A STUDY OF URINARY EXOSOMES IN GENETICALLY DEFINED DENT'S DISEASE

Masataka Inoue, Hiroaki Tamura and Tsutomu Takahashi

Department of Pediatrics, Akita University Graduate School of Medicine

(received 11 December 2019, accepted 6 January 2020)

Abstract

Dent's disease is an X-linked renal tubulopathy characterized by clinical features such as low molecular weight (LMW) proteinuria, hypercalciuria, and progressive renal failure. In Dent's disease, reabsorption of glomerulus-filtered LMW protein in the proximal tubular cells is decreased due to a malfunction of cellular endocytosis caused either by mutation in the Cl^-/H^+ exchanger ClC-5-encoding *CLCN5* gene (in about 60% cases) or the phosphatidylinositol 4,5-biphosphate 5-phosphatase-encoding *OCRL1* gene (in about 20% cases). Three patients diagnosed with Dent's disease using clinical criteria were genetically defined by mutational analyses of the *CLCN5* gene and the *OCRL1* gene. A 20-base pair duplication, c.300_319dup, of the *OCRL1* gene, which is predicted to encode a premature truncated OCRL1 protein, and a single base deletion, c.1726delT of the *CLCN5* gene, which is predicted to encode a premature truncated CLCN5 protein, were identified separately in two of the patients. No pathological gene variant was identified in the third patient.

Exosomes are one of three categories of extracellular vesicles released extracellularly by various cells. They have been identified to play a role in cellular homeostasis and cell communication under both physiological and pathological conditions. To understand the pathological effects of the *CLCN5* and *OCRL1* mutations on urinary exosomes, urinary exosomes were quantified in all three patients with Dent's disease using a double sandwich ELISA. Interestingly, urinary exosomes were significantly increased in the *CLCN5* gene-mutated patient (18.8±3.3 mg/gCr ; $p<0.01$) and in the non-mutated patient (4.73±1.5 mg/gCr ; $p<0.05$) compared to in the three controls (2.5±0.7 mg/gCr). The results suggest a possible pathological role for exosome biology in Dent's disease.

Key words : *CLCN5* gene, Dent's disease, exosome, *OCRL1* gene

Background

Dent's disease is an X-linked renal tubulopathy characterized by clinical features such as low molecular weight proteinuria, hypercalciuria, nephrolithiasis, nephrocalcinosis, and progressive renal failure[1]. Complications such as rickets or osteomalacia may also occur in this disease[2]. These features are found in males only, but female carriers may show a milder phenotype[2].

Approximately 60% of cases are caused by mutations in the *CLCN5* gene that encodes the Cl^-/H^+ exchanger, primarily localized to the early endosome of the proximal tubular cells ; this form of the disease is designated as Dent's disease type 1[1,2]. Mutations in the *OCRL1* gene that encodes a phosphatidylinositol 4,5-biphosphate 5-phosphatase localized at the Golgi apparatus, early endosome, and lysosome, of proximal tubular cells, are responsible for 15-20% of cases and this form of the dis-

Correspondence : Tsutomu Takahashi, M.D., PhD.
Department of Pediatrics, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
TEL : 81-18-884-6159
FAX : 81-18-836-2620
E-mail : tomy@med.akita-u.ac.jp

ease is designated as Dent's disease type 2[1,2]. The *OCRL1* gene is also associated with Lowe syndrome, which is a rare X-linked recessive disorder characterized by congenital cataracts, hypotonia, intellectual disability, proximal tubular acidosis, aminoaciduria, and low molecular weight proteinuria[3]. Patients with Dent's disease do not present symptoms such as severe cataracts or intellectual disability that are associated with Lowe syndrome, consistent with the fact that the *OCRL1* gene mutations associated with Dent's disease do not overlap with gene mutations causing Lowe syndrome[1]. The genetic cause of the remaining 20% of Dent's disease cases is yet to be explained.

