RESEARCH ARTICLE

Human Mutation

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From Lowe Syndrome to Dent Disease: Correlations between Mutations of the *OCRL1* Gene and Clinical and Biochemical Phenotypes

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ABSTRACT: Mutations of *OCRL1* are associated with both the Lowe oculocerebrorenal syndrome, a multisystemic and Dent-2 disease, a renal tubulopathy. We have identified a mutation in 130 Lowe syndrome families and 6 affected by Dent-2 disease with 51 of these mutations being novel. No founding effect was evidenced for recurrent mutations. Two mutations initially reported as causing Dent-2 disease were identified in patients, including two brothers, presenting with Lowe syndrome thus extending the clinical variability of *OCRL1* mutations. mRNA levels, protein content, and PiP_2-ase activities were analyzed in patient's fibroblasts. Although mRNA levels were normal in cells harboring a missense mutation, the OCRL1 content was markedly lowered, suggesting that enzymatic deficiency resulted mainly from protein degradation rather than from a catalytic inactivation. Analysis of a splicing mutation that led to the elimination of the initiation codon evidenced the presence of shortened forms of OCRL1 that might result from the use of alternative initiation codons. The specific mapping of the frameshift and nonsense mutations, exclusively identified in exons 1–7 and exons 8–23, respectively, for Dent disease and Lowe syndrome together with the possible use of alternative initiation codons might be related to their clinical expression, that is, Lowe syndrome or Dent-2 disease.
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KEY WORDS: OCRL1; Lowe syndrome; Dent 2 disease; phosphatidylinositol 4, 5 biphosphate homeostasis

Introduction

Lowe Syndrome

The oculocerebrorenal syndrome of Lowe (OCRL; MIM♯ 309000) is a rare X-linked multisystem disorder presenting with major abnormalities in the eyes, the kidneys, and the central nervous system [Loi, 2006; Lowe et al., 1952]. OCRL is a rare disease with a prevalence estimated between 1 and 2 boys per million people. Ocular abnormalities include a constant prenatal development of cataracts and frequent associated signs such as glaucoma, microphthalmos, decreased visual acuity, and corneal keloid formation. Neonatal hypotonia, intellectual impairment, and areflexia are also cardinal features. The majority of patients have a cognitive delay and behavioral troubles including temper tantrums and aggressiveness are frequently noted. Brain magnetic resonance imaging (MRI) may show periventricular cystic lesions [Loi, 2006; Schneider et al., 2001]. Fanconi syndrome, a generalized impairment of the proximal tubular cells functions, is a major feature.

Onset of the tubular dysfunction can vary between patients, and the severity tends to worsen with age. Low molecular weight proteinuria (LWMP) is invariably present and aminoaciduria, hypercalciuria, and bicarbonaturia are frequently included. Progressive glomerular dysfunction leads usually to renal failure. Skeletal muscle abnormalities may develop as secondary consequences of hypotonia or renal dysfunction. Nontender joint swelling and subcutaneous nodules are also frequently described in affected patients and may reflect a primary abnormality of connective tissue growth. Lowe syndrome results from mutations of the *OCRL1* gene (MIM♯ 300535) that encodes a phosphatidyl inositol 4,5 biphosphate ($PI(4,5)P_2$) phosphatase.

Dent Disease

Dent disease (Dent-1; MIM♯ 300009), is a X-linked proximal renal tubulopathy, characterized by LWMP, hypercalciuria, and

Additional Supporting Information may be found in the online version of this article.

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progressive renal insufficiency. In addition, other features of proximal tubular dysfunction such as glycosuria, aminoaciduria, phosphaturia, uricosuria, or complete Fanconi syndrome may also be present [Dent and Friedman, 1964; Thakker, 2000]. Except for rickets noted in some patients; no extra-renal manifestations of the disease have been reported.

Mutations in the *CLCN5* gene (MIM♯ 300008) encoding the renal voltage-gated chloride channel CLC-5 have been reported in 60% of patients with Dent disease [Hoopes et al., 2004; Lloyd et al., 1996]. ClC-5 is mainly localized in subapical endosomes of epithelial kidney cells and contributes to the acidification of intraendosomal compartments and participates in membrane recycling in the proximal tubule [Dewuyst et al., 1999; Piwon et al., 2000]. A recent study showed that mutations in the *OCRL1* gene can lead to a Dent-like phenotype (Dent-2 disease, DD-2; MIM♯ 300555) that can present as a clinical intermediate between Lowe syndrome and Dent disease [Bökenkamp et al., 2009; Hoopes et al., 2005]. Nevertheless, DD-2 patients harboring an OCRL1 mutation displayed comparable decrease in PI(4,5)P_2ase activity and OCRL1 content to those reported for patients affected by Lowe syndrome ([Hoopes et al., 2005]; this study).

OCRL1

OCRL1 is a type II inositol polyphosphate 5-phosphatase that participates into the PI(4,5) P_2 homeostasis [Attree et al., 1992; Olivos-Glander et al., 1995; Lowe, 2005]. OCRL1 has been originally localized to the trans-Golgi Network and to lysosomes [Dressman et al., 2000; Olivos-Glander et al., 1995; Ungewickell et al., 2004] and more recently to endosomes [Choudury et al., 2005; Erdmann et al., 2007; Hyvola et al., 2006]. Abnormalities in the actin cytoskeleton have been demonstrated in Lowe fibroblasts [Suchy and Nussbaum, 2002] and OCRL1 was recently proposed to play a role in regulating membrane trafficking and actin dynamics [Faucherre et al., 2005; Lowe, 2005].

The OCRL1 gene spans 52,278 bp on chromosome Xq24.26 [ENSG00000122126] and contains 24 exons with exon 18a as an alternatively spliced exon mostly expressed in brain [Attree et al., 1992; Nussbaum et al., 1997]. The ATG start codon was initially assigned to exon 2 [Attree et al., 1992]; however, recent reports mapped the initiation codon in exon 1 [Erdmann et al., 2007; Hyvola et al., 2006; Mazo et al., 2009]. Accordingly the 5,156-bp cDNA (NM_000276.3) encodes a protein monomer of 901 amino acids (MW = 103 226 kDa).

This article reports 51 novel mutations of the *OCRL1* gene associated either with Lowe syndrome or Dent-2 disease. Correlations between the mutations and their expression at the mRNA, functional and protein levels will be discussed in regard to their clinical expression. Based on these data, putative mechanisms to explain how mutations of the OCRL1 protein express as heterogeneous clinical entities will be discussed.

