Wang *et al.*
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Targeted quantitation of CFTR protein expression in vivo using immunoprecipitation & parallel reaction monitoring tandem mass spectrometry

Hui Wang*, Yunxiang Dai, Nicholas Clark, Lianne Boeglin, Caroline Woo, Richard Wooster, Gang Sun* and James C. Sullivan*

Abstract

Background: The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a low-abundance membrane protein. The dysfunction of CFTR protein is the fundamental cause of cystic fibrosis (CF), a fatal genetic disease. In recent years, the novel messenger RNA (mRNA)-based therapy shows high potential to treat CF disease, by delivering CFTR mRNA into lung epithelial cells to generate fully functional CFTR replacement protein. To evaluate mRNA drug efficacy, a targeted quantitative proteomics method is needed to estimate the expression level of mRNA encoded CFTR protein.

Methods: In this paper, a method combining membrane protein extraction, immunoprecipitation (IP), and nanoLC-MS/MS for quantifying CFTR in lung tissue samples was reported for the first time. Absolute quantification was performed by constructing a standard curve by spiking recombinant human CFTR protein in mouse lung tissue matrix.

Results: This method was qualified, with good linearity of standard curve and lower limit of quantification of human CFTR at 1.4 pg per mg tissue. The coefficient of variation of back calculated concentration of all standards and their back-calculation errors were < 20%. The CFTR expression level in mouse lungs dosed with mRNA encapsulated lipid nanoparticle (LNP), and the endogenous level in wild type human lungs were measured successfully.

Conclusions: The result demonstrated high sensitivity, precision and accuracy of this proteomics method for quantifying low-abundance CFTR protein in lung tissue sample.

Keywords: CFTR, CF disease, mRNA therapeutic, Membrane protein, Immunoprecipitation, LC–MS/MS, Tissue, Protein quantification

Background

Cystic fibrosis (CF) is a common, genetic pulmonary disease, which is caused by the mutation of cystic fibrosis transmembrane conductance regulator (CFTR) gene [1, 2]. CFTR is expressed in epithelial cells throughout the body, including the lung, pancreas, and gastrointestinal tract, where it is localized in the apical membrane [3, 4]. There, CFTR acts as an anion channel, where it is responsible for controlling primarily the flux of chloride and bicarbonate ions [5–7]. Patients with CF have mutations that result in a decrease in either the quantity or function of CFTR [8].

The most common CF-causing mutation, F508del, causes a folding defect that results in significantly

*Correspondence: hwang@translate.bio; gangsun_us@yahoo.com; jsullivan@translate.bio

Translate Bio, a Sanofi Company, Lexington, MA 02421, USA

reduced quantity of correctly-folded CFTR and is present in ~ 90% of patients with CF [2, 9, 10]. This phenotype has been determined primarily from cell-based work: the mature, correctly folded and membrane bound version of CFTR is glycosylated and can be differentiated based on molecular weight from the misfolded, non-glycosylated version, for example, grossly by Western blot. CFTR can be over-expressed in cell reporter systems, wherein induction of the F508del variant results in almost complete ablation of correctly folded CFTR protein. Consistent with this, primary human bronchial epithelial (HBE) cell lines have been generated from human lung explants, and where HBE cells derived from patients with the F508del/F508del genotype display little-to-no correctly folded CFTR protein as compared to HBE cells from patients within CFTR variation. In spite of the utility of these cell systems that can qualitatively distinguish between such severe folding defects and wild-type, no methods have been established to quantitate CFTR protein expression, which has hampered basic research in the CFTR protein [11–13].

Much progress has been made therapeutically to modulate the folding of the F508del variant, notably with the approval of multiple pharmacological agents, including Orkambi, Symdeko, and Trikafta. In spite of the benefit these medicines have provided, ~ 10% of CF patients are not amenable to CFTR modulation, including patients with other folding defects or null mutations that result in premature termination codons [14, 15]. An inability to detect low levels of CFTR protein, and the inability to quantitate CFTR has hampered gene therapy efforts for these 10% of patients without access to effective CFTR modulators. As a pioneer for mRNA drug development, Translate Bio has assessed a LNP-mediated mRNA-based therapeutic in CF patients in a first-in-patient safety study. Interim analysis has suggested that this mechanism of treatment may be safe and tolerable [16–18]. These results have led to additional efforts to develop more potent mRNA-based therapeutics for the treatment of cystic fibrosis.

In mRNA-based drug development, the determination of CFTR expression level is important to evaluate the efficacy of drug candidate, especially for animal models in pre-clinical studies. However, CFTR is a low- abundance membrane protein, the quantification of CFTR in real sample, especially in native tissue, is still challenging. Traditional quantitative method is immunoprecipitation and western blot. However, the sensitivity and reproducibility are unsatisfied due to the CFTR protein aggregation and the cross-reactivity of antibodies [19]. For native tissue analysis, extra purification steps were considered to reduce the interference of other proteins and increased the sensitivity of CFTR quantitative assay [20]. As an alternative method, highly sensitive and robust mass spectrometry has been reported for identifying CFTR protein, and its post translational modifications (PTMs) [21, 22], analyzing interaction of CFTR with other proteins [23, 24], as well as quantifying the endogenous expression of CFTR protein [25, 26]. To increase the sensitivity, high specific purification method, such as immunoprecipitation [23–26], gel-based electrophoresis [22], were applied prior to LC–MS/MS detection. However, to our best knowledge, most of the current publications about CFTR quantification are focusing on CFTR expressed cell line samples [21–27]. No application of LC–MS/MS for CFTR quantitative analysis of tissue samples have been reported.

