Japanese-specific CFTR variant L1156F causes mild dysfunction of CFTR and increases the risk of alcoholic chronic pancreatitis in Japanese.

Key words: *CFTR* gene, L1156F, alcoholic chronic pancreatitis, Japanese

INTRODUCTION

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP regulated anion channel that is expressed in the apical membrane of epithelial cells (18). In the pancreas CFTR is found in centroacinar cells, intralobular ducts, and small interlobular ducts and plays a key role in HCO_3^- and fluid secretion in the pancreatic juice (3, 21, 28). CFTR-mediated HCO_3^- secretion dilutes and alkalinizes the protein-rich acinar secretion, which is thought to prevent the formation of protein plugs and blockage of the ducts (12). A recent study using Na^+-H^+ exchanger regulatory factor-1 knock out mice demonstrated that mislocalization of CFTR worsened experimental acute pancreatitis (35) and suggested that CFTR protects from acute pancreatitis.

Over 1,900 mutations and polymorphic loci have now been identified in the *CFTR* gene (Cystic Fibrosis Mutation Database; www.genet.sickkids.on.ca/) and there are considerable regional and ethnic variations in the spectrum (38). Combination of mutations and polymorphisms confer variable phenotypes (11). Loss of CFTR function (< 1%) due to severe mutations on both alleles causes cystic fibrosis (CF) with chronic airway disease and pancreatic insufficiency. Decrease of CFTR function to ~5% due to compound heterozygote of 1 severe mutation and 1 mild mutation causes “pancreatic sufficient” CF. Non-classic or atypical CF presents single organ disease phenotypes, including disseminated bronchiectasis, congenital bilateral absence of the vas deferens (CBAVD), and chronic pancreatitis, which are classified as CFTR-related disorders (5, 36).

Chronic pancreatitis is a progressive inflammatory disease of the pancreas that causes the loss of acinar cells, ductal irregularities often with intraductal stones, and irregular fibrosis. Although alcohol abuse is the major cause of chronic pancreatitis, only a minority of heavy drinkers develop pancreatitis. Thus chronic pancreatitis is thought to result from a

combination of environmental (alcohol, cigarettes, etc.) and genetic factors (*CFTR*, *PRSS1*, *SPINK1*, etc.) (6). In European populations, *CFTR* mutations have been frequently (~ 30%) found in patients with idiopathic chronic pancreatitis (4). Although some of the patients were classified as CF or CFTR-related disorders after search for rare *CFTR* mutations and sweat Cl^- measurement, many of the patients with chronic pancreatitis carrying one *CFTR* mutation were not diagnosed as CF or CFTR-related disorders (5). The risk of chronic pancreatitis increased 4.3 times in CF carriers (8). Subjects carrying one mild/uncommon *CFTR* mutation were also reported to be at an increased (~ threefold) risk of chronic pancreatitis (45).

Although CF is quite rare in Asian populations including Japanese (19, 47), the incidence of chronic pancreatitis in Japan (11.9 per 100,000) (17) is similar to that in the United States (46). Measurement of sweat Cl^- suggested that about one-third to one-half cases of chronic pancreatitis (both idiopathic and alcoholic) in Japanese are related to the dysfunction of CFTR (16, 34). Our previous study (13) indicated the association of 2 types of CFTR variants: c.4056G>C, p.Arg1352His (Q1352H) and c.4357C>T, p.Arg1453Trp (R1453W) and chronic pancreatitis in Japanese. Defects of protein expression and ion transport of Q1352H-CFTR were confirmed by heterologous expression system, while R1453W-CFTR showed mild reduction of open probability (27). In the present study, we have shown that a Japanese-specific CFTR variant c.3468G>T, p.Leu1156Phe (L1156F) is related to alcoholic chronic pancreatitis in Japanese. The risk of L1156F carrier for developing chronic pancreatitis was 9.0 times higher compared to non-carrier. Since L1156F is linked to c.1408A>G, p.Met470Val (M470V), a world-wide CFTR variant, we have examined the protein expression and functional characteristics of M470V+L1156F-CFTR using heterologous expression systems. We have found that combination of M470V and L1156F partially impaired expression and channel function of CFTR and substantially impaired the CFTR-coupled Cl^--HCO_3^- exchange activity.

MATERIALS AND METHODS

Subjects

This study was approved by the ethical committee of Nagoya University Graduate School of Medicine and written informed consent was obtained from each subject prior to the study. Seventy patients with alcoholic chronic pancreatitis (65 males, 5 females; mean age 61.3 years, range 37–82), 18 patients with idiopathic chronic pancreatitis (13 males, 5 females; mean age 55.6 years, range 24–85), and 180 normal subjects (156 males, 24 females; mean age 52.3 years, range 19–87) participated in this study. Diagnosis of chronic pancreatitis was based on the criteria of the Japan Pancreas Society (42). Patients who consumed ethanol over 60 g/day for more than 10 years were considered alcoholic. Patients with no or occasional social alcohol intake were classified as idiopathic following the exclusion of known rare causes of chronic pancreatitis.

Analysis of the *CFTR*, *SPINK1*, and *PRSS1* genes

Genomic DNA was extracted from blood leukocytes. PCR was carried out using the primers shown in Table 1. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). The sequence reaction was carried out using the GenomeLab Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coutler, Fullerton, CA). The reaction products were purified using the Centri-Sep spin columns (Applied Biosystems, Foster, CA) and sequenced by CEQ 8000 system (Beckman Coutler).

All 27 exons and their boundaries (100-300 bp including c.1210-12T (5-9), poly T and c.1210-34GT (9-13), TG repeats in intron 9) and promoter region (up to 1,028 bp upstream of the translation initiation codon of exon 1) of the *CFTR* gene were sequenced for samples from alcoholic and idiopathic chronic pancreatitis. Six *CFTR* variants: c.650A>G, p.Glu217Gly

(E217G); c.1666A>G, p.Ile556Val (I556V); M470V; L1156F; Q1352H; and R1453W were detected. The presence of these variants in normal subjects was screened by SNP typing with Masscode system (Shimadzu, Kyoto, Japan) and confirmed by direct sequencing in positive and equivocal cases. In subjects carrying CFTR variants, the presence of c.101A>G, p.Asn34Ser (N34S) and IVS3+2T>C mutations in the *SPINK1* gene and c.365G>A, p.Arg122His (R112H) and c.86A>T, p.Asn29Ile (N29I) mutations in the *PRSS1* gene were analyzed by sequencing.

Measurement of sweat Cl^- concentration

Cl^- concentrations in insensible sweat were estimated by dividing the amount of Cl^- recovered from one thumb by the amount of sweat measured in the other thumb as we described previously (33, 34). This method is based on the observation that the sweat rates of the right and left fingers are almost identical (44). Sweat rate from one thumb was measured by a perspiration meter (Perspiro 201, Suzuken, Nagoya, Japan) and sweat during the period was collected from the other thumb. The Cl^- content was measured by capillary electrophoresis (Bio-Rad, Hercules, CA).

Evaluation of Pancreatic Exocrine Function

Pancreatic exocrine function was evaluated by the secretin test as we previously described (25). Duodenal intubation was performed to collect pancreatic juice. After an acclimation period, 80 U/body of human secretin (ChiRhoStim, ChiRhoClin, Burtonsville, MD) was administered intravenously and pancreatic juice was collected every 10 minutes for 60 minutes. Total secreted volume (mL/h) and total amylase output (U/h) were measured. The HCO_3^- concentration in each sample was measured and the highest value was set as the maximum HCO_3^- concentration (mEq/L). The lower limits of normal range of volume, amylase output, and the maximum HCO_3^- concentration (MBC) are 183 mL/h, 99,000 U/h

and, 80 mEq/L (25).

Preparation of DNA constructs of CFTR variants

Wild-type human CFTR cDNA in pCMV vector (pcDNA3-CFTR) was provided by Prof. K. Kirk (University of Alabama School of Medicine). CFTR mutations (M470V, L1156F, and both of M470V and L1156F) were introduced by site-directed mutagenesis (QuickChange mutagenesis kit, Stratagene, La Jolla, CA). The primers used for M470V are: sense 5'-GGCAAGACTTCACTTCTAATGGTGATTATGGGAGAACTGG-3', antisense 5'-CCAGTTCTCCCATAATCACCATTAGAAGTGAAGTCTTGCC-3'. The primers used for L1156F are: sense 5'-ATAGATGTGGATAGCTTTATGCGATCTGTGAGCCGAGTCT-3', antisense 5'-AGACTCGGCTCACAGATCGCATAAAGCTATCCACATCTAT-3'.

Expression of CFTR variants in HEK293 and CFPAC-1 cells

Cells were cultured in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% fetal calf serum, penicillin (100 U/ ml) and streptomycin (100 mg/ml) in 35 mm dishes. Cells were transfected with 2.5 μg of each plasmid using Lipofectamin or Lipofectamine LTX & PLUS Reagent (Life Technologies, Carlsbad, CA). Cells were used 24-72 h post-transfection for experiments.

Real-time PCR was performed to confirm the successful transfection. Total RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and reverse transcribed using the PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Otsu, Japan). A fluorogenic SYBR Green and Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA) were used for real time quantification. The primers used for CFTR are: sense 5'- TGCCCTTCGGCGATGTTTTT-3', antisense

5’-GTTATCCGGGTCATAGGAAGCTA-3’.

