MATERIALS AND METHODS

4 MATERIALS AND METHODS

The present study was carried out at the FRIGE's Institute of Human Genetics, Ahmedabad, Gujarat, India.

4.1 Study subjects

4.1.1 Patients and Controls

The patients included in the present study were referred from different Pediatric and Genetic clinics of India. The patients were evaluated by a clinician/pediatrician and referred to the institute for investigation of various lysosomal storage disorders (LSDs). The patients with clinical signs and symptoms of hepatomegaly, splenomegaly, anemia and thrombocytopenia with or without bone abnormalities have been included in the study. On the basis of CNS involvement they are further classified in three groups that is type-I (non neuronopathic), type-II (acute neuronopathic) and type-III (subacuteneuronopathic). Clinical details were noted in a case record form (CRF) (Annexure-I) and an informed written consent from parents (Annexure-II) was obtained for each recruited patienteither from patients or by their legal guardians as per the protocol approved by the IEC (Institutional Ethics committee) as per Helsinki declaration. Normal healthy controls were also enrolled in the present study for biochemical as well as molecular analysis. This are the group of patients used to standardize the range and are used to compare the results obtained.

4.1.2 Sample size

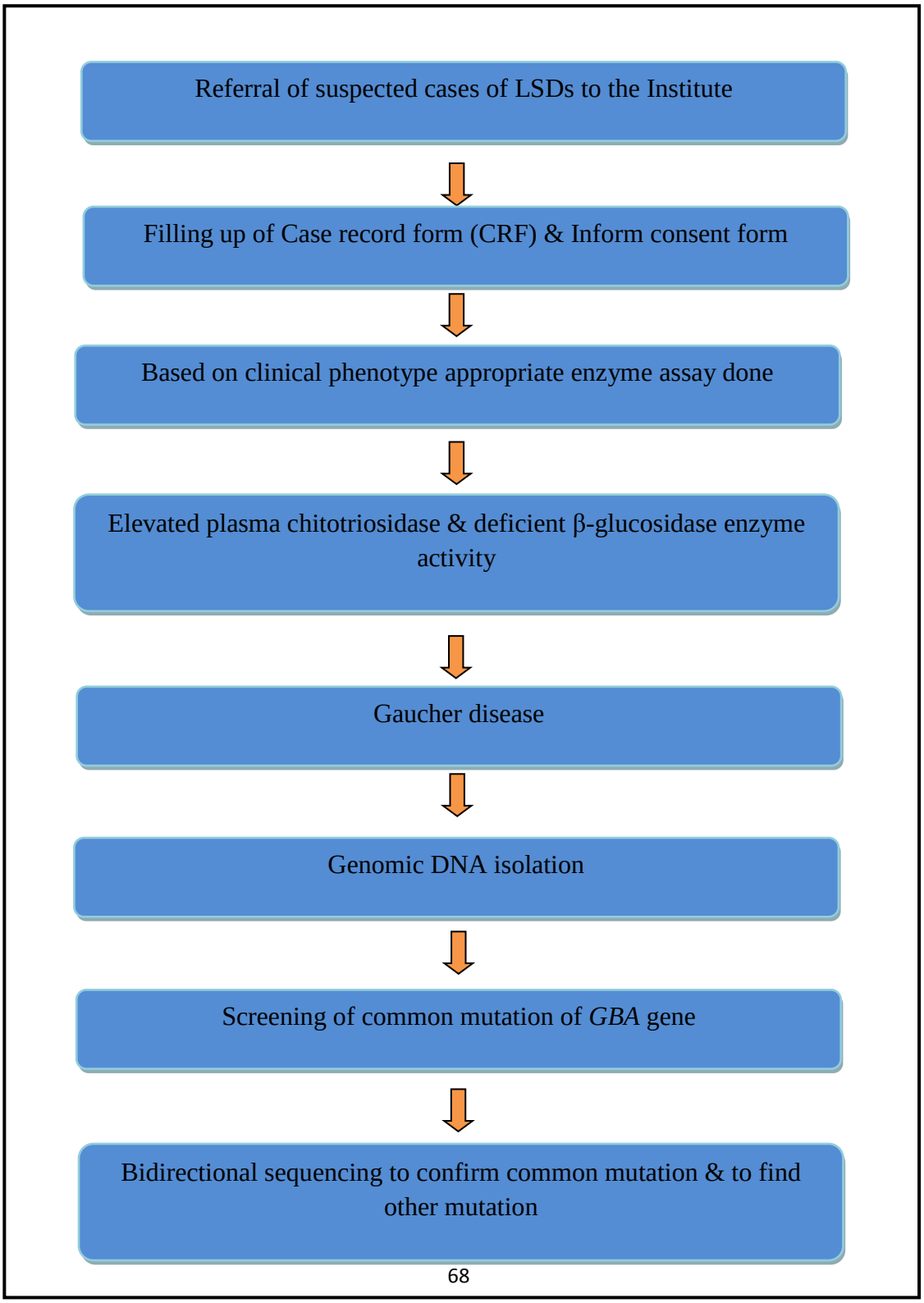
At the institute, we have investigated nearly 747suspected cases for different LSDs based on clinical presentation referred to the Institute during August2010 to March 2014. Plasma chitotriosidase screening and β-glucosidase was carried out in the patients suspected of GD symptoms like hepatomegaly, splenomegaly, anemia and thrombocytopenia with or without bone abnormalities. From these, 50cases of GD were detected. In present study, molecular analysis of the confirmed GD patients was carried out.Carrier analysis was also carried out in 6 parents. In these carrier parent's, analysis was carried as probandwas not alive but was diagnosed as GD earlier by enzyme study. Prenatal diagnosis was carried out in 1case where mutation was known

