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Diagnostic methods for Lysosomal Storage Disease

Armin Mokhtariye[1,3], Lida Hagh-Nazari[1], Abdol-Reza Varasteh[2], Fatemeh Keyfi*[3]

Abstract

Lysosomal storage disorders (LSD) are a class of metabolic disturbance in which manifested by the accumulation of large molecules (complex lipids, glycoproteins, glycosaminoglycans, etc.) in lysosomes. LSDs have a wide range of clinical symptoms that may contain organ dysfunction, neurological and skeletal disorders. The first stage of diagnosis is clinically suspected by a physician. Next stage is enzyme activity assays including Fluorometry and MS/MS methods. These methods usually placed in newborn program screening. The second laboratory diagnostic stage is molecular examination (RFLP-PCR and ARMS-PCR, Mutations Scanning Methods, DNA sequencing, MLPA and NGS methods) that is confirmation of the enzyme assays. In this article, routine diagnostic methods for LSDs were discussed. The gold standard for enzyme activity assay and molecular diagnosis is TMS and NGS, respectively.

Keywords: Diagnostic methods, Enzyme activity, Lysosomal storage disease, Molecular assay.

Introduction

The last operators in the endocytic process are lysosomes that cleavage the large molecules into simpler component (1). Hydrolytic enzymes are proprietary for multiple substrates in the lysosomes-which are activated in the acidic pH (between 4.5 and 5.0) in the organelles' intracellular (1). Lysosomal storage disorders (LSD) are a class of metabolic disturbance due to an absence of more than 40 hydrolytic enzymes in which manifested by the accumulation of large molecules in lysosomes (2). More LSDs are autosomal recessive inherited and generally have an occurrence of about 1 per 7700 live births (3).

Pompe disease (PD), Gaucher disease, Niemann Pick disease, and GM2 gangliosidosis are type of LSDs and due to lack or decrease of acid α-glucosidase (GAA, EC3.2.1.20), acid β-glucocerebrosidase (GBA, EC 3.2.1.45), acid sphingomyelinase (ASM, EC 3.1.4.12), hexosaminidase (HEX, EC 3.2.1.52), respectively (2, 4). LSDs are leading to the accumulation of complex lipids, glycoproteins and glycosaminoglycans as specific substrates. (5). Clinical and laboratory findings of each mentioned LSDs were given in Table 1.

Pompe disease is a rare and neuromuscular disorder due to a lack or decrease of the acid alpha glucosidase (GAA) that leads to a deposit of glycogen in lysosomes of many tissues, particularly heart and skeletal muscle (6, 7). This enzyme is coded by GAA gene, located on chromosome 17 (8). Continuous deposition of glycogen in these tissues leads to the wide clinical range, organ and system damages, and often leading to death (6). The clinical symptoms appear at different ages with a grade of organ damage (9, 10). The acute form of the disease is classic infantile of Pompe with clinical symptoms that present at the first weeks of birth (9), (11, 12). Another form, late-onset Pompe disease appears one year of birth (6).

Gaucher disease (GD) is the most frequent LSD and multisystem disorder due to a lack or decrease of acid beta glucosidase (ABG) that leads to a deposit of glucosylceramide in macrophages (13-15). This enzyme is coded by GBA, located on chromosome 1 (16). GD divided into three subtypes based on the existence or absence of nerve dysfunction. These subtypes including non-nervous dysfunction (type 1), acute nervous dysfunction (type 2) and moderate nervous dysfunction (type 3) (17, 18).

1: Department of Clinical Biochemistry, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran.
2: Immunobiochemistry Lab, Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.
3: Department of Medical Laboratory Sciences, Varastegan Institute for Medical Sciences, Mashhad, Iran.
***Corresponding authors: Fatemeh Keyfi; Tel: +98 51 35091160; Fax: +98 51 35091172; E-mail: keifyf@varastegan.ac.ir.**
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Niemann Pick disease (NPD) is a pan-ethnic and neurological disorder due to a lack or decrease of acid sphingomyelinase (ASM) that leads to an aggregation of sphingomyelin in brain, reticuloendothelial and lung tissue (19, 22). This enzyme is coded by SMPD-1, located on chromosome 11 (23). This disorder divided into two subtypes (A, B) based on the involvement level of CNS dysfunctions (19). Levels of enzyme activity in patients with NPD-A are zero or very low (<5%) that is aggressive NPD and presents in the first months after birth (20). The patients with NPD-B have a longer lifespan and levels of enzyme activity are higher (>5%) than NPD-A (20, 24).

