OCRL-mutated fibroblasts from patients with Dent-2 disease exhibit INPP5B-independent phenotypic variability relatively to Lowe syndrome cells

Rodrick Montjean1,2,3, Rifdat Aoidi1,2,3, Pierrette Desbois1,2,3, Julien Rucci1,2,3, Michaël Trichet1,2,3, Rémi Salomon4, John Rendu5, Julien Fauré5, Joël Lunardi5, Gérard Gacoin1,2,3, Pierre Billuart1,2,3 and Olivier Dorseuil1,2,3,*

1INSERM U1016, Institut Cochin, Paris, France, 2CNRS UMR8104, Paris, France, 3Université Paris Descartes, Paris, France, 4Service de Néphrologie Pédiatrique, Hôpital Necker Enfants Malades, Paris, France and 5Laboratoire de Biochimie et Génétique Moléculaire, CHU de Grenoble, Grenoble, France

Received August 14, 2014; Revised and Accepted October 3, 2014

OCRL mutations are associated with both Lowe syndrome and Dent-2 disease, two rare X-linked conditions. Lowe syndrome is an oculo-cerebro-renal disorder, whereas Dent-2 patients mainly present renal proximal tubulopathy. Loss of OCRL-1, a phosphoinositide-5-phosphatase, leads in Lowe patients’ fibroblasts to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) accumulation, with defects in F-actin network, α-actinin distribution and ciliogenesis, whereas fibroblasts of Dent-2 patients are still uncharacterized. To search for mechanisms linked to clinical variability observed between these two OCRL mutation-associated pathologies, we compared dermal fibroblasts from independent patients, four affected by Dent-2 disease and six with Lowe syndrome. For the first time, we describe that Dent-2 fibroblasts with OCRL loss-of-function (LOF) mutations exhibit decrease in actin stress fibers, appearance of punctate α-actinin signals and alteration in primary cilia formation. Interestingly, we quantified these phenotypes as clearly intermediate between Lowe and control fibroblasts, thus suggesting that levels of these defects correlate with clinical variations observed between patients with OCRL mutations. In addition, we show that Lowe and Dent-2 fibroblasts display similar PI(4,5)P2 accumulation levels. Finally, we analyzed INPP5B, a paralogous gene already reported to exhibit functional redundancy with OCRL, and report neither differences in its expression at RNA or protein levels, nor specific allelic variations between fibroblasts of patients. Altogether, we describe here differential phenotypes between fibroblasts from Lowe and Dent-2 patients, both associated with OCRL LOF mutations, we exclude direct roles of PI(4,5)P2 and INPP5B in this phenotypic variability and we underline potential key alterations leading to ocular and neurological clinical features in Lowe syndrome.

INTRODUCTION

Patients with oculo-cerebro-renal syndrome of Lowe (OMIM 309000) present a rare X-linked disorder that causes mental and physical handicaps. Lowe syndrome is characterized in almost all the cases by a triad of cardinal clinical features, i.e. ocular signs including congenital cataract usually associated with glaucoma and corneal keloids appearing in the first years of life, neurological symptoms, including neonatal hypotonia, intellectual disability, behavioral disturbance and susceptibility to epileptic seizures, and at the kidney level a renal proximal tubulopathy associated with growth delay and usually resulting in slowly progressive chronic renal failure and end-stage renal disease. The vast majority of patients with Lowe syndrome display various types of loss-of-function (LOF) mutations of the OCRL gene with nearly 200 different genetic alterations described so far (1,2). Patients suffering from Dent disease, another X-linked rare genetic condition historically associated with mutations of the CLCN5 gene, present exclusive renal

*To whom correspondence should be addressed at: Institut Cochin, Département de Développement, Reproduction, Cancer, 24 rue du Faubourg St. Jacques, 75014 Paris, France. Tel: +33 144412480; Fax: +33 144412448; Email: olivier.dorseuil@inserm.fr

© The Author 2014. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
features primarily concerning proximal tubular defects, including low molecular weight proteinuria and hypercalciuria (3). More recently, it has been shown that mutations of the OCRL gene may also be associated with the Dent disease, illustrating the genetic heterogeneity of this disease (4–8). Thus, Dent disease caused by OCRL mutations, also called Dent-2 disease (OMIM 300555), has been considered as a mild variant of Lowe syndrome, with minor ocular and cerebral features when present and with renal manifestations such as in the classical Dent disease. In addition, we recently reported two Dent patients without alterations in the CLCN5 gene but carrying mutations in the OCRL gene at the same position as previously reported for Lowe patients, thus extending the phenotypic variability of OCRL mutations (2,5,6,8). Overall, we and others have described a clinical continuum between these two extreme conditions linked to OCRL mutations, with some patients exhibiting intermediate traits at the oculo and cerebral level, thus suggesting existence of various compensating factors and notably a role of the genetic background in such phenotypic variability (2,9).

The OCRL gene encodes a 105 kDa protein exhibiting inositol polyphosphate 5-phosphatase activity that preferentially hydrolyzes the PI(4,5)P2 (10). In accordance with this enzymatic activity, PI(4,5)P2 accumulation is observed as a consequence of OCRL-1 loss, in kidney cells and in fibroblasts derived from Lowe patients (11–13). OCRL-1, which appears central to phosphoinositide metabolism, is also a multi-domain protein able to interact with several Rho and Rab small GTPases, the AP-2 adaptor subunit α-adaptin, clathrin heavy chain, APPL1 and INPP5B A and B (11,14–20). These molecules are associated with the various subcellular localizations of the OCRL-1 protein, such as trans-Golgi network, early and signaling endosomes, clathrin-coated pits and plasma membrane ruffles (11,14,20–23).

A large spectrum of cellular processes has been attributed to OCRL-1, including dynamics of actin polymerization, endocytosis, retrograde vesicular trafficking, cell migration, cell adhesion, cytokinesis and ciliogenesis, which are all in accordance with its diverse subcellular localizations (15,24–28). Analysis of cells from Lowe patients generated important results, e.g. pioneer work on dermal fibroblasts has revealed shortening in β-actinin distribution (26). Subsequent studies using kidney cell lines from patients have also shown alterations in actin cytoskeleton network as well as defects in vesicular trafficking and cytokinesis (15,27). Recently, the Lowe syndrome has been revisited as a particular ciliopathy, since zebrafish ocrl morphants show developmental anomalies observed in Lowe patients (9,30). The paralogous gene present in the human genome, has been suspected in these clinical features characteristic of Dent-2 and Lowe patients (9,31). INPP5B displays inositol 5-phosphatase activity and has already been shown to exhibit functional redundancy with OCRL-1 in ex vivo experiments as well as in vivo in the mouse (32,33), but its implication in human pathology still deserves experimental investigations.