in index case.

4.1.3 Ethics Statement

The study protocol was approved by the Institutional ethics committee of Foundation for Research in Genetics and Endocrinology (FRIGE), Ahmedabad, Gujarat (Registration No-E/13237). (Annexure-III)

4.2 Work Plan



4.3 MATERIALS

4.3.1 Sample types

Four millilitre (4 ml) peripheral blood was collected in sodium heparin and/or EDTA vacutainer from all the patients for leucocytes enzyme assay and 3 ml blood in an EDTA vacutainer for DNA extraction. Chorinic villus sample (CVS) or amniotic fluid (AF) was used for prenatal diagnosis.

4.3.2 Buffers and Reagents

All the buffers and reagents were prepared in autoclaved sterile de-ionized water as below. Buffer pH was adjusted, at ~25°C, with standard calibrations.

4.3.2.1 Leucocyte Separation

(i) 0.9% sodium chloride (normal saline-NS)

Ready to use from Claris Otsuka Pvt. Ltd. (Cat. No-1050002540)

(ii) Acid-citrate-dextran solution (ACD)

Dissolve 24.5 gm anhydrous dextrose (Qualigens, Cat. No-24415, MW 180.16), 22.0 gm trisodiumcitrate dehydrate (Qualigens, Cat. No-14005/1, MW 294.1) and 8.0 gm citric acid monohydrate (Qualigens, Cat. No-22595, MW: 210.14) in 1L normal saline. 1L stock and a smaller aliquot in reagent bottle kept at 4°C in the laboratory refrigerator.

(iii) 6% dextran in 0.9% sodium chloride

Dissolve 60 gm dextran (Acros Organics, Cat No. 406261000, High fraction) in 1 L normal saline.

(iv) 5% D-glucose in 0.9% sodium chloride

Dissolve 50 gm glucose (Qualigens, Cat. No-24415, MW: 180.16) in 1 L normal saline

(v) 3.6% sodium chloride

Dissolve 3.6 gm NaCl (Nice Chemicals Cat. No. S12229, MW 58.44) in 100 ml

water.

4.3.2.2 Protein estimation by Lowry method

(i) Bovine serum albumin (BSA) standards (10, 20. 40, 60, 80 and 100 µg/ml)

Prepare top standard by dissolving 25 mg BSA (Hazleton Biologicals; Cat. No. 84-040-025) in 250 ml water (100 µg/ml) and make dilution for 10, 20, 40, 60 and 80 µg/ml. Store in glass bottles at 4°C.

(ii) "Lowry A" solution (2% sodium carbonate in 0.1 N NaOH)

Dissolve 10 gm sodium carbonate, anhydrous pure (Merck; Cat. No.-MG7M571928, MW 105.99) and 2 gm sodium hydroxide pellets (Merck; Cat.No.-QK2Q622532, MW 40.0) in 500ml water. Store at 4°C.

(iii) 2% sodium potassium tartrate

Dissolve 2 gm sodium potassium tartrate, also called potassium sodium tartrate (Nice Chemicals; Cat. No. S13729, MW 282.22) in 100 ml water. Store at 4°C.

(iv) 1% Copper sulphate

Dissolve 1 gm copper sulphatepentahydrate (Merck; Cat. No. QD2Q620601, MW 249.68) in 100 ml water. Store at 4°C.

(v) FolinCiocalteu's phenol reagent

Ready to use (Sigma; Cat. No. F9252) Store at room temperature.

4.3.2.3 Plasma chitotriosidase enzyme assay

(i) 0.2M Phospate-citrate buffer, pH is 5.2:

Prepare 0.2M disodium hydrogen phospatedihydrate by dissolving3.56 g (analar, BDH cat. No. 10383, MW 179.99) in 100ml water. Prepare0.1M citric acid by dissolving 2.101g (analar, BDH cat. No. 10081, MW 210.14) in 100ml water. Mix 53.6 ml of the former with 46.4 of the later and check that the pH is 5.2. If not adjust with one or other solution.

