

A missense mutation in the chloride/proton ClC-5 antiporter gene results in increased expression of an alternative mRNA form that lacks exons 10 and 11. Identification of seven new *CLCN5* mutations in patients with Dent's disease

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Abstract Mutations in the voltage-gated chloride/proton antiporter ClC-5 gene, *CLCN5*, are associated with Dent's disease, an X-linked renal tubulopathy. Our interest is to identify and characterize disease-causing *CLCN5* mutations, especially those that alter the splicing of the pre-mRNA. We analyzed the *CLCN5* gene from nine unrelated Spanish Dent's disease patients and their relatives by DNA sequencing. Pre-mRNA splicing analysis was performed by RT-PCR. Seven new mutations were identified, consisting of three missense mutations (C219R, F273L, and W547G), one splice-site mutation (IVS-2A > G), one deletion (976delG), and two non-sense mutations (Y140X and W314X). We found that missense mutation W547G also led to increased expression of a new

alternative isoform lacking exons 10 and 11 that was expressed in several human tissues. In addition, we describe another novel *CLCN5* splicing variant lacking exon 11 alone, which was expressed only in human skeletal muscle. We conclude that missense mutation W547G can also alter the expression levels of a *CLCN5* mRNA splicing variant. This type of mutation has not been previously described in the *CLCN5* gene. Our results support the importance of a routine analysis at the pre-mRNA level of mutations that are commonly assumed to cause single amino acids alterations.

Keywords Alternative isoforms · *CLCN5* · Exon skipping · Pre-mRNA splicing · Renal tubulopathy · Splicing mutation

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Introduction

Dent's disease (OMIM 300009) is an X-linked renal tubulopathy characterized by low molecular weight proteinuria (LMWP), hypercalciuria, nephrocalcinosis/nephrolithiasis, and progressive renal failure. The disease may also be associated with aminoaciduria, phosphaturia, glycosuria, kaliuresis, uricosuria, impaired urinary acidification, and can be complicated by rickets or osteomalacia in some patients (Thakker 2000). Inactivating mutations in the *CLCN5* gene cause Dent's disease (Lloyd et al. 1996). Recently, this disorder has also been associated with mutations in the *OCRL1* gene (OMIM #300555) that encodes a phosphatidylinositol-4,5-bisphosphate-5-phosphatase and is correlated with Lowe syndrome (Hoopes et al. 2005).

CLCN5 encodes the voltage-gated chloride/proton antiporter CIC-5 (746 amino acids). A recently identified splice variant with an additional 70 amino acids at the intracellular amino-terminus has been detected only at the mRNA level (Ludwig et al. 2003; Jentsch et al. 2005). CIC-5 is a dimer with two identical subunits, each of which contains a pore and has an internal structural repeat composed of 18 α -helices (A–R) in antiparallel orientation (see Fig. 1). Helices H, I, P, and Q are involved in the formation of the interface between both subunits (Dutzler et al. 2002), and helices D, F, N, and R form the chloride selectivity filter (Dutzler et al. 2002). The carboxy terminus of CIC-5 includes two CBS (cystathionine β -synthase) domains, which constitute an energy-sensing module binding ATP, a PY sorting signal involved in trafficking to acidic endosomes, and a potential protein binding

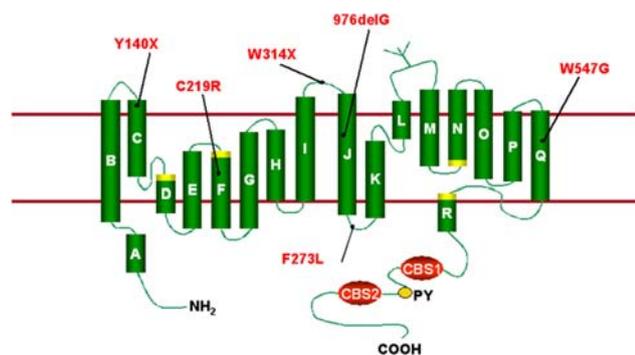


Fig. 1 Schematic representation of the CIC-5 monomer structure, showing the exonic mutations identified in this study. Cylinders depict helices A–R, with the cytoplasmic region below, and the extracellular region above. Regions involved in the chloride selectivity filter are shown in light grey. The CBS (cystathionine β -synthase) domains and the PY motif are also indicated

module, PDZ (Schwake et al. 2001; Scott et al. 2004; Hryciw et al. 2006).