For western blot analysis, cells were lysed in a Passive Lysis Buffer (Promega, Madison, WI). Thirty micrograms of protein were suspended in sodium dodecyl sulfate (SDS) sample buffer (Life Technologies) and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane and probed with a monoclonal antibody against the NBD2 domain of CFTR (M3A7, Millipore, Billerica, MA, or sc-10747, Santa Cruz, Dallas, TX). After treating with the secondary antibody, protein bands were visualized by the enhanced chemiluminescence using ECL Western Blotting Starter Kit (GE Healthcare Bio-Sciences, Piscataway, NJ).

Effects of ethanol (EtOH), acetaldehyde (ALD), palmitoleic acid (POA) (Santa Cruz, Dallas, TX), and palmitoleic acid ethyl ester (POAEE) (Santa Cruz, Dallas, TX) on the protein expression were examined by treating cells for 24-48 hours. POA and POAEE were solubilized according to a previous work (29).

Cl^- channel activity of CFTR variants in HEK293 cells

Macroscopic Cl^--current recordings by whole-cell patch-clamp were performed on HEK293 cells transfected with CFTR variants. The pipette solution contained 150 mM N-methyl-D-glucamine-Cl (NMDG-Cl), 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM ATP, and 10 mM HEPES at pH 7.3. The bath solution contained 150 mM NMDG-Cl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES-Tris at pH 7.4. Cells were stimulated with 10 μM forskolin. The membrane potential was held at -60 mV. CFTR Cl^- current was confirmed by the complete inhibition of the current with 100 μM glibenclamide. Currents were recorded using the Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Data were collected at 5 kHz and filtered at 1 kHz.

HCO_3^- and Cl^- transport by CFTR variants expressed in *Xenopus laevis* oocytes

Oocytes were obtained by partial ovariectomy of anesthetized female *Xenopus*. Healthy oocytes in stages V-VI were injected with 5 ng cRNA of CFTR variants in a final volume of 50 nl. Injected oocytes were incubated at 18°C in a ND96 solution and used 48-120 h after injection.

Intracellular pH (pH_i) and Cl^- concentration (Cl^-_i) were measured at room temperature as previously described (41). In brief, the electrodes were prepared from single-barreled borosilicate glass tubes and vapor silanized with bis (dimethylamino) dimethyl silane. The tips of the pH electrodes were filled with H^+ exchanger resin (hydrogen ionophore I, cocktail B; Sigma-Aldrich, St. Louis, MO). The electrodes were fitted with a holder with an Ag-AgCl wire attached to a high-impedance probe of a two-channel electrometer (FD-223; World Precision Instruments, Sarasota, FL). A second channel was used for the measurement of membrane potential by standard reference microelectrodes. The signal from the voltage electrode was subtracted from the voltage of the pH electrode. HCO_3^- fluxes were calculated from the change of pH_i and the buffering capacity. Cl^-_i was measured with a Cl^--sensitive liquid ion exchanger (477913; Corning, Corning, NY). The tips of vapor-silanized electrodes were filled with the Cl^--selective liquid ion exchanger and backfilled with 3 M KCl. Cl^-_i was calculated according to the equation: $Cl^-_i = Cl^-_{cal} \times 10^{(\Delta V/S)}$, where Cl^-_{cal} is the Cl^- activity of the calibration solutions, ΔV is the difference in voltage between the Cl^- electrode and reference electrode, and S is the slope measured in response to a 10-fold change in Cl^- activity. Membrane current was measured with two-electrode methods using an OC-725C Oocyte Clamp System (Warner Instrument, Hamden, CT).

Cl^--HCO_3^- exchange activity in CFPAC-1 cells transfected with CFTR variants

CFPAC-1 cells transfected with CFTR variants were loaded with BCECF and first bathed in

the standard HCO_3^--CO_2-buffered solution containing 1 μM forskolin. The bath solution was switched to the Cl^--free HCO_3^--CO_2-buffered solution (Cl^- was replaced with glucuronate) in the presence of forskolin. The activity of Cl^--HCO_3^- exchange was estimated from the rate of pH_i increase upon removal of Cl^-.

Molecular modeling of CFTR

A molecular model of CFTR protein was constructed by homology modeling using Discovery studio (Accelrys, San Diego, CA). The sequence alignment of 2 transmembrane domains (TMD1 and TMD2) and 2 nucleotide-binding domains (NBD1 and NBD2) were performed using 3G5U (for TMD1 and TMD2), 1XMI (for NBD1), and 3GD7 (for NBD2) as template-structures according to a previous work (14). Energy minimization and equilibration were performed using the CHARMM22 force field. This atomic model lacks the regulatory domain (R domain, residues 656-857), N-terminal (residues 1-44) and C-terminal (residues 1461-1480) regions.

Statistical analysis

For clinical data, χ^2 test with Yates correction if appropriate was used for statistical analysis. Data of *in vitro* experiments are presented as means ± SEM of the indicated number of experiments. Statistical analysis was carried out by Student's *t*-test or ANOVA followed by Dunnett's test for multiple comparisons.

RESULTS

CFTR variants in Japanese patients with chronic pancreatitis

Established CF-causing mutations were not found. Six variants (E217G, I556V, M470V, L1156F, Q1352H, and R1453W) were identified in coding regions of the *CFTR* gene (Table 2). The allele frequencies of L1156F and Q1352H in alcoholic chronic pancreatitis (5.0 and 7.9 %) were significantly ($p<0.01$) higher than those in normal subjects (0.6 and 1.9 %). The allele frequency of R1453W in idiopathic pancreatitis (11.1%) was significantly ($p<0.01$) higher than that in normal subjects (1.9%). The allele frequencies of E217G, I556V, and M470V were not different among groups.

Genotypes, pancreatic exocrine function, and sweat Cl^- of chronic pancreatitis patients carrying the L1156F

Table 3 shows the characteristics of 8 patients with alcoholic (7 males) and idiopathic (1 female) chronic pancreatitis who carry the L1156F variant. Genotypes of *CFTR* (presence of L1156F and G1352H, M/V470, poly T, and TG repeats), sex, age, etiology of pancreatitis, presence or absence of pancreatic stone, pancreatic exocrine function (secretin test), sweat Cl^- concentration, and mutations in *SPINK1* and *PRSS1* are shown. Age (mean 58.8 years, range 49-73) was not different from that of total patients.

Pancreatic stone was found in 7 of 8 patients. Secretin test was performed in 3 patients. Volume and amylase output of collected pancreatic juice from 3 patients were all below the normal lower limits. The maximum HCO_3^- concentration (MBC) of pancreatic juice from 2 of 3 patients was below the normal lower limits.

There was no homozygote of L1156F. All patients carrying the L1156F had M470V on at least one allele suggesting that L1156F is linked to M470V. Two patients had the Q1352H

variant which is also associated with alcoholic chronic pancreatitis. Two patients had a genotype of 7/7 poly T and 11/11 TG repeats, while 6 patients had a genotype of 7/7 poly T and 11/12 TG repeats. One patient had N34S mutation in *SPINK1*. Sweat Cl^- was measured in 5 patients. Sweat Cl^- of 3 patients was >60 mM and that of 2 patients was in the intermediate range (40-60 mM).

Expression of CFTR variants in HEK293 cells

The protein expression wild-type CFTR and 3 CFTR variants (M470V-CFTR, L1156F-CFTR, M470V+L1156F-CFTR) transfected in HEK293 cells was examined by western blot analysis (Figure 1). The approximately 170 kD bands corresponding to glycosylated mature 'band C' CFTR proteins and another smaller-size fragment (~150 kD) corresponding to immature 'band B' CFTR protein were detected. When the band intensities of mature CFTR were normalized against those of β-actin, the expression of M470V+L1156F-CFTR was reduced to 60 ± 10 % ($p<0.01$) compared to wild-type CFTR. The expression of M470V-CFTR and L1156F-CFTR was not significantly different from that of wild-type CFTR.

Effects of ethanol (EtOH) and acetaldehyde (ALD) on the protein expression of wild-type and M470V+L1156F CFTR were examined (Figure 2). Treatment with combination of EtOH (50 mM) and ALD (200 μM) for 24 hours significantly ($p<0.05$) inhibited the expression of wild-type CFTR, while EtOH and ALD by themselves did not affect the expression. The expression of M470V+L1156F CFTR was not affected by EtOH, ALD, and their combination.

Free fatty acids and fatty acid ethyl esters, non-oxidative metabolites of ethanol have toxic effects on pancreatic acinar cells (9). Previous studies demonstrated that palmitoleic acid (POA) and palmitoleic acid ethyl ester (POAEE) inhibited the protein expression and function of CFTR in pancreatic duct cells (23, 29). Treatment with POA (100 μM) or POAEE (100

μM) for 48 hours significantly ($p<0.05$) inhibited the expression of wild-type CFTR (Figure 3). The expression of M470V+L1156F CFTR was not affected by POA and POAEE.