(ii) 0.022mM 4-methyl umbelliferylβ-D-N, N, N′, N′′- triacetylchitotriosidase in

buffer

Prepare 0.22mM stock solution by dissolving 4-Mu-triacetylchitotriosidase (Sigma cat. No. M5639, MW 785.6) in 0.2M Phospate-citrate buffer to a concentration of 1.728 mg/10 ml (add the appropriate volume of buffer to an accurately weighed amount of substrate). Aliquot in to 0.5 ml amounts and stored frozen. On the day of an assay thaw an aliquot and dilute 1:10 by adding 4.5 ml 0.2M Phospate-citrate buffer.

(iii) 0.5M glycine – NaOH buffer, pH 10.3

Dissolve 37.53 g glycine (Analar, BDH cat. No. 10119, MW 75.07) in about 800 ml water. Adjust at the pH meter to pH 10.3 using 2M NaOH and make up to 1 l with water.

4.3.2.4 β-glucosidase enzyme assay

(i) 0.54M phosphate-citrate buffer, pH 5.5

(ii) 10mM 4-methylumbelliferyl-β-D-glucopyranoside

(iii) 0.5M glycine-NaOH buffer, pH 10.3

Dissolve 37.53 g glycine (Analar, BDH cat. No. 10119, MW 75.07) in about 800 ml water. Adjust at the pH meter to pH 10.3 using 2M NaOH and make up to 1 l with water.

4.3.2.5 DNA Isolation

(i) TE Buffer, pH 8.0

1 M TrisHCL (pH 8.0) (Sigma, Cat. No. T6066, MW 121.1), 0.5 M EDTA (pH 8.0) (Sigma, Cat. No. E5134, MW 186.1) in about 800 ml deionized distilled water. Adjust to pH 8 and make up to 1 L with distilled water.

(ii) RBC lysis buffer, pH 7.6

10mM Tris HCL (pH=8.0) (Sigma, Cat. No.-T6066, MW 121.1), 5mM $Mgcl_2$ (Merck; Cat. No. MK7M561509), 1% Triton 100 (Sigma; Cat. No. X100), 0.032 M sucrose (Sigma; Cat. No. 84097, MW 342.3) in about 800 ml deionized distilled

water. Adjust to pH 7.6 and make up to 1L with deionized distilled water.

(iii) DNA extraction buffer (DEB)

1 M TrisHCL (pH 8.0) (Sigma, Cat. No. T6066, MW 121.1), 0.5 M EDTA (pH 8.0) (Sigma, Cat. No. E5134, MW 186.1), 10% (w/v) Sodium dodecyl sulphate (SDS) (S. D. Fine chemicals; Cat. No. 40175, MW 288.38), 10% N-Lauryl Sarcosine(Sigma; Cat. No. L5125, MW 293.38) in 1 L deionized distilled water

(iv) Proteinase-K (10mg/ml)

10 mg Proteinase-K (Sigma; Cat. No. P2308) in 1 ml deionized distilled water

(v) 5M Sodium chloride (NaCl)

29.22 gm Sodium chloride (NaCl) (Nice Chemicals Cat. No. S12229, MW 58.44) in 100 ml deionized distilled water

(vi) 70% Ethanol

70 ml absolute ethanol and make up the volume to 100 ml using autoclaved deionized distilled water. Store it at 4°C

4.3.2.6 PCR (Polymerase chain reaction)

(i) PCR buffer

First dissolve Gelatin (Gelatinfrom porcine skin, Type-A Cell culture tested; Sigma, Cat. No. G1890) in 3.2 ml triple distilled water (Incubate at 37°C to dissolve Gelatin), add 1.25 ml 2M KCl, 0.5 ml 1M Tris (pH 8.3) and 75 µl 1M $MgCl_2$.

(ii) Ethidium bromide ~95% (HPLC) (Etbr)

1 mg Ethidium bromide (Sigma; Cat. No. E8751, MW 394.31) in 1 ml deionized distilled water

(iii) Gel loading dye

25 mg of Xylene cyanol and 25 mg of Bromophenol blue dissolve in 6.25 ml of deionized distilled water, add 1.25 ml of 10% SDS and 12.5ml of glycerol.

(iv) TAE buffer

2 M TrisHCL (Sigma, Cat. No. T6066, MW 121.1), 50 mM EDTA (Sigma, Cat. No. E5134, MW 186.1) in about 80 ml deionized distilled water. Adjust to pH 8.0-8.3 with glacial acetic acid (Merck (approximately 5.71 ml)) and make up to 100 with distilled water.