The *CLCN5* gene encodes the electrogenic Cl^-/H^+ exchanger ClC-5, which belongs to the CLC family of Cl^- channels/transporters. The ClC-5 exchanger is exclusively expressed in the proximal tubule, the thick ascending limb of the loop of Henle, and the intercalated cells of the collecting ducts. Reabsorption of glomerulus-filtered low molecular weight protein is mediated by the megalin and cubilin receptors in the proximal tubule cells. The multi-receptor complex binds low molecular weight proteins (amongst other ligands) on the apical brush border leading to endocytosis of the low molecular weight proteins. The endosome is acidified by an H^+-ATPase, leading to dissociation of the receptor-ligand complex and subsequent degradation of the ligands in the lysosome. ClC-5 is thought to either dissipate the positive charge gradient in the endosome or drive proton entry directly into the proximal tubular cells where the tubular luminal chloride content is high[4]. These result in proton entry into the endosome. As described above, the ClC-5 plays an essential role in the endocytosis pathway. In addition, it has been hypothesized that ClC-5 plays a role in recycling megalin and cubilin to the brush border, which is critical for normal endosomal function. OCRL1, a phosphatidylinositol 4,5-biphosphate 5-phosphatase, is also thought to be involved in the renal tubular endocytosis pathway. In Dent's disease type 2, an accumulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting from decreased OCRL1 activity is thought to affect cell signaling involved in endocytosis. Despite the different etiologies of Dent's disease, there is little clinical difference between patients with *CLCN5*- and *OCRL1*-associated Dent's disease.

Extracellular vesicles (EVs) are membrane structures released extracellularly by various cells. EVs are classified based on their biogenesis and exosomes comprise one of three categories. Exosomes are 30-150 nm in size and are released into the extracellular space when multivesicular bodies fuse with the plasma membrane. Exosomes are secreted by most cell types under both physiological and pathological conditions and their function is to maintain cellular homeostasis and to mediate cell communication[5]. In kidney, EVs have been shown to be signaling vesicles for different segments of tubules, intra-glomerular, glomerular-tubules, and tubules-interstitial communications[5]. Recently, quantification of exosomes in urine for the analysis of physiological and pathological studies has been made possible[6].

In this study, a molecular diagnosis of three patients with Dent's disease previously diagnosed using clinical criteria was completed to determine the molecular lesions. To uncover possible pathological effects of the *CLCN5* and *OCRL1* mutations on urinary exosome, we quantified urinary exosomes in all three patients with Dent's disease using a double sandwich ELISA specifically designed for quantitative and qualitative analysis of exosomes[6].

Materials and Methods

Blood and urine samples were collected after obtaining written informed consent from the guardians of the three patients and the three control participants. Control participants were children with growth hormone deficiencies being treated with growth hormone replacement therapy. Ethical approval for this study was obtained from the Ethics Committee of Akita University, Graduate School of Medicine in Akita, Japan.

Patients

Case 1 is a 4-year-old boy who was diagnosed with asymptomatic proteinuria at the age of 3 years and 6 months. He was the second child to healthy parents with a healthy elder brother. The family history of the patient included a maternal uncle who had asymptomatic proteinuria with an examination of renal biopsy, resulting

Table 1

	Normal range	Patient 1	Patient 2	Patient 3
Blood				
TP (g/dL)	6.6–8.1	7.0	7.1	7.2
Alb (g/dL)	4.1–5.1	4.3	3.9	4.5
T-Cho (mg/dL)	128–219	141	154	197
BUN (mg/dL)	8–20	11.8	13.4	11.1
UA (mg/dL)	3.6–7.0	2.4	4.2	3.2
Cr (g/dL)	0.65–1.07	0.22	0.26	0.22
Na (mmol/L)	138–145	137	135	137
K (mmol/L)	3.6–4.8	4.2	4.6	3.9
Cl (mmol/L)	101–108	103	102	105
Ca (mg/dL)	8.8–10.1	9.6	9.9	9.4
IP (mg/dL)	2.7–4.6	4.5	4.8	4.9
Cr-eGFR(ml/min/1.73 m^2)	133±27.0	164.20	91.32	123.40
Blood Gas Analyses				
pH	7.32–7.43	N.D.	7.436	7.388
pCO_2 (mmHg)	35–48	N.D.	35.6	36.9
HCO_3 (mmHg)	22–26	N.D.	19.7	26.6
B.E. (mmol/L)	−2–+3	N.D.	−4.6	1.6
Lactate(mmol/L)	0.3–1.8	N.D.	1.6	2.2
Urine				
Protein/Cr (g/gCr)	≦ 0.2	3.0	8.7	3.03
β2MG (μg/L)	≦ 230	1,842	300,494	122,000
NAG (U/L)	≦ 7.0	26.8	N.D.	42.3
Ca/Cr	*	0.18	0.68	N.D.