GM2 gangliosidosis is an extremely rare disorder with deterioration of nerve cells due to a lack or decrease of hexosaminidase (HEX). This disorder leads to an aggregation of ganglioside GM2 and other glycolipids in brain and spinal cord (4). Hexosaminidase A (αβ) and hexosaminidase B (ββ) are isoenzymes of hexosaminidase that encoded HEXA gene on chromosome 15 and HEXB genes on chromosome 5. HEXA gene codes α subunit and HEXB gene codes β subunit (4, 25, 26). Mutations in the HEXA gene leading to Tay Sachs disease (TSD) (27) and mutations in the HEXB gene leading to Sandhoff disease (SD) (28). The wide collection of clinical protests (severe, mild, chronic) exists in these abnormalities (29).

Table 1. Clinical symptoms of Lysosomal storage disorders (LSDs)

Disorder name	Clinical symptoms		Reference
Pompe Disease (PD)	**Infantile form** Hypertrophic cardiomyopathy, hypotonia, hepatomegaly, macroglossia, generalized muscle weakness and death follow up rapid cardiorespiratory failure		Almeida et al, 2017 Kishnani et al, 2006 Rairikar et al, 2017
	Late-onset form: With or without respiratory muscle weakness, slowly progressive axial and/or limb-girdle muscle weakness		Almeida et al, 2017 Kishnani et al, 2014
Gaucher Disease (GD)	**Type 1 (Non-neuronopathic form)** Visceral enlargement such splenomegaly and hepatomegaly, thrombocytopenia, symptomatic anemia, coagulation abnormalities and pain of skeletal system		Zimran et al, 2017 Barton et al, 1991 Devigili et al, 2017
	Type 2,3 (Neuronopathic form) Different degrees of hematological complications similar to type 1 and with involvements of the central nervous system (myoclonus, seizures, ataxia, cognitive impairment, and supranuclear gaze palsy), pulmonary involvement (central apnea)		Sechi et al, 2014 Tezuka et al, 2016
Niemann Pick Disease (NPD)	**Type A (Severe form)** Deterioration of neurological functions, hepatosplenomegaly, psychomotor retardation, recurrent respiratory infections and death during or before the third decade of life		Legnini et al, 2012
	Type B (Milder form) Milder in manifestation, with no neurologic involvement		Legnini et al, 2012
GM2 gangliosidosis	**Tay Sachs Disease (TSD)**	Dysarthria, dysphagia, and hypotonia, amyotrophic lateral sclerosis associated with tremor and cerebellar atrophy, neuromuscular weakness, and cerebellar dysfunction	Utz et al, 2017 Barritt et al, 2017
	Sandhoff Disease (SD)	Decrease in motor, mental and visual functions, alteration of the vision, macrocephaly, seizures, liver enlargement, slight bone deformation and neurodegeneration	Lecommandeur et al, 2017

Diagnosis of LSDs

In the first stage of diagnosis, the physician will be suspected to LSDs according to clinical symptoms are given in Figure 1. Since most LSD are not apparent at birth and have multi-organ involvement and for preventing irreversible damage to affected organ systems, early diagnosis by the laboratory is suggested (30). In the following sections, we

investigate enzyme activity assays and molecular examination for diagnosis of LSDs.

2.1. Enzyme activity assays

The current methods used for enzyme activity assays are fluorometry and MS/MS. These methods are used for a wide range of metabolic disorders especially newborn screening (NBS) for LSDs.

2.1.1. Fluorometry method

Fluorometry is an optical technique that evaluated the emission of fluorescence. The principles of this method on the breakdown of specific 4-MU synthetic substrate by specific enzymes and leads to release of fluorescent 4-methylumbelliferone (4-MU). Therefore, the assay protocol varies from enzyme to enzyme (31). The measured fluorescence is directly proportional to the enzyme activity in a dried blood sample (DBS) (31). This technique was developed by Chamoles et al. to measure lysosomal enzyme activities in DBS (13). After that, numerous modified methods have been described for the enzyme activity of LSDs using fluorometric methods (2, 4, 13, 31-35).

2.1.2. Tandem Mass Spectrometry (TMS or MS-MS) method

Tandem mass spectrometer is a single instrument using two (or more) mass analyzers. A mass spectrometer is a ‘‘molecule smasher’’ that measures molecular and atomic masses of whole molecules. In this technique, molecular fragments and atoms separated according to their mass-to-charge ratio (m/z) and therefore also be used for quantitation of molecular species (36).

Li et al. were pioneered to measuring of lysosomal enzyme activities in DBS by TMS. In the method, the sample extracts combined and purified by liquid-liquid extraction (LLE) followed by solid phase extraction (SPE) to remove the salts, detergents and excess substrates (37, 38).

Since then several technical modifications were carried out focusing on simplifying the sample preparation processes (38).

