ONLINE MUTATION REPORT

Fabry disease: molecular studies in Italian patients and X inactivation analysis in manifesting carriers

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Anderson-Fabry disease (E C 3.2.1.22, MIM 301500) is an X linked lysosomal storage disorder caused by a deficiency of the enzyme α-galactosidase A (GLA).[1 2] The onset of the disease and the severity of clinical manifestations depend principally on residual GLA enzymatic activity.[1] Fabry disease can be classified into two clinical phenotypes: the classical form and the cardiac variant.[1 3] The classical form is mainly characterised, in affected hemizygous males, by angiokeratoma, acroparaesthesias, hypohidrosis, pains, fever crises, and involvement of the kidneys, brain, and heart. Neurological and/or psychological manifestations with personality disturbances can also occur.[1] The cardiac variant is characterised by symptoms restricted to cardiac abnormalities, including conduction defects and/or late onset cardiomyopathy with left ventricular hypertrophy.[1 3 4] A prevalence of Fabry disease in a referral population of male patients with a clinical diagnosis of late onset hypertrophic cardiomyopathy (HCM) has also been reported.[5] The X linked disorders affect males, while the female carriers are generally asymptomatic, owing in part to the random inactivation of the X chromosome.[6] Fabry female carriers can be asymptomatic or clinically affected, usually with a late onset and mild form of the disease. Corneal abnormalities are the most frequent clinical manifestations.[1]

The human *GLA* gene, mapped on Xq22, is organised in seven exons encompassing over 12 kb.[7] So far, about 265 mutations spread throughout the *GLA* gene in all exons have been reported in the Human Gene Mutation Database Web site and, in addition, a further 65 have been published.[8 9]

Recently, Garman and Garboczi[10] constructed a model of the human GLA enzyme, based on the *x* ray structure of the closely related enzyme alpha-N-acetylgalactosaminidase (alpha-NAGAL), in which they mapped more than 190 reported point and stop mutations. They identified two types of *GLA* gene mutations: one disrupts the active site of the enzyme and the other affects the stability of folded protein.

The diagnosis of Fabry disease in male patients can be easily made through enzymatic GLA assay in plasma, leucocytes, or cultured fibroblasts but it is very difficult to determine the carrier status in females. Because of X chromosome inactivation even obligate heterozygous females can show normal GLA enzymatic activity.[1] The only certain method of detecting a female carrier is through molecular analysis.

Fabry, Hunter, and Danon disease are the only X linked lysosomal storage diseases. Most of the Fabry carrier females are symptomatic while the Hunter carrier females, with a normal karyotype, do not usually show clinical manifestations.[11]

Twins heterozygous for the GLA enzymatic defect showing discordant phenotypic expression have been reported.[12] Random X inactivation has been reported for the *GLA* gene.[1] X inactivation studies in two carrier female monozygotic twins showing a discordant expression of the same mutation of the *GLA* gene have been described.[13] So far, no further studies on X inactivation have been carried out on manifesting Fabry female carriers.

GLA enzyme replacement therapy is now available to treat Fabry patients.[14 15] With the aim of genotype/phenotype correlation and in order to develop better patient care and therapeutic management, molecular studies were carried out on 18 Italian patients affected by Fabry disease in 14 unrelated families and in their relatives. In order to determine a correlation between clinical manifestations in Fabry carriers, X inactivation studies on four carrier females from the same family are reported.

Key points

- Our work aimed to carry out clinical, biochemical, and molecular studies in 18 Italian male patients affected by Fabry disease from 14 unrelated families.
- We identified five new (L167P, de novo A352D, c617-618delTT, c126-127insCATG, c946delG) and eight known (P40L, R220X, R227Q, W236C, de novo Y365X, C378Y, IVS3+1G>A, S78X) mutations in the patients' *GLA* gene.
- An aberrant GLA transcript c486-547del62bp, that leads to an early stop codon, was detected in a male patient carrying the IVS3+1G>A splicing defect.
- We also report on X inactivation studies in four female carriers from one family with a history of Fabry disease in males. Two manifesting carriers showed a skewed pattern of X inactivation in favour of the mutant allele while two asymptomatic carriers showed a skewed pattern in favour of the wild type allele. In these carriers, the X inactivation studies suggest a correlation between clinical manifestations and the skewing of X inactivation, which could be helpful in predicting the female phenotype and give useful indications for therapeutic management.

