


ORIGINAL ARTICLE

Novel *GUCY2C* variant causing familial diarrhea in a Mennonite kindred and a potential therapeutic approach

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Abstract

Guanylate cyclase 2C (GC-C), encoded by the *GUCY2C* gene, is implicated in hereditary early onset chronic diarrhea. Several families with chronic diarrhea symptoms have been identified with autosomal dominant, gain-of-function mutations in *GUCY2C*. We have identified a Mennonite patient with a novel *GUCY2C* variant (c.2381A > T; p.Asp794Val) with chronic diarrhea and an extensive maternal family history of chronic diarrhea and bowel dilatation. Functional studies including cosegregation analysis showed that all family members who were heterozygous for this variant had GI-related symptoms. HEK-293 T cells expressing the Asp794Val GC-C variant showed increased cGMP production when stimulated with *Escherichia coli* heat-stable enterotoxin STp (HST), which was reversed when 5-(3-Bromophenyl)-5, 11-dihydro-1,3-dimethyl-1H-indeno[2',1':5,6]pyrido[2,3-d]pyrimidine-2,4,6(3H)-trione (BPIPP; a GC-C inhibitor) was used. In addition, cystic fibrosis transmembrane conductance regulator (CFTR) activity measured with SPQ fluorescence assay was increased in these cells after treatment with HST, indicating a crucial role for CFTR activity in the pathogenesis of this disorder. These results support pathogenicity of the GC-C Asp794Val variant as a cause of chronic diarrhea in this family. Furthermore, this work identifies potential candidate drug, GC-C inhibitor BPIPP, to treat diarrhea caused by this syndrome.

KEYWORDS

BPIPP, diarrhea, *GUCY2C*, Mennonite, Plain community

1 | INTRODUCTION

Diarrhea in children is common, and typically occurs in acute self-resolving episodes most often due to viral infection. However, when diarrhea lasts for over 2 weeks it is termed chronic diarrhea (Avery et al., 1968). Severe forms of chronic diarrhea in children arise from a

variety of etiologies, including dietary protein-induced inflammation, infectious agents, necrotizing enterocolitis, or short bowel syndrome resulting from partial surgical bowel resection due to anatomical disorders (Bhutta et al., 2004). Rarely, chronic diarrhea results from mutations in single genes, referred to as congenital diarrheas and enteropathies (CODES). Gene mutations causing CODES can affect

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many different aspects of the GI tract including nutrient and electrolyte transport, epithelial cell metabolism, epithelial cell trafficking and polarity, enteroendocrine cell function, and immune cell function (Thiagarajah et al., 2018).

Mutations affecting epithelial cell transport proteins are among the best characterized and most prevalent inherited causes of chronic diarrhea. Examples include defects of the Na⁺/glucose transporter SGLT1 (encoded by *SLC5A1*) (Martin et al., 1996), Na⁺/H⁺ exchanger NHE3 (encoded by *SLC9A3*) (Janecke et al., 2015), and guanylate cyclase 2C (GC-C) receptor (encoded by *GUCY2C*) (Muller et al., 2016). While *SLC5A1* and *SLC9A3* mutations are implicated as loss-of-function of their respective protein transporters, *GUCY2C* mutations causing diarrhea are gain-of-function mutations. GC-C falls in the family of guanylate cyclase-coupled receptors that produce cGMP from GTP and serve a diverse range of roles in the body. These receptors are primarily expressed on the apical side of intestinal epithelial cells interfacing with the lumen of the GI tract. They have highest expression in the small intestine with a decrease along the longitudinal axis distally to the colon (Krause et al., 1994). Furthermore, GC-C is most highly expressed in the newborn period and declines over the first 17 months of life (Cohen et al., 1988).

GC-C was first identified as a receptor that is activated in response to heat-stable toxin (HST) produced during an enterotoxigenic *Escherichia coli* (*E. coli*) infection (Basu et al., 2010; Field et al., 1978). HST causes a self-resolving, secretory diarrhea in patients, most prevalent in resource-limited parts of the world. A *Gucy2c*^{-/-} knockout mouse model does not respond to HST due to the lack of functional GC-C receptors (Mann et al., 1997). GC-C also

responds to guanylin and uroguanylin, structurally similar endogenous peptides produced by the intestinal epithelial cells that stimulate GC-C to produce cGMP (Ikpa et al., 2016). cGMP alters intestinal homeostasis through several second messenger systems, acting primarily on the cystic fibrosis transmembrane conductance regulator (CFTR) and NHE3 ion channels to alter chloride and sodium ion transport (Figure 1). Intriguingly, mutations in *GUCY2C* can be inherited in autosomal dominant or autosomal recessive fashion with two different clinical presentations. Autosomal dominant gain of function mutations lead to chronic diarrhea due to increased cGMP production while autosomal recessive loss of function mutations cause meconium ileus (Fiskerstrand et al., 2012; Muller et al., 2016; Romi et al., 2012; Smith et al., 2015; Woods et al., 2019).

Current treatment of diarrhea includes fluid replacement therapy plus medications targeting intestinal motility and/or fluid secretion; however, none directly impact ion channel function (Thiagarajah et al., 2018). GC-C has been identified as a potential therapeutic target for treating diarrheal disorders, but no FDA-approved treatments based on this mechanism exist (Waldman & Camilleri, 2018). Furthermore, no specific treatments for *GUCY2C* mutation-related diarrhea exist. One promising compound is a pyridopyrimidine derivative 5-(3-Bromophenyl)-5,11-dihydro-1,3-dimethyl-1H-indeno[2',1':5,6]pyrido[2,3-d]pyrimidine-2,4,6(3H)-trione (BPIPP), which has been identified as a GC-C inhibitor able to inhibit cGMP production in T84 human colorectal carcinoma cells; it also inhibits chloride cGMP or cAMP ion transport stimulated by accumulation of both cyclic nucleotides, decreasing the accumulation of fluids in an in vivo rabbit intestinal loop model (Kots et al., 2008).

