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Evaluation of Patients Referred to Children's Medical Center Laboratory for Diagnosis of Mucopolysaccharidoses: Eight Years' Experience from Iran

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Abstract

Mucopolysaccharidoses (MPSs) are rare lysosomal storage diseases, resulting from deficiencies of enzymes responsible for Glycosaminoglycans (GAGs) degradation. This leads to accumulation of GAGs in tissues and their excretion in urine, with a wide variety of manifestations. Early diagnosis of MPSs is strictly recommended due to available therapy that can slow down disease progression during the early ages. This study aimed to evaluate patients with suspected MPS referred to Children's Medical Center laboratory over eight years. We also evaluated the usefulness of urine GAG as a screening test for identification of such patients. A total of 1414 patients (40% female, 60% male, with mean age 3.1 ± 4.1years) have participated in this study. The urinary GAG analysis (uGAG) was performed by 1, 9-dimethyl-methylene blue (DMMB) and Berry spot test (BST). All patients with positive and mild positive results or with disease-related symptoms were evaluated in terms of definitive diagnosis, received treatments, morbidity, and mortality rate. In 407 (36.5%) patients uGAG were positive or mild positive, of which 26.3% suffered from one of the types of MPSs, 28.5% suffered from other diseases, 32.9% were undiagnosed, 12.3% were apparently healthy, and 19 died. The negative predictive value of uGAG test in our study was 100%. About 21% of MPSs patients received enzyme replacement therapy, while four patients underwent stem cell transplants. The rest received supportive care. We concluded that a combination of DMMB and BST methods has acceptable sensitivity for screening suspicious MPS patients.

Keywords
- berry spot test
- dimethylmethylene blue
- glycosaminoglycan
- mucopoly-saccharidoses

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Introduction

Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders caused by deficiency of one of the enzymes responsible for Glycosaminoglycans (GAGs) degradation. These deficiencies lead to accumulation of GAGs in tissues and their excretion in urine, resulting in a wide variety of MPS manifestations. It is a heterogeneous disease with a broad spectrum of severity. Most patients appear normal at birth, but in those with a more severe phenotype, clinical symptoms begin before the age of 2 to 4 years and they may die before 10 years of age if untreated. Although the mortality rate varies according to the type and severity of the disorder, life expectancy and quality of life is anticipated to increase due to available therapies.[1] The current MPS incidence is 1.53 per 100,000 live births on an average.[2]

The diagnosis of MPS patients was made for the first time by Dr. Charles Hunter in 1917 when he described two Canadian brothers with MPS phenotypes.[3] Other types of MPS were detected until 1973.[4–9]

The type of storage material was unclear until the mid-twentieth century when the presence of GAGs was confirmed in tissues and urine of MPS patients.[10–13]

In 1962, evaluation of the relative amounts of the different types of GAGs led to the classification of MPSs.[14,15] To date, 11 enzyme deficiencies are detected for different types of MPSs. Scheie syndrome, initially considered as type V, was then recognized to be the attenuated end of the MPS I spectrum. In addition, type VIII was at first considered as a new type of MPS but shortly thereafter recognized as a laboratory pitfall. An up-to-date classification of Mucopolysaccharidoses, enzyme deficiencies, related genes, and available treatments are shown in ►**Table 1**.

Early diagnosis of MPSs is strictly recommended due to available therapies, which can slow down the disease progression in case of early initiation. Current therapies include enzyme replacement therapy (ERT), which is available for MPSs I, II, IVA, VI, VII, and hematopoietic stem cell transplantation (HSCT). More details about the efficacy, effectiveness and safety of available enzyme replacement therapies can be found in the study by Parini and Deodato.[16] Newer therapies such as gene therapy using Adeno Associated Virus (AVV) and substrate reduction using Genistein are also being developed.[17–21]

An available, cost-effective, and fast method for initial MPS screening is urinary GAG analysis, which is available as qualitative and quantitative techniques. Subsequently, samples with elevated GAG can be evaluated for a diagnosis of GAG pattern through chromatography or electrophoresis and confirmed by enzyme activity and molecular studies.[22–25]

The main purpose of this study was to evaluate patients with suspected MPS, referred to Children's Medical Center laboratory from March 2011 to March 2019.

