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Dent Disease with Mutations in *OCRL1*

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Dent disease is an X-linked renal proximal tubulopathy associated with mutations in the chloride channel gene *CLCN5*. Lowe syndrome, a multisystem disease characterized by renal tubulopathy, congenital cataracts, and mental retardation, is associated with mutations in the gene *OCRL1*, which encodes a phosphatidylinositol 4,5-bisphosphate (PIP_2) 5-phosphatase. Genetic heterogeneity has been suspected in Dent disease, but no other gene for Dent disease has been reported. We studied male probands in 13 families, all of whom met strict criteria for Dent disease but lacked mutations in *CLCN5*. Linkage analysis in the one large family localized the gene to a candidate region at Xq25-Xq27.1. Sequencing of candidate genes revealed a mutation in the *OCRL1* gene. Of the 13 families studied, *OCRL1* mutations were found in 5. PIP_2 5-phosphatase activity was markedly reduced in skin fibroblasts cultured from the probands of these five families, and protein expression, measured by western blotting, was reduced or absent. Slit-lamp examinations performed in childhood or adulthood for all five probands showed normal results. Unlike patients with typical Lowe syndrome, none of these patients had metabolic acidosis. Three of the five probands had mild mental retardation, whereas two had no developmental delay or behavioral disturbance. These findings demonstrate that mutations in *OCRL1* can occur with the isolated renal phenotype of Dent disease in patients lacking the cataracts, renal tubular acidosis, and neurological abnormalities that are characteristic of Lowe syndrome. This observation confirms genetic heterogeneity in Dent disease and demonstrates more-extensive phenotypic heterogeneity in Lowe syndrome than was previously appreciated. It establishes that the diagnostic criteria for disorders resulting from mutations in the Lowe syndrome gene *OCRL1* need to be revised.

Introduction

Dent disease (MIM 300009) is an X-linked disorder of renal tubular epithelial function, in which all of the clinical findings may be traced to impaired reabsorption of filtered solutes. Characteristic abnormalities include low-molecular-weight (LMW) proteinuria and other features of Fanconi syndrome, such as glycosuria, aminoaciduria, and phosphaturia, but typically do not include proximal renal tubular acidosis. Progressive renal failure is common, as are nephrocalcinosis and kidney stones. No extrarenal manifestations have been recognized—except for rickets, in a minority of patients—and this may be a consequence of hypophosphatemia from renal losses (Frymoyer et al. 1991; Wrong et al. 1994; Scheinman and Thakker 2000).

Mutations in the *CLCN5* gene encoding the renal chloride channel CLC-5 have been reported consistently in patients with Dent disease (Lloyd et al. 1996). This CLC-5 chloride channel is believed to be critical to acidification of endosomes that participate in solute reabsorption and membrane recycling in the proximal tubule (Lloyd et al. 1996), and it is known to alter membrane trafficking and the megalin-cubulin endocytic pathway. Disruption of the mouse homolog of this gene produces a phenotype resembling the human disease, confirming the role of this gene in the human syndrome (Piwon et al. 2000; Wang et al. 2000). A total of 68 distinct mutations have been reported in 90 families with Dent disease (Hoopes et al. 2004). However, we recently reported 13 additional families with Dent disease in whom mutations in *CLCN5* were excluded, indicating genetic heterogeneity (Hoopes et al. 2004). We now describe mutations in another gene involved in proximal tubular function that account for disease in 5 of these 13 families.

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Subjects and Methods

Patients

We studied the 13 probands reported by Hoopes et al. (2004), all of whom met strict criteria for Dent disease but lacked mutations in *CLCN5*. Details of patient identification and inclusion and exclusion criteria were described in the previous study (Hoopes et al. 2004). All affected males had LMW proteinuria, hypercalciuria, and at least one of the following abnormalities: nephrocalcinosis, nephrolithiasis, hematuria, hypophosphatemia, and renal insufficiency. Probands are identified using the family numbers assigned in the previous study (Hoopes et al. 2004); the 19 families with mutations in *CLCN5* were numbered 1–19, and the 13 families without mutations were numbered 20–32. One family was large enough to allow for linkage analysis. In probands found to have mutations in *OCRL1,* slit-lamp examination was performed. Studies were approved by the Institutional Review Board for the Protection of Human Subjects at the SUNY Upstate Medical University, and informed consent was obtained in compliance with this approved protocol at all participating institutions.

