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The Continuous Challenge of Diagnosing patients with Fabry disease in Argentina: Genotype, Experiences, Anecdotes, and New Learnings

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Abstract

The lysosomal storage disorder Fabry disease (FD) is caused by pathogenic mutations in the α-galactosidase A gene, localized in X chromosome. Deficient enzymatic activity of the product of this gene, the lysosomal hydrolase α-galactosidase A, leads to accumulation of its substrate globotriaosylceramide. Diagnosis of FD starts with clinical suspicion followed by confirmatory laboratory testing. The aim of this work is to report the 14 years' experience and learnings in the diagnosis of patients with Fabry disease in Argentina from a specialized lysosomal diseases diagnosis laboratory and to report the genotype characterization of the 25 families from Argentina with FD detected by us.

Keywords

Fabry disease, α-galactosidase A deficiency, genotype, diagnosis, lysosomal diseases

Introduction

The lysosomal storage disorder Fabry disease (FD) is caused by pathogenic mutations in the α-galactosidase A (*GLA*) gene, localized in X chromosome. Deficient enzymatic activity of the product of this gene, the lysosomal hydrolase α-galactosidase A (αGalA), leads to accumulation of its substrates globotriaosylceramide (Gb3) and lyso-Gb3.[1] Low or absent enzyme activity in men provokes signs and symptoms of classical FD, which is characterized by acroparesthesia, angiokeratoma, hypohydrosis, renal dysfunction, and cornea verticillata. Complications of heart, kidney, and brain are the main cause of death, reducing life expectancy for 20 years when compared to normal population. Heterozygous females are usually also affected, displaying a broader spectrum of phenotypes, from moderate to severe disease.[2] There are also patients with Fabry disease with a different phenotype, called ''late-onset variants,'' characterized by a lack in typical manifestations such as acroparesthesia, angiokeratoma, or cornea verticillata but displaying its main problems in kidney, heart, and/or central nervous system appearing at a later age.[3] Prevalence of FD was previously estimated to be between 1:40 000 and 170 000,[4,5] however pilot newborn screening studies showed higher prevalence of 1 of 3600.[6]

Prior to laboratory studies, diagnosis of FD starts with thorough clinical examination of the patient, his medical, and family history. Based on all these data, if the physician established there is clinical suspicion for FD, laboratory testing is the next step. Laboratory diagnosis of FD in males, as recommended by a consensus of a European expert group, is carried out by determination of αGalA activity. It can be measured in samples such as dried blood spots, plasma, leukocytes, or fibroblasts, however the ''gold standard'' for definite diagnosis is leukocytes or fibroblasts. The following test should be mutation analysis, which is useful to confirm diagnosis, discard possible pseudodeficiency cases (eg, D313Y),[7] and to mainly diagnose female relatives at risk of being heterozygote, and genetic counseling. On the other hand, for females, enzymatic activity determination

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is inconclusive, and only the identification of a pathogenic mutation in *GLA* gene allows definite diagnosis.[8]

The aim of this work is to report the 14 years' experience in diagnosis of patients with Fabry disease in Argentina from a specialized lysosomal diseases diagnosis laboratory. Moreover, we report genotype characterization of the 25 families from Argentina with FD detected by us.

Materials and Methods

Targeted Screening Strategy

Physicians who suspected FD in their patients through clinical manifestations sent blood samples to the laboratory for biochemical and genetic assays. The number of samples received by clinical suspicion was 589. After a new Fabry patient was detected, the screening of the whole family was performed. We have collected 1274 samples from relatives of the different Fabry index cases.

High-Risk Screenings Strategy

Dried blood samples (n = 1401) from males at hemodialysis centers were collected and used for enzyme assay.

Patient Samples

The study period is from January 2002 to June 2015. Blood samples were collected by venipuncture. We received dried blood spots from male patients for enzymatic activity assay. If the result was pathologic, 9 mL of heparin blood and 1 mL of EDTA blood were asked thereafter in order to confirm the diagnosis by measuring α-GalA activity in leukocytes and genetic test. EDTA blood was received from female patients for genetic test.

Enzymatic Activity Determination

The αGalA activity determination was carried out on dried blood filter paper, according to the method of Chamoles et al[9] and described in Ceci et al.[10]

Mutation Detection in GLA Gene

Mutation analysis was done using DNA isolated from EDTA blood samples. Polymerase chain reaction amplification of each exon and adjacent intron–exon boundaries by the use of specific primers was carried out. The amplicons were purified and then sequenced in both directions in a DNA Sequencing device (Applied Biosystems, Foster City, California).