In 2008, Zhang et al. modified Li’s method and Triton X-100 was replaced by CHAPS in GAA activity. The advantage of this modification has minimized the effect of detergent on the MS/MS, which is more effectively removed by solid-phase extraction and results in an increase in GAA activity. In other hand, the addition of ZnCl2 to achieve an optimal activity of ASM was performed. The modified multiplex enzyme assay is an effective method for throughput screening laboratories (39).

Marca et al. used the online trapping and clean up along with liquid chromatography tandem mass spectrometry (LC-MS/MS). In this modified method, all reaction mixtures were injected directly onto the mass spectrometer, and the sample mixture was trapped at first on a preparation column and next enter to the C18 separation column, followed by mass spectrometry measurement. This simple method takes only 4 min as analysis and does not need any time for sample preparation step after the enzymatic reaction (40).

In 2010 Kasper et al. were reported using a multi-dimensional ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) with turbulent flow chromatography (TFC) for online sample clean-up (41).

In 2010, Scott et al. have developed tandem mass spectrometry with electrospray ionization (ESI-MS/MS) for enzyme activity assay in dried blood spots (DBSs). They optimize the ESI-MS/MS assays by exploring buffer conditions that would allow multiple lysosomal enzymes to be assayed with a single DBS punch in a single assay well (42).

In 2016 Elliott and co-workers have used multiplex-tandem mass spectrometry for 6 lysosomal enzyme activity. The advantage of this study was using a single solution and buffers with a pH range of 3.5 to 6.5 achieve to maximum activity of 6 enzymes (43).

2.2. Molecular examination

For confirmation of enzyme activity assays and determination of mutation in the proband, molecular examination would be done.

The molecular methods used for confirmation are reviewed in below.

2.2.1. RFLP-PCR and ARMS-PCR

These techniques are used for identification of known common point mutations in certain areas.

Table 2. Gene features and mutations of LSDs

Disorder	Gene	Mutation	Number of Mutation
Pompe	GAA	Missense/ nonsense	320
		Splicing	69
		Regulatory	1
		Small deletion	78
		Small insertion	32
		Small indel	13
		Gross deletion	15
		Gross insertion	1
		Complex	4
Gaucher	GBA	Missense/ nonsense	351
		Splicing	25
		Regulatory	1
		Small deletion	34
		Small insertion	15
		Small indel	6
		Gross deletion	4
		Gross insertion	3
		Complex	21
Niemann pick	SMPD1	Missense/ nonsense	173
		Splicing	4
		Small deletion	45
		Small insertion	13
		Small indel	4
		Gross insertion	1
Tay sachs	HEXA	Missense/ nonsense	105
		Splicing	35
		Small deletion	29
		Small insertion	10
		Small indel	3
		Gross deletion	1
Sandhoff	HEXB	Missense/ nonsense	52
		Splicing	22
		Small deletion	23
		Small insertion	5
		Small indel	1
		Gross deletion	9

Restriction Fragment Length Polymorphism (RFLP) also known as a cleaved amplified polymorphic sequence (CAPS) that digested specific sequence DNA by restriction endonuclease enzymes. This technique recognizes restriction fragments with different lengths after digestion by gel electrophoresis. The amplification-refractory mutation system (ARMS) has two type primers (normal and mutant) which differ in the 3' terminus nucleotide. The 3' terminal base in normal primer and mutant primer is complementary to normal sequence and certain mutant sequence, respectively (44).

In 1985, Sorge and co-workers optimized RFLP-PCR assays for Gaucher disease in North Torrey Pines. They used 20 different restriction enzymes for GBA gene that PvuII and KpnI endonucleases were more common (45). Mitsuo et al used PvuII and KpnI endonucleases in the Gaucher Japanese

population, but just PvuII polymorphism in this subjects was seen (46).

In 1992, Mistry and co-workers were developed ARMS-PCR assays for Gaucher disease in Jewish and non-Jewish patients (47). In 2000, MING CHENG and co-workers were used DdeI endonuclease for Tay sachs in Ashkenazi population (48).

In 2010, Amarinthnukrowh and co-workers were used BsaHI endonuclease for the check out the c.1935C>A mutation in Thai patients (49) and Mattosova et al. used MboII endonuclease for the c.-32-13T>G mutation in Slovakia patients for Pompe disease (50). In 2016, Mozafari and co-workers were used HpaII endonuclease for the check out the G102S mutation in Iran Gaucher patients (51).

2.2.2. Mutations Scanning

Mutation scanning techniques identify the gene variants without the location of the gene was recognized (52). Common mutation scanning techniques include HRM and DHPLC.

High resolution melting (HRM) is the simple, cost-effective post PCR technique for high throughput mutation scanning, genotyping and methylation profiling (52, 53).

In 2013, Zech and co-workers used HRM method for German's Neimann Pick patient and detected two genetic variants (NPC1 and NPC2) (54).