* normal range in each age group ; 0.5–1.0 y, <0.81 ; 1〜2y, <0.56 ; 2〜3y, <0.5 ; 3〜5y, <0.41 ; 5〜 7y, <0.3 ; 7〜17y, <0.25. TP, total protein ; Alb, albumin ; T-Cho, total cholesterol ; BUN, blood urea nitrogen ; UA, uric acid ; Cr, creatinine ; Na, natrium ; Cl, chloride ; Ca, calcium ; IP, inorganic phosphate ; Cr-eGFR, creatinine estimated glomerular filtration rate ; β2MG, β2-microglobulin ; NAG, N-acetyl-β-D-glucosaminidase ; N.D., not determined.

in no significant pathologic finding. No further information was not obtained about the uncle. Case 2 is a 1-year-old boy who was diagnosed with proteinuria when he was admitted to hospital with respiratory syncytial virus bronchiolitis. The patient did not have any family history of renal diseases. Case 3 is a 3-year-old boy who was diagnosed with proteinuria during a regular medical checkup at the age of 3 years and 6 months. In his family, maternal grandfather had ureterolithiasis during his fourth decade and maternal first cousin showed asymptomatic proteinuria. However, no further information was available in this family.

All the three cases were apparently healthy without any physical complaint. The results of the laboratory tests are shown in Table 1. Three controls in this study were evaluated as normal in laboratory tests for blood and urine (data not shown).

Mutational analyses of the CLCN5 and OCRL1 genes

Genomic DNA was extracted from the peripheral white blood cells of the patients and controls. The genomic DNA of all 11 coding exons and the exon-intron boundaries of the *CLCN5* gene and the genomic DNA of all 22 coding exons and the exon-intron boundaries of the *OCRL1* gene were amplified by PCR. The PCR prod-

ucts were directly sequenced in both directions using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI Prism 310 Genetic Analyzer ; Applied Biosystems). In this study, *CLCN5* and *OCRL1* variants were analyzed and described based on reference sequences with GenBank Accession Number NM_000084.5 and NM_000276.4, respectively.

Determination of CD9-positive exosomes in patients with Dent's disease

Urinary and serum exosomes of the patients were quantified using the ExoTEST™ Ready to Use Kit for ELISA Exosome quantification (Hansa Bio Med, Tallin, Estonia). Briefly, the kit uses a double sandwich ELISA for quantitative and qualitative analysis of exosomes[6]. The protocol uses ELISA plates pre-coated with proprietary pan-exosome antibodies enabling specific capture of exosomes from human biological fluids and additional antibodies for the detection of exosome-specific CD9. Lyophilized Exosome Standards characterized for protein content and particle number allow the quantification of unknown sample by a standard curve. Urine and serum samples were collected for exosome analyses after the laboratory tests (Table 2). Urine and serum samples were measured three times.

Statistical analysis

Data were analyzed using the IBM SPSS statistics 22.0 software package and the results are presented as the mean ± standard deviation (SD). A Students' unpaired *t*-test was used to compare the mean differences between two groups. A p-value < 0.05 was considered statistically significant.

Table 2

	Creatinine (mg/dL)	β2-MG (μg/L)	β2-MG/Cr (μg/gCr)
Patient 1	39.2	98,590	251,633
Patient 2	52.2	15,794	30,286
Patient 3	81.8	153,770	187,960

β2MG, β2-microglobulin ; Cr, creatinine

Results

Mutational analyses of the CLCN5 and OCRL1 genes

In patient 1, analysis of the *CLCN5* and *OCRL1* genes did not identify any genetic variant predicted to cause Dent's disease.