Results and Discussion

Fabry Index Cases and Mutations

In the period of this work, 25 Fabry new index patients were detected and diagnosed. Most of the index cases were males (n = 22), and 3 were females. We have found the mutation in all the 25 families with FD detected by us in Argentina (Table 1). Each family had a different mutation, so the rate of private mutations was 100%. Most of the alleles were missense mutations, accounting for 72% of them, and moreover we found 3 short deletions, 2 short duplications, and 2 nonsense mutations (Figure 1). We have not found any complete exon/gene deletion, splicing, or gross rearrangements. All the duplications and deletions were novel, but only 5 missense and one nonsense mutations were novel.

Average age at diagnosis was 39 years old, and the range was between 15 and 72 years old. Data captured in Fabry registries included worldwide data, such as Fabry outcome survey (FOS), and reported a lower age.[23] However, the age at diagnosis is higher than the age at onset of symptoms, and there is a delay of diagnosis of at least 10 years. The delayed age at diagnosis found in Argentina is completely in agreement with what is happening in other parts of the world, meaning it does not depend on the development or economic status of the country. In spite of multiple efforts invested in dissemination of knowledge among medical community, delay between symptoms onset and age at diagnosis has no changed in the last years.

Absence of the probands' mutations in their mother suggests that in 5 cases, the mutations occurred de novo (20%). However, the possibility of germinal mosaicism could not be ruled out. This percentage is higher than the theoretical value of 3% to 10% based on the reproductive fitness of 0.7 to 0.9,[24] a birth frequency of 1:40.000 to 1:100.000, and a GLA mutation rate of 1×10^{-6}.[25]

Classical Cases

It is well established that FD is still an underdiagnosed disorder worldwide.[27] Regarding both phenotypes of FD, classical cases are easier to suspect than variant ones. The clinical picture in classical cases, meaning the full clinical manifestations and family history altogether, is highly suggestive, and if it is observed by an expert physician in FD, the clinical suspicion is usually made. In our cohort, 17 of the classical cases were detected by clinical suspicion of physicians from different specialties, including nephrologists (59%), dermatologists (17%), cardiologists (6%), neurologists (12%), and clinicians (6%) (Figure 2). Three of the cases are included in the table as detected by the patient itself. One of them was diagnosed because a relative of the patient living in another country was diagnosed, so he contacted a referent specialist in FD. The second one was suspected when the patient went to a commercial shop owned by a previously diagnosed Fabry patient, and he complained about having suffered a renal transplant 3 months ago and with clinical symptoms such as heat intolerance, hypohydrosis, and acroparesthesia. The Fabry patient recognized the symptoms, asked about the presence of angiokeratoma that was confirmed, and derived the patient to a specialist, and the disease was lately confirmed. The last case was detected when his grandmother wrote in a rare disease patient forum, describing the symptoms of his grandchild; a Fabry patient in the forum

Table 1. List of Mutations From 25 Families of Argentinean patients with Fabry disease.[a]