MATERIALS AND METHODS

The α-GLA enzymatic assay was carried out as previously reported.[16] Genomic DNA and total RNA from the patients and their relatives were isolated from lymphocytes. *GLA* cDNA synthesis was generated from 10 µg total RNA using displayTHERMO-RT™ by Display Systems Biotech (Copenhagen, Denmark) with oligo dT and/or a specific antisense oligonucleotide primer RNA7Rest mapping in the 3'UTR region of the wild type GLA cDNA. The *GLA* exons, intron-exon boundaries, and the *GLA* cDNA were amplified by PCR using the following primers synthesised by Roche Molecular Biochemicals (Basel, Switzerland): F1 forward 5' gcccctgaggttaatcttaa 3' (–67/–48nt); R1 reverse 5' aactgttcccgttgagactc 3' (IVS1 +87/+68nt); F2 forward 5' ggaggtacctaagtgttcat 3' (IVS1 –58/–39nt); R2 reverse 5' agcttctgtacagaagtgct 3' (IVS2

Table 1 Clinical features of Italian male patients with Fabry disease

Patient	1.1	1.2	2.1	3.1	4.1	5.1	6.1	7.1	8.1	9.1	9.2	9.3	10.1	11.1	12.1	13.1	14.1	14.2
Present age	39	29	26	44	39	31	11	36	29	52	28	25	23	33	40	40	69	11
Age at diagnosis	32	23	23	40	31	18	10	10	12	40	14	13	14	20	25	27	68	10
Hypohidrosis	+	+	+	+	++	+	+	+	+	+	+	+	++	+	+	+	+	+
Pains	+++	+	+	++	+	+++	+++	+++	+	++	+	++	+++	++	++	++++	++	++
Fever crisis	+	+	No	+	+	+	+++	+++	+	+++	+	++	+++	++	++	+	+	+
Angiokeratoma	No	+	No	+++	No	+	++	++++	++	+++	++	++	+++	++	+	+	+	+
Cerebrovascular involvement	No	No	No	++	No	No	No	No	No	+++	No	++	No	No	No	No	+	No
Ocular manifestations	+	+	+	+	++	+	+	++	++	++	+	+	++	++	++	+++	++	++
Renal insufficiency	++++	+	+	++	++	++++	No	+	+++	+++	+	+	No	++++	+++	++++	+	No
Cardiac involvement	++	No	No	+++	+	++	No	++	++	++	No	No	No	No	+	++	+	No
Lymphoedema	++	+	No	++	+	+	No	+++	No	++	+	++	No	No	No	No	+	No
% of enzymatic activity	5.5	3	12	10	6	3	4	2	9	3	4	5	5	4	3	3	7.8	9.9
Hearing impairment	++	+	+	+++	+	No	No	++	+	++	+	+	No		+	+	+	No