In this work, a highly sensitive targeted proteomic method was developed for absolute quantification of human CFTR (hCFTR) in lung tissue, which combined membrane protein extraction, protein immunoprecipitation followed by nanoLC-MS/MS. The method sensitivity, accuracy, precision, as well as matrix effect, were systematically investigated. The assay was applied to measure hCFTR protein level in human lung samples as well as in mouse lungs transfected with LNP-mediated hCFTR-mRNA. To our knowledge, it is the first mass spectrometry based quantitative proteomics assay for CFTR in tissue matrix. This assay will benefit the preclinical and clinical research for future gene therapy development for CF disease.

Methods

Materials

Minute Plasma Membrane Protein Isolation and Cell Fractionation Kit was from Invent Biotechnologies Inc (Plymouth, MN). Pierce MS-compatible magnetic IP kit, Halt protease inhibitor cocktail, MS-grade Trypsin/LysC protease mix, sequence grade trifluoroacetic acid (TFA), Pierce Protein G magnetic beads, Invitrogen Dynabeads MyOne Streptavidin T1 beads were from Thermo Scientific (Waltham MA). Stable isotope labeled peptide containing $^{13}C_6{}^{15}N_2$ labeled Lysine or $^{13}C_6{}^{15}N_4$ labeled Arginine (> 99%) was synthesized by Vivitide (Gardner MA). 1,4-Dithiothreitol (DTT), Iodoacetamide (IAA), bovine serum albumin (BSA), 1 × phosphate buffered saline (PBS), acetonitrile (ACN, ≥ 99.9%), n-Dodecyl-β-D-maltoside (DDM), and HPLC grade water were all from Sigma (St Louis, MO). Formic acid (FA, HPLC grade), PBS containing 0.05% Tween 20 (PBST) 20x, were from Fluka (Buchs, Switzerland). RapiGest SF surfactant was from Waters (Milford, MA).

Biotinylation of antibody

The purified anti-human CFTR antibody (shown as Table S2) was biotinylated using water-soluble Sulfo

ChromaLink Biotin (Vector Laboratories, Burlingame, CA) via cross-linking biotin succinimidyl ester to antibody primary amine groups according to the manufacturer protocol. To avoid free amine-containing contaminants during the labeling reaction, buffer exchange for antibody solution was firstly performed with amine free modification buffer using Amicon filter with molecular weight cutoff 30 kDa. The desalted antibody was reconstituted to yield a concentration of 1 mg/ml. The sulfo chromalink biotin solution was prepared by dissolving 1 mg biotin reagent powder in 200 µL of deionized water, and then spiked in the antibody solution to achieve molar ratio of 1:20 for Antibody/Biotin. After incubation at room temperature for 2 h, the reaction was stopped by adding 5 µL of 0.1 M pH 8.0 Tris–HCl buffer. Subsequently, buffer exchange was performed again for the resultant biotinylated antibody to remove the unbound biotin. The biotinylated antibody was reconstituted at 1 mg/ml concentration and stored at -80 °C for future use.

Tissue samples

In-life experiments were conducted at Biomere Biomedical Research, Inc (Worcester, MA). In general, male CD-1 mice at age of 6–8 weeks were dosed via intratracheal instillation (15 µg/animal via catheter) with three different in-house novel test articles (LNP-1/LNP-2/LNP-3). After exsanguination and perfusion at 24-h post dose, the mouse right lung was collected and snap frozen in liquid nitrogen, then stored at -80 °C until use. Frozen wild type (WT) CD-1 mouse lung tissue and non-CF male human lung tissue was obtained from BioIVT (Hicksville, New York).

Tissue preparation

Membrane protein extraction was conducted following the Minute plasma membrane protein isolation kit manufacturer protocol. Mouse lung tissue was cut and weighed at ~ 50 mg, then transferred into a 2 mL reinforced tube containing three stainless steel beads (MP Biomedicals, Irvine CA). An 800 µL of extraction buffer A from Minute kit containing 1 × protease inhibitor cocktail was added to the tube. After homogenizing at 6 m/s for 40 s with two cycles on FastPrep-24 5G homogenizer (MP Biomedicals, Irvine CA), the solution was then chilled on ice for 5 min. The resultant homogenate was then transferred to the filter cartridge and centrifuged at 4 °C at 16,000 × g for 30 s. Subsequently, the filter was discarded and the collected flow-through with pellet was vortexed vigorously until the pellet was completely resuspended. Then, the tube was centrifuged at 700 × g for 1 min. The supernatant was transferred to a fresh 1.5 mL tube and centrifuged at 4 °C at 16,000 × g for 30 min. After removing the supernatant, the pellet containing membrane proteins was re-dissolved in 350 µL Pierce IP-MS cell lysis buffer with frequent vortex and sonication. For method qualification, a large volume of membrane protein extracts from WT CD-1 mouse lung tissue was prepared and pooled, then aliquoted for standard curve and quality control (QC). Human lung tissue preparation followed the same protocol, except that the homogenization was performed at a speed of 6 m/s for 30 s with 4 cycles.

Preparation of standards and QCs

Purified recombinant hCFTR protein was used for standard and QC preparation, which was obtained by in-house gene expression of a CFTR-GFP construct in HEK293T cells and purification using GFP-Trap agarose resin (Chromotek). CFTR protein was eluted from column after digestion with PreScission protease (Cytiva) and further purified by Superose 6, 10/300 GL SEC column (GE Healthcare). The final concentration for purified recombinant hCFTR stock solution is 5.8 ng/µL. The standard spiking solution was prepared by serial dilution of neat hCFTR stock in 4% BSA-PBST buffer to get the concentration of 0.9, 2.7, 8.2, 24.7, 74.1 and 222 pg/µL. A 25 µL of standard spiking solution was spiked in membrane protein extract solution containing 50 mg tissue matrix to obtain standard solutions at 0.46, 1.37, 4.12, 12.4, 37.0 and 111 pg hCFTR per mg tissue as standard 1 to standard 6, respectively. The QC solutions were prepared by serial dilution of 5.8 ng/µL human CFTR with 4% BSA-PBST buffer to get QC low (QCL), middle (QCM), and high (QCH) at the concentration of 0.5, 30, 180 pg/µL, respectively. Then 25 µL of QC solution was spiked in 50 mg tissue matrix, resulting in QCL/QCM/QCH at the concentration of 2.5, 15 and 90 pg/mg tissue, respectively. For method qualification, two sets of experiments including standard curve and 3-level QCs were conducted.

