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Evidence for genetic heterogeneity in Dent's disease

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Evidence for genetic heterogeneity in Dent's disease.

Background. Dent's disease (X-linked nephrolithiasis) is a proximal tubulopathy that has been consistently associated with inactivating mutations in the *CLCN5* gene encoding the ClC-5 chloride channel expressed in tubular epithelial cells.

Methods. We performed mutation analysis of the coding region of *CLCN5* by DNA sequencing in 32 unrelated males, all of whom met the following three clinical criteria for the diagnosis of Dent's disease: (*1*) low-molecular-weight (LMW) proteinuria; (*2*) hypercalciuria; and (*3*) at least one of the following: nephrocalcinosis, kidney stones, renal insufficiency, hypophosphatemia, or hematuria.

Results. Sixteen mutations (ten missense, four nonsense, two frameshift) were found in 19 patients. Mutations were confirmed by restriction analysis or allele-specific polymerase chain reaction (PCR), segregated with disease in the families, and were not polymorphisms. In the other 13 patients with Dent's disease, the coding sequence of *CLCN5* was normal. In these 13 patients, we also sequenced two regions of the *CLCN5* promoter (626 and 586 bp, respectively, 2.1 and 1 kb upstream of exon 2) containing regulatory sites [activating protein-1 (AP-1)-like, AP-4, and cyclic adenosine monophosphate (cAMP)-receptor element binding protein (CREB)] and primary and secondary transcription start sites. We found no mutations in these promoter sequences in any of the 13 patients. In one three-generation family, the absence of mutation was confirmed by sequencing in two additional affected family members, and in this family haplotype analysis excluded linkage to the region of the *CLCN5* gene. There were no differences between the 19 patients with *CLCN5* mutations and the 13 without mutations with regard to any clinical features of Dent's disease.

Conclusion. These findings suggest that mutation in other gene(s) may be responsible for the phenotype of Dent's disease in some patients.

Dent's disease, also known as X-linked nephrolithiasis, has been defined by a characteristic syndrome that distinguishes it clearly from other hereditary causes of Fanconi syndrome, kidney stones, or renal failure [1–5]. The typical features include disturbed proximal tubular reabsorption [particularly low-molecular-weight (LMW) proteinuria], hypercalciuria, nephrocalcinosis, nephrolithiasis, renal failure, and rickets. The gene, *CLCN5*, encoding the voltage-gated chloride channel ClC-5, was identified through positional cloning in American and English families [2, 3, 6]. Mutations that inactivate function of this channel have been identified in every reported family with Dent's disease, and mutations segregate with the disease in each family [2, 7–16]. Most of the clinical features of the human disease are reproduced in the mouse when expression of the *CLCN5* is inactivated [17–20]. This evidence establishes a causal relationship between inactivating mutations in *CLCN5* and Dent's disease.

We have performed mutation analysis in probands in 32 families who met strict clinical criteria for the diagnosis of Dent's disease. In 13 of these families, we found no mutation, and we pursued additional studies that revealed the first evidence of genetic heterogeneity in Dent's disease.

Key words: Dent's disease, chloride channel, mutations, nephrolithiasis, hypercalciuria.

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METHODS

Patients

Patients were identified and brought to the investigators' attention by the physicians involved in their care (Table 1). The diagnosis of Dent's disease (X-linked nephrolithiasis) was based on the presence of all three of the following criteria: (*1*) LMW proteinuria [excessive excretion of β_2-microglobulin and/or retinol binding-protein (RBP)]; (*2*) hypercalciuria; and (*3*) at least one of the following: nephrocalcinosis, nephrolithiasis, hematuria, hypophosphatemia, or renal insufficiency.