In the human nephron, CIC-5 is expressed in the proximal tubule, the thick ascending limb, and the intercalated cells of the collecting duct. CIC-5 has been localized in subapical endosomes with vacuolar H^+ -ATPase, suggesting that it may play a role in the ion transport mechanism that facilitates acidification within endosomes (Jentsch et al. 2005). Endosomes are part of the receptor-mediated endocytic pathway that transports proteins such as albumin, and therefore a CIC-5 dysfunction could explain the LMWP in Dent's disease. It is noteworthy that CIC-5 knockout mice exhibit a phenotype similar to that of Dent's disease, with LMWP associated with impaired proximal tubule reabsorption of proteins (Jentsch et al. 2005). Another possible role that has been suggested for CIC-5 is mediating the protein–protein interactions that are involved in the assembly of the endocytic complex (Hryciw et al. 2003).

Different types of disease-causing mutations in the *CLCN5* gene have been reported (Lloyd et al. 1996; Hoopes et al. 1998; Morimoto et al. 1998; Yamamoto et al. 2000; Carballo-Trujillo et al. 2003; Claverie-Martin et al. 2003; Ludwig et al. 2005). Most of these mutations are single nucleotide substitutions that are scattered throughout the coding sequence of the gene. Point mutations are commonly assumed to cause single amino acids alterations or truncations in the encoded proteins. However, increasing evidence shows that mutations that affect pre-mRNA processing cause a considerable number of genetic diseases and that exonic mutations may also alter the splicing pattern of the pre-mRNA (Pagani and Baralle 2004). Non-sense, missense, and silent mutations, as well as coding single-nucleotide polymorphisms and even intronic variants can inactivate genes by inducing exon skipping (Cartegni et al. 2002). Many splicing mutations affect standard consensus splicing signals, but recent studies have shown that there are mutations that alter other elements needed for regulation of the splicing process, i.e., exonic and intronic splicing enhancers or silencers (Cartegni et al. 2002). Therefore, it has been suggested that, in order to determine the full consequences of a mutation in disease, each mutation should be functionally assessed for its potential effect on splicing (Pagani and Baralle 2004). Until now, only a few *CLCN5* mutations affecting pre-mRNA splicing have been described, and only in some cases has the effect been confirmed by mRNA analysis (Morimoto et al. 1998; Yamamoto et al. 2000; Claverie-Martin et al. 2003, 2005; Forino et al. 2004).

In this study, we analyzed the *CLCN5* gene in nine Spanish patients with Dent's disease. We identified

seven new mutations consisting of two nonsense mutations, three apparently missense mutations, one deletion and one splice-site mutation. Our results showed that one of the missense mutations also affects the pre-mRNA splicing pattern. In addition, we characterized two novel *CLCN5* mRNA splicing variants.

Subjects and methods

Patients

Nine unrelated patients diagnosed with Dent's disease, from different geographic areas of Spain (Table 1), and 21 of their relatives were investigated after giving informed consent. All patients had LMWP and hypercalciuria, four had nephrocalcinosis and/or nephrolithiasis, and two suffered from rickets/osteopenia. Venous blood samples were obtained from patients and from affected and unaffected family members for mutational analysis of the *CLCN5* gene. The protocol was approved by the Ethics Committees of all participating centers.

DNA sequence analysis

DNA was extracted from whole blood by using a DNA Blood Mini kit (Qiagen, Hilden, Germany), and all *CLCN5* coding exons and non-coding exons 1a and 1b, including their intronic flanking sequences, were amplified by PCR as described previously (Carballo-Trujillo et al. 2003; Ludwig et al. 2003). Additionally, the *OCRL1* gene was screened for mutations in the two patients without apparent mutations in the *CLCN5* gene using methods described elsewhere (Hoopes et al. 2005). Thermocycling was performed on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The amplified fragments were directly sequenced on both strands using a Big Dye Terminator kit v 3.1 Cycle Sequencing kit and run on an ABI PRISM 310

