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ORIGINAL ARTICLE

Genetic testing of Mucopolysaccharidoses disease using multiplex PCR- based panels of STR markers: in silico analysis of novel mutations

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Abstract

The Mucopolysaccharidoses (MPS) are group of inherited metabolic diseases caused by the deficiency of enzymes required to degrade glycosaminoglycans (GAGs) in the lysosomes. GAGs are sulfated polysaccharides involving repeating disaccharides, uronic acid and hexosamines including chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate (KS). Hyaluronan is excluded in terms of being non-sulfated in the GAG family. Different types of mutations have been identified as the causative agent in all types of MPS. Herein, we planned to investigate the pathogenic mutations in different types of MPS including type I (*IDUA* gene), IIIA (*SGSH*) and IIIB (*NAGLU*) in the eight Iranian patients. Autozygosity mapping was performed to identify the potential pathogenic variants in these 8 patients indirectly with the clinical diagnosis of MPSs. so three panels of STR (Short Tandem Repeat) markres flanking *IDUA*, *SGSH* and *NAGLU* genes were selected for multiplex PCR amplification. Then in each family candidate gene was sequenced to identify the pathogenic mutation. Our study showed two novel mutations c.469 T > C and c.903C > G in the *IDUA* gene, four recurrent mutations: c.1A > C in *IDUA*, c.220C > T, c.1298G > A in *SGSH* gene and c.457G > A in the *NAGLU* gene. The c.1A > C in *IDUA* was the most common mutation in our study. In silico analysis were performed as well to predict the pathogenicity of the novel variants.

Keywords Mucopolysaccharidoses (MPS) · Autozygosity mapping · Linkage · Mutation analysis · Iran

Introduction

Mucopolysaccharidoses (MPS) are a family of inherited metabolic diseases caused by the deficiency of enzymes required to degrade glycosaminoglycans (GAGs) in the lysosome. GAGs are transferred to the lysosomes where they are finally broken down into basic sugars by an array of hydrolytic enzymes. Absent or decreased activity of any of these enzymes causes incomplete digestion of GAGs in individuals with this disease. As a consequence, GAGs accumulate within cells and tissues leading to progressive cellular dysfunction (Peck et al. 2016).GAGs are sulfated polysaccharides involved in repeating disaccharides, uronic acid and hexosamines including chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate (KS). Hyaluronan is a non-sulfated polysaccharide in the GAG family and this feature has made this polysaccharide as an exception in this family (Khan et al. 2017). In Table 1 subtypes of this disease has been shown.

MPS are categorized based on the enzyme deficiencies and mostly inherited as an autosomal recessive form except type II. The estimated incidence of MPS I is 1 in 100,000 live births (Beck et al. 2014). MPS I is categorized into 3 subtypes based on the clinical signs and the severity of the disease (Vazna et al. 2009). The severe form of MPS I, which is called Hurler syndrome (MPS IH; OMIM # 607014), involves in mental retardation, skeletal abnormalities, stiff joints, hepatomegaly, corneal clouding, micrognathia and shortened life

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Table 1 Mucopolysaccharidosis subtypes based on enzyme deficiency

Subtype	Enzyme Deficiency (gene)	inheritance	Accumulating GAGs
MPS I Hurler, Scheie, Hurler/Scheie Syndromes	α-L-iduronidase (IDUA)	AR	HS and DS
MPS II Hunter Syndrome	Iduronate Sulfatase (IDS)	XR	HS and DS
MPS III (A-D) Sanfilippo Syndrome	A: Heparan sulfamidase (SGSH) B: N-acetylglucosaminidase (NAGLU) C: Heparan-α-glucosaminide N-acetyltransferase (HGSNAT) D: N-acetylglucosamine 6-sulfatase (GNS)	AR	HS
MPS IV (A and B) Morquio Syndrome	A: Galactose-6-sulfate sulfatases (GALNS) B: β-galactosidase (GLB1)	AR	A: CS and KS B: KS
MPS VI Marateaux-Lamy Syndrome	N-acetylgalactosamine-4-sulfatase (ARSB)	AR	DS
MPS VII Sly Syndrome	B-glucuronidase (GUSB)	AR	HS, DS, and CS
MPS XI Natowicz Syndrome	Hyaluronidase (HYAL1)	AR	HA

