Reduced sodium/proton exchanger NHE3 activity causes congenital sodium diarrhea

Andreas R. Janecke1,2,†, Peter Heinz-Erian1,†, Jianyi Yin4,5,†, Britt-Sabina Petersen6, Andre Franke6, Silvia Lechner2, Irene Fuchs1, Serge Melancon7, Holm H. Uhlig8, Simon Travis8, Evelyne Marinier9, Vojislav Perisic10, Nina Ristic10, Patrick Gerner11, Ian W. Booth12, Satu Wedenoja13, Nadja Baumgartner3, Julia Vodopiutz14, Marie-Christine Frechette-Duval15, Jan De Lafolie16, Rabindranath Persad17, Neil Warner18, C. Ming Tse4,5, Karan Sud4,5, Nicholas C. Zachos4,5, Rafiquel Sarker4,5, Xinjun Zhu19, Aleixo M. Muise18,20,21,22, Klaus-Peter Zimmer16, Heiko Witt23, Heinz Zoller3, Mark Donowitz4,5,‡ and Thomas Müller1,‡,*

1Department of Pediatrics I, 2Division of Human Genetics, 3Department of Internal Medicine, Medical University of Innsbruck, Innsbruck 6020, Austria, 4Department of Medicine, 5Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA, 6Institute of Clinical Molecular Biology, Christian-Albrechts-University, Kiel 24105, Germany, 7Department of Medical Genetics, McGill University Health Centre, Montreal, Canada H3H 1P3, 8Translational Gastroenterology Unit, Nuffield Department of Medicine, and Children’s Hospital, University of Oxford, Oxford OX3 9DU, UK, 9Service des maladies digestives et respiratoires de l’enfant, Centre de référence des maladies digestives rares, Hôpital R Debré, Paris 75935, France, 10Department of Hepatology and GI Endoscopy, University Children’s Hospital, Belgrade 11000, Serbia, 11Zentrum für Kinder- und Jugendmedizin, Universitätsklinikum, Freiburg 79106, Germany, 12Paediatrics and Child Health, University of Birmingham, Birmingham B4 6NH, UK, 13Department of Medical Genetics, University of Helsinki, Helsinki 00014, Finland, 14Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Wien 1090, Austria, 15Department of Pediatrics, Faculty of Medicine, Sherbrooke University, Sherbrooke, Canada J1H SN4, 16Abteilung Allgemeine Pädiatrie & Neonatologie, Zentrum für Kinderheilkunde und Jugendmedizin, Justus-Liebig-Universität, Gießen 35392, Germany, 17Stollery Children’s Hospital, University of Alberta, Edmonton, Canada T6G 2B7, 18SickKids Inflammatory Bowel Disease Center and Cell Biology Program, Research Institute, Hospital for Sick Children, Toronto, ON, Canada M5G 1X8, 19Department of Medicine, Albany Medical Center, Albany, NY 12208, USA, 20Department of Biochemistry, 21Department of IMS, 22Department of Pediatrics, Division of Gastroenterology, Hepatology, and Nutrition, University of Toronto, Toronto, ON, Canada M5G 1X8 and 23Pädiatrische Ernährungsmedizin, Else Kröner-Fresenius-Zentrum für Ernährungsmedizin, Technische Universität München, Freising-Weihenstephan 85350, Germany

*To whom correspondence should be addressed at: Department of Pediatrics I, Medical University of Innsbruck, Anichstrasse 35, Innsbruck A-6020, Austria. Tel: +43 51250426345; Fax: +43 51250424525; Email: thomas.mueller@tirol-kliniken.at

The authors wish to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

The authors wish to be known that, in their opinion, the last two authors should be regarded as joint Last Authors and joint Corresponding Authors.

