ORIGINAL RESEARCH

The Epithelial Sodium Channel Is a Modifier of the Long-Term Nonprogressive Phenotype Associated with F508del CFTR Mutations

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Abstract

Cystic fibrosis (CF) remains the most lethal genetic disease in the Caucasian population. However, there is great variability in clinical phenotypes and survival times, even among patients harboring the same genotype. We identified five patients with CF and a homozygous F508del mutation in the *CFTR* gene who were in their fifth or sixth decade of life and had shown minimal changes in lung function over a longitudinal period of more than 20 years. Because of the rarity of this long-term nonprogressive phenotype, we hypothesized these individuals may carry rare genetic variants in modifier genes that ameliorate disease severity. Individuals at the extremes of survival time and lung-function trajectory underwent whole-exome sequencing, and the sequencing data were filtered to include rare missense, stopgain, indel, and splicing variants present with a mean allele frequency of $<0.2\%$ in general population databases. Epithelial sodium channel (ENaC) mutants were generated via site-directed mutagenesis and expressed for *Xenopus* oocyte assays. Four of the five individuals carried extremely rare or never reported variants in the *SCNN1D* and *SCNN1B* genes of the ENaC. Separately, an independently enriched rare variant in *SCNN1D* was identified in the Exome Variant Server database associated with a milder pulmonary disease phenotype. Functional analysis using *Xenopus* oocytes revealed that two of the three variants in δ-ENaC encoded by *SCNN1D* exhibited hypomorphic channel activity. Our data suggest a potential role for δ-ENaC in controlling sodium reabsorption in the airways, and advance the plausibility of ENaC as a therapeutic target in CF.

Keywords: cystic fibrosis; ENaC; epithelial sodium channel; genetic modifier; SCNN1D

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Clinical Relevance

There is currently no known association between mutations in the epithelial sodium channel, especially the δ-subunit encoded by *SCNN1D*, and a milder lung disease phenotype in cystic fibrosis (CF). Our data implicate the epithelial sodium channel as a potential genetic modifier of CF in patients with a long-term nonprogressive phenotype.

Cystic fibrosis (CF) remains the most lethal genetic disease in the Caucasian population, affecting between 70,000 and 100,000 people worldwide (1). It is caused by mutations in the *CFTR* gene, which encodes an apical epithelial ion channel that regulates chloride transport across epithelial surfaces. Although CF is a multisystem disease, pulmonary involvement is the leading cause of mortality and reduced lifespan. In the lung, disruption of *CFTR* leads to decreased chloride and bicarbonate secretion, and increased sodium and water absorption across the airway epithelial surfaces. This ion-transport defect results in a both acidic and dehydrated airway surface liquid (ASL) layer, inspissation of the mucus layer, defective mucociliary escalator function, and eventually a chronic vicious cycle of infection and inflammation leading to destruction of the lung tissue and ultimately respiratory failure (2, 3). This phenotype is highly penetrant, with a typical loss of lung function of ∼2.5%/year in the forced expiratory volume in 1 second (FEV_1), which translates into a median life expectancy of ∼37 years (4–6). However, there is great variability in clinical phenotypes and survival times, even among patients harboring the same genotype and colonized with similar microbiota (7–9). This suggests that genetic modulators strongly influence the final CF phenotype, and that genes in addition to *CFTR* need to be addressed (7). Although genome-wide association studies have revealed multiple CF candidate modifier genes, they have not been confirmed definitively (7, 10). This indicates the presence of patient-specific genetic modifiers, which may be better examined by studying patients at extreme ends of the phenotypic distributions. Although the importance of genetic modifiers in patients with extreme phenotypes has long been appreciated, most studies examining this issue have been cross-sectional, stratifying lung function at a point in time instead of evaluating patients from a longitudinal perspective (7, 8, 11, 12).

At our CF registry, we have a cohort of five F508del homozygous patients whose lung function has plateaued with a minimal decline for >20 years. We refer to these patients as long-term nonprogressors (LTNPs). All five patients are colonized with *Pseudomonas aeruginosa*, have pancreatic insufficiency, and are in their fifth or sixth decade of life. Because of the rarity of this phenotype, we hypothesized that these individuals carry rare genetic modifiers that ameliorate the severity of disease.

