

## ORIGINAL ARTICLE

## Reduced sodium/proton exchanger NHE3 activity causes congenital sodium diarrhea

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Received: July 30, 2015. Revised and Accepted: September 3, 2015

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## Abstract

Congenital sodium diarrhea (CSD) refers to an intractable diarrhea of intrauterine onset with high fecal sodium loss. CSD is clinically and genetically heterogeneous. Syndromic CSD is caused by *SPINT2* mutations. While we recently described four cases of the non-syndromic form of CSD that were caused by dominant activating mutations in intestinal receptor guanylate cyclase C (GC-C), the genetic cause for the majority of CSD is still unknown. Therefore, we aimed to determine the genetic cause for non-GC-C non-syndromic CSD in 18 patients from 16 unrelated families applying whole-exome sequencing and/or chromosomal microarray analyses and/or direct Sanger sequencing. *SLC9A3* missense, splicing and truncation mutations, including an instance of uniparental disomy, and whole-gene deletion were identified in nine patients from eight families with CSD. Two of these nine patients developed inflammatory bowel disease (IBD) at 4 and 16 years of age. *SLC9A3* encodes Na<sup>+</sup>/H<sup>+</sup> antiporter 3 (NHE3), which is the major intestinal brush-border Na<sup>+</sup>/H<sup>+</sup> exchanger. All mutations were in the NHE3 N-terminal transport domain, and all missense mutations were in the putative membrane-spanning domains. Identified *SLC9A3* missense mutations were functionally characterized in plasma membrane NHE null fibroblasts. *SLC9A3* missense mutations compromised NHE3 activity by reducing basal surface expression and/or loss of basal transport function of NHE3 molecules, whereas acute regulation was normal. This study identifies recessive mutations in NHE3, a downstream target of GC-C, as a cause of CSD and implies primary basal NHE3 malfunction as a predisposition for IBD in a subset of patients.

## Introduction

Congenital intractable diarrheas with known etiology are monogenic disorders. Clinical and histological criteria as well as molecular testing for mutations in known disease genes are used to classify these disorders (1). The rare diarrheal disorder, congenital sodium diarrhea (CSD; MIM #270420), was first described in two sporadic patients in 1985 (2,3), and subsequently <40 cases have been reported in the literature (4). Jejunal brush-border (BB) membrane studies suggested a defect in sodium/proton exchange activity in three sporadic patients diagnosed with CSD (2,5,6). In 2000, we identified five patients with CSD from a large consanguineous Austrian kindred, demonstrating autosomal recessive inheritance in that family. Linkage analyses in that family excluded all known sodium/proton exchanger genes including *SLC9A3* (solute carrier family 9, subfamily A, member 3; MIM #182307), the gene encoding sodium/proton antiporter 3 (NHE3) (4). Instead, the positional candidate approach identified a homozygous splice-site mutation in *SPINT2* (serine peptidase inhibitor, Kunitz type, 2; MIM #605124) in this family (7). Pathogenic biallelic *SPINT2* mutations were identified in 19 additional families (7–9). Patients with *SPINT2* mutations were clinically characterized as a form of ‘syndromic’ CSD, in which intractable diarrhea was associated with superficial punctate keratitis, choanal atresia, intestinal atresia, as well as other sporadic abnormalities. This form of CSD is also referred to as a syndromic form of congenital tufting enteropathy (intestinal epithelial dysplasia; MIM #613217), as it often shows clustered enterocytes that form ‘tufts’ with branching crypts on histology (8,10).

Classical CSD (also called the non-syndromic phenotype) resembles congenital chloride diarrhea (MIM #214700) (11), but is distinguished by having high fecal loss of Na<sup>+</sup>, and is excluded from enterocyte differentiation and polarization disorders such as microvillus inclusion disease (MIM #251850) (12,13) and non-syndromic tufting enteropathy (10) by histopathology. Very recently, dominant activating mutations in receptor guanylate cyclase C (GC-C; MIM #601330) were found to cause a spectrum of secretory diarrheas including non-syndromic CSD in four patients (14,15). These mutations were associated with elevated intracellular cyclic guanosine monophosphate (cGMP) levels (14,15), an effect of which is the inhibition of NHE3 via its phosphorylation by cGMP kinase II (MIM #601591) as shown in PS120 fibroblasts and Caco-2/Bbe cells (16,17). Here, we identified deletion,

truncating and missense mutations in *SLC9A3*, the gene encoding NHE3, in nine patients including one sib-pair with CSD. Reduced Na<sup>+</sup>/H<sup>+</sup> exchange activity was demonstrated by the *in vitro* expression of identified *SLC9A3* missense mutants. We thus show that recessive mutations in *SLC9A3* are a cause of CSD. Interestingly, inflammatory bowel disease (IBD) developed in a number of patients with dominant GC-C mutations, and we also report IBD in two of nine patients with recessive *SLC9A3* mutations, implicating NHE3 in the pathogenesis of IBD in a subset of patients.