In patient 2 (Fig, 1), analysis of the *OCRL1* gene identified a hemizygous 20-base pair duplication at the cDNA position between 300 and 319, designated as c.300_319dup. The c.300_319dup mutation is predicted to cause a frame shift at codon 107, resulting in a phenylalanine (TTC) to leucine (TTG) substitution. Moreover, as a result of the duplication mutation, codon 111 now encodes a termination signal, and the mutant gene is predicted to encode a prematurely truncated OCRL1 protein, designated p.F107Lfs*6.

In patient 3 (Fig. 2), analysis of the *CLCN5* gene identified a hemizygous single base deletion of T at the cDNA position of 1726, designated as c.1726delT. The c.1726delT mutation is predicted to cause a frame shift mutation at codon 576, which results in a phenylalanine (TTT) to leucine (TTG) substitution. As a result of the frameshift mutation, codon 585 now encodes a termination signal, and the mutant gene is predicted to encode a prematurely truncated CLCN5 protein, designated p.F576Lfs*10.

Determination of CD9-positive exosome in the patients with Dent's disease

Urinary exosomes obtained from the three patients with Dent's disease and three normal controls were quantified using the ELISA kit. The urinary sample from patient 3, who has the *CLCN5* gene mutation, showed significantly increased levels of exosomes : 18.8 ± 3.25 μg/gCr versus 2.5 ± 0.66 μg/gCr in control urine (Fig. 3). The urinary sample from patient 1, who has no mutation in either the *CLCN5* gene or the *OCRL1* gene, also showed significantly increased levels of exosomes in the urine : 4.73 ± 1.54 μg/gCr versus 2.5 ± 0.66 μg/gCr in control urine (Fig. 3). However, the urinary sample from patient 2, who has the *OCRL1* gene mutation, did not show significantly increased levels of exosomes. Using the same method, serum samples

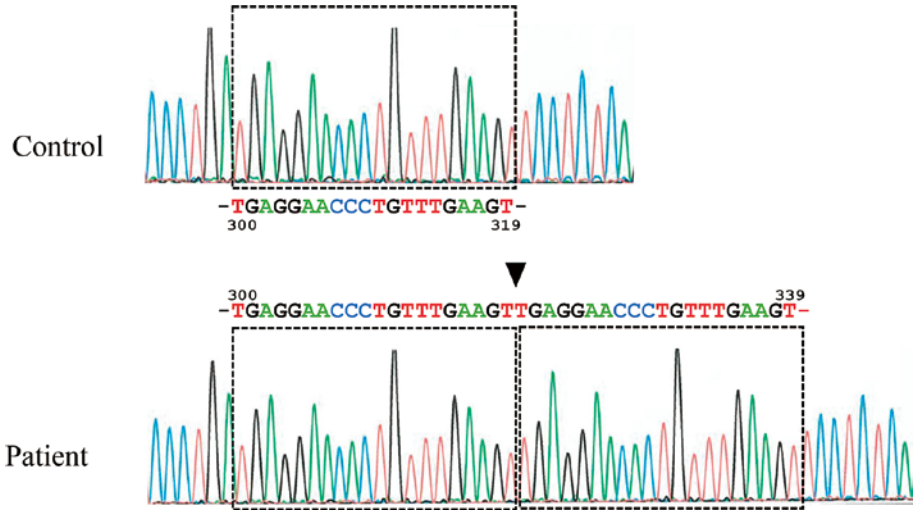

Fig. 1. Genomic exon 5 sequences of the *OCRL1* gene in patient 2 with Dent's disease and a control.
In patient 2, a hemizygous 20-base pair duplication was identified at the cDNA position between 300 and 319 (designated as c.300_319dup).