Interestingly, whole-exome sequencing (WES) revealed that four of the five LTNPs exhibited extremely rare or previously not seen mutations in the epithelial sodium channel (ENaC), a permeable apical membrane ion channel that is responsible for sodium reabsorption in the lung, kidney, colon, and sweat/salivary ducts (13, 14). Three patients exhibited rare mutations in the δ-subunit of ENaC, encoded by the gene *SCNN1D,* and one patient exhibited mutations in the β-subunit of ENaC, encoded by the gene *SCNN1B.* ENaC is typically believed to be composed of three homologous subunits (α, β, and γ), each with cytosolic N- and C-termini and two transmembrane domains connected by an extracellular loop (15). A fourth δ-subunit has been identified in humans, but little is known about its function *in vivo*. Sharing 37% amino acid homology with the α-subunit, the δ-subunit is believed to coassemble with the β- and γ-subunits to form a functional δβγ-ENaC heterotrimer, which exhibits distinct biophysical properties compared with the αβγ-ENaC channel (16–18). The δ-subunit has been identified in human nasal epithelial cells (19), immortalized human respiratory epithelial cells (A549, H441, and Calu-3,16HBE14o−), human alveolar type II cells, and human lung tissues (18, 20–24), establishing the relevance of this newly characterized subunit in lung disease. Our data suggest an important role of ENaC as a potential genetic modifier of CF, and a heretofore-unsuspected role of the SCNN1D gene in modifying lung disease.

Materials and Methods

Participants

We follow ∼600 patients in CF registry at our institution. In the group of homozygous F508del patients, we identified patients who were above the average survival age and whose FEV_1% for age met one of two criteria: (*1*) it was persistently >80% of predicted over 20 years, or (*2*) it showed a lung-function decline of <1%/year over the last 20 years. Exclusion criteria included CF mutations other than the homozygous F508del.

Sequencing

Five individuals with both extremes of survival time and lung-function trajectory were identified from a population of 600 patients with CF who were enrolled in an IRB-approved study at Boston Children's Hospital. Blood samples were collected and processed for DNA extraction by the Research Connection Biobank Core at Boston Children's Hospital. DNA was sent for WES to Axeq Technologies (Rockville, MD). WES and data analysis were performed as previously described (25). Whole-exome sequencing data were filtered to included missense, stopgain, indel, and splicing variants with a mean allele frequency (MAF) of <0.002 (<0.2%) in the databases of the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP, available on the Exome Variant Server [EVS]), the 1000 Genomes Project, and the Exome Aggregation Consortium (ExAC). Several *in silico* missense variant prediction softwares, including PolyPhen-2, MutationTaster, and SIFT were utilized to evaluate pathogenicity. Sequence data from additional individuals with CF from the EPIC (Early *Pseudomonas* Infection Control) cohort participating in the NHLBI ESP were used to identify p.R366W *SCNN1D* in the second stage of this investigation and were previously described in detail (26).

Site-Directed Mutagenesis

Point mutations were generated in ENaC cDNA using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Techologies, Inc., Santa Clara, CA). Mutations were confirmed by direct sequencing. Wild-type (WT) and mutant human δ and β ENaC, and WT human α- and γ-ENaC complementary RNAs (cRNAs) were synthesized using SP6 or T7

RNA Polymerase (Thermo Fisher Scientific, Waltham, MA). All synthesized cRNAs were purified using an RNA purification kit (Qiagen, Germantown, MD) and their concentrations were quantified by spectrophotometry.

ENaC Expression and Two-Electrode Voltage Clamp

ENaC expression in *Xenopus* oocytes and two-electrode voltage clamp were performed as previously described (27). Stage V and VI oocytes free of follicle cell layers were injected with 0.5 or 1.0 ng of cRNA for each hENaC subunit (δ, β, and γ; or α, β, and γ, as indicated) per oocyte and incubated for 20–48 hours at 18°C in modified Barth's saline. The University of Pittsburgh Institutional Animal Care and Use Committee approved the animal protocol. Two-electrode voltage clamp was performed using an Axoclamp 900A amplifier and a DigiData 1440A interface controlled by pClamp 10 (Molecular Devices Corp., Sunnyvale, CA). Oocytes were placed in a recording chamber (Warner Instruments, Hamden, CT) and perfused with bath solutions (110 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4) at a constant flow rate of 12–15 ml/min. Pipette resistances were in the range of 0.2 to ~2 MΩ when the pipettes were filled with 3 M KCl. Oocytes were continuously clamped and data were recorded at −60 mV (for δβγ) or −100 mV (for αβγ) in the presence of the bath solution and bath solution with added 100 μM (for δβγ) or 10 μM (for αβγ) amiloride. The difference in current in the absence and presence of amiloride was considered as the amiloride-sensitive, whole-cell current, which served to indicate ENaC activity.

$CD31^-CD45^-EpCAM^+$ Cell Sorting and mRNA Expression

Human lungs were obtained from the Northern California Donor West Organ Procurement Network. All experiments using cadaver human lungs were approved by the Biosafety Committee of the University of California, San Francisco. The lungs were resected *en bloc* without preservative flush and inflated and transported at 4°C. The lungs were digested in 30% dispase and 3 mg/ml Collagenase D in Hanks' balanced salt solution for 1 hour at 37°C. $CD31^-CD45^-EpCAM^+$ cells were isolated using anti-human $CD45^-$ Pacific Blue (57-0459; eBioscience, San Diego, CA), anti-human CD31-PE (555,446; BD Pharmingen, San Jose, CA), anti-human EpCAM-FITC (347,197; BD Pharmingen), and DAPI staining to eliminate dead cells with a BD FACS Aria. RNA was extracted using Trizol. cDNA was made using the SuperScript III Kit (Invitrogen, Carlsbad, CA) and analyzed using Taqman assays for *CFTR* (Hs00357011_m1), *SCNN1A* (Hs01013028_m1), *SCNN1B* (Hs01548617_m1), and *SCNN1D* (Hs00161595_m1) with an Applied Biosystems Step One Plus (Foster City, CA).