Genetic Analyzer (Applied Biosystems). The primers used for sequencing were the same primers used for the PCR amplification. DNA mutations were determined by comparison to reference sequences (GenBank accession numbers X91906 for *CLCN5* and AL138745 and AL022162 for the 5' and 3' ends of *OCRL1*, respectively), and confirmed by sequencing additional independent amplification products. In addition, in order to demonstrate that mutation C219R was not a common polymorphism in the general population, we designed a primer that annealed adjacent to the affected nucleotide, creating a restriction site for BsmAI only if the mutation was present (5'-GCCCTCTAGTGCACGTGTCT-3'), and amplified a 122 bp fragment with the reverse primer used previously for exon 6. A restriction fragment length polymorphism (RFLP) screen of 100 chromosomes from normal unrelated controls (20 males and 40 females) was performed following the manufacturer's recommendations (not shown).

Pre-mRNA splicing analysis

Total RNA was isolated from whole blood samples using the PAXgene Blood RNA System (Qiagen), digested with RNase-free DNase I (Clontech, Palo Alto, CA) and reverse transcribed with random hexamers using a First-strand cDNA synthesis kit (Roche, Basel, Switzerland). One microliter of cDNA was then used as a template for PCR amplifications to study the effect of the mutations on pre-mRNA splicing. Two segments of *CLCN5* cDNA, from exon 2 to exon 5 and from exon 8 to exon 12, respectively, were amplified using primers and conditions described previously (Claverie-Martin et al. 2005).

Alternative isoforms of *CLCN5* were studied by RT-PCR using total RNA isolated from whole blood of a healthy individual and poly-A mRNA (BD Biosciences Clontech, Palo Alto, CA) from normal human tissues (kidney, lung, pancreas, liver, brain, and skele-

Table 1 Biochemical data and mutations found in Spanish patients with Dent's disease. LMWP Low molecular weight proteinuria

Patient	LMWP (µg/l)	Hypercalciuria (mg/kg per day)	Nephrocalcinosis/nephrolithiasis	Rickets/osteopenia	<i>CLCN5</i> mutation	Consequence	Location
P140	98,000	9.7	+	–	942G > A	W314X	Toledo
P198	4,080	6	+	–	420T > G	Y140X	Asturias
P252	>50,000	12	+	–	976delG	Truncated protein	Murcia
P244	– ^a	6.4	–	–	655T > C	C219R	Barcelona
P374	46,835	7	–	+	817T > C	F273L	Murcia
P253	>50,000	10.8	+	+	1639T > G	W547G	Murcia
P242	42,000	5.9	–	–	IVS2-2A > G	Truncated protein	Madrid

^a LMWP data for P244 were available only as mg/day (1,846 mg/day)

tal muscle), and from mouse kidney. Poly-A mRNA (200 ng) was reverse transcribed as described above. Amplifications were carried out using previously described conditions (Claverie-Martin et al. 2005). To study the coding region in the splicing variant, a primer (5'-ATTCCAAGCAATCGCCTAAG-3') was designed to specifically detect skipping of exons 10 and 11. After testing the specificity of the primer, it was used along with primer CLCN5cDN 2F (5'-GAC-TTCTTGAGGAGCCAATC-3') (Claverie-Martin et al. 2005) to amplify the complete coding region (starting in exon 2) in all tissues. The program for this amplification was 94°C for 2 min, followed by 40 cycles each consisting of three steps, 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s, with a final extension at 72°C for 5 min. In all cases, bands were excised and purified for sequencing using a gel extraction kit (Qiagen). The primers used for sequencing were the same primers used for PCR amplification.

