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【 CASE REPORT 】

Urinary Mulberry Cells as a Biomarker of the Efficacy of Enzyme Replacement Therapy for Fabry Disease

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Abstract:
Mulberry cells are often present in the urinary sediments of patients with Fabry disease (FD). We herein report two patients with FD undergoing enzyme replacement therapy (ERT). A 41-year-old man was diagnosed based on lack of α-galactosidase A activity. ERT was subsequently administered. A 40-year-old woman was diagnosed based on urinary Mulberry cells and genetic testing, and ERT was initiated. While the renal function of the male patient deteriorated, the Mulberry cells disappeared in the female patient after ERT was administered. The detection of urinary Mulberry cells can contribute to the diagnosis as well as serve as a biomarker for the response to treatment.

Key words: enzyme replacement therapy, Fabry disease, Lyso-Gb3, Mulberry body, Mulberry cell, urinary sediment

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Introduction

Fabry disease (FD) is an X-linked hereditary disorder, in which a mutation in the α-galactosidase A (GLA) gene (chromosome Xq22.1) reduces the α-galactosidase A activity, and glycosphingolipids, such as globotriaosylceramide (GL-3), accumulate in the vascular endothelial cells, organ cells, and bodily fluids, resulting in cardiac and renal failure (1).

Since FD is X-linked, its classic symptoms appear in men. FD is categorised as the classical type when almost all symptoms appear and as a subtype when symptoms are limited to one area (renal, cardiac, and central nervous system subtypes) (1). In women, however, FD ranges in severity, from heterozygous patients with almost no symptoms to severe cases (1).

In 2004, α-galactosidase enzyme replacement therapy (ERT) was introduced to treat FD (2). ERT delays the progression of various organ symptoms by breaking down accumulated GL-3. Plasma globotriaosylsphingosine (Lyso-Gb3) is considered a biomarker of the efficacy of ERT for FD (3).

Renal dysfunction in FD occurs due to the intracellular accumulation of GL-3 in glomerular epithelial cells, mesangial cells, and distal convoluted tubule cells. Before the appearance of overt proteinuria, swelling of glomerular epithelial cells and fine vacuoles and zebra bodies are found in these cells (4). The clinical course is defined by proteinuria in adulthood, a gradually decreasing renal function, and end-stage renal disease (ESRD). Mulberry bodies are whirl-shaped fat globules, and Mulberry cells are keratinised, exfoliated, and vacuolated epithelial cells that resemble a mulberry filled with Mulberry bodies. They are often detected through the clinical course of FD and can provide an opportunity for an FD diagnosis (5-10).

We recently encountered a classical male patient and a fe-

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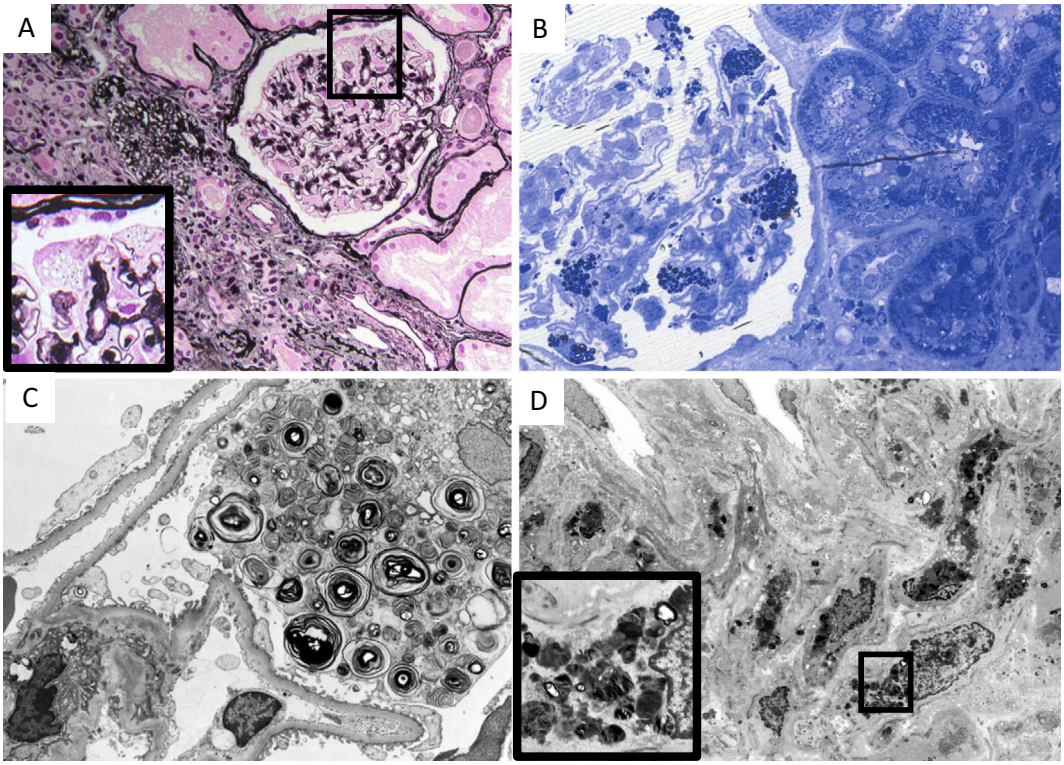