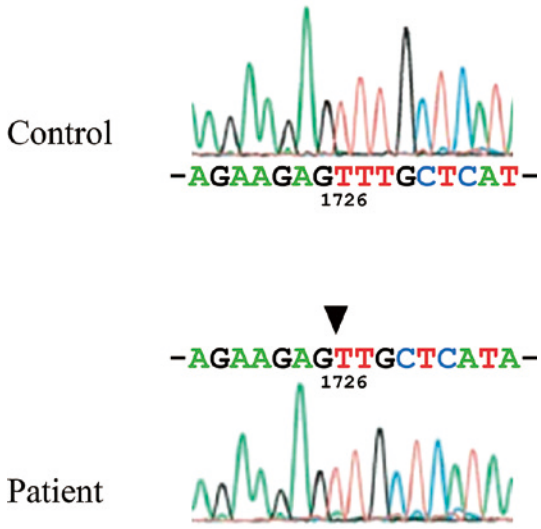

Fig. 2. Genomic exon 10 sequences of the *CLCN5* gene in patient 3 with Dent's disease and a control.
In patient 3, a hemizygous single base deletion of T was identified at the cDNA position of 1726 (designated as c.1726delT).

from the three patients with Dent's disease and the three controls were shown to contain similar levels of exosomes (data not shown).

Discussion

Gene analyses identified *c.300_319dup* in exon 5 of the *OCRL1* gene in patient 2. To the best of our knowledge, *c.300_319dup* in the *OCRL1* gene is a novel mutation identified from the patient with Dent's disease. The *OCRL1* gene is associated not only with Dent's disease but also with Lowe syndrome, which is a neurological and systemic disorder characterize by congenital cataracts, hypotonia, intellectual disability, proximal tubular acidosis, aminoaciduria, and low molecular weight proteinuria (compared to the milder phenotype of Dent's disease). Since the *c.300_319dup* mutation is predicted to result in a prematurely truncated OCRL1 protein, the patient may be expected to show some clinical symptoms associated with Lowe syndrome (Fig. 4). However, the patient is apparently healthy without any neurological or any ophthalmological complications, and thus, the patient was diagnosed with Dent's disease. Interestingly, all of the *OCRL1* truncating mutations associated with Dent's disease (including *c.300_319dup* in patient 2) occur in the first seven exons or within intron 7[1]. By contrast, the *OCRL1* mutations found in Lowe syndrome patients occur primarily in exons 9 to 22, which encode three large functional domains[1]. It is not known why the pheno-

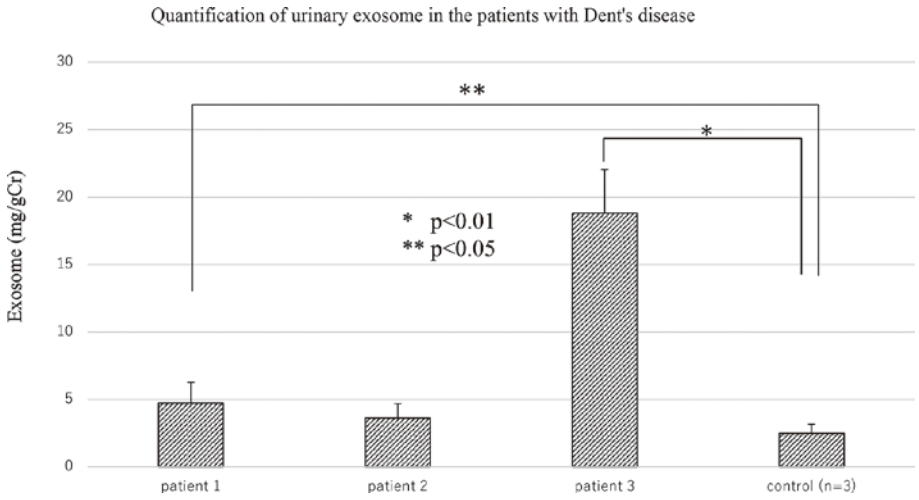

Fig. 3. Quantification of urinary exosomes in patients with Dent's disease.
Urinary exosomes of all three patients were quantified by ELISA. Urine samples were measured in triplicate. Data are shown as average ± standard deviation.