Linkage Analysis

DNA was isolated from peripheral blood by use of a standard protocol (Invitrogen) and was amplified using GoTaq DNA Polymerase (Promega) under standard amplification conditions. Because the inheritance pattern in this pedigree appeared to be X-linked, we studied markers on the X chromosome. PCR amplifications, performed using primers flanking previously identified X-chromosome–linked microsatellite markers (Research Genetics), were run on 8% polyacrylamide gels and were silver-stained, as described elsewhere (Shrimpton et al. 1999). Microsatellite markers were initially selected on the basis of their heterozygosity and spacing approximately every 10 cM along the X chromosome. Additional microsatellite markers were subsequently selected to refine the critical region. Linkage analysis was performed using MLINK software, under the assumptions of full penetrance in males, no penetrance in females, frequency of disease alleles of 0.0001, male mutation rate of 0.0, female mutation rate of 0.000001, and nine alleles of equal frequency.

Mutation Detection

We used intronic primers to amplify the exons and the intron/exon boundaries of candidate genes in the critical linkage region. PCR was performed under standard conditions, by use of genomic DNA isolated from blood leukocytes. PCR products were sequenced using ABI BigDye 3.1 sequencing kits, on an ABI 3100-*Avant* Genetic Analyzer. Both DNA strands were analyzed for each exon, by use of Lasergene software from DNASTAR. Because the pedigree strongly suggested X linkage, we sequenced *CLCN4,* which is a member of the same family of chloride channel genes as *CLCN5* and is located at Xp22.3. We established linkage, however, to a region at Xq25-Xq27.1 that included at least 65 genes. We evaluated those candidate genes on the basis of putative function and information regarding expression in the kidney, and we sequenced first the *SLC9A6* gene, which encodes the NHE6 sodium-proton exchanger, and next the *OCRL1* gene. Sequences of the primers used for sequencing *CLCN4, SLC9A6,* and *OCRL1* are listed in tables A1, A2, and A3 of appendix A (online only).

Screening of Normal Populations for OCRL1 *Mutations*

To establish that the mutations we identified were not common polymorphisms, we screened DNA representing 106–132 unrelated X chromosomes for each of the mutations detected. Four of the mutations were screened by restriction analysis. The exons affected by the mutations were amplified by PCR, along with known normal DNA and that of an individual containing the mutation. The PCR products were diluted in the manufacturer's buffer, which allowed the restriction enzyme to digest the amplicons to completion. The products were analyzed by agarose gel electrophoresis. For one mutation (436insAA), a restriction endonuclease was not available, and we used allele-specific PCR (Bottema and Sommer 1993) to detect the mutation. A 5′-fluorescent-labeled forward primer was designed to amplify DNA containing the mutation, and another was designed to amplify the normal DNA sequence. These were combined with a common unlabeled reverse primer so that there was always at least one PCR product in the reactions. These PCR products were analyzed by an ABI 3100-*Avant* Genetic Analyzer. The two alleles could be distinguished by both size and fluorescence emission. The primers used for this mutation were 6-FAM-TGAATTTGGACAAGAAAAT for the normal allele, VIC-TGAATTTGGACAAGAAAAA for the mutation, and TCACATGGGACTTACAT for the common reverse primer.

Enzyme Assays and Western Blotting for the OCRL1 *PIP_2 5-Phosphatase in Patient Fibroblasts*

Fibroblasts were cultured from skin biopsy specimens taken from each of the probands and, in family 24, from two affected brothers. Measurements of the *OCRL1* PIP_2 5-phosphatase activity for the OCRL1 protein were performed in duplicate in one laboratory by S.F.S., as described elsewhere (Suchy et al. 1995). Western blot

analysis for the OCRL1 protein and β-tubulin was done using anti-OCRL1 antibody (Olivos-Glander et al. 1995) and β-tubulin antibody (Abcam), as described elsewhere (Olivos-Glander et al. 1995).

Results

Linkage Analysis

Family history of Dent disease was seen in a typical X-linked pattern in the relatives of the proband in family 24, whereas all other probands were isolated cases. The initial haplotype analysis in family 24 excluded the X chromosome, except for the 27-cM region between the microsatellite markers *DXS1001* and *DXS1205* on the long arm at Xq24-Xq27.1. Additional markers refined the critical region demonstrating linkage to the disease to the 15-cM region between *DXS8057* and *DXS984* at Xq25-Xq27.1. This 16-Mb critical region formed a haplotype shared by all affected males in this family. None of the unaffected males had this haplotype, which demonstrated a maximum two-point LOD score of 3.31 at 0 cM between the disease and *DXS1192,* under the assumption of an X-linked recessive mode of inheritance, by use of the MLINK software.