Figure 1. **(A) Light microscopy image. No proliferative changes in the glomeruli were noted, but progression of tubulointerstitial injury was seen [left: Periodicacid-methenamine-silver (PAM) staining ×100]. Glomerular podocytes were expanded, and fine vacuolated changes were noted (bottom left: inset ×400). (B) Light microscopy image. A toluidine blue-stained Epon resin-embedded section shows the granular deposition of inclusion corpuscles in the podocytes but not in the tubular epithelium (right: toluidine blue staining ×200). (C) (D) Electron microscopy image. Numerous osmiophilic, lamellated membrane structures, with a concentric pattern called *myelin bodies* or with elongated stripes called *zebra bodies*, were mainly found in the podocytes (C) and the cytoplasm of the vascular smooth cells (D) (C: podocytes ×2,000, D: vascular tunica media ×1,500, inset: ×3,000).**

male patient with FD. In the man, Fabry nephropathy progressed without the disappearance of urinary Mulberry cells despite ERT for 12 years. In the woman, urinary Mulberry cells disappeared within several months of initiating ERT. Urinary Mulberry cells can be used for the diagnosis and potentially for assessing the treatment efficacy. In the present study, we examined their effectiveness for assessing the treatment efficacy and report our findings.

Case Reports

Case 1

A 41-year-old Japanese man had a family history of an uncle who had ESRD at 33 years of age and a cousin who had ESRD in his 20s and died at 48 years of age. His mother was an FD carrier.

Febrile peripheral limb pain first appeared when the patient was nine years old. At 12 years of age, the patient was diagnosed with FD based on decreased α-galactosidase A activity (0.6 nmol/mg/hr). Acroparaesthesia, angiokeratomas, abnormal sweating, and cornea verticillata were present at the time of the diagnosis. He presented with proteinuria for the first time at 25 years of age. Agalsidase β ERT was initiated at 27 years of age. Serum creatinine (Cr) was 0.59 mg/dL, and urinary protein was 1.87 g/g Cr. At 36 years of age, the findings worsened to Cr 1.3 mg/dL; a renal biopsy was performed to assess the degree of Fabry nephropathy. Light microscopy revealed foamy lesions, expansion, and argentaffin granules of glomerular podocytes, which are usually found in FD. High rates of glomerulosclerotic lesions (approximately 75%) and interstitial fibrosis (approximately 60%) indicated disease progression (Fig. 1). On electron microscopy, numerous myeloid bodies and zebra bodies were found in the glomerular podocytes and vascular smooth muscles (Fig. 1). Other organ damage as well as hypertension and cardiac hypertrophy were detected at 36 years old, and anti-hypertensive drug combination therapy (olmesartan at 10 mg and azelnidipine at 8 mg) was initiated.

However, despite ERT being subsequently administered once every two to four weeks, Mulberry cells were still detected, and his renal function decreased gradually during the observation period (Fig. 2). His plasma Lyso-Gb3 levels were 32.6 ng/mL in November, 2018. The IgG antibody to agalsidase β was negative. Recently, genetic testing was performed and indicated a hemizygous nonsense mutation (Tyr

