Chapter -3

Materials and Methods
Ethical approval for the study

The study was approved by the ethical committee of the Institute of Science, Banaras Hindu University. Written informed consent was obtained from the patient or the guardian of the family for the use of their samples, photographs and clinical details for the study. An approval for study on discarded aborted embryonic tissue was also taken from the institutional ethical committee. And a discarded aborted embryonic tissue of ectopic pregnancy was collected after written informed consent.

Subjects

A total of 116 cases with limb defect were recruited for the current study. Cases were recruited at G S Memorial Plastic Surgery Hospital & Trauma Center, Sir Sundarlal Hospital, Institute of Medical Sciences, Banaras Hindu University and Swami Harshankranand Ji Hospital & Research Center, Varanasi, India. These samples were collected within the period of April 2013 to September 2017. The study was approved by the ethical committee of the Institute of Science, Banaras Hindu University (Appendix X). Written consent was obtained from the guardian of the family for the use of their samples, photographs and clinical details for the study (Appendix IX). Detailed clinical history of the patient and their parents were recorded (Appendix VIII). Patient’s clinical history includes age, sex, ethnicity, X-ray photographs of hand and feet, ectodermal problems (hair, nail, teeth etc.), and other congenital anomalies. Clinical history of their parents includes age at the time of pregnancy, complication and infection during pregnancy (Tuberculosis, rubella virus, CMV, herpes simplex virus (HSV) and others), abortion history and familial history of congenital defect. All patients belonged to Eastern Uttar Pradesh and the contiguous regions of Bihar, Jharkhand and Chhattisgarh. By the patients’ clinicians 3 to 5 ml peripheral blood samples were drawn in heparinized syringe under complete aseptic condition from patients and their parents after obtaining a written consent from the patient or their parents. For the familial cases blood samples were also collected from other affected and unaffected members of the family. After written informed consent, a discarded aborted embryonic tissue of ectopic pregnancy of day between 30 and 32 was collected by the concerned gynaecologist (and collaborator of the study) at the Department of obstetrics and Gynecology, IMS, Banaras Hindu University, Varanasi, India. Gestational age of embryo was calculated with
ultrasonography report and morphology of the tissue. The tissue was collected in RNAlater® (Sigma Aldrich) immediately after removal. RNA was isolated separately from limb buds of upper limb, lower limb and the body. RNA was proceed for whole transcriptome analysis using RNA sequencing (RNA-Seq) on Illumina plateform.

**DNA extraction from peripheral blood**

Genomic DNA was isolated from peripheral blood using salting out method (Miller *et. al*, 1988). About 5ml heparinized peripheral blood was taken in 50ml polypropylene tube and mixed with 20 ml of 0.9% NaCl followed by centrifugation at 5000rpm for 5 minutes. Supernatant were discarded without disturbing the pellet and 20 ml of chilled sol A (stored at 4°C) added in the cell pellet, mixed properly and subjected to centrifugation at 5000rpm for 5 minutes (Appendix II). Supernatant were again discarded and 2ml of sol B added in the pellet and mixed well to break the pellet (Appendix II). After breaking the pellet 200µl of SDS (10% w/v) was added without shaking the pellet. 500 µl of Sol C was added and mixed the content carefully (Appendix II). For layer separation 2ml of chilled chloroform added, mixed and centrifuged 5000rpm for 5 minutes. 3 layers were separated and DNA was present in upper, clear aqueous layer which was taken in fresh 15ml falcon tube using pipette. DNA was precipitated using chilled absolute ethanol and DNA washing was performed twice in 70% ethanol. DNA was air dried and dissolved in 200µl 1X TE buffer pH8.0 (10mM Tris-Cl, 1 mM EDTA, pH8.0) (Appendix IV). DNA was stored in 4°C for future use.

**DNA extraction from saliva**

Saliva was taken from those patients who were unable to provide their blood samples. Saliva was collected in collection tube and DNA was isolated using buccal DNA purification kit (HiPurA™ Buccal DNA Purification Kit) according to manufacturer’s protocol. Purification kit relies on the binding of DNA to silica gel membrane of the HiElute Miniprep Spin Column followed by washing and elution of purified DNA.

**DNA quality and quantity assessment**

Quality of genomic DNA was estimated using spectrophotometer (Ultraspec 1100 pro UV/Visible Spectrophotometer, Amersham Biosciences and NanoDrop ND-
Materials and Methods

Genomic Analysis of Congenital Limb Malformations

2000, Thermo Fisher Scientific, USA). Quantity of DNA was measured from optical
density (OD) OD\textsubscript{260} and quality (protein contamination) from OD\textsubscript{260}: OD\textsubscript{280} ratio from
the instruments. Integrity of DNA and no-contamination of RNA was assessed by 0.8
% agarose gel electrophoresis in 1X Tris-Acetate-EDTA (TAE) buffer (Appendix
V).

Polymerase Chain Reaction (PCR)

PCR was performed to amplify the selected region of genome (exons, exon-
intron boundaries and 5’ and 3’ UTRs) of candidate genes (GLI3, TP63, HOXD13,
GJA1, ALX1, ALX3, ALX4, ZRS). For PCR, 50ng of genomic DNA was used in a
reaction volume of 25µl. The PCR reaction mixture was prepared with 0.2mM dNTPs
(Sigma-Aldrich, USA), 1X PCR buffer (10mM Tris-Cl pH 9.0, 50mM KCl, 1.5mM
MgCl\textsubscript{2}, and 0.01% Gelatine) (Appendix VI), 0.5µl unit Taq DNA polymerase
(Sigma), 0.4mM of each of the forward and reverse primers and 10% DMSO if
required. Total reaction volume makeup to 25µl using autoclaved deionized water.
PCR was carried in ABI Verity 96 well thermal cycler and 2720 thermal cycler
(Applied Biosystems, USA). Standard PCR conditions were given in table below
(table 3.1).

