Mucopolysaccharidoses in northern Brazil: Targeted mutation screening and urinary glycosaminoglycan excretion in patients undergoing enzyme replacement therapy

Gustavo M. Viana¹, Nathália O. de Lima¹, Rosely Cavaleiro¹, Erik Alves¹, Isabel C.N. Souza², Raimunda Feio², Sandra Leistner-Segal³, Ida Schwartz³,⁴, Roberto Giugliani³,⁴ and Luiz C. Santana da Silva¹

¹Laboratório de Erros Inatos do Metabolismo, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil.
²Hospital Universitário Bettina Ferro de Souza, Universidade Federal do Pará, Belém, PA, Brazil.
³Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
⁴Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.

Abstract

Mucopolysaccharidoses (MPS) are rare lysosomal disorders caused by the deficiency of specific lysosomal enzymes responsible for glycosaminoglycan (GAG) degradation. Enzyme Replacement Therapy (ERT) has been shown to reduce accumulation and urinary excretion of GAG, and to improve some of the patients' clinical signs. We studied biochemical and molecular characteristics of nine MPS patients (two MPS I, four MPS II and three MPS VI) undergoing ERT in northern Brazil. The responsiveness of ERT was evaluated through urinary GAG excretion measurements. Patients were screened for eight common MPS mutations, using PCR, restriction enzyme tests and direct sequencing. Two MPS I patients had the previously reported mutation p.P533R. In the MPS II patients, mutation analysis identified the mutation p.R468W, and in the MPS VI patients, polymorphisms p.V358M and p.V376M were also found. After 48 weeks of ERT, biochemical analysis showed a significantly decreased total urinary GAG excretion in patients with MPS I (p < 0.01) and MPS VI (p < 0.01). Our findings demonstrate the effect of ERT on urinary GAG excretion and suggest the adoption of a screening strategy for genotyping MPS patients living far from the main reference centers.

Key words: mucopolysaccharidosis, enzyme replacement therapy, mutations, glycosaminoglycans.

Received: July 8, 2010; Accepted: March 23, 2011.

The mucopolysaccharidoses (MPS) are a rare group of lysosomal storage diseases (LSD) with an autosomal recessive inheritance pattern (with the exception of MPS II, which is an X-linked) and characterized by the accumulation of glycosaminoglycans (GAG) in the lysosomes. This accumulation occurs due to low or absent lysosomal enzyme activity, essential to the GAG catabolism, and results in systemic impairment of organs and tissues (Neufeld and Muenzer, 2001).

With an incidence ranging from 1:10000 to 1:25000 newborns worldwide, MPS appear to be among the most frequent LSDs. MPS I and II were found to be the most frequent, while MPS VI is reported to have a lower incidence (Solyom, 1996; Meikle et al., 1999). The MPS Brazil Network, an initiative created to improve the diagnosis and management of these diseases in Brazil between 2004 and 2006, identified 161 MPS patients, among which MPS II showed the highest incidence (n = 82) and MPS VII the lowest (n = 4). Castro et al. (2007) reported a high incidence of MPS II and VI in patients from northern Brazil, followed by MPS I.

So far, eleven different subtypes of MPS have been identified (Neufeld and Muenzer, 2001). Patients with MPS present similar chronic and progressive clinical features, but symptoms may vary depending on the type. Organomegaly, dysostosis multiplex, hepatosplenomegaly, joint contractures and characteristic facial changes are common symptoms described in these patients. Other systemic impairments may also occur, such as hearing deficiencies, cardiorespiratory disorders, low articular mobility and loss of visual acuity (Neufeld and Muenzer, 2001; Muenzer, 2004).

Enzyme Replacement Therapy (ERT) is considered an efficient therapeutic method in MPS treatment. It is based on the periodic replacement of the defective enzyme, leading to higher GAG degradation in tissues and organs,
promoting a significant improvement in some clinical features. However, the influence of ERT on other pathological manifestations, like progressive skeletal disease, arthropathy, central nervous system dysfunction, cardiac involvement and visual impairment, is still not well understood (Miebach, 2005; Arora et al., 2007; El Dib and Pastores, 2007; Pitz et al., 2009).

Previous studies have proposed urinary GAG dosage as a biochemical parameter for evaluating the efficacy of ERT protocols. After some months of treatment, the majority of patients undergoing ERT showed an improvement of many clinical symptoms, which was correlated with a significant reduction in urinary GAG excretion (Harmatz et al., 2005, 2008; Arora et al., 2007; Sifuentes et al., 2007; Muenzer et al., 2009).