Abbreviations: *GAG* Glycosaminoglycan, *HS* Heparan sulfate, *DS* Dermatan sulfate, *CS* Chondroitin sulfate, *KS* Keratan sulfate, *HA* Hyaluronan

expectancy (Kim et al. 2015). An intermediate form which is called Hurler/Scheie syndrome (MPS IH/S; OMIM # 607015) involves in skeletal abnormalities, Splenomegaly, severe organomegaly, Scoliosis,Kyphosis and a variable lifespan with neurological involvement (Chkioua et al. 2011). The clinical presentation of mild form which is called Scheie syndrome (MPS IS; OMIM # 607016) consists of mild skeletal abnormalities, corneal clouding and a long life span without mental retardation (Oussoren et al. 2013; Terlato and Cox 2003). The current treatment strategy of MPS I patients is enzyme replacement therapy (ERT). Because of the high cost of ERT, alternative therapies such as the pharmacological chaperones are being used for these patients (Valenzano et al. 2011). MPSs can also be treated with hematopoietic stem cell transplantation (HSCT) (Hinderer et al. 2016; Lum et al. 2017a, b; Rodgers et al. 2017).

MPS I is caused by a defected α-L-iduronidase (EC 3.2.1.76) an enzyme in the degradative pathway for two types of GAGs, DS and HS (Thomas et al. 2010). This enzyme is encoded by a single gene named "*IDUA*" (NM_000203.3) which has been mapped on 4p16.3(Bie et al. 2013) with 14 exons encoding a 653 amino acids precursor protein (Chkioua et al. 2018; Prommajan et al. 2011; Scott et al. 1991).

MPS III or Sanfilippo syndrome is characterized by developmental delay, seizures, cognitive decline and destruction of the central nervous system leading to progressive neurocognitive impairment (Gaffke et al. 2018). These Patients are usually diagnosed at the age of 2 to 6 years old (Lau et al. 2013). Four subtypes are recognized for MPS III (A–D). MPS IIIA with an incidence of 1 in 100,000, MPS IIIB with an incidence of 1 in 200,000, with an incidence of 1 in 1,500,000 and MPS IIID with an incidence of 1 in 1,000,000 live births (Tebani et al. 2018). Each subtype is due to a defect in a different enzyme that is needed to degrade HS (MPS IIIA: heparan N-sulfatase enzyme (EC 3.10.1.1), MPS IIIB: α-N-acetylglucosaminidase enzyme (EC 3.2.1.50), MPS IIIC: acetyl-CoA: α-glucosaminide acetyltransferase (EC 2.3.1.78) and MPS IIID: N-acetylglucosamine-6-sulfatase (EC 3.1.6.14)). MPS IIIA is the most common of the 4 subtypes. (Knottnerus et al. 2017; Lau et al. 2013; Marco et al. 2016; Muenzer 2011; Scott et al. 1995). In a recent study, MPS IIIA patients were sorted out into 2 phenotypic groups: rapidly (RP) and slowly progressing (SP) (Shapiro et al. 2016). The clinical course of MPS III is divided into 3 phases. The first phase, which usually occurs between 1 and 4 year of age, consists of a developmental delay that often initially affects speech. The second phase generally starts at approximately 3–4 year of age and is followed by severe behavioral problems such as hyperactivity, challenging behavior and progressive cognitive decline. The third phase, which usually occurs after the first decade of life, is associated with the onset of severe dementia (Valstar et al. 2010; Verhoeven et al. 2010).

