JPGN Journal of Pediatric Gastroenterology and Nutrition Publish Ahead of Print

DOI : 10.1097/MPG.0000000000001418

Twelve novel mutations in the *SLC26A3* gene in 17 sporadic cases of Congenital Chloride Diarrhea

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Conflict of interest: the authors declare no conflict of interest.

Acknowledgements: Grants from Regione Campania, DGRC 1901/09 and AIFA MRAR08W002 are gratefully acknowledged.

Word count: 2698

Number of figures:1

Number of tables:1

Supplemental Digital Content: 1

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (*www.jpgn.org*).

Abstract

Objectives: we aimed to improve the knowledge of pathogenic mutations in sporadic cases of congenital chloride diarrhea (CCD) and to emphasize the importance of functional studies to define the effect of novel mutations.

Methods: all *SLC26A3* coding regions were sequenced in 17 sporadic CCD patients. Moreover, the minigene system was used to analyse the effect of two novel splicing mutations.

Results: we defined the *SLC26A3* genotype of all 17 CDD patients and we identified twelve novel mutations. Using the minigene system we confirmed the *in silico* prediction of a complete disruption of splicing pattern caused by two of these novel mutations: the c.971+3_971+4delAA and c.735+4_c.735+7delAGTA. Moreover, several prediction tools

and a structure-function prediction defined the pathogenic role of six novel missense mutations.

Conclusion: we confirm the molecular heterogeneity of sporadic CDD adding twelve novel mutations to the list of known pathogenic mutations. Moreover, we underline the importance, for laboratories that offer molecular diagnosis and genetic counseling, to perform fast functional analysis of novel mutations.

Key words: Chloride Losing Diarrhea, *SLC26A3*, *DRA*, Mutations, Genotype, Splicing effect

Non standard abbreviations: CDD: congenital chloride diarrhea; **SLC26A3:** member 3 of solute carrier family 26; **US:** ultrasonography;

What is known

- About 200 cases of sporadic congenital chloride diarrhea (CCD) have been described so far
- About 80 SLC26A3 mutations have been found in CCD patients
- Some mutations may affect the splicing process

What is new

- We describe 17 novel cases of sporadic CCD
- We describe 12 novel SLC26A3 mutations
- We describe the use of the minigene assay to define the splicing effect of two novel mutations

Introduction

Congenital Chloride Diarrhea or Chloride Losing Diarrhea (CCD, OMIM #214700) is a severe autosomal recessive disease characterized by watery diarrhea with a high fecal chloride loss and metabolic alkalosis. It is characterized by maternal polyhydramnios, dilated fetal bowel loops and preterm birth. After birth, profuse watery diarrhea usually causes electrolyte imbalance with hypochloremic metabolic alkalosis or hyponatremia and life threatening dehydration (1-3). The disease is lethal if undiagnosed or untreated, but an early diagnosis and oral NaCl/KCl replacement may improve growth and the outcome of the disease (4).

Mutation analysis is crucial for the diagnosis of such disease (5, 6) that may be misdiagnosed (7-9) or confounded with a myriad of other causes of chronic diarrhea including other genetic disorders(3, 10). Furthermore, molecular diagnosis may be used for prenatal diagnosis in high-risk couples (11, 12). Finally, the SLC26A3 genotype influences the responsiveness to oral butyrate therapy(13-15). CCD is caused by mutations in the *member 3 of solute carrier family 26* (*SLC26A3*) gene (OMIM #126650), which encodes an intestinal Cl^-/HCO_3^-, Na^+-independent, exchanger (16). In ethnic groups where the disease is more frequent for founder effect, there is a single mutation (17): in Finns (incidence: 1:20-30.000), the p.V317del mutation affects up to 90% of CCD alleles; in Saudi Arabia (incidence: 1:5.000) and Kuwait (incidence: 1:3.200), p.G187X affects more than 90% of altered chromosomes; and in Poland (incidence: 1:200.000) 50% of the CCD alleles carry the I675-676ins mutation (official nomenclature: c.2025insATC). Differently, a large genetic heterogeneity has been reported in about 200 CCD sporadic patients from other ethnic groups and causing mutations spread all over the disease gene (6, 18, 19). However,

the sequencing of the whole coding regions of the *SLC26A3* gene is required for molecular analysis, but frequently novel mutations are identified that may be difficult to classify as pathogenic variant (6). Thus, data on novel mutations found in well-defined cases of CCD are contributory to improve the knowledge of pathogenic mutations. Moreover, functional studies play a key role to define the effect of novel mutations.

