Uneven X inactivation in a female monozygotic twin pair with Fabry disease and discordant expression of a novel mutation in the \( \alpha \)-galactosidase A gene

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Abstract

We describe two female monozygotic (MZ) twins heterozygous for Fabry disease, an X linked disorder resulting from the deficient activity of \( \alpha \)-galactosidase A. While one of the twins was clinically affected, the other was asymptomatic. Enzymatic assay of \( \alpha \)-galactosidase in blood leucocytes, skin fibroblasts, Epstein-Barr virus transformed lymphoid cell lines, and hair follicles of the twins and their parents confirmed the heterozygous status of the twins and indicated that Fabry disease had occurred as a result of a de novo mutation. The son of the unaffected twin sister was shown to be hemizygous. Molecular analysis of the \( \alpha \)-galactosidase A gene permitted the identification of an as yet undescribed point mutation at position 10182 of exon 5 which causes an Asp to Asn substitution at codon 231. Single strand conformation polymorphism (SSCP) analysis again showed the heterozygous status of the twins and a normal pattern in their parents. The basis for the discordant expression of this de novo mutation in the twins was investigated by studying their X inactivation status. Analysis of the inactive X specific methylation at the androgen receptor gene showed unbalanced inactivation in the twins’ fibroblasts and in opposite directions. While the maternally derived X chromosome was preferentially active in the asymptomatic twin, the paternal X chromosome was active in the affected twin and was found in her hemizygotic nephew. These data suggest that the paternal X chromosome carries the de novo \( \alpha \)-galactosidase A mutation and that uneven X inactivation is the underlying mechanism for disease expression in this novel female MZ twin pair. This is the first documented case of female twins discordant for Fabry disease.

Key words: Fabry disease; X inactivation; monozygotic twins.

In female mammals, compensation for sex differences in the dosage of most X chromosomal genes occurs during early embryogenesis through X inactivation.1-3 This phenomenon results in selective, irreversible inactivation of either the maternal or the paternal X chromosome of each somatic cell. Since X inactivation occurs at random and is maintained in all daughter cells, females exhibit a mosaic pattern of expression of X linked genes, each X chromosome being active in about half of all somatic cells. However, if one X chromosome is predominantly inactivated in a female hemizygous for an X linked trait, this may lead to severe manifestation of the X linked disorder.

Such a situation can be observed in female monozygotic (MZ) twins who are heterozygous for an X linked disorder. In all the pairs (22) of heterozygous identical female twins published so far,4-7 there was discordant expression of the disease, that is, only one twin was clinically affected, which is interpreted as the consequence of skewed X inactivation in opposite directions in each of the MZ twins. Since expression of an X linked disorder in both female twins has never been described, an interrelation between twinning and X inactivation has been postulated.8-11

In this study, we report the occurrence of female MZ twins with discordant expression of Fabry disease. Anderson-Fabry disease (MIM 301500) is an X linked disorder caused by the deficient activity of the lysosomal exoglycohydrolase, \( \alpha \)-galactosidase A (EC 3.2.1.22).12 The gene encoding \( \alpha \)-galactosidase A, which contains seven exons and extends over 12 kb, is localised on the long arm of the X chromosome (Xq22).13 14 In addition, the \( \alpha \)-galactosidase A gene has previously been shown to be affected by X inactivation.15 Loss of this enzyme activity results in the progressive lysosomal storage of glycosphingolipids with terminal \( \alpha \)-galactose residues. Affected hemizygous males accumulate mainly globo- triosylceramide and galabiosylceramide within the vascular endothelium. Clinical manifestations include angiokeratoma, pain in the extremities, corneal opacities, renal failure, and vascular disease of the heart and brain. Most heterozygous females are clinically asymptomatic or have minimal symptoms (the most frequent being characteristic keratopathy), although some heterozygotes have been reported to be as severely affected as hemizygous males.16 While Fabry disease in males can be definitively diagnosed by demonstration of...
deficient α-galactosidase A activity, enzymatic identification of female carriers is often inconclusive because of the very wide range of α-galactosidase A activity levels owing to the random X chromosomal inactivation phenomenon.

We report here for the first time the enzymatic and molecular identification, as well as the characterisation of the X inactivation status, of female MZ twins discordant for Fabry disease.