LMW proteinuria was defined as an elevation in the excretion of either β_2-microglobulin or RBP, expressed either per 24 hours or per mg creatinine, by at least five-fold above the upper limit of normal for that laboratory. In all cases in which both proteins were measured, both met this criterion. Hypercalciuria was defined as >4 mg/kg in a 24-hour collection or >0.25 mg calcium per mg creatinine on a spot specimen (all patients collected 24-hour specimens except for six young boys). Nephrocalcinosis was determined radiographically, in most cases by

Table 1. Physicians and patient locations

Patient	Age (years)	Physician	Location
1	15	Andrew Brem, M.D.	Brown University, Providence, RI
2	13	David Kenagy, M.D.	United States Air Force, Dayton, OH
3	7.5	Douglas Blowey, M.D.	The Children's Mercy Hospital, Kansas City, MO
4	10	Avi Katz, M.D.	University of Minnesota, Minneapolis, MN
5	39	Sharon Moe, M.D.	University of Indiana, Indianapolis, IN
6	6	Valerie Langlois, M.D.	Loyola University, Chicago, IL
7	3	Ronald Hogg, M.D.	Dallas, TX
8	16	Scott VanWhy, M.D.	Yale University, New Haven, CT
9	17	Michael Freundlich, M.D.	University of Miami, Miami, FL
10	13	Scott VanWhy, M.D.	Yale University, New Haven, CT
11	6	Barbara Botelho, M.D.	Children's Hospital, Oakland, CA
12	18	Beth Vogt, M.D.	Rainbow Children's Hospital, Cleveland, OH
13	2.5	William Primack, M.D.	University of Massachusetts, Worcester, MA
14	13	Robert Holleman, M.D.	Mary Bridge Children's Hospital, Tacoma, WA
15	5.5	Michael Whyte, M.D.	Shriners' Hospital for Children, St. Louis, MO
16	2	B. Toenshoff, M.D., S. Haas, M.D.	University Children's Hospital, Heidelberg, Germany
17	4.5	Ronald Hogg, M.D.	Dallas, TX
18	6	Beth Vogt, M.D.	Rainbow Children's Hosp., Cleveland OH
19	12	Michael Somers, M.D.	Boston Children's Hosp., Boston, MA
20	9	B. Toenshoff, M.D., S. Haas, M.D.	University Children's Hospital, Heidelberg, Germany
21	17	B. Toenshoff, M.D., S. Haas, M.D.	University Children's Hospital, Heidelberg, Germany
22	7	Craig Langman, M.D.	Northwestern University, Chicago, IL
23	4	Douglas Blowey, M.D.	The Children's Mercy Hospital, Kansas City, MO
24	22	Janos Matyus, M.D., Ph.D.	Universotu of Debrecen Medical School, Hungary
25	8	Bernd Hoppe, M.D.	University of Cologne, Germany
26	9	Ari Simckes, M.D.	The Children's Mercy Hospital, Kansas City, MO
27	15	Horacio Lejarraga, M.D.	Hospital Garrahan, Buenos Aires, Argentina
28	3	William G. Conley, III, M.D.	University of North Carolina, Chapel Hill, NC
29	10	Velibor Tasik, M.D., Ph.D.	Clinic for Children's Diseases, Skopje, Macedonia
30	10	Craig Langman, M.D.	Northwestern University, Chicago, IL
31	(infancy)	Fatih Ozaltin, M.D.	Hacettepe University Faculty of Medicine, Ankara, Turkey
32	9	Robert Holleman, M.D.	Mary Bridge Children's Hospital, Tacoma, WA

ultrasonography. The presence of rickets was determined by radiographic features, and when these were absent the patient was labeled as lacking rickets. Renal failure was defined as a creatinine clearance below the published limits of normal for age [21] or a serum creatinine above the age-related normal ranges of Schwartz, Brion, and Spitzer [22]. Patients were excluded if they had any clinical evidence for other possible causes of proximal tubular dysfunction, hypercalciuria, or renal failure.

Probands from 35 unrelated families met the inclusion criteria for the study. Two were excluded because measurements of leukocyte cystine levels were elevated. One was excluded because of clinical features of Lowe's syndrome. The remaining 32 probands were studied.

Mutation analysis

Genomic DNA was isolated from peripheral leukocytes, and sequencing of the coding sequence of *CLCN5* from genomic DNA was performed as previously described [23]. The primers used allowed examination of flanking intronic sequence harboring splice sites. In each subject whose coding sequence was normal, we also sequenced two regions of the *CLCN5* promoter (626 and 586 bp, respectively 2.1 and 1 kb upstream of exon 2) containing regulatory sites [activating protein-1 (AP-1)-like, AP-4, and cyclic adenosine monophosphate (cAMP)-receptor element binding protein (CREB)] and primary and secondary transcription start sites (Fig. 1). Primer sequences for sequencing the promoter regions were: exon 1a left, 5′-CTT TTG CCA TTT CCT TGC TTC TCT T-3′; right, 5′-AGC CCG CAT TTG GTC ATT CAG GTT 3′; and exon 1b left 5′-TCT CCC CCA TTC CCA CCG TTC AT-3′; right 5′-GGA GGT TGG AGC AGC TTA GGT ATT T-3′.

