X-chromosome inactivation patterns in females with Fabry disease examined by both ultra-deep RNA sequencing and methylation-dependent assay

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Abstract

Background: Fabry disease is an X-linked inherited lysosomal storage disorder related to *GLA* mutations, gene encoding α -galactosidase A. In general, males has severe phenotype, while females has a wide spectrum of sign and symptoms, from asymptomatic to a more classical profile including cardiac, renal, and cerebrovascular manifestations. This variability has been assumed to be derived from organ-dependent skewed X-chromosome inactivation (XCI) patterns in each female patient. Some previous studies examined this correlation using the classical methylation-dependent method; however, conflicting results were obtained. This study was established to determine the existence of skewed XCI in nine females with heterozygous pathogenic variants in the *GLA* gene and its relationship to the phenotypes.

Methods: We present five female patients from one family and four individual female patients with Fabry disease. In all cases, heterozygous pathogenic variants in the *GLA* gene were detected. The X-chromosome inactivation patterns in peripheral blood leukocytes and cells of urine sediment were determined by both classical methylation-dependent *HUMARA* assay and ultra-deep RNA sequencing, the latter being a method that we recently developed.

Results: Among all cases, skewed XCI resulting in predominant inactivation of the normal allele was observed only in one individual case with a severe phenotype. In the other eight cases, no skewing was observed, even among cases with severe phenotypes.

Conclusions: We conclude that skewed XCI could explain the severity of Fabry disease in only a limited number of female cases and is not the main factor in the onset of various clinical symptoms in females with Fabry disease.

Background

Fabry disease (OMIM #301500), is a rare lysosomal disorder that characterized by multi-system involvement due to deficiency of α -galactosidase A, which is required for globotriosyl-ceramide (Gb3) degradation [1]. This enzyme is encoded by the *GLA* gene, located on the X chromosome. Globally, more than 400 private mutations have been defined in patients [2]. The incidence of Fabry disease has been estimated to be approximately 1:117,000 based on live male births, but the incidence can be as high as 1:58,000 when considering heterozygotes [3]. Higher frequencies of 1:7000 and 1:3000 in neonatal mass screening is reported in most recent studies [4–6].

As a result of enzyme deficiency, the accumulation of undegraded substrates, Gb3 and deacylated globotriaosylsphingosine (lyso-Gb3), results in the earliest symptoms in patients with the classic form of this disorder, including neuropathic pain, vascular skin lesions, sweating abnormalities, characteristic ocular changes, gastrointestinal problems, temperature intolerance, or proteinuria [1, 7]. These common signs and symptoms are found in both male patients and female carriers; however, the progression in females may be attenuated, so that they may become symptomatic in a decade or more compare to males patient. It was also reported that the average life expectancy was reduced by 5.75% in females and 22% in males, with the most common cause of death was cardiovascular disease in both sexes [1, 8].

In the past, Fabry disease was known to be transmitted as an X-linked recessive way. It was postulated that the disease may be categorized as X-linked dominant when it was found that heterozygous females could be affected in the same way as hemizygous males [9]; however, at present, Fabry disease is usually described as X-linked [10].

Most X-linked disease only develop in to symptomatic disease in males [11]. However, females demonstrate mosaic expression of X-linked genes through a molecular mechanism known as skewed X-chromosome inactivation (XCI), wherein random transcriptional silencing of one X chromosome takes place in each female cell. While there are various biological events other than skewed XCI that may impact heterozygous females penetrance and phenotype, skew X-inactivation should not be discounted as it can play a significant role in phenotypic expression [12].

Conflicting findings regarding the association between X-inactivation profiles and disease severity in female heterozygotes have been documented in cases of Fabry disease. One study showed that the significant factor in assessing the clinical severity in female heterozygotes was XCI [13, 14], whereas another study showed no correlation between these variables [15]. These studies examined X-inactivation by using a human androgen receptor (*HUMARA*) assay, a polymerase chain reaction-based XCI assay using a methylation-sensitive restriction enzyme [16]. Recently, we developed a novel method for determining X-inactivation patterns by ultra-deep RNA sequencing [17]. The aim of this study is to evaluate whether XCI favoring in the mutant α -galactosidase A exist in our cohort of female heterozygotes with Fabry disease. We applied both the conventional *HUMARA* assay and ultra-deep RNA sequencing using peripheral blood leukocytes and urine- sediments.

Methods

Genomic DNA and total RNA isolation from leukocytes and urine sediment

Quick Gene Mini 80 system (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and Quick-DNA Urine kit (Zymo Research Corporation, Irvine, CA, USA) were used to extract genomic DNA from peripheral blood leukocytes and urine sediment of patient. The obtained genomic DNA was used for targeted sequencing, Sanger sequencing, and *HUMARA* assay.