Table 3.1: Thermal cycler condition for PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>x1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec to 1 min</td>
<td>x25 to 40 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C to 68°C</td>
<td>30 sec to 1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec to 2 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>07 min to 10 min</td>
<td>x1</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

PCR amplification products were subjected to 2% agarose gel electrophoresis
in 1X TAE running buffer. Amplified products were visualized by staining the gel
with Ethidium bromide (EtBr) and pictures were taken using UV transilluminator.
Materials and Methods

**Touch down PCR**

Touch down PCR was performed to amplify specific DNA segments of \textit{ALX3} gene. One reaction of touch down PCR contains 0.2mM dNTPs (Sigma-Aldrich, USA), 1X PCR buffer (10mM Tris-Cl pH 9.0, 50mM KCl, 1.5mM MgCl\textsubscript{2} and 0.01% Gelatine), 0.1\mu l platinum Taq DNA polymerase (Sigma), 0.4mM of each forward and reverse primer and 10% DMSO. PCR conditions were as below in table 3.2-

**Table 3.2: Thermal cycler program for touch down PCR**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>8 min</td>
<td>x14 cycles</td>
</tr>
<tr>
<td>I Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>I Annealing</td>
<td>68°C</td>
<td>40 sec (0.5°C decrease in each cycle)</td>
<td></td>
</tr>
<tr>
<td>I Extension</td>
<td>72°C</td>
<td>60 sec</td>
<td></td>
</tr>
<tr>
<td>II Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>x25 cycles</td>
</tr>
<tr>
<td>II Annealing</td>
<td>63°C</td>
<td>40 sec</td>
<td></td>
</tr>
<tr>
<td>II Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

**DNA sequencing (Sanger dideoxynucleotides chain termination method)**

**PCR product purification**

PCR product purification was performed to remove unincorporated primers and dNTPs. For product purification two methods were used-

**Exonuclease I (ExoI) and Shrimp alkaline phosphatase (SAP) method**

In this method, 3.6U of ExoI and 0.6U of SAP (USB Affimixter, USA) were mixed with 20\mu l of PCR product. The resultant mixture was incubated at 37°C for 45 min followed by heat inactivation at 80°C for 15 min.

**Column purification method**

25\mu l of PCR product loaded on the 2% agarose gel and band of interest was precisely cut using gel cutter. Slice of gel transferred into 1.5ml eppendorf tube. Gel elution was performed using columns (Real genomics Hiyield\textsuperscript{TM} gel/PCR DNA mini
Kit). Sliced gel subjected to gel dissociation, DNA binding, washing and elution were according to manufacturer’s protocol. Elution of DNA was verified by agarose gel electrophoresis.

**Dideoxynucleotides chain termination and end labeling (Cycle Sequencing)**

For Sanger chain termination reaction, ABI Big Dye Terminator V3.1 cycle sequencing kit (Applied Biosystems, USA) was used to label 50ng of purified PCR product. A 10µl of cycle sequencing reaction contains 0.3µl Big Dye Ready Reaction mix V3.1, 1.75µl Big Dye Ready Reaction buffer, 2pM of primer (forward or reverse), 50ng of purified PCR product and nuclease free water. Cycle sequencing was carried in thermal cycler on following condition (table 3.3).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>96°C</td>
<td>1 min</td>
<td>x1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>96°C</td>
<td>10 sec</td>
<td>x25</td>
</tr>
<tr>
<td>Annealing</td>
<td>50°C</td>
<td>5 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>60°C</td>
<td>4 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

**Cycle sequencing product purification**

Cycle sequencing product was purified either in 1.5ml eppendorf tube or in 96 well plate. For purification 10µl cycle sequencing product was transferred into eppendorf tube or in 96 well plate. 12µl of master mix I (10µl MQ, 2µl EDTA pH 8.0) added into the cycle sequencing product and mixed well by vortexing (Appendix VII). 52µl of master mix II (50µl absolute alcohol, 2µl 3M NaOAc pH 4.6) added into the above solution, mixed and incubated at room temperature for 15 min (Appendix VII). Tube or plate was centrifuged at 12000g or 1200g for 45 min, respectively. Supernatant was carefully discarded and pellet washed twice with 250µl (for tube) and 150µl (for plate) 70% ethanol by centrifugation at 12000g (for tube) or 1200g (for plate) for 30 min. Pellet was air dried and dissolved in 10µl of HiDi formamide (Applied Biosystems, USA).
Capillary electrophoresis, base calling and sequence visualization

Dissolved purified cycle sequencing product was denatured at 95°C for 5 min followed by rapid cooling at 4°C in thermal cycler. Denatured product was subjected to automated capillary electrophoresis in ABI 3130 Genetic analyzer (Applied Biosystems, USA) according to manufacturer’s protocol. Sequencing analysis software v5.2 (Applied Biosystems, USA) was used for base calling. Visualization of DNA sequences was performed by Finch TV v1.4.0 (Geospiza, PerkinElmer, USA) software.

Insilico sequence analysis

Sequenced DNA was aligned with human genome and transcript database available on National Center for Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool (BLAST). Sequencing data were analyzed by several in-silico analysis tools as mentioned below. The effect of each SNP was predicted by in-silico analysis using MutationTaster (Schwarz et al., 2014) and other prediction tools like SIFT, Panther, SNAP, Polyphen-1, Polyphen-2, PhD-SNP, MAPP and PredictSNP (Bendl et al., 2014). The Exome variant server (http://evs.gs.washington.edu/EVS/), dbSNP (www.ncbi.nlm.nih.gov/SNP), 1000 genomes and ExAC (http://exac.broadinstitute.org/) were used to determine the frequency of sequence alteration in population.