To better understand the clinical variability observed between Dent-2 and Lowe patients bearing OCRL mutations and to search for molecular mechanisms involved, we isolated and characterized dermal fibroblasts from these two types of patients. We explored actin polymerization, α-actinin distribution, primary cilia formation and PI(4,5)P2 content in a large number of primary cells derived from 10 patients. We observed that OCRL mutations underlying both Lowe syndrome and Dent-2 disease in our patients lead to a dramatic loss in OCRL-1 protein. We next showed unexplained abnormalities from Dent-2 patients’ cells, i.e. significant defects in filamentous actin network, α-actinin staining and primary cilia formation. Since these alterations appear for all of them clearly milder than what we observed in Lowe patients’ cells, we investigated the potential involvement of PI(4,5)P2 and INPP5B in this situation. The role of the various molecular defects observed in triggering ocular and neurological clinical features of Lowe patients is discussed.

RESULTS

Characterization of OCRL gene mutations in Dent-2 and Lowe patients’ skin fibroblasts

In the aim to search for mechanisms linked to clinical variability observed between Dent-2 disease and Lowe syndrome, we explored and compared dermal fibroblasts from both types of OCRL-altered patients. We selected non-related patients in whom we previously reported OCRL mutations, presenting archetypal symptoms, i.e. exclusive kidney traits for Dent-2 patients and oculo-cerebro-renal signs for Lowe patients, and for which skin fibroblasts were available. Cells from patients with Dent-2 disease were obtained from four probands, affected for which skin fibroblasts were available. Cells from patients with Dent-2 disease were obtained from four probands, affected with Dent-2 disease were obtained from four probands, affected with Dent-2 disease were obtained from four probands, affected with Dent-2 disease were obtained from four probands. We also included two patients bearing mutations in the CLCN5 gene but carrying mutations in the OCRL gene at the same position as previously reported for Dent-2 and Lowe patients bearing OCRL mutations, including the ocular manifestations (2,5,6,8). Overall, we and others have described a clinical continuum between these two extreme conditions linked to OCRL mutations, with some patients exhibiting intermediate traits at the ocular and cerebral level, thus suggesting existence of various compensating factors and notably a role of the genetic background in such phenotypic variability (2,9).

The OCRL gene encodes a 105 kDa protein exhibiting inositol polyphosphate 5-phosphatase activity that preferentially hydrolyzes the PI(4,5)P2 (10). In accordance with this enzymatic activity, PI(4,5)P2 accumulation is observed as a consequence of OCRL-1 loss, in kidney cells and in fibroblasts derived from Lowe patients (11–13). OCRL-1, which appears central to phosphoinositide metabolism, is also a multi-domain protein able to interact with several Rho and Rab small GTPases, the AP-2 adaptor subunit α-adaptin, clathrin heavy chain, APPL1 and INPP5B A and B (11,14–20). These molecules are associated with the various subcellular localizations of the OCRL-1 protein, such as trans-Golgi network, early and signaling endosomes, clathrin-coated pits and plasma membrane ruffles (11,14,20–23).

A large spectrum of cellular processes has been attributed to OCRL-1, including dynamics of actin polymerization, endocytosis, retrograde vesicular trafficking, cell migration, cell adhesion, cytokinesis and ciliogenesis, which are all in accordance with its diverse subcellular localizations (15,24–28). Analysis of cells from Lowe patients generated important results, e.g. pioneer work on dermal fibroblasts has revealed shortening in actin stress fibers with defects in α-actinin distribution (26). Subsequent studies using kidney cell lines from patients have also shown alterations in actin cytoskeleton network as well as defects in vesicular trafficking and cytokinesis (15,27). Recently, the Lowe syndrome has been revisited as a particular ciliopathy, since zebrafish ocrl morphants show developmental abnormalities typically associated with defects in assembly of primary cilia, but also atypical signs in eyes and kidneys (24). Independent studies have also described that OCRL-1-depleted canine kidney cell lines, zebrafish ocrl morphants and dermal fibroblasts from Lowe patients display alterations of ciliogenesis (24,28,29). Despite the recent discoveries of all these functions attributed to OCRL-1, the exact molecular mechanisms triggering the clinical features of the Lowe syndrome still remain unclear. Vesicular trafficking defects probably underlie the renal proximal tubulopathy, but the mechanisms involved in ocular and neurological signs remain elusive (9,30). Similarly, the clinical variability associated with OCRL mutations or the restriction of clinical traits to specific organs in Lowe syndrome is still to be explored. A role for the INPP5B gene, the only OCRL

...features primarily concerning proximal tubular defects, including low molecular weight proteinuria and hypercalciuria (3). More recently, it has been shown that mutations of the OCRL gene may also be associated with the Dent disease, illustrating the genetic heterogeneity of this disease (4–8). Thus, Dent disease caused by OCRL mutations, also called Dent-2 disease (OMIM 300555), has been considered as a mild variant of Lowe syndrome, with minor ocular and cerebral features when present and with renal manifestations such as in the classical Dent disease. In addition, we recently reported two Dent patients without alterations in the CLCN5 gene but carrying mutations in the OCRL gene at the same position as previously reported for Lowe patients, thus extending the phenotypic variability of OCRL mutations (2,5,6,8). Overall, we and others have described a clinical continuum between these two extreme conditions linked to OCRL mutations, with some patients exhibiting intermediate traits at the oculo and cerebral level, thus suggesting existence of various compensating factors and notably a role of the genetic background in such phenotypic variability (2,9).

The OCRL gene encodes a 105 kDa protein exhibiting inositol polyphosphate 5-phosphatase activity that preferentially hydrolyzes the PI(4,5)P2 (10). In accordance with this enzymatic activity, PI(4,5)P2 accumulation is observed as a consequence of OCRL-1 loss, in kidney cells and in fibroblasts derived from Lowe patients (11–13). OCRL-1, which appears central to phosphoinositide metabolism, is also a multi-domain protein able to interact with several Rho and Rab small GTPases, the AP-2 adaptor subunit α-adaptin, clathrin heavy chain, APPL1 and INPP5B A and B (11,14–20). These molecules are associated with the various subcellular localizations of the OCRL-1 protein, such as trans-Golgi network, early and signaling endosomes, clathrin-coated pits and plasma membrane ruffles (11,14,20–23).