When a mutation resulted in alteration in a restriction enzyme recognition site, that mutation was confirmed by restriction analysis, and this was also used to screen other members of the family; when no restriction site change resulted, the mutation was confirmed, and other family members screened, by repeat sequencing of the appropriate exon and by allele-specific polymerase chain reaction (PCR). A panel of DNA representing 126 unrelated chromosomes from normal individuals was screened for each mutation by restriction analysis or allele-specific PCR.

Allele-specific PCR was performed according to the method of Bottema and Sommer [24]. Primer pairs were designed to have a melting temperature (Tm) of approximately 48°C, with the last base at the 3′ end of one primer matching the mutation to be tested. Primers were chosen to produce a product size of approximately 150 to 200 bp. Thermocycler conditions were 46 cycles of 94°C for

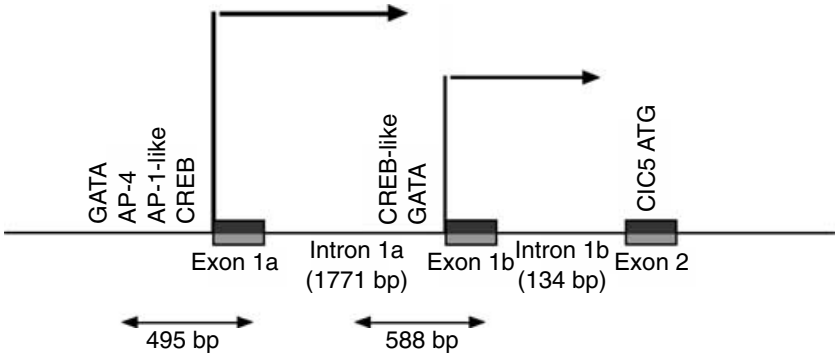

Fig. 1. *CLCN5* gene promoter region. Major promoter activity maps to the 5′ region of exon la, while a weaker promoter maps to the 5′ region of exon lb (bold arrows). Possible regulatory protein binding regions are labeled, 5′ of the transcription start sites. Double arrows indicate regions of the promoters that were sequenced (not to scale) [36].

Table 2. Mutations in 19 patients with Dent's disease

Patient	Base change	Mutation	Helix	Restriction enzyme
Missense mutations				
1	TGT→CGT	C221R	F	Btg I
2	TCG→TTG	S244L	G	–
3	TCG→TTG	S244L	G	–
4	TCG→TTG	S244L	G	–
5	GAA→GCA	E267A	H	Ear I
6	AGC→GGC	S270G	H-to-I loop	Alu I
7	GGT→GAT	G462D	N	SfaN I
8	GGG→AGG	G513R	O	Aar I
9	CGG→TGG	R516W	O-to-P loop	Hinf I
10	AGC→AAC	S545N	Q	–
11	AAG→GAG	K546E	Q	–
12	ACT→AGT	T657S	C-terminal loop	–
Nonsense mutations				
13	TGG→TAG	W45X		Sau96 I
14	TGT→TGA	C221X		Dde I
15	CGA→TGA	R347X		–
16	CGA→TGA	R718X		Hph I
17	CGA→TGA	R718X		Hph I
Frameshift mutations				
18	CCC→CC–	C del 687fs		
19	ACA→TACA	T ins 694fs		

1 minute, 50°C for 2 minutes, and 72°C for 3 minutes. Positive control samples of genomic DNA with the mutation, without the mutation and a heterozygote (or 1:2 diluted mutation containing DNA) were included with each assay (an additional pair of primers that always produced a unique band was added to the PCR reaction to serve as an internal positive control for the PCR). PCR products were separated and visualized on an ethidium bromide-containing agarose gel.

