Disease manifestations and X inactivation in heterozygous females with Fabry disease

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Abstract

Aim: Fabry disease is an X-linked lysosomal storage disorder characterized by an accumulation of neutral glycosphingolipids in multiple organ systems caused by α-galactosidase A deficiency due to mutations in the GLA gene. The majority of heterozygous females show the characteristic signs and symptoms of the disease, and some of them are severely affected. The current hypothesis for the occurrence of disease manifestations in females is skewed X inactivation favouring the mutant GLA allele. Method: We analyzed the patterns of X inactivation in the leukocytes of 28 biochemically and genetically characterized symptomatic Fabry disease heterozygotes and their correlation with clinical and biochemical disease expression. Results: X inactivation patterns in symptomatic females who are heterozygous for Fabry disease did not differ from those of female controls of the same age (p = 0.669). Thirteen (46%) of the 28 females with Fabry disease showed random X inactivation, ten (36%) moderate skewing, and five (18%) highly skewed X inactivation. Segregation analysis was performed in the families of six females who had highly or moderately skewed X inactivation. In four of these females, skewing favoured the wild-type GLA allele and in the other two skewing favoured the mutant allele. Patterns of X inactivation or the extent of skewing were not related to the severity of clinical manifestations or to residual enzyme activity. Conclusion: In this study we provide evidence that heterozygous females with Fabry disease show random X inactivation. Our data do not support the hypothesis that the occurrence and severity of disease manifestations in the majority of Fabry heterozygotes are related to skewed X inactivation.

Key Words: Fabry disease, lysosomal storage disorder, heterozygotes, X inactivation, HUMARA assay

Fabry disease (MIM# 301500) is an X-linked inborn error of glycosphingolipid catabolism resulting from a deficiency of the lysosomal enzyme α-galactosidase A (α-Gal A, EC 3.2.1.22). The enzyme defect leads to a progressive accumulation of neutral glycosphingolipids, mainly globotriaosylceramide, in visceral tissues, body fluids and endothelial cells [1]. The clinical hallmarks of Fabry disease in males comprise characteristic skin lesions (angiokeratoma), acroparesthesias, recurrent crises of excruciating pain, corneal and lenticular opacities, hypohidrosis, cerebral ischaemia and infarction, and cardiac and renal injury. Fabry disease is caused by mutations in the GLA gene, and more than 300 have been reported to date (Human Gene Mutation Database [2]). The GLA gene was mapped to Xq22 and shown to be subject to X inactivation [3]. In the majority of X-linked disorders, heterozygous females are asymptomatic [4,5]. In Fabry disease, however, recent studies revealed that the majority of heterozygous females show the characteristic signs and symptoms of the disease, and many of them are severely affected. Heterozygotes may present with the full spectrum of disease manifestations, but do show a later onset of symptoms, a slower rate of progression and a higher phenotypic variability than male patients [6]. Life expectancy is reduced in both female and male patients, by about 20 and 30 years, respectively [6,7]. α-Gal A activity can be normal, decreased, or undetectable in females [1,6].
Why do females who are heterozygous for an X-linked trait display clinical symptoms? Skewed X inactivation has been shown to correlate with the occurrence and severity of neurological symptoms in females heterozygous for X-linked adrenoleukodystrophy [8]. Similarly, the current hypothesis, which has been extensively referred to in the literature, is that the biochemical and clinical disease manifestations in females with Fabry disease are also related to skewed X inactivation [1,6,9–16]. This assumption appears to be supported by two case reports, on female monozygotic twins and on four females with Fabry disease from one family [17,18], and by a very recent study of 38 symptomatic females with Fabry disease [19]. In the latter study, ten heterozygous females with a preferential inactivation of the wild-type GLA allele showed a more rapid disease progression compared with 27 heterozygous females with balanced X inactivation [19]. In view of these first data, it has been suggested that X inactivation studies could be helpful in predicting the female phenotype and give useful indications for therapeutic management [17,19].

To address this issue, we analysed the patterns of X inactivation in a cohort of 28 clinically, biochemically and genetically characterized heterozygous females with Fabry disease and 56 female controls asking the following three questions: (i) Are females who are heterozygous for Fabry disease prone to skewed X inactivation? (ii) Is the pattern of X inactivation related to the severity of clinical symptoms or the residual enzyme activity? (iii) Is there any evidence for cell selection occurring in favour of the mutant Fabry disease allele?

