



Comparative urinary globotriaosylceramide analysis by thin-layer chromatography-immunostaining and liquid chromatography-tandem mass spectrometry in patients with Fabry disease

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ABSTRACT

In Fabry disease, accumulation of glycolipids, predominantly globotriaosylceramide (Gb3), affects the kidneys, and nephropathy is one of the important disorders that influence the disease severity and prognosis of patients. Urinary Gb3 has been analyzed for diagnosis and monitoring of Fabry disease. In this study, we analyzed urinary Gb3 by thin-layer chromatography (TLC)-immunostaining and liquid chromatography (LC)-tandem mass spectrometry (MS/MS). An improved qualitative method, TLC-immunostaining, revealed excessive urinary Gb3 excretion in 100 (8/8), 88 (14/16), and 74% (45/61) of the classic Fabry males, later-onset Fabry males, and Fabry females examined, respectively. This authentic method is robust, easy, economic, and hardly affected by abundant urinary sediment, and this is useful for diagnosing individual Fabry patients. LC-MS/MS can determine the level of Gb3 in urine with high sensitivity, and it revealed that the Gb3 excretion level was higher in the order of classic Fabry males, later-onset Fabry males, Fabry females, and controls, respectively, and this is expected to be a useful quantitative method not only for diagnosis but also for predicting the progression of Fabry nephropathy. As to the relation of the urinary Gb3 level and renal events, our study revealed that the urinary Gb3 level in Fabry patients experiencing renal events tended to be higher than that in ones who did not have any renal events in each phenotypic group of the disease.

1. Introduction

Fabry disease (OMIM 301500) is an inborn error of metabolism caused by mutations of the X-linked *GLA* gene, which encodes α -galactosidase A (α -Gal, EC 3. 2. 1. 22). Decreased activity of α -Gal results in lysosomal accumulation of glycolipids, predominantly globotriaosylceramide (Gb3), in various types of cells [1,2].

Fabry disease is phenotypically divided into three types: classic Fabry males, later-onset (atypical or non-classic) Fabry males, and Fabry females (most of them, heterozygotes), according to the "Practical guideline for the management of Fabry disease 2020 in Japan", although some investigators further divide Fabry female patients into two subgroups (early-onset and later-onset ones). Classic Fabry males, having

deficient α -Gal activity, exhibit characteristic symptoms, such as neuropathic pain, hypohidrosis, angiokeratoma, gastrointestinal disorders and corneal opacities in childhood or adolescence, and suffer from progressive renal, heart and cerebrovascular disorders in adulthood. Later-onset Fabry males, who usually have low residual enzyme activity, develop manifestations limited to the heart and/or a kidney disorder in adulthood. Fabry females exhibit more variable clinical manifestations and α -Gal activity from almost deficient to normal due to random X-chromosomal inactivation [3].

For diagnosis of Fabry disease, deep clinical insight and reliable physical examinations are required, and a definitive diagnosis is usually reached by measuring α -Gal activity for Fabry males, but requires gene analysis for Fabry females and some later-onset Fabry males [1,2,4]. So

Abbreviations: α -Gal, α -galactosidase A; Gb3, globotriaosylceramide; lyso-Gb3, globotriaosylsphingosine; TLC, thin-layer chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry.

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far, more than 1000 *GLA* variants have been reported, and many genetic variants of unknown significance and possible functional polymorphisms have been found, other than pathogenic mutations, with spread of neonatal screening (Human Gene Mutation Database, HGMD, <http://www.hgmd.cf.ac.uk/>; Fabry-database, <http://fabry-database.org/>; and International Fabry Disease Genotype-Phenotype Database, dbFGP, <http://dbfgp.org/dbFgp/fabry/>). Thus, we sometimes find Fabry cases are hard to diagnose.

Recently, plasma globotriaosylsphingosine (lyso-Gb3), a deacylated form of Gb3, and urinary Gb3 have attracted the interest of clinicians and researchers as possible biomarkers of Fabry disease for diagnosis and monitoring of treatment [5–13]. In this study, we focused on urinary Gb3 excretion in Fabry patients. Nephropathy is one of the main disorders of Fabry disease, and it essentially develops with progressive accumulation of glycolipids, predominantly Gb3, in the kidneys [1,2,14,15]. Although the correlation between renal dysfunction and urinary Gb3 excretion remains obscure, it is important to establish methods for detection/measurement of urinary Gb3 and to properly use them for diagnosis and monitoring of therapy of Fabry disease. For analyzing urinary Gb3, thin-layer chromatography (TLC) has been traditionally used [14]. Recently, liquid chromatography (LC)-tandem mass spectrometry (MS/MS) has been introduced and widely used, because LC-MS/MS can specifically detect target substrates with high sensitivity [10–13]. In this study, we compared a modified authentic method, TLC-immunostaining [4], and a latest one, LC-MS/MS, and investigated their advantages and disadvantages for clinical use.