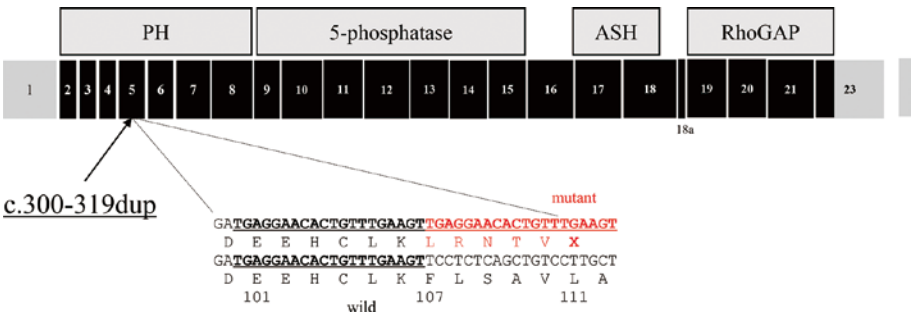

Fig. 4. The location of the c.300-319dup mutation relative to the structure of OCRL-1
A c.300-319dup mutation was identified in exon 5 of the *OCRL1* gene, which is predicted to cause a frame shift at codon 107, resulting in a termination codon at 111. PH : N-terminal pleckstrin homology domain, 5-phosphatase : 5-phosphatase domain, ASH : ASPM–SPD2–hydin domain, RhoGap : C-terminal (catalytically non-active) Rho GTPase activating like domain.

type of Dent's disease (shown to be caused by truncating mutations in the 5' region of the *OCRL1* gene) is much milder than the phenotype of Lowe syndrome. However, a model in which a functioning, truncated form (or isoform) of OCRL1 protein is expressed in Dent's disease type 2, but not in Lowe syndrome, has been proposed to explain the milder phenotypic features in Dent's disease 2[7].

In patient 3, c.1726delT was identified in exon 10 of the *CLCN5* gene and this mutation is predicted to cause a termination at codon 586, resulting in a prematurely truncated CLCN5 protein (Fig. 5). To the best of our knowledge, c.1726delT of the *CLCN5* gene is also a novel mutation identified from the patient with Dent's disease. To date, more than two-hundred inactivating mutation of the *CLCN5* gene have been identified in patients with Dent's disease[4]. The disease-causing mutations are equally distributed along the entire length of the *CLCN5* gene, which comprises 11 exons. Among them, seven frameshift mutations were previously reported in

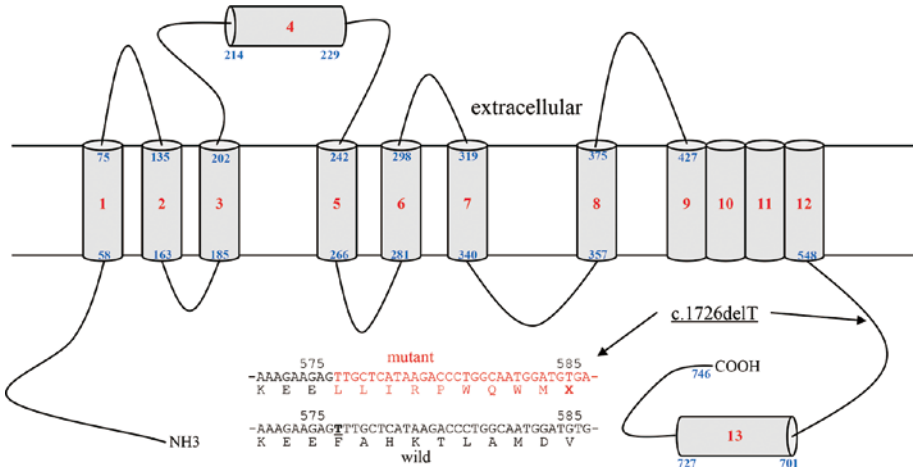

Fig. 5. A The location of the c.1726delT mutation relative to the predicted topology of 746-residue CLC-5.
A c.1726delT mutation was identified in exon 10 of the *CLCN5* gene, which is predicted to cause a frame shift mutation at codon 576, resulting in a termination codon at 585. The predicted topology of the CLC-5 putative transmembrane domains, D1 to D13, is based upon a model that places D4 extracellularly, and in which the hydrophobic core of the D9-D12 region crosses the membrane three or five times and contains a hydrophobic region.

exon 10 of the *CLCN5* gene, suggesting that exon 10 is a hotspot for deletion mutations in this gene.