## Results

### Clinical summary

In our cohort of 18 patients from 16 unrelated families with CSD, we identified *SLC9A3* mutations in 9 patients from 8 unrelated families. One family had two affected children; this sib-pair is denoted as Patients 4 and 5 in Table 1, and both sibs harbored the same homozygous *SLC9A3* mutation. Detailed clinical findings in patients with *SLC9A3* mutations are shown in Table 1, including the two patients (Patients 2 and 3) originally described with this disorder (2,3). There was a uniform history of maternal polyhydramnios, and all CSD patients had watery secretory diarrhea and prominent abdominal distension after birth owing to dilated fluid-filled loops of intestine, indicating that secretory diarrhea begins prenatally.

The patients studied here did not have clinical features associated with the ‘syndromic form’ of CSD. While serum Na<sup>+</sup> was normal in all patients, fecal Na<sup>+</sup> concentrations were high in all patients except one, and urinary Na<sup>+</sup> concentrations, as a marker of body Na<sup>+</sup> depletion, were low. Patient 6 developed IBD at 4 years of age leading to ileo-caecal resection and temporary ileostomy because of recurrent episodes of small bowel obstruction. Patient 8 was diagnosed with IBD at 16 years of age, when he presented with bloody diarrhea and ulceration in the rectum and sigmoid. One year later he had nodular lymphoid hyperplasia with ileal granulomas and colonic ulcers. At present, he passes three to four watery stools per day without bleeding. Intestinal anatomy, histology and transit functions were normal in the other patients.

### Mutation identification

To identify the molecular basis of CSD in patients without GC-C mutations, we performed whole-exome sequencing in Patient 9

**Table 1.** Clinical findings in nine CSD patients with SLC9A3 mutations

Parameter	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5 <sup>a</sup>	Patient 6	Patient 7	Patient 8	Patient 9
Consanguinity	No	No	No	Yes	Yes	Yes	Yes	No	No
Nationality/ ethnicity	Canadian Caucasian	German/ Finnish Caucasian	British Caucasian	Turkish	Turkish	Turkish Kurd Caucasian	German Caucasian	Serbian Caucasian	Canadian Caucasian
Current age (years)	2 ½	37	33	1 ½	Neonate	6 ½	4	19	1 ½
Sex	F	F	F	F	M	M	M	M	M
Gestational age (weeks)	37	33	36	33	32	33	34	34	34
Birth weight (g)	2285	2530	2800	2560	2160	3210	2615	2125	2700
Weight (kg, centile)	12, 25th	n.i.	n.i.	7.34, 90th	2.84, 75th	32.7, >97th	16, 25th	72, 50th	9.02, 10th
Height (cm, centile)	83.2, <10th	n.i.	n.i.	63, 50th–75th	48, 25th– 50th	119, 50th	102, 10th	175, 50th	80, 25th
Polyhydramnios	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Plasma [Na <sup>+</sup> ] (132–155 mmol/l)	138	140	138	135–8	135	132	140	140	138
Plasma pH	7.28	7.34	7.30	7.33	7.30	7.37	7, 25	n.i.	7.33
Plasma [HCO <sub>3</sub> <sup>-</sup> ] (mmol/l)	11.8	n.i.	16.0	21.1	n.i.	24	20,5	n.i.	23
Fecal [Na <sup>+</sup> ] (20–50 mmol/l)	110	145	123	30	144	147	68	64	151
Fecal [Cl <sup>-</sup> ] (5–25 mmol/l)	<50	75	42	31	90	63	33	51	101
Fecal pH (6–7)	8	7	9	6	n.i.	n.i.	8	7	n.i.
Fecal osmolality (320–370 mosmol/kg)	271	n.i.	n.i.	353	n.i.	290	304	n.i.	315
Urinary [Na <sup>+</sup> ] (2–28 mmol/l)	<10	28	n.i.	2	n.i.	19	8	n.i.	<10
Histology	Normal	Not done	Nonspecific focal inflammation	Focal lymphatic hyperplasia	n.d	Partial villous atrophy ileal ulcerations, inflammatory infiltration with increase of eosinophils	Mild nonspecific inflammation	Ulceration in the rectum and sigmoid at 16 years, nodular lymphoid hyperplasia with ileal granulomas colonic ulcers at age 17 years	Normal
Parenteral fluids till (weeks)	Intermittent	None	5	3	2	237	20	None	Current
Current treatment	Oral	Oral	Oral	Oral	Oral	Oral + i.v.	Oral	Oral	i.v.
Current Na supplement (mmol/kg/d)	None	4	n.i.	n.i.	None	6.5	None	None	13
Outcome	Growth retardation	Mild watery diarrhea, otherwise normal life	Mild watery diarrhea, otherwise normal life	Watery diarrhea, hyperaldosteronism	Normal, fully breast- fed	Watery diarrhea, partial PN; ileal ulcerations since the age of 4 years	Mild watery diarrhea, otherwise normal life	Mild watery diarrhea; budesonide treatment; hyperaldosteronism	Total parenteral nutrition, growth retardation

n.d.: not determined; n.i.: no information.

