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Mucopolysaccharidosis VI diagnosis by laboratory methods

<https://doi.org/10.1515/labmed-2018-0048>

Received May 5, 2018; accepted September 5, 2018; previously published online September 29, 2018

Abstract: Mucopolysaccharidosis type VI (MPS VI) results from a defect in arylsulfatase B (ARSB). There are several diagnostic methods using to identify patients; hence, we aimed to review these approaches and consider if one of them could be assigned as the gold standard method. The information of this study was obtained by searching through PubMed and Google scholar databases. In order to collect the most accurate and up to date data, we limited our research to papers in the time period between 2010 and 2017. We collected articles related to our research and extracted the most relevant and accurate data which included the steps of MPS VI diagnosis by routine laboratory approaches. We concluded that an all-inclusive diagnostic approach requires urinary glycosaminoglycan (GAG) analysis, enzyme activity analysis and molecular analysis by mutation scanning through polymerase chain reaction (PCR) and Sanger sequencing or alternative methods such as multiplex ligation-dependent probe amplification (MLPA), real-time polymerase chain reaction, array-comparative genomic hybridization (aCGH) and next generation sequencing (NGS). Reliable classification of patients with MPS VI is necessary for ongoing and future studies on treatments, outcomes and prenatal diagnoses (PNDs). The dependable characterization of patients would be achieved by biochemical techniques and enzymatic assay. However, if a molecular defect is previously identified in the family, PND via mutation scanning is possible.

Keywords: arylsulfatase B; diagnostic techniques; enzyme assay; glycosaminoglycans; mucopolysaccharidosis VI.

Introduction

Mucopolysaccharidosis VI (MPS VI), or Maroteaux-Lamy syndrome (OMIM #253200), is an autosomal recessive lysosomal storage disorder that results from a deficiency of the enzyme *N*-acetylgalactosamine 4-sulfatase (arylsulfatase B, ASB/E.C.3.1.6.12); this enzyme is required for the degradation of glycosaminoglycans (GAGs) dermatan sulfate (DS) and chondroitin 4-sulfate (C4S). The enzyme deficiency results in the aggregation of partially degraded GAGs in tissues and organs, which in turn causes clinical manifestations that gradually gets worse with age [1]. Mutations in arylsulfatase B (ARSB) is observed in patients with MPS VI, the gene which is located on chromosome 5q14.1 spans 2.6 kb and contains eight exons, causing partial or complete enzyme deficiency [2]. A wide, continuous spectrum of symptoms, severity and natural course can be observed in MPS VI which is a heterogeneous disease. Patients with the rapid progressing form of the disease represent skeletal abnormalities, joint stiffness and deformities, short stature with reduced growth rate, cardiovascular symptoms, coarse face and recurrent upper airway obstructions and infections during the first few years of life [3–5]. Patients with the slow progressing form of the disease may show the disease later in life, with variable symptoms and disease progression over several decades. This wide range of phenotypic manifestations and lack of clinician awareness of the disease may contribute to the difficult diagnostic pathway for some patients with MPS VI [6]. Early diagnosis of MPS VI is crucial due to the availability of Galsulfase (recombinant human ASB; rhASB; Naglazyme®), which has been shown to slow the progression of the disease with a more significant impact on clinical outcomes the earlier the treatment is initiated [6, 7]. Early diagnosis also gives vital genetic information to the family, which may influence future reproductive decisions. Unfortunately, lags to or missed diagnoses are prevalent as patients present with ordinary childhood disorders and single organ involvement. Patients with MPS VI do not have cognitive impairment and those with the slow progressing form of the disease may not have the coarse facies characteristic which is mostly observed in

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MPS VI [8]. Slow progressing disease in patients may not be diagnosed immediately even by clinicians aware of the characteristics of MPS diseases [9]. Therefore, it is not out of the question that clinicians who have less experience with MPS diseases may diagnose patients as having another disease with the same symptoms as MPS which are more commonly seen in their profession. Busy specialists may diagnose and cure the patient for the manifestations related to their particular specialty without looking at the patient's complete conditions and hence would not diagnose the underlying MPS disease [6]. Table 1 provides information about the clinical phenotypes of MPS VI [10–13].

Diagnosis of MPS VI happens via a combination of clinical detections and laboratory test results. The quantitative rise of total urinary glycosaminoglycans (uGAGs) is detected by laboratory tests [14], qualitative elevation of DS which is a specific uGAG [15], reduction of ASB enzyme activity [16] and mutations in the *ARSB* gene

[17]. Differential diagnosis in first steps consists of I-cell disease, multiple sulfatase deficiency (MSD) and other MPS disorders.