We analyzed by sequencing the whole coding region of the *SLC26A3* gene in 17 sporadic cases of CCD (three of which had an affected sibling). Finally, using the *in vitro* functional minigene assay, we confirmed the pathogenic effect of two of these novel mutations.

Subjects and methods

Subjects

The Pediatric Gastroenterology Unit at the University of Naples Federico II is an International Reference Center for patients with CCD and other forms of congenital diarrheal diseases (3, 10). From 2005 to 2014, 50 cases of suspected CCD were referred to the Center, and a definitive diagnosis was obtained in 38 patients. Among these, 18 were of arabian origin and were homozygous for the G187X mutation (16) and 20 were sporadic cases of different ethnicity. Of these 20 cases of CCD, 3 included two affected siblings. Finally, we describe the *SLC26A3* (NM_000111) genotype of 17 sporadic, unrelated cases of CDD, 7 of which are enrolled in the study on butyrate treatment (14).

All the participants (guardians in the case of minors) provided written informed consent to anonymously use a DNA sample and clinical data for research purposes.

Molecular analysis of *SCL26A3* gene

Molecular analysis was performed in the laboratory of CEINGE-Advanced Biotechnologies that acts as reference center for molecular diagnosis of inherited diseases in Campania region (about 6 million of inhabitants), located in southern Italy. DNA was extracted from an EDTA blood sample with the Nucleon BACC2 kit (GE Healthcare Europe GmbH, Milan, Italy). The touchdown PCR protocol that enables co-amplification of all exons under the same PCR conditions (primers and conditions are available on request). Sequencing analysis was carried out on both strands with an automated procedure (3100 Genetic Analyzer, Life Technologies, Monza, Italy). All PCR fragments were sequenced with the primers used for PCR. Novel mutations were analyzed in 100 unrelated healthy control subjects whose DNA is available c/o our biological bank by gene sequencing or by DHPLC(20). Procedures are available on request.

***In silico* prediction analysis**

The genomic sequence of interest, wild-type and mutated form, were submitted for splice-site prediction to the Alamut v2.0 and NetGene2 (http://www.cbs.dtu.dk/services/NetGene2) biosoftwares (21, 22).

Databases

The identified c.971+3_971+4delAA and c.735+4_735+7delAGTA mutations have been deposited in the Leiden Open Variation Database (LOVD) at http://databases.lovd.nl/shared/individuals/00018561.

Minigene Construct

To study the effect of the genetic variants on splicing pattern, we amplified a region of 1.2 kb of the *SLC26A3* gene containing the exon 7 and 8 to study the

c.971+3_971+4delAA mutation (Case #2) and a region of 0.6 kb containing the exon 6 for the c.735+4_735+7delAGTA mutation (Case #3). The PCR was performed directly from genomic DNA of heterozygous subject, using the following primers:

- Case #2:

Fw: 5'-CATTCAGGTACCTCCATCGCTACTC-3'

Rev: 3'-CATTCAGGTACCCTGGCCTTACTTTGC-5'.

- Case #3:

Fw: 5'-AGCACGGTACCTGCAAATAAGCCTGTTACTTCTTG-3'

Rev: 5' CAGCTATGGTACCTGATCTCGTGATCCGC-3'.

These primers contained the KpnI restriction sites to cut the PCR products by KpnI restriction enzyme and to clone them into the pMGene vector (23) previously digested with KpnI and dephosphorylated. All clones were sequenced to confirm the insert sequence.