+62/+43nt); F3 forward 5′ aagtaaccttgtctcttccc 3′ (IVS2 –102/–83nt); R3 reverse 5′ ttctttggctcagctaccat 3′ (IVS3 +51/+70nt); F4 forward 5′ agactgaaccccatctcaaa 3′ (IVS3 –113/–94nt); R4 reverse 5′ ccttggtttcctttgttgtc 3′ (IVS4 +71/+52nt); F5 forward 5′ ctgtaaactcaagagaaggc 3′ (IVS4 –102/–83nt); R5 reverse 5′ caggaactttacctgtatttacc 3′ (IVS5 +88/+66nt); F6 forward 5′ ggtttctccatatgggtcat 3′ (IVS5 –62/–81nt); R6 reverse 5′ ttaggcccaagacaaagttg 3′ (IVS6 +65/+46nt); F7 forward 5′ tgaatgccaaactaacaggg 3′ (IVS6 –67/–48nt); R7 reverse 5′ cacctagccttgagctttt 3′ (+76/+57); RNA1F forward 5′ TTCATGTGCAACCTTGACTGC 3′ (c148/c168, exon 1); RNA3F forward 5′ TTCACAGCAAAGGACTGAAG 3′ (c371/c390, exon 3); RNA3R reverse 5′ TTTAGCAGATCTACTCCCCA 3′ (c503/c484, exon 3); RNA5F forward 5′ CTGGCGAAATTTTGCTGACA 3′ (c675/c694, exon 5); RNA5R reverse 5′ TGGTCCAGCAACATCAACAA 3′ (c758/c777, exon 5); RNA7Rint reverse 5′ AGGTCGTTCCCACACTTCAA 3′ (c1029/c1010, exon 7); RNA7Rest reverse 5′ TGGACAGGAAGTAGTAGTTG 3′ (+18/+37nt 3′UTR).

The positions of the *GLA* DNA and cDNA primers used in the experiments described correspond to the nomenclature of den Dunnen and Antonarakis.[17]

Each genomic fragment (F1-R1 348 bp, F2-R2 295 bp, F3-R3 350 bp, F4-R4 276 bp, F5-R5 352 bp, F6-R6 344 bp, F7-R7 434 bp) and *GLA* cDNA fragment (F1-RNA3R 570 bp, RNA1F-RNA5R 629 bp, RNA3F-RNA5R 406 bp, RNA5F-RNA7Rint 354 bp) were amplified under the following conditions: about 200 ng of genomic DNA and *GLA* cDNA were amplified with 2.5 U Ampli*Taq* DNA polymerase (PE=Perkin Elmer Cetus, Branchburgh, NJ), 25 mmol/l of dNTPs, 200 ng of forward primer, 200 ng of reverse primer and 1 × PCR reaction buffer in a total volume of 50 µl. Cycling conditions for these primer sets were: denaturation at 94°C for three minutes, then 28 cycles at 94°C for 30 seconds, 53°C for 30 seconds, and 65°C for four minutes with a seven minute 65°C final extension cycle.

The sequencing reactions were performed using Big Dye Terminator Cycle Sequencing Ready Reaction Kit reagents (PE Biosystems). The reactions were run on an ABI 310 sequencer (PE Biosystems) and were analysed using Sequencing Analysis Software, version 3.3.

The new amino acid substitutions L167P (c500T>C) and A352D (c1055C>A) were also confirmed by *Dde*I (–ACRS) and *Eco*RV (+ACRS) restriction analysis respectively. The following modified primers: L167Prmut 5′ ACTGTCACAGTAACAACCATCAAATCT 3′ (c528-c502/c503A>G) and A352Drmut CGAGGTCCACCAATCTCCTGCCGGTTTATGA (c1088-c1058/c1059G>C) were made to screen the L167P and A352D amino acid substitutions respectively. The fragments F3-L167Prmut (261 bp) and F7-A352Drmut (156 bp), encompassing the region of L167P and A352D mutations, were amplified with the same cycling conditions reported above. A possible benign polymorphism for the new point mutations L167P and A352D was excluded by restriction analysis of 100 Italian normal female controls' genomic DNA.

The polymorphic CAG repeats of the human androgen receptor gene were amplified from peripheral blood DNA and the two chromosomes of each female and their methylation status were determined using methylation sensitive enzymes as previously reported.[18]

RESULTS AND DISCUSSION

The clinical, biochemical, and molecular studies of 18 male patients affected by Fabry disease in 14 unrelated Italian families are reported. The patients' clinical features are summarised in table 1. In all patients, the diagnosis of Fabry disease was confirmed by the reduction or absence of α-galactosidase A enzymatic activity in leucocytes (table 1).