Materials and Methods

Patients and Samples

A panel of 175 nonrelated families addressed to the laboratory for suspicion of Lowe syndrome has been investigated. Among the male patients addressed for suspicion of Lowe syndrome, 124 index cases presented with full OCR criteria of Lowe syndrome. Fifteen mothers of deceased children likely to be affected by Lowe syndrome were investigated for mutations in the *OCRL1* gene. French clinical centers addressed 59% of the families with the remaining families originating from Europe (19%), Middle-East (5%), North America (5%), Asia (6%), and Australia (3%).

Forty-one patients affected by Dent disease were additionally included in the study. All patients originated from France. They did not show cataract or noticed neurological troubles and did not harbor mutations in the *CLCN5* gene.

Blood samples and, whenever possible, fibroblasts, were obtained from the affected patients and/or from their relatives. An informed consent allowing genetic studies was obtained from all patients included in the study.

Mutation Detection

Genomic DNA was isolated using standard procedures. The 24 exons of the *OCRL1* gene were amplified from genomic DNA as described previously [Monnier et al., 2000]. For mutational analysis, PCR-amplified DNA products were subjected to direct automated sequencing on an ABI 3130 DNA Analyser (Life Technologies, Carlsbad, CA).

Alternatively RNA was extracted from 2–5 10^6 fibroblast cells using the Trizol® reagent (Life Technologies). RNA was dissolved in 50 µl of RNAse-free water. Reverse transcription was performed by using 0.2 mg of total RNA at 48°C for 60 min with 50 U of Expand Reverse Transcriptase (Roche, Basel, Switzerland) and in the presence of oligo-dT and the following specific primer (5′-AACTTTGGCTTGGCAATATAAGTC). The resulting cDNA was then amplified and sequenced as previously described [Monnier et al., 2000]. Mutations responsible for aberrant sized transcripts were characterized by direct sequencing of the corresponding exon and intron–exon junctions.

Mutation assignment was based on the cDNA sequence (GenBank NM_000276.3) using the first coding ATG of exon 1 as initiation codon according to international guidelines for description of sequences variants (http://www.hgvs.org/mutnomen). This resulted in an in frame addition of 51 nucleotides at the 5′ end of the cDNA and of 17 amino acids at the N-terminal end of the protein in comparison with the initial nomenclature [Attree et al., 1992]. None of the novel variants identified in this study were found after screening of 200 chromosomes from the general population. Genomic rearrangements were evidenced using data obtained from PCR screening or MLPA analysis in probands. In families harboring a genomic rearrangement, the carrier status of mothers was determined using a homemade MLPA® assay (MRC Holland, Amsterdam, The Netherlands) [Coutton et al., 2010].

Quantitative Transcript Analysis

Quantitative PCR was performed using 100 ng of cDNA in a final volume of 25 µl and specific primers for the *OCRL1* gene (F-5′-CGAGCTGTATCAGCGATGTC; R-5′-GGAGGCCTCAGGAGAAGACT) and the *GAPDH* gene used as control (F-5′-CATCAAGAAGGTGGTGAAGC; R-5′- GAGCTTGACAAAGTGGTCGT). The OCRL1 primers allowed the amplification of a 197-bp fragment spanning exon 21 to 23. Duplicated samples were PCR were run on a iQ Cycler apparatus in presence of the iQ SYBR Green Supermix containing 500 nM primers (Biorad, Hercules, CA).

Enzymatic Assay for PI(4,5)P_2 Phosphatase Activity

Activity was assayed as described previously on cell extracts prepared by freeze–thawing of fibroblasts obtained from affected and nonaffected patients except that each assay included 50 µg of

fibroblast protein and that quantification was performed by direct analysis of the TLC plates using a Beta Imager 2000 (Biospace, Paris, France) [Satre et al., 1999; Suchy et al., 1995].

Western Blot Analysis

For Western blot analysis, aliquots of cell extracts (25 μg of protein) were resolved by an 8% SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon P transfer membrane (Millipore, Billerica, MA). OCRL1 was revealed using polyclonal antibodies raised in rabbit against the N-terminal region (amino acids 18–217) and a peroxidase-labeled mouse antirabbit detection system. As control we used the signals obtained using polyclonal antibodies raised against the β-subunit of the mitochondrial ATPase. The amount of OCRL1 present in each sample was determined by quantitative western blot analysis using a ChemiDoc XRS apparatus and the Quantity One® software (BioRad), after correction of protein loading by Coomassie blue staining.

Results

Mutation Analysis

We have identified a mutation in the *OCRL1* gene in 130 out of the 175 families included for Lowe's syndrome and in 6 out of the 41 Dent disease families. Fifty-one of these mutations were novel (Table 1) with four of them identified in patients affected by Dent-2 disease. Extensive genomic and cDNA sequencing analysis led to the correction of boundaries of exons 8, 9, 11, 12, 13, and 14 in reference to those reported in the Lowe Syndrome Mutation Database [2010] and to the use of a cDNA numbering based on the use of initiation codon in exon 1 (Supp. Table S1).

Near to 200 mutations have been identified so far in the *OCRL1* gene in association with Lowe syndrome or Dent-2 disease (Supp. Table S2). Frameshift, splicing or nonsense mutations leading to a premature termination of the protein represented 63% of the mutations, whereas missense mutations and genomic deletion accounted respectively for 33 and 4% of the molecular variations. No noticeable differences in this distribution were noticed when comparing Lowe syndrome or Dent-2 Disease. Thirty-three recurrent mutations were identified and accounted for the disease in 41% of the genotyped families. Seventeen mutations have been reported in more than three families ($n = 3$: p.Ile274Thr, p.Arg318Cys, p.Arg334X, p.Gln388X, p.Arg822X; $n = 4$: c.940−11G>A, p.Gly421Glu, p.Arg500X, p.Arg541X, c.1694_1695insCCTT, c.2360_2361delTG, p.Arg844X; $n = 5$: p.Arg663X, p.Arg695X, p.Arg810X; $n = 8$: p.Arg500Gln; $n = 10$: c.2581G>A). As it could be inferred from data presented in Supp. Table S2, none of these mutations appeared to be population specific.