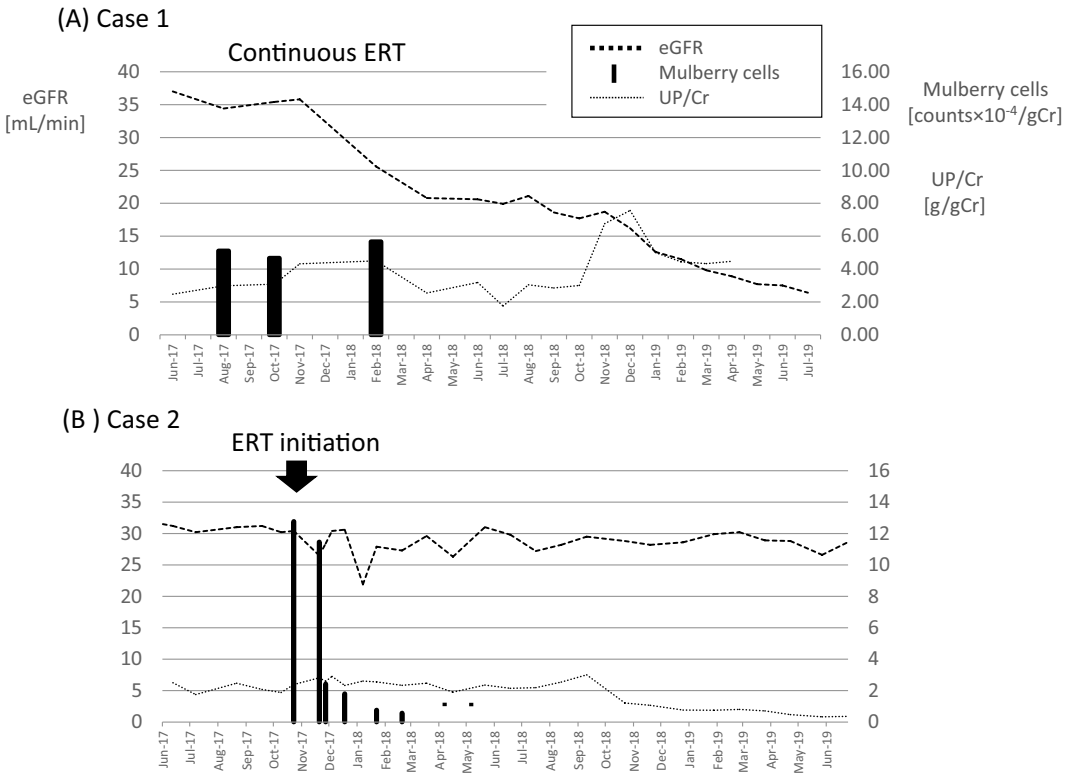

Figure 2. **Clinical course with eGFR and the count of Mulberry cells in two cases. (A) Case 1: Despite long-term ERT, the numbers of Mulberry bodies and cells remained almost constant. (B) Case 2: The numbers of Mulberry bodies and cells decreased promptly after initiating ERT, and the Mulberry cells disappeared altogether after five months. eGFR: estimated glomerular filtration rate, ERT: enzyme replacement therapy, UP/Cr: urinary protein per creatinine, gCr: gram creatinine**

173X) in exon 3 of the GLA gene.

Case 2

A 40-year-old Japanese woman with no medical or family history presented with a chief complaint of proteinuria and renal dysfunction. She had been found to have proteinuria at school medical checkups since she was approximately 8 years old. At 28 years of age, she visited a local physician for persistent proteinuria; however, no renal dysfunction was noted. At 37 years of age, her serum Cr level was 1.36 mg/dL at a company health checkup.

She visited our hospital at 39 years of age. Her findings were Cr 1.39 mg/dL and urinary protein 2.04 g/g Cr. No laboratory findings suggested secondary glomerular disease. As imaging findings revealed thinning of the renal cortex, a renal biopsy was not performed. Mulberry cells were detected in her urinary sediment at an analysis performed on an outpatient basis four months later. She was found to be urinary GL-3-positive, and genetic testing indicated a heterozygous nonsense mutation (Trp61X) in exon 1 of the GLA gene with a low activity of α-galactosidase in leukocytes (patient: 119.13 nmol/mg/h; normal control: 157.25 to 226.23 nmol/mg/h). She was therefore diagnosed with female FD.

The only other organ damage was mild cornea verticillata. After two months, agalsidase-β was initiated. She continued ERT once every two weeks. Urinary Mulberry cells disappeared after five months, and only urinary Mulberry bodies remained. After one year, angiotensin receptor blockade (ARB) with 5 mg of olmesartan was initiated. Her proteinuria decreased, and her renal function was stable during the observation period (Fig. 2). Her plasma Lyso-Gb3 levels were 11.0 ng/mL at the initiation of ERT and 8.04 ng/mL at 1 year after initiation of ERT. IgG antibody to agalsidase β was negative.

