Am J Transl Res 2022;14(10):7612-7620
www.ajtr.org /ISSN:1943-8141/AJTR0144249

Case Report

Personalized medicine approaches in cystic fibrosis related pancreatitis

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Received May 18, 2022; Accepted August 27, 2022; Epub October 15, 2022; Published October 30, 2022

Abstract: We report a rare case of a patient with cystic fibrosis suffering from debilitating abdominal pain due to chronic pancreatitis. This 13-year-old patient was evaluated for surgical intervention to relieve pain from chronic pancreatitis and to improve quality of life. The patient carried two mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene; the most common ΔF508 variant and a second variant, p.Glu1044Gly, which has not been previously described. The patient's condition did not improve despite medical management and multiple endoscopic interventions, and therefore total pancreatectomy with islet autotransplantation and a near-total duodenectomy was offered for definitive management. Patient-derived duodenal crypts were isolated and cultured from the resected duodenum, and duodenal organoids were generated to test CFTR function. Our studies demonstrate that this novel mutation (ΔF508/p.Glu1044Gly) caused severely impaired CFTR function *in vitro*. The Food and Drug Administration (FDA)-approved drug ivacaftor, a CFTR potentiator, was identified to robustly improve CFTR function in the context of this novel mutation. Herein, we describe a personalized medicine approach consisting of performing drug testing on individual patient derived organoids that has potential to guide management of patients with novel CFTR genetic mutations. Identified effective medical therapeutics using this approach may avoid irreversible surgical treatments such as total pancreatectomy with islet autotransplantation in the future.

Keywords: P.Glu1044Gly-CFTR mutation, chronic pancreatitis, total pancreatectomy, personalized medicine, ivacaftor

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR), a protein which plays an important role in maintaining pH and water balance on the apical surface of the epithelium [1-4]. CF was discovered in 1938 [5] and over 2,000 mutations have now been identified [6, 7]. CFTR mutations have been classified into six categories based upon the molecular defect of the CFTR protein: Class I (defect in protein synthesis), Class II (defect in protein trafficking), Class III (defect in channel gating function), Class IV (defect in channel conductance), Class V (defect in mRNA stability) and Class VI (defect in protein stability) [6, 8]. Deletion of the phenylala-

nine codon at position 508 (ΔF508) is well studied overall and causes a trafficking disorder that leads to the most common and severe dysfunction in the CFTR protein. Overall, the ΔF508 mutation is present in over 70% of CF patients [6, 9]. In this report, we present the special case of a patient with a combination of this most common mutant allele (ΔF508) and a second mutation that has not been previously described, p.Glu1044Gly. In addition to the usual CF disease manifestations, this patient was also suffering from severe abdominal pain due to chronic pancreatitis (CP). Interestingly, pancreatitis is a rare complication in CF patients [10]. In 10,071 CF patients from 29 different countries, De Boeck et al. estimated the overall incidence of pancreatitis to be only 1.24%. A recent study in children from the INSPPIRE (International Study Group of Pediatric Pancreatitis: in search for a cure) cohort showed that about 2.6% of children with acute recurrent pancreatitis (ARP) or CP had a diagnosis of CF [11]. In fact, with ΔF508/ΔF508 or other variants associated with severe CF, patients are typically pancreatic insufficient as early as the first 1-2 years of life, and therefore, are not at risk for pancreatitis [10]. Only in the milder forms of CF wherein patients have pancreatic sufficiency is there a risk of developing pancreatitis [10].

In patients suffering from debilitating pain and declining quality of life due to CP or ARP, total pancreatectomy with islet autotransplantation (TPIAT) has been shown to be an effective option to mitigate pain and patient suffering [12, 13]. In this study, we not only report the clinical course of CP in a CF patient with the novel combination CFTR mutations ΔF508/p.Glu1044Gly who underwent TPIAT, but also compare CFTR function and drug response in this patient's cells to those of a non-CF patient with CP and a CF patient with the most common CFTR ΔF508/ΔF508 mutations but not having CP. We obtained surgical remnant specimens under our research protocol to study FDA-approved medications with patient-derived duodenal stem cells *in vitro* to enrich the medical literature with functional data regarding this novel variant, and to serve as a proof-of-concept individualized patient approach to identifying drugs with potential therapeutic efficacy.