Because of the clinical heterogeneity in the presentation of MPS VI, diagnosis is intricate and misdiagnosis have arisen. Patients are distributed over a wide spectrum of slowly and rapidly progressing phenotypes [5]. This phenotypic variation may be a result of the large number of genetic mutations responsible for the disease among other genetic, metabolic and environmental factors. The majority of mutations are either unique to a family or present only in a few number of patients [18]. Different levels of residual enzyme activity, resulting in the alteration of GAG metabolism and therefore leading to a variation in both clinical manifestations and laboratory test results, may be a consequence of different mutations in the *ASB* gene [17]. For an accurate diagnosis, the combination of clinical findings and laboratory test results is critical. The diagnosis must be precise to ensure that the correct advice and care is given to the patient because treatment options, disease management and prognosis are different among lysosomal storage diseases [19].

Table 1: Clinical manifestations of MPS VI.

Appearance and general symptoms	Macrocephaly Coarse facial features Short stature Low endurance
Eyes, ears, nose, throat	Corneal clouding Glaucoma Weak vision Optic nerve disease Recurrent otitis media Weak hearing Recurrent sinusitis
Mouth, teeth	Enlarged tongue Abnormal teeth
Airways, respiration	Sleep apnea Obstructive and restrictive airway disease Low pulmonary function Recurrent pulmonary infections
Heart	Cardiac arrhythmia Cardiomyopathy Pulmonary hypertension
Abdomen	Hepatosplenomegaly Umbilical and inguinal hernias
Bones, joints	Skeletal abnormalities (dysostosis multiplex) Joint stiffness and contractures Hip dysplasia
Brain, nerves	Cervical spinal cord compression Carpal tunnel syndrome Communicating hydrocephalus Normal intelligence

Diagnosis of MPS VI

The degradation of the C4-sulfate ester linkage in N-acetylgalactosamine 4-sulfate residues at the non-reducing ends of the GAGs DS and C4S is catalyzed by the functional N-acetylgalactosamine 4-sulfatase enzyme [20]. These GAGs are long unbranched sulfated polysaccharides comprising repeating disaccharides including an amino sugar (N-acetylgalactosamine) and an uronic sugar. Absence or reduction of ASB enzyme activity leads to the progressive accumulation of DS and sulfated oligosaccharides derived from both DS and C4S in tissues and organs [8]. The phenotypes associated with MPS VI are believed to be particularly as a result of the accumulation of DS [8]. Disease phenotypes are generally observed only in patients with enzymatic activity below 10% of the lower limit of normal activity [8]. Carriers who have one mutant allele do not exhibit phenotypes of MPS VI. Enzyme synthesis, stability or maturation and possibly intracellular transport time or a combination of these factors may be affected as a result of mutations in the *ARSB* gene [21]. Differences in GAG accumulation and clinical manifestation may be observed as a result of different mutations that cause different levels of residual ASB activity [22].

Urinary GAG analysis

Most of the time, the first step during the diagnosis of a patient with suspected MPS VI is uGAG analysis. uGAG analysis can be used for following up a patient's response to treatment. There are two main uGAG analysis approaches available: quantitative and qualitative. It is strictly recommended to perform both assay types simultaneously. It is critical to mention that both uGAG analysis assays are only screening techniques and should never be considered as a diagnostic technique for a specific type of MPS disorder [19].

Quantitative uGAG analysis

The whole level of urinary GAG is evaluated by quantitative uGAG analysis and although it is not unique for any particular MPS disorder and should not be used solely, it is routinely used as an initial screening strategy. Quantitative uGAG analysis is generally carried out by spectrophotometric analysis of urine using a cationic blue dye or by colorimetric analysis of urine using carbazole [19]. Accurate interpretation of quantitative results requires proper reference ranges. The ratio of uGAG to creatinine is higher during infantile and childhood, reduces with age, and then remains stable throughout adulthood [23–25]. It is notable that uGAGs level can be extremely high in normal newborns, so it should not be considered as a valid screening test in this period [26]. Patients with slow progressing manifestations with only slightly elevated urinary DS (as detected by qualitative uGAG analysis) may be “ignored” if the only screening test is used be the quantitative uGAG analysis [27, 28]. Since quantitative uGAG analysis is non-specific, it is essential to identify the exact type of raised GAG through qualitative uGAG analysis to understand type of MPS disorder. While both quantitative and qualitative uGAG analyses are applied simultaneously, the risk of MPS patients being ignored is declined [29].