Table 1 Classification of Mucopolysaccharidoses

MPS	Name	Increased GAGs	Enzyme deficiency	Gene location	Pattern of inheritance	Treatment (ERT)
I	Hurler, Hurler-Scheie or Scheie	HS + DS	α-iduronidase	4p16.3	AR	Aldurazyme® (Laronidase)
II	Hunter	HS + DS	Iduronate sulfatase	Xq28	XR	Elaprase® (idursulfase)
III A	Sanfilippo A	HS	Heparan-N-sulfatase	17q25.3	AR	No approved therapies
III B	Sanfilippo B	HS	α-N-acetylglucosaminidase	17q21.1	AR	No approved therapies
III C	Sanfilippo C	HS	AcetylCoA α- glucosamine acetyltransferase	8p11.2-p11.1	AR	No approved therapies
III D	Sanfilippo D	HS	N-acetylglucosamine 6-sulfatase	12q14	AR	No approved therapies
IV A	Morquio A	KS	Galactosamine-6-sulfate sulfatase	16q24.3	AR	Vimizim® (elosulfase alfa)
IV B	Morquio B	KS	β-galactosidase	3p21.3	AR	No approved therapies
(V)	Scheie syndrome, was initially considered as type V, then was recognized to be the attenuated end of the MPS I spectrum					
VI	Maroteaux-Lamy	DS	N-acetylgalactamine 4-sulfatase	5q11-q13	AR	Naglazyme® (galsulfase)
VII	Sly	HS + DS	β-glucuronidase	7q21.11	AR	Mepsevii® (vestronidase alfa-vjbk)
(VIII)	An enzyme defect was found and considered as MPS VIII, but shortly thereafter recognized as a laboratory pitfall					
IX	Natowicz	Hyaluronan	Hyaluronidase 1	3p21.3	AR	No approved therapies

Abbreviations: AR, Autosomal recessive; DS, Dermatan sulfate; HS, Heparan sulfate; KS, Keratan sulfate; XR, X- linked recessive.

We evaluated the clinical outcome of patients in terms of definitive diagnosis, received treatments, morbidity, and mortality rate. The usefulness of urine GAG level as a diagnostic test for MPS was also evaluated.

Material and Methods

Subjects

In a mixed cohort study, we evaluated all inpatients and outpatients with suspected MPS who were referred to our center from March 2011 and were tested for uGAG. The study was started in 2017 and continued until March 2019. Most of the patients had clinical features of MPS. Skeletal abnormalities, hepatosplenomegaly, short stature, and developmental delay were the main complications of symptomatic patients. Asymptomatic cases included some hospitalized neonates and siblings of affected patients. Information including gender, age, symptoms, consanguinity, and status of each patient was obtained, and all parents were asked for permission for data and sample collection.

uGAG Analysis

Qualitative uGAG analysis using berry spot test (BST) and 1, 9-dimethyl-methylene blue (DMMB) was done on urine samples. Details of procedure and pitfalls are demonstrated in the Laboratory Guide to the Methods in Biochemical Genetics book[26] and Lage et al. study.[24]

Berry Spot Test

For BST we put 25 µl of urine sample on chromatography paper in three steps- twice 10µL and then 5µL, allowing the spots to dry between each step. Filter papers were then kept in Toluidine Blue for 45 seconds, after which they were washed three times with 10% acetic acid, and allowed to dry. We used heparin or a previously confirmed positive sample as a control. Results were reported as negative (which meant there was no excess GAG in urine), mild positive (which meant that the uGAG was a little elevated), and positive (which meant there was excess GAG in urine) and are shown in ►**Fig. 1**.[23,26]

1,9-dimethylmethylene Blue Test

For the DMMB test 1ml of DMMB buffer was added to 100 µl of urine sample, after which the color of the solution was evaluated.[22] Turning the solution from blue to purple was considered positive, changing to violet was mild and no change in color was considered a negative test result.[24,26]

Fig. 1 Results of berry spot test

Enzyme Activity Measurement and Molecular Approach

Most patients were tested for enzyme activity and/or molecular defects. These tests, often performed on dried blood spot samples, were collected at our center and shipped to other laboratories.

We evaluated the enzyme activity for a few samples in our laboratory, using fluorimetric assay, and performed mutation scanning for some of them using PCR-sanger sequencing.[27,28]

Data Analysis using SPSS

All data collected in this study, was analyzed using SPSS version 24 software. Descriptive statistics are presented as Mean, SD, Minimum, and Maximum.

Result

A total of 1391 patients were enrolled, of which 255 (18.3%) had positive, 253 (18.2%) mild positive, and 883 (63.5%) negative uGAG results. The study population was 40% female and 60% male with the mean age of 3.1 ± 4.1years (from 9 days to 23 years old). Among the negative results, 323 (36.6%) were outpatients and 560 (63.4%) were inpatients, of which 446 were hospitalized newborns.

Of the 407 evaluated patients with positive or mild positive results, 107 suffered from one of the MPS types, 116 suffered from other diseases, 134 were undiagnosed at the time of evaluation, 50 were healthy and 19 died. Most of the positive and mild positive results were in males. Parental consanguinity was noted in 66.4% of our patients. Details of data are shown in ►**Table 2**.

