Chapter 4
Materials and methods
This chapter details the clinical investigation and experiments employed throughout the study.

4.1. Patient recruitment for Morquio syndrome and GM1 gangliosidosis

We recruited 100 patients with Morquio syndrome and 53 patients with GM1 gangliosidosis. All patients were diagnosed by clinical and radiologic features and confirmed by enzyme assays. The samples were collected from 18 genetic centers from different geographic regions and ethnicities across India. Two milliliters of peripheral blood was drawn from the probands and their parents after obtaining informed consent from the family. Referring clinicians were asked to send blood or DNA samples of patients and their parents. Clinical features were obtained in a pre-designed clinical proforma from the clinical collaborators. Basic information like age, sex, consanguinity and major clinical findings were documented and analyzed through the clinical proforma. A three-generation pedigree was drawn for all the families. 50 healthy and unrelated individuals from the same ethnic groups were recruited as controls. The study was approved by the Institutional Ethics Committee of Kasturba Hospital, Manipal.

For Morquio syndrome, height and weight were plotted against Centers for Disease Control growth charts (CDC) (Montano et al., 2008b). The severity was assessed as done by Dung et al., (Dung et al., 2013). In this study, we consider a family when parents came for carrier testing.

4.2. Enzyme assay for MPS IVA

4.2.1. GALNS activity was measured with the fluorogenic substrate MU-βGal-6S triethylammonium

The patients suspected to have Morquio syndrome were confirmed to have the condition through the GALNS enzyme assay on leukocytes of their blood samples. Only patients who showed a decreased enzyme activity were recruited for the study. GALNS activity was assayed according to the method described by Van Diggelen et al., (Van Diggelen et al., 1990). Detailed descriptions of the method employed and the pre-made reagents required for the assays are listed below.

4.2.2. Chemicals required

1) Substrate: 10 mM MU-βGal-6S triethylammonium.
2) Substrate buffer: 0.1 M sodium acetate / 0.1 M acetic acid buffer, pH 4.3, containing 0.1 M NaCl, 0.02% sodium azide and 5 mM lead acetate.
3) Pi-buffer: 0.9 M disodium hydrogen phosphate / 0.9 M sodium dihydrogen phosphate, pH: 4.3 + 0.02% (w/v) sodium azide.
4) Stop buffer: 0.5 M sodium bicarbonate / 0.5 M sodium carbonate buffer, pH 10.7, 0.025% Triton X-100.
5) βGal-Ao-stock: (stock solution of β-GAL for 2nd incubation).
6) βGal-Ao-10 U: (Ready to use solution of β-GAL for 2nd incubation).

4.2.3. Preparation of chemicals

1) Substrate: (10 mM MU-βGal-6S triethylammonium)
   1) 2 mg of the substrate MU-βGal-6S triethylammonium was added to 384 µl of milli-Q water.
   2) Aliquots were prepared and stored at -80 °C.

2) Substrate buffer: 0.1 M sodium acetate / 0.1 M acetic acid buffer, pH 4.3, containing 0.1 M NaCl, 0.02% sodium azide and 5 mM lead acetate
   1) To prepare substrate buffer, 0.1 M sodium acetate, 0.1 M of NaCl, 5 mM of lead acetate and 0.02% of sodium azide were added to 40 ml of milli-Q water.
   2) The pH was adjusted to 4.3 by adding 0.1 M glacial acetic acid drop by drop.
   3) After adjusting the pH 4.3, final volume was made up to 50 ml.

3) Pi-buffer: (0.9 M disodium hydrogen phosphate / 0.9 M sodium dihydrogen phosphate buffer, pH 4.3 + 0.02% (w/v) sodium azide)
   1) 50 ml of 0.9 M disodium hydrogen phosphate was prepared by dissolving 6.4 g of disodium hydrogen phosphate in 50 ml of milli-Q water.
   2) 50 ml of 0.9 M sodium dihydrogen phosphate was prepared by dissolving 5.4 g of sodium dihydrogen phosphate in 50 ml of milli-Q water.
   3) The pH of 0.9 M sodium dihydrogen phosphate was adjusted to 4.3 by adding 0.9 M disodium hydrogen phosphate drop wise to it.
   4) After adjusting the pH to 4.3, 0.02% of sodium azide was added.

4) Stop buffer: (0.5 M sodium bicarbonate / 0.5 M sodium carbonate buffer, pH 10.7, 0.025% Triton X-100)
1) To prepare 50 ml of 0.5 M sodium carbonate, 2.65 g of sodium carbonate was added in 50 ml of milli-Q water.