Cl^- channel activity of CFTR variants in HEK293 cells

Cl^- channel current of wild-type CFTR and 3 CFTR variants (M470V-CFTR, L1156F-CFTR, M470V+L1156F-CFTR) was measured by the whole cell configuration (Figure 4). Stimulation with 10 μM forskolin generated a large Cl^- current in cells transfected with wild-type CFTR. The Cl^- current was abolished by glibenclamide (100 μM). The magnitude of Cl^- current by M470V-CFTR and L1156F-CFTR was similar to that by wild-type CFTR. The magnitude of Cl^- current by M470V+L1156F-CFTR was smaller than that by wild-type CFTR, but the difference was not statistically significant.

HCO_3^- and Cl^- transport by CFTR variants expressed in *Xenopus laevis* oocytes

HCO_3^- and Cl^- fluxes were examined in *Xenopus laevis* oocytes expressing wild-type CFTR or 3 CFTR variants (M470V-CFTR, L1156F-CFTR, M470V+L1156F-CFTR) (Figure 5). When oocytes expressing wild-type CFTR were perfused with the HCO_3^--CO_2-buffered solution and stimulated with forskolin, removal of extracellular Cl^- caused a rapid decrease of Cl^-_i (Figure 5B) and elevation of pH_i (Figure 5A), which are probably largely due to Cl^- efflux and HCO_3^- influx via activated CFTR, respectively. Thus the activity of HCO_3^- and Cl^- transport by CFTR can be estimated from the initial rate of pH_i/Cl^-_i changes (Figure 5C). In oocytes expressing M470V+L1156F-CFTR, removal of extracellular Cl^- caused a slower Cl^-_i decrease (Figure 5B) and a slower pH_i elevation (Figure 5A). The activity of HCO_3^- and Cl^- transport by M470V+L1156F-CFTR was significantly ($p<0.01$) reduced to 52 and 57 % of that by wild-type CFTR (Figure 5C). Unexpectedly, membrane current by M470V+L1156F-CFTR was similar to that by wild-type CFTR. M470V and L1156F by themselves did not affect CFTR-mediated HCO_3^-/Cl^- transport.

317 **Cl^--HCO_3^- exchange activity coupled with CFTR variants**

318 It has been known that CFTR and SLC26 Cl^--HCO_3^- exchangers (SLC26A3 and A6) are
319 physically and functionally coupled to work as machinery for HCO_3^- secretion and that the
320 Cl^--HCO_3^- exchange activity of SLC26A3 and A6 is dependent on the presence of functional
321 CFTR (26). CFPAC-1, a human pancreatic duct cell line bearing F508del-CFTR
322 endogenously expresses SLC26A3 and A6 (15). The regulation of SLC26 Cl^--HCO_3^-
323 exchangers by CFTR variants were examined by transfecting the variants to CFPAC-1 cells
324 (Figure 6). The Cl^--HCO_3^- exchange (AE) activity was estimated from the rate of pH_i increase
325 upon removal of Cl^-. In cells transfected with wild-type CFTR, stimulation with forskolin (1
326 μM) increased the rate of pH_i increase by ~4 fold from 0.77 ± 0.10 (n = 18) to 3.28 ± 0.13 (n
327 = 25). The stimulated pH_i response was decreased by only 8.8% to 2.99 ± 0.07 (n = 18) with
328 CFTRinh-172 (10 μM) and thus largely mediated by AE, which is consistent with a previous
329 report that transfection of wild-type CFTR augmented DIDS-sensitive AE activity in
330 CFPAC-1 cells (15).

331 The CFTR-dependent AE activity was compared under forskolin stimulation (Figure 6).
332 Transfection of wild-type CFTR increased the AE activity by ~5 fold (compared with mock).
333 The AE activity in cells transfected with 3 CFTR variants; M470V, L1156F, and
334 M470V+L1156F-CFTR was all much smaller ($p<0.01$) compared to cells transfected with
335 wild-type CFTR. The AE activity coupled with M470V, L1156F, and M470V+L1156F-CFTR
336 was 33%, 35%, and 26% of that coupled with wild-type CFTR. The AE activity coupled with
337 M470V+L1156F-CFTR was significantly ($p<0.05$) smaller compared to that with
338 L1156F-CFTR.

339 **Localization of M470 and L1156 in homology model of CFTR**

340 To examine the possible effects of M470V and L1156F on CFTR function at the molecular

341 level, we constructed a homology model of CFTR in the inward-facing conformation (closed
342 state). Figure 7 indicates M470 in NBD1 and L1156 in the junctional residues between TMD2
343 and NBD2. Thus M470V may affect ATP binding to NBD1 and L1167F may affect
344 conformational transition between closed and open states. However, M470 and L1156F are
345 not close to each other, and it is not clear why the double mutation (combination of M470V
346 and L1156F) but not single mutations affected the transport Cl^- and HCO_3^- (Figure 5).

347

DISCUSSION

Spectrum of CFTR variants in Japanese

The spectrum of *CFTR* mutations/polymorphisms shows considerable regional and ethnic variations (13, 38) and some of the polymorphisms (such as poly T, TG repeats, and M470V) as well as mutations are known to significantly affect CFTR function. Thus the association of CFTR variants/dysfunction and CF/CFTR-related diseases including chronic pancreatitis may vary among different ethnic groups.

Common disease-causing mutations in Europeans such as F508del have never been identified in Japanese CF patients. Most of the mutations found in alleles inherited from Japanese/Asian ancestry are of rare types (22, 30, 48). Our recent data suggest that c.2908+1085_3367+260del7201 (*CFTR*dele16-17b) is the major CF-causing mutation in Japanese (20, 31).

Among polymorphisms, longer TG repeats in IVS8 increase the probability of exon 9 skipping (43) and M470V-CFTR causes mild channel dysfunction to ~60% (10, 27). In a previous study, we have performed haplotype analysis of *CFTR* polymorphisms in Japanese (13). While (TG)10 and (TG)11 are common in Europeans, Japanese had longer (TG)11 or (TG)12. The haplotype frequencies of (TG)n-M/V470 in Japanese were ~51% for (TG)11-V470, ~31% for (TG)12-M470, and ~16% for (TG)12-V470. The data predict that background CFTR function in Japanese is lower than that in Europeans.

The association of chronic pancreatitis and 3 Japanese/Asian types of CFTR variants (L1156F, Q1352H, and R1453W) were demonstrated in our present and previous (13) studies. Twenty-three of 88 patients with chronic pancreatitis (26.1%) carried 1 or 2 of the 3 variants, which was 2.9 times higher than normal subjects (Table 2). None of the CF-causing mutations

was found and thus the spectrum of pancreatitis-related CFTR variants is different from that of CF-related mutations. Although 2 other studies indicated a higher frequency of 5T in Japanese patients with chronic pancreatitis (2, 24), we have not found similar association (Table 2) (13).

Epidemiology of L1156F-CFTR

While Q1352H and R1453W are also found in Koreans (27) and thus categorized to Asian-type CFTR variants, L1156F is probably a Japanese-specific CFTR variant and has not been reported from other countries. Guanine to thymine substitution at nucleotide position 3468 in the *CFTR* gene (c.3468G>T) results in the leucine to phenylalanine substitution at 1156 in the polypeptide. L1156F was previously found in an adult, Japanese healthy female (Cystic Fibrosis Mutation Database), and a recent study reported the association of L1156F and chronic pancreatitis in Japanese (32).

In the present study, we have found that L1156F is associated with alcoholic chronic pancreatitis in Japanese (the odds ratio = 9.0 when compared to normal subjects, Table 2). L1156F was found in 10.0% in patients with alcoholic chronic pancreatitis. While Q1352H was also found in patients with CBAVD (1) and diffuse panbronchiolitis and R1453W in patients with diffuse panbronchiolitis (Cystic Fibrosis Mutation Database), L1156F has not been found in other CFTR-related diseases. Thus L1156F is probably a pancreatitis-specific CFTR variant.

Functional characteristics and molecular modeling of L1156F-CFTR

Although some CFTR variants have been reported to increase the risk for developing chronic pancreatitis, the functional characteristics were examined only for a few variants (10, 27, 39). Since L1156F is most likely linked to M470V (Table 2), we analyzed the protein expression,

Cl^- channel activity, HCO_3^-/Cl^- transport activity, and CFTR-coupled Cl^--HCO_3^- exchange activity of 3 CFTR variants (M470V-CFTR, L1156F-CFTR, M470V+L1156F-CFTR) using heterologous expression systems.

The combination of M470V and L1156F reduced the expression of mature 'band C' form of CFTR protein to 60~70% (Figures 1, 2, and 3). The effects of M470V and L1156F by themselves on the protein expression were not significant due to a considerable variation between experiments. The variable effects of M470V may be related to the different rate of maturation between M470 and V470 CFTR proteins (10).

The M470V+L1156F-CFTR variant expressed in *Xenopus laevis* oocytes showed a reduction of HCO_3^-/Cl^- transport activity to 50~60% of wild-type CFTR (Figure 5), while L1156F by itself caused almost no reduction. However, the M470V+L1156F-CFTR variant expressed in HEK293 cells showed a weak (by ~20%) and non-significant reduction of whole cell Cl^- channel current compared to wild-type CFTR (Figure 4). The difference may be due to the different systems of heterologous expression. To the contrary, CFTR-coupled Cl^--HCO_3^- exchange activity was substantially impaired by M470V, L1156F, and the double mutation (M470V+L1156F, Figure 6).