2. Materials and methods

2.1. Study subjects and samples

In this study, Fabry patients examined were basically classified into three clinical types (classic Fabry males, later-onset Fabry males, and Fabry females), according to the “Practical guideline for the management of Fabry disease 2020 in Japan”, and when necessary, the Fabry female patients were further divided into three subgroups (early-onset, later-onset, and asymptomatic). Urine samples for analysis were obtained from 8 classic Fabry males (age: 12–71 y; mean \pm standard deviation (SD), 38 \pm 21 y), 16 later-onset Fabry males (3–67 y; 44 \pm 20 y), 61 Fabry females (9–80 y; 45 \pm 18 y), and 59 controls comprising non-Fabry subjects with proteinuria and healthy ones (7–80 y; 40 \pm 20 y). Diagnosis of the Fabry patients was performed based on the results of leukocyte α -Gal assaying, measurement of plasma lyso-Gb3, and *GLA* gene analysis, and a pathological examination involving biopsied samples was performed when necessary. The phenotypes of the patients were determined by clinicians who examined them, and information about their renal events (proteinuria and/or renal impairment showing elevated serum creatinine level or decreased eGFR value) was obtained from the clinicians.

This study was approved by the ethical committee of Meiji Pharmaceutical University, and was performed according to the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent for this study was obtained from all the participants.

2.2. Detection of Gb3 in urine by TLC-immunostaining

For detection of Gb3, thirty mL of urine was centrifuged, the urinary sediment being obtained. The glycolipid fraction from the sediment was extracted with an aliquot of chloroform/methanol/water (2/1/0.1, v/v/v). Then, the glycolipid fraction including Gb3 was evaporated to dryness and then diluted with an aliquot of chloroform/methanol/water (60/30/4.5, v/v/v). Then, a pair of HPTLC silica gel 60 plates (Merck, Darmstadt, Germany) were prepared, and fractionation of glycolipids was performed by means of TLC. Basically, aliquots of urinary extracts equivalent to 0.8 mL of urine were applied on the plates, the solvent system used for developing chromatograms being chloroform/

methanol/0.22% CaCl₂ (55/45/10, v/v/v). Then, one of the HPTLC plates was stained with orcinol (NACALAI TESQUE Inc., Kyoto, Japan), which is classically used for staining glycolipids [14,15]. On the other HPTLC plate, the separated Gb3 was immunologically stained with a hybridoma supernatant containing an anti-Gb3 mouse monoclonal antibody [16], as described previously [4]. Color development was performed using horseradish peroxidase-labeled goat anti-mouse IgG (Thermo Fisher Scientific KK, Tokyo, Japan), *o*-phenylenediamine (FUJIFILM Wako Chemical Co., Osaka, Japan), and hydrogen peroxide solution, according to the manufacturers' methods. Purified Gb3 (Larodan AB, Solna, Sweden) was used as a standard, and whether the TLC-immunostaining was positive (+), pseudopositive (\pm), or negative (–) was visually determined (Limit of Gb3 detection: 0.025 μ g/lane).