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

© The Author 2015. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
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+/H+ antiporter 3 (NHE3), which is the major intestinal brush-border Na+/H+ 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+, 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+/H+ 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+ was normal in all patients, fecal Na+ concentrations were high in all patients except one, and urinary Na+ concentrations, as a marker of body Na+ 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5*</th>
<th>Patient 6</th>
<th>Patient 7</th>
<th>Patient 8</th>
<th>Patient 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consanguinity</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Nationality/ethnicity</td>
<td>Canadian</td>
<td>No</td>
<td>German/Finnish</td>
<td>Turkish</td>
<td>Turkish</td>
<td>Turkish Kurd</td>
<td>German</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Current age (years)</td>
<td>2 ½</td>
<td>37</td>
<td>33</td>
<td>1 ½</td>
<td>Neonate</td>
<td>6 ½</td>
<td>4</td>
<td>19</td>
<td>1 ½</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>37</td>
<td>33</td>
<td>36</td>
<td>33</td>
<td>32</td>
<td>33</td>
<td>34</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2285</td>
<td>2530</td>
<td>2800</td>
<td>2560</td>
<td>2160</td>
<td>3210</td>
<td>2615</td>
<td>2125</td>
<td>2700</td>
</tr>
<tr>
<td>Weight (kg, centile)</td>
<td>n.i.</td>
<td>n.i.</td>
<td>7.34, 90th</td>
<td>2.84, 75th</td>
<td>8.2, &gt;97th</td>
<td>16, 25th</td>
<td>72, 50th</td>
<td>9.02, 10th</td>
<td>80, 25th</td>
</tr>
<tr>
<td>Height (cm, centile)</td>
<td>83.2, &lt;10th</td>
<td>n.i.</td>
<td>63, 50th–75th</td>
<td>48, 25th–50th</td>
<td>48, 25th–50th</td>
<td>119, 50th</td>
<td>102, 10th</td>
<td>175, 50th</td>
<td>102, 10th</td>
</tr>
<tr>
<td>Polyhydramnios</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Plasma pH</td>
<td>7.28</td>
<td>7.34</td>
<td>7.30</td>
<td>7.33</td>
<td>7.30</td>
<td>7.37</td>
<td>7.25</td>
<td>n.i.</td>
<td>7.33</td>
</tr>
<tr>
<td>Plasma [HCO₃⁻] (mmol/l)</td>
<td>11.8</td>
<td>n.i.</td>
<td>16.0</td>
<td>21.1</td>
<td>n.i.</td>
<td>24</td>
<td>20.5</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>Fecal [Na⁺] (20–50 mmol/l)</td>
<td>110</td>
<td>145</td>
<td>123</td>
<td>30</td>
<td>144</td>
<td>147</td>
<td>68</td>
<td>64</td>
<td>151</td>
</tr>
<tr>
<td>Fecal [Cl⁻] (5–25 mmol/l)</td>
<td>&lt;50</td>
<td>75</td>
<td>42</td>
<td>31</td>
<td>90</td>
<td>63</td>
<td>33</td>
<td>51</td>
<td>101</td>
</tr>
<tr>
<td>Fecal pH (6–7)</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>n.i.</td>
<td>n.i.</td>
<td>8</td>
<td>7</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>Fecal osmolality (320–370 mosmol/kg)</td>
<td>271</td>
<td>n.i.</td>
<td>353</td>
<td>n.i.</td>
<td>290</td>
<td>304</td>
<td>304</td>
<td>n.i.</td>
<td>315</td>
</tr>
<tr>
<td>Urinary [Na⁺] (2–28 mmol/l)</td>
<td>&lt;10</td>
<td>28</td>
<td>n.i.</td>
<td>2</td>
<td>n.i.</td>
<td>19</td>
<td>8</td>
<td>n.i.</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Histology</td>
<td>Normal</td>
<td>Not done</td>
<td>Nonspecific focal inflammation</td>
<td>Focal lymphatic hyperplasia</td>
<td>n.d</td>
<td>Partial villous atrophy ileal ulcerations, inflammatory infiltration with increase of eosinophils</td>
<td>Mild nonspecific inflammation</td>
<td>Ulceration in the rectum and sigmoid at 16 years, nodular lymphoid hyperplasia with ileal granulomas colonic ulcers at age 17 years</td>
<td>Normal</td>
</tr>
<tr>
<td>Parenteral fluids till (weeks)</td>
<td>Intermittent</td>
<td>None</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>237</td>
<td>20</td>
<td>None</td>
<td>Current</td>
</tr>
<tr>
<td>Current Na supplement (mmol/kg/d)</td>
<td>None</td>
<td>4</td>
<td>n.i.</td>
<td>n.i.</td>
<td>6.5</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>13</td>
</tr>
<tr>
<td>Outcome</td>
<td>Growth retardation</td>
<td>Mild watery diarrhea, otherwise normal life</td>
<td>Mild watery diarrhea, otherwise normal life</td>
<td>Watery diarrhea, hyperaldosteronism</td>
<td>Normal, fully breastfed</td>
<td>Watery diarrhea, partial PN; ileal ulcerations since the age of 4 years</td>
<td>Mild watery diarrhea, otherwise normal life</td>
<td>Mild watery diarrhea; budesonide treatment; hyperaldosteronism</td>
<td>Total parenteral nutrition, growth retardation</td>
</tr>
</tbody>
</table>