Immunoprecipitation

Immunoprecipitation was performed according to the manufacturer instruction using streptavidin magnetic beads (Dynabeads MyOne Streptavidin T1 beads, Invitrogen). A 350 µL of membrane protein extract from 50 mg mouse/human lung tissue sample was aliquoted into the sample well of a Kingfisher 96 deep-well plate. For standard or QC sample, a 350 µL of membrane protein extract from control CD-1 mouse lung matrix was aliquoted, and 25 µL of standard/QC spiking solution was added into each aliquot. Two µg of biotinylated mouse monoclonal anti-human CFTR antibody was added to each sample well. After the plate was incubated at 4 °C with shaking at a 600 rpm overnight, 25

μL of 10 mg/mL pre-washed Streptavidin T1 beads was added and incubated at room temperature with vigorous vortex for 1 h. After that, sample plate was transferred to a KingFisher Flex magnetic particle processor (Thermo Scientific, Waltham MA) to perform the immunoprecipitation. The magnetic beads were washed with 300 μL of 1 × PBST twice, with 300 μL of 1 × PBST/1 × PBS (1/2, v/v) solution once, and then eluted with 100 μL of 0.2% RapiGest in 20 mM Tris–HCl. After IP, the magnetic beads were placed in elution plate followed by on-beads digestion. For comparison, Protein G magnetic beads IP was also performed following the same protocol except for using the un-biotinylated anti-CFTR antibody instead.

Protein digestion

The IP eluate was digested on-beads. In general, the elute plate was firstly heated at 95 °C with vortex at 1000 rpm for 5 min. After cooling down, the eluate was incubated with 10 μL of 50 mM DTT at 55 °C for 45 min with shaking at a 1000 rpm speed, followed by incubating with 10 μL of 150 mM IAA in the dark with shaking at a 1000 rpm speed at room temperature for another 30 min. Finally, 20 μL of 0.1 μg/μL of trypsin/LysC was added to the sample and incubated with shaking at 1000 rpm speed at 37 °C overnight. For comparison, eluting and off-beads digestion was also conducted. After IP, the elution plate was first heated at 70 °C or 95 °C for 5 min, then sat on a magnetic separator for at least 5 min. The eluate was manually transferred to a new tube for trypsin/LysC digestion as described above.

Peptides desalting

After digestion, separation of magnetic beads from sample solution was done using a magnetic separator. Then, each generated digest was manually transferred to a new 1.5 mL protein LoBind tube. 5 μL of 20% TFA was added to the digests, and then the sample was incubated at 37 °C for 40 min. The sample tubes were centrifuged at 4 °C with 20,000 × g for 20 min. Then, the clear supernatant peptide mixture was transferred to a new tube and the pellet of RapiGest was discarded. SOLAμ SPE tips were used for peptide cleanup. By activating the SPE tip with 300 μL of 100% ACN, the cartridge was equilibrated with 300 μL of 0.1% FA for three times. The sample was slowly loaded onto the SPE cartridge, then washed three times with 300 μL of 0.1% FA, and finally eluted slowly with 300 μL of 0.1% FA in 70% ACN. The eluate was dried down in SpeedVac and redissolved in 10 μl of 0.1% FA solution containing hCFTR heavy peptide internal standards at a concentration of 1 fmol/μL.

NanoLC-MS analysis

An EASY-nLC 1200 system coupled to Q Exactive HF-X mass spectrometer (Thermo Scientific, Waltham MA) was used for LC–MS/MS analysis. Separation of tryptic peptides was performed on Thermo PepMap C18, 1.8 μm, 75 μm ID × 50 mm column (Thermo Scientific, Waltham MA) at a flow rate of 300 nL/min with 0.1% FA in DI water as mobile phase A and 0.1% FA in 90% ACN/10% H_2O as mobile phase B, respectively. The gradient was 2% B (0 min)-2% B (5 min)-40% B (44 min)-95% B (48 min)-95% B (53 min)-2% B (58.0 min)-2% B (63 min). The column temperature was set at 45 °C. Targeted proteomics data were acquired by the parallel reaction monitoring (PRM) mode, with settings of MS2 resolution of 45,000, AGC target of 3e6, Maximum IT of 120 ms, isolation window at 1.0 m/z and (N)CE of 30. The mass spectrometry was operated at the positive mode with capillary temperature was set as 320 °C, the spray voltage was 1.6 kV, and the funnel RF level was 40. The precursors of peptide LSLVPDSEQGEAILPR at 862.4649 and 867.4639 were selected for generating the product ions for light and heavy peptide, respectively. Global proteomics data were acquired by the data dependent acquisition (DDA) mode, with full MS resolution of 120,000, AGC target of 3e6, Maximum IT at 60 ms, scan range at *m/z* 375–1950, and MS2 resolution of 7,500, AGC target of 1e5, Maximum IT at 50 ms, isolation window at *m/z* 2.0, and (N)CE at 27. The parameters for nano-spray ionization source were set as same as that of PRM mode.