Materials and Methods

Human studies

We obtained consent from the patient's parent to collect and study duodenum tissue from the surgical remnants according to standard research protocols approved by the Institutional Review Board (IRB: 2013-3309).

History of the patient with the rare CFTR mutations

The patient is a 13-year-old female carrying an unusual, novel CFTR mutation which has not been previously described, p.Glu1044Gly, in combination with the more common mutation, ΔF508. Prior to presentation for evaluation at our Pancreas Care Center, she had undergone 13 endoscopic retrograde cholangiopancreatography (ERCP) procedures. She had nutritional difficulties with associated failure to thrive and weight loss, requiring feeding supplementation by a nasoenteric then a gastrostomy feeding tube to meet nutritional demands. She had already been taking daily opioids for pain alleviation at the time of her evaluation at our center. The patient did not have severe gastrointestinal pathologies or lung disease with minimal pulmonary infections and no requirement for a respiratory regimen at home. In the multidisciplinary review process of her case, the recommendation was made to move forward with TPIAT to relieve the patient's CP dependent pain given that this was the patient's most debilitating symptom.

Isolation, generation and culture of patient-derived duodenal organoids

The procedure of total pancreatectomy with islet autotransplantation has been described previously [13, 14]. During the process, the pancreas was removed and digested to isolate pancreatic islets for infusion into the patient. In addition, the operation involved a near-total duodenectomy. A 2-3 cm long piece of duodenum (about 20% of explanted tissue) was prepared by opening the luminal area using microscissors, with plating the duodenal tissue luminal side down on a homemade-silica gel (polydimethylsiloxane; PDMS; Ells Worth Adhesive; #4019862) coated dish and positioning the stretched tissue on the gel using fine pins

(Fisher scientific; #S13976). The submucosa layer with muscle and serosa layers was removed by microdissection and the tissue was flipped over to a luminal side up orientation and re-pinned onto the silica gel. Villi were removed from the luminal surface of the mucosa layer by gently scraping with curved forceps and washed with cold phosphate buffered saline (PBS; Invitrogen; #20012050; no calcium and no magnesium) until the debris was completely removed. The tissue was covered with 10 mL ethylenediaminetetraacetic acid (EDTA; Invitrogen; #15575; 2 mM) in 15 mL cold PBS followed by shaking the dish gently using a horizontal orbital shaker (Labnet; #ORBIT M60; 15 RPM) for 30 min at 4°C and washing the tissue 5-6 times with cold PBS. To obtain intestinal crypts, 15 mL cold PBS was added and the mucosal tissue surface was gently scraped using curved forceps. The crypt suspension was transferred into a 50 ml conical tube and passed through a 100 μm strainer (Falcon; #352360) with an additional 15 mL cold PBS added to the dish to collect remaining crypts. The crypt suspension was centrifuged at 150×*g* for 3 min (4°C) and the supernatant discarded. The crypts were resuspended in 1 mL cold PBS by pipetting and transferred to a 15 mL tube followed by adding 9 mL cold PBS and centrifuging again at 150×*g* for 3 min and discarding the supernatant. The crypts were then resuspended in cell culture media (Stem Cell Technologies; #IntestiCult™ Organoid Growth Medium) and Matrigel (Corning; #356231) was added at a ratio of cell culture media to Matrigel of 2:3 vol%, mixing gently to avoid bubbles. Approximately 100 crypts in 50 μL Matrigel were plated on a glass bottom dish (MatTek; #P35GC-1.5-14.C) followed by incubation at 37°C for 15 min to solidify the Matrigel. Organoid growth media (IntestiCult) (300 μL) was added to cover the Matrigel. For the first four days, 10 μM Y-27632 of ROCK inhibitor (BD Biosciences; #562822) was added with refreshing the growth media every other day.

To isolate crypts from very small <10 mm^3 duodenal biopsies, the tissue was washed once with 10 mL cold PBS in a 15 mL tube by gently shaking to avoid loss of crypts. The tissue was then immersed in 10 mL of 2 mM EDTA in a 15 mL tube and placed on a horizontal orbital shaker (Labnet; #ORBIT M60; 15 RPM) at 4°C for 30 min. The tissue was gently washed with 10 mL cold PBS once and transferred to a new 15 mL tube containing 10 mL cold PBS. Crypts were separated from the tissue by shaking the tube up and down 20 times and passed through a 100 mm strainer to collect the crypts in a 50 mL tube. To collect additional crypts, the tissue was placed on the strainer with gentle rubbing of the tissue using a sterilized 2 mL Eppendorf tube (flat side down). To aid crypts in passing through the strainer, 3 mL cold PBS was added. The collected crypts were transferred into a new 15 mL tube and centrifuged at 150×*g* for 10 min, followed by carefully discarding the supernatant. The crypts were resuspended in organoid growth media and embedded into Matrigel as described above.