Cell cultures

HeLa cells (ATCC – CCL-2) were maintained in Dulbecco's Minimum Essential Medium (ATCC 30-2003) with 10% heat-inactivated fetal bovine serum (GE Healthcare Europe GmbH); T84 colon cell lines (ATCC – CCL-248) were maintained in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 2.5 mM L-glutamine, 5% heat-inactivated fetal bovine serum, cultures were maintained in 5% (v/v) CO_2 humidified atmosphere at 37°C.

Minigene Expression and Transcripts Analysis

HeLa and T84 cells seeded in 60-mm dishes were transfected with 5 µg of pMGene constructs by lipofectamine reagent (Life Technologies). Transfection medium was replaced with fresh medium 6h after the transfection. 24 hours after transfection, cells were

collected and total RNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), 1 µg of total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Life Technologies). After total RNA retro-transcription, 1/20 of cDNA was PCR amplified using the following primers:

RT-Mgene-For: 5'-CACATGGTCCTGCTGGAGTTC-3'

RT-Mgene-Rev: 5'-CCTGCACTGGTGGGGTGAATTC-3'.

The PCR products were then separated on a 1,5% agarose gel, and individual bands were excised and sequenced using the specific primers.

Results

The *SLC26A3* genotype of the 17 CCD patients is shown in (Supplemental Digital Content 1, Table, *http://links.lww.com/MPG/A788*): 11/17 (64.7%) patients were homozygous for a *SLC26A3* mutation, while 6/17 (35.3%) patients were compound heterozygous for two mutations. In the 11 homozygous patients with CDD we tested both parents (that resulted invariably heterozygous) to exclude the possibility that a large gene deletion would cause a false homozygous mutation result; in 3 cases; in which the parents were not available for the analysis, we excluded the presence of a large deletion by quantitative RT-PCR analysis of the involved exon. Similarly, for all 6 compound heterozygous patients we studied at least one parent to confirm that the two mutations found in the patients were in *trans*. Finally, we identified mutations in 34/34 alleles (100%). In particular, we found 21 different mutations: 9 missense, 3 nonsense, 2 single nucleotide deletion causing frameshift, 2 splicing intronic point mutation, 1 codon duplication, 1 large deletion, 1 codon deletion, 1 intronic four nucleotides deletion and 1

intronic two nucleotides deletion, both these latter causing a splicing effect. Among the 21 mutations, 12 (57.1.%) were novel (6 missense, 3 deletions, 2 frameshift, 1 nonsense).

All the mutations were identified by gene sequencing with the exception of the large deletion (case #1 – Supplemental Digital Content 1, Table, *http://links.lww.com/MPG/A788*). In fact, after the gene sequencing, no gene variants were found in such patient, but exon 18 lack to amplify. To exclude mutations in the primer(s) site, we used a more external set of primers and again, exon 18 did not amplify. Re-amplification using the long-PCR procedure revealed an homozygous large deletion; the heterozygous deletion was present in both parents. The walking primer analysis revealed the first breakpoint in position c.2008-151 and the second breakpoint in position c.2061+1546.

We functionally analysed two novel intronic mutations, i.e., the c.971+3_971+4delAA and the c.735+4_c.735+7delAGTA. Both mutations were found in homozygous patients. First of all, we analysed *in silico* the putative effect of both mutations, predicting a complete disruption of the physiological splicing pattern. Then, we analyzed *in vitro* the splicing pattern using the minigene system assay. Briefly, the exons of interest were cloned in the minigene construct (Figure 1A) and transfected in Hela and T84 cells. The RT-PCR analysis on total RNA extracted from the transfected cells confirmed the *in silico* prediction. Indeed, in both cases, the RT-PCR analysis revealed a complete disappearance of the wild-type band (Figure 1B-1C). In particular, the sequencing analysis of each electrophoretic separated band revealed the following data: for the c.971+3_971+4delAA mutation, we obtained two alternative bands: the upper one due to the skipping of exon 8 and the lower one due to the skipping of both exon 7 and 8. For the

c.735+4_c.735+7delAGTA mutation, the mutated construct resulted in a lower band corresponding to the skipping of exon 6. We confirmed the same splicing aberration pattern in T84 cell line, that expresses a splicing machinery of colon cells and represents a more physiological context for *SLC26A3* gene expression.