Data processing for protein qualification

Biopharma Finder (Version 3.0, Thermo) was used for database searching for data-dependent acquisition (DDA) result. Trypsin was selected with high specificity. Carbamidomethylation at cysteine was set as static modification. Variable modifications included oxidation at methionine and tryptophan, and phosphorylation at serine, threonine, and tyrosine. Mass accuracy was 9 ppm and minimum confidence for MS2 spectra was 0.8. Skyline software (MacCross lab) was used for surrogate peptide selection and IP-MS method optimization. Thermo Xcalibur software (Version 4.0) was used to generate peak area response for each sample within the analytical batch. All peak integrations were visually inspected for an acceptable integration. For qualification, transition 862.4649 (Q1) → 1311.65 (Q3) and 867.4639 (Q1) → 1321.66 (Q3) were used for light and heavy peptide LSLVPDSEQGEAILPR. Peak area ratios (analyte to heavy internal standard) of the calibration standards and their nominal concentrations were used to perform the 1/X-weighted linear regression for the calibration curve. Data were be reported with 3 significant figures.

Results

LC-PRM method development

Peptide selection is an essential step to develop a sensitive and reliable targeted proteomics assay. Surrogate peptides were selected based on the DDA result generated from tryptic/LysC digests of purified full-length recombinant human CFTR protein. Database searching was performed by Biopharma Finder software against human CFTR amino acid sequence (CFTR_HUMAN, UniProtKB, P13569), and the result was shown in supplemental information Figure S1. Common criteria for peptide selection for targeted proteomics analysis, including peptide's uniqueness, MS signal, length, hydrophobicity, modification, mis-cleavage, etc., were considered [28–30]. The top eight peptides with highest mass spectrometry signal were selected as candidates for hCFTR quantitation (supporting information Table S1). The stable isotope labeled corresponding peptides (^{13}C and ^{15} N at C-terminal Arginine or Lysine) were synthesized. Subsequently, the precursor and collision energy (CE) for each peptide was optimized. All the parameters were validated by LC-PRM using recombinant CFTR protein spiked in mouse lung tissue to check the response and matrix interference. By optimization, peptide LSLVPDSEQGEAILPR was finally selected as surrogate peptide for quantifying hCFTR.

IP-MS assay development

The general IP-MS sample preparation workflow is shown in Fig. 1. To determine the feasibility of the IP-MS method for CFTR quantification, membrane protein extract from mouse lung tissue homogenate, and the extracts spiked with 50 ng of recombinant hCFTR protein sample were assessed. Protein IP was performed using protein G magnetic beads binding anti-CFTR antibody, followed by trypsin/LysC digestion and LC-PRM MS analysis. The results demonstrated that human CFTR peptide LSLVPDSEQGEAILPR was detected in hCFTR spiked-in tissue sample with a high intensity at 1.1E6, which suggested a lower spiked-in amount of standard hCFTR protein can be used for the following test. And no hCFTR peptide with peak signal/noise ratio (S/N) > 3 was detected in mouse lung negative control sample, demonstrating the specificity of this assay to human CFTR protein (Fig. 2).

Protein IP optimization

To increase the sensitivity of IP, two types of commonly used magnetic beads, Protein G and streptavidin T1 beads, were assessed, and the data were compared. Protein G beads covalently bind to IgG antibodies, and streptavidin beads usually conjugate to biotinylated

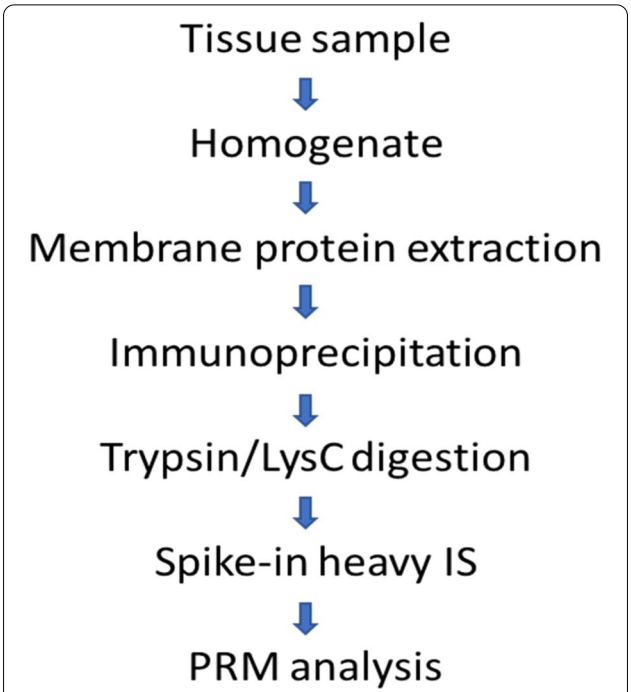

Fig. 1 Workflow of human CFTR protein quantitation in tissue

antibodies. In this study, a test sample using 5 ng hCFTR protein spiked in 50 mg mouse lung was prepared for IP optimization. Figure 3 shows that MS responses of top four hCFTR peptides generated in Streptavidin IP were 7 to 10-fold higher than those in protein G IP, demonstrating the higher specificity of streptavidin beads IP. It was reported that the dissociation constant (K_D) of binding affinity between streptavidin and biotin is 20,000 times stronger than that between Protein G and IgG [31, 32]. Our data further confirmed this difference. Therefore, streptavidin beads were selected for the assay.