M470 and L1156F are not close to each other in the inward-facing conformation (closed state) in a predicted model of CFTR (Figure 7), which do not explain why the combination of M470V and L1156F impaired Cl^-/HCO_3^- transport activity.

L1156F-CFTR variant and chronic pancreatitis

Genotype-phenotype correlation in CF is most evident for exocrine pancreatic status (11). Particular cases of chronic pancreatitis develop as atypical CF with single organ disease phenotypes or CFTR-related disorders (5). CFTR mutations have been frequently found in

patients with chronic pancreatitis (4). Thus pancreas is probably more sensitive to mild CFTR dysfunction than the other organs. The CFTR variant, L1156F, identified in the present study is associated with chronic pancreatitis but not CF, which is consistent with the idea.

The present 8 cases of adult-onset chronic pancreatitis carrying L1156F are probably not categorized to atypical CF. However high level of sweat Cl^- in 3 patients indicates CFTR dysfunction. We may predict total CFTR function *in vivo* from genotypes (Table 3) and the CFTR channel activity of each variant. The activity of M470V+L1156F-CFTR is 50~80% of the wild type CFTR (Figures 4 and 5), that of M470V+Q1352H-CFTR was ~1% of the wild type (27), and that of M470V-CFTR was 60~95% of the wild type (10, 27) (Figures 4 and 5). Thus the predicted total CFTR function of the present 8 cases with chronic pancreatitis carrying L1156F may vary from ~30 to ~90% of the wild type. In addition, CFTR-coupled Cl^--HCO_3^- exchange activity was substantially affected by M470V, L1156F, and the double mutation (Figure 6). Thus CFTR-dependent HCO_3^- transport by pancreatic duct is probably further impaired, which may increase the risk of developing chronic pancreatitis.

In European populations, not only CF carriers (8) but also subjects carrying one mild/uncommon *CFTR* mutation (45) are at an increased risk of chronic pancreatitis. A study from United States reported that a risk of chronic pancreatitis was increased by the presence of p.R75Q, a non CF-causing, HCO_3^--conductance impairing CFTR variant (40). Thus non CF-causing CFTR variants increase the susceptibility to chronic pancreatitis in both Europeans and Japanese. Chronic pancreatitis is now thought to be a complex disease caused by complicated interactions between various genes and environmental factors (7). Mild CFTR dysfunction due to non CF-causing variants may be one genetic factor. A recent study from Germany reported that compound and trans-heterozygosity of *CFTR*, *SPINK1*, *CTRC*, and *PRSS1* variants is an overt risk factor of idiopathic chronic pancreatitis (39).

Although alcohol abuse is the major cause of chronic pancreatitis, the mechanisms by which ethanol induces pancreatic injury have not well been understood. Recent studies demonstrated that ethanol and its non-oxidative metabolites, fatty acid ethyl esters inhibited the protein expression and function of CFTR in pancreatic duct cells (23, 29). In the present study, combination of EtOH and ALD (Figure 2) and POAEE (Figure 3) decreased the protein expression of wild-type CFTR in HEK293 cells. Only a minority of heavy drinkers develop pancreatitis, which suggests the existence of predisposing and protective genetic factors. N34S-SPINK1 allele and *CTRC* variants were reported to be more frequent in patients with alcoholic chronic pancreatitis than in controls, while loss-of-function variants of *PRSS1* and *PRSS2* were overexpressed in controls when compared to patients with alcoholic pancreatitis (7). On the other hand, the association of CFTR variants and alcoholic chronic pancreatitis has not yet been clarified (37).

Although one of 18 patients (5.6%) with idiopathic chronic pancreatitis had L1156F, L1156F was more prevalent (10.0%) in the patients with alcoholic chronic pancreatitis in the present study (Table 2). One patient with alcoholic chronic pancreatitis had M470V+L1156F-CFTR and N34S-SPINK1 (Table 3). In the present study, we have for the first time identified the alcoholic chronic pancreatitis-susceptibility genotype, M470V+L1156F-CFTR. The protein expression of M470V+L1156F-CFTR was not affected by EtOH, ALD, and POAEE (Figures 2 and 3). The association of L1156F and ethanol needs to be further investigated.

In summary, our present data suggest that the Japanese-specific CFTR variant, L1156F, is associated with alcoholic chronic pancreatitis. L1156F is linked with the world-wide CFTR variant M470V. Combination of L1156F and M470V impairs protein expression and HCO_3^-/Cl^- transport activity of CFTR and CFTR-coupled Cl^--HCO_3^- exchange activity. The molecular mechanisms underlying the synergistic effects of L1156F and M470V need to be investigated.

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GRANTS

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638 **FIGURE CAPTIONS**

639 **Figure 1: Protein expression of CFTR variants transfected in HEK293 cells**

640 (A) HEK293 cells transfected with wild-type CFTR and 3 CFTR variants (M470V-CFTR,
641 L1156F-CFTR, M470V+L1156F-CFTR) were lysed and the separated proteins were blotted
642 with antibodies against the NBD2 domain of CFTR (M3A7, Millipore, Billerica, MA) and
643 β-actin. Negative control: non-transfected cells. (B) The staining intensities of CFTR and
644 β-actin bands were analyzed and the CFTR/β-actin ratios were compared. Data are shown as
645 means ± SEM of 8 independent transfection experiments. * $p < 0.01$, compared to wild-type
646 CFTR.

647 **Figure 2: Effects of ethanol and acetaldehyde on the protein expression of wild-type and**
648 **M470V+L1156F CFTR in HEK293 cells**

649 (A) HEK293 cells were transfected with wild-type or M470V+L1156F CFTR and treated
650 with ethanol (EtOH, 50 mM) and/or acetaldehyde (ALD, 200 μM) for 24 hours. The separated
651 proteins were blotted with antibodies against the NBD2 domain of CFTR (sc-10747, Santa
652 Cruz, Dallas, TX) and β-actin. C: non-transfected cells. (B) The CFTR/β-actin ratios were
653 compared. Data are shown as means ± SEM of 5 independent transfection experiments. *
654 $p<0.05$, compared to non-treated control. # $p<0.05$, compared to wild-type CFTR.

655 **Figure 3: Effects of fatty acid and fatty acid ethyl ester on the protein expression of**
656 **wild-type and M470V+L1156F CFTR in HEK293 cells**

657 (A) HEK293 cells were transfected with wild-type or M470V+L1156F CFTR and treated
658 with palmitoleic acid (POA, 100 μM) or palmitoleic acid ethyl ester (POAEE, 100 μM) for 48
659 hours. The separated proteins were blotted with antibodies against the NBD2 domain of
660 CFTR (sc-10747, Santa Cruz, Dallas, TX) and β-actin. (B) The CFTR/β-actin ratios were

compared. Data are shown as means ± SEM of 6 independent transfection experiments. * $p<0.05$, compared to non-treated control. # $p<0.05$, compared to wild-type CFTR.

Figure 4: Cl^- channel activity of CFTR variants expressed in HEK293 cells

Cl^- channel activity of wild-type CFTR (black trace) and 3 CFTR variants (M470V-CFTR: blue; L1156F-CFTR: green; M470V+L1156F-CFTR: red) were measured in the whole cell configuration. After establishing the whole cell configuration with NMDG-Cl rich solutions, CFTR was activated with 10 μM forskolin. Currents were recorded at a holding potential of -60 mV and peak currents were normalized as current densities (pA/pF). After the peak current was observed, glibenclamide (100 μM) was added to the bath solution. Data are shown as representative traces (A) and means ± SEM of 7-9 experiments (B).

Figure 5: HCO_3^- and Cl^- transport by CFTR variants expressed in *Xenopus laevis* oocytes

Xenopus oocytes expressing wild-type CFTR or 3 CFTR variants (M470V-CFTR, L1156F-CFTR, M470V+L1156F-CFTR) were first bathed in HCO_3^--free media. The bath solution was switched to HCO_3^--CO_2-buffered solution as indicated. After the stabilization of pH_i, the bath solution was switched to Cl^--free HCO_3^--CO_2-buffered solution in the presence of forskolin (1 μM). The initial rates of pH_i (A) and Cl^-_i (B) changes were used to calculate the HCO_3^- and Cl^- fluxes. (C) Means ± SEM of HCO_3^-/Cl^- fluxes and membrane current of 3-6 experiments. * $p<0.01$, compared to wild-type CFTR.

Figure 6: Cl^--HCO_3^- exchange activity in CFPAC-1 cells transfected with CFTR variants

CFPAC-1 cells transfected with wild-type CFTR (black trace), 3 CFTR variants (M470V-CFTR: blue; L1156F-CFTR: green; M470V+L1156F-CFTR: red), and vector alone (gray) were first bathed in the standard HCO_3^--CO_2-buffered solution containing 1 μM

forskolin. The bath solution was switched to Cl^--free HCO_3^--CO_2-buffered solution in the presence of forskolin as indicated. (A) Representative traces are shown. (B) The activity of Cl^--HCO_3^- exchange was estimated from the rate of pH_i increase upon removal of Cl^-. Data are shown as means ± SEM of 20-25 experiments. * $p<0.01$, compared to wild-type CFTR. # $p<0.05$, compared to L1156F-CFTR.

