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Application of a diagnostic methodology by quantification of 26:0 lysophosphatidylcholine in dried blood spots for Japanese newborn screening of X-linked adrenoleukodystrophy

Chen Wu[a,b,*], Takeo Iwamoto[c], Junko Igarashi[b], Takashi Miyajima[a,b], Mohammad Arif Hossain[a], Hiroko Yanagisawa[a], Keiko Akiyama[a], Haruo Shintaku[d], Yoshikatsu Eto[a,e,*]

[a] Advanced Clinical Research Center, Institute of Neurological Disorders, Shin-Yurigaoka General Hospital, Kawasaki, Kanagawa, Japan
[b] Rare Disease Research Center, AnGes MG, Kawasaki, Kanagawa, Japan
[c] Core Research Facilities for Basic Science, Molecular Cell Biology, The Jikei University School of Medicine, Tokyo, Japan
[d] Department of Pediatrics, Osaka City University Hospital, Osaka, Japan
[e] Department of Gene Therapy, Institute for DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is a rare inherited metabolic disease that results in the accumulation of very long chain fatty acids (VLCFA) in plasma and all tissues. Recent studies regarding cerebral X-ALD (CALD) treatment emphasize the importance of its early diagnosis. 26:0 lysophosphatidylcholine (LysoPC) is a sensitive biomarker for newborn screening of X-ALD, while its application for Japanese DBS is unclear. Therefore, we evaluated the feasibility of 20:0 LysoPC and 24:0 LysoPC along with 26:0 LysoPC for diagnosing X-ALD in a cohort of newborns (n = 604), healthy adults (n = 50) and patients (n = 4). Results indicated that 26:0 LysoPC had strong significance for discrimination of patients by the amounts of 2.0 to 4.0 and 0.1 to 1.9 pmol/punch for patients and newborns/healthy adults, respectively. Based on these values, we recommend that further diagnostic confirmation is essential if the amount of 26:0 LysoPC in DBS is above 1.7 pmol/punch.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD, OMIM#300100), is the most common peroxisomal disorder affecting approximately 1:30,000 to 1:50,000 boys in Japan [1]. X-ALD is caused by mutation of *ABCD1*, which codes for ALD protein (ALDP) involved in the transportation of very long chain fatty acids (VLCFA)-CoA esters across the peroxisomal membrane [2–5]. Consequently, ALDP deficiency results in the accumulation of VLCFA in plasma and all tissues [6–8]. Patients with X-ALD have several phenotypes, ranging from adrenocortical insufficiency (Addison disease) to slowly progressive adrenomyeloneuropathy (AMN) to rapidly progressive and fatal cerebral X-ALD (CALD) [9]. Several studies have shown the importance of early treatments for childhood cerebral X-ALD (CCALD) by either hematopoietic stem cell transplantation (HSCT) [10] or hematopoietic stem cell gene therapy with a lentiviral vector [11]. Therefore, early diagnosis is crucial.

Increased plasma and/or cultured skin fibroblast VLCFA level and mutation analysis are conventionally used to confirm suspected X-ALD in patients with abnormalities in their clinical presentations or magnetic resonance imaging (MRI) [7,9]. However, due to phenotypic variation in X-ALD [12], misdiagnosis and delayed diagnosis occur frequently. As new technologies for high throughput screening of X-ALD become feasible [13–14], patients can be diagnosed even at birth and treated at a very early stage. Studies on quantification of lysophosphatidylcholines (LysoPCs) in dried blood spots (DBS) for newborn screening have presented satisfactory results, including discrimination of heterogynous females [13–17]. Newborn screening of X-ALD has not been implemented in Japan to date. Therefore, the application of LysoPCs in Japanese newborn DBS (NDBS) is unclear. In this study, we evaluated the feasibility of 20:0 LysoPC, 24:0 LysoPC and 26:0 LysoPC for Japanese newborn screening of X-ALD.

Abbreviations: X-ALD, X-linked adrenoleukodystrophy; VLCFA, very long chain fatty acids; CALD, cerebral X-ALD; AMN, adrenomyeloneuropathy; CCALD, childhood cerebral X-ALD; HSCT, hematopoietic stem cell transplantation; DBS, dried blood spots; NDBS, newborn dried blood spots; MRI, magnetic resonance imaging; IS, internal standard; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; LysoPCs, lysophosphatidylcholines; 20:0 LysoPC, 20:0 lysophosphatidylcholine; 24:0 LysoPC, 24:0 lysophosphatidylcholine; 26:0 LysoPC, 26:0 lysophosphatidylcholine; 26:0-d4 LysoPC, 26:0-d4 lysophosphatidylcholine

* Corresponding authors at: Advanced Clinical Research Center, Institute of Neurological Disorders, Shin-Yurigaoka General Hospital, Asao-Ku, Furusawa 255, Kawasaki City, Kanagawa 215-0026, Japan.
E-mail addresses: chenwu.violet@gmail.com (C. Wu), yosh@sepia.ocn.ne.jp (Y. Eto).