Quantities of urinary Mulberry cells and morphological observations

Urinary Mulberry cells in the urinary sediments of both patients were quantified as follows: a 10-mL urine sample was centrifuged, and the supernatant was removed to obtain 200 μL of concentrated urine sample (equating to a 50-fold urinary concentration rate). A 15-μL aliquot of this concentrated urine sample was then loaded to make the urinary sediment specimen. We counted the number of Mulberry cells in the whole field of the urinary sediment specimen. We then converted the count in the centrifuged urine sample to that in the uncentrifuged urine as follows: (the converted value in uncentrifuged urine) = (the area of the field) × (urinary concentration rate) × (the loading amount on glass slide) / (the area of the cover glass). The area of the whole field is the same as that of the cover glass, which is 324

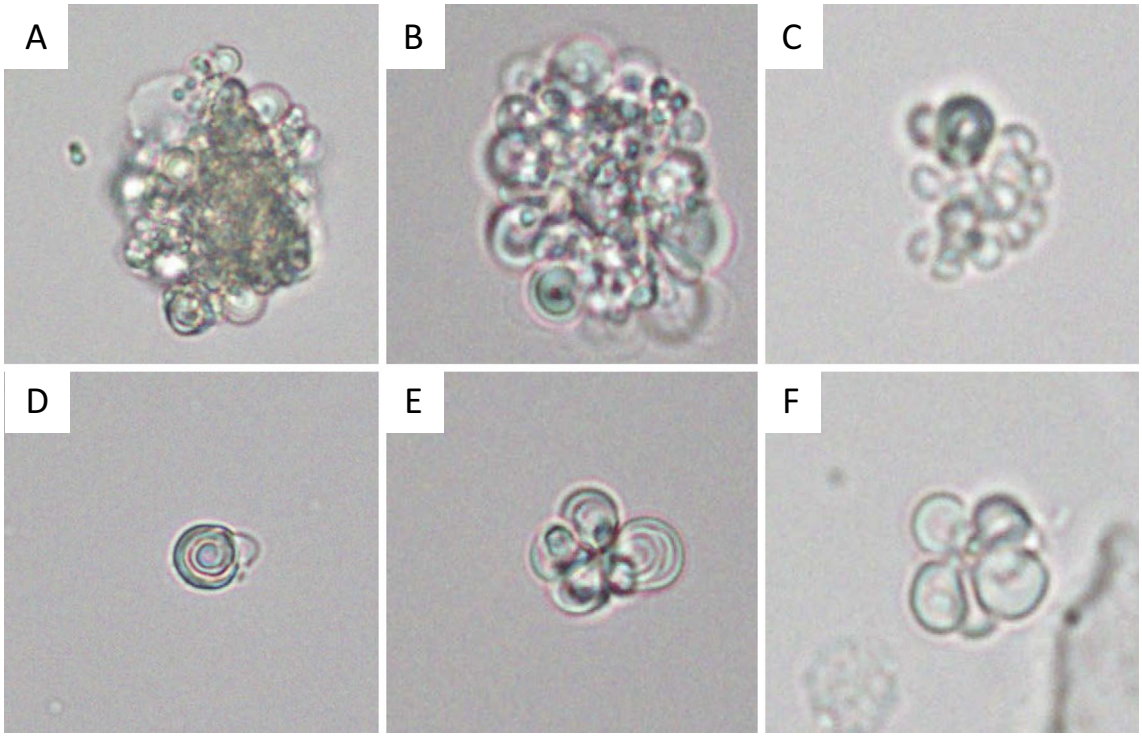

Figure 3. **Morphology of Mulberry bodies and cells. (A) (D) Case 1 (during ERT): Numerous Mulberry cells with typical whirl-shaped and annular ring-shaped fat globules (Mulberry bodies) were found despite many years of treatment (A). Numerous free Mulberry bodies of similar morphology were also found (D). (B) (E) Case 2 (before ERT): Whirl- and ring-shaped fat globules (Mulberry bodies) were found in the Mulberry cells (B). Free Mulberry bodies also formed whirl-shaped structures, annular ring-shaped, and ring-shaped morphologies (E). (C) (F) Case 2 (6 months after initiating ERT): The proportion of whirl-shaped Mulberry bodies inside Mulberry cells decreased, and the proportion of ring-shaped Mulberry bodies increased (C). The majority of free Mulberry bodies were of ring-shaped morphology (F).**

mm^2 (18×18 mm) in Japan. Consequently, the converted value in uncentrifuged urine in the whole field is 750 μL (324×50×15/324). Therefore, the Urinary Mulberry cell creatinine ratio in uncentrifuged urine (/μL/gCr) was calculated by dividing the actual count of Mulberry cells with both 750 (μL) and the urinary creatinine concentration (mg/dL). When using a different field, the count should be corrected by calculating the area of the field as follows: (the area of the field) =π × [(the number of the fields of eyepiece lens) × (the magnification of objective lens) /2]^2.