Statistical Analyses

Two-sample comparisons were performed after integrating BAM files for LTNPs and ESP participants for unified variant calling using the GATK UnifiedGenotyper. The small-sample–adjusted sequence kernel association test (SKAT) was used for the comparisons to obtain valid *P* values for the small case samples (extremes of lung function in CF individuals) versus control samples while adjusting for ancestry.

Results

Patient Clinical Phenotypes

From a population of ~600 patients with CF at our institution, we identified five individuals who are at the extreme ends of both survival time and lung-function trajectory. All five are homozygous F508del males, as confirmed by Sanger sequencing, and living in their fifth or sixth decade (Table 1). All of them have acquired *P. aeruginosa* and are pancreatic insufficient. Their body mass index ranges between 24.7 and 26.2. All of them have been diagnosed with essential hypertension, and four of the five have been diagnosed with CF-related diabetes.

All five individuals have had preserved FEV_1 for at least 20 years (Table 2; Figure 1). LTNP1 has had intermittent exacerbations requiring intravenous antibiotics, but managed to maintain his FEV_1 at 80% from 1992 to 2014, with a rate of decline of 0.32% per year. LTNP2 demonstrated preserved FEV_1% for 24 years until this patient developed allergic bronchopulmonary aspergillosis in 2014. Even taking into account the decline in lung function after LTNP2 acquired allergic

Table 1. Clinical Phenotypes of the Five Long-Term Nonprogressors with Cystic Fibrosis

Patient	Age	CFTR Mutation (Homozygous)	ENaC Mutation	Microbiology	Hyperinflation on Chest X-ray	Sweat Chloride	Body Mass Index	Pancreatic Function	Other Comorbidities
LTNP1	67	F508del	SCNN1D V541L	PsA (mucoid, multi-resistant)	No (6/2015)	121	24.7 (2016)	PI	CFRD, HTN
LTNP2	52	F508del	SCNN1D p579L	PsA (partially resistant), MSSA	No (2,014)	99	25.6 (2016)	PI	Nasal polyps, HTN
LTNP3	66	F508del	SCNN1D V541L	PsA (mucoid, sensitive), *Klebsiella*	No (2,014)	165	25.1 (2016)	PI	CFRD, nasal polyps, HTN
LTNP4	63	F508del	SCNN1B p613L	PsA (sensitive), MSSA	Yes (8/2015)	122	24.6 (2016)	PI	CFRD, sinus disease, HTN
LTNP5	53	F508del	None	PsA (partially resistant), MSSA	No (2,016)	121	26.2 (2016)	PI	CFRD, HTN

Definition of abbreviations: CFRD, cystic fibrosis–related diabetes; CFTR, cystic fibrosis transmembrane conductance regulator; HTN, hypertension; LTNP, long-term nonprogressor; MSSA, methicillin-sensitive *Staphylococcus aureus*; PI, pancreatic insufficiency; PsA, *Pseudomonas aeruginosa*.