2) Also, 50 ml of 0.5 M sodium bicarbonate was prepared by adding 2.1 g sodium bicarbonate to 50 ml of milli-Q water.

3) The pH of 0.5 M sodium carbonate was adjusted to 10.7 by adding 0.5 M sodium bicarbonate drop by drop.

4) After adjusting the pH 10.7, 0.025% Triton X-100 was added.

5) βGal-Ao-stock: (βGal-β-D-Galactoside galactohydrolase (from Aspergillus oryzae))
   1) 10 ml of milli-Q water was added to 25000U of βGal and was dissolved by vortexing.
   2) Aliquots (500 µl) were prepared and stored at -80°C.

6) 0.2% BSA: (BSA-Bovine Serum Albumin, pH and heat inactivated, 0.02% sodium azide)
   The lysosomal enzymes present in commercial BSA were pH and heat denatured as follows:
   1) 5 % BSA was prepared by dissolving 2.5 g of commercial BSA in 50 ml of milli-Q water.
   2) The beaker was sealed with a foil and left overnight at 4 °C for complete dissolution of BSA.
   3) After overnight incubation, 5% BSA solution was brought to room temperature and pH was adjusted to 10.0 by using 6M sodium hydroxide.
   4) The solution was incubated at 50 °C for 4 hr in the water bath.
   5) After incubation, the solution was allowed to cool and pH was adjusted to 7.0 by using 1 N HCL.
   6) The solution was then centrifuged for 10 min at 2000 rpm, supernatant was transferred to a fresh beaker and the pellet was discarded.
   7) The inactivated 5% BSA solution was diluted to 25X in 0.02% sodium azide by adding 2 ml of 5% BSA solution to 48 ml of 0.02% sodium azide.
   8) Aliquots (500 µl) of 0.2% BSA were prepared and stored at -20 °C.

7) βGal-Ao-10U
   1) To prepare 10U of βGal-Ao from βGal-Ao-stock (25000U), 8 µl from 2 ml of 0.2% BSA was taken out and 8µl of βGal-Ao-stock was added to it.
2) Aliquots (200 µl) were prepared and stored at -80 °C.

4.2.4. Preparation of leukocytes from heparinized blood
1) 2 ml of EDTA blood was taken into a 15 ml falcon tube.
2) RBCs were lysed by adding 12 ml of RBC lysis buffer to 2 ml EDTA blood.
3) The falcon tube was mixed properly and incubated in ice for 20 min.
4) It was then centrifuged at 4 °C for 10 min at 2000 rpm.
5) After centrifugation, the supernatant was discarded, RBC lysis buffer was again added and above steps (3-5) was repeated till white pellet was obtained.
6) Once the white pellet was obtained, it was washed with 1 ml of milli-Q water by vortexing and then centrifuged at 4 °C for 5 min at 6000 rpm.
7) The supernatant was discarded and the cell pellet was immediately stored at -20 °C for cell lysis.

4.2.5. Lysate preparation
4.2.5.1. Sonication of WBC pellet
1) 1 ml of milli-Q was added to the leukocytes pellet and vortexed to homogenize the pellet.
2) The sonicator probe was cleaned with 70% alcohol.
3) The amplitude of the machine was set to 40.
4) The sonicator probe was chilled in ice for 10-15 s.
5) The sonicator probe was immersed with great care into the tube containing the homogenized cells without touching the sides.
6) The sonication was done for 10 s.
7) The lysate was kept on ice at all times.

4.2.6. GALNS activity
4.2.6.1. Incubation 1
1) Homogenates were made in milli-Q water by sonication and diluted to the desired protein concentration by 0.2% BSA.
2) For the assay, 10 µl of the homogenate was taken in a 1.5 ml microfuge tube.
3) To the homogenate, 20 µl of the substrate was added and the solution was mixed well and incubated for 17 hr at 37 °C.
4) For the blank, 10 µl of the 0.2% BSA was taken.
4.2.6.2. Incubation 2

1) After incubation, 5 µl of the pi-buffer was added and mixed well.
2) 10 µl of βGal-Ao-10U was then added to reaction mixture and it was incubated for 2 hr at 37 °C.
3) The enzyme reaction was terminated by adding 200 µl of stop buffer and the fluorescence of MU was then measured at λex 360 nm and λem 415 nm.

4.3. Enzyme assay for MPS IVB and GM1 Gangliosidosis

β-GAL enzyme activity was measured with the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside.