Monitoring of CFTR function in the duodenal epithelial cells in vitro

We monitored CFTR function in duodenal organoids using a fluid secretion assay in response to the cyclic AMP-mediated CFTR channel agonist, forskolin (FSK) [15] at day 4 after isolation of organoids. Fluid secretion was calculated by comparison of volumetric fluid change in the ratio of luminal volume to that of the entire organoid pre- and post-treatment with 10 μM FSK for 2 h. For the statistical analysis, we captured at least 20 organoid images pre- and post-FSK treatment and compared their volume changes. The lumen and entire organoid volumes were calculated by measuring the lumen and entire area of the organoids using Image J software provided by NIH. To rescue CFTR function, we used FDA-approved medications, including a CFTR corrector (lumacaftor, VX-809, 2 μM, pre-treatment for 24 h) and a CFTR potentiator (ivacaftor, VX-770, 2 μM, pre-treatment for 24 h or employed at the time of FSK administration without pre-treatment) [5, 6, 16]. In addition, we verified the observed CFTR function in polarized duodenal epithelial cells using a short-circuit current assay (I_{sc}) that is the gold standard method for monitoring of CFTR function by electrical current changes. For this assay, we broke down the Matrigel by pipetting with 1 mL cell culture media and transferred all debris to a 1.5 mL tube, followed by centrifuging the cells at 16,000×*g* for 3 min (Eppendorf Microcentrifuge; #5418) and discarding the supernatant and Matrigel from three layers of cell pellet,

Table 1. Summarized clinical-relevant symptoms prior to TPIAT

First attack pancreatitis	10 years of age
Chronic pancreatitis diagnosis	11.5 years of age
Failure to maintain weight on oral feeds	Requiring intermittent gastric tube feeds
Exocrine pancreatic insufficiency	On pancreatic enzyme replacement therapy since 12 years of age
Respiratory symptoms	FEV1 100% of predicted
	Has daily productive cough
	On pulmonary clearance regimen at home
	Respiratory cultures grew Pseudomonas, Staphylococcus aureus, and methicillin resistant Staphylococcus aureus in the past
Anemia	Requiring intermittent iron infusions
Other conditions	Vitamin D deficiency, adrenal insufficiency, chronic pain

Matrigel and supernatant. Appropriate cell culture media was added to the tube and 20-40 organoids were transferred to a transwell membrane (Corning; #3470) to form polarized monolayers of duodenal epithelial cells. Before the assay, transepithelial electrical resistance (TEER) was measured using an epithelial volt-ohm meter (World Precision Instruments, #EVOM and #STX2). When the TEER was >1000 Ω/cm^2, the cells were mounted in an Ussing Chamber. The buffer solution for the apical and basolateral sides has been previously described [15]. CFTR function was monitored in response to 10 μM FSK, 2 μM ivacaftor and 20 μM CFTR inhibitor, $CFTR_{inh\text{-}172}$. To verify the polarization of the monolayer of duodenal epithelial cells on the transwell membrane, the cells were examined by hematoxylin and eosin (H&E) staining and immunofluorescence microscopy with 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen, #D1306), actin (Invitrogen, #A12379), and CFTR (R1104 monoclonal) [17] immunostaining using a previously described process [15].

Statistical analysis

Data were derived from at least 20 organoids for a fluid secretion assay and three independent replicates for a short-circuit current assay. The level of significance, *p-value*, was calculated by Student's t-test and two-way analysis of variance. A *p-value* <0.05 was considered significant.

