Phenotypic and genetic heterogeneity in Dent’s disease—the results of an Italian collaborative study

Enrica Tosetto, Gian Marco Ghiggeri, Francesco Emma, Giancarlo Barbano, Alba Carrea, Giuseppe Vezzoli, Rossella Torregrossa, Marilena Cara, Gabriele Ripanti, Anita Ammenti, Licia Peruzzi, Luisa Murer, Ilse Maria Ratsch, Lorenzo Citron, Giovanni Gambaro, Angela D’angelo and Franca Anglani

1Division of Nephrology, Department of Medical and Surgical Sciences, University of Padua, Padua, 2Laboratory of Pathophysiology of Uremia, Pediatric Institute G. Gaslini, Genoa, 3Division of Nephrology and Dialysis, Pediatric Hospital Bambin Gesù, Rome, 4Division of Nephrology, Dialysis and Kidney Transplantation, Pediatric Institute G. Gaslini, Genoa, 5Division of Nephrology, Dialysis and Hypertension, IRCCS San Raffaele Hospital, Milan, 6Division of Nephrology, Camposampiero General Hospital, Camposampiero, 7Division of Pediatrics and Neonatology, San Salvatore Hospital, Pesaro, 8Pediatric Institute, University of Parma, Parma, 9Division of Nephrology, Dialysis and Transplantation, Regina Margherita Hospital, Turin, 10Department of Pediatrics, University of Padua, Padua, 11Pediatric Institute, University of Ancona, Ancona and 12Division of Nephrology, Department of Biomedical and Surgical Sciences, University of Verona, Verona, Italy

Abstract

Background. Dent’s disease is an inherited tubulopathy caused by CLCN5 gene mutations. While a typical phenotype characterized by low-molecular-weight (LMW) proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, rickets and progressive renal failure in various combinations often enables a clinical diagnosis, less severe sub-clinical cases may go undiagnosed.

Methods. By single-strand conformation polymorphism analysis and direct sequencing, we screened 40 male patients from 40 unrelated families for CLCN5 gene mutations. Twenty-four of these patients had the prominent features of Dent’s disease, including LMW proteinuria, hypercalciuria and nephrocalcinosis.

Results. We identified 24 mutations in the CLCN5 gene in 21/24 patients with a typical phenotype and in 3/16 patients with a partial clinical picture of Dent’s disease. Overall, 10 novel CLCN5 mutations were identified (E6fsX11, W58fsX97, 267 del E, Y272C, N340K, F444fsX448, W547X, Q600X, IVS3+2G>C and IVS3/C1G>A), extending the number of mutations identified so far from 75 to 85. The CLCN5 coding sequence was normal in three patients. In the group with an incomplete Dent’s disease phenotype, we detected two intronic mutations and one silent substitution leading to the up regulation of an alternatively spliced isoform.

Conclusions. Our data confirm the genetic heterogeneity of Dent’s disease. In most classic cases, the clinical diagnosis is confirmed by genetic tests.

Keywords: CLCN5 gene mutations; Dent’s disease; exonic splicing enhancer; genotype-phenotype correlation; splicing mutation

Introduction

The term Dent’s disease (OMIM 300009), first introduced in 1990 [1], identifies a group of X-linked renal disorders characterized by low-molecular-weight (LMW) proteinuria, hypercalciuria and nephrocalcinosis and/or nephrolithiasis. This triad of manifestations has been variably named in the past as X-linked recessive nephrolithiasis with renal failure, X-linked recessive hypophosphataemic rickets or idiopathic LMW proteinuria of Japanese children [2,3]. The disease usually presents in childhood or early adult life. Due to X chromosome inactivation, carrier females may present some of the manifestations of Dent’s disease [4].

Progression to end-stage renal failure occurs between the 3rd and 5th decades of life in 30–80% of affected males [5]. Early diagnosis may enable the said progression to chronic renal failure to be prevented, but the clinical diagnosis of Dent’s disease is often difficult since patients may have only mild clinical and
biochemical signs and the X-linked inheritance is not always obvious [6]. Dent's disease may consequently go under-diagnosed.

After the early descriptions, it has since been recognized that Dent's disease is caused by mutations in the CLCN5 gene [2,5], which is located on the short arm of the X chromosome (Xp11.22). The CLCN5 gene encodes for the 746-amino-acid CIC-5 chloride channel that belongs to the voltage-gated chloride channel family (CIC1-7, CIC-Ka and CIC-Kb) implicated in membrane excitability, transepithelial transport and possibly the regulation of cell volume [7].