The isolation and characterization of genes involved in the depletion of GAG catabolism enzymes led to the identification of many disease-causing mutations related to MPS. Despite the great variability presented by MPS patients, some mutations were reported to be frequent in some specific populations (Matte et al., 2000; Petry et al., 2005; Alves et al., 2006; Mangas et al., 2008).

The aims of this study were to investigate common MPS mutations in patients from northern Brazil and to analyze the efficacy of ERT by means of urinary GAG measurements.

We studied nine patients with MPS from a region in northern Brazil (state of Pará), two of which with MPS I, four with MPS II, and three with MPS VI. The diagnoses, made by the Medical Genetics Service (SGM) of the Hospital de Clínicas de Porto Alegre, were based on abnormal urinary GAG excretion (before starting ERT) and leukocyte enzyme activity. All patients had clinical manifestations of the disease, and the diagnosis was confirmed by a leukocyte enzyme activity < 20% of the normal range established by our laboratory. Some clinical parameters, such as age, consanguinity, height, corneal opacification and cardiac dysfunctions, were analyzed in each patient, for phenotype determination (Table 1). This study was reviewed and approved by the Human Research Ethics Committee of the Instituto de Ciências da Saúde, Universidade Federal do Pará (protocol no. #172/2008 - November 11th, 2008).

The recombinant enzymes laronidase (Aldurazyme®, recombinant human α-L-iduronidase; BioMarin Pharmaceutical Inc., Novato, CA, USA; Genzyme Corporation, Cambridge, MA, USA) and galsulfase (Naglazyme®, recombinant human ASB; BioMarin Pharmaceutical Inc., Novato, CA, USA) were administered intravenously once a week for 48 weeks, at a dosage of 1 mg/kg bodyweight for MPS I, and 0.58 mg/kg bodyweight for MPS VI, respectively. For MPS II ERT, idursulfase (Elaprase®, Shire HGT) at a dosage of 0.50 mg/kg was intravenously administered.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Origin</th>
<th>Consanguinity</th>
<th>AOS (months)</th>
<th>ALE (years)</th>
<th>Height (cm)</th>
<th>Corneal opacification</th>
<th>Cardiac impairment</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (MPS I)</td>
<td>Belém (PA)</td>
<td>Yes</td>
<td>&lt;12</td>
<td>20</td>
<td>138</td>
<td>++++</td>
<td>IA</td>
<td>P533R / P533R</td>
<td></td>
</tr>
<tr>
<td>P2 (MPS I)</td>
<td>Belém (PA)</td>
<td>No</td>
<td>60</td>
<td>15</td>
<td>152</td>
<td>++</td>
<td>IM</td>
<td>A</td>
<td>P533R / ?</td>
</tr>
<tr>
<td>P3 (MPS II)</td>
<td>Belém (PA)</td>
<td>No</td>
<td>24</td>
<td>10</td>
<td>133</td>
<td>+</td>
<td>IM</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>P4 (MPS II)</td>
<td>Belém (PA)</td>
<td>No</td>
<td>48</td>
<td>08</td>
<td>100</td>
<td>-</td>
<td>IM</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>P5 (MPS II)</td>
<td>Belém (PA)</td>
<td>No</td>
<td>12</td>
<td>08</td>
<td>104</td>
<td>-</td>
<td>IM</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>P6 (MPS II)</td>
<td>Colares (PA)</td>
<td>No</td>
<td>24</td>
<td>08</td>
<td>99</td>
<td>-</td>
<td>IM</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>P7 (MPS II)</td>
<td>Cametá (PA)</td>
<td>No</td>
<td>36</td>
<td>16</td>
<td>110</td>
<td>+</td>
<td>IM</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>P8 (MPS VI)</td>
<td>Cametá (PA)</td>
<td>No</td>
<td>36</td>
<td>14</td>
<td>121.5</td>
<td>+</td>
<td>IM</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>P9 (MPS VI)</td>
<td>Cametá (PA)</td>
<td>No</td>
<td>24</td>
<td>10</td>
<td>97</td>
<td>++</td>
<td>S</td>
<td>V376M / V376M</td>
<td></td>
</tr>
</tbody>
</table>