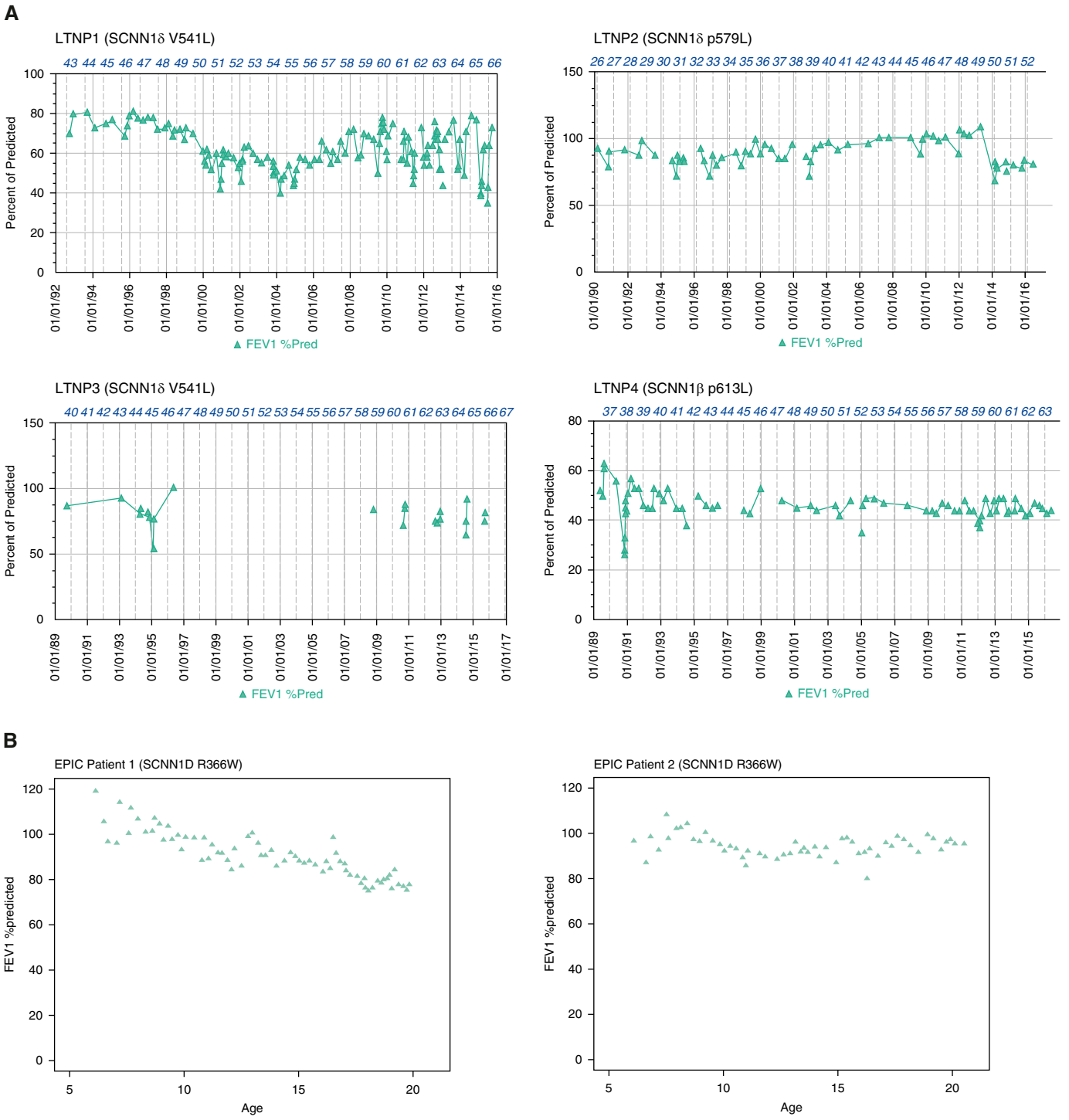

Figure 1. (*A*) Longitudinal analysis of FEV_1 predicted for four F508del long-term nonprogressors with cystic fibrosis and epithelial sodium channel (ENaC) mutations, from the Boston Children's Hospital database. All four patients had demonstrated preserved FEV_1 for >20 years. (*B*) Longitudinal analysis of FEV_1 predicted of the two patients with cystic fibrosis from the EPIC cohort who carry ENaC mutation R366W and whose lung function is in the upper quartile predicted for their age. EPIC, Early *Pseudomonas* Infection Control; LTNPs, long-term nonprogressors.

bronchopulmonary aspergillosis, the decline in FEV_1 was 0.16% per year. LTNP3 was not seen in our institution from 1996 to 2008, but has had preserved FEV_1% in the high 80s consistently after resuming care in 2008, with only a 0.07% per year decline. LTNP4 has had a typical course, with FEV_1 ranging from 40 to 50% in the early 1990s, in the patient's fourth decade of life, but has since flatlined, with a decline of 0.65% per year. For all patients, the slope of FEV_1% change per year was

Table 2. FEV_1 Longitudinal Data for the Five Long-Term Nonprogressors with CF Over a 20-Year Period

Patient	Baseline FEV_1 (%)	Slope (% per year)	95% CI (% per year)
LTNP1	67	−0.32	−0.05 to +0.58
LTNP2	101	−0.65	−0.80 to −0.50
LTNP3	87	−0.07	−0.63 to +0.48
LTNP4	48	−0.16	−0.31 to −0.01
LTNP5	86	+0.29	−0.03 to +0.62

Definition of abbreviations: CF, cystic fibrosis; CI, confidence interval; LTNP, long-term nonprogressor.

consistently less than the 2.5% per year that is typical of patients with CF bearing the homozygous F508del mutation (Table 2).

The independent patients from the EPIC cohort participating in the NHLBI ESP were classified as having a mild pulmonary disease despite carrying a F508del and a deleterious stopgain mutation. Both are female, with pancreatic insufficiency. The patient carrying F508del and Y1092X mutations is 19 years old and her recent FEV_1 was 78.8% of predicted for age. The second patient carrying the F508del and G542X mutations is 21 years old, with FEV_1 95.5% of predicted (Table 3).