In the human kidney, CIC-5 is primarily expressed in proximal tubular cells, in cortical collecting duct intercalated cells and also in the thick ascending limb of Henle's loop. In proximal cells, it is predominantly located in the intracellular subapical endosomes, which are involved in the endocytotic reabsorption of LMW proteins that have passed the glomerular filter. Specifically, CIC-5 was first proposed to provide a shunt conductance in early endosomes permitting an efficient intraluminal acidification by V-type H+/ATPase [8]. Recently, however, two distinct teams have simultaneously demonstrated that CIC-4 and CIC-5 may function as a Cl⁻/H⁺ antiporter when activated by positive voltages [9,10]. This finding renders the interpretation of the physiological role of CIC-5 more complicated [9]. Most likely, a combined defect in megalin and cubilin trafficking to and retrieval from the plasma membrane due to CIC-5 loss, impairs the receptor-mediated endocytosis [8,11]. Like humans, knock-out mouse models have LMW proteinuria, but not all of them develop hypercalciuria and nephrocalcinosis [8,12]. So far, the relationship between impaired endocytosis and renal calcium handling remains unclear [13,14]. It has been suggested that hypercalciuria stems from secondary changes in the regulation of calciotropic hormone caused by urinary loss of key hormone-binding proteins.

To date, more than 70 different nonsense or missense mutations, insertions or deletions in the CLCN5 gene have been reported in the literature meaning that the spectrum of CLCN5 mutations is highly varied [2,3,5,15–25]. Similarly to other X-linked disorders, the number of de novo mutations should be high. An estimate of the de novo mutations in the CLCN5 gene on the basis of the literature data indicates that about 8% (six out of 73 cases whose information about hereditability were available) arise de novo.

Genotype-phenotype correlations have yet to be established [2,26,27]. CLCN5 mutations are scattered throughout the gene's coding sequence and generate truncated or absent CIC-5 in ~70% of cases [20]. In addition, CLCN5 mutations have also been associated with splice sites' disruption, as well as trafficking defects and/or Cl⁻/H⁺ antiporter dysfunction [2,3,24].

Thirty to 40% of the patients with classic symptoms of Dent's disease reveal no CLCN5 gene mutations, suggesting a genetic heterogeneity [16,24,28]. Recently, in a subgroup of patients with the Dent's phenotype, carrying no CLCN5 mutations, Hoopes et al. [29] found mutations in the gene for the Lowe's syndrome, OCRL1, located on chromosome Xq26. Dent's disease caused by mutations in OCRL1 gene is now referred to as Dent's disease 2 (OMIM 300555). Unlike patients with typical Lowe's syndrome, mental retardation was absent or mild, and none of the patients had metabolic acidosis or ocular abnormalities on slit-lamp examination.

The present study is the result of a collaborative Italian investigation. Forty male patients from 12 paediatric and/or nephrology centres with all or some of the symptoms of Dent's disease were screened for CLCN5 mutations.

Subjects and methods

Patients

Dent's disease diagnosis was considered because of the presence, according to Hoopes et al. [28], of the following manifestations:

(i) LMW proteinuria (an at least 5-fold increase in urinary β₂-microglobulin, expressed as β₂-microglobulin excretion in 24-h or creatinine/β₂-microglobulin ratio);
(ii) hypercalciuria (>4 mg/kg in a 24 h urine collection, or >0.25 mg calcium per mg of creatinine in a spot specimen);
(iii) at least one of the following: nephrocalcinosis, kidney stones, hypophosphataemia, renal failure, amino-aciduria, rickets or a positive family history.

The diagnosis was considered likely if a patient met all the three criteria; it was considered possible if only two criteria were fulfilled. To be screened in the study, patients had to meet at least two of the aforementioned criteria. Thus, patients were divided into two groups according to the clinical data available at the time of their initial clinical evaluation, before the mutational analysis became available: 24 patients in group A had the classic Dent's disease phenotype and met all the three aforementioned criteria, while 16 patients in group B met only two of the three criteria. In both groups, no patients had clinical evidence for other possible causes of proximal tubular dysfunction, hypercalciuria or renal failure. None had clinical features of the Lowe's syndrome. All patients, except two (one from Cape Verde Island and another from Colombia), were of Italian origin. Blood samples were taken from all subjects, after their informed consent, and were used for CLCN5 gene mutation analysis and expression studies.

CLCN5 gene mutation analysis

The patients' leucocyte genomic DNA was extracted by NucleoSpin Blood Quick Pure minicolumns (Macherey-Nagel GmbH & Co. KG, Duren, Germany). Thirteen CLCN5-specific pairs of oligonucleotide primers were used for polymerase chain reaction (PCR) amplification of the coding region (exons 2–12) and exon–intron boundaries. Primers and PCR conditions were as reported by Lloyd et al. [15].
Three CLCN5 pairs of primers were used for PCR amplification of the 5′ untranslated region exons i.e. exon 1A [30], exon 1B [31] and 1B1 [23]. Primer sequences, MgCl₂ and annealing temperature are given in Table 1.

The PCR products were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) on 7% polyacrylamide gels. Bands were visualized using the standard silver staining method [23]. Single-strand conformation polymorphism was used to detect DNA mutations, as described elsewhere [23]. Direct automated PCR product sequencing was done using the ABI PRISM GENESCAN 373 A DNA sequencer and the BigDye Terminator v1.1 Cycle Sequencing Kit (PE Applied Biosystem, Foster City, CA 94404 USA). All DNA sequence abnormalities observed were confirmed by independent PCR reactions and DNA samples from unrelated normal subjects, for a total of 188 X chromosomes, were used as controls. The nomenclature of the mutations is based on the CIC-5 mRNA sequence NM_000084.