Table 1  Short tandem repeat (STR) analysis of the twin sisters

<table>
<thead>
<tr>
<th>STR locus</th>
<th>Chromosome location</th>
<th>Patient 1.1</th>
<th>Patient 1.2</th>
<th>Patient II.2</th>
<th>Patient II.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>Xq26</td>
<td>283</td>
<td>287/287</td>
<td>283/287</td>
<td>283/287</td>
</tr>
<tr>
<td>CSF1R</td>
<td>5q33-q34</td>
<td>308/308</td>
<td>308/312</td>
<td>308/308</td>
<td>308/308</td>
</tr>
<tr>
<td>TH</td>
<td>11p15.5</td>
<td>183/193</td>
<td>199/109</td>
<td>193/199</td>
<td>193/199</td>
</tr>
<tr>
<td>PLA2A</td>
<td>12q23-qter</td>
<td>121;127</td>
<td>119;121</td>
<td>121/127</td>
<td>121/127</td>
</tr>
<tr>
<td>F13A1</td>
<td>6p24-p25</td>
<td>281;289</td>
<td>290/290</td>
<td>290/293</td>
<td>290/293</td>
</tr>
<tr>
<td>CYP19</td>
<td>15q21.1</td>
<td>175/175</td>
<td>175/175</td>
<td>175/175</td>
<td>175/175</td>
</tr>
<tr>
<td>LPL</td>
<td>6p22</td>
<td>124/124</td>
<td>124/128</td>
<td>124/128</td>
<td>124/128</td>
</tr>
<tr>
<td>FES</td>
<td>15q25-qter</td>
<td>143/151</td>
<td>147/155</td>
<td>147/151</td>
<td>147/151</td>
</tr>
<tr>
<td>VWF</td>
<td>12p13</td>
<td>166/170</td>
<td>154/162</td>
<td>162/166</td>
<td>162/166</td>
</tr>
</tbody>
</table>

Allele identification was achieved as described in the Methods section. For each member of the family, the length of the PCR products is given (in bp).

Methods

**Isolation of cells and cell culture**

Tissue samples were obtained from subjects with informed consent. Leucocytes from heparinised blood were isolated using Plasmagel® (Roger Bellon Lab, Paris, France). Skin fibroblasts were obtained from forearm skin biopsies. Lymphoid cell lines were established by Epstein-Barr virus transformation of blood B lymphocytes obtained from controls and from our patients. Mononuclear cells were isolated from 10 ml heparinised blood on Ficoll-Histopaque® (Sigma, L’Isle d’Abeau Chesnes, France) gradient and, after washing, suspended in RPMI 1640 medium containing Epstein-Barr virus B95-8 strain. After 24 hours’ incubation, half of this medium was replaced by fresh medium supplemented with cyclosporin A and the culture was continued as previously described. Lymphoid cells and skin fibroblasts were grown in a humidified 5% CO2 atmosphere at 37°C in RPMI 1640 medium (Gibco BRL, Cergy-Pontoise, France) supplemented with L-glutamine (2 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml), and heat inactivated fetal calf serum (10%), as previously reported. Cells were harvested by slow speed centrifugation and washed three times with PBS. Cell pellets were kept frozen at -70°C until use.

**Enzyme studies**

α-Galactosidase activity was assayed in extracts of peripheral blood leucocytes, cultured skin fibroblasts, and Epstein-Barr virus transformed lymphoid cells in the presence of 4-methylumbelliferyl-α-D-galactopyranoside as previously described. α-Galactosidase A activity was specifically determined using heat stability assays or in the presence of α-N-acetylgalactosaminidase. α-Galactosidase activity was also measured on single hair roots as previously reported; hair root activities were expressed as a ratio of α-galactosidase/β-hexosaminidase. The activity of β-hexosaminidase was determined as described previously.

**DNA analysis**

**Preparation of genomic DNA**

Fibroblast and lymphoid cell pellets were suspended in 1 ml of lysis buffer (10 mmol/l Tris-HCl, pH 8, 100 mmol/l NaCl, 1 mmol/l EDTA, 1% SDS) with protease K (200 μg/ml) and incubated overnight at 56°C. Genomic DNA was extracted with phenol-chloroform (two cycles), precipitated with ethanol, and resuspended in deionised water for amplification by the polymerase chain reaction (PCR).

**Polymorphism study**

The twin sisters and their parents were tested for zyosity by short tandem repeats (STR) loci PCR analysis as previously reported (table 1) using the ALF Fragment Manager (Pharmacia).

