Possibilities and Challenges in the Molecular Diagnosis Of Lysosomal Storage Disorders

Csép Katalin[1], Drugan Cristina[2], Bănescu Claudia[1]

[1] Department of Genetics, University of Medicine and Pharmacy, Tîrgu Mureş, Romania
[2] Department of Biochemistry, University of Medicine and Pharmacy, Cluj-Napoca, Romania

In inherited metabolic diseases, the final diagnosis is generally made by classic biochemical methods, despite the monogenic etiology. In lysosomal storage disorders, the suspected clinical diagnosis is confirmed by enzyme assay, and DNA analysis is not mandatory for the diagnosis or initiation of the treatment. Like in most enzyme deficits, the inheritance is recessive (autosomal or X-linked). Genetic heterogeneity is characteristic, and hundreds of alleles of the same gene may exist, caused by various mechanisms or mutations at different nucleotide levels. Besides the targeted analysis of the most frequent mutations (N370S, L444P, R463C, 84GG, recNcil, recTL) in Gaucher disease carried out in the national diagnostic center, often mutation scanning and sequencing is required. Though data must be carefully interpreted, molecular testing may provide important additional information, and it is the basis of carrier testing and prenatal diagnosis. The genotype-phenotype correlation remains inconclusive in most of the cases, though sometimes it can be used as a prognostic marker.

Keywords: lysosomal storage disorders, mutation analysis

Introduction

Lysosomal storage disorders are monogenic disorders. In general, the mutation of a gene coding for a lysosomal enzyme leads to an enzyme deficit, that subsequently causes a substrate accumulation - the basis of cell, tissue and organ dysfunction responsible for the clinical picture. In the pathogenesis of the disorder, every level of modification - from the gene mutation to the organ dysfunction - may be investigated and has a well-defined role, from establishing the diagnosis and identification of the type of the disorder, to the assessment of complications and monitoring of disease progression and therapeutic efficacy. Testing strategies have been developed in certain disorders, including various assays that due to their low cost, simplicity or accessibility on one hand, and their specificity on the other hand explain for their role in the diagnosing process.

Possibilities for Romanian patients

Despite the monogenic etiology, the final diagnosis is generally made by classic biochemical methods. Enzyme analysis is currently the gold standard of diagnosing lysosomal storage disorders, though in certain cases, only mutation analysis may provide the final diagnosis. Assessment of both the level and the activity of the lyosomal enzyme is possible. The techniques currently used are fluorometry and mass spectrometry. The samples are relatively easy to obtain, as usually whole blood is used obtained from venipuncture or a spot dried on filter paper. Most frequently, the enzyme assay is carried out from plasma or isolated leukocytes, though cultured fibroblasts or other cells also may be analyzed. In Romania, biochemical diagnosis of certain lysosomal storage disorders by was introduced in 1997, and has been progressively expanded (for a complete list of disorders in which investigation is possible, please visit http://www.lysosome.ath.cx/index.html). The measurement of enzyme activity using an artificial substrate and the comparison of enzyme levels in a patient sample against normal benchmarks is carried out. Obtaining the results usually takes only a few days, but the tests are complex to interpret and must be analyzed by a specialized laboratory [1]. Recent advances, like the expansion of testing and access to tandem mass spectrometry as well as the development of enzyme assay from dried blood spots further facilitate the access to diagnosis of the Romanian patients.

It should be noted though that enzyme assays have a few limitations. The method is not available for every lysosomal storage disorder, and it does not permit the testing for healthy carriers and patients in certain situations, like in the case of affected heterozygous females in recessive disorders with an X-linked inheritance or atypical variants with residual enzyme activity, because important overlaps in enzyme levels exist with those seen in healthy persons.

Other investigations, more or less specific for a given disorder (like muscle biopsy in Pompe disease, renal biopsy in Fabry disease, the Gaucher cell in the bone marrow, other lab or imagistic investigations) have been used in the past in diagnosing these disorders, when enzyme assay and mutation analysis were not an option. Today, however, these methods, due to their limited sensitivity and specificity, have a reduced importance in establishing the final diagnosis, although in certain stages they may still have a role in assisting the diagnostic process.