### CICN5 transcript analysis

Total RNA was extracted from the patients’ leucocytes using the QIAmp RNA Blood Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

One μl of RNA was used for spectrophotometric quantification at 260 and 280 nm using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA integrity was checked with the Agilent 2100 bioanalyzer (Agilent Technologies, Deutschland GmbH, Waldbronn). The Automated Splice Site Analysis website. The quantitative comparative RT/PCR was used to obtain quantitative data for the normal transcript [23].

Terminator v1.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA 94404 USA). All DNA sequence numbers are given in Table 2. PCR products were analysed from the natural acceptor intron 9 splice site), as identified by the Automated Splice Site Analysis website. The quantitative comparative RT/PCR was used to obtain quantitative data for the normal transcript [23]. Primer sequences and PCR cycle numbers are given in Table 2. PCR products were analysed and quantified using the Agilent bioanalyzer technology. Control negative reactions were performed without reverse transcriptase during the cDNA synthesis step to rule out any genomic contamination. The amplification profile was the same for each primer set and consisted of an initial denaturation at 95°C for 5 min, followed by 35 amplification cycles (45s at 94°C, 45s at 60°C, 1 min at 72°C), and an extension at 72°C for 7 min. The PCR products were gel-purified, and the DNA sequences of both strands were determined.

### Results

Twenty-four CLCN5 mutations were detected, 21 in patients in group A (detection rate 87%) (Table 3 and Figure 1) and 3 in patients in group B (detection rate 0%).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide composition</th>
<th>Expected size of PCR product (bp)</th>
<th>MgCl₂ (mM)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENT 1aF</td>
<td>5′-CTGGTCAGCTGACTCACAAGT-3′</td>
<td>264</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>DENT 1aR</td>
<td>5′-ATGCTTCCAGTCCCTTGGTCAGG-3′</td>
<td>376</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>DENT 1bF</td>
<td>5′-AGGGGACAGTGGTGTAGTTTCTC-3′</td>
<td>359</td>
<td>1.5</td>
<td>60</td>
</tr>
<tr>
<td>DENT 1bR</td>
<td>5′-TTTTTTCCTCCTCCTTCAAATATGTA-3′</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENT 1.2B</td>
<td>5′-TACGACTTTCTGCTATTTAAG-3′</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
rate 19%) (Table 4 and Figure 1). Analysing the CLCN5 coding and non-coding sequences (including intron–exon boundaries and the 5’ UTR exons) failed to identify any causative mutations in the three patients with the classic Dent’s phenotype and in 13 patients with the incomplete Dent’s phenotype.

LMW proteinuria and hypercalciuria were present in all of the 24 patients of group A, whereas nephrocalcinosis, familiarity, rickets or osteomalacia, aminoaciduria, nephrolithiasis and hypophosphataemia were present in 82.6, 56.5, 48, 28.5, 26 and 22% of the patients, respectively (Table 5). Renal failure occurred in six patients (26%). In four of them carrying CLCN5 mutations, decreased creatinine clearance was evident by late childhood.

For 16 out of 24 mutated patients of both group A and B, we had access to urine and/or blood samples (5 and 12, respectively) of probands’ mothers. In 11 of them, the state of heterozygote was established by mutation analysis, and in three, it was suspected because of the presence of LMW proteinuria in urine. Two mothers did not carry CLCN5 mutations (de novo mutations), and in two heterozygous mothers LMW proteinuria was absent.

Eleven mutations had already been reported, while 10 were new (Figure 1). The phenotypic characteristics of patients carrying the novel mutations are reported in Table 6.

All novel mutations were identified in patients in group A and included: two missense mutations (Y272C and N340K), one in-frame deletion (267 del E), one donor splice-site mutation (IVS3 +2G > C), one acceptor splice-site mutation (IVS3/C0 1G > A) and two nonsense mutations (W547X and Q600X) that create premature stop codons resulting in a ClC-5 protein lacking 199 (26.7%) or 146 (19.5%) amino acids of the carboxy terminus domain, respectively.

We further identified three novel frameshift mutations (E6fsX11, W58fsX97 and F444fsX448) that cause a shift in the protein-reading frame and introduce a premature termination of translation at codons 11, 97 and 448, respectively. These mutations are predicted to induce a truncated ClC-5 protein lacking 735 (98.5%), 649 (87%) and 298 (40%) amino acids, respectively, which are likely to result in a loss of function.

For 16 out of 24 mutated patients of both group A and B, we had access to urine and/or blood samples (5 and 12, respectively) of probands’ mothers. In 11 of them, the state of heterozygote was established by mutation analysis, and in three, it was suspected because of the presence of LMW proteinuria in urine. Two mothers did not carry CLCN5 mutations (de novo mutations), and in two heterozygous mothers LMW proteinuria was absent.

Eleven mutations had already been reported, while 10 were new (Figure 1). The phenotypic characteristics of patients carrying the novel mutations are reported in Table 6.