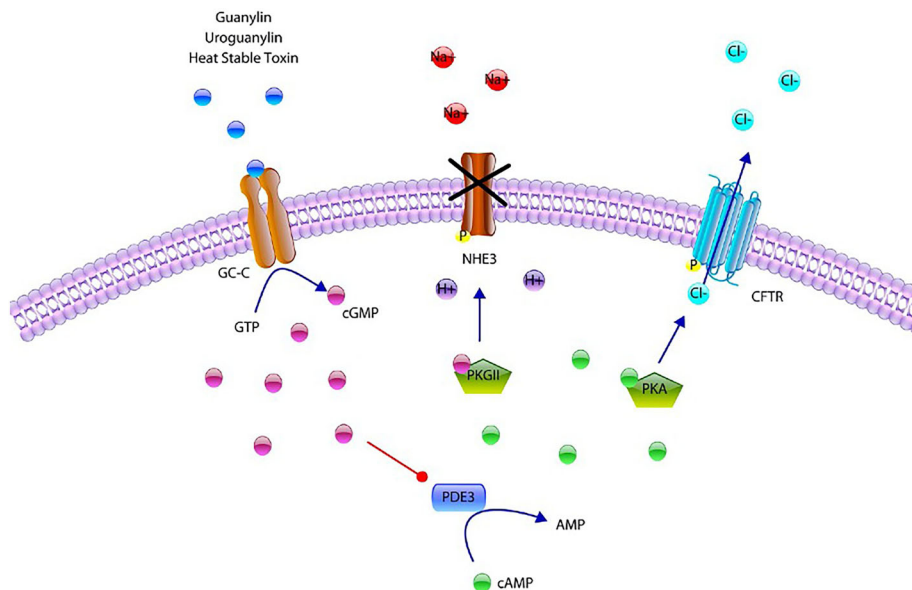


FIGURE 1 Guanylate Cyclase C (GC-C) production of cyclic GMP (cGMP) and signaling to cystic fibrosis transmembrane conductance regulator (CFTR) and sodium hydrogen Antiporter 3 (NHE3) ion channels. Stimulation of GC-C with endogenous ligands uroguanylin or guanylin or by *E. coli* heat stable toxin (blue circles) produces cGMP (pink circles), which directly activates protein kinase GII (PKGII) and indirectly activates protein kinase A (PKA) by decreasing phosphodiesterase 3 (PDE3) degradation of cyclic AMP (cAMP; green circles). Activated PKA and PKGII phosphorylate CFTR and NHE3, respectively. Phosphorylation results in increased Cl⁻ efflux through CFTR (large arrow) and decreased Na⁺ influx/H⁺ efflux through NHE3 (shown by large X). Increased Cl⁻ and Na⁺ in the lumen of the GI tract draw water into the lumen causing diarrhea. Image made with ePath3D

In this study, we have identified a novel *GUCY2C* variant (NM_004963.4, c.2381A > T; p.Asp794Val) in a Mennonite family with an extensive history of chronic diarrhea, including some family members with bowel dilatation at older age requiring tapering enteroplasty surgery. Functional studies verified pathogenicity of this variant in causing diarrhea, and provided a means to examine the *in vitro* use of the GC-C inhibitor, BPIPP. Indeed, BPIPP blocked overproduction of cGMP by the mutant GC-C suggesting that BPIPP analogues are candidates to explore further as novel therapeutics for chronic diarrhea of this etiology.

2 | MATERIALS AND METHODS

2.1 | Case description

A 13-year-old Mennonite male had chronic diarrhea and abdominal bloating beginning in infancy, with some difficulty gaining weight until 5–6 years of age. The patient's linear growth has subsequently been normal and he is currently overweight with body mass index (BMI) of 28. He has 2–3 liquid stools per day containing no blood; he has no nocturnal stooling. He was admitted to the pediatric intensive care unit (PICU) at age 11 years for acute, self-limited, worsening of diarrhea causing multiple electrolyte abnormalities (hyponatremia, hypokalemia, hypochloremia) and dehydration. Infectious studies and fecal occult blood were negative and fecal calprotectin was normal. Stool osmotic gap was consistent with a secretory diarrhea.

Esophagogastroduodenoscopy and colonoscopy with biopsies revealed aphthous ulceration in the duodenum and erythema and

nodularity in the terminal ileum. His colon had diffuse erythema, nodularity, and aphthous ulceration. Histopathologic examination was notable for chronic duodenitis with ileal and pancolonic inflammation including areas of distorted architecture, expansion of the lamina propria with a lymphoplasmacytic infiltrate, and multifocal cryptitis without granulomas. There was a striking family history of chronic diarrhea, a diagnosis of inflammatory bowel disease with poor response to therapy, and progressive bowel dysmotility and dilation requiring tapering enteroplasty in some family members.