4.3.1 Chemicals required

1) Substrate - 0.5 mM 4-MU-β-galactopyranoside
2) 0.1 M Sodium acetate-acetic acid buffer, pH 4.0
3) 5 mM stock solution of 4-MU
4) 5 µM 4-MU solution
5) 1 nmol 4-MU
6) Glycine-carbonate buffer, 0.17 M, pH 9.8
7) Bradford reagent
8) Stock solution of BSA: 10 mg/ml

4.3.2. Preparation of chemicals

1) 0.1 M Sodium acetate-acetic acid buffer, pH 4.0, containing 0.1M NaCl: 100 ml
   1) To prepare this, 0.1M of sodium acetate and 0.1 M of NaCl were added in 80 ml of milli-Q water.
   2) The pH was adjusted to 4.0 by adding 0.1 M glacial acetic acid drop by drop.
   3) After adjusting the pH, final volume was made up to 100 ml with milli-Q water.

2) Glycine-carbonate buffer, 0.17 M, pH 9.8: 100 ml
   1) 0.17 M of glycine and 0.17 M sodium carbonate were added in 80 ml of milli-Q water.
   2) The pH was adjusted to 9.8 with sodium hydroxide and final volume was made up to 100 ml with milli-Q water.

3) 5 mM stock solution of 4-MU: 50 ml
To prepare this, 5 mM of 4 MU was added to 50 ml of 0.17 M glycine-carbonate buffer (pH 9.8) and stored at -20 °C in a 50 ml falcon tube wrapped with aluminium foil.

4) 5 µM 4-MU: 50 ml

1) To prepare this, 50 µl of 5 mM stock solution of 4-MU was taken and made up to 50 ml with 0.17 M glycine - carbonate buffer (pH 9.8).

2) Stored at 4 °C in a 50 ml falcon tube wrapped with aluminium foil.

5) 1 nmol 4-MU: 1.5 ml

To prepare this, 200 µl of the 5 µM 4-MU were added to 1300 µl of 0.17 M glycine-carbonate buffers (pH 9.8).

6) Substrate preparation: 50 ml

1) The substrate was prepared by adding 0.5 mM of 4-MU β-galactopyranoside in 50 ml of 0.1 M sodium acetate-acetic acid buffer, pH 4.0 containing 0.1 M NaCl.

2) Stored at 4 °C in a 50 ml falcon tube wrapped with aluminium foil.

4.3.3. Protein estimation by using Bradford reagent

The total protein was measured by Bradford protein assay (Bradford, 1976) as described below.

The assay involved following steps:

1) It was performed in a 96 well plate.

2) The Bradford reagent in the bottle was gently mixed and brought to room temperature.

3) The protein standards ranging from 0.1 - 1.5 mg/ml were prepared in sterile milli-Q using BSA standard.

4) 5 µl of the protein prepared were added in duplicates to separate wells in the 96 well plate (to the blank well, 5µl of sterile milli-Q was added).

5) 5 µl of the cell lysates was also added in duplicates to the separate wells in the 96 well plate.

6) For each well being used, 250 µl of the Bradford was added and mixed on a shaker for approximately 30 s.

7) The samples were incubated at room temperature for 15 min.

8) The absorbance was measured at 595 nm and then plotted against the protein concentration of each standard.

55
9) The protein concentration of the cell lysates was determined by comparing the absorbance at 595 nm values against the standard curve.

**4.3.4. Determination of fluorescence of 1 nm 4-MU at \( \lambda_{\text{ex}} \) 360 nm and \( \lambda_{\text{em}} \) 415 nm**

1) For fluorescence determination, 200 \( \mu \)l of 5 \( \mu M \) 4-MU solution and 1300 \( \mu \)l of glycine-carbonate buffer were taken in duplicate tubes.

2) The fluorescence of each tube was measured at \( \lambda_{\text{ex}} \) 360 nm and \( \lambda_{\text{em}} \) 415 nm using smallest slit insert 5.

3) Thus, the fluorescence of 1 nm 4-MU at \( \lambda_{\text{ex}} \) 360 nm and \( \lambda_{\text{em}} \) 415 nm at slit insert 5 was determined.

**4.3.5. \( \beta \)-GAL activity**

1) For this assay, 0.020 - 0.030 mg protein for leukocytes was needed.

2) 100 \( \mu \)l of cell lysate (diluted to achieve the necessary protein concentration in the centrifuge tubes) was added.

3) 100 \( \mu \)l of substrate solution was then added to the blank and the test samples (lysate was not added to the blank before incubation).

4) The solution was incubated at 37 °C for 20 min.

5) After incubation, the reaction was stopped by adding 1.3 ml of 0.17 M glycine-carbonate buffer, pH 9.8.

6) For the blank, only the substrate was incubated and the reaction was stopped by adding 1.3 ml of 0.17 M glycine-carbonate buffer and lysate was then added.