To obtain a higher hCFTR peptide recovery, on-beads digestion and off-beads digestion were compared. 0.2% RapiGest solution was used as eluting buffer in on-beads digestion experiment, and 0.1% DDM in 25 mM HCl solution was used as eluting buffer in off-beads digestion. DDM is a non-ionic glycosidic, MS-compatible surfactant, which was added into acidic elution buffer to increase the solubility of membrane protein [33]. Since denaturing protein at high temperature could deactivate the capture antibody and release hCFTR, thereby increase the immunocapture recovery [34], in this experiment, denaturation of IP enriched proteins at higher temperature such as 70 °C and 95 °C was tested. The IP-MS result shows that the lower recovery was obtained in off-beads digestion, even the experiment was conducted at higher denaturation temperature and using acidic DDM solution as eluting buffer. Figure 4 shows that top four

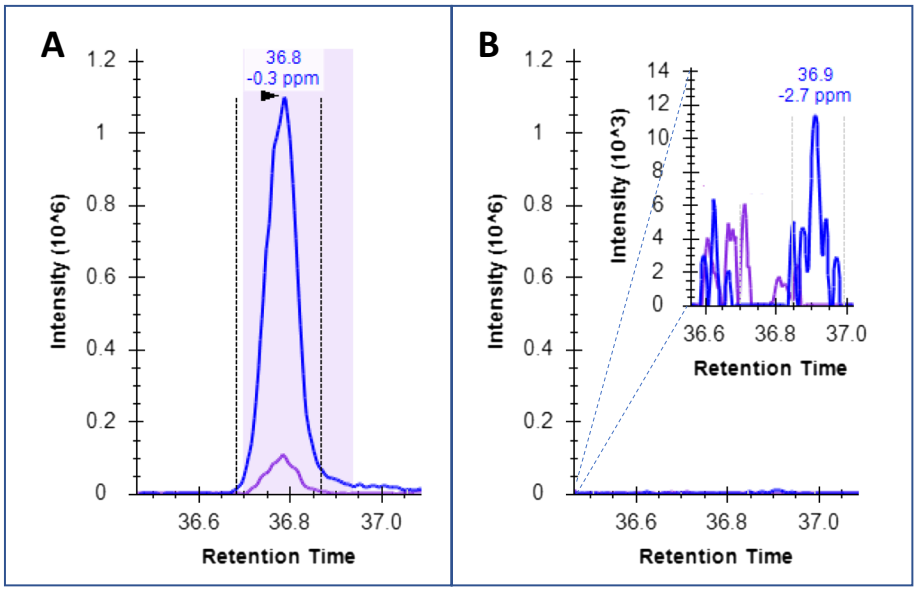

Fig. 2 Human CFTR peptide LSLVPDSEQGEAILPR after IP-MS analysis. **A**, recombinant hCFTR protein (50 ng) spike-in mouse lung membrane protein extracts (50 mg lung tissue); **B**, mouse lung membrane protein extracts only as a negative control (y-axis showed with zoomed-in scale)

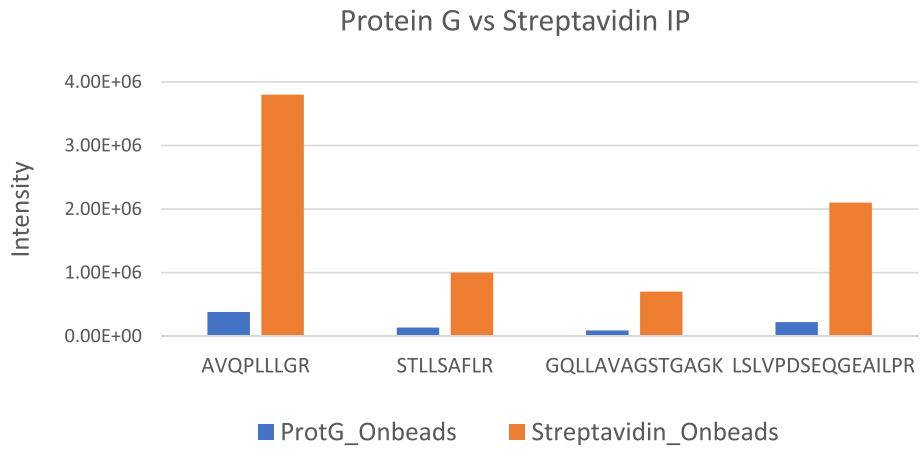

Fig. 3 Comparison of peak intensities of four hCFTR peptides (AVQPLLLGR, STLLSAFLR, GQLLAVAGSTGAGK and LSLVPDSEQGEAILPR) generated by IP using protein G beads vs streptavidin magnetic beads. hCFTR protein (5 ng) spiked in mouse lung membrane protein extracts (50 mg lung tissue) was used as test sample. The data were processed by skyline software

hCFTR peptides generated in on-beads digestion showed higher MS response, and intensities of these four peptides were 3 to 11 times higher than those in off-beads digestion. Since CFTR protein can't be completely eluted using the current elution conditions, on-beads digestion was selected for this assay.

Considering the importance of antibody specificity in immunoprecipitation, eight anti-human CFTR antibodies (Table S2) from various vendors were screened by IP-MS analysis using hCFTR protein spiked in mouse lung tissue and Protein G magnetic beads. 1 ng of hCFTR protein spiked in 25 mg tissue membrane protein extract was used as test sample. Figure 5 shows the peak intensity of hCFTR peptides after IP-MS analysis using different antibodies. hCFTR peptides generated using Ab5 in IP showed the highest MS responses in comparison to using other antibodies, demonstrating the highest specificity of Ab5 to hCFTR protein.

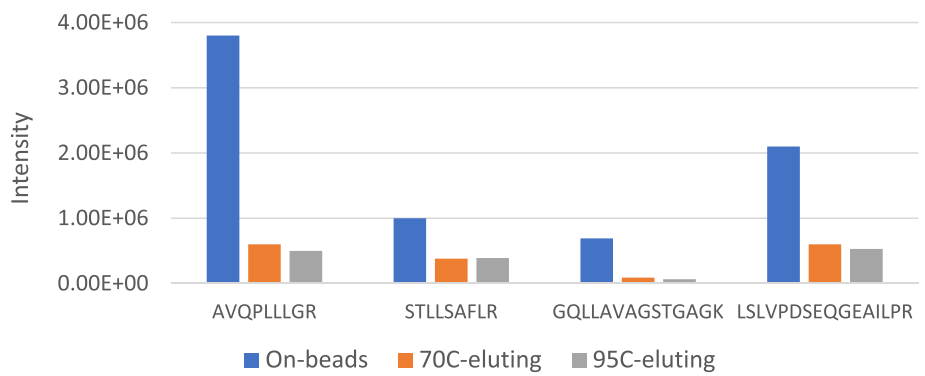

Fig. 4 Comparison of peak intensities of four hCFTR peptides (AVQPLLLGR, STLLSAFLR, GQLLAVAGSTGAGK and LSLVPDSEQGEAILPR) generated by on-beads digestion vs off-beads digestion. hCFTR protein (5 ng) spiked in mouse lung membrane protein extracts (50 mg lung tissue) was used as test sample. Streptavidin magnetic beads and biotinylated antibody were used for IP. Data were processed by skyline software