The urinary Mulberry cells were also morphologically observed. Case 1 completed a treatment course with almost no changes in the numbers of Mulberry cells. In contrast, in Case 2, the Mulberry cells disappeared after initiating ERT (Fig. 2). Morphologically, numerous Mulberry bodies or fat globules in typical whirls and annular ring shapes were found inside and outside the Mulberry cells in Case 1. The morphological findings of Case 2 were similar to those of Case 1 before initiating ERT. However, six months after the initiation of therapy, the numbers of whirl-shaped Mulberry bodies in Case 2 decreased, and the numbers of ring-shaped Mulberry bodies increased (Fig. 3).

Discussion

We reported the changes in urinary Mulberry cells in two patients with FD undergoing ERT. Although there were no marked changes in the male patient with rapid worsening of the renal function, the Mulberry cells disappeared in the female patient with a stable renal function.

The reported effects of ERT on renal failure include decreasing urinary GL-3 levels, suppressing the estimated glomerular filtration rate (eGFR) decrease, and inhibiting the progression of renal pathological findings (2). However, male sex and the onset of proteinuria and a low eGFR at the diagnosis are risk factors of a progressive decline in the renal function (11). Furthermore, the ERT efficacy is poor in patients with urinary protein levels of ≥1 g or glomerulosclerosis ≥50% (12-15).

Although plasma Lyso-Gb3 is thought to be an ideal biomarker of the efficacy of ERT for FD (3), it is not widely performed as a general examination. Instead, previous reports have assessed the effect of ERT on renal lesions based on the presence of oval fat bodies, anti-GL-3 antibodies in urinary sediments, urine Maltese cross particles, and anti-CD77 antibodies for detecting GL-3 (16, 17). Since

urine Maltese cross particles and anti-CD77 antibodies are not specific to FD, tests using anti-GL-3 antibodies are necessary. In our study, urinary Mulberry cells were used as biomarkers of the ERT response. Mulberry bodies, which are produced by GL-3 and accumulate on epithelial cells of the distal convoluted tubules to form Mulberry cells, are specific to FD (9). Recently, podocalyxin, a marker of podocytes, was also reported to be positive on Mulberry bodies (18). Therefore, the origin of the Mulberry bodies and cells is assumed to be both tubular cells and podocytes.

In Case 1, urinary Mulberry cells were still positive despite continued ERT, whereas in Case 2, urinary Mulberry cells disappeared promptly after initiating ERT. In successful ERT, GL-3 clearance is reportedly almost complete in renal tissues after treatment (4, 13), suggesting that the presence of urinary Mulberry cells indicates GL-3 accumulation in renal tissues. In one study, GL-3 levels decreased in podocytes and disappeared from the intima of arterioles at significantly higher rates in the high-dose group (0.2-1.0 mg/kg) than in the low-dose group (0.2 mg/kg) among patients undergoing ERT for a mean of 9.4 years (19). This suggests that despite continuing ERT for many years, the dose for Case 1 may not have been sufficient, so the GL-3 accumulation in the kidneys could not be inhibited; this is further supported by the fact that myeloid and zebra bodies persisted in the renal tissues of Case 1. Going forward, when the amount of enzyme replacement is thought to be insufficient, a dose increase should be considered.

In Case 2, the urinary Mulberry cells disappeared, suggesting that the GL-3 accumulation in the kidneys had been successfully inhibited and the progression of glomerular damage suppressed. A small amount of ARB initiated after one year may also have contributed to the decrease in proteinuria. Therefore, both ARB and the inhibition of GL-3 accumulation by ERT is expected to help the patient maintain her renal function in the future.

Furthermore, morphological changes occurred in the Mulberry bodies of Case 2. While the reason for these changes was not clear, the reduction in GL-3 by ERT might have altered the morphology of the Mulberry bodies.

Although Mulberry bodies and cells detected in urinary sediments are inexpensive and non-invasive biomarkers, one limitation associated with using them as biomarkers for disease progression is that the results may vary depending on the ability and experience of the medical technician. Furthermore, although several reports have proven the efficacy of using Mulberry bodies and cells for the diagnosis (5-9), none have described the relationship between the quantities of Mulberry bodies and cells and the renal failure progression in FD patients or their utility as biomarkers for ERT efficacy.

In conclusion, the detection of urinary Mulberry bodies and cells can be performed as non-invasive and time-dependent assessments, in contrast to a renal biopsy. Although this approach may require adequately training technicians, the counts of urinary Mulberry cells can be used as biomarkers for assessing the ERT efficacy for FD cases.

All procedures performed in the patient study were in accordance with the 1964 Declaration of Helsinki and its later amendments or with comparable ethical standards.

Informed consent was obtained from the patient.

Author's disclosure of potential Conflicts of Interest (COI).

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Yumi Aoyama and Yusuke Ushio contributed equally to this work.

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