2.2 | Study design

The index patient was identified as part of a larger project focusing on the discovery of novel genetic disorders/variants in the Amish and Mennonite communities. Whole exome sequencing performed on a clinical basis identified a variant of unknown significance (VUS) in *GUCY2C* as described below and not previously described in the Mennonite or general population. No other clinically relevant variants were identified. An information and education session on genetic screening for the variant was held for extended maternal family members, followed by an informed consent process for sample collection as approved by the University of Pittsburgh Institutional Review Board. A detailed pedigree, clinical symptoms, physical findings, and specimens were collected from 31 maternal family members including 17 adults and 14 children (see pedigree, Figure 2). Specimen collection included either blood or mouth wash for saliva for DNA extraction.

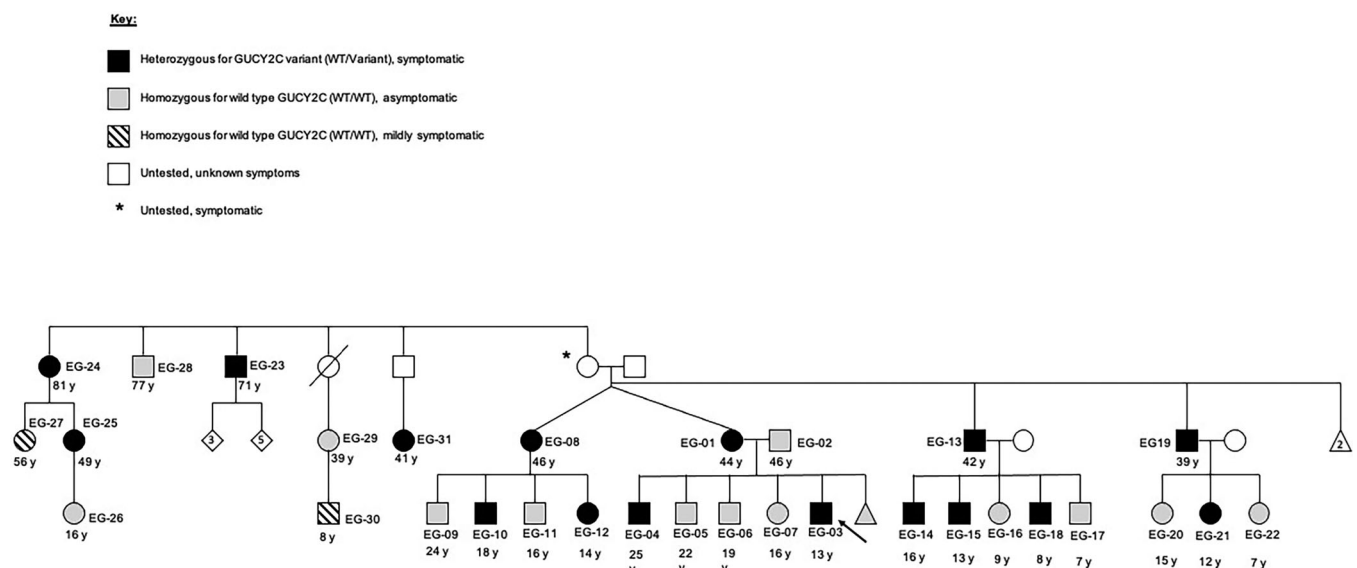


FIGURE 2 Pedigree of the proband's extended family on the maternal side. Family members on the maternal side of the proband (arrow) as well as the father were sequenced in the region of the *GUCY2C* variant; these include symptomatic and asymptomatic individuals. Symbols marked in black represent individuals who described chronic diarrhea symptoms and were heterozygous for the *GUCY2C* (c.2381A > T) variant. Symbols marked in gray represent individuals who did not describe any diarrhea or gastrointestinal symptoms and were homozygous for wild type *GUCY2C*. Symbols marked with a diagonal stripe indicate family members with mild diarrhea or loose stools but were homozygous for wild type *GUCY2C*. Untested individuals are marked with a white symbol. The symbol (*) represents the maternal grandmother of the proband who is symptomatic but not tested; she has diarrhea with several enteroplasty surgeries at older age. Age in years (y) at the time of analysis is indicated

2.3 | DNA extraction and genetic analysis

DNA was extracted from blood and saliva (mouth wash) using a Blood Core Kit B DNA extraction kit (PureGene, Qiagen Germantown, MD) according to the manufacturer's instructions. Whole exome sequencing (INVITAE, San Francisco, CA) was performed on a blood sample from the index patient on a clinical basis and identified a heterozygous variant of unknown significance (NM_004963.4, c.2381A > T; p.Asp794Val) in the *GUCY2C* gene. Targeted Sanger sequencing of the region of interest of the *GUCY2C* gene was conducted in parents and extended maternal family members using custom designed primers (Supplementary Table S1).

2.4 | Cloning and construction of *GUCY2C* control and c.2381A > T variant IRES GFP plasmids

Expression vectors pcDNA3.1(+) containing full *GUCY2C* cDNA sequence with and without the c.2381A > T variant were constructed by BioMatik (BioMatik, Ontario, Canada). Vectors were treated with *NotI* and *AflIII* restriction enzymes (New England BioLabs, Ipswich, MA) and inserts were gel purified using the ZymoClean Gel DNA recovery kit (ZymoResearch, Irvine, CA), then ligated into the multicloning site of a pcDNA3.1(+) IRES-GFP expression vector (Addgene, Watertown, MA). The ligation product was introduced into XL-1 Blue supercompetent cells (Agilent, Santa Clara, CA), and Sanger sequencing was used to confirm the full *GUCY2C* insert sequence (Supplementary Table S1).