MPS IIIA (OMIM# 252900) is caused by a defection in "*SGSH*" gene (NM_000199.3) which has been mapped on 17q25.3 with 8 exons encoding a 502 amino acids residue (Ugrinov et al. 2015). Symptoms of patients with MPS IIIA involved in plenohepatomegaly, skeletal deformations, and gastrointestinal tissues (Duncan et al. 2015). MPS IIIB (OMIM # 252920) is due to the deficiency of "*NAGLU*" gene (NM_000263.3) which is located on chromosome 17q21.1 and it contains 6 exons and encodes a 720 amino acids protein (Weber et al. 1996; Zhao et al. 1996). MPS IIIC (OMIM # 252930) is caused by a defection in "*HGSNAT*" gene (NM_152419.2) located on chromosome 8p11.1 and contains 18 exons and the cDNA encodes a product of 635 amino acids

(Huh et al. 2013). MPS IIID (OMIM # 252940) is due to deficiency of "*GNS*" gene (NM_002076.3) located on chromosome 12q14.3 which contains 14 exons (Jansen et al. 2007).

In Iran and many other countries there are high rates of consanguinity (Shafaat et al. 2018). Consanguineous marriage increases incidence of autosomal recessive disorders. This is most pronounced when rare autosomal disorders are investigated. This study is based on autozygosity mapping, using three panels of short tandem repeat (STR) markers (one panel for each gene) linked to *IDUA*, *SGSH* and *NAGLU* genes, to find the probable defective gene indirectly. The candidate gene was subsequently sequenced to find the exact mutation. In silico analysis was also performed to predict the pathogenicity of the newly identified variants.

Materials and methods

Eight patients with clinical diagnosis of MPS disease (5 for MPS I, 2 for MPS IIIA and 1 for MPS IIIB) were referred to our center by metabolic endocrinologists. The disease diagnosis was based on the clinical presentations and the level of the α-L-iduronidase enzyme for MPS I patients, heparan N-sulfatase enzyme for MPS IIIA and α-N-acetylglucosaminidase enzyme for MPS IIIB. The study was approved by the ethics committee of Kawsar Human Genetic Research Center.

Following genetic counseling and signing the informed consent form by participants, 5 ml peripheral blood was taken from each family member in tubes containing EDTA as an anticoagulant agent. Genomic DNA extraction was done using a DNA extraction kit from Kawsar Biotech Co. (Kawsar Biotech Co., Tehran, Iran, and KBC). In this study, 8 patients were suspicious to three types of MPS disease (I, IIIA, IIIB) were investigated. Autozygosity mapping was performed using 5 STR markers flanking the *IDUA*, 6 for *SGSH* and 6 for *NAGLU* genes (3 panels of multiplex PCR). Fluorescent-labeled primers were purchased from Genetek Biopharma (Genetek Biopharma, GmbH, Berlin, Germany, GT) and used according to manufacture protocols. Fragment analysis was performed using ABI 3130 XL Genetic Analyzer (ABI, Thermo Fisher Scientific, USA, and TFS). Then a haplotype map was drawn for each family. Following indirect identification of the defective candidate gene, Sanger sequencing was done to identify the causative mutations.

The exons and intron-exon boundaries were amplified for the candidate genes for each patient (sequencing primers are available upon request). PCR reaction included an initial denaturation step for 5 min at 95 °C, 1 min at 95 °C, 1 min at 62 °C, and 1 min at 72 °C and final extension for 10 min at 72 °C for 30 cycles. The 25 μl final reaction includes 0.3 μl of each primer (10 μM), 0.66 μl Mgcl2 (100 mM), 1 U Tag DNA polymerase (Kawsar Biotech Co, KBC, Tehran, Iran, KBC), 0.4 μl dNTPs (40 mM), 50–100 ng DNA and enough ddH2O.

The samples were sequenced using Bigdye Terminator kit (Thermo Fisher Scientific, Life Technologies, USA, TS) according to the manufacturer's protocol. The samples were run on an ABI3130XL Genetic Analyzer at the KBC facility. The results were compared with human genomic and cDNA sequences of the gene with the accession number for "*IDUA*" (NM_000203.3), "*SGSH*" (NM_000199.3) and "*NAGLU*" (NM_000263.3). SIFT (Kumar et al. 2009), Polyphen (Adzhubei et al. 2010), mutation tasting (Schwarz et al. 2014) and Mutation@A Glance (Hijikata et al. 2010) were the anticipated tools used for in silico analysis of the newly identified variants.