The data shown correspond to the last clinical evaluation performed. The phenotype classification was based on a clinical evaluation of survival rate, age at onset of symptoms, and growth retardation. Dysostosis multiplex was found in all patients. P1 was not evaluated during the trial. (+) Patients P4 and P9 died during the trial. (?) Data not available; (IA) aortic valve insufficiency; (IM) mitral valve insufficiency; (AOS) age of onset of symptoms; (ALE) age of last evaluation; (S) severe; (I) intermediate; (A) attenuated.
Genomic DNA was extracted from peripheral blood using the phenol-chloroform technique (Sambrook and Russel, 2001). PCR reactions and restriction enzyme tests were designed to screen for eight known MPS mutations: p.Q70X, p.W402X and p.P533R (IDUA gene, MPS I); p.R468W, p.R468Q and p.P467L (IDS gene, MPS II); p.R315Q and 1533del23 (ARSB gene, MPS VI), with the exception of exons 3 and 9 (IDS gene), which were directly sequenced after amplification.

PCR was performed in a total reaction volume of 50 μL, containing 100 ng of DNA, 2 mmol/L of dNTP, 1x PCR buffer (10 mmol/L TRIS-HCl, pH 8.3; 50 mmol/L KCl), 20 pmol of each primer (forward and reverse), 1.5 mmol/L MgCl2, and 1U of Taq DNA polymerase (Invitrogen). Cycling conditions were typically 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55-66 °C, 1 min at 72 °C, and a final extension at 72 °C for 10 min. All amplifications were carried out in an MG96+ Peltier-Based Thermal Cycler (Biosystems). For control, PCR reactions with no DNA (blank) were used during each reaction. The amplified fragments were visualized in 2% agarose gel containing ethidium bromide.

After amplification, the PCR products were subjected to direct sequencing for mutation and/or polymorphism detection, using the BigDye 3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), in an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Some sequence changes were confirmed by digestion with specific restriction enzymes. Targeted mutations, primer sequences, fragment lengths and restriction enzymes are presented in Table 2.

The total GAG concentration in urine samples of MPS patients undergoing ERT was determined by a method based on spectrophotometric detection of metachromatic changes to the 1,9-dimethylmethylen blue (DMB) dye, resulting from GAG binding (Whitley et al. 1989). Urinary creatinine concentrations were also measured separately in order to normalize urinary GAG concentrations (mg GAG/mmol creatinine). Condroitin sulfate was used as standard control in DMB binding measurements.

For the urinary GAG concentrations, all descriptive statistics, including means, standard deviations (SD), and percent change over time were calculated using the Biostat 5.0 Software. Changes in parameters between baseline (before starting treatment) and after 48 weeks of ERT were evaluated using Student’s t test (p = 0.05).

After amplification and direct sequencing of the amplified fragments, two previously described mutations were identified among the nine studied MPS patients: mutation p.P533R, common in patients with MPS I, was detected in patient P1 (homozygous) and patient P2 (heterozygous); another mutation, frequent in MPS II patients (p.R468W), was identified only in patient P3. In addition, two common polymorphisms (p.V358M and p.V376M) were found in MPS VI patients.

A significant decrease in relative urinary GAG concentration was observed in all patients with MPS I undergoing ERT.

Table 2 - Sequence and orientation of primer pairs used for amplification of MPS gene fragments.