4.3.3 Laboratory equipments used

- Cooling centrifuge (R8C, Remi, India)
- Centrifuge (R8C and R24C, Remi, India)
- Table top centrifuge (iFUGE M08VT, Neuaton, India)
- Sonicator (Q55, QSoniaca, India)
- Cyclo mixer (CM-101, Remi, India)
- Digital water bath (CIC-3, Cintexindustrial Corporation, India)
- Spectrophotometer (Shimandzu 1770 UV/VIS, USA)
- Fluorescence spectrometer (LS55, PerkinElmer, USA)
- Thermal cycler 2720 (Applied Biosystems, USA)
- Thermal cycler gradient (MJ Mini, Bio-Rad, USA)
- QIAxpert system (QIAGENE,USA)
- Gel electrophoresis apparatus (Bangalore Genei, India)
- Power pack (Bangalore Genei, India)
- Molecular E-Gel Imager system (Life technologies, USA)
- ABI PRISM ® 3130 Genetic Analyzer (Applied Biosystems, USA)

4.3.4 Bioinformatics tools used

- Pubmed: http://www.ncbi.nlm.nih.gov/pubmed/
- OMIM: http://www.ncbi.nlm.nih.gov/omim/
- GenBank (Entrez Gene): http://www.ncbi.nlm.nih.gov/Genbank/
- SNP database: http://www.ncbi.nlm.nih.gov/SNP/index.html
- Reference Sequences: http://www.ncbi.nlm.nih.gov/refseq/
- ENSEMBL: http://www.ensembl.org/index.html
- Sequence Analysis: http://www.ncbi.nlm.nih.gov/guide/sequence-analysis/
- BLAST: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

- Primer3web version 4.0.0: http://primer3.ut.ee/
- NEBcutter version 2.0: http://tools.neb.com/NEBcutter2/47
- Restriction mapper version 3.0:http://www.restrictionmapper.org/
- Human Gene Mutation Database: http://www.hgmd.cf.ac.uk
- SIFT (Sorting Intolerant From Tolerant): http://sift.jcvi.org/
- Polyphen2 (Polymorphism Phenotyping v2): http://genetics.bwh.harvard.edu/pph2/
- Mutation Taster: http://www.mutationtaster.org/

4.4 Methods

4.4.1 Biochemical analysis

4.4.1.1 Leucocyte separation procedure for postnatal diagnosis

- Leucocytes were isolated from heparinised/EDTA peripheral blood by differential sedimentation at unit gravity in a dense isotonic dextran solution (Skoog and Beck, 1956; Magalhaes et al., 1984b).
- The cells were washed briefly in 0.9% saline to haemolyse the red cells, and then brought back to isotonicity with 3.6% saline.
- Approximately 3-4 ml of blood was centrifuged at 2000 × g for 10 min at 2-8℃.
- The plasma was removed carefully, leaving the buffy coat undisturbed and stored. The leucocytes containing blood was transferred to 50 ml tube; add double volume of mixture containing 1.5 parts of Acid citrate dextran (ACD), 6 parts of 6% Dextran (6%) and 3.5 parts of 5% Glucose (eg. 5 ml blood sample + 10 ml mixture).
- Tubes were mixed gently to avoid bubbles. The tubes were allowed to stand at room temperature for 45 minutes to 1 hour.
- The leucocytes containing supernatant was transferred to a 15 ml tube and centrifuged at 2000 × g for about 10 min at 2-8℃.
- The pellet was washed (the pellet resuspended in 0.8 ml of 0.9% saline and 2.4 ml chilled distilled water, mixed gently by pipetting, after precisely 2 min, 0.8 ml of 3.6% saline was added and mixed gently before centrifuging at 2000 × g for about 10 min at 2-8℃).
- The pellet was washed again to obtain white cell pellet free of red cells.

- All the supernatant was removed and the pellet was frozen at -20℃ until assayed (usually performed within a week).

4.4.1.2 Extraction of cells

- Cell pellets, (leukocytes), were suspended in 100-400 µl double distilled water.
- Cells were extracted by sonication with 20 amplitudes of 5 second, 50 watts, with pauses of 3 second (QSONICASonicator Q55).
- The homogenate was kept for 30-45 min at room temperature and the supernatant (referred to as extract) was collected.
- All these procedures were performed at 4°C.
- The resulting supernatant, referred to as homogenate, served as the source of enzyme in all assays.

4.4.1.3 Estimation of protein concentration in leucocytes using Lowry method

- Protein was measured according to modified Lowry's method (Lowry et al., 1951; Herbert et al., 1974).
- A series of Bovine serum albumin (BSA) standards (0.1 mg/ml to 1.0 mg/ml) was used.
- Final sample protein concentration was adjusted to 2–10 mg/ml and was employed for enzyme studies.

4.4.1.4 Measurement of plasma chitotriosidaseactivity using fluorescencespectrophotometer

Fluorometric assays were performed using 4-methylumbelliferyl (4-MU) derivative artificial substrates, as the procedures described by Galjaard (1980). Enzyme activity was determined by fluorimetric method using specific synthetic substrate (Shapria et al., 1989). Plasma chitotriosidase activity was measured from the hydrolysis of the synthetic substrate 4-methylumbelliferyl triacetylchitotriosidase at acid pH is followed by measuring the fluorescence of the liberated 4-methylumbelliferone after stopping the reaction with alkaline buffer.