The six novel missense mutations (Table 1) were not found in 200 alleles from healthy subjects. Furthermore, the novel mutations were not reported in the 1000 genomes browser (http://browser.1000genomes.org) and in the Exome Variant Server (http://evs.gs.washington.edu/EVS/). Among the missense mutation: the p.S394I involves the random coil/□-helical border changing the neutral polar serine into the apolar isoleucine aminoacid; both the prediction tools indicated a high risk of pathogenicity. The mutation was found in two CCD siblings (in trans with the p.Q48X nonsense mutation). Similarly, the p.S438P involves the random coil/□-helical border changing the neutral polar serine into the apolar proline aminoacid and was found in a CCD patient that had a frameshift mutation on the other allele; both the prediction tools indicated a high risk of pathogenicity for the S438P. The A547E mutation involves the STAS domain and both the prediction tools indicated a high risk of pathogenicity. A missense mutations that abolish the protein function in an adjacent codon (i.e., the p.I544N) was already found in a CCD patient (6, 18). The A547E was found in a patient compound heterozygous for the novel missense Q495P mutation. Such latter causes the change of the neutral polar glutamine with the apolar proline aminoacid. Although the PolyPhen software predicted the mutation as a possibly damaging and SIFT as a tolerated mutation, it involves the same codon of the p.L496R mutation previously found in other CCD patients (6, 18, 19). The p.S654P involves the STAS domain changing the neutral polar serine into the apolar proline

aminoacid; the mutation was found homozygous in a CCD patient and was predicted as probably damaging by PolyPhen, while SIFT predicted the p.S654P as a tolerated mutation, but a mutation in an adjacent codon (i.e., the p.D652N) was previously reported in CCD patients (5, 18). Finally, the p.C508R was found in a patient that had the p.R579X nonsense mutation on the other allele. The mutation changes the neutral polar cysteine with the basic polar arginine aminoacid within the random coil/intracellular domain of the protein. The mutation was predicted as probably damaging by PolyPhen, while SIFT predicted the p.C508R as a tolerated mutation; the p.P506Q mutation that abolishes protein function in an adjacent codon was described in CCD patients (6, 17). Three other missense mutations found in our patients (i.e., the pG120S, the pP131R and the p.P129L), all reported as pathogenic by both the prediction tools (data not shown) had been previously found in CLD patients (6, 18, 19).

Discussion

Molecular analysis of the *SLC26A3* gene revealed the genotype of all the 17 patients with CCD and confirmed the genetic heterogeneity of sporadic CCD (6, 18, 19). In fact, the mutations we identified spread all over the gene, which suggests that the analysis in sporadic CCD patients should be based on sequencing of all *SCL26A3* gene coding regions, differently from ethnic groups where CCD is frequent and the analysis is routinely based on the study of a single mutation (6, 17).

We identified several novel mutations clearly affecting the synthesis or the activity of the protein and thus, resulting in having a pathogenetic role. This is true for the large, 1752-bp deletion in patient #1, that removes the whole exon 18 (55 bp) likely resulting in a

frameshift event (p.I670MfsX17). Similarly, the novel deletions found in patient #2 (found in two affected siblings) and in patient #3 are causing disease because they impair the splicing pattern, as we demonstrate in the present study. Indeed, phylogenetic analysis and *in silico* prediction may help to define the disease-causing effect of splicing mutations, as it is the case for both novel mutations, but only functional analysis permits to finally demonstrate such effect. In this context, the minigene assay we used represents a fast technique that allows, in about two-weeks, to functionally analyze mutations with a putative splicing effect. Noteworthy, 4/21 mutations (19%) found in the present study are deletions among which one was large, confirming that such type of mutation are frequently found in monogenic disorders, as we previously demonstrated for haemophilia A, in which about 19% of 217 mutations are gene deletions (24) and in cystic fibrosis in which large rearrangements are found in about 3% of affected alleles (25).

Furthermore, the single nucleotide deletions observed in patients #6 and #13 (this latter found also in patient #14) cause a frameshift and thus, a truncated inactive protein. The same is true for the novel nonsense mutation found in patient #11.