<sup>a</sup>Patients 4 and 5 are sibs.

and in Patient 2 who was originally reported as having CSD (3). Studies of purified BB membrane vesicles of the proximal intestine from three reported CSD patients had pointed to impaired Na<sup>+</sup>/H<sup>+</sup> exchanger activity previously (2,5,6), but genetic defects in NHE isoforms were not identified. Filtering exome data for private or rare variants in genes encoding Na<sup>+</sup>/H<sup>+</sup> exchangers identified compound-heterozygous SLC9A3 mutations in Patients 2 and 9. Independently, chromosomal microarray analysis revealed a heterozygous, paternally inherited 1.383-Mb deletion on chromosome 5p15.33 encompassing SLC9A3 in Patient 1; Sanger sequencing of the remaining copy of SLC9A3 identified a maternally inherited missense mutation.

We subsequently identified another five SLC9A3 mutations in five unrelated CSD patients by Sanger-sequencing the coding region and flanking intronic regions of SLC9A3 in our patient cohort. A total of eight different SLC9A3 point mutations, a 3-bp deletion and a whole-gene deletion were identified in eight unrelated CSD families (Table 2, Fig. 1). One mutation, p.Arg382Gln, was observed in two seemingly unrelated patients of Canadian origin (Patients 1 and 9). Both affected siblings (Patients 4 and 5) of a consanguineous family were homozygous for the missense mutation p.Ala269Thr. Nine patients from 8 families of our cohort of 16 unrelated CSD families did not harbor either SLC9A3 or GUCY2C mutations indicating that other genes are responsible for the disease in these patients. Sequencing of DNA samples from family members confirmed the bi-allelic inheritance of the NHE3 mutations in six families and demonstrated autosomal recessive inheritance of CSD. A rare disease mechanism of a recessive disorder was revealed in Patient 7, who was homozygous for a SLC9A3 frame-shift mutation that was heterozygous in the mother but absent in the father. SNP array analysis in this family resolved this discrepancy by revealing maternal isodisomy of the entire chromosome 5 in this patient resulting in the maternal SLC9A3 mutation acting as a homozygous mutation in the patient. Monosomy rescue causes isodisomy, in which one chromosome is present in duplicate. In monosomy rescue, a nullisomic gamete is fertilized with a haploid gamete. The single chromosome from the other parent is duplicated and produces isodisomy of the chromosome.

Patients 3 and 8 are heterozygous for one mutation with no second allelic mutation identified after sequencing the complete coding region and multiplex ligation-dependent probe amplification (MLPA) analysis of SLC9A3 exons. Most likely, we failed to detect the two allelic mutations with the current methodology, which does not cover deep intronic or promoter sequences, and which might be identified by whole genome sequencing. With the exception of the observation of mutation p.Arg382Gln in two seemingly unrelated patients, the identified SLC9A3 mutations were all private and not previously identified in several public databases [dbSNP, the 1000 Genomes Project, the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP), the exome aggregation consortium server (EXAC)]. The description of SLC9A3 sequence variants is based on NCBI reference sequence NM\_004174.2.

### Functional studies of NHE3

The apical membrane Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 carries out the majority of intestinal Na<sup>+</sup> absorption. It is regulated by changes in its continual trafficking to and from the brush border, which results in changes in its surface expression (18). When the intestinal mucosa is stimulated by neurotransmitters as part of normal digestion, it is acutely stimulated or inhibited, and when exposed to enterotoxins or drugs that increase intracellular