- All the samples were tested in duplicates. The blanks were freshly prepared at each run. All tubes of samples and blanks were kept in ice.

- In all tubes of test and blanks, add 200µl of 0.022mM 4-methyl umbelliferylβ-D-N, N, N′, N′′-triacetylchitotriosidase.
- Then add leucocytes sample (10 µl) for postnatal diagnosis while CT cells (10 µl) and/or AF cells (10 µl) for prenatal diagnosis into the 'test' tube and 10µl distilled water into the 'blank' tube.
- Incubate the tubes in waterbath for 30 min at 37℃.
- The reaction was stopped by the addition of 3 ml glycine-NaOH buffer (pH 10.7).
- The relative fluorescence of each tube was read on the Perkin-Elmer LS-55 fluorescence spectrometer with excitation wavelength/slit width (366 nm/25 nm), and the emission wavelength/slit width (446/2.5 nm).
- The relative fluorescence readings (RF) were corrected by subtracting the blank reading from each tube and enzyme activity calculated as nanomoles per hour per milligram of protein using the following equation.

$$\text{Specific enzyme activity} = [\text{Sample} - \text{Bl}] \times \frac{2.5}{10} \times 4 \times \left(\frac{1.5 + 0.21}{1.6}\right) \times \frac{1000}{10}$$

4.4.1.5 Measurement of β-glucosidase activity using fluorescencespectrophotometer

Fluorometric assays were performed using 4-methylumbelliferyl (4-MU) derivative artificial substrates, as the procedures described by Galjaard (1980). Enzyme activity was determined by fluorimetric method using specific synthetic substrate (Shapria et al., 1989). β-glucosidase enzyme activity was measured by the hydrolysis of the synthetic substrate 4-methylumbelliferyl-β-D-glucopyranoside at acid pH and in the presence of sodium taurocholate (which inhibits non-specific enzyme activity) is followed by measuring the fluorescence of the liberated 4-methylumbelliferone after stopping the reaction with alkaline buffer.

- All the samples were tested in duplicates. The blanks were freshly prepared at each run. All tubes of samples and blanks were kept in ice.
- In all tubes of test and blanks, add 110 µl of 10mM 4-methylumbelliferyl-β-D-glucopyranoside and 50µl phosphate-citrate buffer (4.1)

- Then add leucocytes sample (20 µl) for postnatal diagnosis while CT cells (20 µl) and/or AF cells (20 µl) for prenatal diagnosis into the 'test' tube and 20µl distilled water into the 'blank' tube.
- Incubate the tubes in waterbath for 30 min at 37℃.
- The reaction was stopped by the addition of 3 ml glycine-NaOH buffer (pH 10.7).
- The relative fluorescence of each tubes was read on the Perkin-Elmer LS-55 fluorescence spectrometer with excitation wavelength/slit width (366 nm/25 nm), and the emission wavelength/slit width (446/2.5 nm).
- The relative fluorescence readings (RF) were corrected by subtracting the blank reading from each tube and enzyme activity calculated as nanomoles per hour per milligram of protein using the following equation.

$$\text{Specific enzyme activity} = [\text{Sample} - \text{Blank}] \times \frac{2.5}{10} \times \frac{(1.5 + 0.18)}{(1.5 + 0.01)} \times \frac{1000}{A}$$

= ___nmol/h/mg protein

A = µg of protein/20 µl sample

4.4.2 Molecular Analysis

4.4.2.1 Genomic DNA Extraction

DNA was isolated by standard salting out method (Miller et al., 1998). The cell suspension was lysed by detergents (SDS) in the presence of high concentrations of chelating agent EDTA. Deproteinization was obtained with Proteinase-K treatment. Ethanol was used to precipitate DNA and dissolved in TE buffer.

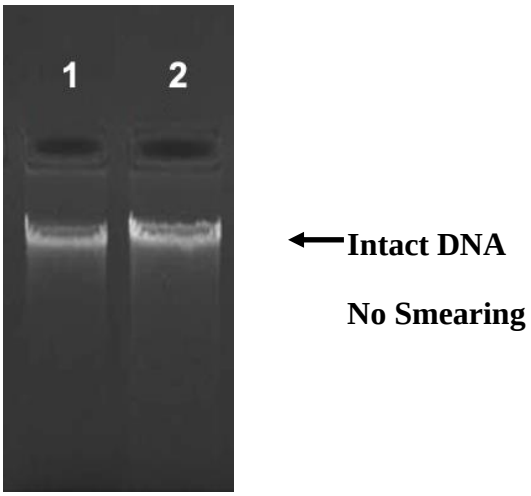
4.4.2.2 Measurement of purity and concentration of isolated DNA

Purity of the isolated DNA was measured by taking ratio of optical density [OD or also known as Absorbance (A)] at 260 nm and 280 nm. If OD_{260} /OD_{280} ratio was within 1.6 to 1.8, then the DNA was considered as pure and was used for further experiments.