Figure 7: Localization of M470 and L1156 in homology model of CFTR

An molecular model of CFTR in the inward-facing conformation (closed state). The TMD1, NBD1, TMD2, and NBD2 are colored in green, lime, blue, and purple, respectively, with ribbon representation. M470 and L1156F are indicated.

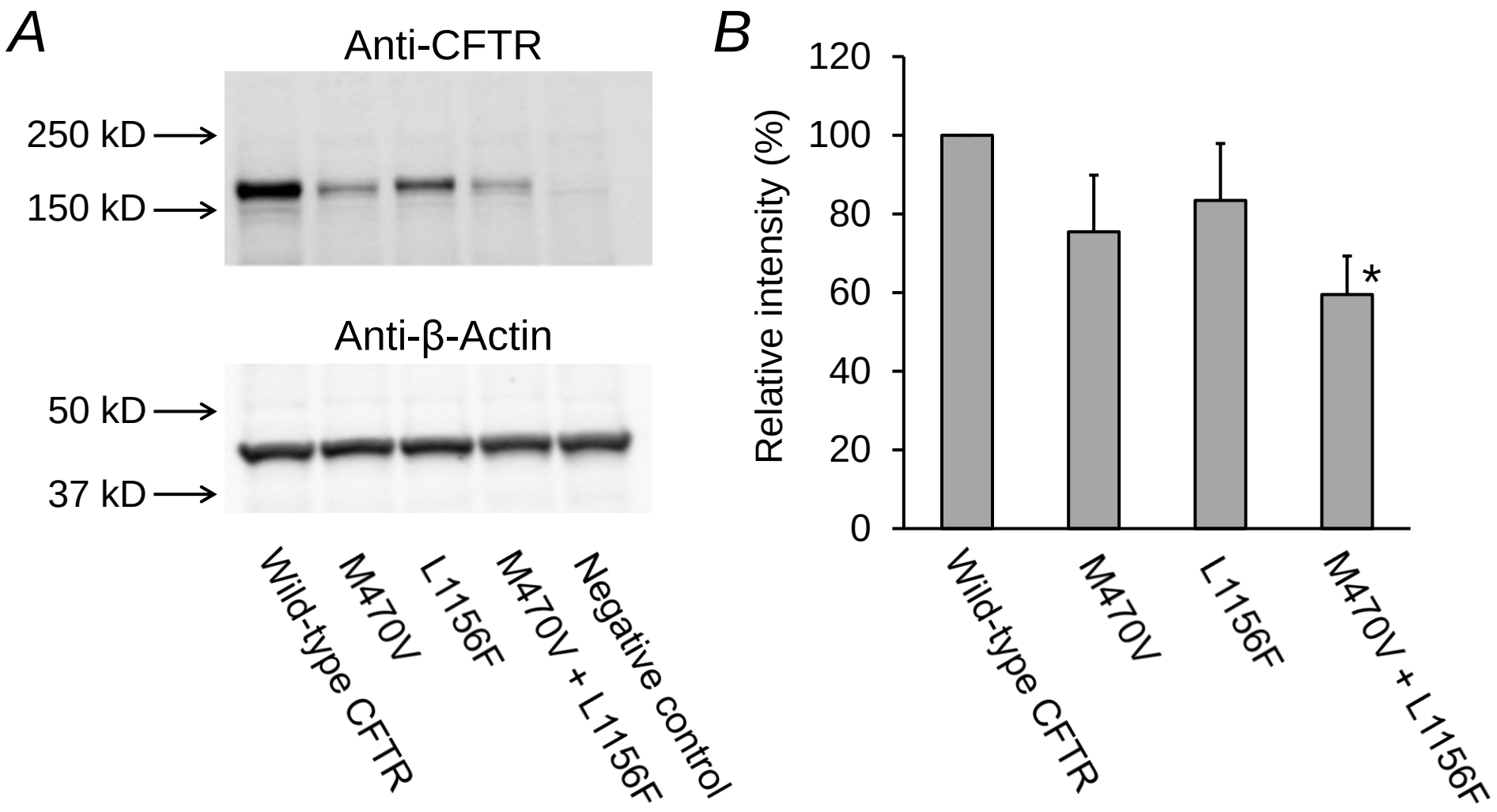
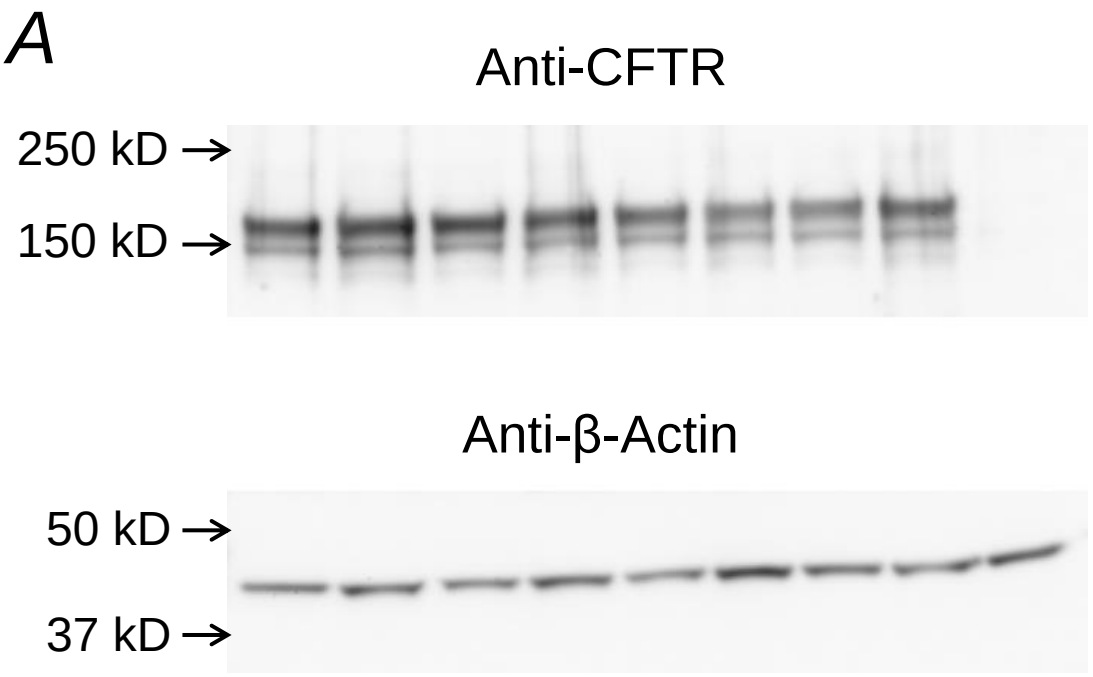

A
Anti-CFTR
250 kD
150 kD
Anti-β-Actin
50 kD
37 kD
Wild-type CFTR
M470V
L1156F
M470V + L1156F
Negative control
B
Relative intensity (%)
120
100
80
60
40
20
0
*
Wild-type CFTR
M470V
L1156F
M470V + L1156F