Results and discussion

Nine patients presenting with LMWP and hypercalciuria and their family members were analyzed for possible mutations in the *CLCN5* gene (Fig. 2; Table 1). The sequenced DNA revealed the presence of seven single nucleotide mutations not previously reported (Fig. 2; Table 1): one splicing mutation and six exonic variants, consisting of two nonsense mutations, three apparently missense mutations and one deletion (Table 1). Two patients presenting with symptoms resembling Dent's disease did not show any mutation in *CLCN5*, as has been observed by others (Morimoto et al. 1998; Hoopes et al. 2005). We also performed a

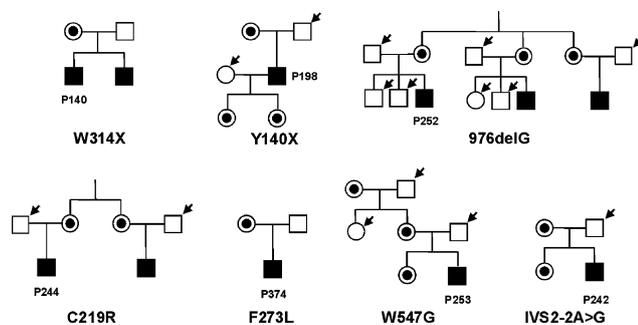
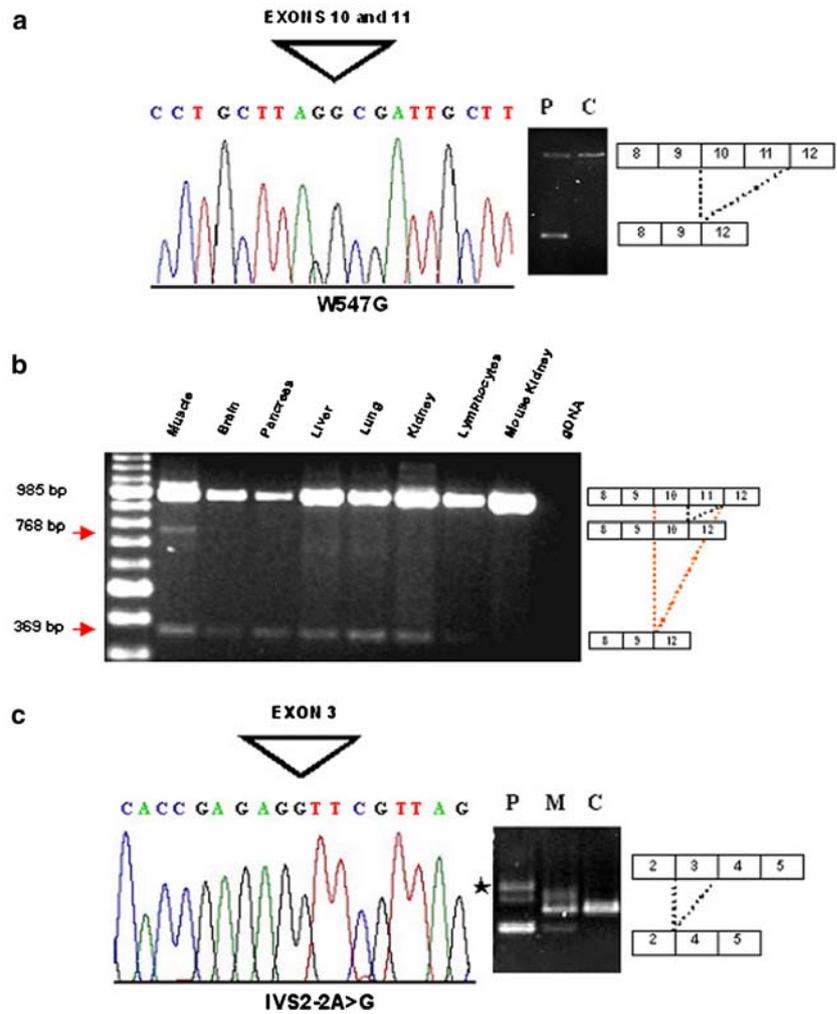


Fig. 2 Segregation of *CLCN5* mutations. Pedigrees of the respective Spanish families are shown with phenotypic findings in males (squares) and females (circles) shown as follows: filled circle female carrier, filled square affected male, open circle unaffected female, and open square unaffected male. Relatives not included in the DNA sequence analysis are indicated by black arrows

sequence analysis of the *OCRL1* gene in these two patients, but no mutations were found (not shown).