7) The average duplicate readings of the fluorescence at \( \lambda_{\text{ex}} \) 360 nm and \( \lambda_{\text{em}} \) 415 nm were determined.

8) The enzyme activity was calculated as:

\[
\text{Lysate activity (nmol/hr/g)} = \frac{\text{fluorescence of sample (sample-blank)} \times 3}{\text{mg of protein per assay} \times \text{fluorescence of 1nmol 4-MU}}
\]

**4.4. Isolation of DNA by phenol chloroform method**

DNA was isolated by following the protocol of phenol chloroform method (Sambrook and Russell, 2006). This protocol required some of the pre-made solutions that are listed below.

**4.4.1. Preparation of chemicals**
1) **IM Tris HCL**
120.14 g of Tris base in 900 ml of dH2O was dissolved. Mixed well and pH was adjusted to 8.0 with concentrated HCL. After the adjusting the pH, final volume was made up to 1000 ml by dH2O.

2) **0.5M EDTA**
186.12 g of EDTA was weighed and poured into 800 ml of dH2O. Stirred slowly and pH adjusted to 8.0 by adding sodium hydroxide pellet. Final volume was made up to 1000 ml by dH2O.

3) **1M NaCl**
58.44 g of NaCl was dissolved in 900 ml of dH2O and volume made up to 1000 ml with dH2O.

4) **RBC lysis buffer**
RBC lysis buffer was prepared by weighing 8.3 g of ammonium chloride, 2.2 g of Tris and 0.036 g of EDTA. All these chemicals were added into 900 ml of dH2O and stirred well. The pH was adjusted to 7.4 with 1N HCL and up to 1000 ml of volume was made by adding dH2O.

5) **WBC lysis buffer**
WBC lysis buffer was prepared by taking 1 ml of 1 M Tris (pH 8.0), 40 ml of 1 M NaCl and 0.4 ml of 0.5 M EDTA (pH 8.0). These solutions were mixed and volume of 100 ml was made by adding dH2O.

6) **20% SDS**
20 gram of SDS was weighed and 100 ml of dH2O was added to it.

7) **Proteinase K**
10 mg/ml of proteinase K solution was prepared by taking 100 mg of proteinase K powder (Sigma-Aldrich, MO, USA) and making up to 10 ml with milli-Q water.

8) **Chloroform: isoamyl alcohol (24:1, v/v)**
To prepare 100 ml of chloroform: isoamyl alcohol (24:1, v/v), 96 ml of chloroform and 4 ml of isoamyl alcohol were taken and mixed well.

9) **3M sodium acetate, pH 5.2**
Weighed 24.61 g of sodium acetate and poured in to 80 ml dH2O. The pH was adjusted to 5.2 with glacial acetic acid and dH2O was added to make up the volume to 100 ml.
10) 70% ethanol
To prepare 100 ml of 70% ethanol, 70 ml of absolute alcohol and 30 ml of dH2O were taken.

11) 1X T<sub>10</sub>E<sub>1</sub> buffer
1 ml of 1M Tris HCl (pH 8.0) and 0.2 ml of 0.5M EDTA (pH 8.0) were taken and the volume was made up to 100 ml with milli-Q water.

12) 5X TBE buffer
To prepare 5X TBE buffer, 54 g of Tris, 27.5 g of boric acid and 20 ml of 0.5M EDTA (pH 8.0) were taken. This solution was mixed and adjusted to the volume of 1000 ml with dH<sub>2</sub>O.

13) 1X TBE buffer
20 ml of 5X TBE buffer was taken and made up the volume to 1000 ml with dH<sub>2</sub>O.

14) 6X gel loading dye
To prepare 6X gel loading dye, 25 mg bromophenol blue (0.25%), 3 ml of 30% glycerol, 10 ml of dH<sub>2</sub>O were mixed.

15) Ethidium bromide 10 mg/ml
50 mg of EtBr was weighed and dissolved in 5 ml of dH<sub>2</sub>O. The EtBr solution was stored in dark colored bottle.

4.4.2 DNA isolation
The protocol involved in the isolation of DNA is described below.
1) 2 ml of peripheral blood was withdrawn from the patient in EDTA vacutainer and poured in to 15 ml falcon tube.
2) To the blood sample, RBC lysis buffer was added and then incubated at 37 °C for 20 min in the water bath.
3) The tube was centrifuged at 3000 rpm for 10 min.
4) After centrifugation, the supernatant was discarded and RBC lysis buffer was added and again incubated at 37 °C for 20 min.
5) The tube was centrifuged at 3000 rpm for 10 min and the steps (2-4) were repeated till a white pellet was observed.
6) To the white pellet, 500 μl of WBC lysis buffer, 15 μl of 20% SDS and 5 μl of Proteinase K were added, vortexed well and incubated overnight at 37 °C.
7) After overnight incubation, all of the contents were transferred to microfuge tube and 500 μl of buffer saturated phenol with pH 7.4 was added.