ENaC Variants

WES showed that four of the five LTNPs exhibited extremely rare or not previously seen mutations in the δ-subunit (encoded by *SCNN1D*, three patients) and β-subunit (encoded by *SCNN1B*, one patient) of ENaC. The ENaC variants in our LTNPs included P613L in *SCNN1B*, and V541L (present in two patients) and P579L in *SCNN1D*. They are described in further detail in Table 4, including their frequencies in publicly available databases and *in silico* pathogenicity predictions. We sought to test whether finding rare variants in genes encoding subunits of ENaC in four of five persons with CF would differ from expectations due to chance. To that end, we performed the small-sample–adjusted SKAT-O test on each individual variant to test for a difference in variant frequency between the LTNPs and 3,302 controls of European ancestry without lung disease (28). Principal component 1 (PC1) and PC2, derived from the ESP CF data, were included as covariates to correct for population stratification. For P613L (*SCNN1B*), V541L (*SCNN1D*), and P579L (*SCNN1D*), the unadjusted *P* values were 2.45e-04, 7.07e-06, and 2.37e-04, respectively. The adjusted *P* values after Bonferroni correction were 7.4e-4, 2.1e-5, and 7.07e-06, respectively. The *P* values were significant after multiple-testing correction for a candidate-gene study and adjustment for ancestry (Table 3). Using the criteria described above to filter out the nonrare variants, we did not find any ENaC variants in any of our typical CF patients or CF patients with the most severe phenotypes of lung disease.

To further test the hypothesis that rare variants in ENaC genes may be enriched in CF patients with milder lung disease, we examined an independent EPIC cohort consisting of 90 CF individuals with lung function in the upper half of the median and exome sequencing data available from the EVS database. Rare variants in ENaC genes with an MAF < 0.002 were determined in these 90 individuals and compared with the 3,210 non-CF controls. One variant in *SCNN1B* and three in *SCNN1D* were identified in 90 individuals after filtering for MAF < 0.002. One of these four variants, p.R366W in *SCNN1D*, was significantly enriched in the EPIC cohort compared with non-CF controls (2/90 CF individuals versus 7/3,210 controls, $P = 0.0033$). Their *CFTR* genotype was evaluated, and both subjects were found to be compound heterozygous for a F508del variant and a deleterious stopgain variant (G542X or Y1092X). These would be considered to be severe disease-causing variants. Although the three remaining ENaC variants came close, they did not achieve a significant *P* value.

Functional Assessment of ENaC Variants

To test the effect of each of these variants on the function of ENaC, we used a *Xenopus* oocyte expression system. We hypothesized that some of these ENaC variants would be hypomorphic alleles, as ENaC hyperactivity is postulated to contribute to CF (29–31). We generated cRNAs bearing specific ENaC mutants, and expressed them in *Xenopus* oocytes. Using a two-electrode voltage clamp, we measured the amiloride-sensitive currents in oocytes injected with δβγ-WT ENaC cRNAs or channels with specific WT β- and γ-subunits and a δ-subunit with a specific mutation. The δP579L variant (Figure 2A) produced a significant reduction in the current ($P < 0.001$, $n = 53$–55/group). Similarly, the variant δR366W (Figure 2B) was also observed to be a hypomorph in the *Xenopus* assay ($P < 0.05$, $n = 64$–72/group). Whole-cell sodium currents in oocytes expressing the δV541L variant were similar to those in the δβγ-W (Figure 2C). Furthermore, currents in oocytes expressing the αβγ-WT were similar in magnitude to those in the βP613L variant (αβP613γ).

Table 3. Clinical Phenotypes of the Two EPIC Cohort Patients Carrying ENaC Mutations

Patient	Age	CFTR Mutations	ENaC Mutation	Last FEV_1%	Microbiology	Sweat Chloride	Body Mass Index	Pancreatic Function	Other Comorbidities
EPIC patient 1	19	F508del; Y1092X	p.R366W	78.8%	PsA	106	33.6	PI	None known
EPIC patient 1	20.5	F508del; G542X	p.R366W	95.5%	—	—	21.6	PI	None known

Definition of abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial sodium channel; EPIC, Early *Pseudomonas* Infection Control; PI, pancreatic insufficiency; PsA, *Pseudomonas aeruginosa*.

Table 4. ENaC Variant in the Long-Term Nonprogressors

Patient	Gene	Genome Assembly	Chromosome	Location	Transcript	cDNA Change	A.A. Change	Polyphen-2	SIFT	MutationTaster	EVS Frequency	X1000GP	ExAC
LTNP1,3	SCNN1D	GRCh37/hg19	1	1223376	NM_001130413	c.1621G>C	pV541L	0.502	0.04	Polymorphism	0.0011	0	0.001
LTNP2	SCNN1D	GRCH37/hg19	1	1225724	NM_001130413	c.1736C>T	p.P579L	1	0.01	Disease-causing	0	0	0.0001
LTNP4	SCNN1B	GRCh37/hg19	16	23392037	NM_000336	c.1838C>T	p.P613L	0.999	0	Disease-causing	0	0	0
EPIC patients	SCNN1D	GRCh37/hg19	1	1222332	NM_001130413	c.1096C>T	p.R366W	0.996	0.01	Polymorphism	0.0008	0.011	0.002

Definition of abbreviations: cDNA, complementary DNA; ENaC, epithelial sodium channel; EPIC, Early *Pseudomonas* Infection Control; EVS, Exome Variant Server; ExAC, Exome Aggregation Consortium; LTNP, long-term nonprogressor; SIFT, sorting intolerant from tolerant.