All novel mutations were identified in patients in group A and included: two missense mutations (Y272C and N340K), one in-frame deletion (267 del E), one donor splice-site mutation (IVS3 +2G > C), one acceptor splice-site mutation (IVS3/C0 1G > A) and two nonsense mutations (W547X and Q600X) that create premature stop codons resulting in a ClC-5 protein lacking 199 (26.7%) or 146 (19.5%) amino acids of the carboxy terminus domain, respectively.

We further identified three novel frameshift mutations (E6fsX11, W58fsX97 and F444fsX448) that cause a shift in the protein-reading frame and introduce a premature termination of translation at codons 11, 97 and 448, respectively. These mutations are predicted to induce a truncated ClC-5 protein lacking 735 (98.5%), 649 (87%) and 298 (40%) amino acids, respectively, which are likely to result in a loss of function.

For 16 out of 24 mutated patients of both group A and B, we had access to urine and/or blood samples (5 and 12, respectively) of probands’ mothers. In 11 of them, the state of heterozygote was established by mutation analysis, and in three, it was suspected because of the presence of LMW proteinuria in urine. Two mothers did not carry CLCN5 mutations (de novo mutations), and in two heterozygous mothers LMW proteinuria was absent.

Eleven mutations had already been reported, while 10 were new (Figure 1). The phenotypic characteristics of patients carrying the novel mutations are reported in Table 6.

All novel mutations were identified in patients in group A and included: two missense mutations (Y272C and N340K), one in-frame deletion (267 del E), one donor splice-site mutation (IVS3 +2G > C), one acceptor splice-site mutation (IVS3/C0 1G > A) and two nonsense mutations (W547X and Q600X) that create premature stop codons resulting in a ClC-5 protein lacking 199 (26.7%) or 146 (19.5%) amino acids of the carboxy terminus domain, respectively.

We further identified three novel frameshift mutations (E6fsX11, W58fsX97 and F444fsX448) that cause a shift in the protein-reading frame and introduce a premature termination of translation at codons 11, 97 and 448, respectively. These mutations are predicted to induce a truncated ClC-5 protein lacking 735 (98.5%), 649 (87%) and 298 (40%) amino acids, respectively, which are likely to result in a loss of function.

For 16 out of 24 mutated patients of both group A and B, we had access to urine and/or blood samples (5 and 12, respectively) of probands’ mothers. In 11 of them, the state of heterozygote was established by mutation analysis, and in three, it was suspected because of the presence of LMW proteinuria in urine. Two mothers did not carry CLCN5 mutations (de novo mutations), and in two heterozygous mothers LMW proteinuria was absent.

Eleven mutations had already been reported, while 10 were new (Figure 1). The phenotypic characteristics of patients carrying the novel mutations are reported in Table 6.

All novel mutations were identified in patients in group A and included: two missense mutations (Y272C and N340K), one in-frame deletion (267 del E), one donor splice-site mutation (IVS3 +2G > C), one acceptor splice-site mutation (IVS3/C0 1G > A) and two nonsense mutations (W547X and Q600X) that create premature stop codons resulting in a ClC-5 protein lacking 199 (26.7%) or 146 (19.5%) amino acids of the carboxy terminus domain, respectively.

We further identified three novel frameshift mutations (E6fsX11, W58fsX97 and F444fsX448) that cause a shift in the protein-reading frame and introduce a premature termination of translation at codons 11, 97 and 448, respectively. These mutations are predicted to induce a truncated ClC-5 protein lacking 735 (98.5%), 649 (87%) and 298 (40%) amino acids, respectively, which are likely to result in a loss of function.

For 16 out of 24 mutated patients of both group A and B, we had access to urine and/or blood samples (5 and 12, respectively) of probands’ mothers. In 11 of them, the state of heterozygote was established by mutation analysis, and in three, it was suspected because of the presence of LMW proteinuria in urine. Two mothers did not carry CLCN5 mutations (de novo mutations), and in two heterozygous mothers LMW proteinuria was absent.

Eleven mutations had already been reported, while 10 were new (Figure 1). The phenotypic characteristics of patients carrying the novel mutations are reported in Table 6.

All novel mutations were identified in patients in group A and included: two missense mutations (Y272C and N340K), one in-frame deletion (267 del E), one donor splice-site mutation (IVS3 +2G > C), one acceptor splice-site mutation (IVS3/C0 1G > A) and two nonsense mutations (W547X and Q600X) that create premature stop codons resulting in a ClC-5 protein lacking 199 (26.7%) or 146 (19.5%) amino acids of the carboxy terminus domain, respectively.

We further identified three novel frameshift mutations (E6fsX11, W58fsX97 and F444fsX448) that cause a shift in the protein-reading frame and introduce a premature termination of translation at codons 11, 97 and 448, respectively. These mutations are predicted to induce a truncated ClC-5 protein lacking 735 (98.5%), 649 (87%) and 298 (40%) amino acids, respectively, which are likely to result in a loss of function.

For 16 out of 24 mutated patients of both group A and B, we had access to urine and/or blood samples (5 and 12, respectively) of probands’ mothers. In 11 of them, the state of heterozygote was established by mutation analysis, and in three, it was suspected because of the presence of LMW proteinuria in urine. Two mothers did not carry CLCN5 mutations (de novo mutations), and in two heterozygous mothers LMW proteinuria was absent.

