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.\(^3\) Fabry disease can be classified into two clinical phenotypes: the classical form and the cardiac variant.\(^4\)\(^5\) 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.\(^6\) The cardiac variant is characterised by symptoms restricted to cardiac abnormalities, including conduction defects and/or late onset cardiomyopathy with left ventricular hypertrophy.\(^7\)\(^8\) 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.\(^9\) The X linked disorders affect males, while the female carriers are generally asymptomatic, owing in part to the random inactivation of the X chromosome.\(^10\) 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.\(^2\) 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.\(^5\)\(^8\)\(^9\) Recently, Garman and Garboczi\(^11\) 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.\(^12\)

Twins heterozygous for the GLA enzymatic defect showing discordant phenotypic expression have been reported.\(^13\) 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.\(^14\) 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.\(^15\)\(^16\) 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.

**MATERIALS AND METHODS**

The α-GLA enzymatic assay was carried out as previously reported.\(^1\) 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 thermodisplaythermo-rt\(^17\) 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’ gctctgagggtttaacct3’ (–67/–48nt); R1 reverse 5’ aatgggctcctgtagac3’ (IVS1 +87/+68nt); F2 forward 5’ ggtgctaatcactgttg3’ (IVS1 –58/–39nt); R2 reverse 5’ aatctcgtcacaagttgct3’ (IVS2

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**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 618delTT, c126-127insCATG, c946delG) mutations in the GLA gene in all exons have been reported.
- 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.
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 AmpliTaq DNA polymerase (PE=Perkin Elmer Cetus, Branchburg, 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 DdeI(−ACRS) and EcoRV (+ACRS) restriction analysis respectively. The following modified primers: L167Prmut 5′-ACTGTCACAGTAACAACTCA 3′ (c528-c502/c503A>G) and A352Drmut 5′-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.

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...
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 also be 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 fever. 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

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</table>

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

The known IVS3+1G>A donor splice site defect 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 acroparasthesias 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, acroparasthesias, 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.
The L167P genetic lesion was also confirmed in the genomic DNA by DdeI (~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 classical Fabry disease. 

Most of the reported amino acid changes (162, 163, 165, and 168) affect the stability of folded protein that is rapidly degraded before cleaving the substrate. 

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 EcoRV (+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. 

The transition c118C>T (exon 1) that leads to the known amino acid substitution P40L 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 distribution. 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 (P405S) has been reported in a Japanese and in a French patient at the same P40 codon. 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 define this a helix terminus mutation. 

The amino acid change, C378Y, caused by the transition c1095T>A (exon 7) was identified in patient 8.1, aged 29 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. 

The R227Q and R220X genetic lesions are the most common mutations described in Fabry patients and correlate with the classical form of the disease. 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 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 was 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 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.1 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. 

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. Clinical heterogeneity, observed in female carriers of fragile X mental retardation, ornithine transcarbamylase deficiency, pyruvate dehydrogenase deficiency, 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. 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. 

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

Acknowledgements
This work was partially supported by grants from the Azienda Ospedaliera Meyer, Florence, Italy and the Association AMMEC.
Fabry disease: molecular studies in Italian patients and X inactivation analysis in manifesting carriers

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J Med Genet 2003 40: e103
doi: 10.1136/jmg.40.8.e103

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