2.3. Measurement of Gb3 in urine by LC-MS/MS

For extraction of glycolipids, five hundred μ L of urine was mixed with 10 μ L of 5 μ g/mL Gb3 (C17:0) (Matreya, LLC, Pleasant Gap, PA) in methanol as an internal standard and 500 μ L of water-saturated *n*-butanol, followed by centrifugation. After recovering the upper phase, the lower phase was extracted again with 500 μ L of water-saturated *n*-butanol. The two upper phases were combined and passed through a 0.22 μ m filter (TERUMO, Tokyo, Japan). Then, the filtered sample was evaporated to dryness and resuspended in a 100 μ L aliquot of chloroform/methanol (1/2, v/v), followed by centrifugation. Then, the supernatant was transferred to a LC vial, and sixty μ L of the sample was used for the LC-MS/MS assay. First, Gb3 isoforms were separated by high performance LC, a Union UK-C8 column (20 \times 3 mm I.D., 3 μ m; Imtakt Co., Kyoto, Japan) being used as the analytical column, and then the isoforms detected by MS/MS. The mass spectrometry was conducted with a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization interface in the positive-ion mode. The assaying was essentially performed according to the method described previously [17]. The calculations for measurement of the major urinary Gb3 isoforms including Gb3 (C16:0), Gb3 (C18:0), Gb3 (C20:0), Gb3 (C22:1), Gb3 (C22:0), Gb3 (C24:1), Gb3 (C24:0), and Gb3 (C24OH) were performed using LabSolutions (Shimadzu), and the total Gb3 contents of the samples were calculated from the sums of those of Gb3 isoforms (Limit of Gb3 measurement: 12.5 ng/mL), according to the methods previously reported [11,12]. The urinary Gb3 value was expressed as μ g/mg creatinine. The measurement of creatinine was performed using a commercially available assay kit.

2.4. Statistical analysis

Data are essentially expressed as means \pm SD (number of cases), and statistical analysis was performed with the Wilcoxon signed-rank test. Values were considered statistically significant at $p < 0.05$.

3. Results

3.1. Urinary Gb3 detected by TLC-immunostaining

Glycolipids in the urine samples were separated by TLC, and Gb3 was qualitatively analyzed by classical orcinol staining and immunostaining with an anti-Gb3 monoclonal antibody. Examples of the results are shown in Fig. 1 (A, orcinol staining; and B, immunostaining). A Fabry female, who had a considerable amount of urinary sediment, exhibited many bands in various positions on the plate (Fig. 1A, lane 2), and another one, who had a small amount of urinary sediment, exhibited several faint bands (Fig. 1A, lane 3) at the position where standard Gb3 migrated to (Fig. 1A, lanes 1 and 6). However, clear bands confirming the existence of Gb3 could be observed in both cases when the plate was stained with the specific antibody against Gb3 (Fig. 1B, lanes 2 and 3).

From the results of the analysis, we found that the percentages of cases exhibiting TLC-immunostaining (+) were 100 (8/8), 88 (14/16),

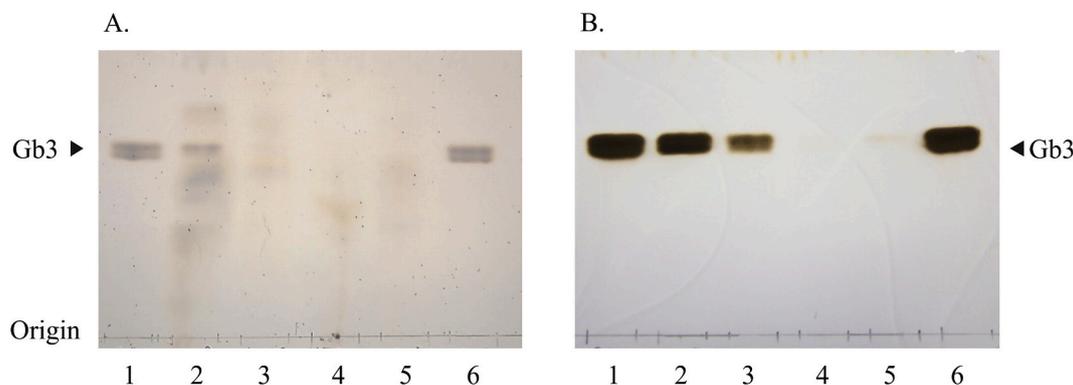


Fig. 1. TLC chromatograms for detection of urinary Gb3 (A. Orcinol staining and B. Immunostaining with an anti-Gb3 antibody). Lanes 1 and 6: Gb3 standard (2 µg), lanes 2 and 3: urine extracts from Fabry females (equivalent to 0.8 mL of urine), and lanes 4 and 5: urine extracts from controls (equivalent to 0.8 mL of urine).

74 (45/61), and 0% (0/59) for the classic Fabry males, later-onset Fabry males, Fabry females, and controls, respectively (Table 1). As to the Fabry females, the percentages of cases exhibiting TLC-immunostaining (+) were 84 (26/31), 73 (16/22), and 38% (3/8) for the early-onset, later-onset, and asymptomatic subgroups, respectively (Table 1). The ages of the two later-onset Fabry males, who exhibited TLC-immunostaining (±) and TLC-immunostaining (–), were 65 and 3 years, respectively. The former exhibited clinical manifestations limited to heart involvement (phenotype: previously called “cardiac type”), and the latter found on a family diagnosis did not exhibit any symptoms at the time of inspection.