<table>
<thead>
<tr>
<th>MPS Subtype</th>
<th>Mutation</th>
<th>Primer (5’→3’)</th>
<th>Product size (bp)</th>
<th>Restriction enzyme</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I</td>
<td>p.Q70X</td>
<td>GGCTTGAACGTGTGTGTCAG (F)</td>
<td>274</td>
<td>-Sau96I</td>
<td>N:122+95+57 M:179+95</td>
</tr>
<tr>
<td></td>
<td>(exon 2)</td>
<td>TCCCATCTGCTCTTGTA (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS I</td>
<td>p.W402X</td>
<td>CTTGGGACTCCTTCACCAAG (F)</td>
<td>354</td>
<td>+BfaI</td>
<td>N:354</td>
</tr>
<tr>
<td></td>
<td>(exon 9)</td>
<td>CAGAGACCTCCTGGAACC (R)</td>
<td></td>
<td></td>
<td>M: 227+ 127</td>
</tr>
<tr>
<td>MPS I</td>
<td>p.P533R</td>
<td>GTGTGGGTGGGAGGTGGA (F)</td>
<td>250</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(exon 11)</td>
<td>TTAGGGGACTGCCACTTGC (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS II</td>
<td>Exon 3</td>
<td>AACGATCTGCTGGTTTCAGG (F)</td>
<td>423</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGACTGCTGGACATGGAGCA (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS II</td>
<td>p.R468W</td>
<td>CATATGAGGCCAGACAGGT (F)</td>
<td>399</td>
<td>-MspI</td>
<td>N:316+83 M:399</td>
</tr>
<tr>
<td></td>
<td>(exon 9 I)</td>
<td>ATGCTGCGTGATGGAAATAGCC (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS II</td>
<td>p.R468Q</td>
<td>CATATGAGGCCAGACAGGT (F)</td>
<td>399</td>
<td>-TaqI</td>
<td>N:241+158 M:399</td>
</tr>
<tr>
<td></td>
<td>(exon 9 I)</td>
<td>ATGCTGCGTGATGGAAATAGCC (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS II</td>
<td>p.P467L</td>
<td>CATATGAGGCCAGACAGGT (F)</td>
<td>399</td>
<td>-NciI</td>
<td>N:316+83 M:399</td>
</tr>
<tr>
<td></td>
<td>(exon 9 I)</td>
<td>ATGCTGCGTGATGGAAATAGCC (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS II</td>
<td>Exon 9 II</td>
<td>CCCGTGAACTGTGTTTCCAT (F)</td>
<td>400</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACTAGCTTCAGGCTGCTTCC (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS VI</td>
<td>p.R315Q</td>
<td>TCAATCCATGCAAAAGACCT (F)</td>
<td>300</td>
<td>-TaqI</td>
<td>N:179+77+44 M:256+44</td>
</tr>
<tr>
<td></td>
<td>(exon 5)</td>
<td>GAAAAGGCCAGGGTGGT (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS VI</td>
<td>1533del23</td>
<td>CCTCTGTGTTTCCTCCCTCA (F)</td>
<td>347</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(exon 8)</td>
<td>CTTCACATTTGGTTTCTTCCC (R)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(F) forward; (R) reverse; N: normal; M: mutant; +/- gain or loss of restriction enzyme site, respectively. All primers were designed by our group, using the Primer3 Software (http://www.genome.wi.mit.edu/cgi-bin/primer3_www.cgi).
ing ERT when compared to baseline (Figure 1). In patient P1, the initial urinary GAG concentration of 76.38 mg GAG/mmol creatinine (single dosage) decreased to 12.34 ± 9.35 mg GAG/mmol creatinine at week 6, representing a reduction of approximately 84% (p < 0.001) in the urinary GAG excretion rate. This reduction became more evident in the subsequent weeks, reaching values of 10.20 ± 6.89 mg GAG/mmol creatinine (86% reduction) during week 12, and this level was maintained until the end of the analyzed period (48 weeks).

Patient P2 (MPS I) also presented a significant reduction in urinary GAG excretion after starting ERT. At baseline, the urinary GAG concentration was 17.27 mg GAG/mmol creatinine, and at week 6 this excretion was reduced to 9.17 ± 4.1, which corresponds to a 47% reduction (p < 0.05). However, at week 12, these values had oscillated, returning to higher levels (16.4 ± 7.6 mg GAG/mmol creatinine) decreasing again by weeks 18 and 24 (11.1 ± 7.2 and 4.3 ± 0.53 mg GAG/mmol creatinine, respectively). In this patient (P2), the urinary GAG concentrations could not be measured after week 24, because at that time his treatment was discontinued.

Patient P5 (MPS II) also presented a significant reduction in urinary GAG excretion after starting ERT. At baseline, the urinary concentration was 12.41 mg GAG/mmol creatinine, decreasing to 4.2 mg GAG/mmol creatinine by week 5, a reduction of approximately 66% (data not shown).

Patients with MPS VI also showed a significant reduction in their urinary GAG excretion during ERT (Figure 2). In patient P7, the mean value of urinary GAG excretion at baseline was 31.9 mg GAG/mmol creatinine. After starting ERT, this concentration had decreased to 6.17 ± 2.79 mg GAG/mmol creatinine by week 6, a reduction of approximately 81% (p < 0.01). This concentration remained stable until week 36, when a discrete but not significant (p > 0.05) increase was observed compared to baseline. Changes in urinary GAG concentration were also observed in patient P8, who presented a significant reduction of 88% (p < 0.01) at week 6 (3.16 ± 0.80 mg GAG/mmol creatinine) as compared to baseline (26.92 mg GAG/mmol creatinine).

