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Original Paper

Systematic DNA Study for Fabry Disease in the End Stage Renal Disease Patients from a Southern Italy Area

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Key Words
End stage renal disease • Fabry disease • P.I91T • GLA

Abstract
Background/Aims: Fabry disease (FD) is a lysosomal storage disorder characterized by pervasive renal involvement. However, this disease is underdiagnosed in patient with chronic kidney disease (CKD), including those with end stage renal disease (ESRD), so their investigation represents an unexploited opportunity for early diagnosis of the disease and for its identification in relatives of affected patients. ***Methods:*** We investigated Fabry disease in a clinical and biological database including ESRD patients of unknown cause in a geographical area with 2 million residents. The study was based on state of art GLA gene sequencing and was extended to relatives of affected ESRD patients. ***Results:*** Among ESRD patients qualified for enrollment into this study, a previously undiagnosed young man harboring the mutation p.I91T was identified. The study of the proband's family led to the identification of 8 additional cases. In another ESRD male patient, we identified the functional polymorphism p.D313Y. Furthermore, in 55 ESRD patients (24.2%) we found intronic polymorphisms of uncertain functional relevance in the non-coding regions of the GLA gene. ***Conclusion:*** A comprehensive survey of ESRD patients in a geographical area of 2 million residents identified one undiagnosed case of Fabry disease and led to the identification of 8 additional cases among his relatives. Screening protocols starting from the dialysis population and upstream extended to families of affected individuals may be an effective strategy to maximize the early identification of subjects with Fabry disease.

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Introduction

Fabry disease is a pan-ethnic rare disease, with an estimated incidence in the general population of ~1:40, 000 in males [1] and ~1:20, 000 in females [2]. Recent studies suggest that the disease might be less rare than estimated in previous surveys because the atypic variant of the disease is underdiagnosed and the actual incidence of the disease may be approximately 1:8.800 individuals [3].

In hemizygous males with the classical form of the disease, clinical symptoms usually begin in childhood or in early adolescence appearing in the form of angiokeratoma, corneal opacities, microalbuminuria or proteinuria, and symptoms that affect the peripheral nervous system, such as episodic crises of acute pain, acroparesthesias, anhidrosis or hypohidrosis [4]. End stage renal disease (ESRD), cardiovascular and neurological complications stand as the main causes of death in patients with Fabry disease and life expectancy in these patients is substantially shorter than in the age and sex matched general population [5].

Progressive renal disease is a pervasive clinical manifestation of Fabry disease. Albuminuria is present in 50% of male patients over 35 years and in 100% of patients over 50 years [6]. In a study of 2017 performed on patients with chronic kidney disease not on dialysis, the prevalence of FD was 0.2% comparable to the results obtained on hemodialysis patients (0.15-1%), which may suggest that Fabry disease should already be considered in young patients with chronic kidney disease with unknown etiology even in the absence of symptoms and signs suggestive of Fabry's disease [7].

In a systematic review of screening studies in high-risk populations, the prevalence of this disease among end stage renal disease (ESRD) patients was 0.33% in men and 0.10% in women [8]. Until now only three large scale, comprehensive surveys on the prevalence of Fabry disease in the ESRD population have been made [9-11] and these studies identified two to four new, undiagnosed, cases (prevalence ranging from 1.7 per 1000 to 3.4 per 1000 dialysis patients).

In this survey, we assessed the prevalence of unrecognized Fabry disease in an ethnically and geographically homogeneous dialysis population and then extended the screening to relatives of affected patients. Since it is well established that the enzymatic analysis could miss the diagnosis in over one-third of affected females, the European Renal Best Practice (ERBP) committee by the European Renal Association, European Dialysis and Transplantation Association (ERA EDTA) recommends DNA sequencing as the primary screening method in females [12]. In the present study we performed the whole screening of the dialysis population by Genomic DNA sequencing.

Materials and Methods

The study protocol was approved by the ethical committee of the coordinating center. All participants gave their informed consent before enrolment.

Study population

The study population is part of a cohort of 1065 dialysis patients enrolled in the PROGREDIRE (Prospective Registry of The Working Group of Epidemiology of Dialysis Region Calabria), a cohort study involving 33 dialysis units in Calabria, a southern Italy region of about 2mln residents. The PROGREDIRE study enrolled 75.6% of the 1408 patients dialyzing in the region between May 2009 and October 2010 and, for the present study, we selected a subpopulation of 227 patients of ESRD patients of unknown cause (Table 1).

Patients had been on regular dialysis [haemodialysis (HD) or peritoneal dialysis (PD)] for a median time of 50, 1 months (inter-quartile range: 24, 6-98, 2 months). HD patients (n=222) were being treated with

standard bicarbonate dialysis with non-cellulosic membrane filters of various type. PD patients (n=5) were either on 4 standard exchanges day or on continuous cycling peritoneal dialysis. The main demographic, somatometric, clinical and biochemical characteristics of the study population are detailed in Table 1.