Patients with a definite diagnosis of MPS ($n = 107$) had a mean age of 6 ± 4.8 years (4 months-23 years), 39% were female and 61% were male. The rate of parental consanguinity was 93% for these patients. Most of the mild positive cases were MPS IVA patients. (IVA = 7; MPS I = 4; III = 3). Patients had different ages of onset from infancy to 4 years of age. Young children suffered from recurrent ear and throat

Table 2 Demographic and laboratory findings of patients with positive/mild positive uGAG

	Urine GAG		
	Positive	Mild positive	Total
Number of cases	209	198	407
Female/Male (n)%	76/133 (36/64)%	82/116 (44/56)%	158/249 (39/61)%
Age Y (mean ± SD Min-max)	5.5 ± 4.6 (1m-23Y)	5.5 ± 4.2 (1m-19Y)	5.5 ± 4.3 (1m-23Y)
Parental Consanguinity	121 (77.6%)	88 (55.4%)	209 (66.4%)
Confirmed MPSs	88 (21.6%)	19 (4.7%)	107 (26.3%)
Other Confirmed Disorders	49 (12%)	67 (16.5%)	116 (28.5%)
Undiagnosed	57 (14%)	77 (18.9%)	134 (32.9%)
Apparently healthy	15 (3.7%)	35 (8.6%)	50 (12.3%)

Table 3 Received treatments, care and outcome of MPSs patients

	ERT* (20.5%)	HSCT** (4%)	Only supportive (67%)	Death (8.5%)	Total (100%)
MPSI	7		13	2	22
MPSII	–	1	5		6
MPSIII	–	–	19	2	21
MPSIVA	10	–	25	1	36
MPSVI	5	3	7	3	18
MPSVII	–	–	1		1
Unspecified MPS	–	–	2	1	3
Total	22	4	72	9	107

*Enzyme Replacement Therapy
**Hematopoietic Stem Cell Transplantation

Table 4 The Chief complaints and clinical symptoms of undiagnosed patients

	Urine GAG		
	Positive	Mild positive	Total
Skeletal disorders	10	15	25 (19%)
Developmental delay*	20	26	46 (34%)
Behavioral difficulties**	7	14	21 (16%)
Cardiac problems	2	3	5 (4%)
Miscellaneous***	15	15	30 (22%)
Death	3	4	7 (5%)
Total	57	77	134 (100%)

*Includes: Speech disorder, Learning disabilities, Hearing Loss.
**Includes: ADHD, autism spectrum disorder.
***Includes: Hepatosplenomegaly, seizure, Cerebral palsy (CP), Asymmetric body growth, bad tempered.

infections and hernia. In older patients, skeletal deformities were the chief complaint.

Two MPS I patients were diagnosed and treated as inflammatory arthritis for a period of time.[29] One of the MPS II patients was successfully transplanted at the age of 8 years. Details of received treatments, care, and outcome of MPSs patients are shown in ►**Table 3**.

In 116 cases, other diseases or syndromes were diagnosed, including, skeletal dysplasia, mucolipidosis ($n = 7$, 3 dead), rheumatoid arthritis, and cardiac problems. Some of these cases were affected by rare diseases such as MONA (Multicentric osteolysis, nodulosis, and arthropathy), MACS (Macrocephaly, Alopecia, Cutis laxa, and Scoliosis), HCS (Hajdu- Cheney Syndrome), FGF-23 (Fibroblast Growth Factor 23), Joubert syndrome, Williams syndrome, Wounderman Disease, CF (Cystic Fibrosis) and SMA (Spinal muscular atrophy). Diagnosis in these patients was based on genetic tests, normal MPS enzyme activity, and data collected from parents. Three cases in this group had died. More details are shown in the ►**Appendix 1**.

In 134 cases, no specific disease was detected until the end of the study. Clinical signs and chief complaints of these cases, seven of whom also died, are shown in ►**Table 4**.

Discussion

In this study, we evaluated the semiquantitative uGAG test results of 1414 patients that were referred to the Children's Medical Center laboratory over eight years. In 36.5%, uGAG were positive or mild positive and in 63.5%, they were negative. Most of the negative results belonged to hospitalized newborns (51%) which seem rational because in our center, available metabolic tests including uGAG are performed for almost all hospitalized neonates as a screening test. In the Mabe et al.[22] study, normal uGAG was noted in 83% of patients with at least one of the MPS symptoms.