We quantified the amounts of urinary exosomes in all three patients with genetically defined Dent's disease. This is the first report describing urinary exosomes in Dent's disease. Interestingly, patient 3 (with Dent's disease type 1) showed increased levels of urinary exosome (as measured by the CD9-utilized ELISA method) in one of two different urines. However, the amount of urinary exosomes was not increased in patient 2 (with Dent's disease type 2). These results suggest that pathophysiological changes caused by *CLCN5* gene mutations in Dent's disease type 1 might be associated with an increase in the amounts of exosomes.

Exosomes are membrane bound extracellular vesicles that are produced in the endosomal compartment of the cell. The multivesicular body is an endosome defined by intraluminal vesicles that bud inward into the endosomal lumen[5]. If the multivesicular body fuses with the cell surface, or plasma membrane, these intraluminal vesicles are released as exosomes. Thus, a malfunction in endocytosis could account for the effects on the metabolism of exosomes observed in Dent's disease.

In addition to preserving parent cell homeostasis, exosomes mediate the dispersal of signals to surrounding and remote cells. Exosome can adhere to the target cells via interactions between adhesion molecules and receptors present on their surfaces, leading to receptor activation of the target cells. Exosomes may then transfer their contents to the target cells via membrane fusion. Therefore, additional study might be to analyze the contents of exosomes to understand the pathophysiological meanings of urinary exosome in patients with Dent's disease.

There have been few reports describing the quantification of urinary exosome in human renal diseases[5]. As for the exosome secretion from kidney cells, an article reported hypoxic-inducible factor-1-mediated production of exosomes during hypoxia from renal tubular cells[8]. This article concluded that hypoxia stimulates exosome production and secretion in renal tubular cells and the exosomes from hypoxic cells are protective against renal tubular cell injury. This study was conducted using cultured cells but suggests the quantitative changes of urinary exosomes during the pathophysiological state or in some kidney diseases.

Our study analyzed only a few urinary samples from small number of patients and controls. In addition, con-

trols were children with growth hormone deficiencies being treated with growth hormone replacement therapy. Those are limitations in this study. Therefore, further analyses of more urinary samples, which contain frequent samplings from more patients and healthy controls, are essential to establish the significance of quantitative changes of urinary exosome in Dent's disease. Then, It could lead to the clinical application of quantification for urinary exosomes in Dent's disease or other kidney diseases.

Conflict of Interest

The authors have no conflict of interest to declare.

References

1) Devuyst, O. and Thakker, R.V. (2010) Dent's disease. *Orphanet J. Rare Dis.*, **5**, 28.
2) Claverie-Martín, F., Ramos-Trujillo, E. and García-Nieto, V. (2011) Dent's disease : clinical features and molecular basis. *Pediatr. Nephrol.*, **26**, 693-704.
3) Bökenkamp, A. and Ludwig, M. (2016) The oculocerebrorenal syndrome of Lowe : an update. *Pediatr. Nephrol.*, **31**, 2201-2212.
4) Bignon, Y., Alekov, A., Frachon, N., Lahuna, O., Jean-Baptiste Doh-Egueli, C., Deschênes, G., Vargas-Poussou, R. and Lourdel, S. (2018) A novel CLCN5 pathogenic mutation supports Dent disease with normal endosomal acidification. *Hum. Mut.*, **39**, 1139-1149.
5) Lv, L.L., Feng, Y., Tang, T.T. and Liu, B.C. (2019) New insight into the role of extracellular vesicles in kidney disease. *J. Cell. Mol. Med.*, **23**, 731-739.
6) Logozzi, M., De Milito, A., Lugini, L., *et al.* (2009) High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*, **4**, e5219.
7) Shrimpton, A.E., Hoopes, R.R., Jr, Knohl, S.J., *et al.* (2009) OCRL1 mutations in Dent 2 patients suggest a mechanism for phenotypic variability. *Nephron Physiol.*, **112**, 27-36.
8) Zhang, W., Zhou, X., Yao, Q., Liu, Y., Zhang, H. and Dong, Z. (2017) HIF-1-mediated production of exosomes during hypoxia is protective in renal tubular cells. *Am. J. Physiol. Renal Physiol.*, **313**, F906-F913.