Eleven mutations had already been reported, while 10 were new (Figure 1). The phenotypic characteristics of patients carrying the novel mutations are reported in Table 6.
in dimer interface formation. The Y272C missense mutation is located to the centre of the loop connecting the helices H and I, which is completely conserved among human ClC-3/4/5 chloride/proton antiporters. The N340K missense mutation is located in the α-helix J which is also highly conserved in other ClC members (>80% identity between ClC-3, ClC-4 and ClC-5) (Figure 1). Furthermore, the affected amino acids are completely conserved among different species or within the ClC-3/4/5 branch of human chloride/proton antiporters.

The two intronic mutations IVS3 +2 G>C and IVS3 −1 G>A affect the splicing donor and acceptor

---

**Table 4.** CLCN5 mutations found in 16 incomplete Dent’s disease phenotype (group B)

<table>
<thead>
<tr>
<th>Family</th>
<th>Exon</th>
<th>Nucleotide substitution</th>
<th>Aminoacidic substitution</th>
<th>Protein localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silent mutation</td>
<td>10</td>
<td>1995 C&gt;T</td>
<td>P568P</td>
<td>α-helix R</td>
</tr>
<tr>
<td>Acceptor splice-site mutation</td>
<td>Intron 2</td>
<td>IVS2 −17T&gt;G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor splice-site mutation</td>
<td>Intron 5</td>
<td>IVS5 +5G&gt;T</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.** Clinical features in patients with the classical Dent’s disease (group A) according to the presence or not of CLCN5 mutations

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>With mutations</th>
<th>Without mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>m 15 range 5–29</td>
<td>m 43.6 range 13–60</td>
</tr>
<tr>
<td>LMW proteinuria</td>
<td>21/21</td>
<td>3/3</td>
</tr>
<tr>
<td>Hypercalciuria</td>
<td>21/21</td>
<td>3/3</td>
</tr>
<tr>
<td>Nephrocalcinosis</td>
<td>19/20</td>
<td>0/3</td>
</tr>
<tr>
<td>Nephrolithiasis</td>
<td>4/20</td>
<td>2/3</td>
</tr>
<tr>
<td>Hypophosphataemia</td>
<td>3/20</td>
<td>2/3</td>
</tr>
<tr>
<td>Renal failure</td>
<td>4/20</td>
<td>2/3</td>
</tr>
<tr>
<td>Aminoaciduria</td>
<td>3/18</td>
<td>3/3</td>
</tr>
<tr>
<td>Rickets</td>
<td>9/20</td>
<td>2/3</td>
</tr>
<tr>
<td>Familiarity</td>
<td>10/20</td>
<td>3/3</td>
</tr>
<tr>
<td>Patient</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>---------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca (mmol/l)</td>
<td>2.45</td>
<td>2.6</td>
</tr>
<tr>
<td>P (mmol/l)</td>
<td>0.65</td>
<td>1.35</td>
</tr>
<tr>
<td>% TRP (%)</td>
<td>&lt;25</td>
<td>NA</td>
</tr>
<tr>
<td>PTH (ng/l)</td>
<td>14</td>
<td>75</td>
</tr>
<tr>
<td>1.25(OH)2 vitamin D (pmol/l)</td>
<td>NA</td>
<td>250</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO3⁻ (mmol/l)</td>
<td>26.5</td>
<td>22.3</td>
</tr>
<tr>
<td>β₂-microglobulin (µg/24h)</td>
<td>11000</td>
<td>47547</td>
</tr>
<tr>
<td>Ca/Cr (mg/mg)</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
<td>Ca (mg/kg/24h)</td>
<td>10.4</td>
<td>5.0</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>2200</td>
<td>1160</td>
</tr>
<tr>
<td>Aminoaciduria</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucosuria</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uricosuria (F. Cl. %; mg/24h)</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>Ccr (mL/min/1.73 m²)</td>
<td>34</td>
<td>140</td>
</tr>
<tr>
<td>Nephrocalcinosis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nephrolithiasis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rickets</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>Familiarity</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mutation</td>
<td>E66fsX11</td>
<td>W58fsX97</td>
</tr>
</tbody>
</table>

Ca, calcium; P, phosphate; TRP, tubular reabsorption of phosphate; PTH, parathyroid hormone; HCO₃⁻, bicarbonate; Cr, urine creatinine; F. Cl., fractional clearance; Ccr, creatinine clearance; M, adult male; +, present; −, absent; NA, not available.

*The normal range in children is variable with the age.
sites of intron 3, respectively. The Automated Splice Site Analysis website predicted that the IVS3 +2 G>C splice-site variant would affect the strength of the donor splice site (initial information content \( R_i \) 7.9 bits; final \( R_i \) 0.4 bits), which has lower \( R_i \) values more than the corresponding natural site. The program also predicted that the IVS3 -1 G>A mutation alters the strength of the acceptor splice site (initial \( R_i \) 8.0 bits; final \( R_i \) 0.4 bits). Lower \( R_i \) values indicate that these sites are either not recognized or they are bound with a lower affinity. The IVS3 +2 G>C mutation is estimated to prevent a correct mRNA splicing by abolishing the donor splice site, whereas the IVS3 -1 G>A mutation abolishes the acceptor splice site (according to the Automated Splice Site Analysis website). In both cases, exon skipping is expected: in the first case exon 3 skipping, in the second exon 4.