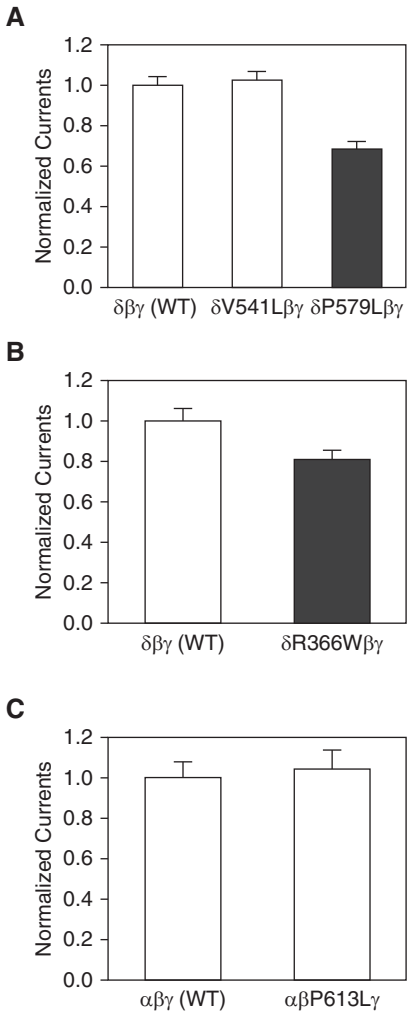

Figure 2. Selected δ-subunit variants alter ENaC activity. Amiloride-sensitive currents were measured in oocytes injected with wild-type (WT) or mutant human ENaC complementary RNAs. Normalized currents represent amiloride-sensitive currents from individual oocytes that were divided by the average of the amiloride-sensitive current of oocytes expressing WT channels from the same batch of oocytes. (*A*) Normalized currents for δR366Wβγ channels ($n = 64$, *solid bar*) were significantly less than those for WT δβγ ENaCs ($n = 72$, $P < 0.05$). Data were pooled from four batches of oocytes. (*B*) Normalized currents for δP579Lβγ ENaCs ($n = 55$, *solid bar*) were significantly less than those for WT δβγ channels ($n = 53$, $P < 0.001$). The currents for δV541Lβγ ENaCs ($n = 55$, *open bar*) were similar to those found for the WT ($P > 0.05$). Data were obtained from three batches of oocytes. For both *A* and *B*, oocytes were clamped at −60 mV and 100 μM amiloride was used to obtain amiloride-sensitive currents. (*C*) Normalized currents in oocytes expressing αβP613Lγ ENaCs ($n = 51$, *open bar*) were similar to those in cells expressing WT αβγ ENaCs ($n = 48$, $P > 0.05$). Cells were clamped at −100 mV and 10 μM amiloride was used. Different clamping voltages and amiloride concentrations were used because δβγ ENaCs have greater channel activity and less amiloride sensitivity than αβγ ENaCs.

Molecular Modeling of ENaC Mutations

We next sought to assess the location of the R366, V541, and P579 variants within the δ-ENaC subunit. A structural model of the α-subunit of mouse ENaC was generated from the PDB file in Kashlan and colleagues (32) using PyMol 1.5 (33). The α-subunit residues homologous to the human δ-subunit residues R366, V541, and P579 were determined by sequence alignment using Vector NTI 11.0 (Invitrogen). δR366 is located in the finger domain of the extracellular region of the channel (Figure 3). δV541 and δP579 are located within loops at the base and top of the thumb domain, respectively.

Expression of ENaC

We confirmed the ENaC subunits' mRNA expression in human lungs by quantitative RT-PCR. We found that *SCNN1A*, *SCNN1B*, and *SCNN1D,* as well as *CFTR*, showed clearly higher expression levels in EpCAM-enriched epithelial cells compared with lung tissue from the same healthy human donor. These data suggest that SCNN1D might have some function as well as three other ENaC subunits in lung epithelial cells (Figure 4).

Discussion

Four out of five homozygous F508del CFTR mutation-carrying individuals with mild lung disease whose lung function has been well preserved over a 20-year observation period also carry variants in ENaC genes. Three of the four ENaC mutations occur in the δ-subunit, whose role in lung disease has not been previously characterized. Further, an independent enriched rare variant (R366) in the δ-subunit was found in the EPIC cohort from the NHLBI EVS database. Two of the three δ-ENaC variants had reduced channel activity when expressed in *Xenopus* oocytes, consistent with the notion that these variants have a protective role in CF, and lending credibility to the idea that ENaC inhibition has a therapeutic role in CF.