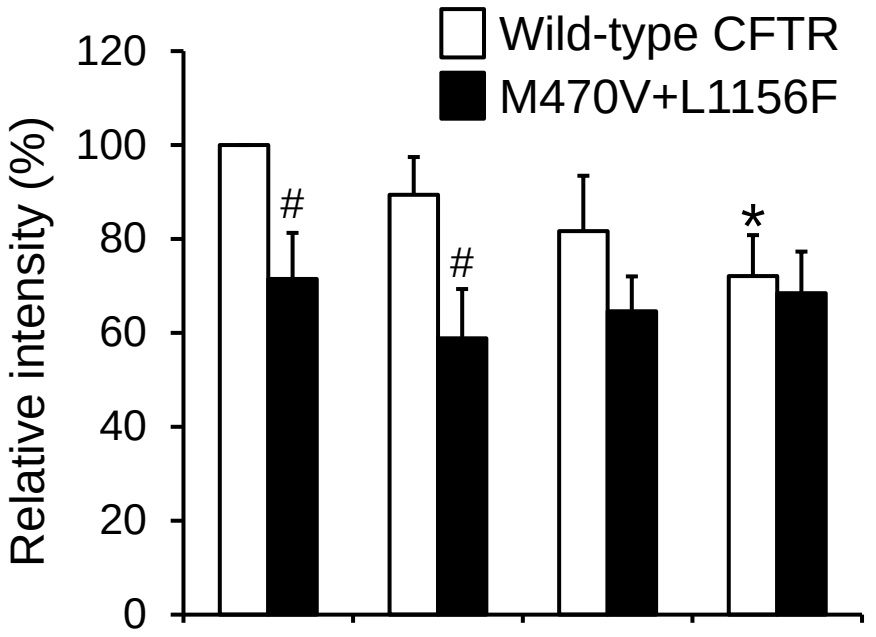
Figure 1

A



EtOH	–	+	–	+	–	+	–	+	–
ALD	–	–	+	+	–	–	+	+	–
	Wild-type				M470V+L1156F				C

B



EtOH	–	+	–	+
ALD	–	–	+	+

Figure 2

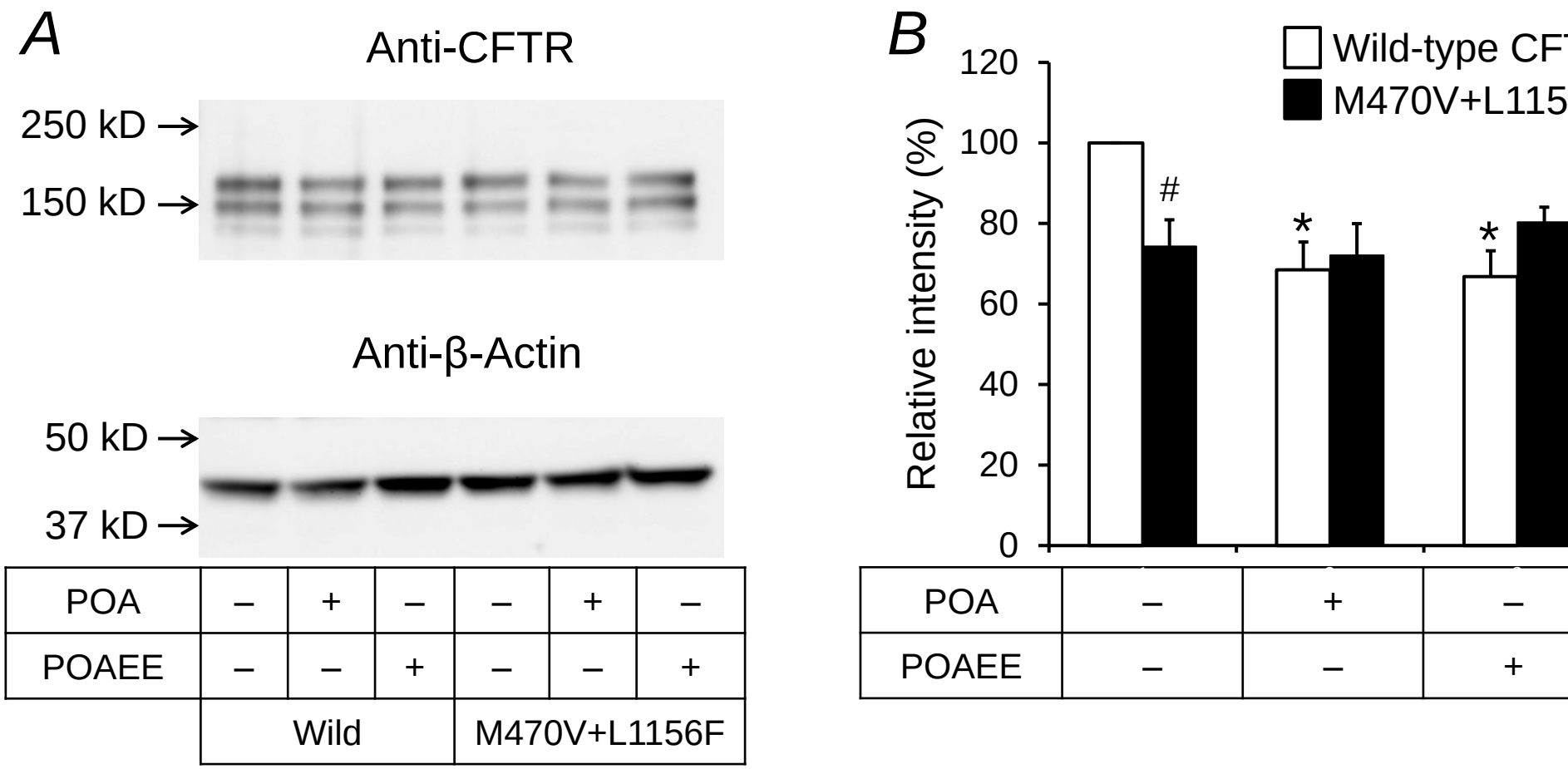

A
Anti-CFTR
250 kD
150 kD
Anti-β-Actin
50 kD
37 kD
POA – + – – + –
POAEE – – + – – +
Wild
M470V+L1156F
B
Wild-type CFTR
M470V+L1156F
Relative intensity (%)
120
100
80
60
40
20
0
#
*
*
POA – + –
POAEE – – +

Figure 3

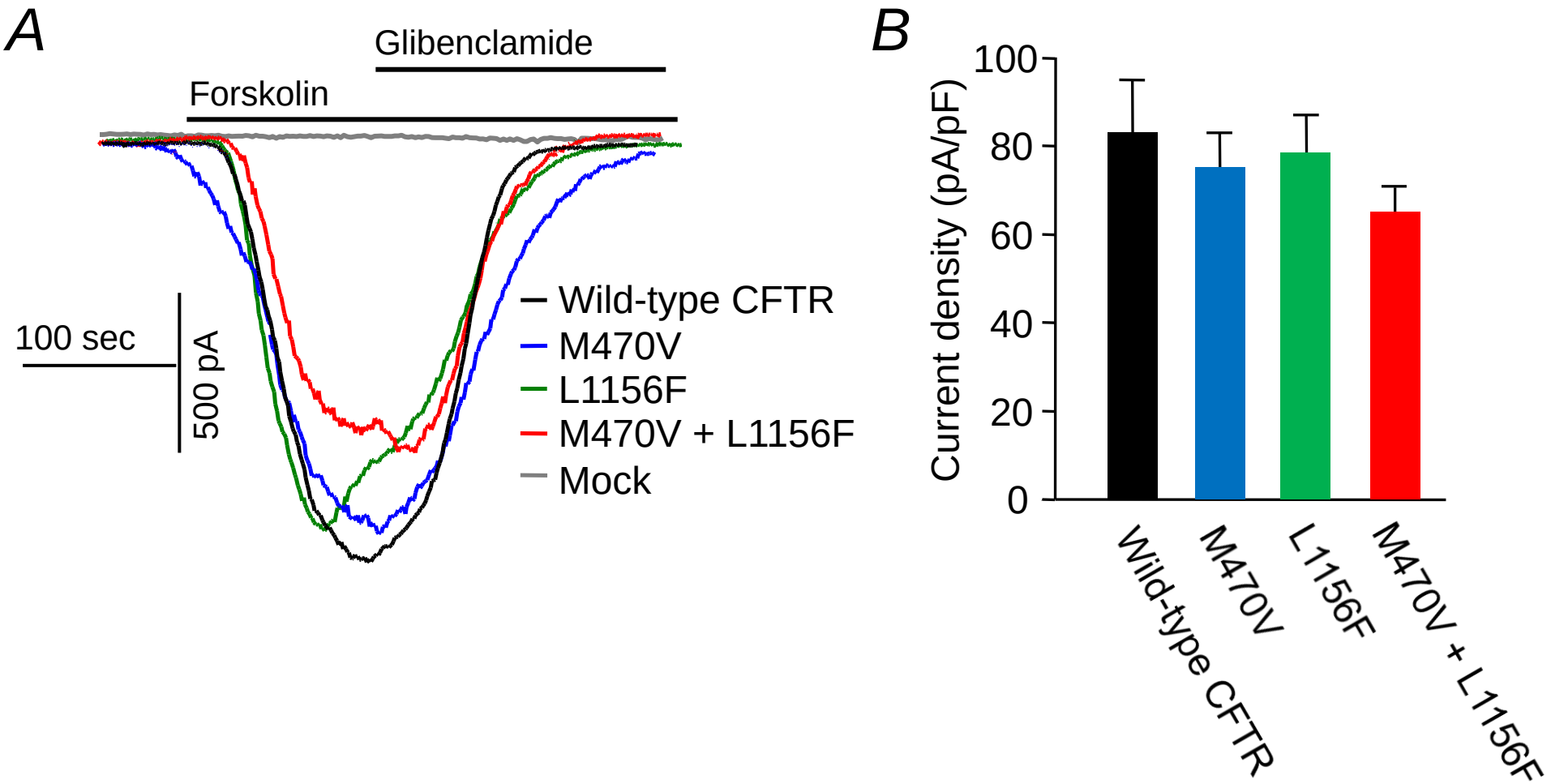

A
Glibenclamide
Forskolin
100 sec
500 pA
Wild-type CFTR
M470V
L1156F
M470V + L1156F
Mock
B
Current density (pA/pF)
100
80
60
40
20
0
Wild-type CFTR
M470V
L1156F
M470V + L1156F