All of our 107 confirmed MPS patients had positive or mild positive uGAG, so the sensitivity and, in other words, negative predictive value of the uGAG test in our study was 100%. If we only take into account the positive uGAG results, the negative predictive value in our study would be 82%. False-negative results of BST have been reported in MPS IVA and MPS III.[22,24] It might be due to the lack of hexuronic acid in the keratin sulfate molecule that is the main urinary GAG in MPS IVA patients. Moderate excretion of GAGs in MPS III and IV may also give a negative result. By using both DMMB and BST methods and reporting mild positive results, we decreased our false-negatives to zero, which was consistent with Mabe's study.[22]

The frequency of confirmed MPSs patients in our study was 26%, and ~28% of positive patients were suffering from non-MPS diseases. The false-positive results of semi-qualitative uGAG tests have been reported in several studies.[30–32] Chondroitin sulfate and oligosaccharides which are excreted in disorders such as mucolipidosis, bone disease, connective tissue disorders, and also interfering agents such as acrylic acid in diapers have been reported to be the cause of false-positive results.[33–35] In some types of MPSs, excretion of chondroitin sulfate is detected with other glycosaminoglycans, but not alone. For differentiation between MPSs types, urine GAGs should be separated by chromatography or electrophoresis[36] which was one of the limitations of our study.

In our study, the number of male MPS patients was greater than females which is in accordance with other studies.[37]

While the most frequent types of MPSs in our study were IVA, it was type I in Fateen et al (2018).[34] and type III in Shawaky[38] and Natale[37] studies. MPS type II was the least reported in our study, which is consistent with Fateen et al. (2014)[39] but different from Zhou J et al study[1]- this may be due to racial diversities. However, it should be considered that determining the most common types of MPSs in a country would be possible if the diagnostic and treatment methods for all types of MPSs are accessible.

The high rate of parental consanguinity in our study is due to the high frequency of consanguineous marriages among

Iranian people which was also reported in Egyptian patients by Fateen et al. (2014).[39]

At the time of evaluation, ~33% of patients did not have a definite diagnosis. The main reason for this was the lack of access to expensive diagnostic methods. We had access to the enzyme and the genetic evaluation methods for MPS types I, II, and VI from the beginning of the study, for type IVA from the middle of 2016, and for types VII and IIIB from late 2018. Considering the common clinical manifestations of undiagnosed patients, which were behavioral difficulties and developmental delay and lesser access to diagnostic methods for type III, it seems that the majority of these patients might be type MPS III.

During the study, the genetic diagnosis became available for a limited number of undiagnosed patients, of which 40% were type III and 30% were type IVA. Although MPS III has no therapy at the moment, the diagnosis is very important for genetic counseling and access to clinical trials around the world.

About 21% of MPSs patients received ERT, which was mainly for types IVA, I and VI respectively. Three of type VI and one of type II patients have received stem cell transplants. The best age for HSCT is under 2 years of age, however due to late diagnosis and lack of access to therapy, our MPS II patient was transplanted at the age of 8 years and is currently 10 years old. HSCT for type III and IV is not approved yet and is still under investigation in clinical trials.[20] The number of transplant patients in our center is increasing, but they were not included in our study. The rest of the patients received supportive care only.

Conclusion

Although new methods like mass spectrometry can evaluate a large number of samples in a short period, the devices are expensive and sample preparation is laborious. The combination of DMMB and BST has acceptable sensitivity for screening suspicious MPS patients. DMMB method also has the potential to be automated which would be useful for larger scales.[40] The use of more convenient, accurate, and cost-effective diagnostic methods along with genetic counseling would lead to appropriate screening, and decreasing morbidity in patients. The uGAG test may also help evaluate the efficacy of ERT and HSCT in MPS patients.

Our study also showed that a few patients received novel therapies, while some still do not have a definitive diagnosis, which requires more attention from the national health policy makers. We suggest the screening for MPSs be included in the national expanded newborn screening program targeting those with consanguineous marriage and having an affected person in the family.

Ethical Approval

The study was approved by the Ethics Committee of Tehran University of Medical Sciences.

The researchers complied with the ethical principles of the Declaration of Helsinki. The Ethics Code is IR.TUMS.CHMC.REC.1399.006.

Informed Consent

All participants or their parents, whenever needed, signed informed consent forms.

Conflict of Interest

None declared.

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Appendix 1 Patients with other diagnosis

	Urine GAG		
	Positive	**Mild positive**	**Total**
Skeletal dysplasia*	9	8	17(14.7%)
Rheumatoid arthritis	19	25	44(37.9%)
Cardiac problems	1	9	10(8.6%)
Miscellaneous**	20	25	45 (38.8%)
Total	49	67	116 (100%)

*Includes:Osteogenesis imperfecta and Spondyloepiphyseal dysplasia·
**includes: Mucolipidosis, MONA (Multicentric osteolysis, nodulosis and arthropathy), MACS (Macrocephaly, Alopecia, Cutis laxa, and Scoliosis), HCS (Hajdu- Cheney Syndrome), FGF-23 (Fibroblast Growth Factor 23), Joubert syndrome, Williams syndrome, Wounderman Disease, CF (Cystic Fibrosis) and SMA (Spinal muscular atrophy).