Although it has long been appreciated that genetic modifiers are responsible for outcomes in patients with CF (34), most studies examining this issue have been cross-sectional, stratifying lung function at a point in time and using candidate-gene approaches (7, 8, 11, 12). To our knowledge, this is the first study to address the question of genetic modifiers of lung disease from a longitudinal perspective over a 20-year period. It remains to be seen whether patient R366 from the EPIC cohort, who also harbors an *SCNN1D* mutation and whose lung function is in the upper quartile for age, will continue to maintain the phenotypes of an LTNP.

To our knowledge, this is also the first time that rare variants in ENaCs have been linked to longevity of the F508del homozygous individuals. There is a growing appreciation for the importance of rare single-nucleotide polymorphisms as crucial factors in many genetic diseases (35, 36). Although the popular common-disease/common-variant hypothesis has largely dominated the genetic studies, there is a growing body of evidence that rare single-nucleotide polymorphisms are actually more likely to have a deleterious effect on protein function than common single-nucleotide polymorphisms (35, 37).

In patients with CF, a defective CFTR leads to derangement of the airways' electrophysiological properties via multiple proposed mechanisms. It has been widely hypothesized that CFTR mediates anionic secretion of Cl^- and $HCO3^-$, the disruption of which leads to increased absorption of Na^+ and water, and dehydration of the ASL. ENaC was found by multiple studies to be upregulated and hyperfunctional in CF. Although the precise mechanism of the CFTR-mediated Na^+ hyperabsorption is unclear (2, 38, 39), enhanced ENaC proteolysis may have a role in channel activation in CF (40, 41). However, findings from other studies do not support the sodium hyperabsorption hypothesis, at least at the onset of disease (42–44). Moreover, it has been shown that deficient secretion of bicarbonate into the airways leads to reduced pH in the ASL, and the resulting acidity in turn leads to impaired airway epithelial antimicrobial activities (44, 45). In CF, both the volume and composition of the ASL are likely impaired, leading to a dehydrated and

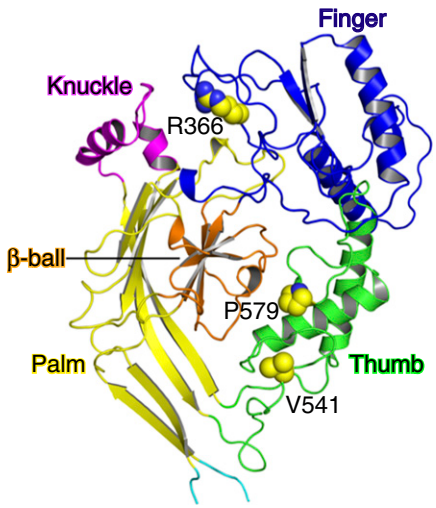

Figure 3. Location of R366, V541, and P579 within the δ-subunit. A structural model of the α-subunit of mouse ENaC was generated from the PDB file in Kashlan and colleagues (32) using PyMol 1.5 (33). The backbone is rendered as ribbons. The α-subunit residues homologous to the human δ-subunit residues R366, V541, and P579 were determined by sequence alignment using Vector NTI 11.0 (Invitrogen, Carlsbad, CA). Side chains are shown in CPK mode, with carbon in yellow and nitrogen in blue. Individual domains within the extracellular region are labeled.

acidic environment that favors the chronic colonization of biofilm-forming organisms.

Given the importance of ENaC in modulating the ASL, genes encoding ENaC subunits have long been considered to be genetic modifiers for CF (46). Indeed, mice overexpressing β-ENaC in the airways were shown to have CF-like lung disease (46, 47). However, mutations of ENaC can be either hypomorphic or hypermorphic, complicating studies to elucidate how ENaC modifies CFTR (48, 49). The fact that the functional effects of ENaC variants can be readily assessed provides opportunities to correlate functional variants with phenotypes.

The δ-subunit of ENaC is less well characterized than the α-, β-, and γ-subunits. Little is known about its function *in vivo*, as rodents do not express δ-subunits, limiting the model organisms available to study the subunit. Our data from sorted lung cells and lung tissue (Figure 4) confirm the expression of the δ-subunit in the lung, in agreement with previous literature showing its presence in human nasal epithelial cells (19), immortalized human respiratory epithelial cells (A549, H441, and Calu-3,16HBE14o−), human alveolar type II cells, and human lung tissues (18, 20, 21). At the message level, *SCNN1D* expression in the lung has been confirmed by northern blot (22), dot-blot (23), gene chip microarrays (24), and immunohistochemistry (21). At the protein level, an anti-δ antibody confirmed the presence of this subunit in a lung adenocarcinoma cell line and established its colocalization with other ENaC subunits (20). A previous study noted that δ-ENaC seems to have lower expression in the lung than α-ENaC; however, the authors found it difficult to make a statistical comparison of the two subunits (21). Studies have also shown that δβγ-ENaC is significantly less sensitive to amiloride than αβγ-ENaC (18, 20, 22, 50), but is more sensitive to protons and thus pH (24, 51). Given the increased sensitivity of δβγ-ENaC to extracellular pH acidification, it is a promising target for CF disease modulation, given the postulation that CF patients have more acidic airways that ultimately lead to impairment of bacterial killing (52, 53). Although ENaC inhibitors such as amiloride analogs have been explored as therapeutics in CF (54–56), these targets are not specific to the δ-subunit and the results to date have been disappointing. To our knowledge, this is the first study to implicate the δ-ENaC subunit in CF.