Qualitative uGAG analysis

Distinctive qualitative evaluation of the GAG type is performed through GAG precipitation followed by electrophoretic separation according to the molecular size and charge on a solid base such as cellulose acetate, polyacrylamide and agarose [15]. After these steps, visualization of bands is done by staining with a dye, such as toluidine blue or 1,9-dimethyl methylene blue (DMB) [19].

Differential diagnoses

Differential diagnoses within the mucopolidoses and MPS consist of:

- MPS I H; I S; I H/S (Hurler syndrome; Scheie syndrome; Hurler-Scheie syndrome)
- MPS II (Hunter syndrome)
- MPS IVA (Morquio syndrome)
- MPS VII (Sly syndrome)
- MSD
- Mucopolidosis I (currently known as sialidosis), II, III and IV.

In the first years of life, the MSD and MPS I, II, IVA, VII may present with the same symptoms as MPS VI. It is possible to distinguish MPS IVA from MPS VI by ligamentous laxity [30]. A combination of common differentiating phenotypes with a composition of GAG substrates in urine may be practical in characterizing different MPSs. DS and heparan sulfate (HS) are usually excreted in the urine of MPSs I, II and VII [19] patients. Corneal clouding is typically less severe in MPS I patients than in MPS VI patients [31]. MPS I may be discriminated from MPS VI by the excretion of both HS and DS in urine in MPS I compared to nearly 100% DS in MPS VI patients. The equal amounts of excreted DS and HS and the absence of corneal clouding are the differentiating features of MPS II which is an X-linked recessive disease affecting mainly boys. MSD may be similar to MPS VI in infants but later mental deficiencies and the ichthyosis help discriminate between MSD and MPS VI [32, 33]. Multiple sulfatase activities are simultaneously impaired in MSD, so patients usually have all types of GAGs present in their urine. MPS VII patients can be differentiated by clinical manifestations *in utero* or at birth, and corneal clouding which is less prevalent at ages below 8 years. MPS VII is also differentiated by uGAG consisting of chondroitin 4- and 6-sulfates, HS and DS. Absence of elevated GAG in urine helps to discriminate between sialidosis (mucopolidosis I), mucopolidosis III, IV and MPS VI. In serum of these patients high levels of beta-hexosaminidase, iduronate sulfatase and arylsulfatase A is observed but in cultured fibroblasts deficiency of the same enzymes can be detected. (Note: the patient's age and the technique used to evaluate uGAG often have an effect on the relevant amount of the identified GAG DS, HS and CS) [8].

Although discriminating between MPS VI and the other MPS disorders causing excess DS excretion seems simple, there are several confusing factors. It is not unusual to observe a very little band of HS and heavy DS in patients with MPS I or II. Therefore, even if only DS is identified, MPSs I and II should not be excluded in favor

of MPS VI. Conversely, it has been mentioned that low amounts of HS may be detected in the urine of MPS VI patients. HS has also been detected in the urine of normal, healthy children and mostly young children (<3 years of age). A patient with naturally occurring low amounts of HS in addition to elevated DS excretion due to MPS VI might be inaccurately diagnosed as an MPS I or II patient. Therefore, the existence of HS and DS simultaneously does not necessarily exclude MPS VI. Conclusively, it is not possible to definitively differentiate MPSs I, II, VI and VII using qualitative uGAG analysis [19]. It is notable to pay attention to the differences between MPSs by raised GAGs, enzyme deficiency, gene location and pattern of inheritance for diagnosis, which is embedded in Table 2 [34].

Enzyme activity assay

MPS VI may be diagnosed by measuring ARSB activity. In healthy individuals during lysosomal deterioration, the hydrolysis of the C4-sulfate ester linkage in N-acetylgalactosamine-4-sulfate residues at the non-reducing ends of dermatan and chondroitin sulfate is catalyzed by ASB [35]. The capability of ASB to catalyze this reaction in MPS VI patients is reduced or lost. The affirmation of this deficiency in ASB activity is required for diagnosis of MPS VI. However, when MPS VI is the suspected disease, many