Results

Patient clinical course before and after TPIAT

The patient was relatively healthy until the age of 10 years when she had her first attack of acute pancreatitis (AP). She subsequently had multiple episodes of AP with eventual development of CP at the age of 11.5 years (**Table 1**). A total of 13 ERCPs were performed along with pancreatic duct stricture dilation and stenting which ultimately failed to provide adequate pain relief resulting in the patient being referred for TPIAT evaluation. Over the two years prior to TPIAT evaluation, she required multiple daily doses of opioids for pain and had been prescribed neuropathy-targeted medications for CP induced chronic pain. Upon expanded genetic testing for hereditary pancreatitis, including gene sequencing for CASR, CEL, CFTR, CLDN2, CPA1, CTRC, PRSS1, SBDS, SPINK1, UBR1 and multiplex ligation-dependent probe amplification as well as analysis for PRSS1 deletion, the patient was shown to have the following CFTR gene variants: CFTR NM-000492.3: c.1521-1523del (p.Phe508del) pathogenic, heterozygous c.3131A >G(p.Glu1044Gly) variant of unknown clinical significance, heterozygous c.1210-34-1210-6TG [10]T [9] heterozygous.

The patient underwent TPIAT successfully at the age of 13 years. Over the first three postoperative months, her pain improved dramatically, and she was able to tolerate a continual wean off opioids. At six months postoperatively, her pancreatitis-type abdominal pain had resolved, and she was off opioid medications. She was also able to tolerate a continual wean off tube feeding supplementation and required only minimal insulin supplementation with ongoing weaning off exogenous insulin.

Improved CFTR function by ivacaftor

Duodenal stem cells were successfully isolated and cultured from the patient with the rare