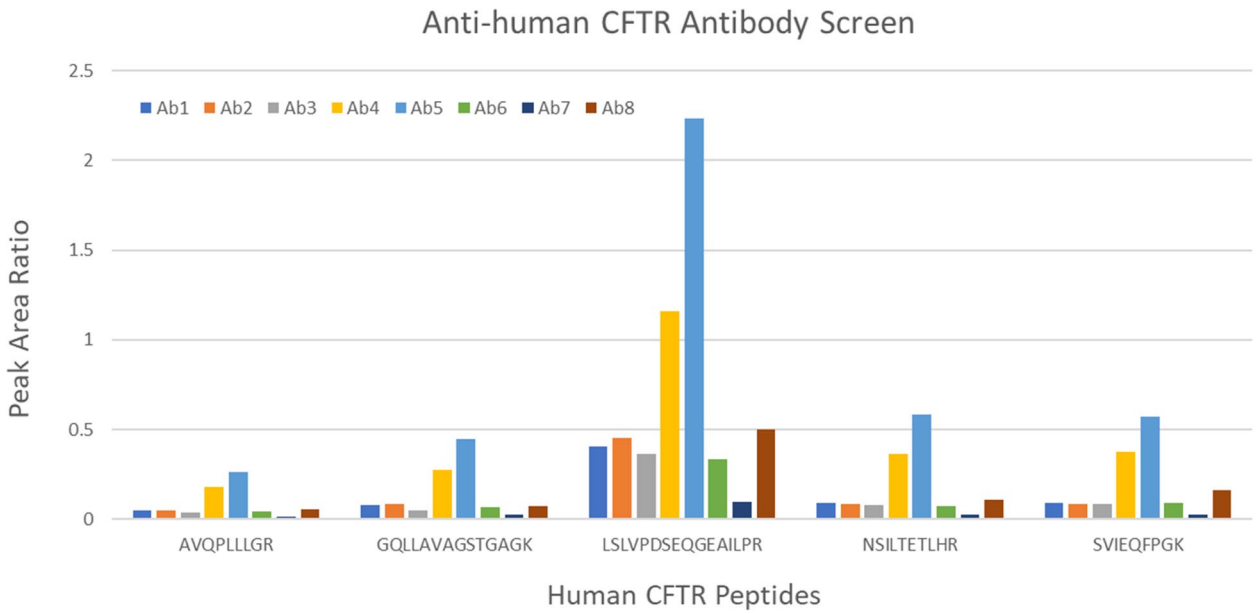

Fig. 5 Comparison of MS responses of five hCFTR peptides (AVQPLLLGR, GQLLAVAGSTGAGK, LSLVPDSEQGEAILPR, NSILNPINSIR, and SVIEQFPGK) generated by IP using 8 different anti-human CFTR antibodies. hCFTR protein (1 ng) spiked in mouse lung membrane protein extracts (25 mg lung tissue) was used as test sample. Protein G magnetic beads and un-biotinylated antibody were used for IP. Data were processed by skyline software. Anti-human CFTR antibody information was shown in Table S2. The reproducible result was shown in Figure S4

Method qualification

The assay performance was evaluated by the linearity, sensitivity, accuracy, precision, and matrix effect. Standard samples at a concentration range of 0.5 to 111 pg/mg tissue were prepared by spiking recombinant hCFTR protein in membrane protein extract of CD-1 mouse lung for generating a calibration curve. A good linearity of peak area ratio of hCFTR peptide to its isotope labeled internal standard was obtained within a dynamic range of 2 orders of magnitudes. Two standard curves prepared in the same batch matched very well with $R^2 > 0.998$ (Fig. 6). The limit of detection (LOD, S/N > 3) is 0.5 pg hCFTR protein per mg tissue, and the lower limit of quantification (LLOQ) is 1.4 pg/mg tissue for hCFTR in mouse lung matrix, based on coefficients of variation (CV) threshold at 20%.

Concentrations of standard samples calculated from 6 replicates in 4 different batches were used for evaluating