Total RNA of patient's blood leukocytes was extracted by A Ribopure Blood Kit (Invitrogen, Carlsbad, CA, USA) by adding RNA stabilization agent (RNAlater; Invitrogen) in to the whole blood. ZR Urine RNA Isolation Kit (Zymo Research Corporation) was used for RNA extraction from urine sediment. Ultra-deep targeted RNA sequencing was performed by using both extracted total RNA.

Detection of pathogenic variants of *GLA*

To detect the pathogenic variant in *GLA*, we conducted comprehensive analysis by next-generation sequencing (NGS) using MiSeq (Illumina, San Diego, CA, USA) for all patients. The sample library for NGS analysis was prepared using HaloPlex Target Enrichment Kit 500 kb for Illumina (Agilent Technologies, Santa Clara, CA, USA), in accordance with the manufacturer's workflow. In brief, for each sample to be sequenced, an individual target enriched, index library is prepared by digest and denature the sample DNA, hybridize probe library to DNA targets, ligate and capture uniquely barcoded targets and amplified fragments by PCR. All indexed DNA samples were amplified by polymerase chain reaction and sequenced using the MiSeq platform. Variant calling was performed by using a SureCall 4.0 software (Agilent Technologies).

Sanger sequencing

Variant that were called by SureCall were confirmed using Sanger sequencing. Primer pairs were constructed to amplify mutational site of *GLA* in each patient. After 40 cycles of amplification, PCR products were separated on 1.5% agarose gel and subjected to direct sequencing using dye terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ, USA) and an automatic DNA sequencer (ABI Prism 3130; PerkinElmer, Applied Biosystems, Foster City, CA, USA). For variant description, NM_000169 was used as a reference sequence.

HUMARA assay

The *HUMARA* assay was performed as described by Allen *et al.* [18]. We used 200 ng of genomic DNA from blood leukocytes and urine sediment to be digested by a methylation-sensitive enzyme (*Hpa2*). Digested and undigested DNA was amplified with FAM-labeled forward primer and reverse primer specific for regions either side of polymorphic CAG repeats (forward primer: 5-TCCAGAATCTGTTCCAGAGCGTGC3 ; reverse primer: 5-GCTGTGAAGGTTGCTGTTCTCAT-3). The product was amplified and mixed with internal size standard (GeneScan 500 LIZ Dye Size Standard; PerkinElmer, Applied Biosystems). Quantification of data and their visualization in graphs were performed using GeneScan software. The XCI pattern was defined as random (from 50:50 to 80:20), skewed (from 80:20 to 90:10), or extremely skewed (more than 90:10) [17].

Ultra-deep targeted RNA sequencing using NGS

We recently developed a novel assay of ultra-deep RNA sequencing for examining XCI patterns [17] that uses the quantification of transcript expression for wild-type and variant alleles. Total RNA from blood leukocytes and urine were reverse transcript by using Ecody Premix (Double Primed; Clontech Laboratories Inc., Mountain View, CA, USA). To ensure the purity of the RNA, we amplified the cDNA by glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers, which were designed to amplify both genomic DNA and cDNA to confirm genomic DNA contamination (forward primer: 5-CCCTTCATTGACCCTCAAC-3, reverse primer: 5-TTCACACCCATGACGAAC-3). Nested RT-PCR was then performed using forward and reverse primers designed according to the mutation site. The cDNA PCR product was purified on 1.5% agarose gel, the ends of the cDNA fragments were repaired and adenyl nucleotides were added using TruSeq Nano DNA Library Prep for Illumina (Santa Clara, CA, USA) following the HaloPlex or SureSelect NGS Target Enrichment Workflow (Agilent Technologies) in the NGS analysis. The results were analyzed using SureCall 3.0 (Agilent Technologies). We analyzed the ratio of variant to wild-type alleles for estimation of the XCI [17].

Results

Patient history

We present five female patients from one family (Cases 2–6, Supplementary Figure 1) and four individual female patients (Cases 1 and 7–9) with Fabry disease (**Table 1**). The average age at onset was 37.5 years old. All cases have a family history of Fabry disease. The manifestations of Fabry disease, including cardiac, renal, and cerebrovascular symptoms, varied among the patients, even within the same family. A very low level of α -galactosidase A was found in one individual case (Case 1).

Detection of pathogenic variants of *GLA*

Targeted sequencing by NGS and Sanger sequencing revealed that all nine cases from the five families were heterozygous for likely pathogenic variants of *GLA* (**Table 1**).