Results

The study included 8 patients with clinical diagnosis of MPS disease (5 for MPS I, 2 for MPS IIIA and 1 for MPS IIIB). All patients were the result of consanguineous marriages. Four patients showed homozygosity for the STR markers flanking the *IDUA* gene and one patient was found to be a compound heterozygote for the *IDUA* gene. Two patients showed homozygosity for the markers flanking the *SGSH* gene and one for *NAGLU* gene. Table 2 shows the clinical features and mutations found in each patient.

Sanger sequencing of *IDUA* gene revealed three different mutations (c.469 T > C, c.903 C > G and c.1A > C) in four patients and one showed a compound heterozygous mutation (c.1 A > G and c. 469 T > C). The c.1A > C mutation was seen in 3 patients in this study which has been reported previously (Atceken et al. 2016). The c.469 T > C and c.903 C > G mutations in the *IDUA* gene were novel and had not been reported previously. Figure 1 showed haplotype analysis of the patients and their families with novel mutation. As it is shown, autozygosity mapping data confirmed the mutation analysis.

Mutation analysis of the *SGSH* gene in 2 patients revealed two different missense homozygote mutations which both of them had been reported previously (c.1298 G > A (Chabas et al. 2001) and c.220 C > T (Weber et al. 1997)). One patient showed homozygosity for markers flanking the *NAGLU* gene and mutation scanning revealed c.457 C > A homozygous mutation that reported previously (Schmidtchen et al. 1998)

Parents in this study showed heterozygous form of the mutations and these mutations were not present in healthy members of the family in homozygote form. The patient with the compound heterozygous mutation had inherited the c.1 A > G variant from the father and the c. 469 T > C from the mother.

Discussion

MPS are a family of inherited metabolic diseases with autosomal recessive inheritance caused by the deficiency of

Table 2 Clinical features, mutations and laboratory test results of patients

Patient No	Age at diagnosis	sex	Current age	Clinical features at diagnosis	Mutation at the nucleotide level	Mutation at the protein level	gene	Laboratory test results	Clinical symptom at Current age
1	6 months	M	7 years	Irregular chest, scoliosis	c.469 T > C	p.S157P	IDUA	α-L-iduronidase 1.18 nmol/spot. The activity of this enzyme is below its reference (450–2600 nmol/spot).	DD, Short stature, Macrocephaly, Coarse face, scoliosis, Hepatomegaly, and Respiratory problems
2	2 years	M	12 years	Cardiomyopathy, scoliosis and Enlarged tongue	c.903C > G	p.D301E	IDUA	α-L-iduronidase 0.5 nmol/ml. The activity of this enzyme is below its reference (1–18.5 nmol/ml).	failure to thrive, DD, Short stature, Enlarged tongue, Normal intelligence, Mitral valve thickening, scoliosis, skeletal deformities, Hepatomegaly and Splenomegaly
3	15 months	M	5 years	Macrocephaly	c.1A > C c.469 T > C	p.M1L p.S157P	IDUA	α-L-iduronidase 15.6 pmol/spot. The activity of this enzyme is below its reference (200–2600 pmol/spot).	Corneal opacity, Hearing loss, Normal intelligence
4	2 years	M	7 years	Extra abnormal body and abdominal inflammation	c.1A > C	p.M1L	IDUA	α-L-iduronidase 6.4 pmol/spot. The activity of this enzyme is below its reference (350–2600 pmol/spot)	failure to thrive, Short stature, Enlarged tongue, Poor eyesight, Hearing loss, scoliosis, and DD
5	1 year	F	3 years	Coarse face with Enlarged tongue	c.1A > C	p.M1L	IDUA	α-L-iduronidase 1.5 pmol/spot. The activity of this enzyme is below its reference (200–2600 pmol/spot)	DD, failure to thrive and skeletal deformities
6	3 years	M	15 years	Scoliosis, Irregular chest, walking with the hardship	c.1298G > A	p.R433Q	SGSH	Heparan sulfamidase 0.22 nmol/mg. The activity of this enzyme is below its reference (4.9–8.5)	failure to thrive, Short stature, The size of the head is more than of the normal range, coarse facies, Enlarged tongue, DD, Seizures, Respiratory problems, Scoliosis, Mild hepatomegaly, and Mild splenomegaly.
7	2 years	F	8 years	failure to thrive	c.220C > T	p.R > 74C	SGSH	Heparan sulfamidase 0.73 nmol/mg. The activity of this enzyme is below its reference(4.5–8.8)	walking with the hardship, failure to thrive, coarse facies, Enlarged tongue, Poor eyesight, DD, skeletal deformities, hepatomegaly, and splenomegaly
8	6 years	M	15 years	failure to thrive, speaking with the hardship, Aggressive behavior	c.457G > A	p.E153K	NAGLU	N-acetylglucosaminidase 0.02 nmol/mg. The activity of this enzyme is below its reference (10–22)	failure to thrive, Short stature, very Poor eyesight, DD, Seizures, Scoliosis, and skeletal deformities, hepatomegaly and splenomegaly