Haplotype analysis

Five microsatellite markers were used for haplotype analysis of the region of the *CLCN5* gene. These were [in order (NCBI human map release build 30)]: telomere-DXS8042-DXS1201-DXS1055-*CLCN5*-DXS1039-DXS988-centromere, spanning a region of approximately 16 cM or 14 Mb. Genomic DNA was used in the PCR with primers for each of these markers (Research Genetics) amplified under conditions as recommended by the supplier. Alleles were identified by PCR product sizes following separation on 8% polyacrylamide gels.

RESULTS

Mutations

The 16 mutations identified in 19 families are indicated on Table 2. These included 10 missense mutations (in 12 unrelated patients), in which a single base change predicts substitution of an amino acid in a conserved region of the protein. Two of these mutations (S244L and R516W) have been previously reported [2, 7] and found not to occur in normal subjects. We studied the other eight mutations and found none of them in 126 chromosomes from normal individuals, indicating that these are not mere polymorphisms.

In addition, we identified four nonsense mutations (in five patients) and two frameshift mutations (in two patients) predicted to result in premature termination of translation. These also would be expected to inactivate channel function. One of the nonsense mutations (R347X) was previously reported in a Japanese family [13]. In each of our families for which samples were available, the mutation segregated with disease, and the mother of the proband was heterozygous for the mutation.

In one patient with a mutation in *CLCN5*, we also identified a silent polymorphism, a C to T transition in codon 568 (CCC → CCT) that predicts no change in the encoded amino acid (proline). One other silent polymorphism (codon 484) has previously been reported [7].

Thirteen subjects had a normal coding sequence for *CLCN5.* In all 13 probands with normal *CLCN5* coding sequence, the promoter regions were sequenced and also found to be normal.

Haplotype analysis

The family of patient 24 included five available affected males. We repeated sequencing of the coding and promoter regions of *CLCN5* in two other affected males

Table 3. Haplotype analysis in five affected and three unaffected males in the family of patient 24

	Affected males			Unaffected males			
Subject	A	B	C	D	E	F	G
Locus							
Telomere							
DXS8042	2	4	4	4	4	3	2
DXS1201	3	4	4	4	4	3	3
DXS1055	3	2	2	2	2	2	3
CLCN5							
DXS1039	1	1	1	2	2	2	1
DXS988	1	1	1	2	2	1	2
Centromere							

Table 4. Clinical features in patients with and without mutations

	With mutations	Without mutation	χ^2	*P*
Nephrocalcinosis	17/19	8/12	2.451	0.117
Stones	5/17	1/12	1.905	0.168
Renal failure	5/19	4/12	0.176	0.675
Hypophosphatemia	9/18	7/12	0.201	0.654
Hematuria	16/19	7/11	1.648	0.199
Aminoaciduria	6/8	5/10	1.169	0.280
Glycosuria	7/18	4/11	0.018	0.892
Rickets	5/13	3/11	0.336	0.562
Hypokalemia	6/17	1/13	3.137	0.077
Hypomagnesemia	2/13	1/9	0.082	0.774

Comparisons performed using Pearson's chi-square test of independence.

who met criteria for Dent's disease (in addition to the proband) and confirmed that these were normal. Haplotype analysis in the five affected males and three unaffected males excluded linkage to the region of the *CLCN5* gene (Table 3).

Clinical features

Every proband in this study met the same inclusion and exclusion criteria for the diagnosis for Dent's disease. Additional data regarding clinical features are given in Table 4. In the group overall, the prevalence of nephrocalcinosis (81%), nephrolithiasis (21%), renal failure (29%), hypophosphatemia (53%), rickets (33%), and other features of the disease was similar to those we have previously reported [25]. There was no difference in any of these features between the groups with and without mutations. The ages of patients (Table 1) were no different in those with (11.0 ± 2.0 years) and without mutations (10.3 ± 1.6 years) (mean $\pm$ SE). Renal failure was present in none of the 17 patients under the age of 10 years in either group, but among patients age 10 years and older, renal failure was present in five of ten patients with mutations and in four of five without mutations. We have reported that nephrocalcinosis is found only occasionally in boys before the teenage years [3, 4], but in this study nephrocalcinosis was present in all of the 11 patients under the age of 7 years in both groups (eight with mutations and three without). White blood cell cystine levels were measured and found to be normal in all 6 of the tested probands lacking mutation in *CLCN5*, and slit-lamp eye examinations were normal in three additional patients without mutations.