In Romania, mutation analysis in lysosomal storage disorders is limited, so far. In Gaucher disease, the most important type of lysosomal storage disorder with available therapy, and the most frequent type of disease affecting 70 % of the Romanian patients diagnosed with lysosomal storage disorder by enzyme assay since 1997, mutation analysis is possible for the six frequent mutations N370S, L444P, R463C, 84GG, recNciI and recTL, by PCR-based techniques (PCR-RFLP). (Figure 1) Mutation analysis for other Romanian lysosomal storage disorder patients has been carried out in collaboration with international laboratories. The rarity of the disorders, the reduced practical

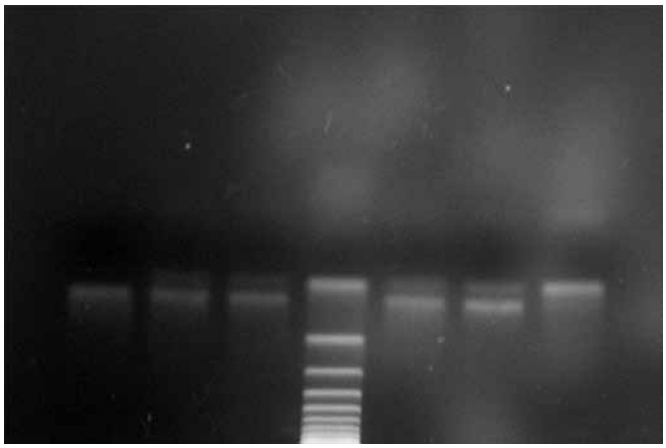

Fig. 1. Agarose gel electrophoresis detecting the N370S mutation in the GBA gene in a group of Romanian patients. From left to right: first lane – absence of mutation in a normal control, lanes 2, 3 and 5, 6 – patients heterozygous for the N370S allele, lane 4 – molecular weight marker pBR322, Hae III digested, SIGMA and lane 7 – patient homozygous for the N370S allele.

implications offered by genotyping, the important genetic heterogeneity, the limited diagnosing infrastructure and high cost of mutation scanning, characterization and sequencing, are the major impediments of the local expansion of mutation analysis currently.

The majority of the approximately 300 reported different mutations, spanning along the whole GBA gene, are point mutations: more than 2/3 lead to missense or splice junction mutations, while small insertions or deletions and complex alleles appear less frequently. The region surrounding the GBA locus is gene rich, and the high homology with the adjacent pseudogene predispose to recombination and may contribute to certain mutations, gene conversion, fusion or duplication [2,3]. Important differences of the mutation frequency exist in various populations. The high frequency of the N370S/L444P places Romania in the second largest group of genotypes reported to the Gaucher Registry, among other European (mainly Balkan and Mediterranean countries) and non-European (Australia, South America) unrelated populations, associated with severe phenotypes [4]. As previously published in 20, 24 respectively 51 Romanian type 1 Gaucher patients, the latter corresponding to approximately a quarter of the expected number of Romanian patients based on general prevalence data in non-Jewish populations, targeted mutation analysis of the most frequent six mutations identified approximately 2/3 of the disease alleles. The N370S mutation had the highest prevalence (50%), followed by the L444P (22.2%) and the recNciI (5.6%) alleles, while the other investigated alleles haven't been found. Rare or novel mutations likely accounted for 22.2% of the disease-producing uncharacterized alleles [1,5,6].

The benefits of molecular analysis

A. Confirming and establishing the diagnosis

Molecular diagnosis allows establishing the diagnosis in pre-symptomatic patients, and confirming the diagnosis in mildly affected cases with residual enzyme activity as well as in manifest heterozygotes. Most lysosomal storage disorders are autosomal recessive, so the identification of two disease-causing alleles provides an additional confirmation of the diagnosis. Molecular diagnosis may elucidate diagnosis in mildly affected patients with residual enzyme activity where enzyme assay is not informative (for example in the cardiac variant of Fabry disease). In the X-linked recessive disorders (like Hunter or Fabry disease), molecular analysis is the method of diagnosing affected female heterozygotes. In Fabry disease, the unusually high number of symptomatic females presenting a broad spectrum of clinical manifestations has raised the question of a different inheritance; lyonization or X-linked dominant inheritance have been proposed as viable explanations. In these patients, demonstration of a markedly decreased enzyme activity confirms the diagnosis; however, in females manifesting the disorder and having an enzyme activity in the normal range, only molecular genetic analysis may confirm the diagnosis [7]. Testing, however, is conditioned by the prior identification of the disease causing mutation in the family.