A large spectrum of cellular processes has been attributed to OCRL-1, including dynamics of actin polymerization, endocytosis, retrograde vesicular trafficking, cell migration, cell adhesion, cytokinesis and ciliogenesis, which are all in accordance with its diverse subcellular localizations (15,24–28). Analysis of cells from Lowe patients generated important results, e.g. pioneer work on dermal fibroblasts has revealed shortening in actin stress fibers with defects in α-actinin distribution (26). Subsequent studies using kidney cell lines from patients have also shown alterations in actin cytoskeleton network as well as defects in vesicular trafficking and cytokinesis (15,27). Recently, the Lowe syndrome has been revisited as a particular ciliopathy, since zebrafish ocrl morphants show developmental abnormalities typically associated with defects in assembly of primary cilia, but also atypical signs in eyes and kidneys (24). Independent studies have also described that OCRL-1-depleted canine kidney cell lines, zebrafish ocrl morphants and dermal fibroblasts from Lowe patients display alterations of ciliogenesis (24,28,29). Despite the recent discoveries of all these functions attributed to OCRL-1, the exact molecular mechanisms triggering the clinical features of the Lowe syndrome still remain unclear. Vesicular trafficking defects probably underlie the renal proximal tubulopathy, but the mechanisms involved in ocular and neurological signs remain elusive (9,30). Similarly, the clinical variability associated with OCRL mutations or the restriction of clinical traits to specific organs in Lowe syndrome is still to be explored. A role for the INPP5B gene, the only OCRL...
producing p.Phe276Ser and p.Ala861Thr) and a splicing defect has been noticed in Lowe2 and Lowe5, which both bear the same splicing mutation (c.940-11G>A) affecting intron 10 and predicted to produce a premature termination of translation. Lowe4 (c.2581G>A) also presents a partial splicing defect as the mutation additionally affects the consensus 5′ splice of intron 22. A frameshift mutation is detected in exon 23 of Lowe3 (c.2622delC), inducing a potential OCRL-1 hybrid protein truncated of 27 residues in C-ter and replaced by an extension of 34 new residues (p.Asn874AsnfsX34). Finally a deletion of exons 19 to 23 of the OCRL gene is detected in Lowe6 and predicted to produce a C-terminally truncated protein.

We carried out characterization of OCRL mutations by measuring the OCRL-1 protein content in lysates from all Lowe and Dent-2 fibroblasts. Using a specific antibody, we observed by western blotting that all mutations induce a dramatic decrease of OCRL-1 as compared with control cells, both in Dent-2 and Lowe fibroblasts (Fig. 1B). Cells even exhibit a complete loss of OCRL-1, except for fibroblasts from Lowe1 and Dent1 that show a residual protein content estimated to be ~5 and 15% compared with control cells. This result is in agreement with our previous observations highlighting that pathological OCRL mutations classically induce messenger RNA and/or protein instability, even for missense mutations (2). Our data are also consistent with reports showing that fibroblasts from either Lowe or Dent-2 patients with OCRL mutations exhibit a marked reduction in PI(4,5)P2 phosphatase activity, 5–20% comparatively to controls, but with no difference between them (2,7). They also confirm that most mutations of Lowe syndrome and Dent-2 disease correspond to loss-of-function (LOF) mutations, explained by disappearance of the OCRL-1 protein. Interestingly, this apparent complete loss of OCRL-1 protein in fibroblasts...
from both Dent-2 and Lowe patients indicates that ocular and neurological features in Lowe patients are unlikely to be due to persistence of an altered OCRL-1 protein, with incomplete functions, but rather mainly suggests existence of modifying factors modulating the clinical consequences of OCRL mutations.

Human dermal fibroblasts (HDF) from Lowe patients have been previously shown to present obvious alterations of their filamentous actin (F-actin) and α-actinin distributions and defects in primary cilia formation, as well as an increase in PI(4,5)P₂ content (24,26,28). We thus characterized HDF from Dent-2 patients with OCRL mutations in order to describe at the cellular level their potential phenotypic defects as compared with cells from controls and Lowe patients.

**Abnormalities of F-actin network in fibroblasts from Dent-2 patients**

We compared cellular phenotypes regarding F-actin network of HDFs from Dent-2 and Lowe patients to controls. Cells in culture were immunostained with phalloidin, which specifically reveals the filamentous actin cytoskeleton. Cells were scored according to their classification into distinct groups as already described for Lowe patient fibroblasts (26). Type ‘a’ cells show primarily densely packed and long transversal actin stress fibers whereas type ‘c’ cells exhibit mainly punctate F-actin staining in the center of the cell and almost no detectable transversal stress fibers. Fibroblasts with an intermediate phenotype, both structures of F-actin detected in significant proportions, were representative of type ‘b’ (Fig. 2A). In four blind and independent experiments, we analyzed the F-actin pattern of the fibroblasts from the three controls (C1–C3), the six Lowe patients (L1–L6) and the four Dent-2 patients (D1–D4). The various cell lines were scored and results are presented with a histogram for each patient (Fig. 2B). Data show an interesting homogeneity according to patient clinical status (Fig. 2C).

Lowe fibroblasts exhibit a significantly altered F-actin network, with a weak proportion of cells with long fibers (14% of type ‘a’ compared with 52% for controls) and enrichment in punctate actin structures (22% of cells of type ‘c’ compared with 7% for controls) (Fig. 2C). These observations are consistent with the abnormalities of stress fibers previously reported in fibroblasts of Lowe patients (26). In contrast, although Dent-2 fibroblasts also present a significant alteration of their actin network compared with controls, with a clear decrease of cells with long fibers (33% of type ‘a’ versus 52% for controls) and an increase of cells with F-actin fibers and punctate structures (61% of type ‘b’ versus 41% for controls), this phenotype appears milder than in Lowe cells. Fibroblasts from Dent-2 patient exhibit an enrichment of long fibers of actin comparatively to Lowe patient (33% versus 14% for cells of type ‘a’) and a decrease of punctate structures (6% versus 22% for cells of type ‘c’). This illustrates that the actin network observed in

**Figure 2.** Actin network is altered in fibroblasts from Dent-2 patients. F-actin is visualized in situ by staining with Alexa555-labelled-phalloidin. (A) Patterns of actin network. F-actin staining allowed us to score in blind experiments the fraction of cells belonging to one of the three subclasses illustrated by images a, b and c, based on differential presence of transversal stress fibers and punctate F-actin staining in the center of the cell. Scale bar: 10 μm. (B) Dent-2 fibroblasts exhibit alteration of the F-actin pattern. F-actin network was analyzed in control fibroblasts (C1–C3), Lowe fibroblasts (L1–L6) and Dent-2 fibroblasts (D1–D4). Histograms represent the average of four independent experiments with >50 cells of each patient examined per experiment. (C) Histograms represent the average ± SEM of data expressed per clinical status, with >600 cells of each status examined. ***χ² test with P < 0.001.
cells from Dent-2 patients is less affected, meaning fibers are thicker, longer and more densely packed than in fibroblasts of Lowe patients. Interestingly, statistical comparison of the actin network distribution from cells of different clinical status significantly indicate that the actin phenotype of Dent-2 cells represents a distinctive phenotype of F-actin dissolution ($\chi^2$ test, $P$ value < 0.001), i.e. an intermediate pattern between control and Lowe patterns (Fig. 2C).