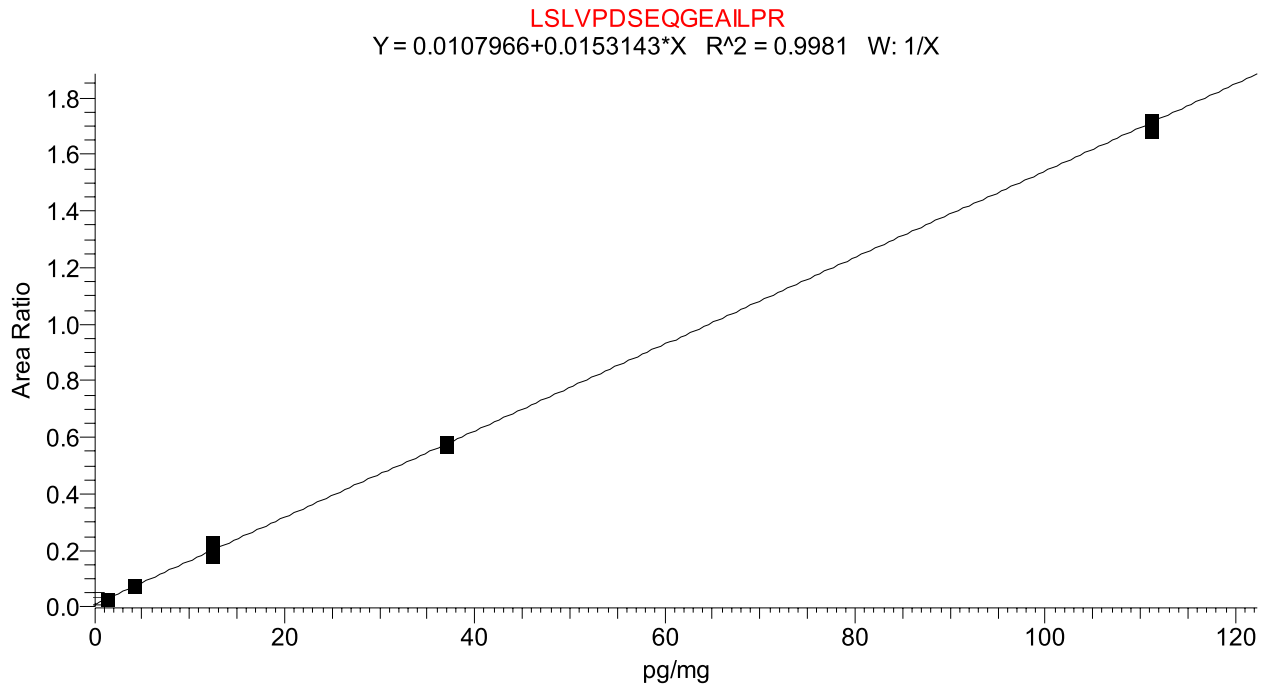

Fig. 6 Standard curve of hCFTR by plotting peak area ratio of light to heavy peptide LSLVPDSEQGEAILPR in mouse lung membrane protein extracts. Streptavidin magnetic beads and biotinylated anti-CFTR antibody were used for IP. hCFTR concentration was 0.5, 1.4, 4.1, 12, 37, to 111 pg per mg tissue for standard 1 to 6, respectively. Calibration curve in linear regression with weighting 1/X was plotted by using Xcalibur software

assay accuracy and precision. The measured standard concentrations were back calculated using the standard curve. The CV is 19% for LLOQ and ≤ 10% for all other standard points. The average absolute difference between estimated concentration and nominal value was 18% for the LLOQ and ≤ 5% for other standard points. This result demonstrates the high reproducibility and accuracy of this assay (supporting information Table S3). In addition, three-level QCs were investigated in two batches. Good accuracy was observed for all QC levels with error within 10%. CV for all replicates was 16% at the QC low level and < 9% at QC middle and QC high levels, further demonstrating the good precision of the assay (supporting information Table S4).

To verify if there is matrix effect on the IP-MS assay, hCFTR standard solutions in mouse lung tissue and in 4% BSA-PBST control buffer were investigated. After incubation with anti-human CFTR antibody overnight, protein IP was performed in tissue matrix and in BSA buffer, respectively, using magnetic streptavidin beads followed by nanoLC-PRM analysis. Five standards at a concentration range of 0.5 to 111 pg/mg tissue were tested. The result shows that at a same concentration, MS responses of hCFTR peptides in BSA buffer are much higher than those in mouse lung tissue, demonstrating the substantial matrix impact on the detection of hCFTR in lung tissue. This observation could be caused by two possibilities: 1) lung tissue interferences may reduce hCFTR enrichment efficiency during IP; 2) MS signal could also be suppressed by lung tissue interferences in LC–MS analysis, leading to the lower sensitivity for hCFTR peptides detection (supporting information Figure S2). As a result, for hCFTR quantification in tissue sample, if blank tissue is not available, a surrogate tissue matrix is required for standard curve and QCs to mimic the real tissue sample condition.

Tissue analysis

The developed IP-MS assay was then further tested to quantify hCFTR expression level in 15 mouse lungs that were previously treated with three different mRNA-LNPs formulations at a same dose. 50 mg of mouse lung tissue was used in each sample analysis. Figure 7 shows that hCFTR peptide LSLVPDSEQGEAILPR can be detected in all 15 mouse lungs and the measured concentrations of hCFTR protein varied from2 to 20 pg/mg tissue. These results demonstrated that the hCFTR mRNA was successfully delivered into lung epithelial cells and translated into hCFTR protein in the mouse lung.

The same assay was also successfully applied to human lung tissue analyses. By using mouse lung tissue as surrogate matrix, hCFTR endogenous levels in two normal

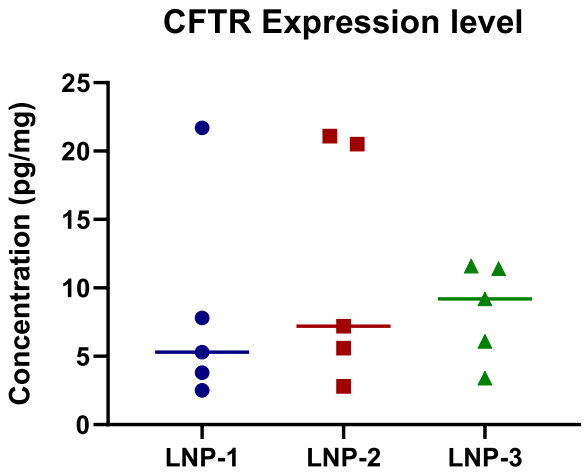

Fig. 7 hCFTR level measured by the developed method in mouse lung treated with three different LNPs formulations. 50 mg of mouse lung tissue was analyzed. Five animals were dosed for testing each formulation. Streptavidin magnetic beads and biotinylated anti-CFTR antibody were used for IP. Absolute quantification of hCFTR protein was based on the calibration curve of CFTR peptide LSLVPDSEQGEAILPR

human lung tissues were measured to be 13.5 and 4.9 pg/mg tissue, respectively. Figure S3 in supporting information shows chromatograms for hCFTR peptide after IP-MS analysis. These results clearly demonstrated the potential use of this assay in low abundant hCFTR quantification in both human lung tissues and in preclinical animal lung tissues.