Subjects and Methods

Subjects

Twenty-eight symptomatic heterozygous females with Fabry disease from a total of 19 families were analysed. Two patients were monozygotic twins (H22 and H25). All patients were seen in a tertiary referral centre. Blood samples for genetic analyses were donated in accordance with consent principles. Patients with Fabry disease were aged between 3 and 71 years (mean age, 36.3 years). In order to minimize bias, all consenting females were included in the study. Heterozygote status was confirmed by mutation analysis of the GLA gene [16]. All but two families sharing the mutation R220X had ‘private’ mutations. In view of identical haplotypes defined by three intragenic DNA polymorphisms (data not shown), these two kindreds may be remotely related.

Controls were matched according to age-groups of 10 years and consisted of 56 females aged between 2 and 73 years (mean age, 36.7 years). Blood samples from these subjects were obtained anonymously from the central laboratory of the Ludwig-Maximilian University Hospital ‘Klinikum Innenstadt’.

X inactivation analysis in leukocytes

DNA was extracted from peripheral blood leukocytes using a standard salting out procedure [20]. Patterns of X inactivation were determined by polymerase chain reaction (PCR) analysis of a polymeric CAG repeat in the first exon of the human androgen receptor gene (AR) as previously described (HUMARA assay) [8].

X inactivation patterns were divided into three groups: random X inactivation (a ratio of between 50:50 and 64:36), moderate skewing (a ratio of between 65:35 and 80:20), and highly skewed X inactivation (a ratio of greater than 80:20) [21].

Parental origin of AR alleles

The parental origin of AR alleles was analysed in heterozygous females with highly or moderately skewed X inactivation [22]. In order to determine the correct phase, this analysis was performed only if a DNA sample from the father of the heterozygote was available.

Biochemical and clinical evaluation

α-Gal A activity in leukocytes was quantified in all Fabry heterozygotes by standard procedures [23]. None of the heterozygotes had been treated with agalsidase alfa enzyme replacement therapy before, or at the time of blood sampling. All heterozygous females underwent comprehensive diagnostic evaluations performed by physicians or nurse specialists experienced in Fabry disease. The individual clinical phenotypes were assessed using the Mainz Severity Score Index (MSSI) [24]. The MSSI was specifically developed for Fabry disease and consists of four components comprising the specific general, neurological, cardiovascular and renal signs and symptoms of the disease. General and renal components have a maximum score of 18, whereas neurological and cardiovascular components have a maximum score of 20. The four components are summed to give the total MSSI score ranging from 0 to 76. The severity of Fabry disease is classified as mild (total MSSI score <20), moderate (total MSSI score 20–40) and severe (total MSSI score >40) [24].

Statistics

Differences between the distribution of patterns of X inactivation in heterozygous females with Fabry disease and controls were analysed using a non-para-
metric Mann–Whitney U test. To establish a correlation between patterns of X inactivation and α-Gal A activity and clinical findings (MSSI scores), respectively, a non-parametric Spearman’s rho correlation was applied. Statistical significance was set at p < 0.05. Statistical analyses were performed using SPSS version 6.1.3 for Windows.

Results

Pattern of X inactivation in heterozygous females with Fabry disease and controls

We addressed the issue of whether symptomatic Fabry heterozygotes are prone to skewed X inactivation in order to explain the high incidence of clinical signs and symptoms in these individuals. Thirteen (46%) of the 28 symptomatic females showed random X inactivation, ten (36%) moderate skewing, and five (18%) highly skewed X inactivation (Figure 1A). The distribution of X inactivation patterns was of a bell-shaped Gaussian type and did not differ from the control group. Random X inactivation was found in 29 (52%) of the 56 controls, moderate skewing in 16 (29%), and highly skewed patterns of X inactivation in 11 (20%) (p = 0.649; Mann–Whitney U test) (Figure 1B).