laboratories routinely assess other MPS disease-related enzymes in addition to ASB [19]. It is recommended to analyze another lysosomal enzyme in the same sample used for ASB assessment to evaluate the quality of the sample. If the reference enzyme is normal and ASB activity is decreased, another sulfatase (e.g. iduronate-2-sulfatase) should be measured to rule out MSD, a different lysosomal disease in which reduced levels of all sulfatases are found, including ASB [36]. Fibroblasts, dried blood spots (DBS) and leukocytes (including lymphocytes) are the sample types routinely used to assess enzyme activity. Amniocytes, cultured chorionic villus cells and dissected chorionic villi are the other potential sample types which are used for pre-natal diagnosis (PND) through enzyme activity assay. The level of arylsulfatase C activity in chorionic villi is high and hence could interfere with the result if this material is used for PND of MPS VI; therefore the sample of choice for the biochemical PND of this type of MPS is amniocytes. Table 3 describes the best approaches of MPS VI diagnosis through uGAG and enzyme activity analysis [19].

Molecular analysis of the *ARSB* gene

The *ARSB* gene is located at 5q11-q13 [2], extends approximately 2.6 kb and contains eight exons [37]. The first step in identification of the *ARSB* gene mutations is

Table 2: Classification of mucopolysaccharidosis.

MPS	Name	Increased GAGs	Enzyme deficiency	Gene location	Pattern of inheritance
I	Hurler, Hurler-Scheie or Scheie	HS + DS	α -iduronidase	4p16.3	AR
II	Hunter	HS + DS	Iduronate sulfatase	Xq28	XR
III A	Sanfilippo A	HS	Heparan-N-sulfatase	17q25.3	AR
III B	Sanfilippo B	HS	α -N-acetylglucosaminidase	17q21.1	AR
III C	Sanfilippo C	HS	AcetylCoA α -glucosamine acetyltransferase	14p21	AR
III D	Sanfilippo D	HS	N-acetylglucosamine 6-sulfatase	12q14	AR
IV A	Morquio A	KS	Galactosamine-6-sulfate sulfatase	16q24.3	AR
IV B	Morquio B	KS	β -galactosidase	3p21.3	AR
V	Scheie syndrome, initially proposed as type V, was recognized to be the attenuated end of the MPS I spectrum				
VI	Maroteaux-Lamy	DS	N-acetylgalactamine 4-sulfatase	5q11-q13	AR
VII	Sly	HS + DS	β -glucuronidase	7q21.11	AR
VIII	An enzyme defect was found and proposed as MPS VIII, but shortly thereafter recognized as a laboratory pitfall; the proposal was withdrawn				
IX	Natowicz	Hyaluronan	Hyaluronidase 1	3p21.3	AR

HS, heparan sulfate; DS, dermatan sulfate; KS, keratan sulfate; AR, autosomal recessive; XR, X-linked recessive.

Table 3: Best approaches in uGAG and enzyme activity analysis.

Best practices	Considerations
Urine GAG analysis A first morning void of 10–20 mL is considered the optimal sample Urine GAG analysis should include both qualitative and quantitative assessment Urine should be shipped frozen or via dried filter paper Laboratories should participate in and submit positive samples to proficiency testing schemes	Elevated GAGs may be missed in diluted urine samples High normal to mildly elevated quantitative uGAGs might be observed in patients Laboratories should try to standardize their units and list the units and normal ranges with clarity on reports Clinicians should pay attention to units and reference ranges, especially when using a new or different laboratory Comparison of uGAG test results from more than one laboratory may not be reliable because multiple test methods are commonly used
Enzyme activity analysis The current gold standard diagnostic method for MPS VI is performed using leukocyte or fibroblast samples; however, enzyme activity may be assessed in dried blood spots as a screening test (see considerations) A reference lysosomal enzyme has to be assayed in the same sample simultaneously to affirm sample accuracy When ASB activity is found to be reduced, a second sulfatase must be assessed to exclude MSD. If fibroblasts are used, a mannose-6-phosphate-guided reference enzyme must be assessed to exclude I-cell disease Blood samples should spend the least amount of time in shipment as possible. Next day arrival is strictly recommended Laboratories should participate in and submit positive samples to proficiency testing schemes	Assessment of multiple enzymes is necessary to exclude multiple enzyme defects and lysosomal trafficking deficiencies It is strictly recommended to evaluate a reference enzyme with similar stability if a dried blood spot is used Following diagnosis of reduced ASB activity by an initial dried blood spot screening assay, in situations where there exists restricted capability to acquire and ship quality whole blood or tissue for diagnostic enzyme activity testing in leukocytes or fibroblasts, it is recommended to affirm activity of multiple enzymes as described in a second, independently acquired dried blood spot

collecting DNA sample from the patient. It is common to use blood samples; however, saliva samples or blood spot samples are also acceptable. After DNA purification from the sample, generally a polymerase chain reaction (PCR) is performed to amplify the coding regions of the *ARSB* gene. PCR products are sequenced using prevalent methods such as Sanger Di-deoxy terminator chemistry. The patient sequence is compared to a reference (normal) sequence and any changes should be noted [19].