α-Galactosidase A DNA amplification

The seven exons encoding α-galactosidase A and their flanking sequences were PCR ampli-
fied using previously described primers. The amplification was performed in 33 cycles, each cycle consisting of one minute denaturation at 94°C, one minute annealing at 55°C, and two minutes extension at 72°C. The reaction mixture contained 400 ng of high molecular weight DNA, 400 ng of each primer, 200 μmol/l of each dNTP, 100 μg/ml bovine serum albumin, 67 mmol/l Tris-HCl (pH 8.8), 6.7 mmol/l MgCl₂, 10 mmol/l β-mercaptoethanol, 6.7 μmol/l EDTA, 16.6 mmol/l (NH₄)₂SO₄, 10% dimethylsulphoxide, and 40 U/ml of Taq DNA polymerase (Cetus Corp, Emeryville, CA, USA). The amplified fragments were analysed by electrophoresis on 2% agarose gels.

**Nucleotide sequence determination**

The amplified DNA was purified by ultralow gelling temperature gel electrophoresis. The excised fragments were directly sequenced by the dideoxy chain termination reaction with α-[35S]dATP (600 Ci/mmol). The amplification primers were used as primers in the sequencing reaction.

**Single strand conformation polymorphism (SSCP)**

SSCP was identified by automated electrophoresis on 12.5% homogeneous PhastGels (Pharmacia-LKB) with native buffer strips for 200 Vh at 4°C according to the manufacturer's instructions. The DNA was visualised by silver staining.

**Analysis of X inactivation patterns**

The X inactivation pattern analysis was performed by studying the methylation of the HhaI sites in the first exon of the human androgen receptor locus as previously reported. DNA samples (1 μg, 20 μl) were digested with 20 U of HhaI (Gibco BRL Life Technologies), or incubated in the same buffer without enzyme, for 10 hours at 37°C. Ten microlitre aliquots were amplified by PCR (100 μl final volume) with 0.5 μmol/l of each primer (synthesised by Eurogentec, Seraing, Belgium), 200 μmol/l dNTPs, 2.5 U Taq DNA polymerase (Eurobio, Les Ulis, France), 10 mmol/l Tris-HCl (pH 9), 50 mmol/l KCl, 0.1% Triton X-100, and 2.5 mmol/l MgCl₂. Samples were amplified using a TRIO-Thermoblock (Biometra, Göttingen, Germany) for 35 cycles, each consisting of a denaturing step at 95°C for one minute, an annealing step at 60°C for 30 seconds, and an extension step at 72°C for 30 seconds. Aliquots of the PCR products were analysed on a 12% polyacrylamide (29:1) non-denaturing gel. Relative intensity of bands after ethidium bromide staining was assessed by densitometry using a Biocom scan. The size of the amplified fragments averaged 280 bp, which corresponds to 11-31 CAG repeat units.

**Results**

**CYTOTOGENIC AND POLYMORPHISM STUDIES**

Cytogenetic analysis showed normal karyotypes in both twins. The sisters shared the same alleles for all STR markers tested. Product length of each marker is shown in table 1 for both parents and the sisters. The PLA2A, VWF, and FES markers were informative for both parents, the CYP19 marker was not informative, and the other six markers were informative for a single parent. From these data it can be calculated that the sisters II.2 and II.3 are monozygotic twins with a probability of 99.98%.

**α-GALACTOSIDASE ENZYME STUDIES**

As illustrated in table 2, both the clinically affected woman (patient II.2) and her clinically unaffected MZ twin sister (patient II.3) exhibited clearly reduced levels of α-galactosidase activity in various cell types, indicating their heterozygous status for the Fabry trait. The fact that the non-manifesting twin was indeed a carrier for Fabry disease was confirmed by the finding that her son (III.2) had severe α-galactosidase activity deficiency, consistent with a hemizygous status. Normal total α-galactosidase and α-galactosidase A² activities were found in both the mother and father of the twins (table 2) as well as in other members of this family. The heterozygous status of the twins was further confirmed by analysing the enzyme activities in single hair follicles. As shown in fig 2, the twin sisters had some hair follicle values in the normal range (fig 2 left) for female controls but also values distinctly below the normal range, that is, in the range of the hemizygous son (III.2). These data provide further evidence for the heterozygous status of the twins. In contrast, none of the 25 hair roots analysed in their mother (fig 2) showed abnormal (intermediate or deficient) α-galactosidase activity (fig 2, right). The enzymatic data therefore suggest that Fabry disease in the twins occurred as a result of a de novo mutation.