Taken together, these data demonstrate for the first time that fibroblasts from Dent-2 patients with OCRL mutations show an alteration of the actin cytoskeleton network that is distinct from control cells but also from defects observed in cells of Lowe patients. The phenotype of Dent-2 HDFs is intermediate compared with Lowe and controls, suggesting a correlation between severity of this defect and clinical variations associated to OCRL mutations. We conclude that fibroblasts from patients represent an appropriate model to further study the molecular basis of the mechanisms involved in the compensatory phenomenon existing between Dent-2 and Lowe patients.

**Alteration of alpha-actinin distribution in fibroblasts from Dent-2 patients**

We next analyzed $\alpha$-actinin, an actin binding protein involved in F-actin network regulation, with a particular focus on its distribution in fibroblasts from controls (C1–C3), Lowe patients (L1–L2, L4–L6) and Dent-2 patients (D1–D4). Cells were scored into two classes based on the pattern of $\alpha$-actinin labeling as previously described for Lowe fibroblasts (26). HDFs displaying an $\alpha$-actinin signal mainly in focal adhesion sites and along actin stress fibers were categorized as type ‘a’ cells whereas those showing a distinctive accumulation of punctate staining in the middle of the cell were cataloged as type ‘b’ (Fig. 3A). Results are expressed, similarly to previous analyses, as the mean of four independent experiments performed blind to clinical status and are presented with histograms for each patient (Fig. 3B). The average values are also presented per clinical status (Fig. 3C). We first noticed that Lowe HDFs display a dramatic decrease of type ‘a’ staining (17% versus 85% in controls) corresponding to a massive increase of punctate $\alpha$-actinin staining (83% versus 15% in controls), in line with the published abnormal accumulation of punctate labeling in fibroblasts from Lowe patients (26). We noticed that Dent-2 HDFs were also altered, although they appear less affected than Lowe HDFs (Fig. 3C). Indeed, 41% of Dent-2 fibroblasts show the aggregated pattern (type ‘b’) while this pattern is present for 15% and 83% in controls and Lowe HDFs, respectively. Accordingly, statistical comparisons confirmed that Dent-2 HDFs present a distinctive and robust alteration of the $\alpha$-actinin staining, which is significantly different from the pattern observed in controls and Lowe HDFs ($P < 0.001$).

Consistent with F-actin disorganization described above, these data demonstrate that Dent-2 fibroblasts show an alteration of the actin cytoskeleton network. This alteration is distinct from control cells but also from defects observed in fibroblasts of Lowe patients. The phenotype of Dent-2 HDFs is intermediate compared with Lowe and controls, suggesting a correlation between severity of this defect and clinical variations associated to OCRL mutations. We conclude that fibroblasts from patients represent an appropriate model to further study the molecular basis of the mechanisms involved in the compensatory phenomenon existing between Dent-2 and Lowe patients.

**Figure 3.** Alpha-actinin staining is disordered in fibroblasts from Dent-2 patients. (A) Profile of $\alpha$-actinin distribution. $\alpha$-Actinin staining was analyzed using a specific antibody to determine the fraction of cells exhibiting an even distribution of signals (a) or punctate stainings (b). Scale bar: 10 $\mu$m. (B) Dent-2 fibroblasts display perturbation of $\alpha$-actinin staining. The staining of $\alpha$-actinin was analyzed in fibroblasts from controls (C1–3), Lowe patients (L1–2 and L4–6) and Dent-2 patients (D1–4). Cells of each patient were scored into ‘a’ and ‘b’ subclasses for their pattern of $\alpha$-actinin staining as illustrated above. Histogram represents the average of four independent experiments with >50 cells of each patient examined per experiment. (C) Histograms represent the average ± SEM of data expressed per clinical status, with >600 cells of each status examined. ***$\chi^2$ test with $P < 0.001$. 

Downloaded from https://academic.oup.com/hmg/article-abstract/24/4/994/617269 by guest on 24 November 2018
unprecedented disorganization of the α-actinin staining, which we classify as an intermediate level between the pattern of controls and the pattern of Lowe cells.

**Defect of primary cilia formation in dermal fibroblasts from Dent-2 patients**

We then investigated primary cilia formation, another cellular process altered in fibroblasts from Lowe patients (24,28). We triggered cilia formation from HDFs in culture by a short serum starvation. The presence of the primary cilium was detected by immunofluorescence using anti-acetylated-tubulin antibody (Fig. 4A). HDFs from controls (C1–C3), Lowe (L1–L6) and Dent-2 patients (D1–D4) were scored for two parameters, the percentage of ciliated cells (Fig. 4B) and the length of cilia (Fig. 4C). Cilia length was classified into three groups as reported before (39). In the first class, cells exhibit a cilium shorter than 2 μm, the second class corresponds to an

---

**Figure 4.** Primary cilia abnormality in fibroblasts from Dent-2 patients. Primary cilia are revealed by immunofluorescence using an acetylated-tubulin antibody. Cilia were analyzed in fibroblasts from controls (C1–3), Lowe (L1–6) and Dent-2 patients (D1–4). (A) A representative primary cilium is shown for fibroblasts of controls and of Lowe and Dent-2 patients. Scale bar: 10 μm. (B) Dent-2 fibroblasts show ciliation defect. The percentage of ciliated cells was analyzed among a population of fibroblasts. Histograms on the left represent the average of three independent experiments with >50 cells of each patient examined per experiment. Histograms from the right panel show the average value ± SEM of data expressed per clinical status, with >450 cells of each type examined. (C) Dent-2 fibroblasts present shorter cilia. Cilia length was subdivided into three classes, the first for cilia shorter than 2 μm, the second for cilia between 2 and 4 μm and the third for cilia longer than 4 μm. Histograms on the left show the average of three independent experiments, with >50 cells of each patient examined per experiment. Histograms from the right panel represent the average ± SEM of data expressed per clinical status, with >450 cells of each type examined. **χ² test with P < 0.001.**
intermediate size with cillum length between 2 and 4 μm, while the third class represents cells with a cillum longer than 4 μm. The results are expressed as the mean of three independent experiments for HDFs of each patient (Fig. 4B and C, left panels) and as the average values per clinical status (Fig. 4B and C, right panel).