3.2. Urinary Gb3 level measured by LC-MS/MS

The urinary Gb3 concentration in the Fabry patients and control subjects was quantitatively determined by LC-MS/MS, and the results are summarized in Table 1. The levels of urinary Gb3 in the classic Fabry males, later-onset Fabry males, Fabry females, and controls were 1.56 ± 1.05 (8), 0.81 ± 1.11 (16), 0.23 ± 0.24 (61), and 0.03 ± 0.03 (59) µg/mg creatinine, respectively. The mean urinary Gb3 value was higher in the order of the classic Fabry males, later-onset Fabry males, Fabry females, and controls, and there were statistical differences between the control group and all the Fabry groups ($p < 0.05$). As to the Fabry females, the urinary Gb3 levels in the early-onset, later-onset, and asymptomatic subgroups were 0.26 ± 0.25 (31), 0.25 ± 0.26 (22), and 0.08 ± 0.07 (8) µg/mg creatinine, respectively. The results of the qualitative analysis by TLC-immunostaining were compared with those of the quantitative analysis by LC-MS/MS. This comparative analysis revealed that there

Table 1
Results of urinary Gb3 analysis by TLC-immunostaining and LC-MS/MS.

	Detection of urinary Gb3 by TLC-immunostaining			Total
	+	±	–	
Classic Fabry males	1.56 ± 1.05 (8)	–	–	1.56 ± 1.05 (8)
Later-onset Fabry males	0.90 ± 1.17 (14)	0.30 (1)	0.06 (1)	0.81 ± 1.11 (16)
Fabry females	0.26 ± 0.26 (45)	0.19 ± 0.23 (5)	0.15 ± 0.18 (11)	0.23 ± 0.24 (61)
Early-onset	0.29 ± 0.26 (26)	0.18 (1)	0.13 ± 0.11 (4)	0.26 ± 0.25 (31)
Later-onset	0.25 ± 0.26 (16)	0.58 (1)	0.20 ± 0.25 (5)	0.25 ± 0.26 (22)
Asymptomatic	0.10 ± 0.09 (3)	0.06 ± 0.09 (3)	0.05, 0.08 (2)	0.08 ± 0.07 (8)
Controls	–	0.04 ± 0.04 (6)	0.03 ± 0.03 (53)	0.03 ± 0.03 (59)

The levels of urinary Gb3 determined by LC-MS/MS are basically expressed as means ± SD (n), and the unit is µg/mg creatinine.

was a tendency that the urinary Gb3 level was higher in the TLC-immunostaining (+) cases than in the TLC-immunostaining (±) and TLC-immunostaining (–) ones among the later-onset Fabry males and Fabry females.

3.3. Urinary Gb3 excretion and renal events in the Fabry patients

Urinary Gb3 excretion was examined as to renal events (+) and renal events (–) in each phenotypic group of the Fabry patients. The relation between the urinary Gb3 excretion detected on TLC-immunostaining and the renal events is summarized in Table 2. Of the subjects who exhibited renal events, the percentages of the cases of TLC-immunostaining (+) were 100 (6/6), 91 (10/11), 86 (19/22), and 0% (0/22) in the classic Fabry males, later-onset Fabry males, Fabry females, and controls, respectively. Of the subjects who exhibited TLC-immunostaining (+), the percentages of the cases who did not show any renal events were 25 (2/8), 29 (4/14), and 58% (26/45) in the classic Fabry males, later-onset Fabry males, and Fabry females, respectively. The ages of the two classic Fabry males who did not exhibit any symptoms of renal disorder although urinary Gb3 was detected on TLC-immunostaining were 12 and 16 y, i.e., apparently younger compared with other classic Fabry males who exhibited renal events. The urinary Gb3 concentration determined by LC-MS/MS in the individual Fabry patients is shown in Fig. 2. The relation between the urinary Gb3 level and the occurrence of renal events is summarized in Table 3. Although there was a tendency that the urinary Gb3 level in the renal event (+) cases was higher than that in the renal event (–) ones in all the phenotypic Fabry groups, a statistical difference was found only in the Fabry female group ($p < 0.05$). As to the Fabry females, an apparent statistical difference was found between the renal event (+) cases and the renal event (–) ones in the later-onset subgroup ($p < 0.05$). The urinary Gb3 levels in the renal event (+) cases and the renal event

Table 2
Urinary Gb3 detection by TLC-immunostaining and renal events in Fabry patients.