**α-GALACTOSIDASE A MOLECULAR STUDIES**

The seven exons of the α-galactosidase A gene of the proband (III.2) were shown to be ampli-
Discordant expression of Fabry disease in MZ twins

Figure 2  α-Galactosidase activity in hair follicles from control female subjects (left panel) and members of the present Fabry family (right panel). The activity of α-galactosidase (α-GAL) was determined as described in the Methods section and is expressed as the ratio to β-hexosaminidase (β-HEX) activity. Each point corresponds to a different hair root.

Figure 3  DNA sequence gel showing the G10182A mutation. The G, A, T, and C reactions of the proband III.2 (P) and a normal control (N) have been loaded alternately. The arrows indicate the point mutation.

Figure 4  Single strand conformation analysis of exon 5 of the α-galactosidase A gene. The gel shows a mobility shift for the G10182A transition that results in a D231N amino acid substitution. The arrows indicate the shifted bands.

α-Gal/β-Hex

0-12

0-10

0-8

0-6

0-4

0-2

0

C-1 C-2 C-3 C-4 C-5 C-6

II-2 II-3 II-2 II-2

This suggests that if the gene defect of the proband is located in the amplified fragments, it is too small to be detected by agarose gel electrophoresis. We then determined the nucleotide sequence of all the amplified fragments. A single alteration was found. At position 10182 of exon 5 of the α-galactosidase A gene (GenBank accession No X14448) a G to A transition has occurred (fig 3). This alteration causes an Asp to Asn amino acid substitution at codon 231 of the gene.

The segregation of the G10182A mutation was followed in the pedigree by SSCP. Fig 4 depicts the SSCP analysis of the G10182A transition. The proband III.2 showed a mobility shift for both DNA strands of the exon 5 PCR product. Both his mother (II.3) and his aunt (II.2) exhibited the normal and abnormal patterns, showing heterozygosity for the abnormality. The grandparents (I.1 and I.2) had the normal pattern.

Since neither maternal grandparent (I.1 or I.2) had the mutant allele, and since non-paternity of the grandfather was unlikely because of HLA typing and polymorphism studies (table 1), both the enzymatic and molecular findings are consistent with a de novo mutation.

ANALYSIS OF THE X INACTIVATION PATTERN

Analysis of the methylation of the HhaI sites adjacent to the highly polymorphic CAG repeat (90% heterozygosity) of the human androgen receptor gene was informative and was performed on cultured skin fibroblasts (fig 5A) and lymphoid cells (fig 5B) from the twins. In this assay, DNA is digested with HhaI before PCR amplification of the polymorphic repeat. Only methylated alleles, present on an inactive X chromosome, can be amplified. This semi-quantitative method allows reliable
Figure 5 Study of X inactivation patterns in the present Fabry family by using analysis of methylation of HhaI sites at the human androgen receptor locus. DNA samples from cultured skin fibroblasts and lymphoid cell lines of the patients were PCR amplified as described in Methods, after preincubation in the absence (lanes -) or presence (lanes +) of HhaI. The androgen receptor alleles in the twins' fibroblasts (A) or lymphoid cells (B) are compared to those of the lymphoid cells from the rest of the family. In fibroblasts, densitometric analysis of digested alleles indicated ratios of 0:100 and 97:3 for the paternal:maternal alleles in the affected (II. 2) and unaffected (II. 3) twin sisters, respectively. In lymphoid cells (B) after digestion, ratios of 12:88 and 37:63 were obtained for the manifesting and unaffected twins, respectively. Similar results were obtained in five to 10 separate experiments.

Interpretation of the X inactivation status only when perfectly random or almost 100% skewed patterns are obtained.

Figure 5A shows the various alleles in the Fabry twins' fibroblasts. PCR without previous HhaI digestion was also performed on lymphoid cells from the parents and the affected child. The alleles in the undigested samples were of approximately equal intensity. Methylation patterns of the twins were determined by comparing the intensity of the paternal and maternal alleles in samples that had been digested with the restriction endonuclease. A skewed methylation pattern in opposite directions was apparent in the fibroblast DNA samples from the twins (fig 5A). In one twin (II. 2), the ratio of paternal:maternal alleles after HhaI digestion was 0:100 while this ratio was 97:3 in the other twin (II. 3). From this methylation pattern, it can be concluded that the paternal X chromosome is preferentially active in the Fabry diseased twin and that the maternal X chromosome is active in the unaffected sister. Since the X chromosome active in the affected twin (II. 2) and the Fabry hemizygote (III. 2) is of paternal origin, our data also suggest that the de novo mutation occurred in the twins' father.