Parental origin of X-chromosomes

We aimed to determine whether the wild-type or the mutant GLA allele was preferentially inactivated in the 15 heterozygotes displaying highly or moderately skewed X inactivation. As samples were not available for analysis from nine of the fathers (in six out of nine cases because of the advanced age of the female patient), the parental origin of the AR alleles was analysed in the remaining six heterozygotes with skewed X inactivation (Figure 2). In heterozygotes H4, H6 and H13, the mutant GLA allele was inherited from the father and was preferentially inactivated in H4 and H6. Heterozygotes H2, H3 and H9 inherited the wild-type GLA allele from their fathers and it was preferentially inactivated in H2. In summary, the mutant GLA allele was preferentially inactivated in four of the six heterozygotes, namely H3, H4, H6 and H9.

Clinical scores, α-Gal A activity and X inactivation in heterozygote females with Fabry disease

The clinical findings in Fabry heterozygotes included in this study have previously been reported [16,25–27]. Median clinical scores were 21 for total MSSI (5.5 for the general, 7.0 for the neurological, 1.5 for the cardiovascular and 4.0 for the renal components). Two of the females were severely affected, 16 moderately affected and ten mildly affected (Table I).

Two of the patients (H8 and H10) were severely affected and showed moderate skewing, whereas carriers displaying highly skewed patterns of X inactivation showed only mild to moderate clinical symptoms (H1 to H5), although patient H2 had the wild-type GLA allele preferentially inactivated. Likewise, mild and moderate phenotypic expressions were found in patients with random X inactivation patterns. Both members of the monozygotic twin pair showed random X inactivation despite discordant phenotypes, with only mild symptoms in patient H22 and moderate symptoms in patient H25. No significant correlation between the extent of skewing of X inactivation and the severity of clinical symptoms could be established in a Spearman’s rho correlation; either for the total MSSI score (p = 0.413), or for the four separate components (general MSSI [p = 0.627], neurological MSSI [p = 0.563], cardiovascular MSSI [p = 0.838] and renal MSSI score [p = 0.432]) (Table I).

As both skewing of X-inactivation and severity of Fabry disease manifestations have been shown to increase with age, heterozygotes older than 40 years of age with more severe symptoms (total MSSI >30) might be expected to show skewed X inactivation. In our cohort, however, the X inactivation patterns of the six females who fell into this group were variable, with
random inactivation in two (H18 and H28), moderately skewed inactivation in three (H8, H10, and H14) and highly skewed inactivation in one female (H5). The age of females with Fabry disease showed no correlation with the patterns of X inactivation \( (p = 0.634) \), whereas age correlated strongly with the severity of clinical symptoms as assessed by total MSSI score \( (p < 0.001) \).

\( \alpha \)-Gal A activity was determined in leukocytes of all Fabry disease heterozygotes. Only 6 of the 28 patients (H2, H5, H7, H17, H20, and H21) showed decreased activity (Table I). There was no significant correlation between X inactivation in leukocytes and \( \alpha \)-Gal A activity \( (p = 0.768; \) Spearman’s rho correlation).

**Discussion**

Females who are heterozygous for X-linked traits are usually asymptomatic due to random inactivation of one of the two X chromosomes in somatic cells leading to a mosaic of cell populations, which express either the wild-type or the mutant allele. Thus, in females who are heterozygous for X-linked diseases, the wild-type allele is active in approximately 50% of cells, and the gene product provided by these cells is usually sufficient to spare heterozygous females from the clinical effects of the disease [4]. The manifestation of clinical signs and symptoms in the majority of females who are heterozygous for Fabry disease [6] indicates a disease penetrance of close to 100%, an
### Table I. Genotype, X inactivation, enzyme activity and clinical scores of females who are heterozygous for Fabry disease.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Mutation</th>
<th>X inactivation</th>
<th>α-Gal A activity (mU/mg)</th>
<th>Total (0–76)</th>
<th>General (0–18)</th>
<th>Neurological (0–20)</th>
<th>Cardiovascular (0–20)</th>
<th>Renal (0–18)</th>
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<td>1</td>
<td>31</td>
<td>IVS2 + 1G &gt; A</td>
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<td>17</td>
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<td>12</td>
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<td>2</td>
<td>23</td>
<td>Q321X</td>
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<td>5</td>
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</tr>
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<td>14</td>
<td>R220X</td>
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<td>0</td>
</tr>
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<td>8 (M11)</td>
<td>68</td>
<td>W236C</td>
<td>76:24</td>
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<td>76:24</td>
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<td>75:25</td>
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<td>37</td>
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<td>74:26</td>
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<tr>
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<td>3</td>
<td>7</td>
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<td>N320I</td>
<td>63:37</td>
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<td>22</td>
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<td>0.190</td>
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<td>32</td>
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</table>