HDFs from Lowe patients clearly display an abnormal phenotype, with most of the cells displaying no cillum in our experimental conditions (62% versus 29% for controls, Fig. 4B, right panel). When present, cilia are shorter than in controls, e.g. only a few cells display a cillum longer than 4 μm (1.5% versus. 25% for controls, Fig. 4C, right panel). These results illustrate defects in primary cilia formation of Lowe HDFs and are similar to previous reports about the same type of cells from Lowe patients (24,28). However, we noticed that HDFs from Dent-2 patients also present ciliation and ciliogenesis defects, leading to shorter primary cilia than in controls, which have not been reported so far. Dent-2 fibroblasts display no primary cilia in 41% of the observations versus 29% for controls (Fig. 4B, right panel) and a cillum longer than 4 μm is observed in only 11% of cells versus 25% for controls (Fig. 4C, right panel). In addition, these defects in primary cilia formation from Dent-2 cells seem clearly weaker than what we described in Lowe HDFs. Statistical analysis strengthen the observations by showing that fibroblasts from Dent-2 patients display significant defects in ciliation and ciliogenesis compared with controls (P < 0.001) and Lowe cells (P < 0.001).

These data, demonstrating that Dent-2 fibroblasts show an obvious cellular alteration of the primary cillum, illustrate again that cells from Dent-2 patients display a phenotype of intermediate severity between control and Lowe phenotypes.

Equivalent PI(4,5)P2 accumulation in fibroblasts from Dent-2 and Lowe patients

PI(4,5)P2 is known to be involved in actin remodeling through modulation of various actin-binding proteins (40,41), it inhibits activity of α-actinin (42) and it is thought to play a role in ciliogenesis (43–45). We thus explored whether fibroblasts from Dent-2 patients, consistent with the phenotypes described above, display an abnormal PI(4,5)P2 accumulation as already demonstrated for Lowe patients (11,12).

PI(4,5)P2 content was specifically revealed by immunofluorescence on fixed cells, using a well characterized anti-PI(4,5)P2 antibody recently reported by Hammond et al. (46,47). The intensity of PI(4,5)P2 staining was analyzed in HDFs from controls (C1–2), Lowe (L2, L3, L6) and Dent-2 (D1, D2, D4) patients. A PI(4,5)P2 accumulation is easily detected in OCRL-1-deficient fibroblasts, either from Lowe or Dent-2 patients, as compared with signal from controls (Fig. 5A). The results, i.e. the average intensity of PI(4,5)P2 per cell in three independent experiments, are expressed either for fibroblasts of each patient (Fig. 5B) or for fibroblasts of the same clinical status (Fig. 5C). Lowe HDFs show a significant 1.8-fold increase in

Figure 5. Accumulation of PI(4,5)P2 staining in fibroblasts from Dent-2 patients. (A) Pattern of PI(4,5)P2 repartition. PI(4,5)P2 was revealed by immunofluorescence with a specific antibody used on fibroblasts. Scale bar: 10 μm. (B) Dent-2 fibroblasts show an accumulation of PI(4,5)P2 staining. PI(4,5)P2 staining was analyzed in fibroblasts from controls (C1–2), Lowe patients (L2, L3 and L6) and Dent-2 patients (D1, D2 and D4). Histograms represent the average intensity of PIP2staining + SEM from three independent experiments, with at least 20 cells of each patient examined per experiment. (C) Histograms show the average + SEM of data expressed per clinical status, with at least 120 cells of each type examined. Mann–Whitney test with ns: non-significant or with ***P < 0.001.
PI(4,5)P2 staining compared with controls ($P < 0.001$), which is an accumulation level similar to previous descriptions showing a 2.1-fold increase (12). Dent-2 cells also present a significant accumulation in PI(4,5)P2 level, with a 1.6 fold increase as compared with controls ($P < 0.001$). Interestingly, the accumulation of PI(4,5)P2 that we detect in HDFs from patients with Dent-2 disease presents a similar subcellular localization to what we observe in cells from Lowe patients.

Dent-2 fibroblasts therefore exhibit a new phenotype of PI(4,5)P2 accumulation, which appears similar in distribution and level to the accumulation observed in fibroblasts from Lowe patients.

Altogether our current descriptions of the various cellular defects observed in fibroblasts from Dent-2 patients, with their milder and distinguishable deficiencies compared with cells from Lowe patients and controls, jointly with the similar OCRL LOF mutations harbored by Dent-2 and Lowe patients, suggest that some compensatory mechanisms are operating in the Dent-2 fibroblasts relatively to Lowe cells. The INPP5B protein, which shows a structural domain organization and an inositol 5 phosphatase activity comparable to OCRL-1, has thus been suggested to be involved in the clinical variability associated with OCRL mutations (14,16,21,48). In addition, a compensatory role of INPP5B for OCRL-1 defect has been recently demonstrated in ciliogenesis (33) and it has been long known that murine ocrl and inpp5b exhibit functional overlap since none of the knockout mice shows any phenotype whereas the double knockout results in an early embryonic lethality (32). While our results show that cellular PI(4,5)P2 accumulation seems not to differ between Lowe and Dent-2 HDFs, we still asked whether INPP5B could partially compensate the absence of OCRL-1 in Dent-2 fibroblasts. Indeed, INPP5B and OCRL-1 share interaction partners, including various Rab GTPases, APPL1, IP1P27A and B (Ses1 and 2), proteins all involved in vesicular trafficking, which may be essential to compensate for OCRL-1 function in Dent-2 HDFs (16–18,48).