Genetic investigation of 98 mothers of patients affected by Lowe syndrome showed the occurrence of a de novo mutation in 36 cases (37.2%) whereas three cases of mosaicism were characterized in our panel of genotyped families indicating that, although not frequent, mosaicism must be taken in account for genetic counselling.

As shown in Figure 1, 53 out of the 66 missense mutations reported to date mapped to the PI(4,5)P_2 phosphatase domain spanning exons 9–15. They involved amino acids defined as major determinants for specificity of the inositol polyphosphate 5-phosphatase activity [Tsujishita et al., 2001; Whisstock et al., 2001]. The remaining missense mutations were identified in the C-terminal ASH-RhoGAP-like domain spanning exons 16 to 23 [Choudury et al., 2005; Erdmann et al., 2007; Faucherre et al., 2003; Hyvola et al., 2006; Peck et al., 2002] and involved amino acids located in well-conserved domains (Supp. Fig. S1). Noticeably, no missense mutations have been identified so far in exons 1 to 8.

Frameshift mutations or nonsense mutations leading to premature termination of the protein have been characterized in all exons but exons 2 and 3 (Fig. 1). Although nine substitutions affected directly the consensus acceptor or donor sites at the exon–intron junctions, seven additional intronic mutations induced abnormal splicing: c.40−14A>G, c.238+4701G>A, c.940−11G>A, c.1467–3C>G, c.1879+5G>A, c.1880−5del16, c.2469+2_6delinsA, c.2581+4A>G. Transcript analysis showed that three substitutions affecting the last nucleotide of exons 9, 14, and 22: c.824G>C, c.1466G>A, c.2581G>A affected the splicing. Thus, the p.Gly275Ala, p.Ser489Asn, p.Ala861Thr mutations, initially reported as missense mutations [Kawano et al., 1998; Monnier et al., 2000] led thus primarily to a premature termination of the protein.

Noticeably, although frameshift and nonsense mutations associated with Lowe syndrome concentrated in exons exons 8 to 23, all frameshift and nonsense mutations causative of Dent 2 disease have been characterized so far only in the first seven exons.

Functional Expression of Mutations

mRNA expression

As shown in Figure 2 no significant differences in the level of mRNA expression of the *OCRL1* gene could be evidenced in patients harboring a missense mutation when compared to controls. At difference, patients harboring stop, frameshift, or splicing mutations showed a very low mRNA content that indicated either a decreased transcription or more likely a mRNA instability resulting from a nonsense-mediated decay mechanism. No difference was observed between patients affected by Lowe syndrome or Dent-2 disease. Noticeably, one patient harboring a c.[40−14A>G] splicing mutation showed a normal amount of mRNA (Fig. 2, arrow). As shown in panel A of Figure 3, the c.40−14A>G mutation resulted in the production of two abnormal transcripts. Sequencing analysis showed that whereas both transcripts could be amplified and quantified, they both lacked the 3′ end of exon 1 that contains the ATG initiation codon (Supp. Fig. S2).

PIP2ase activity

PI (4,5)P_2 phosphatase activity was measured in 12 patients harboring a missense mutation and in 11 patients harboring a nonsense or a frameshift mutation that led to a premature termination of the protein. As presented in Table 2 the enzymatic activities measured in patients with either a missense mutation or a mutation leading to a premature termination of the protein showed a mean inhibition of 85–90% when compared to PI (4,5)P_2 phosphatase activity determined in fibroblasts originating either from healthy individuals or from patients addressed for a suspicion of Lowe syndrome and for whom no mutation had been identified in the *OCRL1* gene. No significant differences between the residual activities measured in fibroblasts harboring a missense mutation and those harboring a nonsense or frameshift mutation were evidenced. Likewise no differences of PI (4,5)P_2 phosphatase activities could be evidenced between Lowe syndrome or Dent-2