True to the prediction, RT/PCR analysis of the mRNA from the patient carrying the IVS3 +2 G>C donor splice-site mutation revealed an aberrantly processed mRNA; the mutation caused exon 3 (100 nt) to be skipped, with a loss of 33 amino acids and a consequent shift in the protein-reading frame that introduces a premature stop codon at codon 71, thus inducing a 90.5% amino acid loss (Figure 2A and B).

Since the RNA from the patient carrying the IVS3 -1 G>A mutation was not available for mRNA analysis, we were unable to demonstrate the effect of this mutation on CLCN5 transcript. According to the results of the Automated Splice Site Analysis website, we hypothesize that this mutation could cause exon 4 skipping.

Two intronic (IVS2 -17 T>G and IVS5 +5 G>T) and one silent mutation (P568P) were identified among patients in group B.

Here again, the Automated Splice Site Analysis website was used to predict the effects of these mutations on mRNA splicing. The patient carrying the IVS2-17 T>G mutation has already been described in one of our previous article [23]. Briefly, the patient was male aged 26, suffering from familial nephrolithiasis, who had no tubular proteinuria or nephrocalcinosis, but did have mild hypercalciuria and renal failure. The mutation was a de novo mutation since it was not inherited from his mother, but it did not affect the correct splicing of ClC-5 mRNA at least in leucocytes.

Fig. 2. (A) RT/PCR analysis of ClC-5 mRNA in leucocytes of the patient carrying the IVS3 +2 G>C mutation (lane 1) and of two normal controls (lanes 2 and 3). RT/PCR analysis of ClC-5 mRNA in leucocytes of the patient carrying the IVS5 +5 G>T mutation (lane 5), his brother and mother (lanes 6 and 7) and of two normal controls (lanes 8 and 9). Lane 4, control negative reactions. Gel-like image obtained after a chip run with the Agilent 2100 bioanalyzer. Molecular weight marker (lane L). (B) Schematic representation of the effects of the IVS3 +2 G>C and (C) IVS5 +5 G>T mutations on ClC-5 mRNA.
The IVS5 +5 G>T mutation was detected in a 14-year-old boy with a partial phenotype (with LMW proteinuria and nephrocalcinosis, but no hypercalciuria). The mutation was inherited from his mother and was also documented in the proband’s brother, who was 8 years old when tested and had LMW proteinuria and hypercalciuria. According to the Automated Splice Site Analysis website prediction, this functionally relevant donor splice-site mutation induces the skipping of exon 5 (123 nt) with an in-frame loss of 41 amino acids (Figure 2A and C).

The silent P568P mutation, corresponding to a C→T transition in nucleotide 1995 (1995 C>T), was found in one patient and in his brother. They were two children (10 and 11 years old), from the Islands of Cape Verde (Africa), with LMW proteinuria and border-line hypercalciuria but no nephrocalcinosis. No additional CLCN5 mutation was found. Since silent mutations can affect exonic splice site regulatory regions (ESE: exonic splicing enhancer) [33], we thought that this P568P mutation would be located in one ESE sequence. Using the ESE finder and RESCUE-ESE websites, we found that the silent mutation does not directly affect an ESE motif of CLCN5 exon 10, rather it strengthens a neighbouring cryptic splice site located in exon 10, 180 bp downstream the natural acceptor splice site of intron 9. The natural site (R, 11.4 bits) is 5.7 bits stronger than the strengthened cryptic site, which corresponds to at least a 2AR, or 52-fold, difference in affinity. These calculations indicate that the exon using this cryptic splice site would not account for more than 2% of the total mRNA. To detect any alternatively spliced transcripts, we used a reverse boundary-spanning primer encompassing CLCN5 exons 9–10 at the cryptic site level. Semi-nested PCR was used because the amount of alternatively spliced transcript was expected to be much lower than the normal transcript. We succeeded in detecting the alternative transcript (expected size of the PCR product: 456 bp) in the mRNA pool extracted from the leucocytes of the two brothers carrying the silent mutation, but the transcript was also detected in normal individuals (Figure 3C). As expected, DNA sequence analysis of the PCR product revealed a deletion of 180 bp from the nucleotide 1826 to nucleotide 2005 that coincides with the in-frame loss of 59 CIC-5 amino acids (codons 513–571) encompassing α-helices P, Q and R. Quantitatively measuring the minor transcript expression in the leucocytes of normal males (n = 4), normal females (n = 3) and mutated patients (n = 2) showed that the alternatively spliced transcript is 1.5 times more abundant in mutated patients than in normal males (Figure 3E), thus confirming the Automated Splice Site Analysis website prediction (of a 1.6-fold increase). Real-Time PCR was not used, because of the difficulty to find out suitable primer pairs for real-time analysis with SYBR Green I (the reverse primer must be specific for the cryptic site) in such a short sequence. We believe that such a quantification, though not as precise as the one obtained with real-time PCR, is anyway adequate to reveal the different levels of transcripts in nested PCR. Indeed, in a normal RT/PCR reaction, the aberrantly spliced mRNA was visible carrying the PCR reaction to 42 cycles. However, because of the very low abundance of the isoform, the specific PCR product was not always amplifiable in control male samples. On the contrary, in the two patients carrying the P568P mutation, the specific RT/PCR product was always obtained, confirming indirectly the higher abundance of this isoform in these patients with respect to controls. The low number of patients and controls, however, did not allow any definitive answer. Furthermore, we demonstrated that the mRNA levels of the normal transcript are similar in both control males and females and in mutated patients (Figure 3B and D).