**Absence of contribution of INPP5B in compensatory phenomenon observed between fibroblasts of Dent-2 and Lowe patients**

To provide insights into the underlying molecular mechanisms involved in the compensation explaining the distinctive phenotypes of Lowe and Dent-2 fibroblasts, we investigated the potential contribution of INPP5B, the OCRL paralogous gene, in such phenomenon. We first studied INPP5B mRNA expression level in fibroblasts from controls (C1–C3), Lowe (L1–L6) and Dent-2 (D1–D4) patients. INPP5B mRNA was quantified from the various cells in three independent experiments of real time RT-PCR performed on total RNA. Results are expressed as the average mRNA level observed per patient or per clinical status (Fig. 6A). We next quantified the INPP5B protein level by western blot and the results of three independent experiments are also expressed as the average level observed per patient or per clinical status (Fig. 6B). We observed that INPP5B mRNA and INPP5B protein levels in fibroblasts are similar in controls and Lowe patients. These results are in agreement with a previous report on similar cells (25). However, we also observed that fibroblasts from Dent-2 patients did not exhibit quantitative variations in INPP5B mRNA or protein amounts as compared with controls or Lowe HDFs, thus excluding the role of INPP5B expression level in the differential compensation of OCRL mutations. To further assess INPP5B role, we searched for allelic variants in the coding DNA sequence (CDS) of the gene that may modulate its protein enzymatic activity and therefore account for its hypothetical involvement in compensatory mechanisms. Sequencing of INPP5B from all the HDFs studied here revealed no variations in the gene CDS, neither in Dent-2 patients nor in Lowe patients (not shown). We did identify a few sequence variations in the untranslated regions of the INPP5B RNA messenger (not shown), therefore potentially affecting mRNA stability, but we did not considered them as relevant since Dent-2 and Lowe patients fibroblasts display similar INPP5B expression level. These observations do not suggest a role of INPP5B as a differentiating factor between OCRL-mutated HDFs from Lowe and Dent-2 patients and they are in agreement with the similar PI(4,5)P2 accumulation level that we observe in cells from both types of patients.

We conclude that a phenotypic variability is present between fibroblasts from patients with Dent-2 disease and Lowe syndrome, thus suggesting that compensatory mechanisms are operating between these cells harboring LOF OCRL mutations. Altogether, our data demonstrate, unexpectedly, that neither INPP5B nor PI(4,5)P2 are directly involved in the phenotypic variability that we describe here, and they also strongly suggest that the INPP5B gene is not contributing to compensatory mechanisms explaining the absence of oculo-cerebral clinical traits in our Dent-2 patients.

**DISCUSSION**

Dent-2 disease, mainly a renal proximal tubulopathy associated with OCRL mutations, appears to represent a mild variant of the oculo-cerebro-renal syndrome of Lowe, thus suggesting the existence of a clinical continuum between these two OCRL-associated pathological conditions. This continuum was not only observed between patients with different OCRL mutations but also occurred between patients carrying the same OCRL mutation as we previously described (2). The molecular basis of this clinical variability is currently unknown, although genetic background and in particular INPP5B has been suggested to be involved (2,7,9,31). In order to describe phenotypes associated with fibroblasts from patients with Dent-2 disease, we first explored in these cells the occurrence of various deficiencies previously described in Lowe fibroblasts, i.e. alterations in the actin and the alpha-actinin distributions and in the primary cilia formation. We next investigated the mechanisms involved in the phenotypic variability associated with OCRL mutations through the analysis of PI(4,5)P2 accumulation and through the evaluation of the role of INPP5B gene.

We described for the first time the presence of significant alterations in actin cytoskeleton and alpha-actinin distribution in fibroblasts from Dent-2 patients. These defects are clearly intermediate between those observed in cells from Lowe patients and control cells. We speculate that this differential level of defects generates Lowe clinical signs compared with Dent-2 symptoms. Indeed, actin reorganization is known to be critical for transparency of the lens, for dendritic spine dynamics as well as for the ability of proximal tubular cells to reabsorb...
extracellular solutes \((27, 49, 50)\). In line with this, homozygous deletion of murine \textit{abi-2}\footnote{Abl interactor-2}, a molecule belonging to the WAVE complex known to be directly involved in the regulation of actin dynamics, leads to defects of cell morphogenesis in the lens and to aberrant neuronal spine morphogenesis resulting in deficits in learning and memory \((51)\). It has also been reported that proximal tubular cells of Lowe patients exhibit F-actin defects associated with impaired recycling of various receptors including Megalin. It thus suggests that a pathophysiological mechanism for the proximal tubular dysfunction in Lowe syndrome is an actin dynamics defect inducing dysfunctions of a large spectrum of receptors and channels \((27)\). We can therefore speculate that the actin defect that we described as more intense in Lowe fibroblasts than in Dent-2 cells, triggers more severe oculo-cerebro-renal features in Lowe patients than in Dent-2 patients.

Ciliogenesis defects are also detected in fibroblasts from Dent-2 patients. These abnormalities being significantly milder than those observed in Lowe cells, they also correlate with variability of clinical traits described between Lowe and Dent-2 conditions. We therefore assume that these alterations of ciliation could participate differentially, between both types of \textit{OCRL}\footnote{Ovular Cystinosin-Like} associated patients, to the pathogenesis of the oculo-cerebro-renal traits. Primary cilia have been shown to express a multitude of receptors, such as the PDGF receptor or the Patched receptor \((52, 53)\) and a recent observation showed that primary cilia are central for triggering receptor endocytosis in kidney tubular cells. It suggests that a ciliation defect may be a molecular basis for renal dysfunctions in Lowe syndrome and Dent-2 disease \((54)\). In addition, loss of primary cilia in neurons has been shown to result in impaired glutamatergic synapse formation, an alteration classically associated with intellectual disability.

---

**Figure 6.** \textit{INPP5B} expression in fibroblasts from Dent-2 patients. Analysis in fibroblasts from controls \((C1–3)\), Lowe patients \((L1–6)\) and Dent-2 patients \((D1–4)\). (A) \textit{INPP5B} mRNA level in \textit{OCRL}-mutated fibroblasts. The \textit{INPP5B} mRNA was quantified in HDFs by real-time RT-PCR. Histograms represent the average \(\pm\) SEM of \textit{INPP5B} mRNA level (arbitrary units) of three independent experiments, expressed per patient or per clinical status. (B) \textit{INPP5B} protein content in \textit{OCRL}-deficient fibroblasts. \textit{INPP5B} protein is explored by western blot using a specific antibody. A representative \textit{INPP5B} protein analysis is shown with alpha-Tubulin signal as reference. Histograms represent the average \(\pm\) SEM of \textit{INPP5B} level from three independent experiments, expressed per patient or per clinical status. ns, non-significant Mann–Whitney test.
Lowe syndrome, which is described as an atypical ciliopathy is characterized by alteration of lens differentiation, as well as subtle brain cysts and renal proximal tubulopathy (56). Therefore, a mild perturbation in primary cilia formation such as detected in Dent-2 HDFs, may participate to the renal signs in both diseases while only stronger ciliation defects may trigger intense oculo-cerebral features such as in Lowe syndrome.