HUMARA assay analysis

HUMARA assay was performed using genomic DNA derived from blood leukocytes and urine sediment (**Table 2**). Cases 1 and 8 had homozygous CAG repeats, for which examination of XCI patterns could not be performed. For the *HUMARA* assay, we determined the XCI pattern in each allele divided by molecular size after running capillary electrophoresis. Therefore, when the CAG repeat number was the same in each allele, we could not examine the pattern. For Case 7, we failed to extract genomic DNA from the urine. For Cases 8 and 9, we could not obtain fresh urine samples because these individuals lived far from our hospital. No skewed pattern of XCI was revealed in this assay, except for in Case 1, in which low α -galactosidase A activity was also exhibited. Case 8, in which relatively low α -galactosidase A activity was identified, had a homozygous CAG repeat number, for which the XCI pattern could not be examined by *HUMARA* assay.

Ultra-deep targeted RNA sequencing

We conducted targeted RNA sequencing for all cases. The depth of sequencing in the patients ranged from 102,834 to 694,692 and from 2032 to 3323 for Haloplex and SureSelect NGS Target Enrichment Workflow in the NGS analysis, respectively, representing ultra-deep sequencing. A skewed pattern of XCI involving predominant inactivation of the wild-type allele was revealed in only one individual case (Case 1), in which a very low α -galactosidase A level was exhibited (Table 2).

Discussion

Recently, we have developed a novel method of analyzing XCI using ultra-deep RNA sequencing. Using this assay, we directly measure the ratio of mutated allele to wild-type allele in the *GLA* gene at the transcript level, which functionally and significantly reflects the influence of XCI on clinical manifestations [17].

The presumption for female heterozygous for X-linked diseases is that they will be nearly asymptomatic due to the contribution of the non-mutated allele. It is recognized that the vast majority of heterozygotes female with Fabry disease will suffer from the classical signs and symptoms, but with a delayed onset relative to male hemizygous patient. This phenomenon is thought to be underlying by non-random XCI [12].

Female patients with Fabry disease may display wide spectrum in progression, severity, and disease onset [19], which was also found in this study. Our patients suffered from a wide range of Fabry-related manifestations.

Despite the small number of patients in our study, the ranges of the ages upon the onset of the first Fabry symptoms and diagnosis were nearly consistent with those in a larger study encompassing patients enrolled in the Fabry Registry (32 years vs. 40 years) [20, 21]. In the present study, all patients except Case 1 showed random X-chromosome inactivation in the range of 45:55 to 78:22, irrespective of the disease severity. Our findings are accordance with the study by Maier *et al.* [15], who found that only 18% of female cases showed highly skewed XCI ratios, and with other studies with slightly higher ratios [14]. The skewed XCI pattern in Case 1 was concordant with the disease severity, revealed by a very low level of α -galactosidase A. This case shows that skewed XCI might be one reason for female cases being severely affected. Case 3 and Case 8 were recognized to be severe by their symptoms, having random XCI by both *HUMARA* assay and the ultra-deep RNA sequencing method. From these results, in the presented analysis, the clinical involvement of Fabry disease in females did not correlate with the XCI profiles. This is consistent with the results presented by Maier *et al.* [15] and Elstein *et al.* [12]. Nevertheless, most recent research has shown that Fabry disease progression correlated with the direction of skewing in females with skewed XCI [14, 22]. The measurement of α -galactosidase A enzyme activity has been reported to be unable to predict the clinical phenotype of heterozygous females [13]. However, it was previously reported that there was a clear correlation between XCI and the accumulation of lyso-Gb3. Lyso-Gb3 measurement has been used to be a prognostic marker to clarify disease progression and severity in males [23]. The accumulation of Lyso-Gb3 is considered to be a potential biomarker as it can improve the initial diagnosis of clinically relevant Fabry disease, particularly in females. Nonetheless, in female patients, this accumulation is not reliably correlate with progression and disease burden in females patient [19]; the levels of lyso-Gb3 may be very low or normal, especially with later onset of symptoms [23].

The data obtained in this study suggest that the patterns of XCI in blood leukocytes and urine cells were similar. Although a previous report by Sharp *et al.* described that XCI in older adult women (aged 60 or more) showed marked variation between tissues, they showed that there was severe skewing present in the blood and a random inactivation pattern in buccal epithelial samples [24].

It should be noted that, given our limited number of patients and the finding of only one patient with skewed XCI, our results do not enable the prediction of all female cases.

Conclusions

Our female Fabry cases presented with a wide range of clinical severity. This phenotypic variability cannot be explained only by the phenomenon of XCI because in most of our patients, a random pattern of XCI was detected. Further investigations are needed to elucidate the reason for the variable clinical expression of Fabry disease in females.

Abbreviations

XCI: X-chromosome inactivation

NGS: next-generation sequencing

Declarations

Ethics approval and consent to participate:

All procedures performed in this study were reviewed and approved by the Institutional Review Board of Kobe University Graduate School of Medicine (IRB approval number 019-301). Written informed consent was obtained from all participants for conducting this study

Consent for publication:

Written informed consent was obtained from all participants for publication.