B. Predictive testing

Predictive testing may identify at-risk asymptomatic family members, but it is conditioned by the knowledge of the disease causing mutations in the family. If the parents are carriers, prenatal diagnosis for at risk pregnancies is possible both by enzyme assay or molecular genetic testing, while preimplantation genetic diagnosis is possible only by molecular testing. It should be noted, though, that in these situations, diagnosis is possible only if the disease causing mutation in the family have been previously identified.

C. Identification of healthy carriers

The interpretation of enzyme activity in healthy carriers is difficult because considerable overlap exists between the lower end of the normal range and enzyme activity in affected persons. Molecular diagnosis is the method that allows the screening for the presence of the mutation in healthy carriers, both in the family of a patient and high-risk populations with an increased carrier frequency (like Gaucher disease in Ashkenazi Jews).

D. Genotype-phenotype correlations

Genotyping may have practical implications in the assessment of the prognosis and selection of the best therapeutic option. Unfortunately, genotype-phenotype correlations are limited, and frequently inconclusive. As many non-recurrent alleles have been identified, the ability to accurately predict phenotype based on results of DNA analysis may be difficult, particularly in the early stages of a disease.

Sometimes the genotype is informative, and can be used as a prognostic marker. The genotype may inform about disease severity and evolution, neurological or other manifestations.

In Romania, like in other non-Jewish populations, the majority of Gaucher patients, whatever their genotype, exhibit various degrees of hematological and skeletal involvement, and genotype-phenotype correlations were similar to those reported for other Caucasian non-Jewish populations.

The N370S substitution is considered a frequently occurring mutation associated with a relatively benign prognosis. Compound heterozygotes for the N370S allele and an unknown mutation display a large degree of clinical variability. The most severe bone complications were found in N370S/L444P or N370S/? heterozygous patients [5]. The frequent N370S/ L444P compound heterozygotes present type 1 disease, usually with a severe phenotype, including an important skeletal involvement [6]. So the absence of neuronopathic disease in patients presenting at least one copy of the N370S allele was confirmed, but the relative mildness of N370S homozygotes was not a constant feature among Romanian patients [5,6]. It is an observation recently confirmed also on a large number of patients from the Gaucher Registry. N370 homozygosity does not consistently confer a mild adult onset phenotype, and patients may exhibit important phenotypic heterogeneity, including early onset and severe clinical picture [3,8]. The presence of the L444P or of an uncharacterized sporadic mutation was always associated with severe clinical manifestations, even in compound heterozygotes presenting the N370S allele [5,6]. The L444P allele is often correlated with the development of neuronopathic disease. One homozygous patient for the L444P mutation manifests a type 3 disease, the subacute neuronopathic form, that also imposed an increase in the dose of rhGC. Another patient, a possible type 3b, a L444P/? compound heterozygote, died before manifesting detectable neurological involvement, not benefitting from substitution therapy with the placentally derived enzyme, available at that time.

A large degree of phenotypic variability is observed in patients displaying the same genotype; unidentified genetic and non-genetic factors may explain these genotype-phenotype relationship inconsistencies. After carefully interpreting the results of DNA analysis, however, the knowledge of the mutation in the family may have further practical implications in the management. In the case of two Romanian siblings, for example, enzyme replacement therapy has been initiated in the younger asymptomatic sibling of a severely affected N370S/? child, based on the older sister's clinical picture and the identical genotype of the siblings. In an another case, however, close monitoring but not treatment has been elected in a nearly asymptomatic N370S/N370S adult, sister of an affected and treated patient, where the milder clinical form and the genotype usually associated with a more benign evolution assisted the decision making process. It should be emphasized that personalized management based on close monitoring has remained the principal guiding rule also in these situations.

E. Research purposes

Mutation analysis contributes to the clarification of the etiology and pathogenesis of lysosomal storage disorders at the molecular level. The various localizations and types of mutations explain the characteristic structural and functional disturbances in the coded enzymes. Beyond the scientific importance, the acquired information may lead to the development of novel therapeutic strategies and the elaboration of optimal personalized management, based on the genotype-phenotype relationships. Gene expression profiling could also help identifying novel biomarkers that could be used as surrogate measures of disease activity and therapeutic efficacy [9].