2.5 | Cell transfection

The wild type or variant *GUCY2C*/IRES-GFP containing expression vectors or control IRES-GFP expression vector was transfected into HEK-293 T cells either alone or along with equal amounts of wild type *CFTR* pcDNA3.0 (a gift from the Bertrand lab) using Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. After 24 h in transfection media, wells were aspirated, and growth medium was added to each well. GFP signal was visualized at 24 h to confirm transfection efficiency. All experiments were performed 72 h post-transfection.

2.6 | BPIPP preparation

One-milligram of BPIPP (Cayman Chemical, Ann Arbor, MI) was dissolved in 1.1 ml of 100% cell culture grade DMSO by incubating in an ultrasonic water bath for approximately 30 min, to obtain a stock concentration of 2.0 mM. BPIPP was prepared the day prior to the experiment and stored at room temperature. About 250 μ l of each aliquot was added to 10 ml of 1x phosphate buffered saline (PBS) for a final concentration of 50 μ M BPIPP in 2.5% DMSO and 2.5% DMSO

vehicle. Solutions were mixed thoroughly before application to cell cultures.

2.7 | HST preparation

About 0.5 mg of Enterotoxin STp (*E. coli*) trifluoroacetate salt (Bachem Cat # H-6248.0500, Torrance, CA) was reconstituted at room temperature in 250 μ l of 1.0% acetic acid and 0.5% bovine serum albumin (BSA) solution in deionized water to a stock concentration of 1 mM of *E. coli* heat-stable enterotoxin STp (HST). In addition, 0.1 mM aliquots were stored at -20°C . Aliquots of vehicle (1% acetic acid and 0.5% BSA solution in deionized water) were also made for use as a control treatment and stored at -20°C . Prior to treatment, aliquots were thawed at room temperature. Ten-microliter of each aliquot was added to 10 ml of PBS, pH 7.4 for a final concentration of 0.1 μ M HST in 0.001% acetic acid and 0.0005% BSA, and 0.001% acetic acid and 0.0005% BSA. Solutions were both filtered via vacuum-driven filtration systems before application to cell cultures.

2.8 | Treatment of HEK-293 T cells with BPIPP and HST

For all experiments, HEK-293 T cells were seeded into the appropriate plate size, transfected as above, then at 72 h were treated with either 50 μ M BPIPP in 2.5% DMSO, 2.5% DMSO vehicle, 10^{-7} M HST in acetic acid/BSA, or acetic acid/BSA vehicle prepared as above. First, HEK-293 T were treated with either 50 μ M BPIPP solution in 2.5% DMSO or 2.5% DMSO vehicle and incubated for 10 min at 37°C , 5% CO_2 . The solutions then were aspirated and each well was treated with either 10^{-7} M HST or acetic acid/BSA vehicle for 15 min at 37°C , 5% CO_2 . Finally, the solutions were aspirated, and cells were prepared for analysis as described below.

2.9 | Western blot analysis

Transfected HEK-293 T cells in 12-well plates were treated with 150 μ l radioimmunoprecipitation assay (RIPA) Buffer (ThermoFisher Scientific) and 1x Protease Inhibitor Cocktail (PI) (Roche, St Louis, MO), added to each well for 15 min at room temperature. The contents of each well were transferred to Eppendorf tubes, and allowed to incubate on ice for another 15 min, followed by centrifugation at $14,000 \times g$ for 15 min at 4°C . The supernatants were subjected to electrophoresis on 4–15% Criterion SDS-polyacrylamide gels (Bio-Rad, Hercules, California) and transferred to PVDF membranes for western blotting. Primary antibodies used included Anti-GAPDH (1:25000 Abcam, ab8245), Anti-GC-C (1:200, Santa Cruz, sc-100,302), and Anti-CFTR #596 (1:1000, UNC CF Research Center) (Supplementary Table S2). Blots were visualized with horseradish peroxidase-conjugated secondary antibodies.

2.10 | cGMP ELISA assay

HEK-293 T cells were seeded at 400,000 cells/well into 24-well plates and transfected with *GUCY2C* wild type IRES-GFP, *GUCY2C* D794V variant IRES-GFP, or IRES-GFP control expression vectors, then treated with BPIPP and HST at 72 h post-transfection as described above. Without aspirating the 500 μ l of HST or vehicle solutions, 300 μ l of 1% Triton X-100 in 1x PBS was applied to each well for 10 min. Cell lysates were separated by centrifugation as above, then analyzed for cGMP using the Direct cGMP ELISA kit according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). The concentration of protein in each individual sample was measured in triplicate with the DC protein assay (BioRad).

2.11 | 6-Methoxy-N-(3-sulfopropyl) quinolinium (SPQ) fluorescence assay

HEK293T cells were transfected as described above. In addition, equal amount of CFTR pcDNA3.0 was used. At 48 hours post-transfection, HEK-293 T cells were seeded onto 35 mm MatTek glass bottom dishes (MatTek, Ashland, MA) coated with poly-L-lysine at approximately 250,000 cells/dish. At 72 h post-transfection, SPQ (Fisher Scientific, Grand Island, NY) was introduced into the cells via hypotonic shock, as previously described (Silvis et al., 2009). The dish was mounted on an Olympus IX81 epifluorescent microscope (Olympus Life Sciences, Tokyo, Japan), and continuously perfused with solution warmed to 32°C through an inline solution heater (Warner Instruments). SPQ fluorescence was monitored at 445 nm with excitation at 340 nm. Each experiment started with isotonic iodide solution for ~5 min. After signal stabilization, the perfusate was switched to nitrate solution (Silvis et al., 2009) combined with acetic acid/BSA vehicle for 4 minutes, followed by nitrate solution combined with 0.1 μ M HST for 15 min. Iodide solution then was perfused for 10 min. Regions of interest (ROIs) were drawn around individual cells and fluorescence intensity for each ROI was recorded every 15 s throughout the experiment. The maximum slope of iodide efflux for each condition measurement was calculated.