n.d.: not determined; n.i.: no information.
*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+/H+ 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+/H+ 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 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+/H+ exchanger NHE3 carries out the majority of intestinal Na+ 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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Allele 1 (exon)</th>
<th>Protein alteration/ consequence</th>
<th>Polyphen2</th>
<th>SIFT</th>
<th>CADD PHRED</th>
<th>Allele 2 (exon)</th>
<th>Protein alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.1146G&gt;A (4)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>2</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>3</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>4</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>5</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>6</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>7</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>8</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
<tr>
<td>9</td>
<td>c.1153G&gt;A (6)</td>
<td>p.Ala269Thr</td>
<td>n.a.</td>
<td>Tolerated</td>
<td>29.9</td>
<td>c.805G&gt;A (5)</td>
<td>p.Ser582Leufs*6</td>
</tr>
</tbody>
</table>
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, Ca²⁺ or cAMP and mimic the pathophysiology of diarrhea, electroneutral NaCl absorption and the NHE3 contribution are inhibited (18). NHE3 consists of 12 N-terminal transmembrane domains that carry out Na⁺/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 Na⁺/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.Ala269Thr had normal Na⁺/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 Na⁺/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 ß-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 ± 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 ß-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, ß-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 ± 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).
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−/− mice exhibit diarrhea, metabolic acidosis, low blood pressure and reduced body fat and die rapidly when placed on a low Na+ 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+ 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+/H+ 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+/H+ 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−/− 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−/− 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−/− mice (30).

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

<table>
<thead>
<tr>
<th>Point mutation</th>
<th>Amino acid alteration</th>
<th>Basal activity Vmax (µM/s)</th>
<th>Basal activity Vmax (%WT)</th>
<th>K′(H+)i (µM)</th>
<th>K′(H+)i (%WT)</th>
<th>Surface NHE3 expression (%)</th>
<th>% Surface NHE3 expression</th>
<th>Total NHE3 expression (%)</th>
<th>% Total NHE3 expression</th>
<th>Basal activity/surface NHE3 expression (%WT)</th>
<th>% Basal activity/surface NHE3 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>474.9 ± 40.7</td>
<td>100</td>
<td>0.43 ± 0.05</td>
<td>100</td>
<td>11.6 ± 1.8</td>
<td>100</td>
<td>159.8 ± 29.3</td>
<td>100</td>
<td>25.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Patients 1 and 9</td>
<td>c.1145G&gt;A p.Arg382Gln</td>
<td>123.6 ± 47.4</td>
<td>26.5 ± 10.2</td>
<td>0.76 ± 0.18</td>
<td>37.2 ± 11.1</td>
<td>3.2 ± 0.6</td>
<td>25.4</td>
<td>192 ± 71.9</td>
<td>100</td>
<td>25.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Patient 2</td>
<td>c.932C&gt;T p.Ala311Val</td>
<td>13.8 ± 1.9</td>
<td>3.0 ± 0.6</td>
<td>0.94 ± 0.10</td>
<td>208.7 ± 53.7</td>
<td>5.7 ± 0.6</td>
<td>192 ± 71.9</td>
<td>135.8 ± 29.3</td>
<td>100</td>
<td>25.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Patient 3</td>
<td>c.379G&gt;pA p.Ala127Thr</td>
<td>393.8 ± 78.9</td>
<td>81.5 ± 19.4</td>
<td>0.5 ± 0.06</td>
<td>152.4 ± 41.1</td>
<td>12.1 ± 1.7</td>
<td>192 ± 71.9</td>
<td>117.3 ± 17.5</td>
<td>100</td>
<td>25.4</td>
<td>25.4</td>
</tr>
<tr>
<td>Patients 4 and 5</td>
<td>c.805G&gt;A p.Ala269Thr</td>
<td>131.1 ± 18.3</td>
<td>28.5 ± 5.6</td>
<td>0.77 ± 0.11</td>
<td>177.3 ± 63.3</td>
<td>66.6 ± 20.8</td>
<td>192 ± 71.9</td>
<td>108.3 ± 18.1</td>
<td>100</td>
<td>25.4</td>
<td>25.4</td>
</tr>
</tbody>
</table>

Human Molecular Genetics | 7

Downloaded from http://hmg.oxfordjournals.org/ at University of Toronto Library on October 6, 2015
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+ 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 Trueq Exome Enrichment Kit and HiSeq2000 sequencer. Chromosomal microarray analysis (Genome-wide copy-number variant detection) and loss-of-heterozygosity analysis was performed with CytoSNP-12xv2 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 Ca2+, cAMP and cGMP (16,35–37). Na+/H+ exchange activity was determined fluorometrically using the intracellular pH-sensitive dye BCECF-AM, as described

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+/H+ 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+-dependent intracellular alkalinization. (B) Quantitative analysis of Na+/H+ 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).
Immunoblotting was performed with primary antibodies to NHE3 (rabbit polyclonal 6347, 1:500) (35) GAPDH (mouse-monoclonal Abcam, Cambridge, MA; 1:5000) and β-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

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, RO1DK 099566, T32 DK200763 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.

References