Comparison of protein sequence to genomic DNA sequence was performed using GeneWise (http://www.ebi.ac.uk/Tools/psa/genewise/) to find out the altered amino acid due to nucleotide substitution. In-silico nucleotide to protein translation for comparing normal and mutant protein, was done using online tool EMBOSS Sixpack (http://www.ebi.ac.uk/Tools/st/emboss_sixpack/). Tertiary structure of protein was downloaded from PDB (http://www.pdb.org) and visualized on standalone tool DeepView - Swiss-PdbViewer (http://spdbv.vital-it.ch/) (Guex et al., 1997) and UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) (Pettersen et al., 2004). Effect of mutation on protein microenvironment like hydrophobicity and secondary structure formation capacity was studied using ProtScale web server (https://web.expasy.org/protscale/) (Gasteiger et al., 2005).
In-silico protein structure determination

A. Protein structure was determined using two methods


2. Energy minimization using GROMACS for HOXD13 and GLI3 protein structure determination and analysis (Pronk et al., 2013).

1) Protein structure determination and analysis using Homology modelling

In this method following steps involved –

a) Protein searched against structure database of protein (Protein Data Bank) using PSI-BLAST to find out the homologous of protein (Fig. 3.1).

![Fig. 3.1: PSI-BLAST result showing query coverage against Protein Data Bank](image)

b) Templates were selected based on the BLAST result.

c) Installed Modeller 9.15 and python-3.3.4.amd64 on the system. Made a folder and put all templates and four file python file named align2d_mult.py, model_mult.py, multiple_template.py and evaluate_model.py into it.

d) Put query protein sequence in PIR format and save the file as .ali extension in the same folder. PIR format is given in the Fig. below (Fig. 3.2).

![Fig. 3.2: Protein sequence in PIR format to be save in .ali extension](image)
e) Opened multiple_template.py script with editplus having following script (Fig. 3.3)

```python
from modeller import *
log.verbose()
env = environ()
env.io.atom_files_directory = '../.../atom_files/'
mdl = model(env, file='code', model_segment=('FIRST:'+chain, 'LAST'+chain))
mdl.append_model(mdl, atom_files='code', align_codes='code,chain')
for (weights, write_fit, whole) in [(1, 0, 0, 0, 1, 1, 0, True),
                                    (1, 0.5, 1, 1, 1, 0, False, True),
                                    (1, 1, 1, 1, 1, 0, True, False),
                                   ]:
    aln = alignment(env)
    aln.add_template(file='template.mol2', atom_file='code.mol2',
                     align_codes='code,chain')
    aln.append_model(mdl, atom_files='code,align_codes=code,chain')
    aln.align_sequence(alignments[1],
                       sequence[2],
                       chain, weights, write_fit=write_fit,
                       write alignments=True, fit=True, write_fits=True,
                       write_all=True, output='ALIGNMENT QUALITY')
```

**Fig. 3.3:** Script of multiple_template.py file opened with editplus

Opened modeller 9.15 terminal and run the multiple_template.py script file using following command line. Result was shown in Fig. 3.4.

**Fig. 3.4:** Showing modeller terminal result of multiple_template.py run
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f) Opened align2d_mult.py script with editplus having following script (Fig. 3.5).

```python
from modeller import *
log.verbose()
env = environ()
env.libs.topology.read(file='${LIB}/top_heav.lib')

# Read aligned structure(s):
aln = alignment(env)
aln.append(file='tm00495.ali', align_codes='all')
aln_block = 1:n(aln)

# Read aligned sequence(s):
aln.append(file='Seq.ali', align_codes='Seq')

# Structure sensitive variable gap penalty sequence-sequence alignment:
aln.align(output='', max_gap_length=20,
gap_function=True, # to use structure-dependent gap penalty
alignment_type='PAIRWISE', align_block=aln_block,
feature_weights=(1.0, 0.0, 0.0, 0.0, 0.0), overhang=0,
gap_penalties_id=(-400, 0),
gap_penalties_2d=(0.35, 1.2, 0.5, 1.2, 0.6, 1.6, 1.2, 0.6),
similarity_flag=True)

aln.write(file='seq-mult.ali', alignment_format='FIR')
aln.write(file='seq-mult.pap', alignment_format='FAP')
```

Fig. 3.5: Script of align2d_mult.py file opened with editplus

The script align2d_mult.py was run on terminal using following command line. Result was shown in Fig. 3.6.

![Mod9.15 align2d_mult.py](image)

Fig. 3.6: Showing modeller terminal result of align2d_mult.py run
g) The model_mult.py script file is given below. This file contains all the three templates on the basis of which structure of query protein will be generated (Fig. 3.7).

```python
from modeller import *
from modeller.automodel import *

env = environ()
a = automodel(env, alnfile='seq-mult.all',
              knowns=('1r8aA', '2l12A', '2zw3A'), sequence='Seq')
a.starting_model = 1
a.ending_model = 5
a.make()
```

**Fig. 3.7:** Script of model_mult.py file opened with editplus

The model_mult.py script file was run using the following command and result was shown in Fig. 3.8.

```bash
Mod9.15 model_mult.py
```

**Fig. 3.8:** Showing modeller terminal result of model_mult.py run

After this run five protein models were generated which was validated by running the script named evaluate. The evaluate script is given below (Fig. 3.9).

```python
from modeller import *

log.verbose()  # request verbose output
env = environ()
env.libs.topology.read(file='./LIB/top_heavy.lib')  # read topology
env.libs.parameters.read(file='./LIB/par.lib')  # read parameters

mdl = complete_pdb(env, '899998001.pdb')

# assess all atoms with DOPE:
s = selection(mdl)
s.assess_dope(output='ENERGY_PROFILE NO_REPORT', file='Seq.profile',
              normalize_profile=True, smoothing_window=15)
```

**Fig. 3.9:** Script for evaluating the model generated. Highlighted region is the name of predicted protein
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The script was run of Modeller 9.15 platform using following command

A log file was generated with a DOPE score for the predicted protein in the same folder. Protein with the minimum DOPE score was selected for further analysis although all proteins were also analyzed using other methods like RAMPAGE, PDBsum and ERRAT server before choosing the best PDB structure.