8) The sample was mixed in the rotospin for 10 min and centrifuged at 12,000 rpm for 15 min at 4 °C.

9) After centrifugation, the supernatant was collected in another microfuge tube and equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added.

10) The mixture was mixed in the rotospin for 10 minutes and centrifuged at 12,000 rpm for 15 min at 4 °C.

11) After centrifugation, the supernatant was collected in another microfuge tube, 1/10th volume of 3M sodium acetate and double volume of 100% chilled absolute alcohol was added.

12) The solution was mixed by inverting the tube till white wool like structure appeared.

13) The tube was kept at -20 °C overnight or at -80 °C for 2 - 3 hr.

14) The tube was then centrifuged at 12,000 rpm for 15 min at 4 °C to obtain the pellet.

15) Decant the supernatant, 500 μl of 70% ethanol was added, the pellet was dislodged and centrifuged at 12,000 rpm for 15 min at 4 °C.

16) The supernatant was discarded, the pellet was semi dried and 30-40 μl 1X T10E1 buffer was added.

17) It was mix properly by short spin and was allowed to dissolve at room temperature for overnight.

18) After dissolving, the extracted DNA was quantified in Spectrophotomer (by measurement of A260:A280 ratios) and the quality was assessed in 0.8% agarose gel.

4.5. Primers for GALNS and GLB1 genes

Primers were designed using the Primer 3 tool (Rozen and Skaletsky, 1999) (http://bioinfo.ut.ee/primer3/) to amplify all 14 exons and 16 exons of GALNS and GLB1 genes respectively. Primers were designed in such a way that the exon and exon-intron boundaries were covered. The lists of primers are given in the Table 4.1 and Table 4.2.

4.6. Polymerase chain reaction (PCR) of GALNS and GLB1 genes

PCR amplifications were performed on the thermocycler (Life Technologies, Foster City, CA, USA) with a reaction volume of 25 μl for all the exons except exon 1 of GALNS and
### GLB1.

**Table 4.1: Primers used for sequencing of exons of GALNS gene.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL_1</td>
<td>ACTGGTACGAGGCGACTGC</td>
<td>GCGTGTGGATGAGGCTGTA</td>
<td>552</td>
</tr>
<tr>
<td>GAL_2</td>
<td>GTGCTGCTCCACCGTGC</td>
<td>CAGAGTCGAGGCCTGGAAGGA</td>
<td>315</td>
</tr>
<tr>
<td>GAL_3</td>
<td>GCTGCTGCTTACCTGGTTGG</td>
<td>CAGCTGACCCACCCGAGTC</td>
<td>265</td>
</tr>
<tr>
<td>GAL_4</td>
<td>GAGGGGCCCAGCTGCTGTT</td>
<td>AAGACACCCCTCCTATTTG</td>
<td>321</td>
</tr>
<tr>
<td>GAL_5</td>
<td>CCATCTCAGTCTGGAGGGG</td>
<td>ATGAGTTGCCACCTGAGCC</td>
<td>369</td>
</tr>
<tr>
<td>GAL_6</td>
<td>ATGGCTCTCTGCTGAAATC</td>
<td>GGTGAGTTGAGTCATTCTC</td>
<td>254</td>
</tr>
<tr>
<td>GAL_7</td>
<td>GACCGCACAAATCTGCC</td>
<td>TGAAGGACAGACGCAACC</td>
<td>463</td>
</tr>
<tr>
<td>GAL_8</td>
<td>ACTCCGTGAATCAAGTAGC</td>
<td>CTTCATGCTGTCACCCACC</td>
<td>335</td>
</tr>
<tr>
<td>GAL_9</td>
<td>CTTTTCTCTATATGCAGGACCTC</td>
<td>ATGCTCAGCCTCTTTTAT</td>
<td>377</td>
</tr>
<tr>
<td>GAL_10</td>
<td>TGAGGCTCTCTGCTTACACA</td>
<td>AGCACCCCTGTTGACGAGC</td>
<td>437</td>
</tr>
<tr>
<td>GAL_11</td>
<td>GCTAGAGGCACTGAGACAG</td>
<td>CTGTCTACCCCTCTGTCG</td>
<td>299</td>
</tr>
<tr>
<td>GAL_12</td>
<td>TCCAGGACACAGGGACAGAAC</td>
<td>AATAGCAACAGCAGATGACG</td>
<td>378</td>
</tr>
<tr>
<td>GAL_13</td>
<td>GACTGCTCTCGTCTGGTCTCAGC</td>
<td>GGCCTACCAGTGAGGAG</td>
<td>366</td>
</tr>
<tr>
<td>GAL_14</td>
<td>AATCCACAGCAGCTACTCACT</td>
<td>GTCTGACAGTTGCTGTCTG</td>
<td>426</td>
</tr>
</tbody>
</table>