Family	cDNA	Exon	Protein	Type of Mutation	Reference	De novo	Specialist	Phenotype	Age at Diagnosis	Relatives Studied	Number of Positive Relatives
1	c.679C>T	5	p.Arg227X	Nonsense	[11]		Nephrologist	Classic	24	253	8
2	c.728T>G	5	p.Leu243Trp	Missense	[12]		Nephrologist	Classic	39	54	5
3	c.463G>C	3	p.Asp155His	Missense	[13]		Dermatologist	Classic	23	319	73
4	c.1244T>C	7	p.Leu415Pro	Missense	[14]		Dermatologist	Classic	32	67	18
5	c.281G>A	2	p.Cys94Tyr	Missense	[3]		Nephrologist	Classic	46	13	8
6	c.572T>C	4	p.Leu191Pro	Missense	Cooper, 1998		Patient	Classic	25	5	2
7	c.1088G>A	7	p.Arg363His	Missense	Cooper, 1998		Screening hemodialysis	Variant (Cerebrovascular)	62	9	5
8	c.286-287dupA	2	Truncated	Frameshift			Nephrologist	Classic	46	3	2
9	c.581C>T	4	p.Thr194Ile	Missense	[15]		Patient	Classic	44	59	29
10	c.874G>A	6	p.Ala292Thr	Missense	[16]		Clinician	Classic	27	8	8
11	c.1145G>A	7	p.Cys382Tyr	Missense	[17]	De novo	Nephrologist	Classic	30	3	1
12	c.520 T>G	3	p.Cys174Gly	Missense			Cardiologist	Variant (Cardiac)	56	279	51
13	c.680G>A	5	p.Arg227Gln	Missense	[18]		Dermatologist	Classic	26	15	8
14	c.790G>T	5	p.Asp264Tyr	Missense	[19]		Neurologist	Classic	62	3	2
15	c.1122_1125delAGGA	7	Truncated	Frameshift			Nephrologist	Classic	31	5	2
16	c.160C>U	1	p.Leu54Phe	Missense			Nephrologist	Variant (renal)	50	6	3
17	c.644A>G	5	p.Asn215Ser	Missense	[11]		Nephrologist/pathologist	Variant (renal)	61	75	15
18	c.647A>G	5	p.Tyr216Cys	Missense	[20]		Neurologist	Classic	26	11	6
19	c.448delG	3	Truncated	Frameshift		De novo	Nephrologist	Classic	30	4	1
20	c.772G>T	5	p.Gly258Stop	Nonsense		De novo	Nephrologist	Classic	45	13	2
21	c.782-783dupG	5	Truncated	Frameshift		De novo	Nephrologist	Classic	46	5	1
22	c.718_719delAA	5	Truncated	Frameshift		De novo	Patient	Classic	15	3	1
23	c.335G>A	2	p.Arg112His	Missense	[21]		Screening hemodialysis	Variant (renal)	72	24	15
24	c.902G>A	6	p.Arg301Gln	Missense	[22]		Cardiologist	Classic	48	35	17
25	c.100A>G	1	p.Asn34Asp	Missense			Nephrologist	Classic	16	3	3

Abbreviation: cDNA, complementary DNA.

[a]Also shown is the phenotype of the index patient, the specialist who made the clinical suspicion, the reference if the mutation has been reported, the age at diagnosis of the index case, and the number of members of each family that were studied and the number of positive ones.

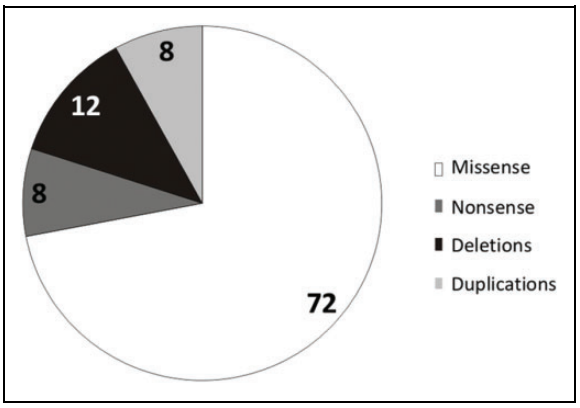

Figure 1. Type of mutations detected in patients with Fabry disease from Argentina (%).

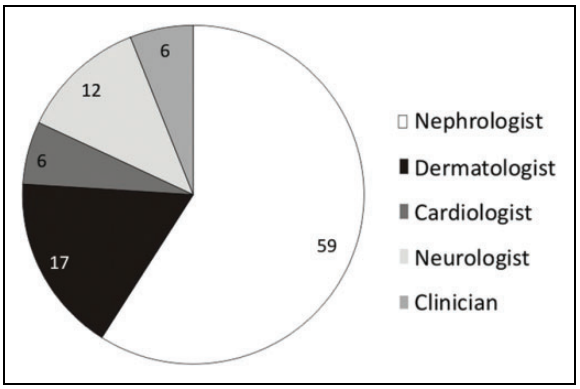

Figure 2. Specialists who made clinical suspicion (%).

realized he could have FD and made the contact with the physician to initiate diagnosis process confirming the disease. Average age at diagnosis among classical cases is 34 years old, and the range is between 15 and 62 years old.

Variant Cases

Variant cases are the most difficult to suspect and identify. Among the phenotypes found in the cohort of 25 Fabry families, 5 were variant cases. Two of them were the ones detected by high-risk screenings. The cardiac variant case was detected by a cardiologist specialized in echocardiography and suspected FD through echo image after he attended a conference about FD. One of the renal variants detected by a nephrologist was suspected after kidney biopsy. The biopsy revealed foamy cells in the glomerulus, and the pathologist suggested FD as probable diagnosis. Average age at diagnosis among variant cases is 60 years old, and the range is between 56 and 72 years old. This higher age is not surprising taking into account the lack of manifestations at younger age.