Table 2. SLC9A3 mutations in nine CSD patients

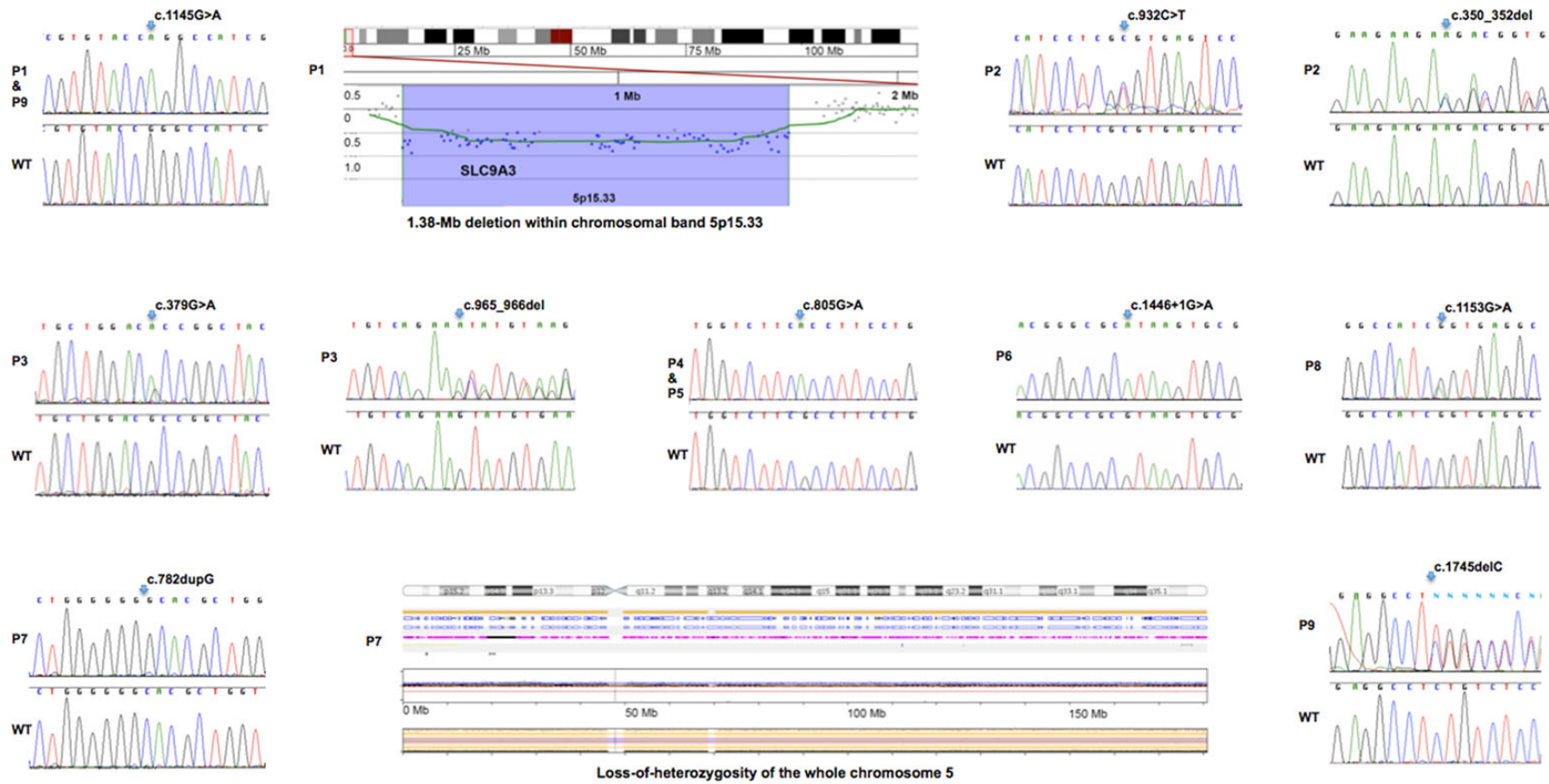
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5 <sup>a</sup>	Patient 6	Patient 7 <sup>b</sup>	Patient 8	Patient 9
Allele 1 (exon)	c.1145G>A (6)	c.932C>T (5)	c.[379G>A; 963_964delGT] (2; 6)	c.805G>A (5)	c.805G>A (5)	c.1446+1G>A (8)	c.782dupG (5)	c.1153G>A (6)	c.1145G>A (6)
Protein alteration/consequence	p.Arg382Gln	p.Ala311Val, splicing <sup>c</sup>	p.[Ala127Thr; Tyr322Argfs*83]	p.Ala269Thr	p.Ala269Thr	Splicing, truncation	p.Thr262Hisfs*144	p.Gly385Ser, splicing <sup>c</sup>	p.Arg382Gln
Polyphen2	Probably damaging	Probably damaging	p.Ala127Thr: possibly damaging	Probably damaging	Probably damaging	n.a.	n.a.	Probably damaging	Probably damaging
SIFT	Not tolerated	Not tolerated	Tolerated	Not tolerated	Not tolerated	n.a.	n.a.	Tolerated	Not tolerated
CADD PHRED	29.9	29.7	24.3	34.0	34.0	23.3	n.d.	27.3	29.9
Allele 2 (exon)	Gene deletion	c.350_352del (2)	n.d.	c.805G>A (5)	c.805G>A (5)	c.1446+1 G>A (8)	c.782dupG (5)	n.d.	c.1745delC
Protein alteration	No protein	p.Phe117del	n.d.	p.Ala269Thr	p.Ala269Thr	Splicing, truncation	p.Thr262Hisfs*144	n.d.	p.Ser582Leufs*6

<sup>a</sup>Patients 4 and 5 are sibs.

<sup>b</sup>homozygous mutation owing to maternal isodisomy of the entire chromosome 5.

<sup>c</sup>DNA mutation affects the most 3' nucleotide in the exon and might interfere with proper splicing in addition to creating an amino acid change. n.d., not determined; n.a., not applicable.