**Table 4.2: Primers used for sequencing of exons of GLB1 gene.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLB_1</td>
<td>TGAGCAAGGCGGAGGGGCTGCG</td>
<td>GTGGCTCAGGCCCCCAGGCCGGTT</td>
<td>276</td>
</tr>
<tr>
<td>GLB_2</td>
<td>GCTACTCTCAAAGGATCGGCTTC</td>
<td>GCCACCTGAGAAAAATACAGTTG</td>
<td>325</td>
</tr>
<tr>
<td>GLB_3</td>
<td>AAGGCGCTTCTCCCTCTTAC</td>
<td>CAGGCCCAGTGCCTCTG</td>
<td>377</td>
</tr>
<tr>
<td>GLB_4</td>
<td>ATACCCCTCTTGCTAGAGC</td>
<td>ATTTTGATGGGGGCGAGGTT</td>
<td>347</td>
</tr>
<tr>
<td>GLB_5</td>
<td>ACCAGACCCAAAGACTGAGA</td>
<td>GCACATCTGTCGGTGACATCCT</td>
<td>363</td>
</tr>
<tr>
<td>GLB_6</td>
<td>CCAGCAGTCATTAAGGCTGTGCT</td>
<td>CTCTTGCCACAGATAGCCTCCT</td>
<td>488</td>
</tr>
<tr>
<td>GLB_7</td>
<td>ATCTGACTGCTAAGGGGCTC</td>
<td>AGAATGGCTATGACTCCACTCACA</td>
<td>305</td>
</tr>
<tr>
<td>GLB_8-9</td>
<td>GTTGTAGAAGCTGAGAGTAAACA</td>
<td>GCAACCCCCCTCCAAAATA</td>
<td>512</td>
</tr>
<tr>
<td>GLB_10</td>
<td>AAGGCTACGGCTGTGCTTCC</td>
<td>GTCTAGCTGATGCTCCTCCTC</td>
<td>392</td>
</tr>
<tr>
<td>GLB_11</td>
<td>GGCACTGTGTTACTTGGACTCT</td>
<td>CAGCAGCAGACTTTACTCCT</td>
<td>273</td>
</tr>
<tr>
<td>GLB_12</td>
<td>ATGGGAGGAGGCTAAGGAGAAGACTG</td>
<td>CACGCCAGCCTTTAAAGCTG</td>
<td>436</td>
</tr>
<tr>
<td>GLB_13</td>
<td>CGCGGAGTGGAGGAAAGATT</td>
<td>AAGATGATGGTGGGCGAGTGG</td>
<td>368</td>
</tr>
<tr>
<td>GLB_14</td>
<td>CCAAGGTCTGGTACACTTCAAGG</td>
<td>GGGCTCCCAGATGCTGAGATTAC</td>
<td>355</td>
</tr>
<tr>
<td>GLB_15</td>
<td>TCTCGAGGTTACCTTCTGTTGG</td>
<td>CCTCTGGAAGAATGCTGCCAAAC</td>
<td>520</td>
</tr>
<tr>
<td>GLB_16</td>
<td>GCCATTTCTTTCTCTCCATTGC</td>
<td>CATTTCACATTTCAATCAGTG</td>
<td>504</td>
</tr>
</tbody>
</table>

**PCl components/reagents**

<table>
<thead>
<tr>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q water</td>
</tr>
<tr>
<td>10X Ex Taq Buffer (Takara, Ohtu, Japan)</td>
</tr>
</tbody>
</table>
For exon 1 of GALNS and GLB1, we used 2X GC buffer I (Takara) as the PCR enhancer because exon 1 of both the genes has a high GC content. Reagents used for the PCR of exon 1 of GALNS and GLB1 are as follows:

<table>
<thead>
<tr>
<th>PCR components/reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q water</td>
<td>1.125</td>
</tr>
<tr>
<td>2X GC Buffer I (Takara)</td>
<td>6.25</td>
</tr>
<tr>
<td>dNTP Mixture (Takara, 2.5 mM of each dNTP)</td>
<td>2</td>
</tr>
<tr>
<td>Forward primer (100 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer (100 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>TaKaRa LA Taq (Takara, 5 U/µl)</td>
<td>0.125</td>
</tr>
<tr>
<td>Genomic DNA (100 ng/µl)</td>
<td>1</td>
</tr>
</tbody>
</table>

The thermal parameters for exons 2, 3, 6, 7, 8, 9, 10, 11, 12, 13 and 14 of GALNS consisted of initial denaturation at 94°C for 5 min, 35 cycles of amplification at 94°C for 45 s, annealing at 63°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. For exons 1, 4 and 5, of GALNS, the thermal profile was 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, annealing at 63°C for 45 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Optimal thermal conditions for exons 1, 2, 3, 4, 5, 7, 10, 11, 12, 13 and 14 of GLB1 were attained by an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, annealing at 61°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 10 min. For exons 6, 8-9, 15 and 16, thermal parameters were 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, annealing at 63°C for 45 s and 72°C for 45 s, followed by a final extension at 72°C for 10 min.

4.6.1. Agarose gel preparation

Depending on the PCR product size, agarose was weighed and poured into 100 ml of 1X TBE buffer in a conical flask. The mixture was heated in the microwave until the agarose
dissolved. After dissolving, it was allowed to cool down to around 50-45 °C. Once it cooled down, 4 µl of Etbr was added and mixed well. The gel tray was cleaned, sealed and a comb was placed in the slot. The melted agarose solution was poured in to the tray and allowed to solidify for 25 - 30 min. The tray was placed in to the electrophoretic tank containing 1X TBE buffer and the comb was carefully removed. Samples were mixed with 6X loading dye and loaded in to the gel wells. The gel was run for approximately 20 - 30 min or till the dye traveled down 3/4\(^{th}\) of the gel. Gel images were viewed under a UV trans-illuminator.

4.6.2. Purification of PCR products by gel elution

The PCR products were eluted and purified by the following protocol as described by Sambrook and Russell (Sambrook and Russell, 2006).

1) Agarose gel of certain concentration depending on the product size was prepared as mentioned above with well sizes good enough to load approximately 20 µl of the sample.

2) The electrophoresis was run for around 30 - 45 min or till the tracking dye reached 3/4\(^{th}\) of the gel.

3) After the tracking dye reached 3/4\(^{th}\) of the gel, gel was kept on UV trans-illuminator and the specific fragments were cut using a sharp surgical blade.

4) The chopped pieces were transferred into a microfuge tube.

5) 300-400 µl buffer saturated phenol pH 7.4 was added to the microfuge tube. The tube was kept at - 20 °C overnight or at - 80 °C for 2 - 3 hr.

6) After overnight incubation, the tube was thawed completely and 200 µl of milli-Q water was added. The contents of the tube was mixed well and centrifuged at 12,000 rpm for 10 min at 4 °C.

7) The supernatant was transferred to another microfuge tube and an equal volume of 24:1 (v/v) chloroform Isoamyl alcohol was added. The mixture was then mixed for around 10 min by inverting by hand or in rotospin and then centrifuged as done above.

8) After centrifugation, the supernatant was transferred to another microfuge tube and 1/10\(^{th}\) of its volume of 3M sodium acetate and double its volume of 100% chilled ethyl alcohol were added.
9) The solution was mixed by inverting the tube and the tube was kept at -20 °C overnight or at -80 °C for 2 - 3 hr.
10) The next day or after 2 - 3 hr of -80 °C incubation, the tube was centrifuged at 12,000 rpm for 15 min at 4 °C to obtain the pellet.
11) The supernatant was discarded and 500 µl of 70% ethanol was added.
12) The pellet was dislodged and then centrifuged as mentioned above.
13) The supernatant was completely discarded, the pellet was allowed to semi dry and 10 µl of milli-Q water was added.
14) The contents of the tube was mixed properly through a short spin in the centrifuge and allowed to dissolve at room temperature for 1 - 2 hr.
15) After dissolving, the purified PCR product was quantified in a spectrophotometer.

### 4.6.3. Sanger sequencing of the purified PCR product

After purification of the PCR product, Sanger sequencing was performed as per the following steps:

Sequencing PCR was carried out in a reaction volume of 10 µl using the following reagents.