2. Ab-initio modelling using I-TASSER for HOXD13:

For structure generation protein sequence was submitted onto the I-TASSER web server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) which generated 5 protein models (Fig. 3.10) (Roy et al., 2010).

Fig. 3.10: HOXD13 protein structure prediction result on I-TASSER web server

B. Structure validation and selection of best model

All the protein models generated either by homology modelling or I-TASSER web server were validated using four different web servers and tools i.e. RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) for Ramachandran Plot Analysis, ERRAT program (http://services.mbi.ucla.edu/ERRAT/) for verifying protein structures determined by crystallography, PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) structural analyses tool and modeller9.15 to find out the best model among all.
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C. MD Simulation: Protein in Water

Molecular dynamics (MD) is a computer simulation method for studying the physical movements of atoms and molecules. The atoms and molecules are allowed to interact for a fixed period of time, giving a view of the dynamic evolution of the system.

I have correctly installed and use the script written for GROMACS 4.5.4. to run MD simulation. MD simulation steps includes following steps.

1. GROMACS Coordinate and Topology Files

The first step in MD simulation with GROMACS is to create GROMACS compatible coordinate (.gro) and topology (.top) files. The command line execution looks like this:

```
gmx pdb2gmx -f 1AKI.pdb -o 1AKI_processed.gro -water spce
```

There are three output files resulting from pdb2gmx:

1.) protein.gro # gromacs-format coordinate file
2.) topol.top # gromacs-format topology file
3.) posre.itp # position restraint file

2. Box Preparation

This step will define a box size for the system, center the protein in the box, solvate, and finally, add counter ions to the system. The three files generated in last step will be needed to start this step.

Editconf

The GROMACS tool editconf is very useful to change the format of coordinate files, to rotate and translate coordinate files, to define the box size, among other things.

```
gmx editconf -f 1AKI_processed.gro -o 1AKI_newbox.gro -c -d 1.0 -bt cubic
```

Genbox

The next GROMACS command, 'genbox', will fill the box with solvent molecules.

```
gmx solvate -cp 1AKI_newbox.gro -cs spc216.gro -o 1AKI_solv.gro -p topol.top
```
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The 'cp' flag specifies the coordinate file of the system to be solvated, and 'cs spc216.gro' tells genbox to use an SPC (a simple threepoint water model) coordinate file to populate the box. The output coordinate file is called'protein_sol.gro'. The topology file, 'topol.top', is also provided on the command line.

Genion

The final step before simulation is to add enough ions to the system to neutralize the net charge or, alternatively, add enough ions to neutralize the net charge and reach some physiological concentration. To create the input file, execute:

```bash
gmx grompp -f ions.mdp -c 1AKI_solv.gro -p topol.top -o ions.tprgenion_input.tpr
```

The net charge of the ubiquitin system is already 0 so instead of neutralizing the system, add enough NaCl to reach 100 mM salt concentration. To do so, execute:

```bash
gmx genion -s ions.tpr -o 1AKI_solv_ions.gro -p topol.top -pname NA -nname CL -nn 8
```

When prompted, choose group 13 (SOL). Genion then chooses 22 solvent molecules and replaces half with sodium ions, and the other half with chloride ions. The topology file is also provided on the command line so that it may be updated accordingly. The '[ molecules ]' section has been modified by removing 22 SOL molecules and adding 11 each of NA and CL molecules.

3. Energy Minimization

Prior to running actual dynamics, energy minimization was performed. The purpose of a minimization was to relax the molecular geometry of the system in order to get rid of any atomic clashes or other irregularities that might exist. Energy was minimized according to steepest descent energy minimization method not to exceed 2,000 steps. For energy minimization we executed the following command-

```bash
gmx grompp -f minim.mdp -c 1AKI_solv_ions.gro -p topol.top -o em.tpr
```

Once the minimization is complete (which should take around 500 steps), several files have been written to disk, all with the prefix protein_min'. They are as follows:
1.) Protein_min.gro # system coordinates at the final step
2.) Protein_min.trr # trajectory file
3.) Protein_min.log # log file
4.) Protein_min.edr # energy file

Plot the potential energy curve using following command

```
gmx energy -f em.edr -o potential.xvg
```

```
xmgrace potential.xvg
```

Potential energy of system as a function of minimization step will be plotted.

4. **Equilibration MD**

Equilibration will be carried out in two steps. First, an NVT (constant Number of atoms, Volume, and Temperature) simulation was performed for 1ns in order to bring the system to the target temperature. Second, an NPT (constant Number of atoms, Pressure, and Temperature) simulation was performed for 1ns to allow the system to find the correct density.

**NVT Simulation**

Executed the command

```
gmx grompp -f nvt.mdp -c em.gro -p topol.top -o nvt.tpr
```

An output file was created and then proceed for next command

```
gmx mdrun -v -deffnm nvt
```

After the completion of run the plot was generated using the command below to confirm the system equilibration around the give temperature.

```
gmx energy -f nvt.edr -o temperature.xvg
```

```
xmgrace temperature.xvg
```

**NPT Simulation**

Executed the command:

```
gmx grompp -f npt.mdp -c nvt.gro -t nvt.cpt -p topol.top -o npt.tpr
```
Materials and Methods

An output file was created and then proceed for next command

```bash
gmx mdrun -deffnm npt -v
```

After the completion of run the plot was generated using the command below to confirm the system equilibration around the give temperature.

```bash
gmx energy -f npt.edr -o pressure.xvg
```

```
xmgrace pressure.xvg
```

5. Production MD

Executed the command to perform the production MD for 10ns. CHARMM force field was selected to run the MD.

```bash
gmx grompp -f md.mdp -c npt.gro -t npt.cpt -p topol.top -o md_0_1.tpr
```

No error occurred then begin simulation with command

```bash
gmx mdrun -v -deffnm md_0_1
```

6. Analysis

```bash
gmx trjconv -s md_0_1.tpr -f md_0_1.xtc -o md_0_1_noPBC.xtc -pbc mol -ur compact
```

Select 0 ("System") for output.