We identified a missense mutation, W547G, which is located at the beginning of exon 10 and is predicted to lead to the substitution of a tryptophan residue in helix Q (Fig. 1), which is involved in the formation of the interface between both CIC-5 subunits. Residue W547 is conserved throughout mammalian CIC-5 proteins but not in the CIC family (results not shown). There is increasing evidence that missense mutations also alter pre-mRNA splicing, and we are especially interested in characterizing this type of mutation in the *CLCN5* gene. Therefore, we examined the RNA from blood of this patient by RT-PCR analysis and found that, after amplifying from exon 8 to exon 12, two different splicing products were found: the expected 985-bp product from normal pre-mRNA processing together with a shorter product of 369 bp of similar intensity suggestive of an exon skipping event. Sequencing of the 369-bp fragment confirmed that it lacked exons 10 and 11, and contained exons 9 and 12 precisely spliced together (Fig. 3a). This leads to a change in the open reading frame, generating 11 new codons (DCLESLPKRMC) at the 3' end of residue 511, followed by a premature termination codon. However, mRNA molecules carrying premature stop codons are expected to be degraded through nonsense-mediated mRNA decay, resulting in decreased expression of the protein. The normal spliced mRNA would be translated into a mutant protein with a glycine instead of a tryptophan at position 547. Therefore, the phenotype of this patient would be due to the combined effect of decreased CIC-5 expression and the presence of a mutant CIC-5 protein. To determine if the spliced form lacking exons 10 and 11 was present in normal tissues, we PCR amplified cDNAs from human kidney, muscle, brain, pancreas, liver, lung, and lymphocytes. This experiment revealed that the alternative product was present in all human tissues examined, albeit in small amounts (Fig. 3b). This product was not detected in mouse kidney when we used either human primers (not shown) or mouse-specific primers (Fig. 3b). In addition, a 768-bp band corresponding to the skipping of exon 11 alone, was observed in human skeletal muscle (Fig. 3b). In an attempt to characterize the 5' coding part of this alternative *CLCN5* mRNA, we amplified the complete coding region spanning from exon 2 to the junction between exons 9 and 12, in all human tissues tested. RT-PCR analysis showed no evidence of additional splicing products other than the skipping of exons 10 and 11 (not shown). The loss of these two exons predicts a truncated protein without the transmembrane helices O, P and Q (P and Q are involved in

Fig. 3a–c RT-PCR analysis and DNA sequence of the cDNA products. **a** Effect of mutation W547G in *CLCN5* pre-mRNA splicing in the patient’s lymphocytes. Lanes *P* and *C* show the products from the patient and a normal control, respectively. **b** Expression pattern of *CLCN5* isoforms from different human tissues. Poly-A mRNA from mouse kidney and human genomic DNA, gDNA, were used as controls. **c** Effect of mutation IVS2-2A > G in *CLCN5* pre-mRNA splicing in the patient’s lymphocytes. Lanes *P*, *M* and *C* show the products from the patient, the patient’s mother and a normal control, respectively. Artifacts generated in the RT-PCR are indicated by a *star*



the formation of the interface between both subunits), the cytoplasmic helix R (a component of the chloride selectivity filter) and the carboxy-terminus of the ClC-5 protein.

Our results suggest that mutation W547G leads to an alteration in the expression levels of the alternative isoform lacking exons 10 and 11 (Fig. 3a). The mechanism by which exons 10 and 11 are skipped simultaneously is not known. It has been reported that some mutations alter cis-elements needed for correct splicing (Cartegni et al. 2002). We analyzed mutation W547G using ESE-finder (Cartegni et al. 2002) and found that it does not predict the disruption of any exonic splicing enhancer (ESE). However, ACESCAN2 (Yeo et al. 2005) analysis showed that the T to G substitution leads to the creation of four overlapping exonic splicing silencers (ESS) elements (AAGGGG, AGGGGG, GGGGGT, and GGGGTG) within the exon 10 sequence. ESSs are known to suppress the recognition of exons in splice site definition. Recent analysis of alternative splicing processes in *Drosophila* and humans