When treatment first became available in Gaucher disease, the disease Registry was also initiated, in 1991. Now the more than 6000 patients registered worldwide make possible solid statistical analysis in such a rare disorder, and have already led to important conclusions. Treating physicians from the national reference centers from the First Pediatric Clinic, Center of Genetic Diseases and Nephrology Clinic, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj, are contributing to our better understanding of mucopolysaccharidosis, Gaucher, Pompe and Fabry disease by registering the Romanian patients in the international disease registries. It should be underlined, that to benefit to the maximum of the gathered molecular data, a standard format for genotype is recommended.

The challenges of molecular analysis

A. Heterogeneity

Genetic heterogeneity leads to technical difficulties and requires attention in the interpretation of data. Besides the approximately 50 types of different lysosomal storage disorders, genetic heterogeneity in the distinct diseases complicates the etiology and the diagnosing process. Genetic heterogeneity can be allelic or non-allelic, when mutations of different genes cause the same or similar phenotypes. In most disorders allelic heterogeneity is present. Both types of allelic heterogeneity are characteristic: variable mutation mechanisms (point mutations, large deletions, etc.) may affect the same gene leading to different isoalleles, or mutations occurring at variable nucleotide sequences may cause various heteroalleles. Tens or hundreds of iso- or heteroalleles of the same gene have been described and may be found in mutation databases. Luckily, de novo mutations are infrequent. Due to the important genetic heterogeneity, most patients, considered recessive homozygous, are in fact compound heterozygotes, carrying two different disease-causing mutations.

The relative rarity of each mutation, as well as the fact that many mutations are unique to single families may cause further challenges in the interpretation of the results. Newly identified mutations must be evaluated for pathogenicity, and non-pathological polymorphisms and rare sequence variants must be differentiated.

In certain lysosomal storage disorders, like the mucopolysaccharidoses, non-allelic or locus heterogeneity is also possible, as the group comprises various forms characterized by similar clinical traits but caused by mutations in genes coding for different enzymes.

It may also be noted, that significantly different phenotypes may appear among the patients presenting the same mutations. Atypical manifestations like parkinsonism in Gaucher disease has led to interesting findings regarding its genetic background. It is a good example of how a mutation leading to a monogenic disorder may determine an increased risk of developing a complex disease in healthy mutation carriers [10]. Thus, it supports the importance of elucidating the etiology of various rare or very rare inherited disorders, because the insights gained may also contribute to our understanding and optimal management of common multifactorial disorders.

B. Technical difficulties

The targeted analysis of frequent mutations by various techniques is possible. However, complex mutation mechanisms, genetic heterogeneity, and allele specific or family specific mutations frequently make mutation screening and sequencing necessary.

Targeted mutation analysis is recommended before proceeding to full sequence analysis. In Gaucher disease for instance, the type and frequency of mutations depends on ethnicity. Four mutations account for the disease in 90% of the Ashkenazi Jewish patients, but in non-Jewish populations, these mutations account for less than 60% of the cases, as seen also in the Romanian patients. More than that, the patients are usually compound heterozygotes possessing one common and one rare or unique mutation.

Mutation scanning implies the use of a variety of methods (SSCP, DHPLC, etc.), but may allow for rapid detection of alterations. However, mutation detection must be followed by the characterization of the alteration. The high number of various mutations makes frequently necessary the combination of different assays, and may ultimately lead to sequencing of the entire gene, coding region or of select exons. Deletions/duplications not readily detectable by sequence analysis are identified by a variety of methods (MLPA, targeted/full array GH, etc.).

Mutational overlaps may further complicate the testing and interpretation. In Gaucher disease for example the 55-bp del and N370S are overlapping, so compound heterozygotes may appear homozygous for N370S. In practice, testing the parents or routinely testing all N370S/N370S patients for the 55-bp del clarify these cases.

Where mutation analysis locally is impossible, collaboration with foreign laboratories may lead to mutation identification. In Fabry disease more than 400 different mutations have been described in the GLA gene, many unique to single families. New mutations are continuously reported, and the novel c.874G>A (p.A292T) substitution was first described in a Romanian family [11]. Identifying a mutation may further complicate the situation, and complex interpretation of data is required to clarify its role in the etiology, and differentiate between polymorphisms, rare sequence variants and disease-causing mutations).

In conclusion, mutation analysis has been initiated in Romanian lysosomal storage disorder patients by targeted identifications of frequent mutations causing Gaucher disease, and continuous efforts are undertaken to expand the diagnostic possibilities. The data acquired may contribute to the optimal management of the patients and families, but should be carefully interpreted due to its complexity.

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