RMSD calculation

```bash
gmx rms -s em.tpr -f md_0_1_noPBC.xtc -o rmsd_xtal.xvg -tu ns
```

Radius of gyration

```bash
gmx gyrate -s md_0_1.tpr -f md_0_1_noPBC.xtc -o gyrate.xvg
```

Control sample screening

SNP screening in control population

Control samples were screened to study the frequency of SNPs in a particular population. For control screening two methods were used –

Tetra-primer Amplification-refractory mutation System (ARMS) PCR

ARMS is based on the use of sequence specific primers that allow amplification of DNA with specific allele. For the present study four allele specific
primers were designed using Primer BLAST tool. 120 control individuals were screened for the observed variation g.176958118A>G in *HOXD13* gene. For the screening 4 primers were used in a single reaction and designing of ARMS-PCR were given in Fig. 3.11.

![Designing of ARMS-PCR](image)

**Fig. 3.11:** Designing of ARMS-PCR for the screening of g.176958118A>G variant (*HOXD13*) in control population

Primer sequences were given in table below (Table 3.4).

**Table 3.4:** Allele specific primer sequences for ARMS-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Amount used in each reaction</th>
<th>PCR Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Set 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOXD13_F</td>
<td>5’-CAGCCAAAGAGTGCCCAG-3’</td>
<td>0.8mM</td>
<td>210bp, 383bp, 555bp</td>
</tr>
<tr>
<td>HOXD13_WF</td>
<td>5’-GGCTTTCCCGTGAGAGTA-3’</td>
<td>0.4mM</td>
<td></td>
</tr>
<tr>
<td>HOXD13_MR</td>
<td>5’-GGCCTGACACGTCCATGC-3’</td>
<td>0.4mM</td>
<td></td>
</tr>
<tr>
<td>HOXD13_W</td>
<td>5’-GGGGCGCATGACTCTTAGC-3’</td>
<td>0.8mM</td>
<td></td>
</tr>
<tr>
<td><strong>Set 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOXD13_F</td>
<td>5’-CAGCCAAAGAGTGCCCAG-3’</td>
<td>0.8mM</td>
<td>210bp, 383bp, 555bp</td>
</tr>
<tr>
<td>HOXD13_MF</td>
<td>5’-GGCCTGACACGTCCATGC-3’</td>
<td>0.4mM</td>
<td></td>
</tr>
<tr>
<td>HOXD13_WR</td>
<td>5’-GGGCGCATGACTCTTAGC-3’</td>
<td>0.4mM</td>
<td></td>
</tr>
<tr>
<td>HOXD13_W</td>
<td>5’-GGGGCGCATGACTCTTAGC-3’</td>
<td>0.8mM</td>
<td></td>
</tr>
</tbody>
</table>

PCR reaction for tera-primer ARMS PCR contains 0.2mM dNTPs (Sigma-Aldrich, USA), 1X PCR buffer (10mM Tris-Cl pH 9.0, 50mM KCl, 1.5mM MgCl2, and 0.01% Gelatine), 1µl Taq DNA polymerase (made in house, CGD, BHU) and 4 primers according to table for each set. Reaction volume was makeup to 25µl using nuclease free water. PCR conditions were given in table below (Table 3.5).
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Table 3.5: Thermal cycler program for tetraplex ARMS-PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>64°C</td>
<td>1 min</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

PCR was followed by 2% agarose gel electrophoresis and visualized on Gel-doc using EtBr staining.

Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP screening method in controls was used to screen those variants which affect a particular restriction site. In this method specific DNA sequence with variant was amplified using specific primer by PCR and followed by restriction digestion using specific restriction enzyme according to manufacturer’s protocol. In the present study 4 different restriction enzymes were used to screen 4 different variants in patient and control population. List of restriction enzymes and primer used for amplification of specific DNA sequences containing desired variation is listed in table below (table 3.6). Digested products were resolved in 2% agarose gel electrophoresis in 1X TAE after staining with EtBr.

Table 3.6: List of primers used for amplification of specific DNA sequences and restriction enzymes used for digestion

<table>
<thead>
<tr>
<th>SN</th>
<th>Variation</th>
<th>Primers</th>
<th>Restriction enzyme</th>
<th>Digestion product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GLI3 g.42004239_42004240insA</td>
<td>CGTCAAGCTTGGCAGTTGTC</td>
<td>DdeI</td>
<td>Wild allele- 105bp, 205bp, 137bp, 255bp Ins A allele-105bp, 255bp, 343bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAACAGCCAACCAAAAGTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>GLI3 g.42007251G&gt;A</td>
<td>ATTTGGCTCTCCTTCTTGTAC</td>
<td>BstUI</td>
<td>G allele- 136, 333bp, A allele- 469bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACATAAAAACTGAGGGCCTGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>No</th>
<th>Symbol</th>
<th>Mutation</th>
<th>Primer 1</th>
<th>Enzyme</th>
<th>Allele 1 Length</th>
<th>Allele 2 Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>GLI3</td>
<td>g.42005678G&gt;A</td>
<td>TAGTTGTGAGGCAGGCAATG</td>
<td>BseY1</td>
<td>G allele - 463bp, 362bp, 25bp</td>
<td>A allele - 459bp, 220bp, 146bp, 25bp</td>
</tr>
<tr>
<td>4</td>
<td>GLI3</td>
<td>g.42088222T&gt;C</td>
<td>CCCAAACAAATTGCATAGCG</td>
<td>BtsI</td>
<td>T allele - 312bp, 278bp, 6bp</td>
<td>C allele - 590bp, 6bp</td>
</tr>
</tbody>
</table>