Abbreviations: *DD* Developmental delay, *F* Female, *M* Male

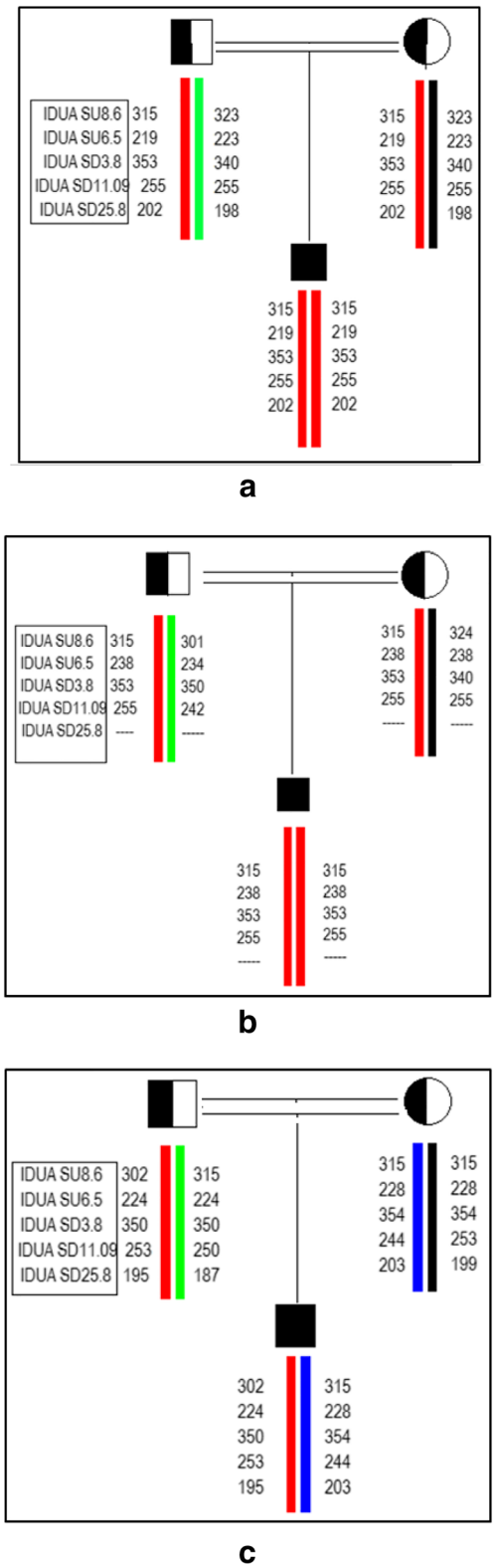

Fig. 1 The figures show the haplotype analysis of the 2 families with novel mutations (**a** and **b**) and also the compound heterozygote mutation (**c**). S: STR, U: upstream, D: downstream. **a** The affected child showed homozygosity for STR markers flanking IDUA gene. Mutation analysis showed c.469 T > C mutation. **b** The affected child showed homozygosity for STR markers flanking IDUA gene and mutation analysis showed c.903C > G mutation. **c** The affected child with a compound heterozygote mutations in the IDUA gene (C.1A > C and c.469 T > C) also showed heterozygosity in the studied markers

enzymes required to degrade GAGs in the lysosome. This category of diseases is more prevalent in countries with a higher rate of consanguineous marriages such as Iran, with an approximate rate of 38.6% consanguineous marriage (Shafaat et al. 2018). Autozygosity mapping with the help of STR markers have been shown to be a very helpful approach to validate the result of the clinical diagnosis (Abiri et al. 2017; Shafaat et al. 2018).