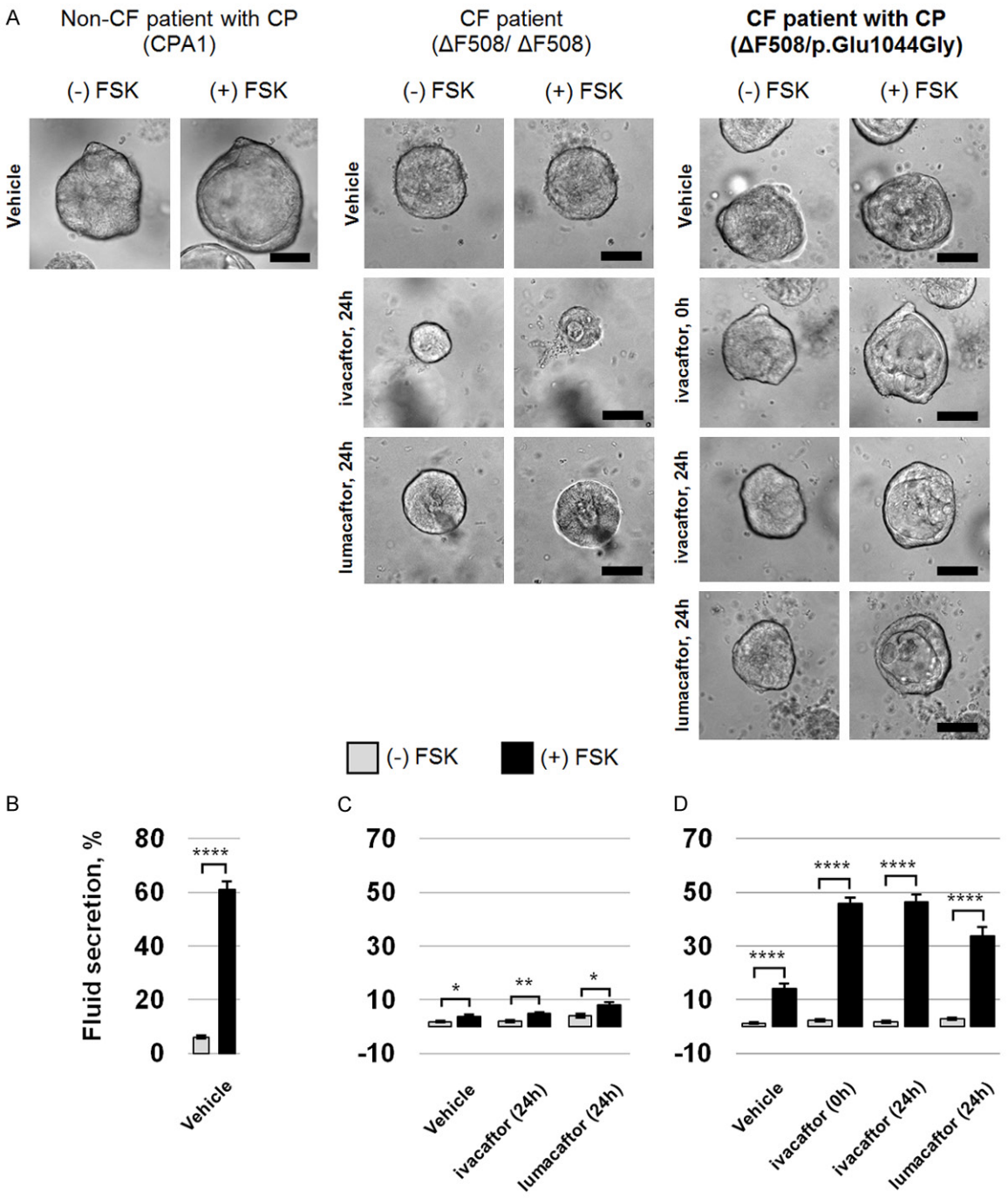

Figure 1. Fluid secretion assay. CFTR channel function was monitored using a fluid secretion measurement in response to 10 μM FSK for 2 h (A). Bar graphs show CFTR function in non-CF patient with CP (B: CPA1), CP-patient with the most common CF (C: ΔF508/ΔF508) without CP and CF-patient with CP (D: ΔF508/p.Glu1044Gly). The organoids were treated with ivacaftor (2 μM, 0 h and 24 h), and lumacaftor (2 μM, 24 h) prior to the assay. Scale bars: 100 μm. (n ≥20 organoids; data are mean ± SEM; *p-values* from two-way analysis of T-test: *<0.05, **<0.001, ***<0.0001, ****<1.0×10^{-6}).

combination of CFTR mutations (ΔF508/p.Glu1044Gly). CFTR function was monitored from 3-dimensional organoids using a fluid secretion assay (**Figure 1A**). As controls, CFTR functional testing was performed in organoids created from two controls: 1.) a non-CF patient

with CP carrying a mutation in the Carboxypeptidase gene A1 (CPA1) (**Figure 1B**) and 2.) a CF-patient with severe ΔF508/ΔF508 mutant disease but lacking CP (ΔF508/ΔF508; no CP; **Figure 1C**). Organoids from the non-CF patient demonstrated highly efficient CFTR function, with fluid secretion increasing from basal secretion of 6.1% to 60.9% following FSK treatment (**Figure 1B**). As expected, organoids from the CF-patient with ΔF508/ΔF508 mutations showed impaired CFTR function (**Figure 1C**). Basal secretion was 1.8%, and fluid secretion increased only marginally to 3.7% with FSK treatment. This finding is consistent with the lack of CFTR on the apical membrane of epithelial cells in the setting of homozygous ΔF508 mutation due to a trafficking disorder of CFTR protein from the Golgi apparatus to the apical membrane [6]. CF patients with homozygous ΔF508 mutations have been successfully treated clinically with the FDA-approved medications, lumacaftor and ivacaftor [5, 6, 16]. Thus, we pre-treated organoids with 2 μM ivacaftor or 2 μM lumacaftor for 24 h and monitored CFTR function. Although the fluid secretion was still low in organoids from the patient with homozygous ΔF508 mutations, CFTR function was significantly improved by drug pre-treatment (**Figure 1C**). We then similarly pre-treated the organoids with the novel mutation combination ΔF508/p.Glu1044Gly with both the CFTR potentiator and corrector for 24 h. Fluid secretion in these organoids showed severely impaired CFTR function with basal secretion of 1.3%, increasing to only 14.2% with FSK treatment. However, CFTR function was significantly and robustly improved in ivacaftor or lumacaftor-treated organoids (**Figure 1D**). Fluid secretion increased from 2.4% to 45.7% with addition of ivacaftor at time of FSK administration (no pre-treatment with ivacaftor), from 1.9% to 46.3% after ivacaftor pre-treatment for 24 h, and from 3.0% to 33.7% after lumacaftor pre-treatment for 24 h (**Figure 1D**). We observed that ivacaftor treatment resulted in conspicuously improved CFTR function *in vitro* in the organoids with the novel mutation combination.