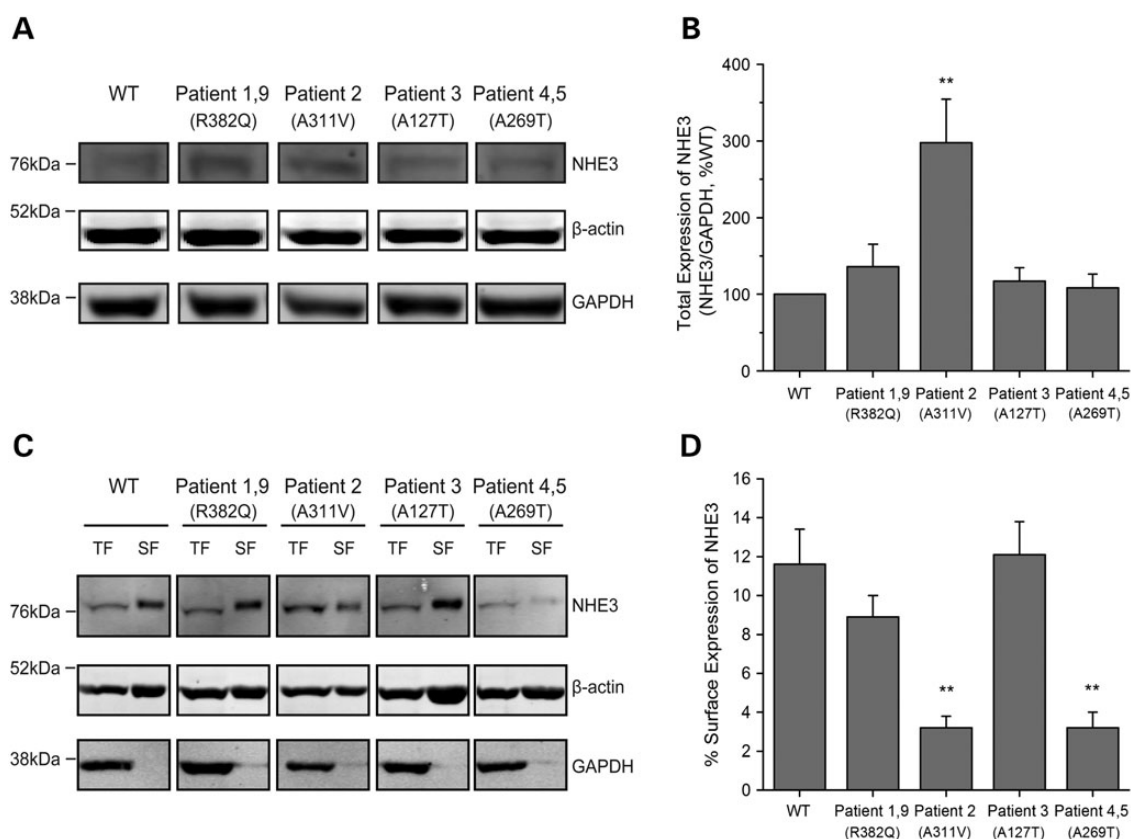
**Figure 1.** Detection of *SLC9A3* mutations and that of a whole *SLC9A3* gene deletion in CSD patients. Identified compound-heterozygous and homozygous mutations in *SLC9A3* identified by Sanger sequencing in eight unrelated patients, and a heterozygous whole-gene deletion identified in Patient 1 (Table 1) by array comparative genomic hybridization, and loss-of-heterozygosity for the whole chromosome 5 owing to uniparental isodisomy identified in Patient 7.

cGMP,  $\text{Ca}^{2+}$  or cAMP and mimic the pathophysiology of diarrhea, electroneutral  $\text{NaCl}$  absorption and the NHE3 contribution are inhibited (18). NHE3 consists of 12 N-terminal transmembrane domains that carry out  $\text{Na}^+/\text{H}^+$  exchange, followed by a long cytoplasmic domain that interacts with the cytoskeleton and confers acute regulation of NHE3. This NHE3 regulation is critical for systemic volume and acid-base homeostasis (18,19).

SLC9A3 mutations identified included one whole-gene deletion, one splicing and two frame-shift mutations, all of which would be expected to abolish protein production from these alleles. We further investigated the effect of four identified missense mutations or variants, p.Arg382Gln, p.Ala311Val, p.Ala269Thr, p.Ala127Thr on NHE3 activity. Wild-type and variant forms of NHE3 were stably expressed in PS120/Flag-NHERF2 fibroblasts that lack all endogenous plasma membrane NHEs (20). NHERF2 was also expressed because much of NHE3 regulation is NHERF2 dependent (18). NHE3 total protein expression was comparable in all cell lines, except for the p.Ala311Val cell line, which had increased protein expression (Fig. 2, Table 3). The percentage of surface expression of NHE3, determined by cell surface biotinylation, was reduced in

cells expressing p.Ala311Val and p.Ala269Thr and was normal in the other two mutant cell lines studied (Fig. 2, Table 3). Basal  $\text{Na}^+/\text{H}^+$  exchange activity was significantly decreased in three of the four cell lines studied (Fig. 3, Table 3). NHE3 activity was normal in the cell line expressing p.Ala127Thr. Note that p.Ala127Thr was present *in-cis* with a p.Tyr322Argfs\*83 frame-shift mutation indicating that the latter compromised NHE3 activity and p.Ala127Thr represents a benign variant.

For normalization of the basal activity with the amount of surface-expressed NHE3, the relative number of plasma membrane NHE3 molecules (compared with wild-type NHE3) was calculated as total expression of NHE mutant times the percentage of total NHE3 on the plasma membrane. p.Arg382Gln and p.Ala311Val had reduced, and p.Ala127Thr and p.Ala269Thr had normal  $\text{Na}^+/\text{H}^+$  exchange function per surface molecule expressed compared with wild-type function (Table 3). Acute stimulation by dialyzed serum and inhibition by forskolin occurred normally on all point mutations except p.Ala311Val in which the  $\text{Na}^+/\text{H}^+$  exchange rate was so low that accurate changes could not be determined (Fig. 3).