2.12 | Statistical analysis

Statistical significance was assessed with 2-way ANOVA using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California). A *p* value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Identification of a novel *GUCY2C* mutation causing chronic diarrhea

Whole exome sequencing identified a novel heterozygous missense variant of unknown significance (c.2381A > T; p.Asp794Val) in the

GUCY2C gene (GenBank: NM_004963.4) in the proband. Sanger sequencing was performed on both parents: The affected mother was found to be heterozygous for the same variant while the asymptomatic father was not. Targeted Sanger sequencing on extended family members with chronic diarrhea demonstrated co-segregation with the *GUCY2C* Asp794Val variant. Two individuals lacking the variant were reported to have mild diarrhea episodes and frequent loose stools that were felt by the family to differ from family members with the variant. Clinical descriptions of each family member as well as *GUCY2C* genotype status are detailed in Supplementary Table S3.

Phylogenetic analysis of the GC-C protein shows that Asp794 is an invariant residue among the vertebrate species examined (Figure 3(b)), occurring within the highly conserved linker region of the GC-C receptor (Saha et al., 2009). Indeed, two other activating mutations causing chronic diarrhea that occur in this linker region have previously been reported (Figure 3(a); Table 1). Each activating mutation is a heterozygous missense mutation, one in a Dutch family (c.2376G > C; p. Leu775Pro) and another in a German family (c.2376G > C; p. Arg792Ser). The GC-C linker region affected by these mutations includes a predicted coiled-coil domain involved in receptor dimerization (Biswas et al., 2009) and may also transduce conformational changes from ligand binding to the catalytic domain (Bose et al., 2020). The Paircoil2 program (<http://cb.csail.mit.edu/cb/paircoil2/>) (McDonnell et al., 2006) identifies motifs potentially forming α -helical coiled-coil supersecondary structure with *p*-scores below 0.025, as seen for the linker region of wild type GC-C (Figure 3(c)), but the three chronic diarrhea mutations in this region are predicted to weaken (Figure 3(d),(e)) or abolish (Figure 3(f)) the ability to form the predicted α -helical coiled-coil supersecondary structure.

3.2 | GC-C Asp794Val leads to increased cGMP production

cGMP production in response to treatment with 0.1 μ M HST increased by approximately 200-fold when HEK-293 T cells were transfected with a vector encoding wild type GC-C compared to a control vector (Figure 4(a)). Use of a vector encoding the GC-C Asp794Val variant further increased cGMP production 3-fold over transfection with wild type GC-C (Figure 4(a)). In contrast, cGMP production was unchanged when each transfected cell line was treated with the vehicle. Pretreatment of transfected cells with 50 μ M of the GC-C inhibitor BPIPP prior to HST stimulation decreased cGMP production by approximately 4-fold for cells transfected with the variant *GUCY2C* expression vector, while cells transfected with wild type *GUCY2C* decreased cGMP production by approximately 1.7-fold. (Figure 4(b)). Cells were transfected equally with the wild type and variant *GUCY2C* containing vectors as shown by western blotting (Figure 4(c)). Quantitation of the relative density of the GC-C bands (*n* = 3 for each variant) was compared after normalization to a loading control (GAPDH). No statistically significant differences were seen in the expression of GC-C (Supplementary Figure S3).