Discussion

The present study identified 24 CLCN5 gene mutations in a heterogeneous group of patients with a classic (n = 24) or incomplete (n = 16) Dent’s disease phenotype. As expected, the majority of mutations (21/24) were identified in the first group of patients. Only three mutations occurred in the second group.

Of the total, 54.2% of the mutations are mutations that introduce in the CIC-5 protein, premature stop codons (37% nonsense and 16.7% frameshift), followed by missense (21%) and deletion mutations (12.5%).

No clear correlation between the type of mutations and the severity of the phenotype was observed. Scheinman et al. [27] and Norden et al. [26] had presented data supporting the lack of genotype-phenotype correlation, and Scheinman and Thakker [34] more recently summed up the prevailing experience by stating that there is ‘no correlation between the nature of these mutations and the phenotypic severity of the disease, or with the presence or absence of variable features such as rickets’. In essence we confirm this conclusion, with a possible exception that, due to the low number of patients, need to be confirmed by others. We found few hypophosphataemic patients, who were all affected by rickets as contrary to Hoopes et al. [28] whose hypophosphataemic patients were not always affected by rickets. This association was not unexpected due to the fact that phosphate is an important constituent of bone and that a number of inherited forms of hypophosphataemic rickets and osteomalacia have been described. Approximately 48% of our group A patients had rickets which does not correlate with any particular mutations, except for the mutation S244L. Similarly to what was observed in two European families, the mutation S244L, found in two unrelated patients, resulted to be associated with hypophosphataemic rickets [35,36]. This seems to indicate that a genetic and/or environmental background could contribute to the onset of this particular phenotype in Dent’s patients carrying S244L mutation.
The 10 newly identified mutations extend the molecular spectrum of Dent’s disease to 85 CLCN5 gene molecular defects.

Their functional significance appears clear for the two nonsense mutations and the three frameshift mutations, since they give rise to severely truncated ClC-5 proteins. Although the Q600X mutation only induces the loss of 199 amino acids, it is located in the long cytoplasmatic carboxy-terminus domain, which contains two cystothionine tetra synthase (CBS) domains needed for accurate intracellular targeting and protein trafficking [37,38]. Heterologous nonsense expression of mutations located in the CBS1 and CBS2 domains in *Xenopus* oocytes was found to abolish Cl−/H+ conductance [37]. These data suggest that the Q600X nonsense mutation of the CBS1 domain inactivates the Cl−/H+ antiporter in much the same way as the previously identified mutations.

Two intronic mutations affecting the canonical consensus splicing donor and acceptor sites of intron 3 were detected in two patients with the classic signs of Dent’s disease indicating that they are disease-causing mutations. In one patient, the presence of an aberrantly spliced ClC-5 mRNA in leucocytes, leading to a truncated or absent protein, confirmed the functional significance of the mutation. As previously reported by others [39] and our group [23], in leucocytes the level of expression of ClC-5 mRNA was similar to that in kidney. Furthermore, ClC-5 5′ UTR isoforms have been found to be common to both tissues [23]. For this reason, leucocytes may represent a good model for ClC-5 mRNA expression studies. The fact that an aberrantly spliced mRNA has been detected in leucocytes as a consequence of splice-site mutations, accordingly to the Automated Splice Site Analysis prediction, confirms the worthiness of the model.

The functional significance of the two novel missense N340K and Y272C mutations appears less clear-cut. The Y272C mutations are predicted to produce a rather conservative substitution whereas the N340K mutation results in the replacement of an uncharged

---

**Fig. 3.** (A–C) Gel-like image obtained after chip runs with the Agilent 2100 bioanalyzer of ClC-5 RT/PCR products from leucocyte mRNA of three normal females (lanes 1–3), 4 normal males (lanes 4–7), and of two patients carrying P568P mutation (lanes 8 and 9). L. molecular weight marker; UM upper marker; LM lower marker. (A) Glyceraldehyde-3-phosphate dehydrogenase as internal standard; (B) normal spliced transcript; (C) alternatively spliced transcript. Quantitative data generated by the Agilent 2100 bioanalyzer of normal (D) and alternatively spliced transcripts (E).
Heterogeneity in Dent’s disease in Italy

Among 16 patients with a partial phenotype (group B), affecting non-canonical splicing regulatory elements, some had classic Dent’s disease, indicative of a functional impairment of the CIC-5, and one of these mutations (N340K) segregates in affected patients and carriers in the family pedigree.