**Figure 2.** Total and surface expression of NHE3 in PS120/FLAG-NHERF2 cells stably transfected with human NHE3-WT and mutations. Total expression (above). (A) A representative immunoblot with total cell lysates. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Protein expression was detected with primary antibodies to NHE3 (1:500, AB 6347) and  $\beta$ -actin (1:5000) and GAPDH (1:5000) as the loading controls. (B) Quantitative analysis of total expression of NHE3. The expression of NHE3/GAPDH was calculated, and NHE3-WT was set as 100% for each experiment. Results are mean  $\pm$  SEM of nine independent experiments. Of the four NHE3 missense variants, one (Patient 2) had higher expression whereas the other three had comparable expression to NHE3-WT. Analysis of the same data normalized to  $\beta$ -actin (1:5000) gave similar results and is shown in Supplementary Material (see Supplementary Material, Fig. S3). \*\* $P < 0.01$  compared with NHE3-WT (unpaired Student's *t* test). Surface expression (below). (C) A representative immunoblot with biotinylated cell lysates. Cells were biotinylated with NHS-SS-biotin and washed with quenching buffer to remove unbound NHS-SS-biotin. Cells were then lysed and divided into the total fractions and surface fractions. The surface fraction was incubated with avidin-agarose beads, and biotinylated proteins were eluted into SDS buffer. Total and surface fractions of each cell line were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with primary antibodies to NHE3,  $\beta$ -actin and GAPDH as mentioned above. TF, total fraction; SF, surface fraction. (D) Quantitative analysis of surface expression of NHE3. The percentage of surface expression of NHE3 was determined as in Supplementary Material. Results are mean  $\pm$  SEM of five independent experiments. Two mutant forms of NHE3 (Patient 2 and Patients 4 and 5) showed significantly lower plasma membrane expression than NHE3-WT. \*\* $P < 0.01$  compared with NHE3-WT (unpaired Student's *t* test).

Table 3. Comparison of transport characteristics in NHE3 wild type and mutations

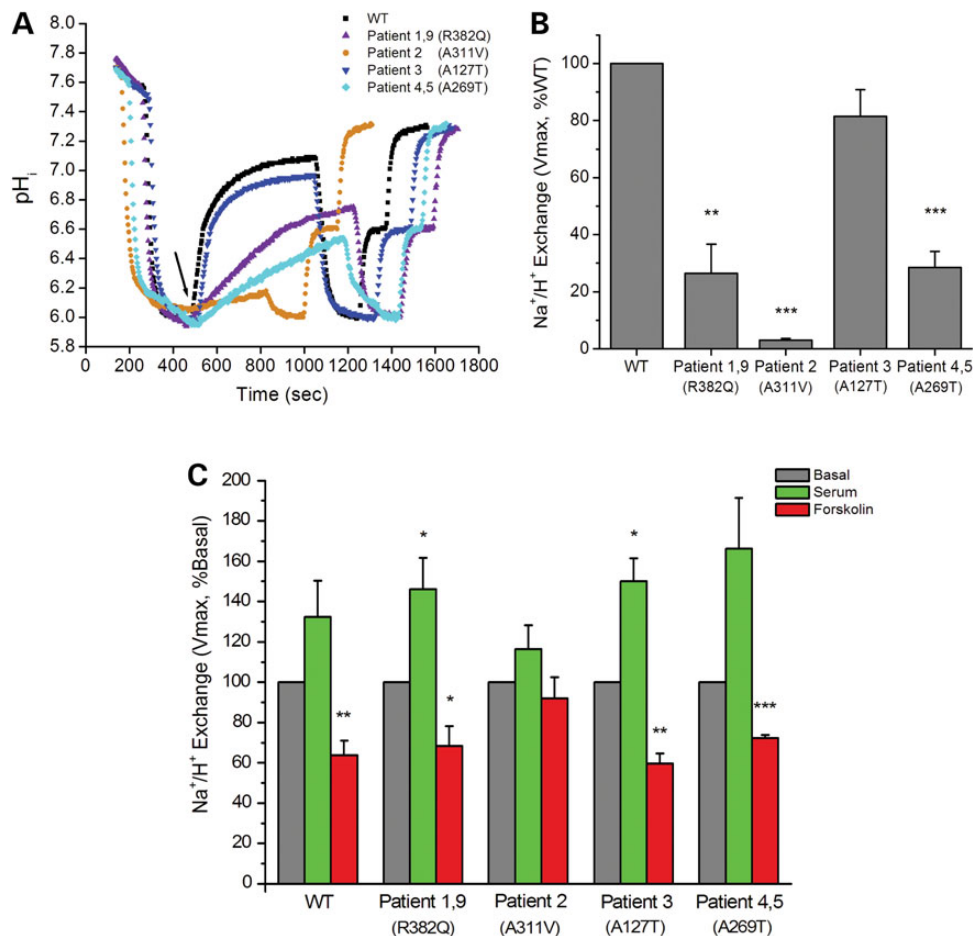
	Point mutation	Amino acid alteration	Basal activity V <sub>max</sub> (μm/s)	V <sub>max</sub> (%WT)	K <sub>t</sub> (H <sup>+</sup> ) <sub>i</sub> (μm)	K <sub>t</sub> (H <sup>+</sup> ) <sub>i</sub> (%WT)	Total NHE3 expression (%WT)	% Surface NHE3 expression	Basal activity/ surface NHE3 expression (%WT)
WT	-	-	474.9 ± 40.7	100	0.43 ± 0.05	100	100	11.6 ± 1.8	100
Patients 1 and 9	c.1145G>A	p.Arg382Gln	123.6 ± 47.4	26.5 ± 10.2	0.76 ± 0.18	192.7 ± 71.9	135.8 ± 29.3	8.9 ± 1.1	25.4
Patient 2	c.932C>T	p.Ala311Val	13.8 ± 1.9	3.0 ± 0.6	0.84 ± 0.10	208.7 ± 53.7	297.7 ± 56.6	3.2 ± 0.6	3.7
Patient 3	c.379G>pA	p.Ala127Thr	393.8 ± 78.9	81.5 ± 9.4	0.5 ± 0.06	115.2 ± 4.1	117.3 ± 17.5	12.1 ± 1.7	66.6
Patients 4 and 5	c.805G>A	p.Ala269Thr	131.1 ± 18.3	28.5 ± 5.6	0.77 ± 0.11	177.3 ± 6.3	108.3 ± 18.1	3.2 ± 0.8	95.4