In this study Autozygosity mapping was applied using three panels of Multiplex-PCR (one panel for each gene) for possible confirmation of a causative gene involvement in patients suspected to MPS. This technique helped us to identify the probable mutated gene in heterogeneous and autosomal recessive disorders quickly and indirectly. Biochemical tests are known to be a less accurate method of detection compared to molecular tests (e.g. homozygosity mapping and Sanger sequencing) therefore, there is a possibility of false positives reported in the biochemical enzymatic diagnosis tests carried out for a number of MPS patients. Also, some individuals have either never been biochemically tested, or have no access to the results of their previous enzymatic tests. Due to this, for multigenic disease homozygosity mapping and STR markers are preformed prior to Sanger sequencing in order to verify that the individual is homozygote for the candidate gene. Only after STR analysis verifies that the individual is homozygote, Sanger sequencing is performed for the candidate gene. Therefore, this study used Sanger sequencing and autozygosity mapping to confirm the clinical diagnosis of MPS patients and both techniques confirmed the obtained results. Using the mentioned approach is very useful in prenatal diagnosis (PND) and pre-implantation genetic diagnosis (PGD) of the at-risk families.

287 different mutations were reported in HGMD database (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=*IDUA*) till March of 2018 for *IDUA* gene and missense/nonsense mutations are most common mutations in this gene such as p. W402X and p.Q70X mutations. The frequency of the p. W402X mutation in MPS I patients has been estimated to be 4% in Russia (Voskoboeva et al. 1998), 38.8% of mutant alleles in Spain and 24% in Poland (Bertola et al. 2011) and 39% in the United States (Li et al. 2002). The p.Q70X mutation is also common in Europe, accounting for 35% of mutant alleles (Bunge et al. 1994). In this study 2 mutations (c. 469 T > C, p. S157P and c.903C > G, p.D301E) in *IDUA* gene were novel and had not been reported previously. c.469 T > C mutation leads to a substitution of proline instead of serine. Serine is a polar and hydrophilic amino acid, while proline is a nonpolar and hydrophobic residue. Substitution of serine to