	TLC-immunostaining	Renal events (n)	
		+	–
Classic Fabry males	+	6	2
Later-onset Fabry males	+	10	4
	±	1	0
	–	0	1
Fabry females	+	19	26
	±	0	5
	–	3	8
Controls	+	0	0
	±	1	5
	–	21	32

n: number of cases.

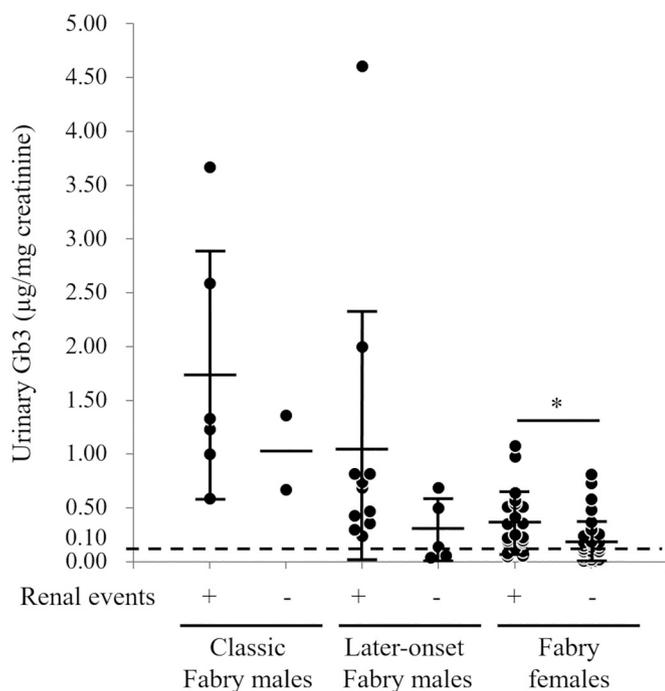


Fig. 2. Urinary Gb3 levels and renal events in the Fabry patients. Urinary Gb3 concentrations of the patients were determined by LC-MS/MS. *A statistical difference was found between the renal event (+) group and renal event (–) one in the Fabry females ($p < 0.05$). The dotted line shows the upper limit of control range ($< 0.1 \mu\text{g/mg creatinine} = \text{mean} + 2\text{SD}$).

Table 3
Urinary Gb3 measured by LC-MS/MS and renal events in Fabry patients.

	Renal events	
	+	–
Classic Fabry males	1.74 ± 1.16 (6)	$0.67, 1.36$ (2)
Later-onset males	1.04 ± 1.28 (11)	0.29 ± 0.29 (5)
Fabry females	0.36 ± 0.28 (22)	0.17 ± 0.19 (39)
Early-onset	0.37 ± 0.27 (11)	0.21 ± 0.22 (20)
Later-onset	0.35 ± 0.30 (11)	0.16 ± 0.17 (11)
Asymptomatic	–	0.08 ± 0.07 (8)
Controls	0.04 ± 0.03 (22)	0.02 ± 0.04 (37)

The levels of urinary Gb3 are basically expressed as means \pm SD (n), and the unit is $\mu\text{g/mg creatinine}$.

(–) ones in all the Fabry patients examined were 0.76 ± 0.96 (39) and 0.22 ± 0.27 (46) $\mu\text{g/mg creatinine}$, respectively. There was a statistical difference between the two groups ($p < 0.05$).

4. Discussion

In this study, we compared two methods for analyzing urinary Gb3, being widely used: the authentic TLC and the latest LC-MS/MS, and investigated their advantages and disadvantages. The TLC method has been traditionally used for detecting glycolipids accumulated in cells and tissues of lysosomal storage diseases including Fabry disease. In this method, urinary sediment is usually used as a sample [18], because a considerable amount of organic solvent is required for extraction and the evaporation takes a long time, when whole urine is used as a sample. Furthermore, it has been reported that more than 80% of urinary Gb3 is adsorbed on the sediment [19]. In this study, we modified the classic TLC method, and used immunostaining with an anti-Gb3 mouse monoclonal antibody, instead of orcinol staining, to increase the specificity for the detection of Gb3. On the other hand, in the LC-MS/MS assaying, we used whole urine as a sample, because LC-MS/MS is

highly sensitive and can specifically detect the target substrates.