DISCUSSION

Until this report, a total of 54 mutations had been reported in 71 families with Dent's disease, including missense, nonsense, frameshift, splice-site, in-frame insertion, and microdeletion mutations [2, 7–16, 26–29]. The 16 mutations identified in our 19 patients with Dent's disease represent the largest single published collection of mutations. They bring the total number of reported mutations to 68 in 90 families.

One of these mutations, S244L, has been reported in four other families in addition to the three described here [2, 10, 16, 23], and this is the most common mutation in *CLCN5* thus far described. Lloyd et al [2] expressed the S244L mutation in *Xenopus* and demonstrated that it substantially reduces the chloride conductance of the channel. The nonsense R347X mutation has also been expressed and found to inactivate function of the chloride channel [13].

Nine of the ten missense mutations described here occur within (C221R, S244L, G462D, G513R, S545N, K546E, and E267A) or near (S270G and R516W) transmembrane domains. The T657 mutation occurs in the C-terminal cytoplasmic domain within ten amino acids of a PY domain that appears to be an internalization signal that interacts with ubiqitin-protein ligases [30]. The functional effects of this specific mutation have not been studied.

Wu et al [31] have analyzed the previously reported mutations relative to the recent characterization of the 3-dimensional structure of bacterial ClCs reported by Dutzler et al [32]. Table 2 indicates the position of each of the missense mutations we found in our patients relative to this model; the transmembrane domains are identified using letters as assigned by Wu et al. ClC chloride channels are homodimers, and helices H, I, P and Q are the major helices involved in establishing the dimer interface [31]; mutations E267A (helix H), S545N and K546E (both helix Q) are in these domains. Helices D, F, N, and R come together to form the chloride-selectivity filter near the center of the channel. Mutations C221R (helix F) and G462D (helix N) are the first mutations reported in these helices.

Another 13 patients met the same entry criteria for the diagnosis of Dent's disease, yet lacked any mutation in the coding sequence for *CLCN5*. No mutations in promoter regions for this gene have been reported. We sequenced the two promoter regions in each of these 13 subjects, and found no mutations. The presence of a family history

of renal failure, kidney stones, or other features of this disease in three of these subjects indicated that the disease occurred on a familial rather than a sporadic basis. In the one family large enough to perform haplotype analysis, we excluded linkage to the region of the X chromosome harboring the *CLCN5* gene. A comparison of clinical findings between patients with mutations and those lacking mutations indicated no difference between the two groups, although the power of this analysis to detect a difference is limited by the small numbers of patients. This evidence suggests that there is genetic heterogeneity in Dent's disease.

The voltage-gated chloride channel ClC-5 is expressed in the kidney in subapical endosomes of the cells of the proximal tubule and the medullary thick ascending limb of Henle's loop. Dysfunction of proximal tubular endosomes is consistent with the nearly universal finding of LMW proteinuria. Consequent abnormalities in membrane recycling could explain other defects in proximal tubular function such as phosphaturia, aminoaciduria, and glycosuria. Abnormal trafficking of the sodium-phosphate cotransporter $NaPi_2$ [18] and of megalin and cubulin [33] has been observed in *CLCN5* knockout mice, and the low urinary levels of megalin observed in knockout mice [18] and in patients with Dent's disease [34] would also be consistent with abnormal membrane recycling. Other important features of Dent's disease, including absorptive hypercalciuria (possiby the consequence of excessive production of 1,25-dihydroxyvitamin D by renal epithelial cells), and renal failure, remain unexplained. If the ClC-5 chloride channel is intact in some patients with Dent's disease, it is reasonable to speculate that the phenotype could be produced by defects of other proteins necessary for endosomal function in the proximal tubular epithelium. In these cells, the chloride channel is thought to provide a voltage shunt that allows maximal acidification of the endosomal lumen, accomplished through a proton-andenosine triphosphatase (ATPase). The endosomal proton-ATPase is a complex multimeric protein consisting of 13 subunit types, and these ATPase subunits could be important candidates for Dent's disease in these patients. To date, no proton ATPase subunit has been found to be expressed predominantly in proximal tubule. Other candidates could include proteins involved in regulation, processing, or trafficking of ClC-5 or other endosomal membrane components, or any of a range of proteins that are necessary for normal proximal tubular reabsorptive function. Identification of other gene(s) that are mutated in patients with Dent's disease will require mapping work in families.

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