In this study, we found two targeted mutations: p.P533R in two patients with MPS I, and p.R468W in one patient with MPS II. Moreover, two previously described polymorphisms (p.V358M and p.V376M) were detected in patients with MPS VI. None of the other investigated mutations were found.

Mutation p.P533R, identified in homozygosis in patient P1 and in heterozygosis in patient P2, has been previously reported in Brazilian patients by Matte et al. (2003), who described different phenotypes in patients with the same mutation. Previously, Gatti et al. (1997) and Venturi et al. (2002) had also reported variable phenotypes in the presence of the p.P533R mutation in Italian MPS I patients.

Patient P3 (MPS II), who presented the p.R468W mutation, had a phenotype classified as severe, differing from results reported in the literature.

This difficulty in establishing genotype/phenotype correlations may result from some unaccounted factors, such as the rarity of this disease and the inheritance profile of the mutations.

Some studies suggest that certain mutations involving C>T or G>A transitions in CpG dinucleotides are due to in situ methylation-deamination processes and, therefore, these regions are considered hotspots for mutation identification. The p.R468W mutation is caused by a C>T change in CpG regions and is, therefore, considered a hotspot. However, its role in phenotype determination is not yet well understood, due to the high heterogeneity of this gene.

According to Froissart et al. (2007), seven out of eight patients with attenuated phenotypes carried a missense mutation, including p.R468W. Jonsson et al. (1995)
had also described three patients with this mutation, all presenting a mild phenotype. In contrast, Froissart et al. (2007) themselves, besides other authors (Isogai et al., 1998; Lin et al., 2006), reported severe phenotypes associated to this mutation in different patients. On the other hand, Li et al. (1999) did not report the phenotypic classification of their patients, due to lack of clinical data.

The polymorphisms p.V358M and p.V376M were detected in patients P8 and P9, respectively, both presenting a severe phenotype. These polymorphisms are also produced by transitions at CpG nucleotides and, when associated with some disease-causing mutations, can contribute for a decrease in enzymatic activity, thus influencing the clinical development of the patients (Karageorgos et al., 2004).

Petry et al. (2005) established a correlation between the presence of polymorphisms p.V358M and p.V376M in MPS VI patients with a severe to intermediate phenotype. Although their patients presented the same polymorphisms and phenotypes, their mutations were variable. This suggests that the presence of p.V358M and p.V376M is not necessarily linked to a specific mutation, but patients with these polymorphisms can present similar phenotypes.

Our study demonstrated that ERT was capable of significantly reducing urinary GAG excretion in all MPS patients undergoing this treatment. Although some data regarding the clinical evaluations were not available, this reduction, according to the literature (Harmatz et al., 2005, 2008; Arora et al., 2005, 2007; Sifuentes et al., 2007), is strictly related to the improvement of the patients’ clinical picture.

We observed a significant reduction (p < 0.01) in urinary GAG excretion in MPS I and MPS VI patients. In MPS I, the overall reduction was of about 94% for patient P1, and 41% for patient P2. In MPS VI patients, the overall reduction was of about 77% for patient P7 and 73% for patient P8. Similar total reduction rates were also reported in the literature, where reduction levels of 54% to 60% during the initial weeks, 59% during treatment, and between 69% and 88% after six years were described (Wraith et al., 2004, 2007; Sifuentes et al., 2007). Furthermore, higher enzyme doses have been reported to be more efficient than lower doses in achieving clinical improvements and reduction of urinary GAG in MPS patients undergoing ERT (Harmatz et al., 2005, 2008).

This is the first genetic and biochemical report on MPS patients from northern Brazil. The mutations and polymorphisms identified in these MPS patients were all previously reported in Brazilian patients. Urinary GAG excretion was significantly reduced in all MPS patients undergoing ERT, confirming the responsiveness of this treatment. A complete molecular screening and a comprehensive epidemiological investigation of all patients, performed at health care centers near to their birth places, could contribute to earlier diagnosis and treatment, as well as to a better understanding of the etiology of the disease.

Acknowledgments

We would like to thank the Hospital Universitário Bettina de Ferro Souza (HUBFS/UFPA), the Instituto de Genética Médica Populacional (INAGEMP) and the MPS Brazil Network for their important collaboration. We also would like to thank Fundação de Amparo à Pesquisa do Estado do Pará (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant number 573993/2008-4) for the financial support provided.

References


License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.