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Table 1
CVs [%] of 20:0 LysoPC, 24:0 LysoPC and 26:0 LysoPC measured by tandem mass.

Sample	20:0 LysoPC CV [%]	24:0 LysoPC CV [%]	26:0 LysoPC CV [%]
Mixture of LysoPCs	4.1[a]	3.0[a]	5.2[a]
(n = 1)	8.7[b]	7.2[b]	6.6[b]
Newborns (n = 4)	7.3–12.3[c]	7.4–12.4[c]	4.6–9.1[c]
	23.2–44.3[d]	7.1–31.1[d]	7.4–22.7[d]
Healthy adults	6.3–12.9[c]	1.8–15.9[c]	0.6–6.7[c]
(n = 3)	5.3–31.4[d]	6.8–37.5[d]	7.8–25.7[d]
X-ALD male (n = 1)	8.1[c]	8.1[c]	5.5[c]
	33.1[d]	22.4[d]	16.9[d]

[a] Mean of ten measurements within a day.
[b] Mean of three independent days with ten measurements per day.
[c] Mean of triplicate measurements of a punch.
[d] Mean of three independent punches with triplicate measurements per punch.

2. Materials and methods

2.1. DBS samples

Six hundred four anonymous NDBS were provided by Osaka City University, Japan. DBS of 50 anonymous controls (healthy adults), an AMN male and his symptomatic mother were obtained from Southern TOHOKU General Hospital, Japan. The DBS of a 12-year-old X-ALD male (without clinical profile) was supplied by the National Center for Child Health and Development, Japan, and the DBS of a 16-year-old AMN male was obtained from St. Marianna University School of Medicine, Japan.

2.2. Extraction of LysoPCs

A 3-mm punch of DBS/NDBS was put into a 1.5 mL tube containing 10 μL of MilliQ water. After mixing for 5 min by micro tube mixer (Tomy, Japan), 240 μL of methanol (HPLC grade, Kanto, Japan), including 10 μL of internal standard (IS) 26:0-d4 LysoPC (Avanti Polar Lipids, Inc., USA), was added. Next, the extraction was sonicated for 15 min at room temperature followed by centrifuging for 5 min at 10,000*g*. Next, 200 μL of supernatant from the extraction was dried under air stream and reconstituted with 20 μL of methanol. The final solution was transferred to autosampler micro vials for tandem mass measurement.

2.3. Calibration set of LysoPCs

A calibration set consisted of 20:0 LysoPC, 24:0 LysoPC and 26:0 LysoPC (Avanti Polar Lipids, Inc., USA) standards with the same amount of IS in the methanol solvent. The calibrated amounts of 20:0 LysoPC, 24:0 LysoPC and 26:0 LysoPC were 0.1–0.7 ng (R^2 = 0.993), 0.1–0.9 ng (R^2 = 0.998) and 0.1–0.7 ng (R^2 = 0.999), respectively. One calibration set was performed for every 50 samples.

2.4. MS/MS measurement

Five microliters of solution was measured by high-performance liquid chromatography (HPLC) connected to tandem mass (LCMS-8040, Shimadzu, Japan). The mixture was eluted with mobile phase A (10 mM NH_4COOH, Wako, Japan) and phase B (10% 10 mM NH_4COOH/MeOH) through C8 column (Inertsil C8–3, 3 μm, 2.1 × 50 mm, GL Sciences Inc., Japan) connected with an in-line filter unit (ACQUITY, Waters, USA). The multiple reaction monitoring (MRM) transitions monitored were 552.5 > 104.1 *m/z* for 20:0 LysoPC, 608.5 > 104.1 *m/z* for 24:0 LysoPC, 636.6 > 104.1 *m/z* for 26:0 LysoPC and 640.6 > 104.1 *m/z* for 26:0-d4 LysoPC, respectively. The quantification of LysoPCs was performed by comparing the peak molecular target area with the IS area.