The population of healthy control subjects was composed by 483 individuals who underwent the standard health screening contemplated for blood donors. These subjects had no clinically apparent diseas and no clinical history of hereditary diseases, renal diseases in particular. The average age of this population was 50 years and the sex distribution was well balanced (Males 48%; Females 52%).

Table 1. Characterization of the patients recruited in this study. BMI is for Body Mass Index, CRP is for C-reactive Protein, CV is for Cardiovascular

Parameter	PROGREDIRE group (n=1065)	Subpopulation of ESRD patients of unknown cause n=227
Age (years)	65±14	45±7.9
Male gender (%)	687 (65)	148 (65)
Smokers (%)	525 (50)	112 (49)
Diabetes (%)	270 (27)	38 (17)
BMI (kg/m2)	25±5.0	25±5.5
Systolic pressure (mmHg)	136±22	134±22
Diastolic pressure (mmHg)	74±11	79±12
Cholesterol (mg/dL)	154±39	157±43
Hemoglobin (g/dL)	11.3±1.5	11.4±1.4
Albumin (g/dL)	4.0±0.50	4.0±0.53
Calcium (mg/dl)	9.00±1	9.13±0.90
Phosphate (mg/dl)	5.0±1.6	5.7±1.66
PCR (mg/dL)	4.0 (3.0-11.0)	3.6 (2.8-8.9)
Dialysis vintage (months)	47 (21-90)	50.1 (24.6-98,2)
With CV comorbidities (%)	539 (51)	83 (37)

DNA isolation

Genomic DNA was extracted from peripheral blood leucocytes by salting-out technique [13] and the concentration was determined using a spectrophotometer.

Polymerase Chain Reaction and sequencing of GLA

Eight pairs of primers were designed for the analysis of target regions containing the seven exons of the GLA gene, the regulatory sequences flanking them and part of intron 4 including the criptic exon. PCR products were purified and sequenced using an automated DNA sequencer at BMR Genomics laboratories.

Alpha-galactosidase activity assay

Alpha-galactosidase A activity was measured by Dried Blood Filter Paper test described by Chamoles et al. [14], with minor modifications [15]. Normal values of this test are > 3.0 nmol/h/ml

Statistical Analysis

Data were expressed as mean ± standard deviation (normally distributed data) or median and interquartile range (not normally distributed data) or as percent frequency (categorical data). Differences in proportions were tested by the adjusted chi squared test with Yates correction for continuity, as appropriate. All Statistical Analysis were performed by a commercially available statistical software (SPSS ver.22 USA).

Results

In the whole study cohort, 227 dialysis patients were ESRD patients of unknown cause. The demographic and clinical characteristics of this population are described in Table 1. The proportion of males was 65%. 17% were diabetic and 37% had background cardiovascular comorbidities. In no case Fabry disease was indicated as the cause of ESRD.

The survey identified one male patient of 39 years, with a mutation in the GLA gene causative of Fabry disease and minor of 0.3 nmol/h/ml activity of alpha-galactosidase A (Table 2). The mutation is a transition of a thymine to a cytosine in position 272 of the cDNA (c.272 T>C) causing the substitution of an isoleucine to a threonine at amino acid 91 in the protein (p.I91T) [16]. Our patient had a family history of cardiomyopathy, and he suffered from hypertrophic cardiomyopathy and severe kidney failure leading to dialysis at 38 years. Moreover, starting from childhood, he has been suffering from burning pain episodes in hands and feet, especially after exercise, suggesting early neural involvement (Table 2).

Table 2. Clinical and molecular information of the proband and his relatives with mutation in GLA. Normal values of α-galactosidase A activity assayed in whole blood are > 3.0 nmol/h/ml. In bold, values below the normal range

Patient	Kinship	Enzimatic activity	Gender	Mutation in GLA	Clinical information	Age at diagnosis
1	Proband	<0.3	M	c.272T>C (p.I91T)	Severe renal impairment, hypertrophic cardiomyopathy, acroparaesthesias	38
2	Mother	6	F	c.272T>C (p.I91T)	Ischemic heart disease, angina, shortness of breath, heart attack, hypertension	61
3	Sister	3,3	F	c.272T>C (p.I91T)	Asymptomatic	26
4	Aunt	6.2	F	c.272T>C (p.I91T)	Acroparaesthesias, anhidrosis, angina	59
5	First cousin	2,1	F	c.272T>C (p.I91T)	Asymptomatic	33
6	Second cousin	1,5	F	c.272T>C (p.I91T)	Heat and cold intolerance, abdominal pain	10
7	Aunt	1	F	c.272T>C (p.I91T)	Anhidrosis, tinnitus, stroke and hypertension	66
8	First cousin	<0.3	M	c.272T>C (p.I91T)	Recurrent fever, tinnitus, fatigue	40
9	First cousin	<0.3	M	c.272T>C (p.I91T)	Recurrent fever, tinnitus, fatigue	28