Paternity Issues

X-linked inheritance of FD in which male reproductive fitness is normal could expose out paternity issues. Genetic counselors have to be extremely cautious in this aspect when talking with relatives of patients with Fabry disease during family screening. Daughter of a Fabry male should be obligate heterozygote. We avoid using the word obligate inheritance when talking about daughters who do not have Fabry sons, yet. We always explain the families the genetic test is mandatory, and it is the solely way of confirmation of Fabry diagnosis. In our cohort, we had the experience of 2 families with paternity issues. We tested daughters from Fabry males but the genetic test was negative.

Oocyte Donation

One interesting anecdote showed up during screening for FD in a family after detection of a new patient. Two female relatives of the index Fabry patient were donating eggs for purposes of assisted reproduction as third-party reproduction. To our knowledge, 30 births arose from eggs of these Fabry women during 7 years. One of the families came to us for diagnosis of their male baby, and unfortunately it was positive. As conclusion of this case, physicians should ask every new patient if he or she is donating sperm/eggs in the anamnesis.

Detection of Patients by Clinical Suspicion

Since we opened the service of lysosomal disorders, we have assayed 3264 samples for Fabry diagnosis. From the total number of samples assayed, 589 samples (18% from total samples assayed for Fabry diagnosis) were sent by physicians after clinical suspicion of FD during the first visit of the patient to his consultation. Among them, diagnosis of FD was confirmed in 23 patients, a percentage of 4%. In a simplistic point of view, it could be thought that the yield of 4% could be low. However, taking into account it is a rare disease, the constellation of unspecific signs and symptoms, the low awareness among medical community, this point of view changes to a more positive one. Most of the index cases were males, but 3 of them were females. There are a lot of advantages of this result. Twenty-three persons finally obtained their confirmed diagnosis, after being consulting from more than 10 years with more than 10 medical specialists in a process called "odyssey diagnostic." Confirmatory diagnosis finishes this unpleasant process for the patient and his family. Moreover, each patient could now benefit from instauration of a specific treatment now available since 2003.

Detection of Patients by Family Screening

One of the greatest benefits of detection of undiagnosed patients with Fabry disease is for his or her family. FD is a genetic condition, and the mutation is transmitted from parents to children, so generally once a new patient is detected, the whole family could be screened and more previously undiagnosed patients are confirmed with FD. Diagnosis of relatives arrives earlier as compared as if the patients would have been diagnosed by clinical suspicion. Moreover, some patients are

diagnosed at an earlier age, before irreversible complications in organs, and are the most benefitted with this family screening process. Family screening starts with pedigree analysis. Geneticists should build the genealogic tree together with the family and give genetic counseling to the family. Genetic counseling includes, among others, telling the risk of affected couples to have affected children at each pregnancy and underscore which relatives could be at risk of being patients and therefore who should be tested.

Working with all the index cases detected and building each family tree, we offered the relatives at risk the possibility of being tested in order to obtain confirmatory diagnosis. And our experience is the following. We have taken 1274 samples from relatives, corresponding to 39% of the samples analyzed in our laboratory for Fabry diagnosis. And the proportion of positive diagnosis is 22%, representing 286 new patients with Fabry disease with definitive diagnosis, who benefit from avoiding suffering the odyssey and from an early instauration of treatment if necessary as decided by medical judgment. It is obvious to say, family screening in FD is the higher performance way to detect patients with Fabry disease and for many of the cases diagnosis is obtained at a lower age as compared to the average.

Detection of Patients by High-Risk Screenings

As we said earlier, detection of patients with Fabry disease is a hard and difficult task. There are many patients still undiagnosed in the world. Together with the advent of specific therapy whose benefit may be better when initiating early have prompted researchers to perform screening studies for FD in high risk populations. The meaning of high risk includes patients who express at least one sign or symptom that could be caused by FD. Several studies of this type have been carried out, and the high risk populations included patients on or with hemodialysis, renal transplant, left ventricular hypertrophy, and stroke, as the main ones, and others were patients with small fiber peripheral neuropathy, angiokeratoma, or cornea verticillata.