3. Results and discussion

3.1. Performance of MS/MS for LysoPCs measurement

MS/MS measurement provides precise values of LysoPCs. The repeatability of MS/MS on both standard mixture and DBS is considered reliable when the inter- and intra-day CVs are < 15.9% (Table 1). Bias from instrumental errors in our sample was therefore deemed insignificant. Our developed methodology using a simple extraction process provided acceptable results for LysoPCs to the same degree as other reported data (Table 2).

Nonuniformity of the LysoPCs distribution in one spot was such that normalization of LysoPCs should be considered when punching. Three independent punches from another spot were also extracted, measured and evaluated (Table 1). The dramatic variation in the amount of LysoPCs in these spots, ranging from 5.3 to 44.3% (CV), suggests that LysoPCs are not homogeneous in these spots. Study of this variation has not been reported to date; hence, in the present study, DBS was punched in the same manner. However, the normalization process for sampling in future studies should be evaluated.

3.2. Newborn screening

In this study, 26:0 LysoPC was superior for diagnosing Japanese X-ALD, whereas 24:0 LysoPC and 20:0 LysoPC overlapped (Fig. 1) in keeping with previous studies on LysoPCs [13–14,17]. The heterogynous female (red dot) in our study did not present obvious differentiation of newborns but did differentiate healthy adults (Fig. 1C). This was probably due to the number of samples, as neither LysoPCs nor hexacosanoic acid (C26:0) vary with age [13–14,19]. Otherwise, diet is an alternative explanation for higher levels of LysoPCs in newborns from western Japan in comparison to healthy adults from eastern Japan

Table 2
Amounts of LysoPCs in newborns, X-ALD patients and adult controls.

C20:0 LysoPC [pmol/punch]			C24:0 LysoPC [pmol/punch]			C26:0 LysoPC [pmol/punch]		
Newborns	Patients	Adult controls	Newborns	Patients	Adult controls	Newborns	Patients	Adult controls
0.03–8.02	1.62–2.21	0.78–4.32	0.01–1.44	0.58–1.65	0.01–0.66	0.05–1.92	2.00–3.98	0.01–0.56
0.11–0.63[a]	0.18–0.29[a]	0.14–0.45[a]	0.23–0.43[a]	0.56–0.74[a]	0.15–0.28[a]	0.19–0.36[a]	0.74–1.05[a]	0.20–0.68[a]
0.09–0.31[b]	0.05–0.29[b]	–	0.02–0.13[b]	0.07–0.51[b]	–	0.02–0.06[b]	0.08–0.98[b]	–
–	–	–	–	–	–	0.66[c]	4.69–7.37[c]	–
–	–	–	–	–	–	0.08–1.18[d]	3.80–7.37[d]	–

[a] [16]: transferred the unit of [μg/mL] to [pmol/punch]. Newborns: n = 130; patients: newborn X-ALD, n = 4; adult controls: n = 20.
[b] [13]: transferred the unit of [ng/punch] to [pmol/punch]. Newborns: n = 19; patients: X-ALD and AMN, n = 25.
[c] [14]: newborns: n = 1; patients: newborn X-ALD, n = 5.
[d] [18]: newborns: n = 4689; patients: newborn X-ALD, n = 16.