### RNA isolation from peripheral blood

Total cellular RNA was extracted from blood using TRI reagent (Sigma-Aldrich, USA). For RNA extraction 750µl of TRI reagent was added into 250µl blood (3:1). After mixing 20µl of 5N acetic acid was added into the solution. Mixed properly by vortexing and incubate it for 5 minutes at room temperature. After incubation 200µl of chloroform was added, mixed properly and incubated for 5 minutes at room temperature. Incubation was followed by centrifugation 12,000g for 15 minutes at 4°C. 3 layers were separated and upper aqueous layer contains RNA which was transferred into a fresh eppendorf tube. For RNA precipitation, 500µl of isopropanol was added into the aqueous phase and incubated at room temperature for 5 minutes followed by centrifugation at 12,000g for 8 minutes at 4°C. Supernatant was carefully decanted and precipitate was washed twice using 75% ethanol. For washing centrifugation was performed at 7500g for 5 minutes at 4°C. Pellets were air dried and dissolved in 20µl of nuclease free water. RNA was stored in -80°C for future use.

### RNA isolation from fetal tissue

Carefully examined under light microscope and both upper and lower limb bud were separated and transferred to eppendorf tubes. 1ml of TRI reagent was added into each tube and minced the tissues properly using homogenizer followed by centrifugation at 12,000g for 5 minutes at 4°C. Supernatant was transferred into a fresh eppendorf tube and 200µl of chloroform was added to separate the three layers with RNA in upper aqueous layer. Precipitation was done by isopropanol and washing was carried out twice using ethanol. RNA pellet was dissolved in 20µl nuclease free water.
RNA quality and quantity assessment

Quality of RNA was estimated using spectrophotometer (NanoDrop ND-2000, Thermo Fisher Scientific, USA). Quantity of RNA was measured from optical density (OD) OD\(_{260}\) and quality (protein contamination) from OD\(_{260}/\text{OD}_{280}\) ratio from the instruments. Integrity of RNA was assessed by 1% agarose gel electrophoresis in 1X Tris-Acetate-EDTA (TAE) buffer.

DNase I treatment of RNA

DNase I (RNase free) (Ambion) treatment of RNA was performed according to manufacturer’s protocol which was necessary to remove unwanted DNA from RNA sample. In brief, 30µl of reaction volume contains 6U DNase I enzyme (RNase free), 1X reaction buffer, 30µg of total cellular RNA and nucleases free water. This reaction was incubated at 37°C on water bath for 30 minutes. After incubation 70µl of nucleases free water was added to make the volume 100µl followed by addition of 100µl of TRI reagent. Chloroform was used to separate the three layers and precipitation was performed by isopropanol. Pellet was dissolved in 10µl of nucleases free water and stored at -20°C.

cDNA synthesis from RNA

For cDNA synthesis from 2µg of DNA, random priming method was used with the help of High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer’s protocol.

Next Generation Sequencing

Whole genome sequencing was performed from DNA samples isolated from peripheral blood as well as saliva (Shendure et al., 2008). For RNA sequencing, total RNA was isolated from upper limb bud, lower limb bud and whole body of 32 days embryo of ectopic pregnancy.

Library preparation

TrueSeq DNA sample preparation kit V2 (Illumina) was used for library preparation of DNA and TrueSeq RNA sample preparation kit V2 (Illumina) was used for RNA library preparation. Agilent Bioanalyzer 2100 was used to monitor the quality control (QC) of library preparation which was further followed by
Materials and Methods

Genomic Analysis of Congenital Limb Malformations

Quantification by real time PCR using KAPA NGS library preparation kit (KAPA biosystem). cBot instrument (Illumina) was used to generate cluster and Illumina HiSeq 2000 platform was used for sequencing by synthesis.

Raw read filtering

Illumina HiSeq 2000 generated base call file (.bcl) and CASAVA software v1.8.2 (Illumina) was used to convert this base call file into nucleotide sequence with quality score FASTQ (FASTA with quality score) followed by adapter trimming. Bases with QC>30 was selected for further study.

Read alignment, variation detection and transcript assembly

For annotation of DNA sequencing reads, filtered reads were aligned with human genome sequence hg19 (UCSC) build using Avadis NGS v1.5.1 (Strand scientific intelligence, Inc). This alignment resulted into the identification of single nucleotide variations (SNVs) and small indel (Insertion and deletion). NCBI-dbSNP-build 139, Oct 2013 database was used to annotate the SNVs observed during alignment. For SNV call, minimum coverage depth of reads was set as >4 and minimum percentage of supporting reads to call for a valid SNP was set at >25%.

For annotation of RNA sequencing reads, filtered reads were aligned with human genome and transcriptome data hg19 (UCSC) build using Avadis NGS v1.5.1 (Strand scientific intelligence, Inc) followed by normalization and quantification using DEseq algorithm. Avadis NGS v1.5.1 software was further used for pathway analysis of differentially expressed gene.

Gene Ontology

Gene ontology analysis of upregulated and downregulated genes were carried out using DAVID-GO slim online tool (Huang da et al., 2009b, Huang da et al., 2009a).