In contrast to fibroblast samples, analysis of DNA extracted from lymphoid cell lines of the twins did not show opposite inactivation patterns, but a slightly preferential inactivation of the maternal X chromosome in both twins (fig 5B). These data are in good agreement with the α-galactosidase activity levels measured in the two types of cells (table 2); while a clear difference in the activity levels between the twins was apparent in the fibroblasts, quite similar values (slightly below the heterozygote range) were observed in lymphoid cell homogenates.

Discussion

This study reports a French family with Fabry disease resulting from an as yet undescribed mutation. A series of molecular lesions in the α-galactosidase A gene causing Fabry disease have been reported. Most of these mutations are private and include exonic gene rearrangements, small deletions and insertions, mRNA processing defects, and single base substitutions. The single point mutation we identified causes a change in the charge of the amino acid chain and is therefore very likely to affect the α-galactosidase structure or function or both. A similar substitution has already been described in exon 5 at codon 244.

The findings of our enzymatic and molecular studies suggest that in the present family, the mutant allele arose in a gamete of the grandfather. To our knowledge, this report is the third example of Fabry disease resulting from a de novo mutation. Indeed, out of the many mutations identified in Fabry disease, only two instances of de novo mutations have been reported, one partial deletion and one complex mutation in exon 5. Analysis of the polymorphism at the human androgen receptor locus indicated that the Fabry mutation found in the twins and the hemizygous child was carried by the X chromosome inherited from the grandfather, suggesting that the de
Discordant expression of Fabry disease in MZ twins

The discordant expression of Fabry disease in MZ twins was established on the basis of several criteria. In addition to the physical similarities and identity of erythrocyte and HLA groups, the twin sisters shared the same pattern of polymorphic STR markers (table 1).

Although monozygotic, the twins exhibited discordant expression of Fabry disease. While they were both shown to be heterozygous for α-galactosidase A deficiency, only one twin had clinical signs and symptoms of Fabry disease. Two other female twin pairs heterozygous for Fabry disease have previously been reported in an English\(^2\) and a Dutch\(^3\) family. Although their monozygosity has not been established, these patients also seemed to exhibit different expression of the disease.\(^4\)

The observed phenotypic discordance between the twins could be explained by X inactivation dissimilarities. Indeed, uneven lyonisation between the twin sisters, that is, a different pattern of X inactivation at the androgen receptor locus, was found in skin fibroblasts. This type of analysis clearly showed that the paternally derived X chromosome was active in most skin cells of the Fabry diseased twin while the maternally derived X was active in the unaffected twin. This X inactivation pattern is likely to occur also in the target cells of Fabry disease, that is, endothelial and smooth muscle cells,\(^12\) since the latter cells and skin fibroblasts have the same mesodermal origin.

While completely opposite inactivation patterns were noted in skin fibroblasts of our twins, the patterns of methylation in the lymphoid cells were not perfectly random but were comparable in both sisters. Several hypotheses may account for the discrepancy between the two cell types. First, different tissues may show different patterns of X inactivation. Second, a selective effect in vitro may have resulted in significant clonal outgrowth of lymphoblasts in culture, as previously reported.\(^3\) Finally, it has been postulated that the random inactivation status in lymphocytes could result from shared fetal circulation of lymphocyte precursors.\(^4\) Accordingly, analysis of the X inactivation patterns in a cell type other than cultured fibroblasts or lymphoblasts, for example, peripheral blood leucocytes or lymphocytes, was not performed because concordant patterns of methylation in lymphocytes (but not in the skin) have previously been reported in a twin pair\(^4\) and because one of our twins is no longer available.

Discordant expression of X linked disorders in monozygotic twin females has been reported in at least 22 female twin pairs heterozygous for Duchenne muscular dystrophy, deuteranomaly, G6PD deficiency, haemophilia B, fragile X syndrome, and Hunter disease.\(^4\) Our observation adds another twin pair with markedly different X inactivation patterns for an hitherto undescribed X linked trait, α-galactosidase A deficiency. Thus, as previously noted,\(^6\) it appears that concordant expression of (at least seven) X linked disorders in MZ female heterozygotes has never been reported. This finding has led to the postulation that MZ twinning events and uneven X inactivation are connected.\(^3\)\(^6\)\(^4\)\(^6\)\(^7\) Indeed, if X inactivation occurred independently in each female MZ twin, creation of opposite patterns of non-random X inactivation would be unlikely. Accordingly, it seems that X inactivation precedes MZ twinning, and that predominant inactivation of the normal X in one of the twins frequently accompanies the twinning process. One possible mechanism for non-randomness would be the asymmetrical splitting of the inner cell mass.\(^10\)\(^6\)