Fabry disease shows wide phenotypic variability both in clinical manifestations and biochemical parameters. The presence of skin lesions alone is not always an indication of the

Table 2 *GLA* gene mutations identified in Italian patients with Fabry disease

	Phenotype	Exon/intron location	Nucleotide change	Effect on coding sequence	Genotype	Reference
1.1	Classical	Exon 1	c126-127insCATG	Frameshift/stop codon	c126-127 insCATG	This work
1.2	Classical	Exon 1	c126-127insCATG	Frameshift/stop codon	c126-127 insCATG	This work
2.1	Classical	Exon 4	c617-618delTT	Frameshift/stop codon	c617-618delTT	This work
3.1	Classical	Exon 6	c946delG	Frameshift/stop codon	c946delG	This work
4.1	Classical	Intron 3	IVS3+1G>A	Splicing defect	IVS3+1G>A	21
5.1	Classical	Exon 3	c500T>C	Amino acid change	L167P	This work
6.1	Classical	Exon 7	c1055C>A	Amino acid change	A352D (de novo)	This work
7.1	Classical	Exon1 (helix terminus)	c119C>T	Amino acid change	P40L	21
8.1	Classical	Exon 7 (disulphide)	c1133G>A	Amino acid change	C378Y	29
9.1	Classical	Exon 5 (active site)	c680A>T	Amino acid change	R227Q	22
9.2	Classical	Exon 5 (active site)	c680A>T	Amino acid change	R227Q	22
9.3	Classical	Exon 5 (active site)	c680A>T	Amino acid change	R227Q	22
10.1	Classical	Exon 7	c1095T>A	Stop codon	Y365X (de novo)	31
11.1	Classical	Exon 5	c658C>T	Stop codon	R220X	30
12.1	Classical	Exon 2	c233C>G	Stop codon	S78X	28
13.1	Classical	Exon 2	c233C>G	Stop codon	S78X	28
14.1	Classical	Exon 5 (buried)	c708G>C	Amino acid change	W236C	19
14.2	Classical	Exon 5 (buried)	c708G>C	Amino acid change	W236C	19

disease even if in this study they were present in 14 out of the 18 patients. In all patients, pronounced hypohidrosis, fever crises, ocular manifestations, and pain are present. Most of the patients (12/18) also showed hearing impairment. With increasing age, the major morbid symptoms are progressive renal impairment and cardiac involvement. Cerebrovascular manifestations (strokes and transient ischaemic attacks) can be also present.

It is not easy to determine the clinical onset and the natural history of Fabry patients, especially if angiokeratoma is absent, because clinical signs may be confused with other diseases such as rheumatoid disease or fevers. Diagnosis is often not made until adulthood when renal or cardiac deterioration are evident. In most of the reported patients a late diagnosis was made. Molecular analysis was carried out in order to find a possible genotype-phenotype correlation and to obtain a more accurate assessment of prognosis.

Five new (c126-127insCATG, c617-618delTT, c946delG, L167P, de novo A352D) and eight known (IVS3+1G>A, P40L, C378Y, R227Q, R220X, de novo Y365X, S78X, W236C) mutations were identified in the patients' *GLA* gene. An aberrant *GLA* transcript c486-547del62bp, that leads to an early stop codon, was detected in a male patient carrying the IVS3+1G>A splicing defect (table 2).

The new insertion duplication, c126-127insCATG (exon 1), was detected in the *GLA* genomic DNA of two brothers (1.1 and 1.2) affected by the classical form of the disease with kidney involvement and also in their mother.

The *GLA* cDNA analysis of the brothers 1.1 and 1.2 identified only one aberrant transcript, carrying the c126-127insCATG 4 bp insertion duplication that leads to a frameshift and a premature stop codon. Patient 1.1 is a 39 year old with severe renal failure. He has been treated with haemodialysis three times a week for five years and is awaiting renal transplantation. The Fabry diagnosis was made two years after he started dialysis. He does not show any skin lesions or neurological impairment. Only a mild hypertrophic cardiomyopathy associated with conduction abnormalities is present. His younger brother, patient 1.2, 29 years old, has a mild angiokeratoma on his back, very mild lymphoedema of the extremities, and a mild proteinuria but he does not show any cardiac involvement. The patients' clinical manifestations are very different, showing great intrafamilial heterogeneity. This phenomenon, observed also in other patients, might be explained by the presence of modifying genes that cooperate in the expression of GLA in patients with the same mutation. Two other genetic lesions have been reported in the same CATG region of exon 1: the amino acid substitution M42V[19] and the small deletion c125-137del13[20]; all of them appear to be private.