OCRL1 *Mutations*

The sequences of *CLCN4* and *SLC9A6* were normal. Five distinct mutations in *OCRL1* were identified in 5 of the 13 families (table 1), none of which have been reported previously in patients with Lowe syndrome (MIM 309000). Two of these were missense mutations in which a base substitution predicted a single–amino acid change in exons 11 and 14, in the PIP_2 5-phosphatase domain of the OCRL1 protein, where many known mutations map (see Lowe Syndrome Mutation Database Web site). One of these, the mutation R301C in family 24, clearly segregated with the disease (fig. 1). The other three mutations, an intronic acceptor splice-site mutation, a 2-base insertion, and a 4-base deletion, are in exons 5 or 7 or intron 7 and are predicted to result in frameshifts and premature translational termination. The only mutations previously described in exons 1–8, which encode a region of the OCRL1 protein with no known homology to other proteins, have involved multiexon deletions.

Functional Consequences of the Mutations

Enzyme activity of the OCRL1 PIP_2 5-phosphatase was substantially reduced in skin fibroblasts from all five probands to the range typically seen in patients with Lowe syndrome (table 1). Enzyme activity was slightly less reduced in the two families with missense mutations (families 20 and 24) than in the three with truncating mutations and no detectable OCRL1 protein. By western blot analysis, protein was detectable at a reduced level in the two families with missense mutations but was undetectable in the other three families, consistent with the nature of the mutations (table 1 and fig. 2).

Both missense mutations would be expected to alter function. The Y462C mutation in exon 14 substitutes cysteine for tyrosine, an aromatic amino acid residue within the PTYKYD domain that is highly conserved among all known phosphoinositide 5-phosphatases. The R301C mutation inserts a cysteine in the place of a highly basic residue, arginine, in exon 11 (this residue is either arginine or glutamine in the highly related phosphatidylinositol and inositol 5-phosphatases INPP5B, PIB5PA, synaptojanin, and INPPL1).

Clinical Features

Slit-lamp examinations showed normal results in all five probands. None had the characteristic facial appearance of patients with Lowe syndrome, although one patient was thought to be borderline dysmorphic because of a narrow skull without scaphocephaly, asymmetric ears, and a suggestion of periorbital fullness. Slit-lamp examinations of an affected sibling of the proband in family 24 and the parents of the proband in family 20 showed normal results. Formal mental-developmental testing was performed for four of the five probands. In three, there was evidence of mild developmental delay. The proband in family 24 showed mild mental retardation when tested by MAWI (Magyar Wechsler Intelligencia Teszt) (IQ 80; VQ 87; PQ 76), as did his brother (IQ 67; VQ 70; PQ 70); the proband in family 20 also showed mild mental retardation when tested by HAWIK-R (Hamburg-Wechsler Intelligence Test for Kinder-Revised) (IQ 83; verbal 82; action 87). In these tests, scores between 80 and 90 are considered low average, and those between 70 and 80 are considered to reflect borderline mental retardation. The proband in family 20 was also tested by CFT-2 (Cattell Culture Fair Test-2), for which his score was normal (IQ 95). The proband in family 29 had reduced scores by BHTHM testing (VIQ 72; MIQ 57; GIQ 62). The patient in family 25 was normal by the Kaufmann Assessment Battery for Children (60%) and Raven's Colored Progressive Matrices (63%). The proband in family 26 was not tested formally but is an honor student enrolled in regular school. It is worth noting that the two patients with normal intelligence, as assessed by history or by developmental testing (patients in families 25 and 26), had a deficiency of the *OCRL1* PIP_2 5-phosphatase as profound as that seen in patients with classical Lowe syndrome and showed no protein by western blotting.