As has been observed by others [16,24,28], no CLCN5 mutation (including in the 5′ UTR promoter region) was detected in 12% of the patients with classic Dent’s disease (group A). The extent of locus heterogeneity that we found in our series, however, is much lower than that previously reported. The clinical pictures of patients with and without a CLCN5 mutation were apparently indistinguishable, though the number of patients without mutations was too small to identify any minor differences in the frequency of symptoms (Table 5). Hoopes et al. [29] have shown that OCRL1 gene mutations account for the 15.6% of patients with classic Dent’s disease, whereas other genes, yet to be identified, may be involved in the remaining 25% of the patients. No CLCN5 mutations were found in 13 patients of group B. These patients, however, did not meet the strict criteria, adopted also in the work of Hoopes et al. [29], to define Dent’s disease.

In addition to the mutations in patients with classic Dent’s disease, we also detected one splice-site mutation and two nucleotide substitutions probably affecting non-canonical splicing regulatory elements among 16 patients with a partial phenotype (group B).

Our findings show that the IVS5 +5 G>T mutation causes abnormal splicing of CIC-5 mRNA in leucocytes. That this is a disease-causing mutation was already shown by Scheinman [40], who demonstrated that heterologous expression of this mutation in Xenopus oocytes yields chloride currents indistinguishable from water-injected controls, indicating a loss of channel function. These observations support the idea that leucocytes may indeed be a valuable model for CLCN5 mRNA expression studies. Calciuria was borderline in our proband, and for this reason he was included in group B. On the contrary, calciuria was high in his brother, who carried the same mutation. Hypercalciuria is the intermediate phenotype that cannot be invariably reproduced in all CIC-5 knock-out mice models, however. It has been suggested that hypercalciuria might be diet-dependent in transgenic mice with a reduced CIC-5 expression [41]. At the time of the DNA analysis, the proband’s brother was clinically asymptomatic (in fact, no familiarity—third criterion—was indicated for that proband). Familial DNA analysis revealed that both, the proband’s mother and brother carried the same mutation, suggesting the state of carrier for the mother and that of affected for the brother. Subsequent urinary analyses revealed that the brother indeed had LMW proteinuria and hypercalciuria and, therefore, a typical Dent’s disease phenotype since he satisfied all three of the inclusion criteria.

On the contrary, as elsewhere already described and discussed, the IVS2 –17 T>G mutation did not appear to modify mRNA splicing, although the nucleotide substitution was a de novo mutation. Selective abnormal splicing in the renal tissue cannot be ruled out, however [23]. The variant was not screened in a normal population since, being de novo, it was not inherited and it should not be present in the normal population as a polymorphism occurring with a frequency >1%.

The silent P568P mutation was believed to represent a polymorphism because it was reported in association with another CLCN5 mutation in a patient with Dent’s disease [28]. Hypothesizing a linkage disequilibrium, we tested for other CLCN5 mutations but no other mutation in the coding as well as the 5′ UTR regions of CLCN5 was found. This substitution was also detected in the proband’s brother who had some of the manifestations of Dent’s disease. This silent mutation did not appear to induce any major changes in the expression of different mRNA transcripts in comparison with the controls. The functional significance of the shorter isoform, however, remains to be established along with the potential pathogenic role of its over-expression. We did not look for such a polymorphism in our Caucasian control population because of the African origin of these two subjects. However, it is puzzling that in this couple of brothers with a clinical picture suggesting Dent’s disease, the only variant we found was this. We cannot exclude that these cases are also an example of Dent’s disease heterogeneity.

In this case, the silent mutation might be in linkage disequilibrium with a mutation in another gene, and the OCRL1 gene is a good candidate. However, though there is no clear-cut evidence that the P568P variant is a mutation, we still believe that the issue whether this variant is a mutation rather than a polymorphism remains. More work needs to be performed.

In conclusion, our data indicate that the clinical diagnosis of Dent’s disease, based on the concurrence of the relevant symptomatic triade LMW proteinuria, hypercalciuria and nephrocalcinosis (which indeed is much more frequent than previously observed, as noted by Hoopes et al. [28]) is accurate, leading to a more than 85% detection rate of CLCN5 mutations. A thorough clinical and biochemical examination of family members may be very helpful to implement the clinical criteria to confirm the diagnosis, though mothers may not present LMW proteinuria.

A few Dent’s disease patients do not appear to carry CLCN5 mutations, confirming the role of genetic heterogeneity in this disorder.

Acknowledgements. We thank Prof. Peter Rogan (University of Missouri-Kansas City Schools of Medicine and Computer Science and Engineering Division of Medical Research Children’s Mercy Hospital and Clinics, Kansas City, USA) for his helpful suggestions.
This study was supported by grant No. 2002062925_003 from the Italian Ministry of Education, University and Research.

Conflict of interest statement. None declared.

References


Received for publication: 2.12.05
Accepted in revised form: 20.4.06