Competent Cell preparation

A plate of Luria-Bertani (LB) agar was streaked with E. Coli DH5α host cell and incubated at 37°C for overnight. After incubation, single colony of E. Coli was inoculated into a 5ml of LB broth media (HiMedia, India) and incubated at 37°C for overnight with shaker at 180rpm. 1 ml of the grown culture was inoculated into 100ml
of fresh autoclaved LB broth media and incubated at 37°C with shaking at 200rpm for
two hours till OD\text{550nm} reached to 0.5. Chilled the culture on ice for 20min and
transferred the culture into pre-cooled 50ml sterile centrifuge tube followed by
centrifugation at 6500rpm at 4°C for 10min. Supernatant was discarded and pellet was
suspended into 20ml of chilled 100mM Calcium Chloride (CaCl\text{2}) solution at 4°C till
the solution become smooth and silky. Solution was incubated on ice for 20min
followed by centrifugation at 6500rpm at 4°C for 10min. Supernatant was discarded
and pellet was suspended in 1-2 ml of 100 mM CaCl\text{2}, left on ice for 1 hour. For
longer storage pellet was dissolved in 1-2 ml of 100 mM CaCl\text{2} and 20% glycerol.
200µl of competent cells were prepared and stored at -80°C for longer use.

**Bacterial Transformation**

For transformation, 10-25ng of desired plasmid was added in 100µl of
competent cells in 1.5ml microcentrifuge tube and incubated on ice for 30min. After
incubation, heat shock at 42°C was given for 2min followed by 2min incubation on
ice. 500µl of LB broth (without antibiotic) was added into the tube followed by
incubation at 37°C for 1 hr. After incubation 100 µl of transformed cells were spread
on LB agar plate with 100 µg/ml of Ampicillin. LB agar plate was incubated
overnight at 37°C.

**Plasmid DNA isolation by alkaline lysis with SDS**

Plasmid DNA was isolated according to minipreparation protocol described in
Molecular Biology cloning: A laboratory manual, 3rd edition (Sambrook and Russell,
2001). 5ml of LB broth containing appropriate antibiotic was inoculated by a single
 colony of bacteria and incubated overnight at 37°C with shaking at 200rpm.
Transferred the culture into 1.5ml microcentrifuge tube and centrifuged at 7000rpm
for 5min at 4°C. Discarded the supernatant and pellet was resuspended in 100µl of ice
cold Alkaline Lysis Solution I (50mM glucose, 25 mM tris-Cl pH 8.0, 10mM EDTA
pH 8.0) by vortexing (Appendix III). Incubated on ice for 5 min and followed by
addition of 200µl of freshly prepared Alkaline Lysis Solution II (0.2N Sodium
Hydroxide, 1% (w/v) SDS) and mixed thoroughly by inverting the tube 5 to 6 times
(Appendix III). Incubated on ice for 3 min and 150µl of ice-cold Alkaline Lysis
Solution III was added into the cell lysate, mixed by inverting the tubes 8-10 times
(Appendix III). Lysate was incubated on ice for 20 min followed by centrifugation at
Materials and Methods

12,000 rpm at 4°C for 20 min. supernatant containing plasmid DNA was transferred into a fresh eppendorf tube and incubated with RNase A at 37°C for 3 hours. After incubation equal volume of chloroform:Isoamylalcohol (24:1) was added into the lysate and mixed properly and incubated at 37°C for 10 min followed by centrifugation at 12000 rpm for 10 minutes at 4°C. Supernatant was discarded and pellet was washed twice with 1ml of 70% alcohol, air dried and dissolved in 200µl of 1X TE buffer pH 8.0.

Plasmid DNA isolation by Column purification method

Midipreparation of plasmid was performed using Qiagen plasmid midi kit (Qiagen, USA) following manufacturer’s protocol. Briefly 100µl of bacterial culture was grown overnight and cell lysate was passed through Qiagen column which bind plasmid to the column. Column washed twice and plasmid DNA was eluted. Eluted DNA was again precipitated using isopropanol and washed plasmid twice with 70% ethanol. Dried plasmid and dissolved in 500µl 1X TE buffer pH 8.0.

Site directed mutagenesis

In vitro site directed mutagenesis to introduce any nucleotide change within the cloned DNA, was performed using QuickChange Site-Directed Mutagenesis Kit (Stratagene, USA) according to manufacturer’s protocol. Mutagenesis was confirmed by sequencing.

Mammalian cell culture

HEK293: These are transformed human embryonic kidney cells and widely used for transfection to study the expression of mammalian gene.

COS 7: This cell line is an African green monkey kidney fibroblast-like cell line and used to study the expression of mammalian gene, localization and protein isolation.

Both the cell lines were cultured in DMEM medium (Dulbecco’s Modified Eagle’s Medium) (Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, USA) and antibiotic (100U/ml Penicillin, 100µg/ml Streptomycin) (HiMedia, India) in T25 flask. Culture was incubated at 37°C in 5% (v/v) Carbon dioxide (CO₂) in a humidified incubator (Thermo fisher Scientific, USA) until cells reached up to 70-80% confluence. Media was changed after 3 days. Regular splitting
of cell line was performed which involved the treatment of cells with 0.1%-Trypsin-0.1%-EDTA solution for 2 min followed by addition of DMEM media containing 10% FBS. From this step cells were either freeze for storage or proceeded for further experiments. For freezing cells suspended in DMEM media were collected in 15ml tube and centrifuged it for 2500 rpm for 5 min, supernatant was discarded and cell pellet was resuspended in cold freezing medium (DMEM, 10% FBS, 10% DMSO). Suspension was gradually cooled from 4°C to -80°C and stored up to 1 year.

**Transfection**

For transfection, cells were seeded in appropriate density in 6-well plate or 12-well plate and were allow to grow for 48 hours in incubation under optimal condition. Before proceeding for transfection experiment, DMEM media was replaced with Opti-MEM medium (Invitrogen, USA). Appropriate expression vectors and reporter vectors were transfected into the cells using transfecting agent Lipofectamine 2000 (Invitrogen, USA) according to manufacturer’s protocol. A total of 2.5µg of vectors were transfected per well of 6-well plate using Lipofectamine 2000 followed by 40 hours of incubation under optimal condition before proceeding for further experiments.