## Discussion

This study shows that absent or mutated NHE3 protein, owing to recessive SLC9A3 mutations, is a cause of CSD. The loss or reduced NHE3 function acts similarly to the NHE3 inhibition observed in the post-prandial state or in diarrhea. Similar to these NHE3-deficient patients, *Nhe3*<sup>-/-</sup> mice exhibit diarrhea, metabolic acidosis, low blood pressure and reduced body fat and die rapidly when placed on a low Na<sup>+</sup> diet (21,22). Interestingly, activating GC-C mutations can result in a CSD phenotype indistinguishable from patients with recessive NHE3 mutations (15). Owing to the small number of CSD patients with GUCY2C or SLC9A3 mutations known, a genotype-phenotype correlation cannot be provided. As activating GC-C mutations were shown to cause elevated cGMP levels (14,15), they are expected to result in inhibition of NHE3 at the apical enterocyte membrane (16), thereby providing an explanation for the secretory diarrhea by abrogated Na<sup>+</sup> absorption (23).

There were two patterns to the abnormal transport function attributed to the NHE3 missense mutations, reduced transport function of individual NHE3 molecules present on the plasma membrane (abnormal turnover number) (Patients 1, 2 and 9) and reduced surface expression of NHE3 (Patients 2, 4 and 5). The explanation for the reduced surface expression could be due to either abnormal trafficking to the membrane (reduced exocytosis or increased endocytosis) or reduced BB stability. The functionally abnormal missense mutations that compromised NHE3 function all are present in one of the twelve membrane-spanning domains (MSD) of NHE3, based on homology modeling with bacterial Na<sup>+</sup>/H<sup>+</sup> antiporters, which have had their structures solved (see Supplementary Material). The mutations are in putative MSD II (Patients 2 and 3), VI (Patients 4 and 5), VIII (Patient 2) and X (Patients 1, 8 and 9). How these mutations compromise NHE3 function at an atomic level has not been determined. However, MSD II and VIII form the funnel through which Na<sup>+</sup>/H<sup>+</sup> exchange occurs, MSD II on the cytoplasmic and MSD VIII on the extracellular surfaces (24-26).

An additional important component of the phenotype associated with the NHE3 mutations was early and adolescent IBD in two of nine patients indicating a role of NHE3 deficiency as a predisposition to IBD. Interestingly, large-scale IBD genome-wide association studies have shown a strong replicated association between ulcerative colitis and the SLC9A3 locus (27,28). This association between compromised NHE3 function and IBD is further supported by a number of rodent and human studies. *Nhe3*<sup>-/-</sup> mice develop spontaneous distal chronic colitis and have increased susceptibility to dextran sulfate (DSS)-induced mucosal injury (29). An abnormal microbiome contributes to the *Nhe3*<sup>-/-</sup> colitis with no colitis reported in a very clean facility and an altered microbiome documented in mice in a facility in which colitis occurred (29-31). Consistent with the animal studies, NHE3 activity is reduced in both ulcerative colitis and Crohn's disease with activity reduced in areas of inactive colitis as well as in the areas of active inflammation (32,33). The mechanism is somewhat controversial with reports of both reduced message and protein (32) and reduced NHE3 activity with normal message, amount of protein and surface expression (33). In addition, 6 out of 36 patients with chronic diarrhea owing to activating mutations of GCC including 1 of 4 patients with CSD had IBD (14,15) further supporting the role of reduced sodium absorption in the pathogenesis of IBD possibly following changes in the intestinal microbial composition owing to alterations in electrolyte transport and in mucosal pH as demonstrated in *Nhe3*<sup>-/-</sup> mice (30).