**b** α-Gal A reference range: 0.23–1.14 mU/mg protein.

**c** Mild disease: Total MSSI <20; moderate disease: Total MSSI 20–40; severe disease: Total MSSI >40 [24].

**d** Nonparametric Spearman’s rho correlation.

D, Daughter; α-Gal A, α-Galactosidase A; M, Mother; MSSI, Mainz Severity Score Index; MZT, Monozygotic twins; S, Sister.
uncommon feature for females who are heterozygous for an X-linked trait [28]. Significant disease manifestation in females who are heterozygous for X-linked diseases has often been attributed to an unfavourable skewing of X inactivation, with the mutant allele being active in most cells [29]. At least three major mechanisms leading to unbalanced X inactivation are known [30–32]. (i) Skewing of X inactivation may occur as stochastic variation of a random process. (ii) Skewing may be due to genetic factors influencing the X inactivation process itself. (iii) Skewed X inactivation may be the result of a post-inactivation selection mechanism. The first two mechanisms are rare and occur independently of the underlying defective GLA allele. Therefore, these approaches would explain the manifestation of clinical signs and symptoms in only a small proportion of Fabry heterozygotes. To explain the occurrence of clinical signs and symptoms in the majority of heterozygotes, one would expect that skewed X inactivation is a frequent finding in females with Fabry disease and is related to post-inactivation selection mechanisms favouring the mutant GLA allele. In our study, however, the incidence of skewed X inactivation was not higher in symptomatic females who are heterozygous for Fabry disease than in healthy controls of the same age. Highly skewed X inactivation, i.e., X inactivation ratios greater than 80:20, that are considered to be pathogenic [21, 33,34], were identified in only 18% of symptomatic females with Fabry disease.

Moreover, if there was a relationship between the clinical phenotype and X inactivation, pronounced skewing of X inactivation would be expected to occur particularly in the more severely affected heterozygotes [35]. In our cohort, however, severely affected heterozygous females (H8 and H10) showed only moderate skewing, whereas females with highly skewed patterns of X inactivation were only mildly (H1 and H2) or moderately (H5) affected. We found no significant correlation between the X inactivation pattern and the clinical phenotype, either in terms of severity, or in terms of affected organ systems. Likewise, there was no correlation between X inactivation and α-Gal A enzyme activity in leukocytes. It is important to consider, however, that there are certain limitations in the instrument used to assess the severity of symptoms in heterozygotes. It is known that the MSSI score increases progressively with age and that this system has not been independently validated. However, taking into account these limitations, this scoring system is still useful for reflecting the severity of disease.

Our data indicate that symptomatic heterozygous females with Fabry disease are not more likely to have skewed X inactivation, but show random X inactivation, as has been suggested for heterozygotes for most X-linked diseases [29,33]. Consistent with these findings, among females with skewed X inactivation, equal numbers showed preferential inactivation of the wild-type and the mutant alleles, indicating no evidence for selection mechanisms favouring the mutant allele (Figure 2). Thus, the occurrence and severity of clinical signs and symptoms in heterozygous females with Fabry disease is not attributable to a high frequency of skewed X inactivation. X inactivation studies will therefore not, as previously proposed [17,19], be helpful in predicting the female phenotype and cannot give useful indications for therapeutic management.

These data are in agreement with that of a very recent study on symptomatic Fabry heterozygotes from the Czech and Slovak Republics [19]. In both cohorts, symptomatic females were not more likely to have unbalanced X inactivation, with skewing (ratio of greater than 75:25) being observed in 29% in the Czech/Slovak and in 32% in the German study. These figures correspond to the incidence of skewed X inactivation found in the normal female population [31] and to that of our control group (21%). Nonetheless, the observation that ten of the 11 females with skewed X inactivation showed preferential inactivation of the wild-type GLA allele in the Czech/Slovak cohort appears to be in contrast with our finding of only two of six females showing preferential inactivation of the wild-type GLA allele. This difference, however, might be due to the small numbers of individuals with skewed X inactivation studied in both cohorts. Dobrovolny et al. [19] reported that the ten females with skewed X inactivation favouring the mutant GLA allele showed a more rapid clinical deterioration compared with the symptomatic females with balanced X inactivation and a symptomatic female with skewed X inactivation favouring the wild-type GLA allele. In spite of the fact that skewing (>75:25) occurred only in 29% of Fabry disease heterozygotes analysed, the authors concluded that X inactivation was a major factor determining the morbidity of Fabry disease in females.