Table 1. Novel Mutations in the OCRL1 Gene

Patient ID	Exon	Nucleotide change[a]	Protein change[b]	Enzyme activity[c]	Disease status	Age[d]	Ocular symptoms[e]		Neurological symptoms[e]			Renal symptoms[e]						
							CC	G	NH	DD	MR	FS	PT	RF	AA	LMWP	P	Ca
05den10	1	c.40−14A>G	Splicing defect	1.6	Dent	7	−		−						+		+	
09ls009	i1	c.238+4701A>G	Splicing defect		Lowe	7	+			+			+			+		
04ls15	8	c.632delT	p.Val211AspfsX38		Lowe	6	+	+	+	+	+/−				+		+	
06ls37	8	c.688C>T	p.Arg230X		Lowe	2	+		+	+			+		+			
05ls26	8	c.702T>G	p.Tyr234X		Lowe	1												
02ls42	9	c.725T>C	p.Phe242Ser	2.6	Lowe	30	+	+	+	+	+		+			+		+
02ls25[f]	9	c.821T>C	p.Ile274Thr		Lowe	43	−			+	+/−			+		+		
02ls26[f]					Lowe	34	+			+	+/−			+		+		
08ls008	i9	c.825−2A>G	Splicing defect		Lowe	17	+			+	+		+			+		
06ls59	10	c.830A>G	p.Gln277Arg		Lowe	7	+			+	+		+		+	+	+	
06ls54	11	c.952C>T	p.Arg318Cys		Lowe	23	−	−	+	+	+	+	+	+	+	+	+	
07den01					Dent	7	−			−						+		
10ls011	11	c.1005C>G	p.Tyr335X		Lowe	5	+	−	+	+	+		+			+	+	
06ls32[g]	11	c.1009C>T c.1082G>T	p.Arg337Cys p.Arg361Ile	1.4	Lowe	1	+		+							+	+	
04den01	12	c.1060A>C	p.Asn354His		Dent	27	−		−								+	
98ls44	12	c.1115T>G	p.Val372Gly		Lowe	22	+	+		+		+	+					
06ls15	12	c.1117A>T	p.Asn373Tyr	1.4	Lowe	13	+		+	+	+	+	+		+	+		
98ls48	12	c.1121C>T	p.Ser374Phe		Lowe	3	+		+	+		+	+		+	+	+	
10ls016[f]	12	c.1241A>G	p.His414Arg		Lowe	2	+	+	+	+	+	+				+	+	+
10ls017[f]					Lowe	5	+	+	+	+	+	+				+	+	+
09ls014	13	c.1341_1342delCT	p.Leu448GlufsX17		Lowe	1	+		+			+				+	+	
03ls38	13	c.1351G>A	p.Asp451Asn		Lowe	30	+	+		+		+	+					
00ls37	13	c.1355delA	p.Gln452ArgfsX1		Lowe	12	+			+								
00ls03	14	c.1369C>G	p.Arg457Gly	0.6	Lowe	20	+			+	+	+						
06ls17	14	c.1378A>T	p.Lys460X		Lowe	1	+	+	+		+	+	+		+	+	+	
03ls35	14	c.1402G>A	p.Glu468Lys		Lowe	5	+		+		+	+	+			+	+	
07ls20	14	c.1403A>G	p.Glu468Gly		Lowe	5	+		+	+		+				+	+	
04ls11	14	c.1440−1441delCT	p.Asp463AspfsX1		Lowe	0.1	+	+	+			+				+	+	
06ls19	i14	c.1467−3C>G	Splicing defect		Lowe	7	+								+		+	
02ls50	15	c.1484C>A	p.Pro495Leu	1.8	Lowe	3	+		+				+			+		
04ls05	15	c.1495G>C	p.Asp499His		Lowe	5	+		+	+		+						
06ls18	15	c.1507T>C	p.Trp503Arg		Lowe	0.4	+	+	+	+					+			
05ls21	16	c.1681_1682insGACT	p.Phe561X		Lowe	2	+	+	+	+	+	+			+		+	
00ls29	16	c.1692_1693insCCTT	p.Leu565ProfsX11		Lowe	12	+	+		+	+	+						
07ls20	17	c.1773C>A	p.Asn591Lys		Lowe	13	+	+	+	+	+				+	+		
03ls23	17	c.1780C>T	p.Gln594X		Lowe	0.9	+		+	+	+	+					+	
04ls21	i17	c.1879+5G>A	Splicing defect	0.3	Lowe	32	+				+/−							
00ls23	18	c.1927_1928delGT	p.Val643AsnfsX8	1.8	Lowe	0.3	+		+	+			+		+	+		
02ls35	18	c.2086G>T	p.Glu696X		Lowe	20	+			+	+		+			+		+
07ls10	18	c.2092delC	p.Pro681LeufsX66		Lowe	dead	+		+									
08ls011	19	c.2194dupC	p.Leu732PfsX38		Lowe	1	+	+	+	+	+	+	+			+		
02ls11	19	c.2200delG	p.Val734PhefsX8		Lowe	6	+		+			+						
08ls15	19	c.2224_2226delGTA	p.Val742del		Lowe	10	+	+	+	+	+	+	+	+	+	+	+	+
98ls54	20	c.2269C>T	p.Gln757X		Lowe	23	+		+	+	+	+/−	+	+	+	+	+	
05ls29	20	c.2311−2312insT	p.Cys771LeufsX8		Lowe	7	+		+	+	+	+	+			+	+	
09ls001					Lowe	2	+		+	+						+		
04ls17	21	c.2464C>T	p.Arg822X		Lowe	2	+	+	+	+			+			+		
05ls17	i21	c.2469+2T>G	Splicing defect	0.3	Lowe	1	+		+	+	+/−		+	+		+		
01ls19	i22	c.2581+1G>C	Splicing defect		Lowe	1.5	+	+										
03ls14	i22	c.2582−1G>A	Splicing defect		Lowe	23	+				+		+			+		
06ls65	i22	c.2582−2A>G	Splicing defect		Lowe	6	+		+	+	+	+	+		+	+		
08ls03	23	c.2591_2595del6	p.Gln879_Thr880>HisfsX4		Lowe	28	+	+	+	+	+		+		+	+		
02ls08	23	c.2637_2638del	p.Gln862HisfsX3		Lowe	12	+				+							
09ls013	23	c.2672T>G	p.Leu891Arg		Lowe	10	+		+	+			+		+			
06den06	3–4	del exon 3–4	Frameshift deletion		Dent	9	−		−	−	−		+			+		+
00ls05	19–23	del exons 19–23	Frameshift deletion	1.8	Lowe	30	+		+	+	+	+						

[a]Numbering was based on the cDNA sequence with +1 corresponding to the A of the ATG initiation codon of translation in the reference sequence.
[b]Expected consequences of the mutations with codon 1 corresponding to the ATG initiation codon of translation in exon 1.
[c]Enzyme activity measured in controls was $N = 9.6 \pm 2.3$ nanomoles $PI(4,5)P_2$ hydrolyzed/min/mg.
[d]Age at the time of the molecular investigation.
[e]CC, congenital cataract; G, glaucoma; NH, neonatal hypotonia; DD, developmental delay; MR, mental retardation; FS, Fanconi syndrome; PT, proximal tubulopathy; RF, renal failure; AA, amino-aciduria; LMWP, low molecular weight proteinuria; P, hypophosphatemia/hyperphosphaturia; Ca, hypercalciuria (+) sign present; (−) sign absent; ( ) sign not documented at the time of the last examination.
[f]Patients 02ls25 and 02ls26, and 10ls06 and 10ls017 are brothers.
[g]Variants c.1009C>T and c.1082G>T are on the same allele.