Concentration was measured by taking standard as OD_{260} =1 for 50µg/ml dsDNA (Double stranded DNA). Thus the DNA concentration in µg/ml was calculated by the formula = A 260 of experimental DNA × dilution factor × 50.

4.4.2.3 Checking the integrity of DNA

Agarose gel electrophoresis of DNA: High molecular weight DNA was checked for its integrity. DNA was run on 2% ethidium bromide (EtBr) stained agarose gel to check its integrity. Electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook et al., (1989). DNA samples (200 ng) were mixed with 1.0 µl of the DNA gel loading buffer. The gel was run under constant voltage (100 V) for 30 minutes. The bands were viewed under ultra violet (UV) light in gel documentation system (Figure 4.1).



Lanes 1- 2 showing yield of intact DNA

Figure 4.1: Agarose gel of genomic DNA extracted from whole blood

4.4.2.4 Screening Procedure for Common Mutations by PCR

Mutation screening was carried out for the common mutant allele N370S (c.1226A>G), L444P (c.1448T>C), R463C (c.1504C>T) and Ivs2 (+1) G>A) by PCR-RFLP (Restriction fragment length polymorphism) using forward and reverse primer.

4.4.2.4.1 Primer designing

Primers were designed using online software Primer3web version 4.0.0 (http://primer3.ut.ee/). All primers selected were checked for all essential parameter as per universal standard guideline (e.g. primer length, GC content, Tm, complimentary of primer etc.) (Abd-Elsalam, 2003) and use for PCR consistency. The forward and reverse primers designed are as shown in Table 4.1.

4.4.2.4.2 PCR Reagents and conditions

All PCR reactions were performed in thermal cycler 2720 (Applied Biosystem, USA) and thermal cycler gradient (MJ Mini, Bio-Rad, USA) using 0.2 ml thin-walled PCR tubes. PCR reactions were carried out in total volume of 20ml, containing 250ng of genomic DNA, 20 pmol of each primer, 10x Cetus buffer, dNTPs (2 mM) and 1 U Taq polymerase with negative control included. The PCR conditions for N370S (c.1226A>G), L444P (c.1448T>C), R463C (c.1504C>T) and Ivs2 (+1) G>A)as shown in Table 4.2.

4.4.2.4.3 Restriction enzyme (RE) selection

The MspI, AvaI and HpHI restriction enzyme (RE) was selected using online softwereNEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) and/or Restriction Mapper version 3 (http://www.restrctionmapper.org/).

All the patients who were found negative after screening using these PCR based techniques were later analyzed by DNA sequencing of exons to rule out the presence of other mutations in the exonic region which cannot be analyzed using PCR based techniques.

Table 4.1: Primers for identification of common mutations

Mutations	Primer Sequences (5'-3')	Restriction sites	Size (bp)
L444P (c.1448T>C)	F.P. 5' -CTGAACCCCGAAGGAGGACC-3' R.P. 5' -GGGCTTACGTCGCTGTAAGCTCACAC**C**GGC-3'	MspI	931
R463C (c.1504C>T)	F.P 5' -CTGAACCCCGAAGGAGGACC-3' R.P 5' -GGGCTTACGTCGCTGTAAGCTCACAC**C**GGC-3'	MspI	931

N370S (c.1226A>G)	F.P 5'-TGTCTCTTT GCCTTTGTCCTTACCCT**C**GA-3' R.P 5' -GACAAAGTTACGCACCCAATT-3'	AvaI	115
IVS2 + 1G>A (c.115 +G>A)	F.P 5'-GCATCATGGCTGGCAGCCTCACAGGACTGC-3' R.P 5'-GCCCAGGCA ACAGAGTAAGACTCTGTTTCA-3'	HpHI	255

L444P- Bold base(C) in reverse primer altered to create MspI site (underlined)
N370S- Bold base C in forward primer is the base altered to create Ava1 site(Underlined)

Table 4.2: PCR conditions for common mutations

Mutations	PCR conditions	PCR cycles
L444P (c.1448T>C)	Initial Denaturation 95°C ,5mins	1 cycle
	Denaturation 95°C ,45 s Annealing 61°C ,45 s Extension 72 °C ,45 s	35 cycle
	Final elongation 72 °C ,10 mins	1 cycle
R463C (c.1504C>T)	Initial Denaturation 95°C ,5mins	1 cycle
	Denaturation 95°C ,45 s Annealing 61°C ,45 s Extension 72 °C ,45 s	35 cycle
	Final elongation 72 °C ,10 mins	1 cycle
N370S (c.1226A>G)	Initial Denaturation 95°C ,5mins	1 cycle
	Denaturation 95°C ,45 s Annealing 61°C ,45 s Extension 72 °C ,45 s	35 cycle
	Final elongation 72 °C ,10 mins	1 cycle
IVS2 + 1G>A (c.115 +G>A)	Initial Denaturation 95°C ,5mins	1 cycle
	Denaturation 95°C ,45 s Annealing 68°C ,45 s Extension 72 °C ,45 s	35 cycle
	Final elongation 72 °C ,10 mins	1 cycle