supports the hypothesis that splice-site recognition is more efficient across the intron, and that alternative splicing is less likely for exons flanked by short introns (Fox-Walsh et al. 2005). Notably, splice-site recognition across the intron results in enhanced inclusion of exons with weak splice sites. By using either GENESCAN (<http://www.genes.mit.edu/GENSCAN.html>) or the splice sites predictor (http://www.fruitfly.org/seq_tools/splice.html) we found that *CLCN5* exon 11 has a low matching score compared to the consensus acceptor site sequences (data not shown). This, together with the small size of intron 10 (155 bp) could explain the simultaneous skipping of exons 10 and 11 in the presence of the W547G mutation. The creation of the new ESS elements may decrease exon 10 incorporation into the mature mRNA, and the subsequent loss of cis elements that may reside within exon 10 could disable the splicing machinery for cross-intron recognition of exon 11, resulting in increased skipping of both exons. In summary, the missense mutation W547G can also alter the expression levels of a *CLCN5* mRNA splicing

variant. This type of mutation has not been previously described in the *CLCN5* gene. Previously, Yamamoto et al. (2000) reported a *CLCN5* missense mutation, D601V, which also results in the creation of a new donor splice site and leads to the skipping of part of exon 10.

Another two new missense mutations were found in this study: C219R and F273L. Mutation C219R, located on exon 6, affects CIC-5 helix F (Fig. 1), which is one of the helices involved in the formation of the chloride selectivity filter, and would lead to a change in the amino acid polarity. Ludwig et al. (2005) used functional studies in *Xenopus* oocytes to demonstrate that the C221R missense mutation, also located in helix F, not only interfered with protein function but also abolished CIC-5 trafficking to the plasma membrane (Ludwig et al. 2005). C219 is a conserved amino acid among mammalian CIC-5 homologues, but it is not located in the conserved domain GREGP, which is found at the amino terminus of helix F and is directly involved in anion binding (Estevez and Jentsch 2002), or in regions of high homology described for the CIC family (Dutzler et al. 2002). The third missense mutation, F273L, is located at the beginning of exon 8 and introduces a change in the loop between helix H and I (Fig. 1). Residue F273 is conserved between mammalian CIC-5 proteins and also in the CIC family. These changes in conserved amino acids probably affect essential properties of the protein.

In one of our patients, we identified a mutation located at the acceptor splice site of intron 2, IVS2-2A > G, which disrupted the highly conserved AG dinucleotide sequence. As expected, RT-PCR analysis of the patient's RNA showed an aberrant *CLCN5* pre-mRNA splicing product of 340 bp instead of the normal 440-bp product (Fig. 3c). Sequence analysis of the smaller fragment revealed skipping of the entire exon 3, which results in a change in the reading frame that introduces two new codons (VR) and a premature termination codon in exon 4. RNA from the patient's mother was also analyzed and showed both normal and aberrant products (Fig. 3c). Simultaneous expression of both splicing forms in the mother can be explained by the fact that the multigene domain in Xp11.21-p11.22, where *CLCN5* gene is located, has been shown to escape X inactivation (Miller and Willard 1998).

We also identified two new nonsense mutations: Y140X, which is located in exon 5 and predicts a disruption of helix D, one of the transmembrane domains, and W314X, found in exon 8, which introduces a disruption in the loop between helix I and J (Fig. 1). A novel single-base deletion, 976delG in codon 325 of exon 8, was also identified, resulting in a frame-shift

leading to the addition of 32 new amino acids (AYLVVCGEHCYSAQTLPGVGSERPPSWASILL) and the premature termination of translation at codon 358. These three mutations would be expected to inactivate channel function, either by truncation or by degradation through nonsense-mediated mRNA decay.

In conclusion, we identified seven new *CLCN5* mutations that cause structural alterations in the CIC-5 protein and are likely to result in a loss of function. Missense mutation W547G was shown to alter the pre-mRNA splicing pattern. In addition to expanding the spectrum of *CLCN5* mutations associated with this renal tubulopathy, our study reveals two new *CLCN5* splicing variants, and supports the importance of a routine analysis at the RNA level of mutations that are commonly assumed to cause single amino acids alterations. The ideal source of RNA to study the effect of mutations on *CLCN5* pre-mRNA splicing would be kidney biopsies from patients. However, as this source is not always available, one can use RNA obtained from lymphocytes, which has been shown to be valid for this type of study (Forino et al. 2004; Claverie-Martin et al. 2005).

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