Figure 4

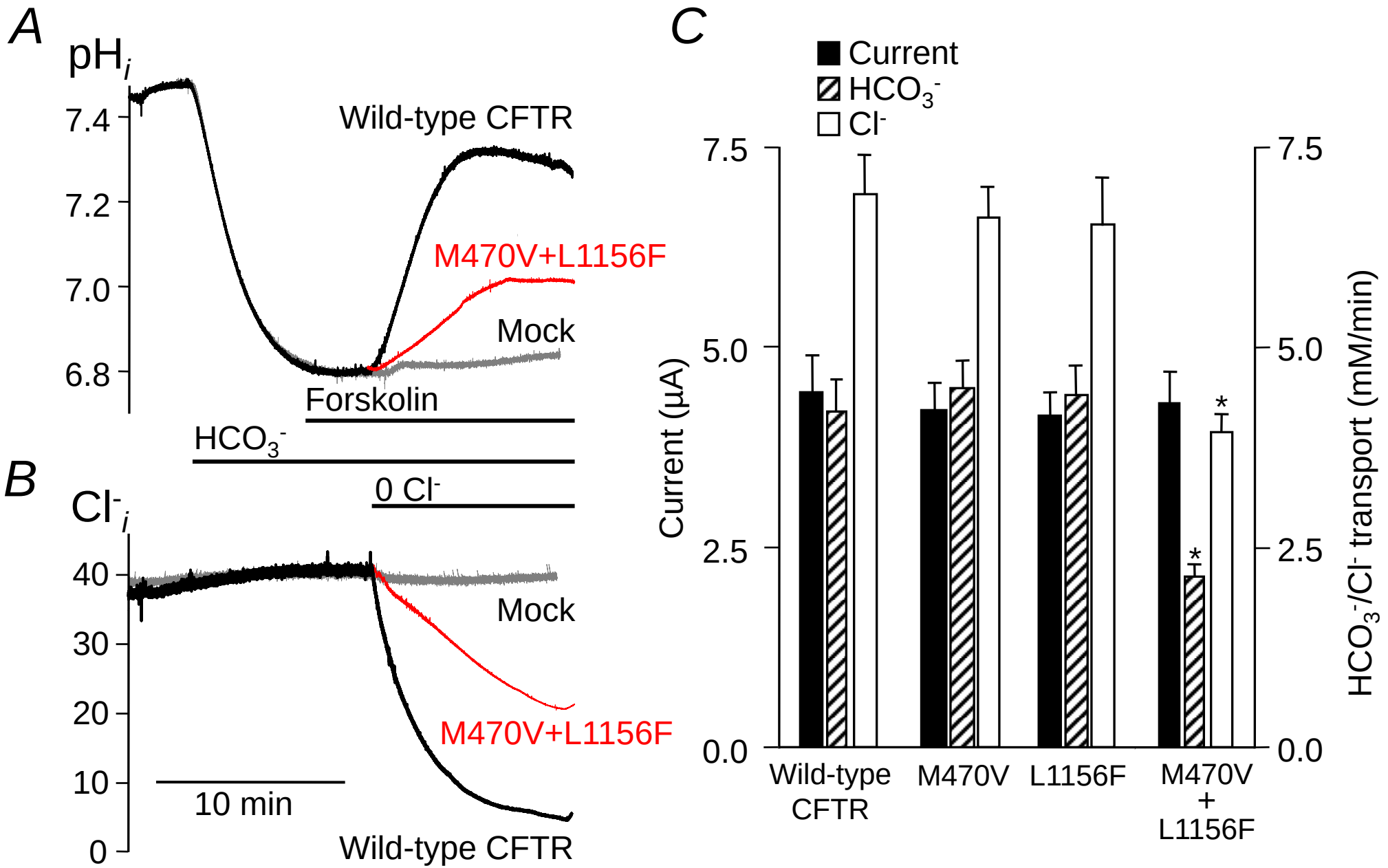

A
pH$_i$
7.4
7.2
7.0
6.8
Wild-type CFTR
M470V+L1156F
Mock
Forskolin
HCO$_3^-$
B
Cl^-_i
40
30
20
10
0
0 Cl$^-$
Mock
M470V+L1156F
10 min
Wild-type CFTR
C
Current
HCO$_3^-$
Cl$^-$
7.5
5.0
2.5
0.0
Current (μA)
HCO$_3^-$/Cl$^-$ transport (mM/min)
*
*
Wild-type CFTR
M470V
L1156F
M470V + L1156F

Figure 5

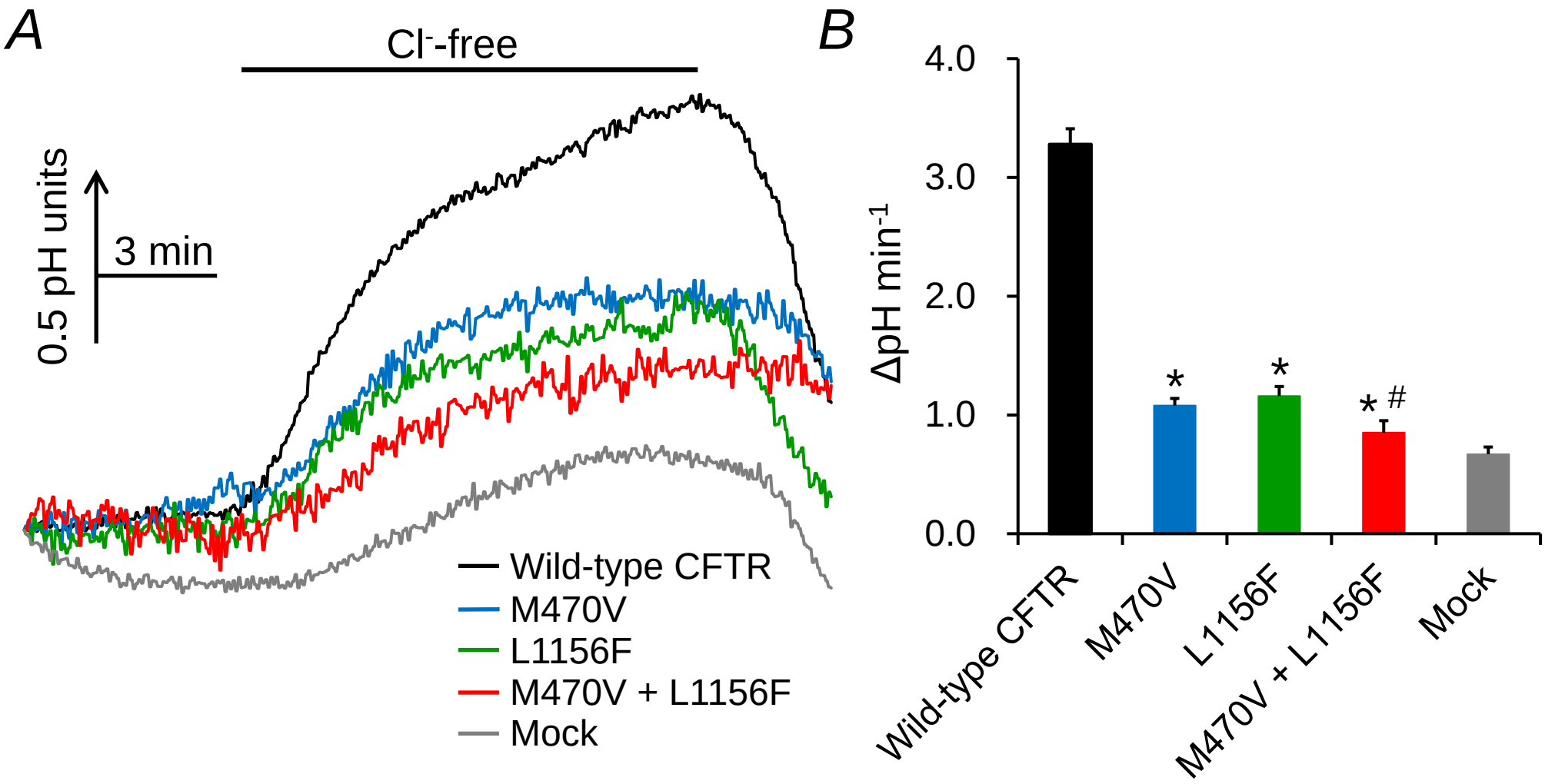

A
Cl-free
0.5 pH units
3 min
Wild-type CFTR
M470V
L1156F
M470V + L1156F
Mock
B
ΔpH min-1
4.0
3.0
2.0
1.0
0.0
*
*
* #
Wild-type CFTR
M470V
L1156F
M470V + L1156F
Mock