Renal involvement is one of the most important disorders that influence the disease severity and prognosis of patients with Fabry disease [20,21]. Progressive accumulation of glycolipids, predominantly Gb3, in all renal cell types, including podocytes, endothelial cells, epithelial cells, and tubular cells, contributes to the renal symptoms that manifest as early hyperfiltration, albuminuria, proteinuria, and progressive deterioration of renal function [20,21]. Gb3, a component of the cellular membrane, consists of three sugars and a ceramide residue ($\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$), the ceramide being composed of a sphingosine residue and a fatty acid of various types. Therefore, there are many Gb3 isoforms in organs and tissues, differing in the structure of fatty acids, due to their own metabolic pathways, as animal experiments [17,22,23] and a clinical examination [24] have revealed. The metabolism of globo-based glycolipids in the kidneys is active, and the content of Gb3 in the kidneys is relatively higher than those in the liver and heart in both wild-type and Fabry mice [17].

The origin of urinary Gb3 is thought to be tubular cells and the urinary collecting system [25], and it has been used as a diagnostic biomarker of Fabry nephropathy for both genders [21,25–28]. Previously, detection of urinary Gb3 had been performed by means of classic TLC followed by orcinol staining [18]. This method is robust and easy, and it does not require any expensive equipment. However, it is not quantitative and glycolipids other than Gb3 are also detected, especially in cases having a large amount of sediment. To avoid non-specific detection, we performed immunostaining with an anti-Gb3 monoclonal antibody as described above, and could clearly and specifically detect bands indicating the existence of Gb3, as shown in Fig. 1. Using this method, we detected excessive urinary Gb3 excretion in 100, 88, and 74% of the classic Fabry males, later-onset Fabry males, and Fabry females examined, respectively. This TLC-immunostaining for detecting urinary Gb3 is clinically useful for diagnosing individual Fabry patients, although it cannot be used for many samples at one time.

As to quantitative measurement of Gb3 in urine, an LC-MS/MS method has been rapidly spreading [10–13]. LC-MS/MS can accurately measure each Gb3 isoform, although expensive equipment is required and the method is relatively susceptible to various factors, i.e., contamination by bacteria and leukocytes [29], and abundant urinary sediment. Our analysis revealed that the level of urinary Gb3 excretion was higher in the order of classic Fabry males, later-onset Fabry males, Fabry females, and controls, and that there was a tendency that the mean urinary Gb3 level in the TLC-immunostaining (+) cases was higher than that in the TLC-immunostaining (\pm) and (–) ones in both the later-onset Fabry males and Fabry females.

There have been a few studies examining the correlation between urinary Gb3 excretion and renal events. Some investigators reported that they could not find any correlation between urinary Gb3 levels and various renal function parameters/clinical symptoms in Fabry patients [30,31]. Our study revealed that most of the Fabry patients who experienced renal events exhibited TLC-immunostaining (+), and that the urinary Gb3 level in Fabry patients exhibiting renal events tended to be higher than that in Fabry patients who did not show any renal events. In the case of Fabry patients exhibiting high levels of urinary Gb3 but not showing any renal symptoms at the time of inspection, there should be careful monitoring to prevent further development of nephropathy. In this study, there was the limitation that detailed information about renal function in the Fabry patients could not be obtained from the clinicians unfortunately. So, more detailed examination is required to elucidate the relation between the urinary Gb3 level and the renal function/degree of a kidney disorder in Fabry disease.

5. Conclusion

We analyzed urinary Gb3 excretion in Fabry patients by means of TLC-immunostaining and LC-MS/MS. Excessive urinary Gb3 excretion was detected in most of the Fabry patients for whom TLC-

immunostaining was performed. The quantitative analysis of urinary Gb3 by LC-MS/MS revealed that the urinary Gb3 level was higher in the order of classic Fabry males, later-onset Fabry males, Fabry females, and controls, and that the urinary Gb3 level in the Fabry patients exhibiting renal events tended to be higher than that in those who did not show any renal events in each phenotypic group of the disease.

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Declaration of Competing Interest

We declare that none of the authors have competing interests.

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