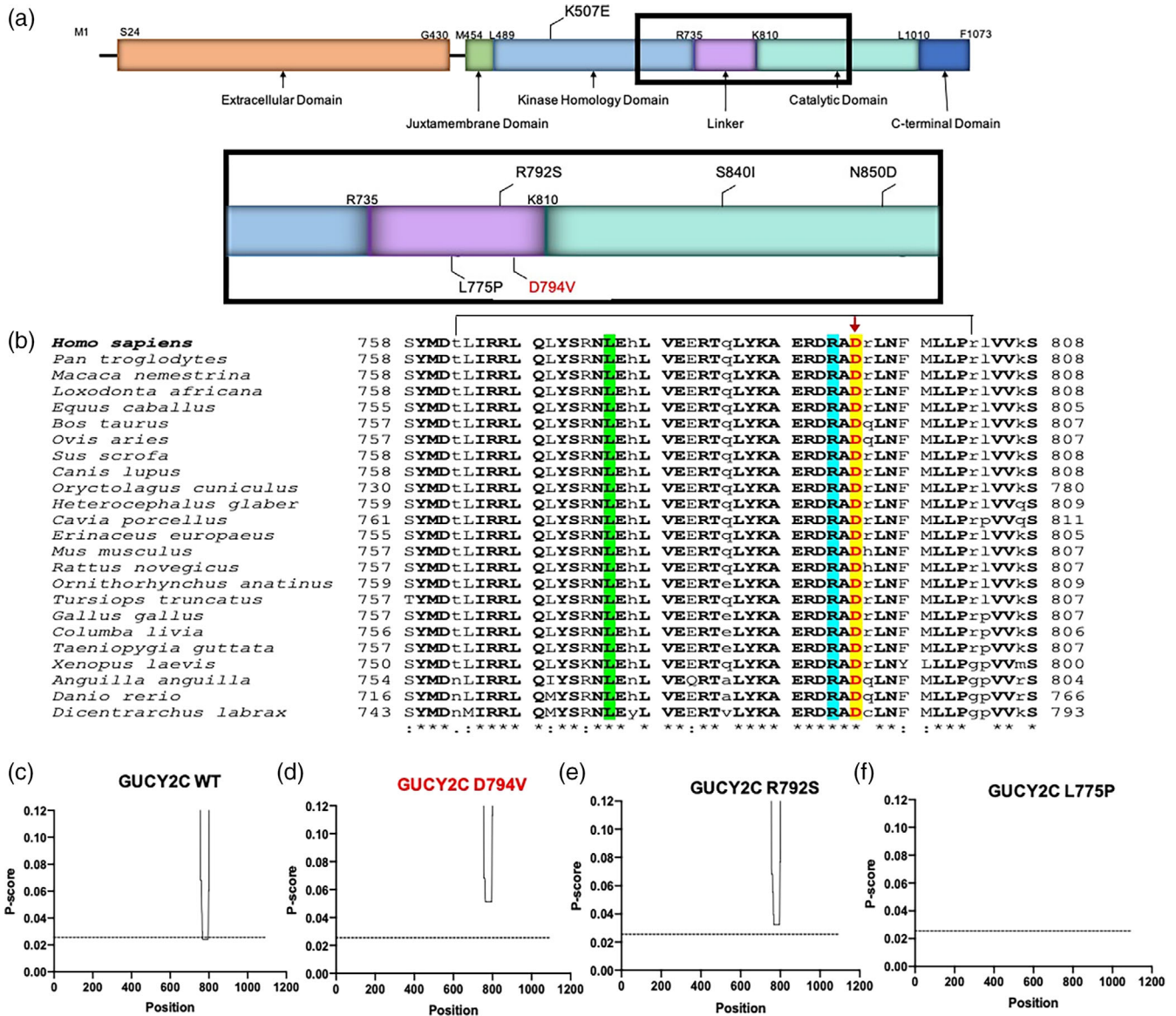


FIGURE 3 Mutations in GUCY2C predicted to affect a highly conserved GC-C linker domain. (a) Map of GC-C protein marked by first and last amino acid residue for each region of the polypeptide, with expanded inset. Each known activating GUCY2C mutation identified by amino acid change is marked on the map with an arrow pointing to location (the mutation identified in this study is in red). (b) Multisequence alignment of the predicted α -helical coiled-coil supersecondary structure (indicated with the horizontal bracket on top of sequence) within the linker region. The position of Asp794 is highlighted in yellow and the residues for two other chronic diarrhea mutations are highlighted in green (p.Leu775Pro) and blue (p.Arg792Ser). Invariant residues are capitalized, bolded, and marked with an asterisk; highly conserved residues are capitalized with a double dot; chemically similar residues are lowercase and marked with single dot. (c)–(f) Paircoil2 plots predict amino acid sequence region forming an α -helical coiled-coil supersecondary structure for (c) wild type human GC-C, however the two chronic diarrhea mutations in this region are predicted to weaken the ability to form a coiled-coil α -helix as in (d) Asp794Val and (e) Arg792Ser or abolish it as in (f) Leu775Pro mutation

3.3 | GC-C Asp794Val increases activation of CFTR

The CFTR channel is activated by cGMP but is not normally expressed in HEK-293 T cells. We therefore co-transfected a wild type CFTR expression vector with variant (c.2381A > T; p.Asp794Val) or wild type GUCY2C vector or control vector and measured CFTR activity with and without stimulation with HST. The presence of wild type GC-C protein increased CFTR activity \sim 5-fold over the control when

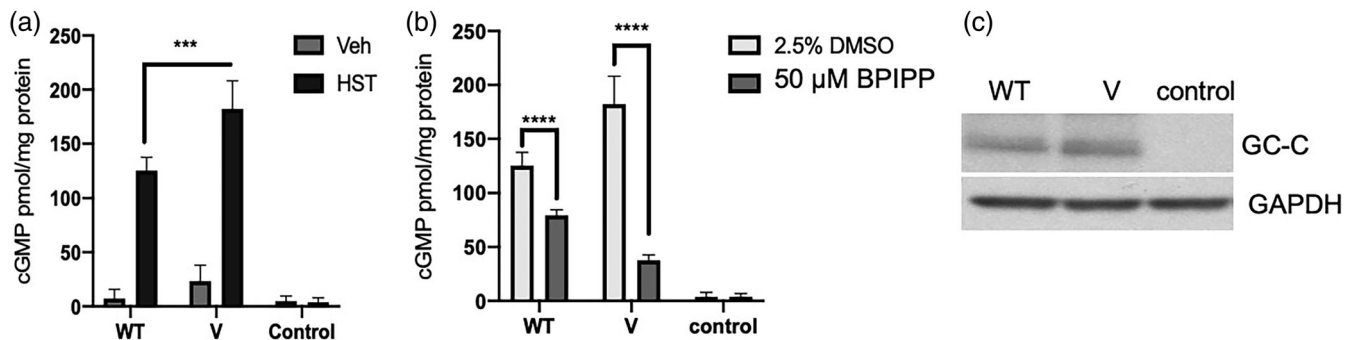
stimulated with 0.1 μ M HST, while the variant GC-C protein increased CFTR activity \sim 2-fold over wild type GC-C. Vehicle treatment did not increase CFTR activity from baseline (Figure 5).