As genetic abnormalities began to be described [18], there was a growing attempt to find the genotype-phenotype correlations, but it has been disappointing most of the time, as a consequence of the vast molecular heterogeneity present in this gene, including disease-causing mutations and several polymorphisms [36].

Evaluation of genotype-phenotype correlations in compound heterozygotes, which have two different disease-causing alleles or when the existence of polymorphisms or two disease-causing mutations on the same allele may have an effect on the disease phenotype, is notably ambiguous [8]. More than 145 disease-causing mutations in the *ARSB* gene have been identified in the

Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>), of which 114 are missense mutations, 20 are deletions, 10 are splicing mutations, two are insertion/deletion and four are insertions. Several polymorphisms have been also reported and expression studies have proved that one of them, p.V358M, could weaken the residual activity of *ARSB*, depending on the disease-causing mutation present. Those patients presenting both alleles with mutations which contribute to the synthesis of a non-functional protein such as nonsense, small insertions and/or deletions tend to present a more severe phenotype, and most mutations are unique [18]. Some mutations cannot be detected by traditional sequencing methods and may require the use of quantitative methods. Alternative methods such as multiplex ligation-dependent probe amplification (MLPA), real-time polymerase chain reaction and array-comparative genomic hybridization (aCGH) can be useful to check for deletions or duplications of the *ARSB* gene in the case of initial sequencing not detecting two mutations. Mutations located deep inside introns or in the *ARSB* promoter will be missed using routine laboratory methods [19].

Nowadays, modern approaches allow for the identification of a wide number of sequencing reactions in a single assay, allowing for a perfect analysis of the whole gene (or even several genes). This technology is based on next generation sequencing (NGS) and can be used as panels of certain genes or even whole exome and genome sequencing and it is becoming an extremely advantageous technique due to its speed and validity. Although this kind of analysis is still expensive, there is a decreasing cost trend as already shown with other novel technologies. NGS can even be appropriate for diagnosis of patients without a definitive biochemical diagnosis [36].

For a specific group of mutations such as complete gene deletions including contiguous genes and gene inversions/rearrangements, this strategy would fail to identify the disease-causing mutations. In gene inversions/rearrangements, sequencing or even NGS would not permit to map deletion break-points, and other methods such as comparative genomic hybridization (CGH) array would be required as subsidiary methods [38].

If a molecular defect is known in the family, PND is possible by mutation scanning using several sources of biological material such as amniocytes, umbilical cord blood or chorionic villi and can be performed simultaneously with an enzyme assay or alone.

Early diagnosis and newborn screening

There is evidence that storage of GAGs begins very early in life [39] and that this storage activates a cascade of pathogenic events [40, 41], a process that, once started, could be difficult to reverse. The experience of many physicians indicates that early diagnosis and early treatment bring a better outcome in MPS patients. This opinion has been well documented by Gabrielli et al. [42] for two MPS I sibs, and by McGill et al. [43] for two MPS VI sibs. In both reports, one sib was initiated on the enzyme replacement therapy (ERT) prior to the other, and observations made when the patients were at the same age portended a better outcome for the early-treated sib compared to the late-treated one [34].

Conclusions

During the last 100 years, substantial advancements in the clinical, biochemical and molecular characterizations of MPS have led to successful preventive and therapeutic approaches.

The path to MPS VI diagnosis is often supposed to be an undemanding path moving from uGAG analysis through enzyme activity assay and molecular confirmation. Urinary GAG assay is not always abnormal, enzyme activity analysis includes several caveats and needs the appropriate reference enzymes usage, and molecular testing does not always identify two previously described pathogenic mutations. Therefore, the path to diagnosis is not certainly facile and must include several checks and balances with thoughtful consideration and evaluation of each stage. Clinicians have to keep in touch with laboratories and be perceptive of possible misdiagnoses during the whole process of the diagnosis.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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