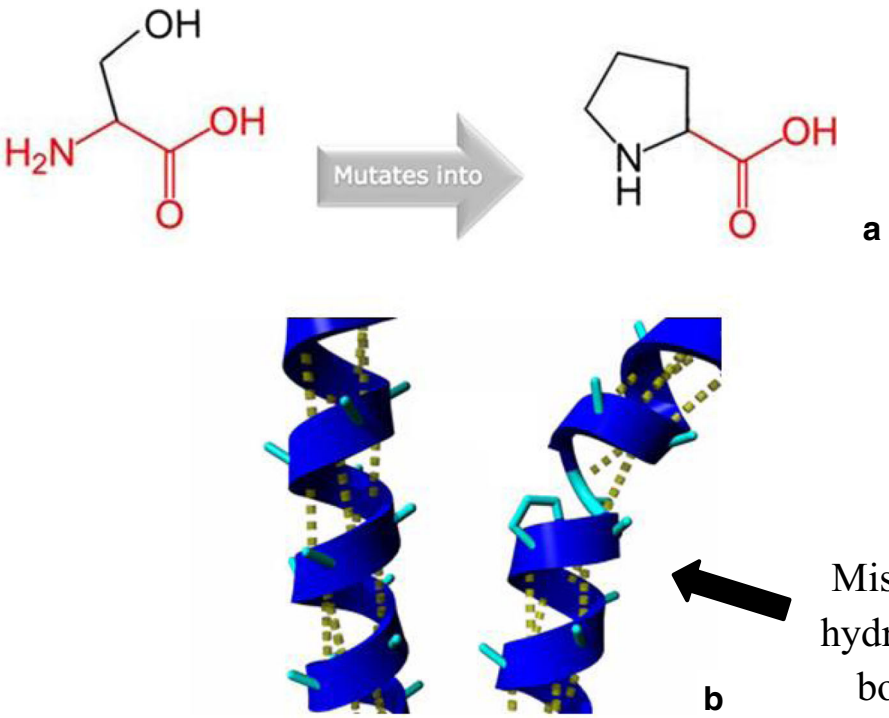

Fig. 2 **a** shows the schematic structures of the serine and proline amino acids. **b** 3D-structure shows the missing hydrogen bond in Lysine at position 153 and Glycine at position 208 in a- helix

proline could cause a huge change in the functional and spatial structure of the protein. Figure 2a shows the schematic structures of the serine and proline amino acids. The mutant residue is bigger and more hydrophobic than the wild-type one. In addition, the wild-type residue forms a hydrogen bond with Lysine at position 153 and Glycine at position 208. The size

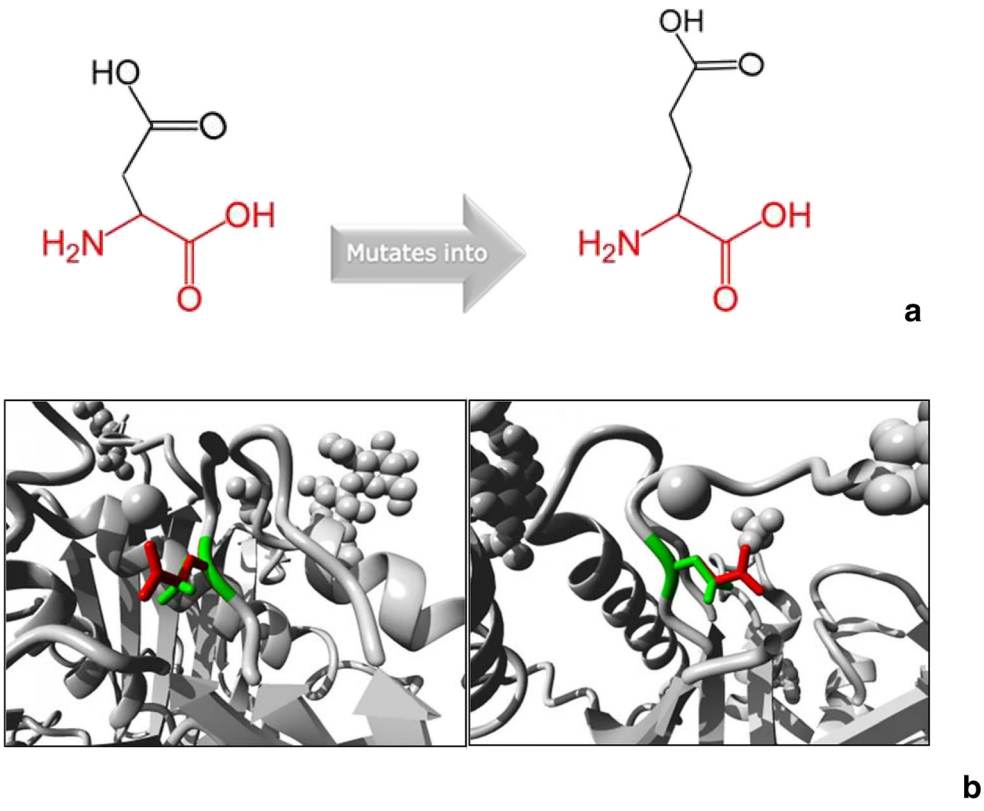

Fig. 3 A shows the schematic structures of the glutamic acid and aspartic acid in IDUA gene. Fig. B shows two different angle of the protein. The protein is colored grey, the side chain of wild type residue is colored green and mutant residue is red. The mutation will disrupt the hydrogen bond between Lysine at position 264 and Aspartic Acid at position 349

difference between wild-type and mutant residue makes that the new residue is not located in the correct position to make the same hydrogen bond as the original wild-type did. The difference in hydrophobicity affects hydrogen bond formation (Fig. 2b). Also, the wild-type residue was buried on the core of the protein. The mutant residue is bigger and probably may not fit in its position. The mutation may cause loss of hydrogen bonds in the core of the protein and as a result, disturbs the correct folding (Venselaar et al. 2010). In addition, polyphen-2 and FATHMM sites confirmed that this mutation (c.469 T > C in *IDUA* gene) is a pathogenic but SIFT and Provean sites could not certainly confirmed that this mutation is pathogenic. Although the predictions are not compatible, but based on the clinical features of the patient, it seems that our prediction is more compatible.