Table 3. Numbers and percentages of dyalisis patients and healthy control subjects with mutations in the GLA gene

Aplotype	Mutations description	Dialysis patients (n)	%	Healthy control subjects (n)	%
	c.272 T>C (p.I91T)	1	0.4	0	
	c.937 G>T (p.D313Y)	1	0.4	1	0.2
1	-10 C>T; IVS2-77_81del5; IVS4-16 A>G; IVS6-22C>T	16	7.04	15	3.1
2	IVS2-77_81del5; IVS4-16 A>G; IVS6-22C>T	15	6.60	30	6.2
3	-12G>A; IVS4+68 A>G; IVS6-22C>T	11	4.84	6	1.24
4	-12G>A; IVS4+68 A>G; IVS4+866_867delAG; IVS6-22C>T	2	0.88	6	1.24
5	IVS6-22C>T	1	0.44		
6	-10 C>T; IVS6-22C>T	0		6	1.24
7	-10C>T	1	0.44		
9	-30G>A	1	0.44	3	0.6
10	IVS4+771C>T; IVS6-22C>T	2	0.88		
11	-10C>T; IVS4+739C>T; IVS6-22C>T	3	1.32		
12	IVS4+715C>T	1	0.44		
14	IVS4-16 A>G; IVS6-22C>T	1	0.44		
15	-10 C>T; IVS3-7C>T; IVS4+739C>T; IVS6-51_54del4; IVS6-22C>T	1	0.44		
Total intronic polymorphism mutations		55/227	24.2	66/483	13.6

Study extension to members of the family of the INDEX case

The study of the disease was extended to the proband's family. This screening identified 8 additional subjects with the p.I91T mutation (Table 2). The proband inherited the mutation from his mother, a woman of 61 years suffering from ischemic heart disease and heart failure. This woman transmitted the mutation also to a 26 years old daughter that was asymptomatic. Extending the study to other maternal line relatives, we identified the mutation in two sisters of the proband's mother: a 59 year-old woman who suffered from acroparaesthesias, anhidrosis and angina, and a woman of 66 years who suffered from anhidrosis, tinnitus, stroke and hypertension. In the proband's mother and sister and in one of two aunts alpha-galactosidase A activity was normal, while in the other aunt was below the normal range (1 nmol/h/ml). The p.I91T mutation was found in the younger sister of the proband's mother who had in turn an asymptomatic daughter of 33 years with the same mutation associated with enzyme activity below the normal range (2.1 nmol/h/ml). This woman transmitted the mutation to a child that suffered from heat and cold intolerance and abdominal pain and exhibited enzymatic activity below the normal range (1.5 nmol/h/ml). The variability of alpha galactosidase A activity values, identified in these females, is probable due to the chromosome X inactivation. Thus, on the basis of which chromosome is silenced, (mutated one or wt one) the resulting protein will be a functional or an inactive enzyme [17]. The other sister of the proband's mother had two sons, one of 28 years and the other of 40 years, both suffering from recurrent fever, tinnitus and fatigue. The genetic and enzymatic study, performed in these two subjects, detected the p.I91T mutation and showed minor of 0.3 nmol/h/ml activity of alpha-galactosidase A.

D313Y mutation

In the same cohort, we identified a 55 years old male patient showing a functional polymorphism in the GLA gene, i.e. a polymorphism located in exon 6 of GLA dictating a substitution of guanine to thymine in position 937 of the cDNA (c.937 G>T) that causes the amino acid variant p.D313Y (Table 3) [18, 19]. In this patient the alpha-galactosidase activity assay showed levels in the normal range (7.5 nmol /h/ml).

Intronic mutations

In the same survey, we also systematically studied intronic portions of the GLA gene. In 55 of 227 patients (24.2%), we found mutations in non-coding GLA regions, the most of those are grouped in aplotypes (Table 3) [20]. In all of these patients, the analysis of enzymatic activity was inside the normal range. The prevalence of intronic mutations in patients with ESRD [55/227 (24.2%)] was 1.64 times higher than the prevalence observed in the healthy population [66/483 (13.6%)] (P = 4, 5 x 10-3) (Table 2).