Figure 6

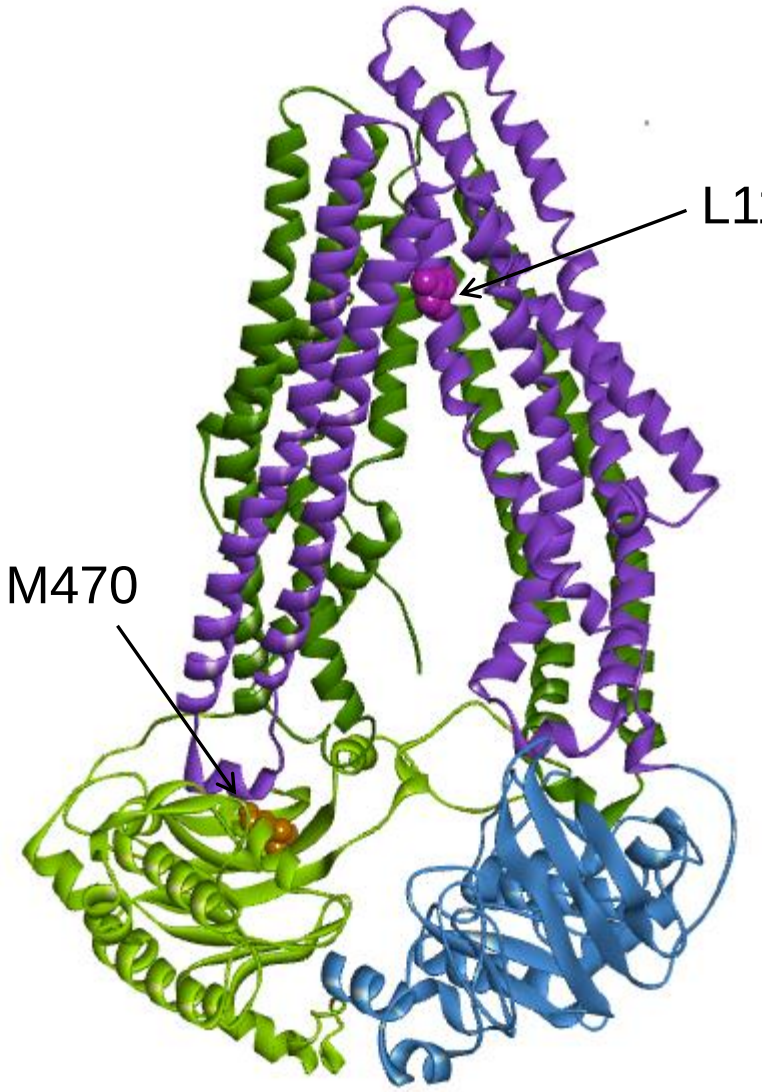


Figure 7

Table 1. Primer sequences for conventional PCR analysis of *CFTR*, *SPINK1*, and *PRSS1* genes

Exon/Intron	Forward primers (5'-3')	Reverse primers (5'-3')
CFTR		
5' UTR	GCCCCTCAGAGAGTTGAAGA	TGATCCTAGTCGGGTTCCTG
5' UTR	AAACGTAACAGGAACCCGACTA	CTCAACCCTTTTTCTCTGACCT
Exon 1	GGAGAAAGCCGCTAGAGCAAA	GTGGCTCTCTATTCAATCAGC
Exon 2	TATTCCCTCCCAATCCCTTT	TGGGATTACAGGCATTAGCC
Exon 2	TGTAAGAGATGAAGCCTGGTA	GCTCCTATTTTTAAATATAAG
Exon 3	CCATGAGATTTTGTCTCTATA	GAGTTGGATTCATCCTTTATA
Exon 4	AAGAGTTTCACATATGGTATG	TGCCATTTATTTAATAGGCAT
Exon 5	GAAGATAGTAAGCTAGATGAA	AATTGACCTTTCTTAGTTTCC
Exon 6a	TGCTCAGAACCACGAAGTGT	ATTAGCTGGGTGTGGTGCAT (1st)
		CTGAGGCAGGAGAATTGCTT (2nd)
Exon 6b	TGGAATGAGTCTGTACAGCG	TGCATGAATATTGACAGAACT
Exon 7	TATAGGCAGAAAGACTCTAGA	TCCTAGTATTAGCTGGCAACT
Exon 8	TGGGTAATTCAGGGTTGCTT	CGCCATTAGGATGAAATCCA
Exon 9	GGCCATGTGCTTTTCAAACT	TCGCCATGTGCAAGATACAG
Exon 10	TTGTGCATAGCAGAGTACCTGAAA	ATTGATCCATTCACAGTAGCT
Exon 11	TCAGCAATGTTGTTTTTGACC	CCAAGATACGGGCACAGATT
Exon 12	CTTCTGCACCACTTTTGAGAA	GCTACATTCTGCCATACCAA
Exon 13	GGTACCAATTTAATTACTACAG	ATTTGTAAGGGAGTCTTTTGC
Exon 13	TGGGATGTGATTCTTTCGACC	GGGAAGAGATATGTCCATTGC
Exon 13	CATTAGAAGGAGATGCTCCTGT	TCGTAATCCTATGATTTTAGT
Exon 14a	ACACTTAGATTCAAGTAATACT	CAGAAGCTAAGAACTATATGA
Exon 14b	GACCCAGGAACACAAAGCAAA	TTCCACTACCATAATGCTTGG
Exon 15	GGTTAAGGGTGCATGCTCTTC	AAGCCAGCACTGCCATTA
Exon 15	TAGACTCAAGTTTAGTTCCATT	TAGACTCAAGTTTAGTTCCATT
Exon 16	TCTGAATGCGTCTACTGTGA	GCAATAGACAGGACTTCAAC
Exon 17a	CACCAGCATGGCACATGTAT	CCAAAATGAAGTCACATGGTCA
Exon 17a	AGAAATAAATCACTGACACAC	CCATGTGTACTTTGTAATATAG
Exon 17b	CAAAGAATGGCACCAGTGTG	CGACAATCTGTGTGCATCG
Exon 17b	TTTAACCAATGACATTTGTGA	ATACCGATTTCAAGGAAATTA
Exon 18	TAGGAGAAGTGTGAATAAAG	GATACACAGTGACCCTCAATT
Exon 19	GCCCGACAAATAACCAAGTG	TGCTTCAGGCTACTGGGATT
Exon 20	CAGGATTGAAAGTGTGCAACA	CTATGAGAAAACTGCACTGGA
Exon 21	TGTTCACAAGGGACTCCAAA	AGGGGTAGGTCCAGTCAAAA
Exon 22	AGAGCCATGTGCCACGTATT	TCCACTGGGCAATTATTTCAT
Exon 23	CCATGGTTGAAAAGCTGATTG	TGAGTAAAGCTGGATGGCTGT
Exon 24	TTTCTGTCCCTGCTCTGGTC	TCTGGCTTGCAAAACACAAG (1st)
		ACTATTGCCAGGAAGCCATTT (2nd)
SPINK1		
Intron 3-Exon 3	CCAATCACAGTTATTCCCCAGAG	GTTTGCTTTTCTCGGGGTGAG
PRSS1		
Exon 2-Exon 3	CCGACAGCAACAGAATAGCA	TGTGTAATGGGCACTCGAAA (1st)
Exon 2	TCCCAACTCCTATCCCACTG	TACCACCCACTGTTCGTTGA (2nd)
Exon 3	CCTCCAGAGCTGTCCATGAG	ATGGGCACTCGAAATGTGTC (2nd)

Table 2. The allele frequencies of polymorphisms in the coding regions of *CFTR* gene

	ACP N=140	ICP n=36	NS n=360
E217G (Exon 6a)			
Glu	137 (97.9%)	36 (100%)	354 (98.3%)
Gly	3 (2.1%)	0 (0%)	6 (1.7%)
M470V (Exon 10)			
Met	60 (42.9%)	14 (38.9%)	143 (39.7%)
Val	80 (57.1%)	22 (61.1%)	217 (60.3%)
I556V (Exon 11)			
Ile	138 (98.6%)	36 (100%)	348 (96.7%)
Val	2 (1.4%)	0 (0%)	12 (3.3%)
L1156F (Exon 18)			
Leu	133 (95.0%)	35 (97.2%)	358 (99.4%)
Phe	7 (5.0%) *	1 (2.8%)	2 (0.6%)
Q1352H (Exon 22)			
Gln	129 (92.1%)	35 (97.2%)	353 (98.1%)
His	11 (7.9%) *	1 (2.8%)	7 (1.9%)
R1453W (Exon 24)			
Arg	138 (98.6%)	32 (88.9%)	353 (98.1%)
Trp	2 (1.4%)	4 (11.1%) *	7 (1.9%)

*: $p<0.01$ vs. NS by Yates Chi square test
ACP: alcoholic chronic pancreatitis
ICP: idiopathic chronic pancreatitis
NS: normal subjects

Table 3. Characteristics of 8 patients who carry L1156F

	CFTR genotypes		Sex	Age	Etiology	Pancreatic Stone	Secretin test* Volume	MBC	Amylase	Sweat [Cl^-]	*SPINK1* mutation	*PRSS1* mutation
1	L1156F + Q1352H	+ V/V470 + 7/7T + 11/11(TG)	M	59	alcoholic	+					-	-
2	L1156F	+ V/V470 + 7/7T + 11/11(TG)	M	65	alcoholic	+	174.5	96.9	21524	42.0	-	-
3	L1156F	+ V/V470 + 7/7T + 11/12(TG)	M	49	alcoholic	-					-	-
4	L1156F + Q1352H	+ M/V470 + 7/7T + 11/12(TG)	M	57	alcoholic	+				88.9	-	-
5	L1156F	+ M/V470 + 7/7T + 11/12(TG)	M	60	alcoholic	+	53.0	50.9	1627	67.0	-	-
6	L1156F	+ M/V470 + 7/7T + 11/12(TG)	M	56	alcoholic	+	149.0	71.3	6891	98.9	-	-
7	L1156F	+ M/V470 + 7/7T + 11/12(TG)	M	51	alcoholic	+					N34S	-
8	L1156F	+ M/V470 + 7/7T + 11/12(TG)	F	73	idiopathic	+				58.9	-	-

Genotypes of CFTR indicate presence of L1156F and G1352H, M/V470, poly T, and TG repeats.

* The lower limits of normal range of volume, the maximum HCO_3^- concentration (MBC), and amylase output are 183 mL/h, 80 mEq/L, and 99,000 U/h.