4 | DISCUSSION

A number of GUCY2C mutations have been characterized as causing chronic diarrhea syndromes. Gain of function autosomal dominant

TABLE 1 Summary of currently known mutations in *GUCY2C*. Familial diarrhea syndrome mutations are heterozygous while mutations associated with meconium ileus are homozygous or compound heterozygous

Reported phenotype	Nucleotide change	Amino acid change	Family ethnic background	<i>GUCY2C</i> protein domain	Reference
Familial diarrhea syndrome	c.2519G > T	p.(Ser840Ile)	Norwegian	Catalytic	(Fiskerstrand et al., 2012)
	c.2548A > G	p.(Asn850Asp)	German/Polish	Catalytic	(Muller et al., 2016)
	c.1519A > G	p.(Lys507Glu)	French/Algerian	Juxta-membrane	
	c.2324 T > C	p.(Leu775Pro)	Dutch	Linker	
	c.2376G > C	p.(Arg792Ser)	German	Linker	
	c.2381A > T	p.(Asp794Val)	Mennonite	Linker	Current study
Meconium ileus	c.2008G > A	p.(Ala670Thr)	Lebanese	Kinase homology	(Smith et al., 2015)
	c.2782 T > C	p.(Cys928Arg)	Lebanese	Catalytic	
	c.1160A > G	p.(Asp387Gly)	Bedouin	Extracellular	(Romi et al., 2012)
	c.2270dupA	p.(Asn757Lysfs*2)	Bedouin	Truncated protein	
	2575A > G, c.2864_2865delCCinsTA	p.(Ile859Val), p.(Ser955Leu)	Asian/Caucasian	Catalytic	(Woods et al., 2019)

**FIGURE 4** Variant GC-C (Asp794Val) leads to increased cGMP production compared to wild type and BPIPP decreases cGMP production. cGMP production of HEK-293 T cells transfected with wild type *GUCY2C*-(WT)-IRES-GFP, variant *GUCY2C*-(V)-IRES-GFP, or IRES-GFP (control) vector. (a) Cells were stimulated with acetic acid/BSA vehicle or HST. (b) Cells were pre-treated with either 2.5% DMSO vehicle or 50 μ M BPIPP, then exposed to HST. Data from one experiment are shown ($n = 3$). The experiment was repeated twice. Error bars indicate SD. Ordinary two-way ANOVA with Tukey's. Heat-stable toxin (HST) post-hoc test for pairwise comparisons was performed as indicated. Bars show the mean and SD of three measurements. A p -value < 0.05 was considered significant. p value: **** $p < 0.0001$; *** $p < 0.001$. (c) Equivalent expression of variant (V) and wild type (WT) GC-C was observed on western blot analysis; no GC-C was observed in the control. In addition, GAPDH was equivalent in all three groups (see Supplementary Figures S1 and S2 for full blots). The relative density of the western blotting GC-C bands ($n = 3$ for each WT and variant) was compared after normalization to a loading control (GAPDH) and did not show statistically significance difference in the expression of GC-C (see Supplementary Figure S3)

GUCY2C mutations in unrelated families of Norwegian, French/Algerian, German, and other ethnicities have been previously reported in patients with chronic diarrhea in infancy, with variable severity and complications including sepsis, volvulus, and bowel dilatation (Table 1) (Fiskerstrand et al., 2012; Muller et al., 2016). These mutations localize to three of the predicted polypeptide domains, including cytoplasmic juxtamembrane, catalytic, and linker domains. Notably, mutations causing increased GC-C activation and chronic diarrhea have been reported in the linker region, an approximately 75-amino acid region adjacent to the catalytic domain near the C-terminus of GC-C (Saha et al., 2009). Though *GUCY2C* mutations have been associated with familial diarrheal syndromes, the relationship between GC-C mediated

cGMP production and CFTR and NHE3 activity has not previously been investigated. Interestingly, four unrelated kindreds have been reported with autosomal recessive meconium ileus due to inactivating *GUCY2C* mutations, a frequent indicator of cystic fibrosis in newborns (Table 1) (Romi et al., 2012; Smith et al., 2015). This suggests that *GUCY2C* mutations may cause pathogenic changes due to their effect on CFTR activity.

Here, we report a novel mutation c.2381A > T in the *GUCY2C* gene leading to an Asp794Val residue change in GC-C linker region identified in a Mennonite family with familial chronic diarrhea, with co-segregation with the disease as an autosomal dominant trait of varying severity among individuals. None of the affected individuals