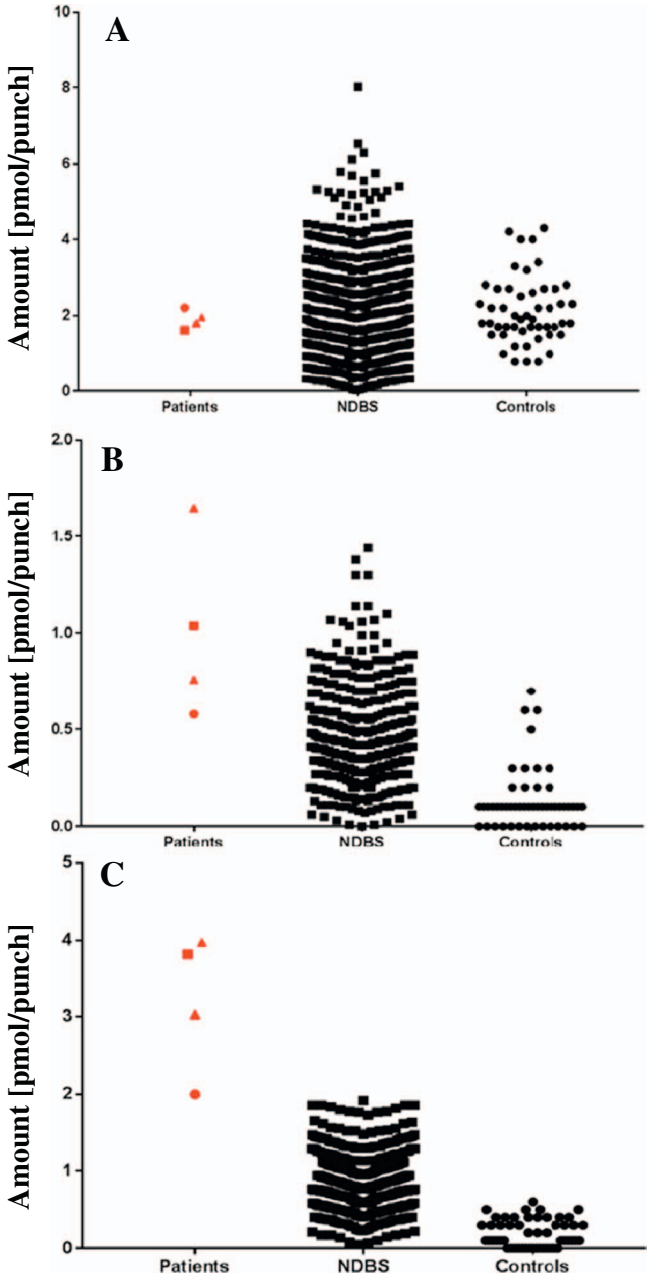

Fig. 1. Comparison of LysoPCs in X-ALD patients, newborns and healthy adults (controls). The plots are the amount of: A) 20:0 LysoPC; B) 24:0 LysoPC; C) 26:0 LysoPC. X-ALD patients (n = 4) are in red; newborns (n = 604) and healthy adults (n = 50) are in black. The red dot is a symptomatic female; the red square marker is an X-ALD male; the other two triangle markers are two AMN males. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[20]. Patients with lower amount of 20:0 LysoPC usually had higher 26:0 LysoPC and 24:0 LysoPC (Fig. 1). The ratio of 26:0 LysoPC to 20:0 LysoPC, furthermore, does not present significant discrimination of newborns as we had expected (Fig. 2), possibly due to the unstable and unreliable molecular structure of 20:0 LysoPC. These findings should be confirmed in subsequent research.

Samples containing amounts of 26:0 LysoPC exceeding 1.7 pmol/punch warrant further diagnostic confirmation. This recommended value is based on 95% confidence limits in our patients, yearly male birth (0.5 million, 2014, Japan) and incidence of X-ALD newborn male [1]. This value will be revised as our investigation proceeds.

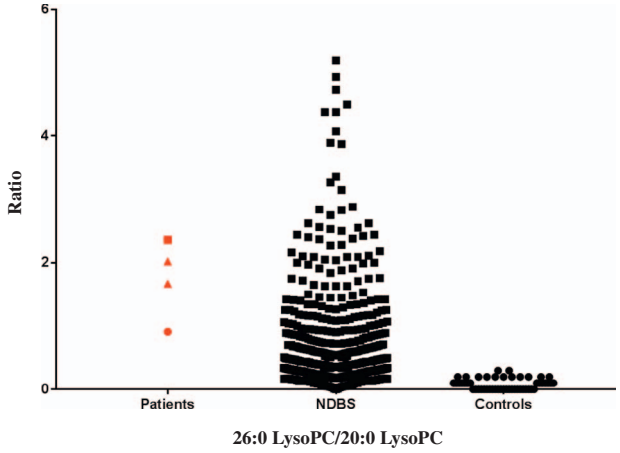

Fig. 2. Ratios of 26:0 LysoPC to 20:0 LysoPC (26:0 LysoPC/20:0 LysoPC) in X-ALD patients, newborns and healthy adults (controls). X-ALD patients (n = 4) are in red; newborns (n = 604) and healthy adults (n = 50) are in black. The red dot is a symptomatic female; the red square marker is an X-ALD male; the other two triangle markers are two AMN males. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The data that we presented in this article strongly indicate that our methodology is applicable for Japanese newborn screening of X-ALD. In comparing three kinds of LysoPCs, quantification of 26:0 LysoPC demonstrated significance for diagnosis. However, 26:0 LysoPC/20:0 LysoPC did not display the same attribution. Our findings, such as the difference between newborns and healthy adults, western and eastern Japanese and heterogeneous distribution of LysoPCs in the spot, will be investigated further.

Conflict of interests

The authors have declared no conflict of interests.

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