In contrast to the typical patient with Lowe syndrome, none of the affected males in these families required therapy with bicarbonate. Four of the five were able to acid-

Table 1

Mutations and Expression Data in Affected Males from the Five Families

Family	Patient's Age at Examination (years)	PIP_2 Phosphatase Activity[a] (nmol/min/mg)	Level of OCRL Protein (Western Blot)[b]	Exon	Mutation Type	Nucleotide Change	Effect on Translation	Screening Method	No. of Normal X Chromosomes Screened
20	9	.63	++	14	Substitution	1385A→G	Y462C (Tyr→Cys)	RFLP (*Tsp*451)	120
24[c]	22/27	.76/.85	+++/+++	11	Substitution	901T→G	R301C (Arg→Cys)	RFLP (*Hha*I)	132
25	8	.42	−	7	2-base insertion	436insAA	I147K (stop)	Allele-specific PCR	106
29	10	.49	−	5	4-base deletion	del259-262 (TGTT)	C87 (stop)	RFLP (*Eco*571)	106
26	9	.54	−	7 (5′)	3′ splice-site mutation	389-2, A→G	Unknown (likely frameshift)	RFLP (*Bfa*I)	112

[a] Control fibroblast, 4.71 nmol/min/mg; Lowe fibroblast, 0.52 nmol/min/mg.

[b] Control fibroblast, ++++; Lowe fibroblast, −.

[c] There were two affected brothers in this family.

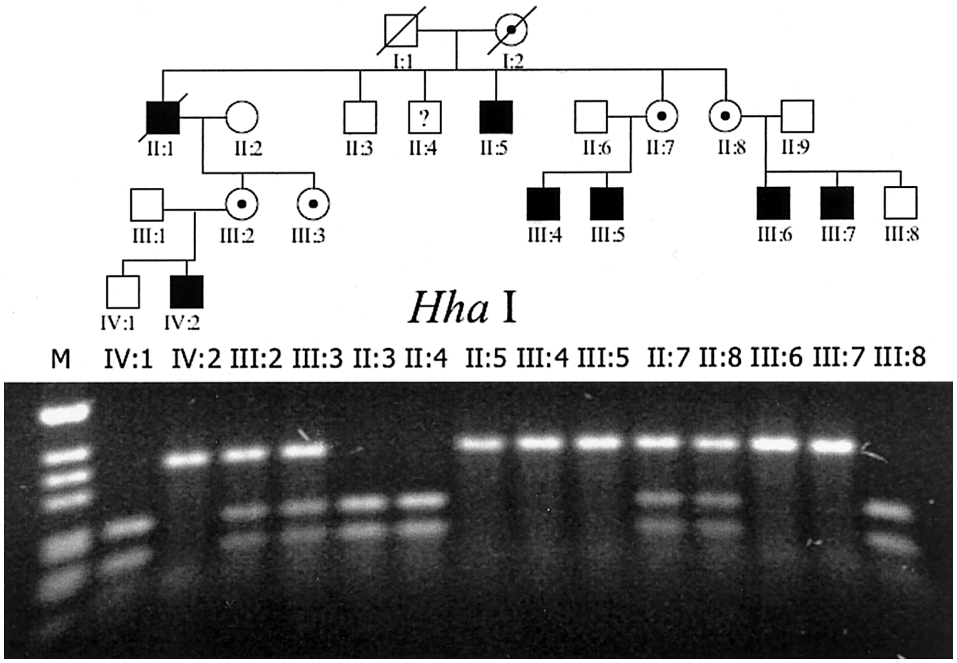

Figure 1 Segregation of the R301C mutation in family 24. The pedigree is shown, with affected subjects identified as blackened symbols. Digestion of the 298-bp exon 11 amplicons with the restriction endonuclease *Hha*I in normal subjects results in two products of 158 bp and 140 bp (subjects IV:1, II:3, II:4, and III:8). The mutated sequence produces a single detectable digest of 298 bp (subjects IV:2, II:5, III:4, III:5, III:6, and III:7). Heterozygous females display all three products (subjects III:2, III:3, II:7, and II:8). The lane containing *Phi*X174/*Hae*III molecular weight markers is indicated by "M."

ify urine to <pH 5.8, either on acid loading or on a first-morning voided specimen; a test of urine acidification was not available for the fifth patient. Only one of the patients had growth retardation, which was mild, and that patient was able to acidify urine to <pH 5.5 on ammonium chloride loading.