To verify the effect of ivacaftor in the ΔF508/p.Glu1044Gly mutant patient, we prepared polarized duodenal epithelial cells on a transwell membrane (**Figure 2A**, **2B**) mounted to an Ussing chamber to monitor CFTR function using a short-circuit current measurement (**Figure 2**). We applied 10 μM FSK followed by 2 μM ivacaftor and 20 μM CFTR inhibitor, $CFTR_{inh-172}$, to the apical side of the transwell membrane (**Figure 2C**). In general, duodenal organoids from the non-CF patient with CP had functional CFTR chloride channels which resulted in a robust response to FSK (Δ24.79±1.40 μA/cm^2) (**Figure 2D**, **2E**). In epithelial cells derived from the patient with the rare mutant, the corresponding current was dramatically lower at Δ1.77±1.56 μA/cm^2 post-FSK treatment. However, the CFTR dependent current was significantly increased by ivacaftor treatment (Δ6.21±0.59 μA/cm^2) (**Figure 2C**, **2E**). Our results demonstrate that ivacaftor robustly improved CFTR function in these rare ΔF508/p.Glu1044Gly mutant patient-derived stem cells.

Discussion

Our report is novel as it shows the application of a potential personalized medicine approach in patients with CP from a new CFTR variant combined with the known pathogenic ΔF508, through the understanding of molecular and functional genetics. We herein show that culturing individual patient-derived organoids *in vitro* is a useful technique for studying cellular functions in the context of rare, novel genetic variants and allows for direct assessment of the efficacy of therapeutic interventions.

CP is an ideal complex disorder to be studied by personalized medicine approaches as a new framework for medical care that incorporates disease simulation based on the underlying mechanisms of disease and genetic variants. With the era of huge data sets, incorporation of genetics, clinical expertise, and advanced laboratory experimentation must all come together as integrated tools to optimize advancement of patient care [18-21]. While CFTR is a gene known to be involved in development of pancreatitis, there is unclear risk assessment of the known and unknown (and novel) CFTR variant effects on end organ damage or disease progression. Understanding genetic variant specific function, together with identifying therapeutic response on a patient-by-patient basis is suitable for CFTR related diseases given the >2000 known genetic variants identified in CF patients [6, 7]. The future focus should be on designing platforms that incorporate genetic,

