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Analysis of globotriaosylceramide (Gb_3) isoforms/analogs in unfractionated leukocytes, B lymphocytes and monocytes from Fabry patients using ultra-high performance liquid chromatography/tandem mass spectrometry

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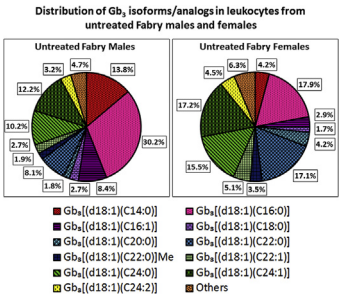
HIGHLIGHTS

- Methylated Gb_3 in blood cells might be part of a pathway between Gb_3 and lyso-Gb_3.
- High abundance of Gb_3[(d18:1)(C16:0)]Me in B lymphocytes and monocytes.
- Similar Gb_3 group distributions between Fabry patients and healthy controls.
- The severity of Fabry disease mutations affects biomarker levels in blood cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder with marked variability in the phenotype and genotype. Glycosphingolipids such as globotriaosylceramide (Gb_3) isoforms/analogs, globotriaosylsphingosine (lyso-Gb_3) and analogs, and galabiosylceramide (Ga_2) isoforms/analogs may accumulate in biological fluids and different organs. The aims of this study were to: 1) develop/validate a novel UHPLC-MS/MS method for relative quantitation of Gb_3 in leukocytes (unfractionated white blood cells), B lymphocytes and monocytes; 2) evaluate these biomarkers in a cohort of Fabry patients and healthy controls; and 3) assess correlations between these biomarkers, treatment and genotype. Whole blood, plasma and urine samples from 21 Fabry patients and 20 healthy controls were analyzed. Samples were purified by liquid-liquid extraction and analyzed by UHPLC-MS/MS in positive electrospray ionization. Methylated Gb_3 isoforms were detected, showing that a methylation process occurs at the cellular level. Our results show that there were no significant differences in the distribution of the different Gb_3 isoforms/analogs in blood cells between Fabry patients and healthy controls. In leukocyte, Gb_3[(d18:1)(C14:0)], Gb_3[(d18:1)(C16:0)], Gb_3 [(d18:1)(C16:0)]Me, Gb_3 [(d18:1)(C16:1)], Gb_3 [(d18:1)(C18:0)], Gb_3 [(d18:1)(C18:1)], Gb_3 [(d18:1)(C20:1)], Gb_3 [(d18:1)(C24:2)], Gb_3 [(d18:1)(C26:1)] and total Gb_3 allowed good discrimination between male Fabry patients and male controls, patients

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having higher biomarker levels than controls. Regarding B lymphocytes and monocytes, the same tendency was observed without reaching statistical significance. A positive concordance between mutation types and biomarker levels in white blood cells was established. Our results might provide a deeper mechanistic comprehension of the underlying biochemical processes of Gb_3 biomarkers in white blood cells of Fabry patients.

1. Introduction

Fabry disease (OMIM no. 301500) is an X-linked, panethnic, inborn error of metabolism caused by mutations of the gene *GLA* leading to α-galactosidase A (α-GAL A, EC 3.2.1.22) enzyme deficiency [1–4]. This multisystemic lysosomal storage disorder causes the accumulation of glycosphingolipids such as globotriaosylceramide (Gb_3) and related isoforms and analogs [5,6], globotriaosylsphingosine (lyso-Gb_3) and related analogs [7–11] and galabiosylceramide (Ga_2) and related isoforms and analogs [12]. Patients affected with classic Fabry disease develop early symptoms, such as acroparesthesia, hypohidrosis, angiokeratomas, gastrointestinal symptoms and psychosocial manifestations [13,14]. Older patients develop cardiac manifestations, including left ventricular hypertrophy, arrhythmia, ischemia, and mitral valve complications. Renal complications occur, which may lead to progressive renal impairment leading to dialysis. Neurological symptoms are also part of the clinical spectrum, as well as ocular manifestations such as cornea verticillata and corneal opacity [15–17]. Typically, Fabry males are more severely affected than females, although in some cases, females can be as severely affected as men [18,19]. The estimated incidence of Fabry disease is between 1/40,000 to 1/117,000 in the general population, but owing to the complexity of obtaining a final diagnosis of the disease, it is probably underestimated [20–22]. In Taipei, a newborn screening study showed that the incidence in the male population is approximately 1/1,600 with the specific cardiac late-onset mutation IVS4 + 919G → A [23,24]. This study suggests that Fabry disease is more prevalent in some populations than initially suspected.