More difficult it is to define the pathogenic role of six novel missense mutations found in our CCD patients. Some criteria suggest their pathogenic role: i) they were absent from 200 normal alleles and in the 1000 genomes browser (http://browser.1000genomes.org) and in the Exome Variant Server (http://evs.gs.washington.edu/EVS/); ii) in all cases, each of the parents was heterozygous for one of the mutations identified in the proband; iii) no other mutations were identified in the *SLC26A3* gene in these patients; and iv) the amino acids affected by the mutations are highly conserved. In detail, both novel missense mutations identified in patient #15,

namely, p.Q495P and p.A547E, lie within the mutation-bearing hotspots characteristic of most sporadic CCD cases (19). The p.Q495P mutation could affect the correct folding of the last but two transmembrane domain of the protein (according to the 14 transmembrane □-helices model) (26-28) thereby altering the charge distribution. Alternatively, the mutation could alter the sulphate transporter family domain (19). The p.A547E mutation also involves the first □-sheet of the "STAS-like" domain. Glutamic acid is a powerful □-sheet interrupter and probably also induces a strong charge distribution change. Indeed, mutations involving the STAS-like domain have been associated to the absence of residual activity of the protein (29, 30). Patient #16 is homozygous for the novel missense mutation p.S654P. The mutation involves a highly conserved amino acid belonging to the conserved loop between the third and fourth □-helix of the STAS-like domain. We suggest p.S654P is a disease-causing mutation, since the proline residue is a powerful □-sheet interrupter and mutations affecting amino acids within the "STAS-like" domain are very likely to be disease-causing. Moreover, the p.S654P involves a palindromic sequence (c.GACTTTTCAG -> c.GACTTTCCAG), which could be more susceptible to mutations. The p.S438P mutation (patient #13) involves serine 438, the last amino acid of the random coil motif between the 11^{th} and 12^{th} transmembrane helix (according to the 14 transmembrane □-helices model) and proline is a powerful interrupter of □-helices. Alternatively, the mutation could alter the sulphate transporter family domain. Also in this case, the mutation lies in the hotspot region (19).

To conclude: our data confirm the high genetic heterogeneity of CCD in ethnic groups where the disease is sporadic. This highlights the importance to analyze the whole coding region by gene sequencing in patients suspected for sporadic CCD, particularly

when born to consanguineous parents. In case of novel, missense mutations, other mutations must be excluded and the novel mutation must be tested in a large number of unaffected chromosomes. Phylogenetic analysis and *in silico* prediction may help to confirm the disease-causing effect of novel mutations but functional analysis, here performed for two splicing affecting mutations, is very helpful to conclude on the pathogenetic effect.

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Supplemental Digital Content

SDC 1: SLC26A3 (NM_000111) genotype of 17 patients with CCD. The asterisk marks novel mutations; the double asterisk marks the cases that have an affected sib-pair with the same genotype.

Legend to the figure

Figure 1: A. Schematic structure of the pMGene empty and of the pMGene-DRA constructs showing the position of the novel genetic variants. B and C. RT-PCR analysis of mRNA extracted from cell transfected with the indicated pMGene constructs (M=mutated, W=wild type). The white arrows indicate the position of the PCR products with a schematic view in the right side. T84 (Human lung metastase from colorectal carcinoma cells) and HeLa (cervical cancer cells)