Our data contrast with the reports by Redonnet-Vernhet et al. [18] and Morrone et al. [17]. These groups described an association between X inactivation and clinical phenotype in a pair of female monozygotic twins and in four females with Fabry disease from one family, respectively. However, the observed skewing was presumably not related to the mutant GLA allele in these females. Variable disease expression with divergent skewing of X inactivation is an apparently common phenomenon in monozygotic female twins heterozygous for X-linked disorders [31] and has been observed in various X-linked traits [36–39]. In addition, skewed patterns of X inactivation have been shown to run within families [30,40–42].
We and others [17–19] have studied X inactivation patterns in leukocytes of females who are heterozygous for Fabry disease. Beside the fact that leukocytes are easily accessible, they are suitable for use to study the hypothesis that selection mechanisms may occur in Fabry disease. These mechanisms take place in tissues that express the mutant gene, and are most pronounced in tissues such as rapidly dividing blood cells, which are exposed to high selective pressure [29,30,33]. In females who are heterozygous for Fabry disease, no significant difference has been found so far between the individual X inactivation patterns seen in peripheral blood cells, and urinary and buccal epithelia [19]. Similarly, ultrastructural examination of cardiac and renal tissue from symptomatic women with Fabry disease showed two cell populations, normal cells and cells with lipid accumulation [7,43,44]. Nonetheless, patterns of X inactivation have been described to vary between different tissues [45,46]. Therefore, it cannot be excluded that a correlation between X inactivation and disease phenotype may be revealed in some tissues or organs affected by Fabry disease such as the kidneys or nervous system.

In the light of these considerations, how do random patterns of X inactivation correlate with clinical signs and symptoms in females who are heterozygous for Fabry disease? Females heterozygous for X-linked traits are usually spared from symptoms due to their proportion of functionally normal cells. Alternatively, growth differences between two cell populations may lead to a loss of the deficient cell line due to a selective disadvantage [4,29]. Pronounced differences result in an early loss of the disadvantaged cell population and in marked skewing of X inactivation, whereas more subtle differences lead to a gradual loss of the disadvantaged cell population throughout life and to skewing of X inactivation in older heterozygotes [29]. Indeed, subtle differences between the maternal and the paternal X chromosomes lead to an increase of skewing of X inactivation with age in the healthy female population [46,47]. In the symptomatic female patients in our study, skewing was not a frequent finding compared with females of the same age. A significant correlation between age and X inactivation was not established in the Fabry disease cohort, most likely due to the small sample size. However, we found a strong correlation between age and severity of disease manifestation as seen in hemizygous male patients [6]. Remarkably, two of the elderly female patients (H18 and H28) showed severe manifestations but random patterns of X inactivation. Thus, it seems that disease progression with age reflects the natural course of the disease and is due to the detrimental effect of the mutant GLA allele itself rather than being a result of skewing of X inactivation with age arising from a selective advantage of the mutant GLA allele.

After random inactivation, cells expressing the mutant GLA allele proliferate unhindered and the negative effects of lysosomal lipid accumulation increase with age. Although cells with an active wild-type GLA allele produce fully functional enzyme, part of which is secreted and can be taken up through endocytosis by α-Gal A-deficient cells, this cross-correction is of limited efficiency and can only postpone or ameliorate the pathology – and the manifestation of the signs and symptoms of Fabry disease in females. Consistent with this hypothesis, signs and symptoms in females can be as severe as in males but occur at a later age [6,7].

In conclusion, we provide evidence that females with Fabry disease show random X inactivation. Our data do not support the hypothesis that the occurrence and severity of disease manifestations in the majority of females with Fabry disease are related to unfavourable skewing of X inactivation.

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