One of the main issues is the type of laboratory assays that should be carried out in screenings.[28] Determination of enzymatic activity is used when analyzing male samples, mainly in dried blood spots. When a pathogenic result is obtained, it should be confirmed in leukocytes. The final diagnosis is accompanied by genetic test establishing the presence of a pathogenic mutation. When females are included, genetic test is mandatory. In many cases, the pathogenicity of the mutation can't be established, they are called genetic variants with unknown significance (GVUS).[29] In cases with GVUS, there is an uncertain diagnosis of FD, and enzyme replacement therapy should not be started. Van der Tol et al suggest using an electron microscopy analysis of the biopsy of the affected organ to look for Gb3 deposits. Gb3 and Lyso-Gb3 in urine and/or plasma could also be measured, however the usefulness is limited in variant cases or females.[30] Another useful strategy it is to undergo a thorough family testing, especially to find males with low αGalA activity and the same mutation that would support the mutation being pathogenic.[31] And if the diagnosis cannot be confirmed, the patient remains in study until a confirmatory result, positive or negative, could be given.

In Argentina, we have carried out screenings in hemodialysis patients. We have assayed 1401 samples representing 43% of the samples assayed for FD. By this studies, we confirmed Fabry diagnosis in 2 patients, giving a yield of 0.1%. This type of looking for patients with Fabry disease is the one with poor performance. But again, the positive view is that 2 new families with various relatives have benefit from correct diagnosis.

Clinical Suspicion Versus High-Risk Screenings: Analysis of Yield and Cost–Benefit

The cost–benefit analysis is difficult to undergone when talking about medicine, disease, and patients. Ethical issues may arise when this discussion is taking place. And it is our opinion that all suffering patients deserve to obtain the better up to date attention from biomedical specialists. No patient should be left apart from the best care of the moment.

If we only put attention on numbers and proportion of positives, there is no doubt that detection of patients with Fabry disease by medical suspicion is the best option. The classical way to detect patients in clinical visit is still the best. For this reason, awareness in medical community about FD should be one of the main activities from reference professionals in the field of lysosomal diseases. Different specialists could suspect FD in their patients, such as nephrologists, cardiologists, rheumatologists, dermatologists,[23] and also searching with Google may lead to a diagnosis.[32] On the other side, in our experience, both patients detected by high risk screening were variant cases, the most difficult to find among the phenotypes of FD. And, surely we wouldn't find those cases without this screening. The main disadvantages of screening in populations with one of the symptoms of Fabry's are to find cases in which the final diagnosis can't be established. Moreover, the worst thing is when this process is undergone and analyzed by nonreference professionals that may make a mistake in the interpretation of the results. There are examples in literature considering diagnosis of FD in patients whose pathogenicity of the mutation could be wrong or couldn't be finally established.[33] The mutation D313Y causes a reduced enzymatic activity of αGalA toward the artificial substrate in the in vitro test, however expression studies demonstrated high residual activity and no significant accumulation of the substrate Gb3. Now, mutation D313Y is considered a polymorphism, it is present in 1% of control population, and causes a "seudodeficiency."[34]

Over Suspicion

A new emergence challenge in the field of Fabry diagnosis is over suspicion. The benefit of continuing medical education in terms of better awareness that is so important in rare diseases could have the potential impact of being over suspected. Moreover, the idea of having a specific therapy to offer to a potential patient made the physician to put FD as one of the first in the

list of diseases for differential diagnosis when only one sign of symptom is present in his or her patient. This aspect was detected by us as a reference lab with personal specialized in Fabry diagnosis. In the last couple of years, we are receiving more samples from too many different physicians, and the proportion of positive cases that was 8% in our first years of work has declined to the 4% we reported above. Having detected this over suspicion, we are more cautious and ask the physicians to send a more complete clinical picture, medical history, and family history in order to decide, especially in females, which samples should be tested.

Conclusion

In this work, we presented our 14 years' experience in diagnosis of Fabry disease in Argentina from a specialized lysosomal diseases diagnosis laboratory. Moreover, we report genotype characterization of the 25 families from Argentina with FD detected by us. Although there are reports of mutations in patients with Fabry disease from Argentina, it is the biggest report of mutations of FD in Argentina. The mutations were missense in 72% of the cases, 3 short deletions, 2 short duplications, and 2 nonsense mutations. All the duplications and deletions were novel, but only 5 missense and 1 nonsense mutations were novel. We have no found exon or gene deletions.

This challenge was approached by medical education, close contact with the physicians who suspect FD in their patients, and working with affected families in order to offer diagnosis. All this work is carried out in close collaboration with a group of physicians of different specialties, who are gathered in AADELFA, a medical association for study of Fabry disease in Argentina.

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