The new 2 bp deletion, c617-618delTT (exon 4), was detected in patient 2.1. This small deletion leads to a frameshift and a premature stop codon. The patient's *GLA* cDNA analysis did not show any additional aberrant transcript except the transcript carrying the c617-618delTT.

The new c946delG (exon 6) was detected in patient 3.1. This deletion gives rise to a premature stop codon and a truncated protein. More than 70 small deletions or insertions, spread throughout the *GLA* gene, have been reported showing that these types of mutations often occur in this gene.

The known IVS3+1G>A donor splice site defect[21] was identified in the *GLA* gene of patient 4.1, in his mother, and in his sister. Patient 4.1 is a 39 year old man who was suspected of suffering from Fabry disease because of the constant presence of proteinuria from early childhood. The diagnosis was confirmed by GLA enzymatic assay at 31 years of age. He does not manifest any skin lesions, but has painful crises associated with acroparaesthesias and hypohidrosis with attacks of fever during the summer. Cardiac conduction defect is present. This mutation has been reported in genomic DNA but its effect on *GLA* mRNA has not been analysed. We studied the *GLA* mRNA isolated from leucocytes and we amplified three different fragments encompassing exons 1-7, 3-5, and 3-7, in order to detect a possible aberrant transcript owing to the IVS3+1G>A splicing junction defect. Surprisingly, in all fragments amplified a partial deletion of exon 3, c486-547del62bp, and not the predicted skipping of exon 3 and/or exon 4 was detected. Apparently no new donor site is generated and this new aberrant *GLA* transcript leads to a frameshift and an early stop codon. Splice defects are not often reported in Fabry patients. Of about 330 *GLA* genetic lesions reported up to now only 17 are splice defects and most of these have not been studied at the RNA level.

The new transition, c500T>C (exon 3), that leads to the new L167P amino acid substitution was detected in patient 5.1. The patient has shown a gradual deterioration in renal function with proteinuria from 18 years of age. A kidney biopsy allowed the diagnosis of Fabry disease that was confirmed by enzymatic assay of α-galactosidase A. The patient started peritoneal dialysis three years ago because of renal failure. He has corneal opacities, acroparaesthesias, painful abdominal crises, and mild mitral valve insufficiency. The family history of patient 5.1 shows that retrocapsular cataract, renal failure, and hypertrophic cardiomyopathy were present in his 68 year old mother.

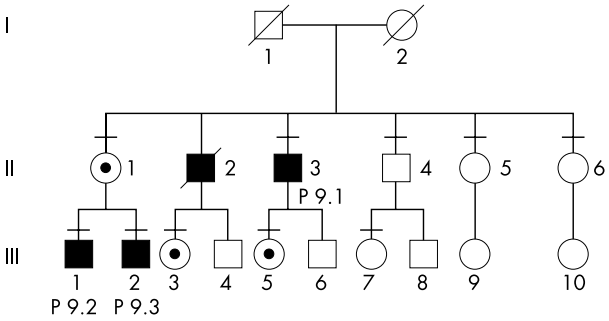


Figure 1 Pedigree of family 9.