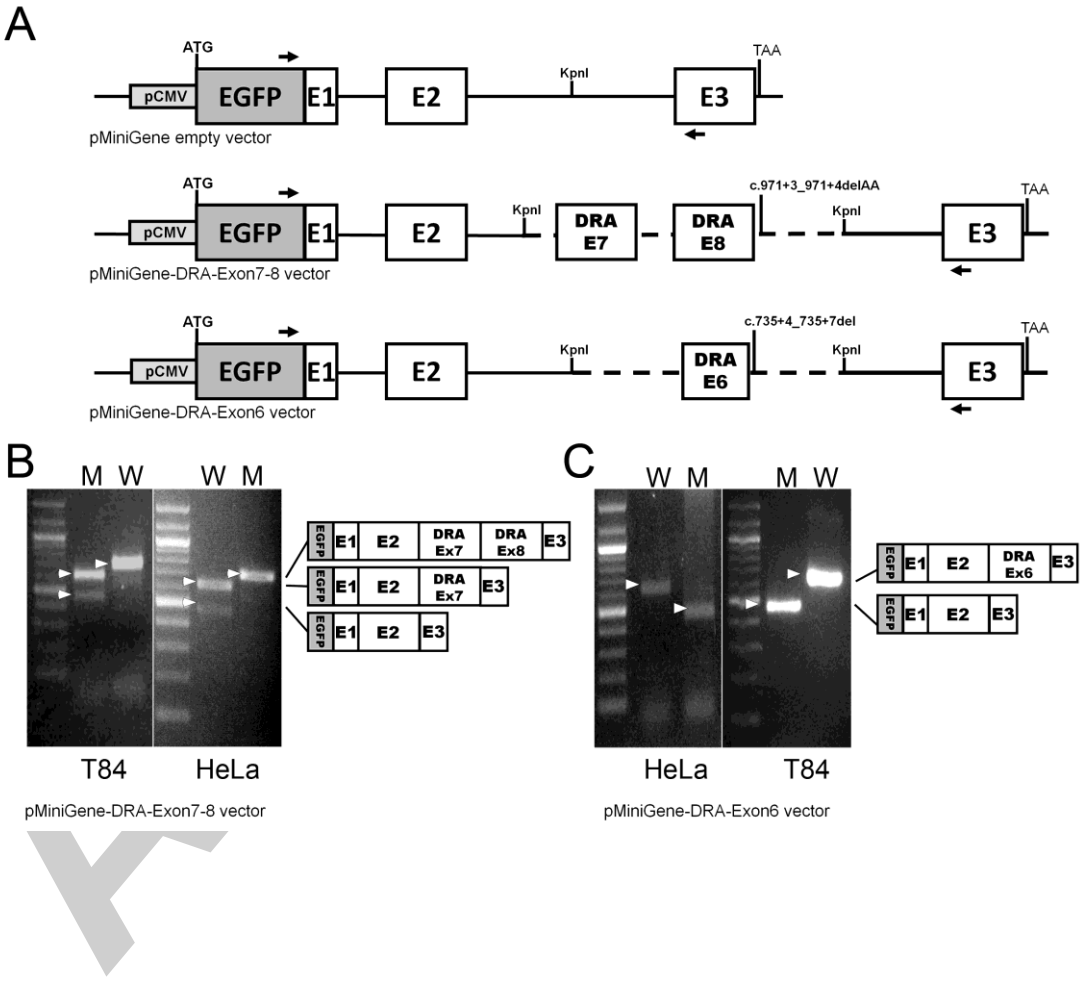

Table 1: Possible pathogenic role of missense mutations. For each of the 6 mutations, the Table reports: i) the protein domain involved by the mutation; ii) the PopyPhen prediction score of pathogenicity; iii) the SIFT prediction score of pathogenicity; iv) the list of mutations previously described involving adjacent aminoacids.)

Mutation cDNA	Mutation protein	Domain	PolyPhen prediction score	SIFT prediction score	Adjacent mutations
c.1181G>T*	p.S394I*	random coil/α-helical border	1 (probably damaging)	0 (deleterious)	
c.1312T>C*	p.S438P*	random coil/α-helical border	0.998 (probably damaging)	0 (deleterious)	
c.1640C>A*	p.A547E*	STAS domain	0.993 (probably damaging)	0 (deleterious)	I544N (6,7)
c.1960T>C*	p.S654P*	STAS domain	0.93 (probably damaging)	0.07 (tolerated)	D652N (6,7)
c.1522T>C*	p.C508R*	random coil/intracellular domain	0.713 (possibly damaging)	0.12 (tolerated)	P506Q (7,24)
c.1484A>C*	p.Q495P*	random coil/intracellular domain	0.696 (possibly damaging)	0.09 (tolerated)	L496R (6,7,19)