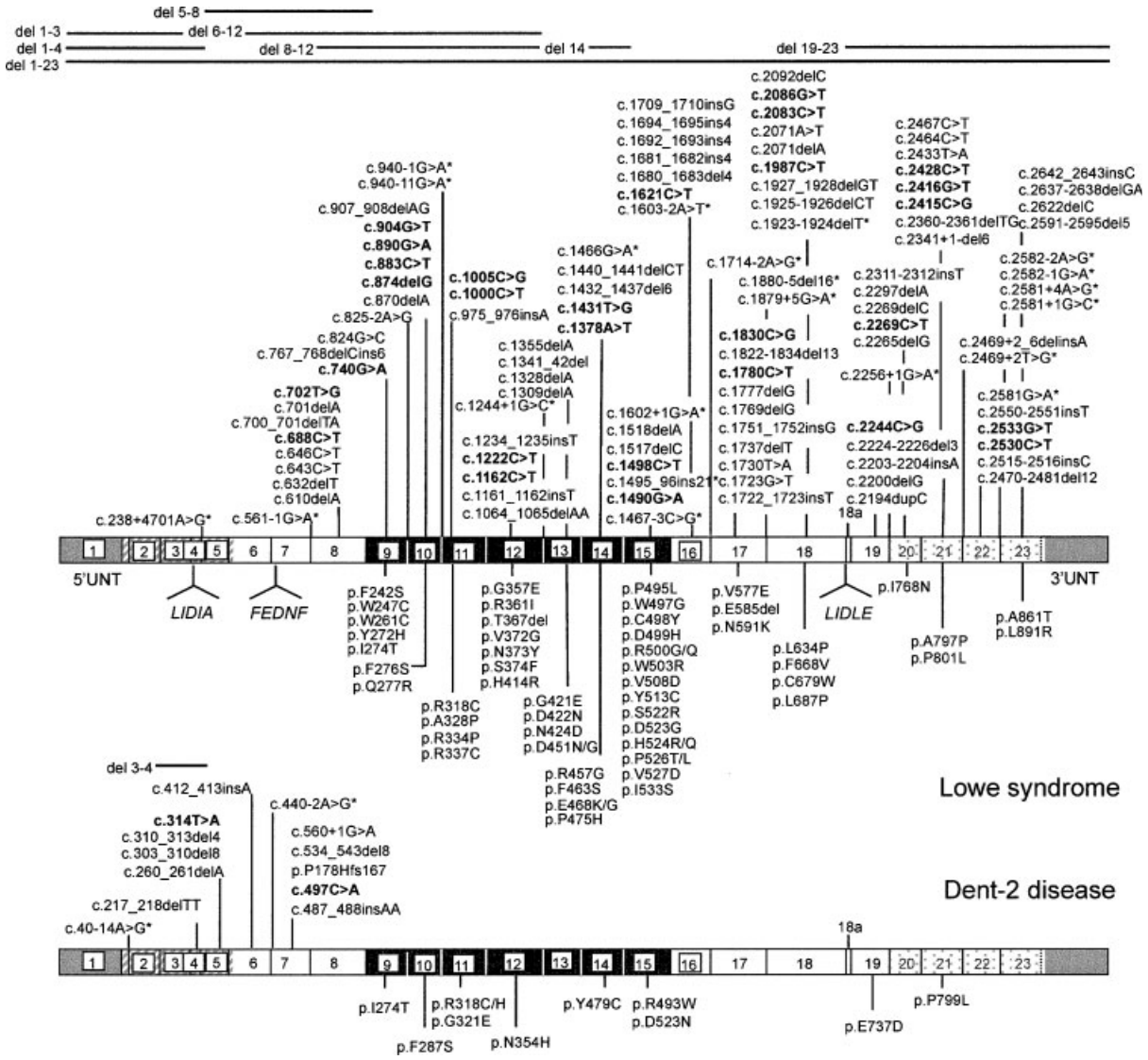

Figure 1. Mapping of mutations associated with Lowe syndrome and Dent disease in the *OCRL1* gene. Exons, including the alternatively spliced exon 18a, are numbered 1 to 23 and are drawn to scale at the exception of the noncoding sequence of exon 23. The catalytic phosphatase domain is indicated in black, the PH domain in hatched, the ASH-Rab binding domain in light gray, the Rho-GAP-like domain as a punctuated pattern and the 5′ and 3′ untranslated regions are shown in dark gray. LIDIA and LIDLE refer to motives involved in clathrin binding [Mazo et al., 2009] and FEDNF to amino acids involved in AP-2 clathrin adaptator binding [Ungewickell et al., 2004]. Frameshift and nonsense mutations are indicated using their cDNA numbering above the schematic representation of the cDNA, whereas missense mutations and in-frame deletions are presented below the cDNA and designated by their protein numbering. Mutations indicated in bold correspond to nonsense mutations. Stars refer to intronic mutations leading to splicing defects. Horizontal bars indicated gross genomic deletions.

patients (not shown). Residual activities measured in mutant fibroblasts were likely to correspond to nonspecific hydrolysis of PI $(4,5)P_2$ by other inositol polyphosphate phosphatases present in fibroblasts because they were also determined in cells fully devoid of OCRL1 as a consequence of a genomic deletion of the *OCRL1* gene ([Lin et al., 1997; Monnier et al., 2000]; this article).

OCRL1 protein analysis

The amount of OCRL1 was estimated in total cell extracts using either the total amount of protein or the β-ATPase signal for normalization. As shown in Figure 4, patients harboring a missense mutation in the *OCRL1* gene (lanes 2–6) had a markedly reduced content of OCRL1 when compared to controls (lanes 1, 7, 8, and 12). Two bands were revealed by antibodies, the upper band at ≈105 kDa corresponds to the native OCRL1, whereas the lower band at ≈75 kDa corresponds to a proteolytic product [Lichter-Konecki et al., 2006]. As expected, cells harboring stop and frameshift mutations and characterized by a low mRNA content showed an almost complete absence of OCRL1 (lanes 9, 10, and 11). For quantification studies of OCRL1 presented in Table 3, both bands were taken in account. Unexpectedly, most missense mutations resulted in a drastic decrease of the OCRL1 content, although their mRNA content was unaffected (Fig. 2). The decrease in OCRL1 was observed whether missense mutation mapped to the central phosphatase domain or to the C-terminal Rho-Gap domain (Fig. 4). This indicated that missense mutations not only could affect the catalytic properties of OCRL1 but also could affect the stability of the protein. Noticeably, expression of the c.40−14A>G resulted in the presence of a protein doublet at ≈80 kDa with no detectable bands at 100 or 75 kDa (Fig. 3B, lane 2).

Clinical Expression of Mutations

All but 2 of the 124 male patients that presented with classical features of the disease, that is, congenital bilateral cataract, central nervous system symptoms, and kidney troubles harbored a mutation in the *OCRL1* gene. However, as no cDNA analysis could be performed for these two patients, we cannot exclude the presence of a mutation not detected by genomic sequencing. At contrast, no mutation in the *OCRL1* gene was identified in the 36 additional male patients presenting with an incomplete panel of oculocerebrorenal symptoms although highly suggestive of Lowe syndrome. When tested, $PI(4,5)P_2$ phosphatase activity also excluded the diagnosis of Lowe syndrome in these patients.

Six out of the 41 patients included on the basis of diagnosis of Dent disease not associated to the *CLC5* gene carried a mutation of the *OCRL1* gene. None of these six patients had ocular or CNS symptoms.