None of the patients had nephrolithiasis, and only one of the five probands had nephrocalcinosis on ultrasound examination. None had clinical rickets, although one had mildly reduced bone mineral density. As is commonly seen in Dent disease, Fanconi syndrome was incomplete in these patients. Three of the five had inappropriate urinary phosphate loss with hypophosphatemia. Two had aminoaciduria; none had glycosuria. Although the prevalence of some of these features was higher in the 19 probands with *CLCN5* mutations in our recent study describing genetic heterogeneity in Dent disease (Hoopes et al. 2004) and in previously published studies (Scheinman 1998; Scheinman and Thakker 2000), the number of cases in the present study is too small to allow any statistical comparisons.

Two of the probands had mild elevations in muscle enzymes, although without muscle weakness. These patients (from families 20 and 29) had elevations of creatine kinase, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase that were 1.1–2.0-fold elevated above the upper limit of normal, but there was no clinical evidence of muscle weakness. Mild elevations in muscle enzymes have been described in patients with Lowe syndrome (Charnas et al. 1991).

Discussion

Of the 32 families with the clinical diagnosis of Dent disease reported by Hoopes et al. (2004), 19 (60%) had mutations in *CLCN5*. We have now demonstrated that another five (16%) have mutations in *OCRL1,* in patients for whom the diagnosis of Lowe syndrome had been excluded because cataracts were absent. Since these two genes do not account for all patients with the phenotype of Dent disease, it is likely that other genes involved in proximal tubular function will be found to be mutated in such patients. This appears analogous to another inherited renal tubulopathy, Bartter syndrome, for which there are now five genes known to be responsible in subgroups of patients (Vargas-Poussou et al. 2002).

Although it is formally possible that some of the patients described here had the relatively mild phenotype of Dent disease, despite having mutations in the *OCRL1* gene (because of somatic mosaicism of their *OCRL1* mutations), there are three lines of evidence that argue against this explanation. First, the mutations were seen

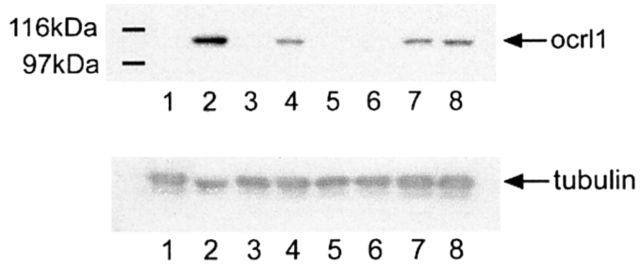

Figure 2 Western blot analysis of OCRL1 in control and patient fibroblasts. Fibroblast supernatant (20 μg), which was prepared as described in the text, was loaded in each lane. *Lane 1,* a Lowe syndrome patient known to not express OCRL1; *lane 2,* an unaffected individual (control fibroblasts); *lane 3,* proband from family 25; *lane 4,* proband from family 20; *lane 5,* proband from family 29; *lane 6,* proband from family 26; and *lanes 7* and *8,* two affected brothers from family 24. The positions and sizes of broad-range protein markers (Bio-Rad) are shown on the left. The presence of adequate protein loading in all lanes is demonstrated in the lower panel by a western blot analysis of the same blot with the use of antibody against β-tubulin as a control.

clearly in the sequences of PCR products made from DNA extracted from white blood cells, without any evidence of admixture of normal and mutant gene sequence. Second, the enzyme assays and western blotting were both performed on cultured fibroblasts, a different tissue, with clear evidence of deficient enzyme activity and, in some cases, absent protein. Thus, if somatic mosaicism were present, it would have to be absent in two independently derived tissues, white blood cells and skin fibroblasts. Finally, the evidence of multiple affected individuals in family 24 argues against mosaicism, at least in this one family.

Both Dent disease and Lowe oculocerebrorenal syndrome involve disordered proximal tubular function, and their clinical renal findings are similar but not identical. Both are associated with extreme degrees of LMW proteinuria (Norden et al. 2001), as well as other features of proximal tubular reabsorptive failure, such as renal glycosuria, aminoaciduria, and phosphaturia. Renal failure is common in both (Charnas et al. 1991; Frymoyer et al. 1991; Wrong et al. 1994; Scheinman and Thakker 2000). Hypercalciuria, nephrocalcinosis, and nephrolithiasis—common features of Dent disease—have been reported, but only rarely, in patients with Lowe syndrome (Sliman et al. 1995). In these five families, hypercalciuria was present in all five probands, nephrocalcinosis was present in only one proband, and none of the probands had kidney stones. Two of the five probands had some degree of reduction in glomerular filtration rate.