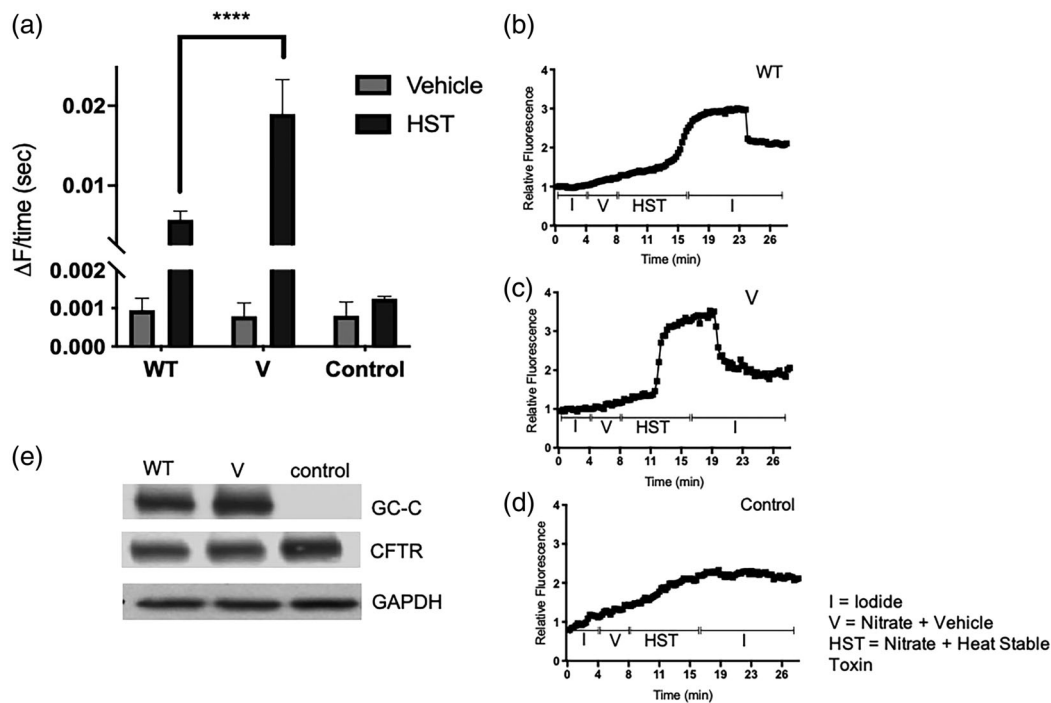


FIGURE 5 Variant GC-C (Asp794Val) leads to increased Cl^- efflux compared to wild type. (a) Average change in SPQ fluorescence (ΔF) measuring Cl^- efflux over time from HEK-293 T cells co-transfected with wild type CFTR pcDNA3.0 and either wild type *GUCY2C*-(WT)-IRES-GFP, variant *GUCY2C*-(V)-IRES-GFP, or IRES-GFP (control) vector. Note that the y-axes are discontinuous as marked by the double hash mark. Ordinary two-way ANOVA with Tukey's post-hoc test for pairwise comparisons was performed as indicated. Bars show the mean and SD of three measurements. **** $p < 0.0001$. SPQ fluorescence measurements of Cl^- efflux from single HEK-293 T cells that were co-transfected with a CFTR vector and (b) wild type *GUCY2C*-(WT)-IRES-GFP, (c) variant *GUCY2C*-(V)-IRES-GFP or (d) IRES-GFP (control) vector were performed. (e) Expression of variant (V) and wild type (WT) GC-C was observed on western blot analysis; no GC-C was observed in the control. In addition, CFTR expression is observed in all groups including the control. GAPDH was equivalent in all three groups (see supplementary figures S4, S5 and S6 for full blots). CFTR, cystic fibrosis transmembrane conductance regulator

required continued parenteral nutrition; however, several required tapering enteroplasty surgery at an older age due to bowel dilatation. Our study and the others aforementioned demonstrate that mutations in the highly conserved linker region of GC-C lead to increased HST-stimulated cGMP production. Co-expression of mutant GC-C and CFTR in HEK-293 T cells led to an increased CFTR response to HST compared to wild type GC-C. This finding would translate into increased chloride ion efflux from intestinal epithelial cells, consistent with CFTR overactivation as one of the mechanisms of diarrheal illness in patients with *GUCY2C* mutations. Additional studies on GI epithelium will be necessary to confirm this conclusion. *NHE3* mutations are another well recognized cause of chronic diarrhea. It will be important in future studies to assess the functional interrelationships between GC-C, *NHE3*, and CFTR in chronic diarrhea, and whether genetic variation in any of these genes modifies the phenotype.

Our findings suggest that inhibiting GC-C activity is a potential therapeutic approach at the cellular level for chronic diarrhea syndromes associated with gain of function *GUCY2C* mutations. BPIPP is a non-competitive GC-C inhibitor that has previously been shown to inhibit GC-C production of cGMP via HST in T84 human colorectal carcinoma cells and suppress fluid accumulation in a rabbit intestinal loop model (Kots et al., 2008). Our study extends these observations

by demonstrating that BPIPP inhibits the hyperactive Asp794Val mutant GC-C receptors in transfected HEK-293 T cells, decreasing cGMP levels to a similar level as when wild type receptor is stimulated with HST. Additional studies on different mutations are needed to further study the effect of this compound. Combined, the data indicate the potential to calibrate the small-molecule concentration to achieve normal levels of GC-C function. Thus, BPIPP analogues are promising candidates to treat this condition, and potentially for other gain of function mutations in *GUCY2C*.

In conclusion, we have identified a gain of function c.2381A > T mutation in the *GUCY2C* gene causing a Asp794Val substitution that co-segregates with a phenotype of chronic diarrhea and bowel dilatation at older age in a Mennonite family. CFTR activity is increased in HEK-293 T cells expressing this mutation, indicating a key role for this transporter in causing disease symptoms. Finally, we demonstrated that BPIPP reverses the increased cGMP production in these cells expressing the GC-C Asp794Val protein suggesting that use of GC-C inhibitors is a viable approach to treat this disorder.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Rachel M. Wolfe reviewed literature, completed most experiments, and drafted the manuscript. Rachel M. Wolfe and LGG developed the experimental design with assistance of Al-Walid Mohsen, Robert D. Nicholls, and Jerry Vockley. Rachel M. Wolfe, Cate Walsh Vockley, and Lina Ghaloul-Gonzalez obtained consent of participants and collected samples after informational session. Cate Walsh Vockley helped with collecting clinical data and communicated the results of the study to the family members. Paige Heiman assisted with laboratory studies. Carol A. Bertrand conducted the SPQ experiments with assistance of Rachel M. Wolfe. Leah M Seibold drafted the clinical description of the patient and follows the patient clinically in the gastroenterology clinic. All authors discussed the results and contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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