A few mutations that mapped to the same amino acid or to amino acids located at close vicinity were identified in patients addressed either for diagnosis of Lowe syndrome or Dent disease (Table 4). The p.Ile274Thr initially described in a patient presenting with the phenotype of Dent disease [Utsch et al., 2006] was identified in two families: one presenting with a classical Dent disease and one diagnosed as a Lowe syndrome family in which the two affected brothers had mental retardation and developmental delay with one of them having a congenital cataract. Likewise, the p.Arg318Cys mutation previously reported in two families with Dent disease [Hoopes et al., 2005; Sekine et al., 2007] was identified in a patient presenting with Dent disease and in another patient initially diagnosed as Lowe syndrome patient on the basis of renal and CNS symptoms although ocular symptoms at 24 years of age were restricted to a severe myopia. Three mutations mapped to amino acids closely located in the Rho-GAP domain, that is, p.Ala797Pro, p.Pro799-Leu, and p.Pro801Leu. Although all the three mutations were associated with proximal tubulopathy, only two of them (p.Ala797Pro and p.Pro801Leu) were associated with congenital cataract and SNC symptoms, typical features of the Lowe syndrome.

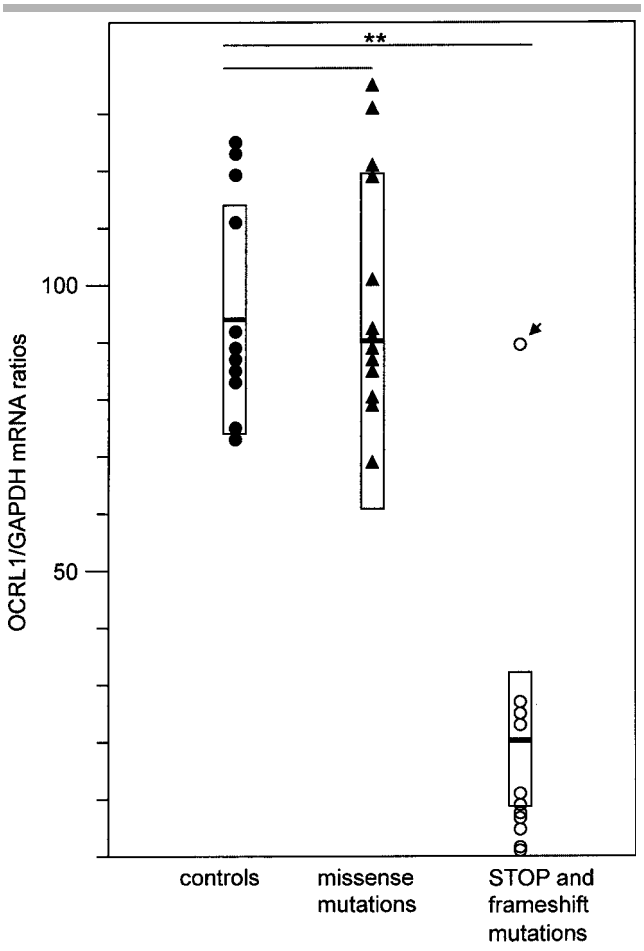

Figure 2. Quantification of mRNA OCRL1 expression in patients harboring a mutation of the *OCRL1* gene. OCRL1 and GAPDH mRNAs were isolated from fibroblasts and quantified as described in Materials and Methods. Horizontal black bar represent the mean and upper and lower boxes the 2 SD limits. Arrow points to the ratio value measured in a patient affected by Dent Disease and harbouring a c.40−14G > A mutation.

Table 2. $PI(4,5)P_2$ Phosphatase Activity in Patients with OCRL1 Mmutations

	Enzyme activity[a] (nanomol $PI(4,5)P_2$ hydrolyzed $mg^{-1}\,min^{-1}$) ($\pm$SEM)
Patients with missense mutations[b]	1.39 ± 0.99 ($n = 12$)
Patients with nonsense or frameshift mutations[b]	1.54 ± 0.63 ($n = 11$)
Patients with no OCRL1 mutation identified[c]	9.43 ± 1.47 ($n = 12$)
Control individuals	9.62 ± 2.31 ($n = 13$)

[a]*n* value represent the number of independent patients tested.
[b]Nineteen patients were affected by Lowe syndrome; 4 presented with Dent-2 disease.
[c]These patients were adressed to the laboratory for molecular and biochemical investigations on the basis of suspicion of Lowe syndrome.

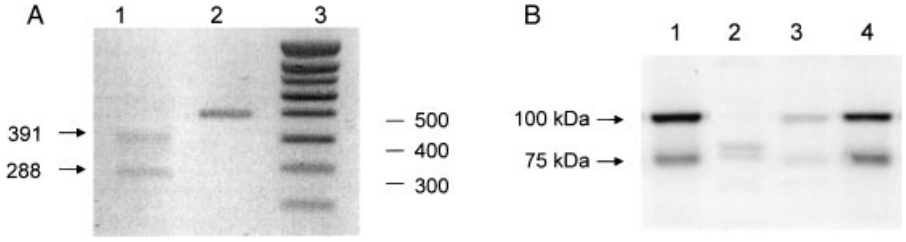

Figure 3. mRNA and protein expression of the c.40−14 A > G mutation. **A:** cDNA obtained from fibroblasts harbouring the c.40–14A > G mutation was amplified using primers allowing amplification of a sequence encompassing exons 1, 2, and 3. Amplification products were analyzed using a 2% agarose gel. Amplification of the mutant cDNA (lane 1) generated two bands respectively long of 288 and 391 bp (arrows), whereas amplification of the normal sequence (lane 2) generated a 488-bp fragment as expected. **B:** Western blotting of fibroblast extracts was performed as described in Materials and Methods. Lanes 1 and 4: control fibroblasts; lane 2: c.40−14A > G mutant fibroblasts; lane 3: p.Ile274Thr mutant fibroblasts.