Renal tubular acidosis is a prominent feature of Lowe syndrome, usually requiring alkali therapy to ameliorate the growth retardation. In contrast, it is not a feature of Dent disease (Scheinman 1998). Despite having mutations in *OCRL1,* none of patients in these five families had acidosis.

The predominant renal manifestation of both Lowe syndrome and Dent disease is Fanconi syndrome, particularly LMW proteinuria but also other abnormalities in proximal tubular reabsorption, such as renal wasting of phosphate and amino acids. The ClC-5 chloride channel is located in subapical endosomes that are critical to the process of degradation of reabsorbed proteins by the proximal tubular epithelium. Mutations that impair chloride flow through this channel lead to impaired acidification of the endosomal lumen, and, as a consequence, trafficking of endosomal membrane back to the apical surface is disrupted (Christensen et al. 2003). The role of *OCRL1* mutations in causing Lowe syndrome is still unclear. Impaired function of the *OCRL1* phosphatase leads to elevation in cellular levels of PIP_2—which is known to be involved in vesicle trafficking at the Golgi apparatus, where OCRL1 is localized (Dressman et al. 2000)—but a defect in Golgi trafficking has yet to be demonstrated in Lowe syndrome. Elevated PIP_2 levels are also believed to be responsible for the alterations in the actin cytoskeleton observed in fibroblasts from patients with Lowe syndrome (Suchy and Nussbaum 2002). Actin remodeling is closely connected to both Golgi trafficking (Randazzo and Hirsch 2004) and endosomal membrane trafficking (Apodaca 2001), which suggests that abnormalities in the actin cytoskeleton could act on a number of cellular processes in the renal epithelium of patients with Lowe syndrome.

Although phenotypic variability in the renal and neurological phenotypes is a well-known feature of Lowe syndrome, dense congenital cataracts are considered a cardinal finding required for diagnosis. They are always present at birth and can be found at 20–24 wk of gestation (Charnas and Nussbaum 2001). Cataracts, usually clinically insignificant, are consistently present even in carrier females and are used as a screening method for the carrier state (Roschinger et al. 2000). The present findings demonstrate that the absence of cataracts does not exclude the possibility of mutations in *OCRL1*. The lack of cataracts in the affected members of family 24 suggests further that this particular mutation, R301C, may be an allele that causes renal disease only, although a modifier gene closely linked to the *OCRL1* locus cannot be ruled out. In contrast, three of the patients reported here have deficient *OCRL1* PIP_2 5-phosphatase activity and complete absence of the OCRL1 protein by western blotting—results that are indistinguishable from those seen in classic Lowe syndrome with dense cataracts. However, all three patients lacked cataracts, and two patients had no detectable mental retardation. These results indicate that the variability in Lowe syndrome cannot be entirely due to allelic differences in

the *OCRL1* gene itself; instead, these results more likely point to modifying loci and/or unknown environmental factors that can significantly alter the phenotypic consequences of a deficiency of the OCRL1 protein.

OCRL1 encodes a PIP_2 5-phosphatase that is widely distributed in human tissues, so it is not clear why deficiency of this enzyme results in a phenotype that is specific to the eye, brain, and kidney. A clue to the answer may come from studies of a mouse model of PIP_2 5-phosphatase deficiency. Knockout mice in which expression of the *Ocrl1* gene is prevented by targeted disruption have no evidence of cataracts, neurological abnormalities, or renal dysfunction. However, simultaneous deficiency of both *Ocrl1* and another phosphatase gene that is highly homologous to *Ocrl1* (*Inpp5b*) results in an embryonic lethal phenotype. This strongly suggests that the *Inpp5b* phosphatase can compensate for absence of the *Ocrl1* enzyme. If the same or a similar phenomenon occurs in humans and if there is variability among tissues and individuals in the expression of the compensating enzyme, then this could explain the phenotypic variability, such as the absence of cataracts and the normal urinary acidifying ability, in some patients with *OCRL1* mutations (Janne et al. 1998).

These findings demonstrate that Dent disease can be caused by mutations at at least three loci and also that not all mutations in the *OCRL1* gene cause Lowe syndrome. Mutations in the *OCRL1* gene cannot be excluded on the basis of the absence of cataracts in patients with Fanconi syndrome.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Lowe Syndrome Mutation Database, http://research.nhgri.nih.gov/lowe/ocrl1_mut_db.shtml

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for Dent disease and Lowe syndrome)

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