Table 4.3: Restriction Digestion of PCR product

Component	Volume (µl)
PCR product	10.0
Restriction Enzymes (5U/ µl)	1.0
Total:	11.0

Digest at 37^0C for 3hrs or overnight as shown in Table 4.3.

4.4.2.4.4 Electrophoresis of PCR amplicons

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook et al., (1989). Electrophoresis of all PCRamplicon was carried out on 2% agarose gel to separate PCR amplicon by molecular size. The agarose was dissolved in 1 X TAE buffer by microwaving on the high setting for 1-2 min followed by cooling to approximately 50 C. Etbr (100 µg/L) was added and the gel left to set for about 30 min before the PCR products were loaded. The PCR products (10 µl) were mixed with 1 µl loading dye (0.25% bromophenol blue, 30% glycerol) and 100bp ladder also loaded to monitor the movement of amplicons in the gel system. This dye runs at approximately 40 bp and therefore did not obscure the amplicon being tested. The electrophoresis was carried out at 100 V for 30 mins in a Bangalore Genei gel electrophoresis system (Bangalore Genei, India) and the gel was visualised using a UV transillumination (λ=302nm) and the image captured on E-Gel imager system (Life technologies, USA).

4.4.2.5 DNA sequencing

DNA sequencing is the process of determining the exact order of the bases A, T, C and G in a piece of DNA. In essence, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by size, and the bases at the end are identified, recreating the original sequence of the DNA. The most commonly used method of sequencing DNA - the dideoxy or chain termination method - was developed by Fred Sanger in 1977. The key to the method is the use of modified bases called dideoxy bases; when a piece of DNA is being replicated and a dideoxy base is incorporated into the new chain, it stops the replication reaction.

The exonic and intronic flanking sequences of the *GBA* gene were PCR amplified into 11 fragments using the primer pairs as described earlier (Ankleshwaria et al., 2014) (Table 4.4). Amplification was performed in ain thermal cycler 2720 (Applied Biosystem, USA) and thermal cycler gradient (MJ Mini, Bio-Rad, USA) using 0.2 ml thin-walled PCR tubes. PCR was performed in a total volume of 20 ml, containing 250 ng of genomic DNA, 10 pmol of each primer, 10x Cetus buffer, dNTPs (2 mM) and 1 U Taq polymerase. The amplification protocol for exons 2-11 as shown in Table 4.5.

PCR products were run on agarose gel to look amplified PCR product visualized under UV transillumination (λ=302nm) and the image captured on a Geldoc system (E-Gel imager, Life technologies, USA) (Figure 4.2). Sequencing of PCR amplicon required purification of the amplicon using Exo-sap treatment. Exo-sap was used to remove extra dNTPs and primer dimmer. This is based on the utilization of two hydrolytic enzymes, Exonuclease I and shrimp alkaline phosphatase which removes unwanted dNTPs and primers. These clean-up PCR products ranging in size from less than 100bp up to over 20kb. The DNA is now ready for direct sequencing using manual or automated methods.

Table 4.4: Primers for DNA sequencing of *GBA* Gene

Exons	**Primer Sequence (5'→3')**	**Fragment Size (bp)**
Exon 2	F.P. 5'-GGAGAGGGGCTTGCTTTTCA-3' R.P. 5'-GGAGGCAGAGGTTGGAATGA-3'	371
Exon 3-4	F.P. 5'- CAAGGGGTGAGGAATTTTGA-3' R.P. 5'-CACCACTGCACTCCTGTCTC-3'	696
Exon 5-6	F.P. 5'-TGGCCCTGACTCAGACACTA-3' R.P-5' -CTGATGGAGTGGGCAAGATT-3'	788
Exon 7	F.P. 5'-GGCTGTTCTCGAACTCCTGA-3' R.P. 5'-ATAGTTGGGTAGAGAAATCG-3'	473
Exon 8	F.P. 5'-AGTTGCATTCTTCCCGTCAC-3' R.P. 5'-ATCATGGTTCCCCAGAGTTG-3'	466
Exon 9	F.P. 5'-CAGCTGCCTCTCCCACAT-3' R.P. 5'-GTGTGCCTCTTCCGAGGTT-3'	381
Exon 10-11	F.P.5'-GAGAGCCAGGGCAGAGCCTC-3' R.P.- 5'-CTCTTTAGTCACAGACAGCG-3'	569