**Figure 3.** Functional study of NHE3-WT and mutations. (A) The basal activity of NHE3 was studied fluorometrically using the pH-sensitive dye BCECF. A representative experiment is shown demonstrating that the mutations detected in five patients (Patients 1, 2, 4, 5, 9) had reduced Na<sup>+</sup>/H<sup>+</sup> exchange activities compared with NHE3-WT. In contrast, the NHE3 mutation identified in Patient 3 had similar activity with NHE3-WT. The black arrow indicates the start of Na<sup>+</sup>-dependent intracellular alkalinization. (B) Quantitative analysis of Na<sup>+</sup>/H<sup>+</sup> exchange activities. Basal NHE3 activity (Vmax, μM/s) of each cell line was determined, and NHE3-WT was set as 100% for each experiment. Results are mean ± SEM of three independent experiments. \*\*P < 0.01, \*\*\*P < 0.001 compared with NHE-WT (unpaired Student's t test). (C) PS120/FLAG-NHERF2 cells stably transfected with human NHE3-WT and mutations were treated with dialyzed serum (10%) or forskolin (10 μM) for 30 min following serum starvation for 3 h. NHE3 activity was determined and compared with the basal activity (Vmax, μM/s) of each cell line which was set as 100%. All cell lines responded normally by up-regulating activity following serum stimulation and down-regulating activity following forskolin inhibition except for the mutation identified in Patient 2. Results are mean ± SEM of three independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared with basal activity (unpaired Student's t test).

In conclusion, we demonstrate genetic locus heterogeneity for CSD; CSD can either be caused by recessive mutations in NHE3 or by dominant mutations in GC-C, thereby stimulating further search for mutations in these genes as well as future research on drugs and peptides to restore or enhance Na<sup>+</sup> absorption of diarrheal disorders.

## Materials and Methods

### Ethics statement

The study was approved by the ethics committees of the Medical University of Innsbruck, the Johns Hopkins University School of Medicine and the Hospital for Sick Children.

### Patients

CSD was diagnosed in 18 patients from 16 unrelated families by gastroenterologists on characteristic clinical findings, which are described in the results section.

### Genetic screening and verification

Exome capture was performed with the Illumina Truseq Exome Enrichment Kit and HiSeq2000 sequencer. Chromosomal microarray analysis (Genome-wide copy-number variant detection) and loss-of-heterozygosity analysis was performed with CytoSNP-12v2 BeadChip SNP arrays (Illumina, Little Chesterford, UK). Primers and conditions for SLC9A3 Sanger sequencing and MLPA analysis are provided in Supplementary Material, Tables S1 and S2.

### Molecular characterization

PS120 cells stably expressing triple FLAG-tagged human NHERF2, as described, were stably transfected with human wild-type and four NHE3 mutants (20,34). Trafficking of NHE3 has been characterized in PS120 cells and shown to be similar to that in Caco-2 and OK cells as related to regulation of NHE3 exocytosis and endocytosis by elevated Ca<sup>2+</sup>, cAMP and cGMP (16,35–37). Na<sup>+</sup>/H<sup>+</sup> exchange activity was determined fluorometrically using the intracellular pH-sensitive dye BCECF-AM, as described



(38). Immunoblotting was performed with primary antibodies to NHE3 (rabbit polyclonal 6347, 1:500) (35) GAPDH (mouse-monoclonal Abcam, Cambridge, MA; 1:5000) and  $\beta$ -actin (rabbit polyclonal, Sigma).

Experimental procedures are described in detail in Supplementary Material, Methods.

## Supplementary Material

Supplementary Material is available at HMG online.

## Web Resources

dbSNP: <http://www.ncbi.nlm.nih.gov/projects/SNP/>, Ensembl: <http://www.ensembl.org/>, Exome Aggregation Consortium (ExAC) Browser: <http://exac.broadinstitute.org/>, NHLBI Exome Sequencing Project (ESP) Exome Variant Server: <http://snp.gs.washington.edu/EVS>, Combined Annotation Dependent Depletion (CADD) score: <http://cadd.gs.washington.edu/score>.

*Conflict of Interest statement.* None declared.

## Funding

This work was supported by grants from Jubiläumsfonds der Österreichischen Nationalbank (grants no. 14496 and no. 15627), the Tiroler Wissenschaftsfonds (grant no. UNI-0404/1286), Else Kröner-Fresenius-Stiftung Nr. 2013\_A230. This work was also supported in part from NIH/NIDDK grants RO1DK 26523, RO1 DK 61765, P01DK 72084, KO8DK 088950, R03DK 099566, T32 DK2007632 and Conte GI Core Center grant P30DK 89502, The Hopkins Digestive Diseases Basic and Translational Research Core Center. A.M.M. is funded by a CIHR – Operating Grant (MOP119457) and by the Leona M. and Harry B. Helmsley Charitable Trust to study VEOIBD.

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