Competing interests:

Kandai Nozu has received lecture fees from Sumitomo Dainippon Pharma. Atsushi Fukunaga has received lecture fees from Sumitomo Dainippon Pharma and Sanofi K.K. Shoichi Maruyama has received grant supports from Sumitomo Dainippon Pharma and Sanofi K.K.. Kazumoto Iijima has received lecture fees from Sanofi K.K. Hideki Fujii has received lecture fees from Sumitomo Dainippon Pharma, Sanofi K.K., and Amicus Therapeutics. the results presented in this paper have not been published previously in whole or part, except in abstract format.

Data Availability Statement:

All data are available on request.

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Authors contributions:

R.R, A.F, N.K, S.M and K.I. designed the study concept and wrote the manuscript. K.N interpreted the data and wrote the manuscript. C.N, T.H, T.Y, N.S, S.M, S.I, Y.A, E.O, and T.N conducted the genetic studies. S.N and H.F critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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Tables

Table 1 Clinical characteristics of nine female cases with Fabry disease

Case No./ Patient ID	Case 1 FAB1	Case 2 FAB 5-1	Case 3 FAB 5-2	Case 4 FAB 5-3	Case 5 FAB 5-4	Case 6 FAB 5-5	Case 7 FAB-7	Case 8 FAB 10-D	Case 9 FAB 12-M
Age (years)	59	69	75	45	43	40	59	47	58
Family history	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Age at onset (years)	40	39	27	42	40	37	55	40	55
HT	(-)	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)
DM	(-)	(-)	(+)	(+)	(-)	(-)	(-)	(-)	(+)
CAD	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
CHF	(-)	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(-)
Stroke	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
Acroparesthesia	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)
Hypohidrosis	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Angiokeratoma	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
Corneal opacities	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
Angina-like chest pain	(-)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
Hearing loss	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(+)	(-)
Vertigo	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(+)	(+)
White matter lesion	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(+)
LVH	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(+)
eGFR (mL/min/1.73 m ²)	72.3	54.2	52.2	64.4	69.7	78.8	81.3	86.2	69.6
Albuminuria (mg/g.Cr)	665.0	868.01	2607.78	86.02	2.73	24.14	12.8	110.2	235.49
Mulberry body	(+)	(+)	(-)	(+)	(+)	(+)	(-)	(-)	(+)
BNP (ng/ml)	95.13	261.13	430.94	20.26	11.97	28.11	11.47	233.0	141.0
Lyso-Gb3 (ng/mL, normal<2)	36.3	18.0	41	9.90	5.69	5.86	0.858	9.81	10.6
β-Galactosidase A (nmol/mg prot./h, normal>49.8)	5.8	43.5	25.2	38.1	58.2	35.2	74.8	20.8	25.4
Mutation site	Exon 6 c.928 C>T	Exon 2 c.281 G>A	Exon 2 c.281 G>A	Exon 2 c.281 G>A	Exon 2 c.281 G>A	Exon 2 c.281 G>A	Exon 7 c.1124 G>A	Exon 5 c.749 A>G	Exon 5 c.658 C>T
Transcript	p. (Leu310Phe)	p. (Cys94Tyr)	p. (Cys94Tyr)	p. (Cys94Tyr)	p. (Cys94Tyr)	p. (Cys94Tyr)	p. (Gly375Glu)	p. (Gln250Arg)	p. (Arg220*)
ACMG/AMP criteria	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	Pathogenic

HT, hypertension; DM, diabetes mellitus; CAD, coronary artery disease; CHF, chronic heart failure; LVH, left ventricular hypertrophy; eGFR, estimated glomerular filtration rate; BNP, brain natriuretic peptide; lyso-Gb3, lyso-globotriaosylceramide

Table 2 X-chromosome inactivation patterns: comparison between *HUMARA* assay and ultra-deep RNA sequencing

Method	Tissue	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
HUMARA assay	Blood leukocytes	NA	68:32	44:56	60:40	69:31	85:15	68:32	NA	63:37
	Urine sediment	NA	43:57	70:30	53:47	69:31	69:31	ND	ND	ND
Ultra-deep RNA sequencing	Blood leukocytes (read depth)	10:90 (628,218)	51:49 (215,173)	45:55 (326,836)	52:48 (460,749)	52:48 (190,181)	78:22 (403,103)	61:39 (347,461)	54:46 (2,032)	71:29 (3,323)
	Urine sediment (read depth)	15:85 (683,772)	60:40 (135,634)	46:54 (142,706)	65:35 (109,933)	66:34 (282,213)	57:43 (331,774)	78:22 (694,692)	ND	ND

NA: Not available because of homozygosity in CAG repeat numbers.

ND: Not determined because samples were not available.

These ratios are shown as activities of wild-type : variant alleles.

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