The L167P genetic lesion was also confirmed in the genomic DNA by *Dde*I (−ACRS) restriction enzyme analysis. In the GLA protein region close to the L167 residue, several mutations were reported on residues 162, 163, 165, 166, and 168 in patients with the classical form of the disease.[8 9 22–25] These data show that this region is an important domain for GLA protein. Most of the reported amino acid changes (162, 163, 165, and 166) affect the stability of folded protein that is rapidly degraded before cleaving the substrate.[10]

The new amino acid substitution A352D caused by the transversion c1055C>A (exon 7) was detected in the *GLA* gene of patient 6.1. This mutation was not present in the patient's mother, sisters, grandmother, or maternal aunt, even though all of them share the same 278 bp androgen receptor allele, detected by gene scan analysis (data not shown). This result suggests that the A352D is a de novo mutation. This mutation was also confirmed in the patient's genomic DNA by *Eco*RV (+ACRS) restriction enzyme analysis. The A352D amino acid substitution is located in the region encompassing codon 334-430, in which most of the reported mutations occur, and many of them are correlated with severe renal failure.[26]

The transition c118C>T (exon 1) that leads to the known amino acid substitution P40L[21] was detected in patient 7.1. This patient, at 10 years, manifested frequent upper respiratory tract infections and severe angiokeratoma on the palm of his hands, the dorsal region of his feet, his back, and scrotal regions. He was classified as being affected by angiokeratoma corporis diffusum. The enzymatic diagnosis was performed later. The residual enzymatic activity of α-galactosidase A in leucocytes was 2% of normal controls. He is now 36 years old and his skin lesions have greatly increased in number and diffusion. He has mild renal impairment and mild left ventricular hypertrophy. At an ocular level, there is mild tortuosity of the retinal and conjunctival vessels. Lymphoedema of the legs has been present since childhood. Another amino acid substitution (P40S) has been reported in a Japanese and in a French patient at the same P40 codon.[27 28] The high frequency of mutations at this P40 codon is the result of the presence of the CpG site in which mutations often occur. Garman and Garboczi[13] define this a helix terminus mutation.

The amino acid change, C378Y, caused by the transition c1133G>A[29] was detected in patient 8.1, aged 29 years. Residual α-galactosidase A enzymatic activity detected in leucocytes was 9% of normal controls. The first sign of the disease was the angiokeratoma observed on his buttocks and hips at the age of 12 years. Since then cardiac and renal functional parameters have also been impaired. C378Y disrupts one disulphide bond of the GLA protein and reduces its stability.[10]

The known (c680G>A exon 5) R227Q mutation[22] that disrupts a residue of the *GLA* active site[13] was identified in two brothers (patients 9.2 and 9.3), in their mother, and in their 52 year old uncle 9.1 (fig 1).

These patients showed a gradual deterioration in renal function. Severe renal failure was present in patient 9.1 whose older brother died at 53 years of age from renal failure without being diagnosed as suffering from Fabry disease. Patient 9.1 showed the most severe clinical phenotype with angiokeratoma and severe renal and cardiac involvement. He also shows severe neurological deterioration, with personality changes and psychotic behaviour, owing to cerebral lesions caused by numerous ischaemic attacks. Recently he had a cerebrovascular ischaemic attack leading to hemiparesis.

Patient 9.2 is the 28 year old nephew of 9.1. At 14 years he had a cluster of angiectases around his genitalia. This pattern of distribution is constant and has not increased. He has mild deterioration in renal function, pain, mainly in the extremities, and fevers less frequent and intense than those of his younger brother, patient 9.3. Patient 9.3, 25 years old, had two cerebrovascular ischaemic attacks that resulted in hemiparesis of his left eye and an ataxic gait. His renal function was borderline to normal. His clinical manifestations were worse than those of his older brother, 9.2. These data clearly show the presence of clinical heterogeneity in this family.

The known (c658C>T exon 5) R220X nonsense mutation[30] was detected in a 33 year old patient 11.1 who presents renal failure, angiokeratoma, fevers, and pain. The α-galactosidase A activity was 4% of normal controls. The molecular analysis extended to the family members at risk allowed two of the patient's sisters to be identified as carriers.

The R227Q and R220X genetic lesions are the most common mutations described in Fabry patients and correlate with the classical form of the disease.[9 22 30] Their high frequency can be explained by the presence of a CpG site in the *GLA* gene.