Discussion

hCFTR protein is a low abundant membrane protein with ~ 150 k Da molecular weight. Its detection and quantitation in lung tissue are extremely challenging due to its high hydrophobicity, extremely low endogenous level, and large protein size. In our method, for improving hCFTR detection, membrane protein extraction was used as the first protein enrichment step. Since this step can separate hCFTR fraction that is inserted in plasma membrane from the hCFTR fraction in cytosol, our method is powered to quantitate membrane hCFTR only, not total hCFTR in epithelial cell. Therefore, the level of hCFTR measured in human lung tissue by this method should directly represent its physiological function, or the level measured in the treated animal lung tissue will represent the potency of mRNA-based therapeutics accurately for CF drug evaluation and development.

In LC-PRM MS analysis, a surrogate peptide is usually selected and used for protein quantification. In this hCFTR assay, LSLVPDSEQGEAILPR was selected as the surrogate peptide because it is unique to human hCFTR and is not present in CFTR protein of preclinical species such as mouse, rat, and monkey. Since its high specificity, this peptide allows us to distinguish hCFTR protein from CFTR protein of other species and accurately assess the potency of mRNA-based therapies with hCFTR protein expression in preclinical animal models.

This assay was applied to three groups of mice lung tissue treated with three different mRNA-encapsulated LNPs formulations at a same dose. The hCFTR protein was successfully quantified in all samples. Due to variation of biological samples and the small cohort for each group ($n=5$), this preliminary result didn't show statistically significant difference for dosing conditions. And a large cohort might be needed for further confirmation. Using mouse lung as surrogate matrix, the endogenous level of hCFTR in normal human lung tissue was also successfully measured. To our best knowledge, it is for the first time that the endogenous level of hCFTR in normal human lung tissue is reported. These data clearly demonstrated the potential application of this IP-MS assay in both preclinical and clinical drug development for CF treatment.

Conclusions

CFTR research has been hampered by an inability to quantitate CFTR protein expression, a key parameter for evaluating drug efficacy. Its determination in tissue matrix remains a challenge due to its low abundance and membrane protein property. To address this problem, a highly sensitive and selective assay combining membrane protein extraction, protein immune-affinity enrichment and nanoLC-MS/MS was developed. The method shows a good linearity in a 2 orders of magnitude dynamic range for hCFTR quantitation in tissue matrix. The LLOQ is 1.4 pg hCFTR per mg tissue, meets the need of determination of the low abundant hCFTR protein generated by mRNA-based therapeutics. The CV% of back-calculated standard curve and QCs and the back calculation error for both the intra batch and inter batch was within 20%, demonstrating high precision and accuracy for hCFTR quantification. This assay was then successfully applied to quantifying hCFTR expression in mouse lung tissue and endogenous level in normal human lung tissues. This assay shows high potential to preclinical study in drug development in addition to allowing for further basic research on CF pathophysiology.

Abbreviations

CF: Cystic fibrosis; CFTR: Cystic fibrosis transmembrane conductance regulator; hCFTR: Human CFTR; mRNA: Messenger RNA; WT: Wild type; MS: Mass spectrometry; LC–MS/MS: Liquid chromatography-tandem mass spectrometry; PRM: Parallel reaction monitoring; CE: Collision energy; IP: Immunoprecipitation; QC: Quality control; LOD: Limit of detection; LLOQ: Lower limit of quantification; CV: Coefficient of variation; S/N: Signal to noise ratio; LNP: Lipid nanoparticle; Ab: Antibody.

Supplementary Information

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Additional file 1: Table S1. Peptide candidates for human CFTR PRM analysis. **Table S2.** Anti-human CFTR antibody. **Table S3**. Back calculated CFTR standard concentration measured for intra- and inter- batches (n =6). **Table S4**. Back calculated CFTR QC concentration (n =3).

Additional file 2: Figure S1. Peptide mapping for trypsin/LysC digests of recombinant human CFTR protein (UniProtKB-P13569). **Figure S2.** Standard curve of hCFTR peptide LSLVPDSEQGEAILPR peak area ratio vs protein centration in 50 mg mouse lung membrane protein extracts and 4% BSA-PBST buffer. hCFTR concentration was 0.5, 1.4, 4.1, 12, and 111 pg per mg tissue. **Figure S3.** Human CFTR peptide LSLVPDSEQGEAILPR in wild type human lung tissue after IP-MS Analysis. A and B represent two different human lungs. **Figure S4.** Antibody screening using targeted quantification of peptide LSLVPDSEQGEAILPR with replicates.

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Authors' contributions
HW and GS designed the experiment. HW collected the data. HW and SD optimized MS assay. NC prepared standard protein. CW, LB, JS, GS, RW conceived the in vivo study. HW, GS, JS analyzed and interpreted the data. HW, JS, GS contributed to the discussion and preparation of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated and analyzed during this study are included in the published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
Experiments were carried out on CD1 mice (6–8 weeks old males) obtained from Charles River Laboratories (Kingston, New York, USA) and housed at Biomere Biomedical Research Models (Worcester, MA, USA) accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animal experiments were conducted in accordance guidelines for care and use of laboratory animals in accordance with policies for the ethical treatment of animals established by the National Institutes of Health and were approved by the IACUC committee (Biomere). Animals were house in AAALAC accredited facilities in concordance with all local, institutional requirement under an animal care and use committee approved protocol.

Consent for publication
Not applicable (this study does not contain any individual person's data).

Competing interests
The authors declare that they have no competing interests. All authors are Translate Bio employees and may hold shares and/or stock options in the company.

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