Fibroblasts from Dent-2 patients display an accumulation of PI(4,5)P2, the main inositol phosphate substrate of OCRL-1. Surprisingly, this particular defect appears similar in level and pattern to what we observed in cells from Lowe patients. PI(4,5)P2 accumulation does not appear as a differential feature between both types of fibroblasts, but correlates with renal pathological signs of the diseases. This suggests that i/ PI(4,5)P2 does not constitute a direct actor for the pathogenesis of the ocular and neurological signs of patients with Lowe syndrome or ii/ a differential sensitivity of the cells to the accumulation of PI(4,5)P2 explains the phenotypic variability between patients. Loss of OCRL-1 phosphoinositide phosphatase activity in Lowe patients has been described as the main initial dysfunction for pathogenesis, suggesting that accumulation of PI(4,5)P2 is essential for physiopathology (15,25,27,28). Interestingly, Suchy et al. have shown an increased calcium response in HDFs from Lowe patients suggesting an alteration of the PI(4,5)P2/IP3/calcium signaling pathway (57). In addition, IP3/calcium pathway is implicated in actin dynamics of neuronal dendritic spines (58) and accumulation of calcium in human lens epithelial cells is linked to genesis of cataract (59). We thus propose that, it is not PI(4,5)P2 directly, but its downstream signals that may be differentially implicated in patients affected by Lowe syndrome or Dent-2 disease and in their ocular and cerebral clinical features.

We also explored the potential involvement of INPP5B as a candidate gene differentially compensating for OCRL mutations in our fibroblasts, thus explaining the clinical variability observed between Lowe and Dent-2 patients. We noticed neither difference in INPP5B content (mRNA and protein) nor in alleles of INPP5B when comparing fibroblasts from healthy controls or patients affected by either Lowe syndrome or Dent-2 disease. Thus, although it has been demonstrated that Inpp5b and Ocrl murine genes have overlapping functions in the mouse (32), we conclude that in fibroblasts from patients, INPP5B does not differentially compensate for OCRL-1 loss. This result is in agreement with several observations illustrating that cellular functions of INPP5B and OCRL-1 do not totally overlap in cells and are thus suggesting that proteins are not compensating for each other in humans. INPP5B exhibits specificities in subcellular localization, molecular partners and in trafficking regulation in HeLa cells relatively to OCRL-1 (60) and a migration defect detected in fibroblasts from Lowe patients is not rescued by INPP5B (25). We thus conclude that INPP5B is probably not involved in clinical variability associated with OCRL mutations and observed between patients affected by Lowe syndrome or Dent-2 disease.

In conclusion, fibroblasts from Dent-2 patients exhibit phenotypes of intermediate severity between Lowe and control fibroblasts, showing that intensities of these defects correlate with clinical variations observed between conditions with OCRL mutations. Fibroblasts from patients represent therefore a relevant model to study the molecular basis of the mechanisms involved in the compensatory phenomenon existing between OCRL-altered patients affected by the oculo-cerebro-renal syndrome of Lowe or by the Dent-2 disease. We suggest that the observed abnormalities of F-actin, of alpha-actinin, of primary cilia and possibly ofPIP2 downstream signaling, contribute to this clinical variability between patients. Regarding the molecular mechanisms underlying the compensatory phenomenon operating in ocular and cerebral tissues of Dent-2 patients, we suggest that INPP5B is not the factor involved. Shrimpton et al. previously proposed a model explaining the phenotypic variability relying on the nature of OCRL mutations with (i) persistence of altered OCRL-1 protein in some patients and (ii) existence of a shorter OCRL-1 isoform not altered in Dent-2 patients (7). We show here that Dent-2 patients with OCRL gene mutations present a complete loss of OCRL-1 function similarly to Lowe patients, suggesting that persistence of an altered OCRL-1 protein with preserved functions is unlikely to systematically compensate for ocular and cerebral symptoms. Nevertheless, in some particular patients as we observed in a Lowe patient carrying the pAla797Pro OCRL mutation and presenting only moderate neurological signs, a preserved residual OCRL-1 phosphoinositide phosphatase activity may also participate in clinical variability (2,35). We also described in a previous study that the use of an alternate initiation codon and the production of a truncated OCRL-1 protein due to a specific splicing OCRL mutation, may also account for the clinical variability (2).

We propose here a complementary explanation for compensatory mechanisms operating between both types of patients, based on the individual genetic background, with differential ‘activity’ of modifying factors modulating phenotypes linked to OCRL-1 loss and thus triggering appearance of either Lowe syndrome or Dent-2 disease. Further investigations of the molecular mechanisms involved in these compensatory phenomena are still needed and would require comparative large-scale genomic analysis using tissues from both Lowe and Dent-2 patients.

**MATERIALS AND METHODS**

**Cell culture**

Primary culture of HDFs from six Lowe (Lowe 1–6) and four Dent-2 (Dent 1-4) affected boys were derived from dermal biopsy. Informed consent from parents allowing genetic studies was obtained according to local institutional review board guidelines. Control fibroblasts were derived from three healthy children (Control 1–3). Mutations in OCRL gene were previously revealed by genomic and cDNA sequencings. Blood samples and DNAs from affected individuals and controls were also obtained from all participants. Primary fibroblasts were grown for the same limited period of time for all genotypes, with standard conditions in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 units/mL penicillin–streptomycin (Invitrogen), 1 mM sodium pyruvate (Invitrogen) and 0.25 µg/mL fungizone (Invitrogen) at 37°C with 5% CO₂.

**Antibodies**

The primary antibodies used for western blot analysis were rabbit anti-OCRL-1 serum that we previously described (61), rabbit polyclonal anti-INPP5B antibody (Epitomics) and mouse...
monoclonal anti-α-tubulin antibody (Merck Millipore). Mouse monoclonal IgM anti-α-actinin antibody (Sigma), mouse monoclonal IgM anti-PiP2 2C11 antibody (Santa Cruz Biotechnology) and mouse monoclonal IgG anti-acetylated-tubulin antibody (Sigma) were used in immunofluorescence analysis of HDF. The secondary molecules were Cy3 conjugated donkey anti-mouse IgG antibody (Jackson IR), Alexa 488 conjugated goat anti-mouse IgM antibody (Invitrogen) and Alexa 555 conjugated phalloidin (Molecular Probes). Cross hybridizations of secondary antibodies were checked in each assay with co-staining experiments.