Discussion

The clinical diagnosis of Fabry disease is notoriously difficult because clinical signs and symptoms of this disease amply overlap with those of other diseases. Analyses in existing databases show that this disease is often diagnosed at a late life-stage in about 40% of male and 70% of female patients and only after consultations of about 10 specialists with experience on the syndromic phenotype of the same disease [21]. Thus, the variability of the clinical phenotype of Fabry disease and the overlap with other conditions represents an objective barrier to the timely identification of affected individuals which may lead to an underestimation of the true prevalence of this condition at population level.

Kidney involvement is a hallmark of Fabry disease. As alluded to before, microalbuminuria and proteinuria have a 50% and 100% prevalence in 35-year and 50-year patients affected by this condition, respectively [6]. After the fifth decade of life, end stage renal disease is a frequent outcome in these patients [6] and in a survey in Japan the 1, 2% of male subjects with ESRD attribute to chronic glomerulonephritis on specific testing resulted to be affected by Fabry disease [22, 23]. Of note, the majority of patients (83%) in this survey in Japan did not show the classical clinical manifestations of the disease emphasizing the potential underestimation of the prevalence of the disease in studies based on clinical algorithms.

Even though a consensus document by experts of the European Best Practice committee of the European Renal Association European Dialysis and Transplantation Association (ERA EDTA) recommends screening for Fabry disease in male (<50 year) and female (irrespective of age) patients with CKD of unknown etiology and to then extend the screening to relatives [24], to our knowledge only one study [11] reported on the yields of a screening protocol extended to relatives of the index case. Furthermore, in this study the screening of the dialysis population was performed by the dried blood spot method both in male and female patients while it is well established that this method could miss the diagnosis in over one-third of females where mutation analysis is the recommended method of screening [11]. Our study protocol contemplated sequencing of the GLA gene in all patients, thus maximizing the diagnostic yield in some female patients that, even though harboring functionally relevant mutations may exhibit normal enzymatic activity [17].

In the present study the systematic genotyping of 227 ESRD patients on regular dialysis treatment in nephrology units in a geographic area with a 2 million population, identified one previously undiagnosed case with full-blown Fabry disease [1/227 (0.4%)]. This 39-year male patient presented the c.272 T>C mutation in the exon 2 of the GLA gene. This mutation, previously described in the literature as responsible of the atypical variant of Fabry disease [16] caused in the same patient a marked reduction of α-galactosidase enzymatic activity. Importantly, the subsequent family screening found 8 proband's relatives with the same disease. Like in the index case, some of his relatives complained of symptoms that typically occur in Fabry disease including acroparaesthesias, anhidrosis, angina and stroke, but the diagnosis of FD was overlooked. Furthermore, in this survey, we identified a subject with a functional polymorphism (p.D313Y) [18] in the GLA gene. To determine the pathogenicity of a GVUS in an individual, Lyso Gb3 accumulation and null or strongly decreased enzyme activity are needed [25-27]. In our patient such as in previous studies in two Danish families

[19], this male patient had normal enzymatic activity and no accumulation of Lyso Gb3 was found, which negate the diagnosis of Fabry disease.

Of interest, we also observed that as much as the 22.4% of the whole dialysis population displays intronic polymorphisms of the GLA gene [20] which is a prevalence significantly higher than that registered in the corresponding general population. Even though some studies try to associate these intronic mutations in GLA gene with Fabry disease [28], the involvement of intronic mutation in Fabry disease remains unclear. In our cohort, all patients with intronic mutations the enzyme activity was always normal and accumulation of GB3/Lyso-GB3 was never found. These results suggest that the intronic mutations detected in our study do not affect to a significant extent the catalytic activity of the GLA enzyme or other cellular processes that can influence this enzymatic function. Nonetheless, the observation that intronic mutations in the GLA gene are more frequent in ESRD patients than in the general population is of interest. If confirmed in other CKD populations and in other ethnicities, this finding suggests that these mutations may be either causally involved in the pathogenesis of CKD or be in linkage disequilibrium with other genes implicated in CKD. Further studies are necessary to clarify if complex intronic haplotypes are involved in mechanisms of GLA regulation and in alterations of glycosphingolipids metabolism.

Conclusion

Our observations further again confirm the inherent difficulty of diagnosing Fabry disease on clinical grounds and show the potential of enzymatic and genetic tests in the dialysis population and in relatives of affected dialysis patients.

The surveys targeting the dialysis population may represent an effective opportunity for identifying patients with undiagnosed Fabry disease and to trigger family screenings aimed at discovering the disease in asymptomatic or pauci-symptomatic relatives of affected dialysis patients. The cost-effectiveness of dialysis population study for the identification of patients with Fabry disease deserves to be assessed in specifically designed studies taking into full account the benefits and the risk of screening this rare disease [29].

Disclosure Statement

The authors declare that they have no competing interests.
The authors did not receive economic contribution for the submission of this paper.

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