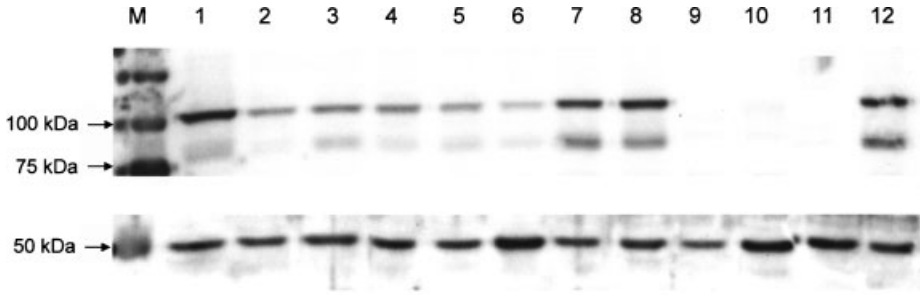

Figure 4. Western blot analysis of OCRL1 in control and patient fibroblasts. Fibroblasts extract (25 μg) prepared as described in Materials and Methods was loaded in each lane. Upper panel: Western blot analysis using polyclonal antibodies directed againts OCRL1. Lane M: molecular weight markers, lanes 1, 7, 8, and 12: control patients, lane 2: p.Glu468Gly, lane 3: p.Arg318Cys, lane 4: p.Asn373Tyr, lane 5: p.Pro801Cys, lane 6: p.Pro799Leu, lane 9: p. Arg810X, lane 10: c.2469+2T>G, lane 11: c.825−2A>G. Lower panel: protein loading in the different lanes was demonstrated by a Western blot analysis of the same blot with the use of antibody against the β-subunit of the mitochondrial ATPase.

Table 3. OCRL1 Content in Fibroblasts Originating from Patients with OCRL1 Mutations

	OCRL1/β ATPase[a] relative units (±SEM)
Patients with nonsense or frameshift mutations	0.05±0.02 ($n=11$)
Patients with missense mutations	0.35±0.14 ($n=12$)
Control individuals	1.70±0.36 ($n=13$)

[a]*n* value represents the number of independent patients tested.

Conclusions and Discussion

A major consequence of mutations affecting the *OCRL1* gene was the loss of PI(4,5)P_2ase activity regardless of the Lowe or Dent-2 clinical phenotypes associated with mutations in patients and regardless of the pathogenic mechanisms.

Nonsense mutations, genomic deletions, frameshift, or splicing mutations leading to the occurrence of a premature termination codon represent 2/3 of the OCRL1 mutations identified to date. Expression studies of these mutations indicated that all but one of the mutations tested resulted in an almost complete absence of mRNA and protein (Fig. 2 and Table 3).

Interestingly, the splicing c.40−14G>A mutation allowed an apparently normal quantitative production of mRNA. However, and as shown in Figure 3A, the mutation led to the synthesis of two abnormal transcripts lacking the normal initiation codon in exon 1 (see Supp. Fig. S2). As shown in Figure 3A, two peptides with a molecular weight close to 80 kDa were evidenced in fibroblasts harboring the c.40−14A>G mutation. Based on bioinformatics analyses, it has been recently proposed that a differential splicing of the *OCRL1* gene associated with the possible use of an alternative initiation of translation in exon 8 that would allow the synthesis of a shortened OCRL1 might occur in different tissues and at different developmental stages [Shrimpton et al., 2009]. Remarkably, frameshift and nonsense mutations associated so far with Dent-2 disease were exclusively identified in the first 7 exons of the OCRL1 gene, whereas all frameshift and nonsense mutations causing Lowe syndrome mapped to exons 8–23 (Fig. 1). There are only three methionyl residues in the sequence spanning exon 3 to 10 at position 158, 187, and 206, respectively in exons 7 and 8, that will keep the OCRL1 reading frame. As suggested by Shrimpton et al. [2009], use of these methionyl residues as alternative initiation codons will allow the synthesis of 85.425, 82.159, and 79.976 kDa peptides, respectively, molecular weights compatibles with the observed bands at ≈80 kDa in Figure 3. Such proteins will lack the PH domain and a major clathrin binding site [Mazo et al., 2009] and are likely to display modified interactions with membranes and other proteins.

Although most missense mutations mapped to the phosphatase domain of OCRL1 spanning exons 9–15, 14 missense mutations or in-frame deletion have been identified in the C-terminal ASH (ASPM, SPD-2, Hydin)-RhoGAP-like domain spanning exons 16 to 23 (Fig. 1). Eight mutations mapped to the ASH/Rab binding domain spanning exons 17–19 (amino acids 539–752) close to residues that have been shown to modulate Rab binding and targeting of OCRL1 to Golgi and endosomes [Hyvola et al., 2006]. The six missense mutations in exons 20–23 affected amino acids that mapped to a region in close interaction with the ASH domain [Erdmann et al., 2007]. Mutations in exon 21 clustered to a domain showing significant homologies with GAP related proteins (Supp. Fig. S1). Although interaction with Rac 1 a member of the Rho family has been reported [Erdmann et al., 2007] and low Rac GAP activity measured in vitro [Faucherre et al., 2003], whether OCRL1 functions as a GAP in vivo is still debated [Lowe, 2005]. Expression studies of the p.I768N and p.A797P mutations in COS7 cells suggested that the Rho–GAP-like domain is important for the enzymatic activity of OCRL1 while being catalytically inactive by itself and showed that this domain could also interact with small G-proteins of the Arf family [Lichter-Konecki et al., 2006]. Therefore, and as suggested by their effect on activity and OCRL1 content, these last two residues might play a critical role for the folding of the protein. The ASH–Rho–GAP-like domain has been recently shown to interact with the Rab5 effector APPL1 [Erdmann et al., 2007; McCrea et al., 2008] and with Ses1 and Ses2, two closely related endocytic proteins [Swan et al., 2010]. Isothermal titration calorimetry studies performed using peptides corresponding to the interacting domains of Ses1 and APPL1 with OCRL1 suggested that binding of Ses and APPL1 are mutually exclusive and abolished by the same mutations at positions 591, 634, 799, and 801 in the ASH–Rho–GAP-like domain that also disrupt APPL1 binding. This suggested that Lowe syndrome and Dent disease might result from perturbations at multiple sites within the endocytic pathway [Swan et al., 2010]. Noticeably, most variants affected amino acids conserved among OCRL1 species and INPP5B but much less conserved among other polyphosphate phosphatases. This may suggest that interaction with Rab proteins is a specific feature of OCRL1 and INPP5B.