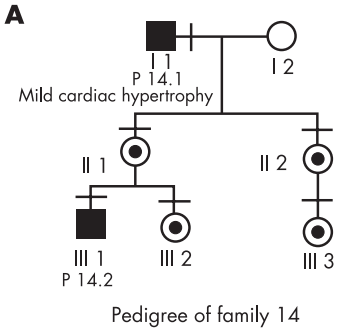
The genetic lesion c1095T>A (exon 7) that leads to the Y365X nonsense mutation was detected in patient 10.1, a 23 year old, with mild clinical symptoms. This known mutation[31] was absent in the patient's mother and in his sister suggesting a de novo mutation event.

The known (c233C>G exon 2) S78X nonsense mutation[28] caused by a transversion was detected in two unrelated patients, 12.1 and 13.1. Even if these male patients are not related they live in the same small village in central Italy. Patient 12.1 had a kidney transplant as a consequence of his renal failure. Patient 13.1 shows severe renal failure. The molecular analysis extended to the family of patient 12.1 allowed the identification of the S78X mutation in the patient's sister.

The transversion c708C>G (exon 5) that leads to the known W236C[19] was detected in patient 14.1 and his nephew, 14.2. The molecular analysis extended to the relatives allowed the identification of four heterozygous carriers: the two obligate carrier sisters (II.1 and II.2) and their daughters (III.2 and III.3). Only II.2 and her daughter, III.3, were manifesting carriers with pains, while II.1 and her daughter, III.2, were asymptomatic (fig 2A, B). W236 is a conserved amino acid between GLA and α-NAGAL. This W236C amino acid change disrupts the folding of the protein altering its hydrophobic core.[13]

Severe clinical manifestations in female carriers of X linked disorders are the result of skewing of X inactivation. It is also known that 5-10% of normal females show skewing of X inactivation.[32] Clinical heterogeneity, observed in female carriers of fragile X mental retardation,[33] ornithine transcarbamylase deficiency,[34] pyruvate deidrogenase deficiency,[35] etc, has been correlated with clonal variability of X inactivation, while in several disorders, such as X linked immunodeficiencies, post-inactivation cell selection is observed in affected tissue, and non-random X inactivation of the X carrying the mutant allele is observed.[32] Only a few X inactivation studies have been performed in manifesting Fabry carriers. Fabry heterozygous asymptomatic and symptomatic monozygotic female twins showing discordant phenotypes and unbalanced X inactivation in opposite directions have been described.[13]

We carried out X inactivation studies on the four carriers of family 14. The two sisters (II.1 and II.2) were obligate carriers.



B

Carrier female	II 1	II 2	III 2	III 3
Age at present	39 y	38 y	8 y	6 y
Age at diagnosis	38 y	37 y	7 y	5 y
Hypohidrosis	No	No	No	No
Pains	No	Yes, since 6 y	No	Yes, since 4 y
Fever crisis	No	No	No	No
Angiokeratoma	No	No	No	No
Cerebrovascular involvement	No	No	No	No
Cornea verticillata	+/-	+++	No	+
Renal insufficiency	No	No	No	No
Cardiac involvement	No	No	No	No
Lymphoedema	No	No	No	No
Enzymatic activity(%)	Normal	45	53	24
Hearing impairement	No	No	No	No