Enzyme replacement therapy (ERT) is one of the treatments for Fabry disease [25,26]. There are currently two common ERTs, using recombinant enzymes: agalsidase alfa (Replagal®, Shire) and agalsidase beta (Fabrazyme®, Sanofi Genzyme), being infused at 0.2 mg/kg biweekly and 1 mg/kg biweekly, respectively. It has been established that ERT slows the progression and the severity of the disease [27–29]. Unfortunately, there were several cases where patients developed antibodies against the enzyme [30,31]. The benefits of ERT must be greater than the potential risks of secondary effects. In order to be eligible for treatment in Canada, patients must respect the indications of the Canadian Fabry Disease Initiative guidelines [32,33]. Nonetheless, even among patients treated by ERT, there were cases with progression of the disease to premature death or organ failures [34].

The monitoring and follow up of patients by quantitation of Gb_3 is currently performed in urine and in plasma by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [6,7]. A method to analyze Gb_3 in urine collected on a filter paper was also developed to facilitate the transport and storage of samples [35]. The discovery of novel Gb_3 isoforms and analogs in urine of patients with Fabry disease was also done by our group using a metabolomic approach [36], and the analysis of Gb_3 isoforms and analogs is now routinely applied for follow up of patients [6]. Gb_3 isoforms are characterized by modifications on the fatty acid chain of the molecule and analogs by modifications on the sphingosine moiety. Gb_3 isoforms and analogs are divided into five groups: 1) Gb_3-related isoforms with saturated fatty acids; 2) Gb_3-related isoforms/analogs with one double bond; 3) Gb_3-related isoforms and analogs with two double bonds; 4) Gb_3-related analogs with a hydrated sphingosine; and 5) methylated Gb_3-related isoforms; [36,37]. Metabolomic studies also demonstrated the existence of lyso-Gb_3 and related analogs [8,11], and galabiosylceramide (Ga_2) with isoforms and analogs in Fabry patient urine samples [12,38]. Several other studies reported the quantitation of Gb_3, lyso-Gb_3 and Ga_2 in plasma [9,39].

In order to localize Gb_3 in different tissues, an immunohistological study was performed in biopsies, autopsies and cell cultures of Fabry patients treated by ERT [40]. Results of this study show an important heterogeneity in the distribution of Gb_3 between organs. Qualitative analysis of the staining demonstrated a significant concentration of Gb_3 in the heart and the kidneys. The subcellular immunohistology highlighted the accumulation of Gb_3 in lysosomes, as expected, but also in the nuclei, the endoplasmic reticulum and the cell membranes. Finally, the study demonstrated that even after five years of ERT treatment for patients, there was a considerable amount of Gb_3 in various cells and tissues [40]. This highlighted the urgent need to investigate the accumulation and distribution of Gb_3 in different organs and cell structures.

A previous study demonstrated the perturbations of the distribution of leukocyte subtypes in Fabry patient [41]. The authors quantified the leukocyte subpopulations by flow cytometric analysis and the intracellular Gb_3 was quantified using fluorescence of monoclonal Gb_3-specific-antibodies. Their results show that Fabry patients tend to have higher levels of lymphocytes and a lower concentration of monocytes. The lymphocytes of Fabry patients showed a significant increase of intracellular Gb_3 compared with controls [41]. These results demonstrate differences between Fabry patients and controls and show that the analysis of intracellular Gb_3 may lead to a better follow up of patients.

Regarding the quantitation of the five aforementioned groups of Gb_3 isoforms and analogs, we have developed and validated a tandem mass spectrometry methodology allowing their analysis in different tissues of NOD/SCID/Fabry mice [37]. These results show a high level of saturated Gb_3 isoforms compared to the other groups. Moreover, Gb_3 isoforms and analogs were particularly increased in the spleen compared to other organs which showed progressively decreasing levels in the small intestine, the kidneys, the lungs, the heart, the liver to the brain [37].

The main objectives of this study were thus to: 1) develop and validate a method by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the relative quantitation of Gb_3 and related isoforms and analogs in white blood cells (unfractionated leukocytes, B lymphocytes, and monocytes); 2) evaluate these biomarkers in a cohort of ERT-