The decrease of the PI(4,5)P_2ase activity in mutant cells harboring a missense mutation was usually associated with a deleterious effect of the missense mutations on phosphatase

Table 4. Clinical Expression of OCRL1 Mutations in Patients Affected by Lowe Syndrome or Dent Disease

Patient ID	Clinical diagnosis[a]	Mutation	Age[b]	PiP_2ase activity[c]	Ocular symptoms	SNC symptoms	Renal symptoms
98ls18	Lowe	del exons 1–4	3		Congenital bilateral cataract	Neonatal hypotonia, convulsions	Fanconi syndrome
06den06	Dent	del exon 3–4	14		None	None	LMW proteinuria, hypercalciuria
02ls25[d]	Lowe	p.Ile274Thr	43		None	Moderate mental retardation allowing an autonomous life	Nephrotic syndrome, proteinuria, renal failure
02ls26[d]	Lowe	p.Ile274Thr	34		Congenital unilateral cataract	Moderate mental retardation allowing an autonomous life	Renal failure, proteinuria
05den11	Dent	p.Ile274Thr	2		None	Developmental delay	Proximal tubulopathy, proteinuria, hypercalciuria
06ls59	Lowe	p.Arg318Cys	24		Severe myopia	Neonatal hypotonia, severe mental retardation	Fanconi syndrome, proteinuria, hyperphophaturia, renal failure
07den01	Dent	p.Arg318Cys	4		None	None	Tubular proteinuria, hypercalciuria
98ls80	Lowe	p.Ala797Pro	57	2.9	Congenital bilateral cataract	Moderate mental retardation and mild psychomotor troubles	Fanconi syndrome
04den05	Dent	p.Pro799Leu	21	0.8	None	None	LMW proteinuria, hypercalciuria, nephrocalcinosis, hyperphosphaturia, aminoaciduria
06ls27	Lowe	p.Pro801Leu	1	1.6	Congenital bilateral cataract	Neonatal hypotonia, moderate developmental delay, no psychomotor troubles	Glomerular and tubular proteinuria

[a]Initial clinical diagnosis.
[b]Age at the time of the molecular investigation.
[c]Enzyme activity measured in controls was $N = 9.6 \pm 2.3$ nanomoles $PI(4,5)P_2$ hydrolyzed/min/mg.
[d]Patients 02ls25 and 02ls26 are brothers.

activity, whether they mapped to the catalytic $PI(4,5)P_2$ase domain or to the Rho–GAP domain [Hyvola et al., 2006; Lin et al., 1997; Shrimpton et al., 2009]. However, and as illustrated in Figure 4 and Table 3, the consequence of missense mutations is mostly a decrease of the amount of OCRL1 present in the cell. This is likely to result from an abnormal processing of the protein since OCRL1 mRNA levels were normal in the cells harboring these missense mutations (Fig. 2). The protein degradation might be the consequence of the activation of endoplasmic reticulum-associated degradation (ERAD) or of unfolded protein response (UPR) as an answer to the accumulation of unfolded or misfolded mutant OCRL1 in the endoplasmic reticulum [Schröder and Kaufman, 2005]. Conclusions of functional studies based on expression mutant OCRL1 in cellular models must thus take in account protein degradation processes when analyzing the physiological relevance of the different mutations. Nevertheless, a small amount (20–30%) of OCRL1 was detected in a few mutant cells although $PI(4,5)P_2$ase activity was almost totally absent. In these situations, the overall decrease of $PI(4,5)P_2$ase activity is likely to result from two processes: a decrease of the amount of OCRL1 through protein degradation and a catalytic inactivation. At contrast a few missense mutations, for example, p.Phe242Ser and p.Ala797Pro, resulted in the presence of both a small amount of protein and a residual PiP_2ase activity. Noticeably, although the Lowe patients harboring these mutations presented with classical eye and kidney features, their neurological symptoms were less severe and their degree of mental retardation was moderate. This may reflect a differential dependence of tissues or of developmental stages toward $PI(4,5)P_2$ homeostasis. Differences in clinical expression that were observed between patients harboring different mutations might thus reflect variability in protein synthesis and/or catalytic properties in the different tissues depending on the nature of the mutation beside variability of the individual genetic background.

Mutations of the *OCRL1* gene have been associated with two distinct clinical phenotypes, that is, Lowe syndrome and Dent disease. Although apparently undistinguishable from the Dent phenotype associated to the *CLCN5* gene, Dent phenotype associated with OCRL1 mutations has been referred as Dent-2 disease to specify the genetic cause of the disease and may represent a mild variant of Lowe syndrome [Bökenkamp et al., 2009]. Accordingly, it has been proposed that a phenotypic continuum exists between Dent-2 disease and Lowe syndrome. This continuum was not only observed between patients harboring different OCRL1 mutations but also occurred between patients harboring the same mutation as presented in Table 4. Clinical phenotypes range from patients affected by severe Lowe syndrome with typical ocular, neurological, and renal features to Dent-2 patients presenting only with renal impairment and comprise atypical forms of Lowe syndrome presenting with incomplete eye symptoms or moderate neurological troubles. Presence of a residual $PI(4,5)P_2$ase activity has been documented during this study in patients presenting usually with a moderate clinical expression. However, it must be noted that ocular and neurological symptoms were documented even in Lowe patients showing a residual $PI(4,5)P_2$ase activity up to 20% of the normal values in their fibroblasts. On the other hand, it must be kept in mind that mild and severe presentations of Lowe syndrome together with pure renal forms of Dent disease were associated with a complete loss of the OCRL1 enzymatic activity. A possible explanation of this clinical variability might be the presence of modifying factors (compensatory phosphatases, interacting proteins, etc.) whose expression will depend on the genetic

background of the different patients. The main clinical difference observed between two brothers presenting with a Lowe syndrome and harboring the same p.Ile274Thr mutation, for example, a documented unilateral cataract for the youngest and no signs of cataract for the eldest one would thus likely result from such modifying factors. Along this line we have investigated a possible role of INPP5B, a closely related phosphatase. No significant variations in the INPP5B content could be evidenced when comparing fibroblast extracts originating from healthy controls or patients affected either by Lowe syndrome or Dent-2 disease. This was in agreement with our data showing that no significant differences of the PiP2-ase activity could be evidenced between Lowe syndrome and Dent-2 disease patients (Table 2).

The report of truncated forms of OCRL1 that might result from the use of alternative initiation codons open also new perspectives to explain clinical variability associated with OCRL1 mutations. However, these investigations were performed on skin fibroblasts and using antibodies that may not recognize all forms of truncated or alternatively spliced OCRL1. It will be very important to extend these studies to other cell types that are more affected by the disease, for example, renal and ocular cells and to use a larger panel of antibodies directed against different domains of the protein in order to gain new insights regarding the differential pathological mechanisms leading to the different clinical phenotypes.

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