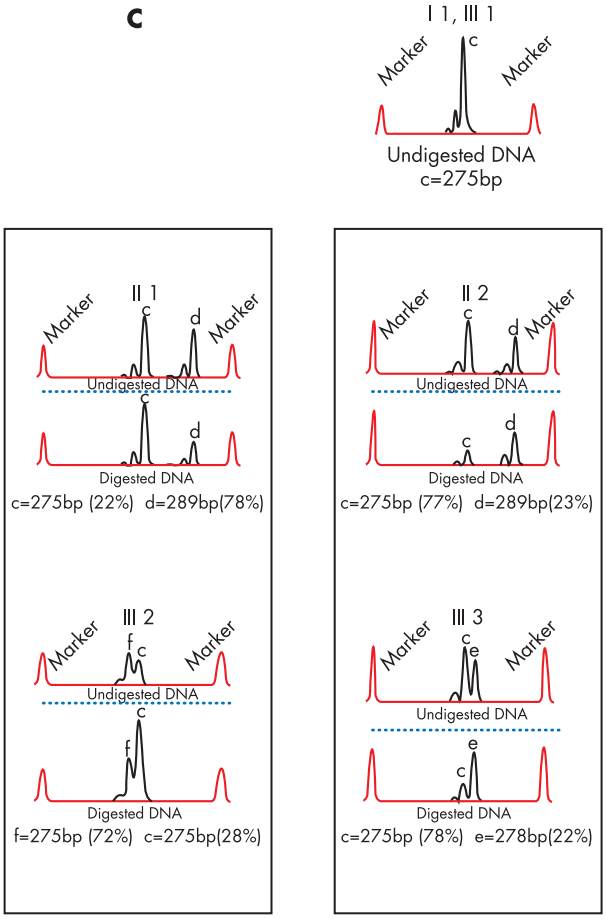


Figure 2 (A) Pedigree of family 14. (B) Clinical features of family 14's female carriers. (C) X inactivation studies were carried out on the two obligate sisters (II.1, II.2) and their daughters (III.2, III.3). The mutant allele of 275 bp was detected in both patients P14.1 (I.1) and 14.2 (III.1). A skewed X inactivation pattern in favour of the mutant allele (275 bp) was detected only in the manifesting carriers (II.2 and III.3), while a skewed pattern in favour of the wild type allele (289 bp and 272 bp respectively) was detected in the two asymptomatic carriers (II.1, III.2).

The paternally mutated derived X chromosome was preferentially active in the symptomatic sister (II.2) and the maternal X chromosome was active in the asymptomatic sister (II.1). Both their daughters (III.3 and III.2) were heterozygous carriers. The manifesting carrier II.2 and her manifesting daughter, III.3, show the same clinical symptoms and a skewed X inactivation pattern in favour of the mutant allele. The carrier II.1 and her daughter, III.2, were asymptomatic and showed the same skewed X inactivation pattern in favour of the unaffected chromosome (fig 2C). These data suggest a correlation between clinical phenotype and X inactivation in this family.

Inherited factors determining the familial skewed X inactivation have been reported for some disorders which are not typically characterised by non-random X inactivation, such as Duchenne muscular dystrophy, haemophilia A and B, and Lesch-Nyhan syndrome.

Evidence of preferential X inactivation has also been reported in a family with Fabry disease.[36] Familial X inactivation can be excluded in our carriers because only one of the two obligate sister carriers was a manifesting carrier. Further studies will be needed to establish a correlation between the X inactivation pattern and the clinical phenotype in Fabry carriers.

In conclusion, molecular analysis performed on a small group of Fabry patients and their families allowed us to identify several mutations in all exons of the *GLA* gene. Some of them are correlated with severe renal failure, such as S78X, c126-127insCATG, and A352D and others to neurological involvement as in R227Q. However, owing to the presence of intrafamilial heterogeneity, it can be hypothesised that other factors can influence the expression of mutations and make a genotype-phenotype correlation more difficult. Molecular studies on females, until now considered as normal even if they are obligate heterozygotes, are very important owing to the poor assessment of enzymatic assay in such subjects. We would like underline the importance of screening for GLA enzymatic activity in the *GLA* gene in a selected group of patients affected by idiopathic cardiac involvement and/or renal failure but without any skin lesions or other typical Fabry clinical manifestations. The detection of de novo mutations shows that a high frequency of spontaneously genetic lesions occur in the *GLA* gene.

Molecular studies are essential for the identification of heterozygous females especially those for whom GLA enzymatic assay does not allow the assessment of their carrier status and for prenatal diagnosis.

The preliminary results of our X inactivation studies suggest a correlation between the clinical manifestations of heterozygous females and the skewing of X inactivation detected in peripheral blood DNA. We would also like to stress the importance of X inactivation studies that could be helpful in predicting the female phenotype and give useful indications for therapeutic management.

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