Western blotting
Lysates were generated with NaCl 150 mM, Triton X-100 1%, NP40 0.5%, HEPES 50 mM and protease inhibitor cocktail « Complete » (Roche); proteins were resolved on SDS–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Signals were revealed using peroxidase-labelled swine anti-rabbit (Dako) or rabbit anti-mouse immunoglobulins (Dako) and Supersignal West Pico detection system (Pierce). Signals were acquired by direct measurement of chemiluminescence using a digital camera (Image Quant LAS 4000 mini, GE Healthcare) and quantification was done using Image J software from at least three independent experiments.

F-actin and alpha-actinin distribution
F-actin distribution was analyzed on HDFs, seeded on 14 mm diameter glass, fixed in 4% PFA for 20 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with BSA 1% /0.02% Triton X-100 for 1 h at 4°C. Cells were then incubated in the same solution with Alexa Fluor 555-phalloidin 0.25 units/ml for 30 min at room temperature. Alpha-actinin staining was investigated on cells fixed with methanol and treated as described previously on fibroblasts from Lowe patients (26). Coverslips were mounted using Fluoromount (Interchim) with Hoechst (Sigma). Cell preparations were observed under a Zeiss Axiophot epifluorescence microscope, images were processed using Adobe Photoshop and ImageJ softwares. The various fibroblast cultures were scored for pattern of actin and alpha-actinin in at least three independent blind experiments, with >50 cells from each patient per experiment.

Ciliogenesis assays
Of note, 3 × 10³ cells were seeded onto 14 mm diameter glass coverslips and grown in complete media. Then media was exchanged with 0% serum DMEM for 17 h (overnight starvation) to induce cilia formation. Cells were then fixed for 20 min in 4% paraformaldehyde followed by treatment with 50 mM NH₄Cl for 10 min and permeabilized in 0.2% Triton X-100 for 5 min. HDFs were finally immunolabeled with anti-acetylated-tubulin antibody. Confocal images were digitally acquired with a Leica Spinning disk laser microscope coupled to a Coolsnap HQ2 camera (Photometrics). Ciliation was quantified by scoring the percentage of cells containing primary cilia marked by acetylated tubulin. Ciliogenesis was quantified in the various fibroblast cultures by tracing the length of each cilium, tip-to-tip using ImageJ’s segmented line tool, in at least three independent blind experiments.

Data are expressed as an average size of cilia with >50 cells of each patient measured per experiment.

PI(4,5)P₂ distribution
This is a modification from a previously published protocol described by Hammond et al. (47). Cells were fixed in 4% formaldehyde, 0.01% Triton X-100 and 0.2% glutaraldehyde at room temperature before rinsing with PBS containing 50 mM NH₄Cl. Plates were then placed in an ice bath and chilled for at least 2 min. All subsequent steps were performed on ice, with all solutions prechilled. Cells were blocked and permeabilized with a solution containing 5% normal goat serum, 50 mM NH₄Cl and 0.5% saponin. Mouse monoclonal IgM anti-PiP2 and Alexa 488 conjugated goat anti-mouse IgM antibodies were applied in buffer containing 5% normal goat serum and 0.1% saponin. Fibroblasts were post-fixed in 2% formaldehyde for 10 min on ice, before warming to room temperature for an additional 5 min. Formaldehyde was removed by rinsing with PBS containing 50 mM NH₄Cl. Cell preparations were observed under a Leica epifluorescence microscope coupled to a Coolsnap Cf camera (Photometrics). PI(4,5)P₂ signals for each cell were quantified using ImageJ software, in at least three independent blind experiments, with >50 cells of each patient per experiment.

Gene expression analysis by real-time PCR
Total RNA from HDF was purified with a RNA-Plus Extraction Kit (Qiogene) and 1 μg of total RNA was reverse-transcribed using Superscript II (Invitrogen). Relative expression level of the INPP5B mRNA was determined by quantitative RT-PCR using LightCycler 480 SYBR Green I Master (Roche) and a set of primers specific for human INPP5B gene (forward primer: 5′-TAGTCACTGTCCCGTAACCC-3′, reverse primer: 5′-TG GCCATCTTCCGTGGCT-3′). INPP5B expression was normalized to GAPDH (forward primer: 5′-TGACCCACCAACTGCTTACG-3′, reverse primer: 5′-GGCATGGACTGTGGTCATG-3′) and EEF1G (forward primer: 5′-AGATGCGCCATTTGTATGCTAA-3′, reverse primer: 5′-GGTTCTCCTCCTCGTGAAACCT-3′) mRNA expression. Data were analyzed with the 2⁻ΔΔCt method and values are expressed as the average of triplicate.

INPP5B cDNA sequencing
Total RNA was extracted from HDFs with a Trizol Plus RNA Purification Kit (Invitrogen), cleaned up with NucleoSpin RNA clean up XS (Macherey-Nagel) and 0.5 μg of total RNA was reverse-transcribed using Superscript III One-Step RT-PCR System Kit (Invitrogen). PCRs were performed on a fraction of cDNAs generated previously using Advantage HD Polymerase (Clontech) and a set of primers specific for human INPP5B gene (forward primer: 5′-TAGTCACTGTCCCGGAACCT-3′, reverse primer: 5′-GCTAAACATTGGTTGGCTTAA-3′). The PCR products of 2800 bp were cleaned up with PCR-clean up NucleoSpin Extract II (Macherey-Nagel) and fully sequenced (Eurofins MWG). Data from the open reading frame were compared in sequences in databases (NCBI) with Sequencher software (Genecodes).
ACKNOWLEDGEMENTS

We would like to thank all family members and ‘Association du Syndrome de Lore’ (ASL) for their complete contributions to this work. Pr. Jamel Cheilly for helpful discussion and Justine Gallissant for technical assistance.

Conflict of Interest statement. None declared.

FUNDING

R.M. was supported by research fellowships from the ‘Conseil Regional de la Martinique’, the EU Gencodys program (FP7, grant number 241995) and by the Fondation Orange. This work was supported by INSERM, CNRS and by grants to O.D. and J.L. from ‘ANR Maladies Rares’ and the ASL.

REFERENCES