In 2013, Hopfner et al. investigated the relationship between SNPs in SCARB2 gene (mediates GBA) and Parkinson disease by HRM method in a German and Austrian population. They didn't detect a novel mutation in this gene (55).

Kiong ER and co-workers used HRM technique in Pompe patients for identifying seven known GAA mutations in Taiwanese population (56).

Denaturing high performance liquid chromatography (dHPLC) as a post-PCR method for screening of DNA samples by analysis on reversed phase columns. This technique was performed by temperature changes for denaturing of DNA strands (57, 58).

In 2005, Pomponio and co-workers used dHPLC for Gaucher patient and finding three novel mutations in Colombia population. This method was reported as a reliable and sensitive method to detection of fragments between 100 to 1500 bp (59).

In 2007, Pittis and co-workers scanned PCR products of 45 Italian Pompe patients by dHPLC and detected 12 novel mutations. According to their reports, dHPLC technique has high precision, cost- and time -effective method, but not very good for investigation of highly polymorphic genes such as GAA gene (60).

2.2.3. DNA sequencing

DNA sequencing is a technique that identifies the precise position of nucleotides in the genes. (53). In 1996, Redonnet-Vernhet and co-workers investigated HEX genes by DNA sequencing and reported two single point mutations in the Portuguese family (61). In 2003, Sikora and co-workers investigated mutation analysis in SMPD1 gene. They identified eight mutations in a Turkish population. These are including one common mutation, six single base substitutions and one nucleotide deletion. All mutations were present in different parts of the whole gene (62). In 2011, Haghighi and co-workers used DNA sequencing for three Tay Sachs patients of the Persian population. They reported two different point mutations that previously reported in American black and French infant (63). In 2015, Tamhankar and co-workers performed sequencing of 22 Sandhoff patients in Indian population and detected 13 mutations with eight novel mutations (64). Feng et al. used sequencing method for 22 Gaucher patient in Southern China. Their result showed 22 different mutations with four novel mutations (65). In 2018, Fukuhara and co-workers used DNA sequencing method for 38 Pompe patient in the Japanese population. They reported seven novel mutations which six mutations were detected in exon 2 and just one mutation observed in exon 14 (66).

2.2.4. MLPA

Multiplex ligation-dependent probe amplification is a variation of multiplex-PCR. In this technique, some different DNA sequences polymerized in a microtube with the same conditions and just a single primer pair. MLPA includes a few probes and each probe has two parts, one part complementary to the target sequence and the other part connects to the single primer pair (67). Only when both probes are hybridized to their targets, complete probe is formed and finally PCR amplification occurs (67).

In 2012, Zampieri and co-workers used MLPA method for analysis of HEXB gene. They designed 17 pair probes to identify large deletions in this gene. They reported nine novel mutations consist of 4 sequence variation, 3 intronic mutations, 1 nonsense mutation and 1 small in-frame deletion (68).

In 2015, Basgalupp and co-workers used MLPA method for 33 Brazilian GD patients. The MLPA kit used for identifying deletion/duplication in GBA. In this study, they reported no deletion/duplication, but just one patient had a heterozygous deletion that it's detected by sequencing (69)

2.2.5. Next Generation Sequencing

Next Generation Sequencing (NGS) is a method for investigating DNA sequencing in large regions and very cost- and time -effective method. NGS method consists of platforms that doing massively parallel sequencing and a million copies of the DNA fragments are distributed (70, 71). In 2013, Hoffman and co-workers applied NGS method for Tay sachs patients and found the 7.6 kbp deletion in some patient and concluded that NGS technique was superior to other genotyping methods and false-positive/false-negative results did not show (72). Yoshida et al. demonstrated three mutations in the Gaucher Japanese family by NGS method and two different mutations were found in parents. In this study, NGS is known as prenatal diagnosis method without needing genetic pedigree information (73). In 2016, Tsai and co-workers used NGS for Pompe patient. They detected one-point mutation and one deletion in GAA exon by NGS (74). Zampieri et al. used NGS for 38 patients with Gaucher disease and identified different mutations in GBA gene. In this study, they reported 10 variants in the intron region of GBA by NGS method (75).

Conclusion

In this paper, we discuss the conventional methods for enzyme activity assays and genetic analysis used in the LSDs. In the first laboratory diagnostic stage is enzyme activity assays and usually placed in newborn screening, because of cost-effective and simple. The biochemical diagnosis techniques are Fluorometry and TMS. The second laboratory diagnostic stage is molecular examination. The molecular assays that we discussed in this study are ARMS PCR, RFLP PCR, Mutation scanning, Sequencing, MLPA and NGS. These techniques have several advantages including; confirmation of enzyme assays, determination of mutations, family mutation study and prenatal diagnosis. According to conducted studies, the gold standard for enzyme activity assay and molecular diagnosis is TMS and NGS, respectively.

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