**Dual Luciferase Assay**

For dual luciferase assay, cells were seeded in 6 well plate at appropriate density and allowed to grow at 70 -80% confluence in DMEM media supplemented with 10% FBS. When cells reached to confluence, cells were co-transfected with 600ng pcDNA3.1-hisB vector containing GLI3 cDNA sequence, 600ng PGL4.0 vector containing firefly luciferase coding sequence downstream of GLI1 promoter and 200ng pRL-Tk vector containing renilla luciferase coding sequence. Lipofectamine 2000 was used to transfect the vectors into cell line according to manufacturer’s protocol. Transfected cells were incubated under optimal condition for 40hours. After incubation, media was removed and attached cells were washed twice with Dulbecco's phosphate buffer saline (PBS) (Thermo Fisher Scientific). Cells were lysed in freshly prepared 1X passive lysis buffer (PLB) according to manufacturer’s protocol. Cells lysates were treated with luciferase assay reagent (LAR II) followed by Stop and Glow reagent and readings were taken in luminometer.
Whole blood culture and Karyotyping (G-Banding of Human chromosome)

For short-term whole blood culture, 10 to 12 drops 0.3 ml of blood was added into 5ml of RPMI-1640 pH 7.2 (Sigma-Aldrich, Inc., St. Louis, MI, USA) culture media supplemented with 10% fetal bovine serum (Himedia, India), 50 μg/ml of antibiotic (Gentamycine) and stimulated by phytohaemagglutinin-M (Sigma-Aldrich, Inc., St. Louis, MI, USA). Culture was incubated at 37°C for 70 hours and then treated with colchicine (Sigma-Aldrich, Inc., St. Louis, MI, USA) at a concentration of 0.02μg/ml of culture to arrest the cells at metaphase. Culture was centrifuged at 1500 rpm for 5 minutes, supernatant was discarded and the pellet was broken down. Cells were treated with prewarmed hypotonic solution (44.9mg/ml Potassium Chloride and 4.0 mg/ml Sodium Citrate) at 37ºC for 12 minutes (Appendix I). Cells were fixed with 4-5 drops of fixative (Methanol and Acetic Acid in 3:1 ratio) and centrifuged at 1500 rpm for 5 minute (Appendix I). The supernatant was discarded and pellet was resuspended into fixative by adding drop by drop to 8 ml with fixative (Appendix I). The above step was repeated for 3-5 times until clear cell suspension is obtained. A drop of cell suspension was dropped on a clean, grease-free slide from 6 inches height and subjected instant drying by exposing to flame. Slides were incubated at 60ºC for overnight.

For G-banding, slides were treated with trypsin solution (1ml 3% trypsin (prepared in 0.9% NaCl) was mixed in 49 ml 0.9% NaCl in coupling jar pH 7.5 by 1N NaOH) for 8-20 seconds depending upon results. After trypsin treatment, slides were transferred in a coupling jar containing 50 ml of phosphate buffer (1:1 ratio of 9.073% KH₂PO₄ and 11.87% Na₂HPO₄.2H₂O) and rinsed with distilled water in a separate coupling jar (Appendix I). Subsequently, slides were stained with 5% Giemsa for 5 minute and mounted with DPX.

Cytogenetic microarray

The Cytogenetics 2.7M array was performed to identify reported and novel chromosome imbalance across the entire genome. Cytogenetic microarray experiment was performed using Cytogenetics 2.7M array and Affymetrix microarray work station (Affymetrix, Inc. Santa Clara, CA, USA) following the Affymetrix Cytogenetics Assay Protocol User Manual. The Cytogenetics 2.7M array provided whole genome coverage with the density of 2.7 million markers to enable superior
resolution and 400,000 single nucleotide polymorphisms (SNPs) to enable the detection of loss of heterozygosity (LOH), uniparental disomy (UPD), and regions identical-by-descent.

For cytogenetic microarray, whole genome amplification with 100 ng of genomic DNA from each sample was performed using reagents provided by Affymetrix (Affymetrix, Inc. Santa Clara, CA, USA). Genomic DNA was purified using cyto magnetic bead, cyto wash buffer and cyto elution buffer according to manufacturer’s protocol. Optimum quality and quantity of eluted DNA was confirmed by OD260/OD280 ratio between 1.8-2.0 and DNA concentrations >0.55 μg/μL. 2 μg of purified DNA was used for fragmentation and labeling using cyto fragmentation and labeling buffers and enzymes. Working of fragmentation reaction was confirmed by running the fragmented DNA on 3% agarose gel. DNA fragments ranging from 50bp to 150bp was considered optimum fragmentation before proceeding to hybridization reaction. Denaturation was performed in thermocycler after Cyto hybridization buffer was mixed with fragmented samples. Denatured sample was immediately loaded into the arrays and checked for any bubble (if there it should be movable). Arrays in trays were rotated at 60 rpm in hybridization oven at 50°C for 16 to 19 hours. After incubation, hybridization arrays were washed and stained using GeneChip Fluidics Station 450. Before using Fluidics Station for processing Cytogenetics Arrays, standard bleaching protocol was run on the GeneChip Fluidics Station. Bleaching, washing and staining were performed by GeneChip Fluidics Station 450 under control of software Affymetrix GeneChip Command Console (AGCC). For washing and staining of hybridized arrays, cyto stain buffer 1, cyto stain buffer 2, cyto holding buffer, cyto wash buffer A and cyto wash buffer B was used. After washing and staining, arrays were scanned by GeneChip Scanner 3000 7G under control of software AGCC scan control and cell files were generated. Chromosomal Analytical Suit (ChAS) software was used to run and analyzed these files. Genomic duplication and deletion analysis filter was set at 100 kbp for analysis.