Regarding the molecular structure of ENaC, δR366 is located in the finger domain of the extracellular region of the channel, and δV541 and δP579 are located within loops at the base and top of the thumb domain, respectively (Figure 3). As the least conserved of the five extracellular domains (palm, β-ball, knuckle, finger, and thumb) (32), the finger domain has been suggested to impart functional properties that are not shared with other ENaC/Degenerin family members. Although the functional roles of the finger domain of the δ-subunit are unknown, previous studies of ENaCs offer some clues. Finger domains contain functional sites for channel regulation by proteases, transition metals, extracellular Na^+, shear stress, and reactive oxygen species (57–63). Finger domain interactions with other domains are likely involved in channel transitions in response to extracellular cues that affect the channel open probability (64). In addition, selected finger domain mutations alter channel surface expression (65). The activity of δV541L channels was similar to that of the WT, suggesting that this mutation is well tolerated

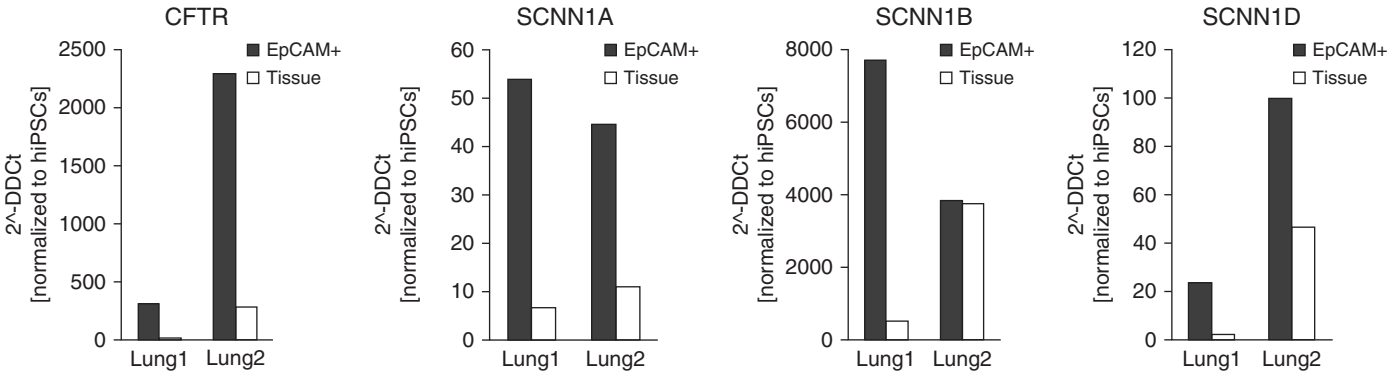

Figure 4. CFTR and ENaC (α-, β-, and δ- subunits) expression in human lungs by quantitative RT-PCR. $EpCAM^+$ (*solid bar*) represents $CD45^-CD31^-EpCAM^+$ cells sorted from healthy human fresh lung tissue. Tissue (*open bar*) represents human lung tissue from the same donor. Lungs 1 and 2 are from two different donors. Healthy human induced pluripotent stem cells were used as a negative control. CFTR, cystic fibrosis transmembrane conductance regulator; DDCt, delta-delta-Ct; EpCAM, epithelial cell adhesion molecule.

There are limitations to our study. Only two of the ENaC variants showed reduced sodium channel function. Two patients bearing the variant R366W from the EPIC cohort are at the upper quartile of lung function, and it remains to be determined whether these patients will maintain the LTNP phenotype. Even though these two EPIC cohort patients are heterozygous for F508del, they each carry a nonsense mutation (G542X and Y1092X, respectively) that is predicted to have a deleterious effect on the protein, as well as severe pulmonary phenotypes. It is possible that the other ENaC variants that did not alter channel activity in the *Xenopus* oocytes might affect channel activity in native tissues, where cell-specific factors might influence ENaC expression and activity (66). Furthermore, we did not assess message stability or protein stability and trafficking of the ENaC variants. It would be important in future studies to evaluate both the ASL volume and pH of the LTNPs as compared with the normal CF phenotype. In addition, we do not have information on the levels of ENaC expression in these LTNPs.

Our data implicate ENaC as a potential genetic modifier of CF and suggest a heretofore-unsuspected role of δ-ENaC in lung disease. Additionally, our studies advance the plausibility of ENaC as a therapeutic target in CF. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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