Regarding c.903C > G mutation in the second patient (Table 2) leads to substitution of glutamic acid instead of aspartic acid. The mutant residue is bigger than the wild-type (Fig. 3a). The wild-type residue forms a hydrogen bond with Lysine at position 264 and Aspartic Acid at position 349. The size difference between wild-type and mutant type makes that the mutant residue is not located in the correct position to make the same hydrogen bond as the original wild-type did. The wild-type residue is located in its preferred secondary structure, a turn. The mutant residue prefers to be in another secondary structure; therefore the local conformation will be slightly destabilized (Fig. 3b). The mutated residue is located in a domain that is important for the activity of the protein and in contact with another domain that is also important for the activity. The interaction between these domains could be disturbed by the mutation, which might affect the function of the protein (Venselaar et al. 2010). The prediction that this mutation can be pathogenic is also supported by the Sift (Kumar et al. 2009), Polyphen-2(Adzhubei et al. 2010), Provean, mutation tasting (Schwarz et al. 2014) and Mutation@A Glance (Hijikata et al. 2010) softwares.

Our study is in concordance with the study by (Atceken et al. 2016) with reported the c.1A > C pathogenic mutation in Turkish patient (p.M1L). We observed this mutation in 3 patients and this mutation was the most frequently identified mutation in our study. Two patients showed homozygote pattern of this mutation and one patient was heterozygote for this mutation. Wang X et al. (Wang et al. 2012) also reported another pathogenic mutation (c.2 T > C) in this site. The patient with the compound heterozygous mutation in this study had inherited the c.1A > C variant from the father and the c.469 T > C from the mother. The Fig. 1 shows the haplotype of STR markers in this family.

Regarding c.220C > T (p.R74C) mutation in *SGSH* gene which was reported by Weber B et al. in Australian population for the first time (Weber et al. 1997). They proposed that amino acid residues R74 is likely to be involved in the formation of the active site of sulfamidase enzyme. Harboring this mutation will result in abolishing the active site of the enzyme. In Spanish group of patients (c.1298G > A. (p.R433Q) mutation was reported by Chabás A et al. (Chabas et al. 2001). In this study, we found c.1298G > A mutation in one patient. c.1297C > T (p.R433W) is other pathogenic mutation in this codon which reported by C. Beesley in 2000(Beesley et al. 2000). Unfortunately they did not mention the clinical feature of their studied patients.

In this study one patient showed homozygosity for markers flanking the *NAGLU* gene and sequencing of the exons and exon/ intron boundaries of this gene in patient revealed c.457G > A (p.E153K) pathogenic mutation. For the first time this mutation had been reported by Schmidtchen in 1998(Schmidtchen et al. 1998).

We think that our findings, though not a large sample size, will benefit others working in this field and will help others to conduct similar studies. Our combined methods using direct DNA sequencing and autozygosity mapping will enable medical genetics lab to perform even more accurate and reliable prenatal diagnosis for MPS. Further studies with more sample size are recommended to establish genotype - phenotype correlations. These findings will extend our molecular understanding of MPS disease in Iran and other populations.

Compliance with ethical standards

Conflict of interest Mehdi Shafaat declares that there is no conflict of interest. Dr. Mehrdad Hashemi declares that there is no conflict of interest. Dr.Ahmad Majd declares that there is no conflict of interest. Dr. Maryam Abiri declares that there is no conflict of interest. Dr. Sirous zeinali declares that there is no conflict of interest.

Informed consent Informed consent was received from 8 patients for participation in this study.

Animal rights This study does not contain any animal study.

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