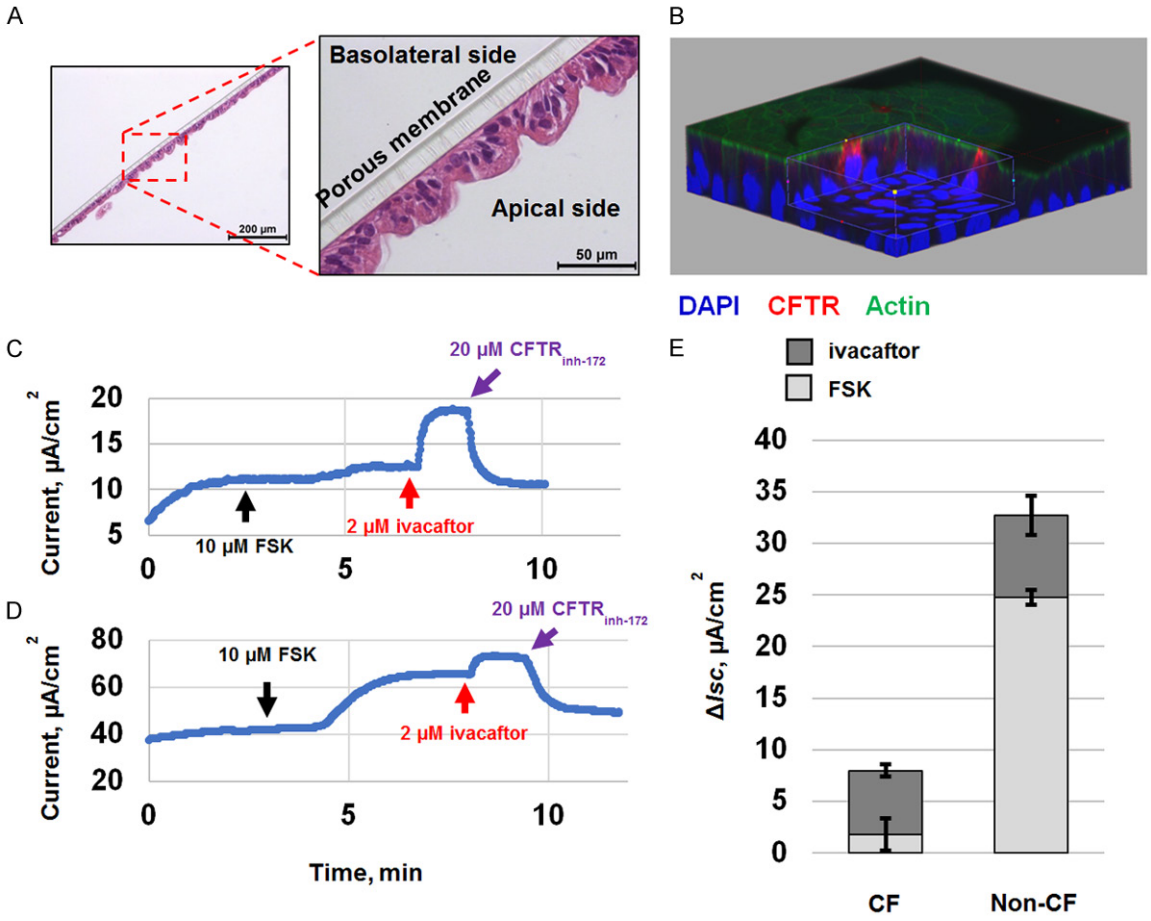

Figure 2. CFTR function using polarized epithelium. Duodenal epithelial cells polarized on trans-well membranes (A) and were examined with immunofluorescent microscopy for CFTR (B). CFTR is randomly expressed on an apical membrane of the epithelial cells. CFTR function was monitored using short-circuit current assay from CF-patient with CP (C: ΔF508/p.Glu 1044Gly) and non-CF patient with CP (D: SPINK1) in response to 10 µM FSK, 2 µM ivacaftor, and 20 µM $CFTR_{inh-172}$ (C-E). (n=3 sample preparation from the same patient).

functional, and clinical data through bioinformatic approaches to best serve our patients.

The reported patient experience raises the possibility that a personalized medicine approach in CF-related pancreatitis could help substantiate or alleviate the need for TPIAT surgery by defining variant specific functional data on a patient-by-patient basis to provide rationale for personalized treatment decisions. This possibility becomes particularly relevant in diseases such as CF where genetic alteration related therapies are available. The FDA has approved Ivacaftor (a CFTR potentiator) and combination therapy of two CFTR-correctors, elexacaftor and tezacaftor, and the potentiator (ivacaftor) that provide targeted treatment for almost 90% of CF patients [22, 23], but excludes the remaining ~10% of patients with rare, complex and novel mutations for whom targeted treatments are not available [22]. The patient described in the present study with a very rare form of CFTR variant (combination of ΔF508 and p.Glu1044Gly) falls into this later group given the presence of a CFTR variant of unknown clinical significance. Our studies demonstrate that the CFTR potentiator, ivacaftor, significantly and robustly improved CFTR function in intestinal stem cells derived from this patient (**Figure 1**) with a novel mutation. These studies highlight the potential valuable role of incorporating theratyping as part of patient evaluation to provide rationale to support medical interventions with therapeutic utility for patients with rare mutations of unknown clinical significance.

Our study, while novel, has limitations including the small patient size and single center design.

Measuring the end point for therapeutic efficacy for pancreatitis is also very challenging. While in lung disease forced expiratory volume 1 (FEV1) can serve as an objective measure, in CP objective measures are not clearly defined with potential outcomes including patient reported outcomes, exocrine function, number of hospitalizations, as well as many others given that it is still not known which clinical parameters are ideal for assessing disease progression.

In conclusion, we present a case of CP due to CF disease with a novel CFTR variant with unknown function and clinical significance. We propose that patient-derived cell platform studies have potential to guide rational design of therapies not previously incorporated into patient care due to the unknown genetic variant function. Acquiring knowledge of variant specific effects on cellular function together with testing efficacy of available therapeutics in individual patient derived *in vitro* systems is a logical approach to personalize medicine in pancreatitis and has potential to avoid unnecessary operations or invasive treatment options for CP patients. Future studies should be geared towards personalized medicine approaches to optimally care for patients with pancreatic diseases including CP.

Acknowledgements

The subject was enrolled in the INSPPIRE (International Study Group of Pediatric Pancreatitis: in search for a cure), study group under the CPDPC consortium, which has different aims and objectives than this case study. This research was supported by the National Institutes of Health [DK080834, DK093045, P30 DK117467], CF Foundation grants to K.S.M. [MUN18F0] and A.P.N. [NAREN14XX0]. MAH is supported by NIDDK, [grant number 1K23DK118190-01].

Disclosure of conflict of interest

None.

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