Table 4.5: PCR conditions for exons DNA sequencing

Exons	PCR conditions	PCR cycles
Exon-2	Initial Denaturation 94°C ,4mins	1 cycle
	Denaturation 94°C ,30s Annealing 65°C ,30 s Extension 72 °C ,30 s	33 cycle
	Final elongation 72 °C ,10 mins	1 cycle
Exon 3-11	Initial Denaturation 96°C ,2mins	1 cycle
	Denaturation 96°C, 30 s Annealing 58°C, 30 s Extension 74°C, 60 s	33 cycle
	Final elongation 74°C ,5 mins	1 cycle

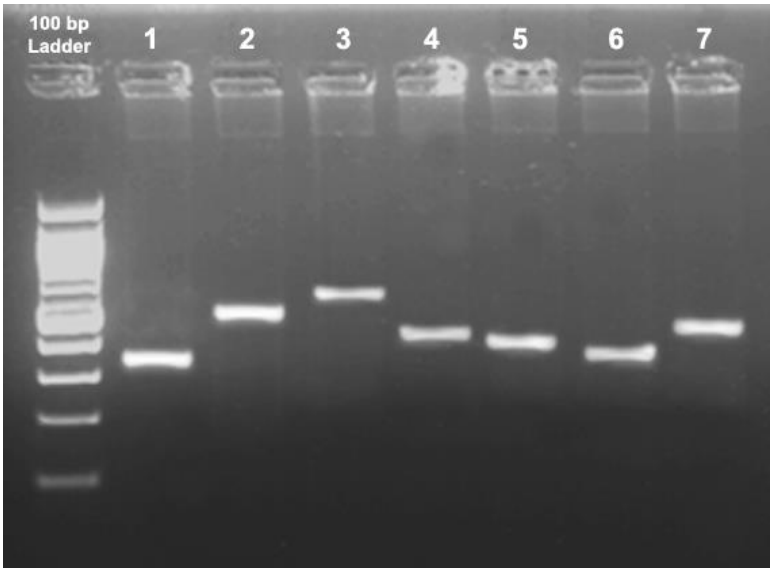


Figure 4.2: Agarose gel of amplified PCR product for *GBA* gene. 100bp Lader as marker & Lane 1-7 showing Exon 2-11 bands, respectively.

Sequencing reaction was performed using 2-5 µl purified PCR product, 3 pmol primer forward or reversed) and 4 µl of ABI Dye 2 Terminator mix in a total volume of 20 µl with nuclease free water. Amplification was performed in a thermal cycler 2720 (Applied Biosystems, CA, USA). Sequencing product was cleaned up by using Sodium acetate and ethanol, dry final pallet at 56 °C in DNA dryer, dissolved in

formamide and run in sequencer (model-3110 from Applied Bio-system). Sequenced alignment was performed using the Blast program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul et al., 1997).

The nucleotide numbers arederived from *GBA*GenBank reference sequence NM_000157.2. Heterozygosity for these mutations was confirmed in parents. The mutations identified were then looked up in public domain of Human Gene Mutation Database (http://www.hgmd.cf.ac.uk).Novel variants were further confirmed for their pathogenicity by using Human Gene Mutation Database (http://www.hgmd.cf.ac.uk) and other bioinformatics tools.

4.4.2.6 Bioinformatics analysis

Various bioinformatics software is used for prediction of effect of sequence variations on the structure and function of protein. This software allows prediction of changes in DNA structure as a consequence of sequence variations as well predict their effect on protein structure and sequenced based on function. The sequence variations are predicted as deleterious or not depending upon their nature e.g. affecting folding, structure, function or conservation. The various methods used for analyzing sequence variations are as follows:

4.4.2.6.1 Bioinformatic tools for predicting non-synonymous single nucleotide substitutions

Prediction of functional effects of non-synonymous single nucleotide substitutions (nsSNPs) was done using various software programs; SIFT (Sorting Intolerant From Tolerant) (available from: http://sift.jcvi.org/), Polyphen2 (Polymorphism Phenotyping v2) (available from: http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (available from: http://www.mutationtaster.org/) (Kumar et al., 2009; Adzhubei et al., 2010; Schwarz et al., 2010) to compare the finding and to confirm the obtained results.

4.4.2.6.2 Protein modeling

The mutants (G289A, I466S) were built using build mutant protocol of Accelrys Discovery studio 2.0 using the 1OGS native and 1OGS mutant PDB structure of acid β-glucosidase. It showed that G289A (c.866 G>C) mutant allele located in β4 strand

and root-mean-square deviation (RMSD) value for superimposition was very small (0.009 Angstrom), which suggests that this mutation has little effect on the structure and mutant allele I466S(c .1397T>G) has created extra turn in α8 helices. Both these mutation were found to destabilize the protein structure.