<table>
<thead>
<tr>
<th>PCR components/reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers (forward or reverse)</td>
<td>1 (20 ng/µl)</td>
</tr>
<tr>
<td>Purified DNA</td>
<td>1 (approximately 40-50 ng/µl)</td>
</tr>
<tr>
<td>ABI reaction mix (Life Technologies)</td>
<td>0.5</td>
</tr>
<tr>
<td>ABI dilution buffer (Life Technologies)</td>
<td>1.75</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>5.75</td>
</tr>
</tbody>
</table>

Sequencing PCR was performed on the thermocycler (Life Technologies) and with the thermal profile of initial denaturation at 96 °C for 1 min, then 25 cycles at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min.

1) After PCR, 2 µl of 125 mM EDTA, 10 µl of milli-Q water and 50 µl of 100% absolute alcohol were added.
2) PCR tube was incubated at room temperature for 15 min in dark.
3) The tube was then centrifuged at 12,000 rpm for 15 min at 4 °C.
4) After centrifugation, supernatant was discarded and 150 µl of 70% ethanol was added to the pellet.
5) The tube was again centrifuged at 12,000 rpm for 10 min at 4 °C and supernatant was discarded and pellet was semi dried.

6) 12 μl of formamide was added and the tube was incubated at room temperature for 30 min in dark.

7) After incubation, denaturation was done at 95 °C for 3 min and then the tube was immediately kept on ice.

8) The sample was loaded in the sequencer sample plate and run on ABI Prism 3130 Genetic Analyzer (Life Technologies).

4.7. Sequence analysis

1) The sequences were obtained from ABI Prism 3130 Genetic Analyzer and analyzed by comparing with the references sequences of GALNS gene; NM_000512.4 and GLB1 gene; NM_000404.2.

2) The variations identified were confirmed with the current literature and HGMD to check whether the variations were novel or reported previously.

3) The novel variations were checked in the Exome Aggregation Consortium (ExAC) browser, 1000 genome browser (Abecasis et al., 2010), and 537 Indian next generation sequence data from Strand Life Sciences to confirm whether the novel variations were pathogenic or neutral polymorphisms.

4) All the mutations identified in this study were confirmed by bidirectional sequencing of the exons harboring the mutations.

5) Whenever possible, parental DNA was checked for carrier status by sequencing of exons carrying the mutations.

6) The identified exons harboring the novel mutations were also sequenced in 50 (100 chromosomes) healthy unrelated Indian individuals.

4.8. Bioinformatics analysis for GALNS and GLB1 mutations

In order to analyze the putative causal and functional impact of all the novel missense mutations identified in this study, each novel mutation was evaluated using the following in silico prediction tools: Mutation Taster (Schwarz et al., 2010), SIFT (Kumar et al., 2009), PolyPhen-2 (Adzhubei et al., 2010) and PROVEAN (Choi et al., 2012).

Complete human GALNS and GLB1 gene sequences were downloaded from ensembl GALNS: ENST00000268695 and GLB1: ENST00000307363. Using the Expasy translate
tool (http://web.expasy.org/translate/), *in silico* translation of cDNA sequences of **GALNS**: NM_000512.4 and **GLB1**: NM_000404.2, containing deletion, insertion and nonsense classes of mutations were performed. Conservation of the GU donor site (5' end of the intron), branch site containing A (near the 3' end of the intron) and AG acceptor site (3' end of the intron) were examined in case of splice mutations. In addition, the impact of splice mutations in the **GALNS** and **GLB1** genes on pre-mRNA splicing was assessed by MaxEntScan (Yeo and Burge, 2004).

The homologues for Human **GALNS** (NP_000503.1) and **GLB1** (NP_000395) proteins were identified using PSI-BLAST (Altschul et al., 1990). Among the homologues, only those with a minimum of 95% sequence coverage and 80% sequence identity were chosen and subjected to Multiple sequence alignment (MSA) with **GALNS** and **GLB1** using clustalW (Larkin et al., 2007). The MSA was used for investigating the conservation of amino acid residues at all the missense mutation sites.

The structural analysis of the novel missense mutations was carried out using the available crystal structure of human **GALNS** (PDB code: 4FDI) (Rivera-Colón et al., 2012). The structural features viz., % solvent accessibilities and hydrogen bonds of the relevant amino acid residues were computed using the programs PSA and HBOND respectively, available as a part of JOY suite (Mizuguchi et al., 1998). Swiss-PDB viewer (SPDBV) (Guex and Peitsch, 1997) was used to aid in the structural analysis and for modeling some of the missense mutations. The residues that lose even 1% of their solvent accessible surface areas due to dimer formation were identified as the residues at the dimer interface. Gribskov scores (Gribskov et al., 1987) were calculated using Emboss - prophecy tool (http://emboss.sourceforge.net/) for the wild-type and mutated residues at the missense mutation sites.