Summary
Genomic analysis of Congenital Limb Malformations

Congenital limb malformations are among the most frequent congenital malformations in human caused by genetic mutations or teratogenic effects resulting either in abnormal, loss of, or additional skeletal elements with a frequency of about 1 in 500 live births for upper limbs. Up to 18% of all children with a congenital limb malformation die before the age of 6 years, usually because of associated, more serious organ malformations and/or dysfunctions. The human congenital malformations caused by alterations affecting the morphoregulatory gene networks that control early limb bud patterning and outgrowth. Specific genes are involved in a number of signaling pathways regulate different aspects of limb bud growth and patterns of development in three axes: dorsal–ventral, anterior–posterior, and proximal–distal. Disruptions in such genes have been associated with limb malformation. Many mutations that cause limb malformation also affect the development of other organ systems. Clinical geneticists use these specific patterns and combinations of malformations to delineate specific syndromes and identification of the underlying molecular basis which helps to refine this process and provides new developmental links between apparently disparate organ systems. Furthermore studies on inherited human limb malformations throw up new genes not previously suspected to play a role in limb development. Much works remain to be done to delineate the precise role of these genes to analyze the underlying developmental mechanisms. The identification of causative gene mutations is important for genetic counseling and also provides insights into the mechanisms controlling limb development.

In the present study, a total of 116 cases with limb defect were recruited and registered during the period of April 2013 to September 2017. Limb defect cases were characterized on the basis of clinical phenotypes. Most common limb defects were polydactyly (12.06%) and syndactyly (29.31%) followed by ectrodactyly (6.89%) and polysyndactyly (4.31%). Polydactyly is generally more common than syndactyly but higher frequency of syndactyly was observed in the current study. 24 cases with syndromic and non-syndromic polydactyly were analyzed to study the correlation among the position of extra finger, other anomalies and gender which revealed that polydactyly of hand (47.82%) was more common than feet (17.39%) and unilateral polydactyly of hand (57.89%) and bilateral polydactyly of feet (91.66%) was more common. Syndromic polydactyly (73.91%) was more common than non-syndromic (26.08%) and male (69.56%) was affected more than the female (30.43%). Analysis
also revealed that right hand (40.42%) was affected more than other limbs (20%) and postaxial polydactyly (61.70%) is more frequent than preaxial polydactyly (38.29%).

Gene expression profiling during human limb development was studied by whole transcriptome analysis of human limb bud from discarded aborted embryonic tissue from an ectopic pregnancy, and revealed that TGF-beta signaling, FGF signaling, ROR signaling, transcription factors, Homeobox transcription factors, BMP signaling, Wnt signaling and T box genes were slightly upregulated in both human embryo and murine whereas Hedgehog signaling was not expressed in limb at this stage. A total of 1388 genes were upregulated (> 2 folds) in upper limb and 1190 in lower limb as compared to rest of the body in human.

GLI3 mutational screening in 15 syndromic and non-syndromic polydactyly revealed 26 variants including 5 pathogenic mutations. Three pathogenic mutations were identified in GCPS cases (two familial cases and one sporadic case), one in sporadic case of non-syndromic post-axial polydactyly and one in familial case of polysyndactyly. First case was a familial Greig cephalopolysyndactyly (GCPS) syndrome with finger like thumb as novel feature in an affected member caused by a novel mutation g.42085059delG (p.Y251Mfs59) that lead to haploinsufficiency of GLI3. Second case was again a familial case of Greig cephalopolysyndactyly (GCPS) syndrome in which nonsense mutation g.42007251G>A (p.R792X) was identified. A sporadic case with post-axial polydactyly in all four limb was screened and a novel mutation g.42004239_42004240insA (p.E1478X) was discovered. Another case was a familial case of polysyndactyly and mutational analysis revealed a novel mutation g.42005257_42005257delG (p.H1138Qfs*68) present in all affected members but none in unaffected members. Furthermore a sporadic case with Greig cephalopolysyndactyly (GCPS) syndrome was screened for GLI3 gene and a novel mutation g.42012177G>A (p.P621L) was identified that lies in DNA binding domain of protein. Two mutations p.H1138Qfs*68 and p.P621L were selected for further analysis which revealed that the mutant protein have reduced transcriptional activity in-vitro which lead to haplo-insufficiency of GLI3 and might be causal for clinical manifestation in patients. Case control study for SNP rs929387 and rs929387 were performed in limb malformation cases and healthy control from same population. The
study revealed no significant association for both the SNP as risk for congenital limb malformation in Indian population.

In this study role of \textit{HOXD13} and \textit{GJA1} were also investigated in 23 syndactyly which revealed 7 different variants of which 4 variants were synonymous and 2 were pathogenic non- synonymous and one in 3'UTR region of \textit{HOXD13}. Two pathogenic mutations \textit{g.176958118A>G} (\textit{p.Y167C}) and \textit{g.176959387A>C} (\textit{c.961A>C; p.T321P}) were identified. The first mutation \textit{g.176958118A>G} (\textit{p.Y167C}) was identified in two independent patients with limb defect and oral cleft, supporting the role of \textit{HOXD13} in rostrum development. The second mutation \textit{g.176959387A>C} (\textit{p.T321P}) was identified in patient with non-syndromic syndactyly that lie in DNA binding domain of protein. Mutational analysis of \textit{GJA1} revealed two non-synonymous and one variant in 3'UTR region. No pathogenic variant was observed in \textit{GJA1} in the present study.

Mutational analysis of \textit{TP63} gene in 10 patients with limb defect and oral cleft was also performed which revealed 9 variants of which 8 variants were present in either intron, synonymous variant in exon or present in 5' UTR region. The pathogenic mutation \textit{g.189585692G>A} (\textit{p.R318H}) was identified in ectrodactyly cleft lip/palate (ECP) syndrome. As the single \textit{TP63} mutation is causal for ectrodactyly ectodermal dysplasia-cleft lip/palate (EEC) syndrome, Rapp–Hodgkin syndrome (RHS) and ECP syndrome, it can argued that RHS and ECP might be clinical variability of EEC syndrome. Existence of three different syndrome EEC syndrome, RHS syndrome and ECP syndrome suggested be reviewed and redefined again.

In the present study a 5-generation family with split hand/foot malformation (SHFM3) was studied showing autosomal dominant pattern of inheritance. Cytogenetic analysis and Next Generation Sequencing analysis revealed a gain of 4Mb region on chromosome location 10q24.31. This duplicated region contains 4 genes \textit{LBX1}, \textit{BTRC}, \textit{POLL} and \textit{FBXW4} in all affected member and was absent in unaffected members. Analysis revealed that \textit{BTRC} and \textit{FBXW4} might have role in SHFM3 manifestation in the family.

The present study also reported a novel and unique combination of frontonasal dysplasia (FND) spectrum manifesting severe craniofacial developmental disturbance along with mild mental retardation, clinodactyly and cryptorchidism. The patient
manifested overlapping features among FND type 2, FND type 3 and AFND. Cytogenetic microarray analysis of patient and his parents revealed an intermittent deletion of 125 kb on 1p13.3 involving two genes $SLC25A24$ and $NBPF4$ and one uncharacterized gene LOC400768 in the patient. $NBPF4$ gene seems to be a strong candidate for clinical manifestation in the patient because $NBPF4$ belongs to a neuroblastoma breakpoint family and copy number variation in this family members reported to be involved in several developmental and neurogenetic diseases.

Therefore the whole study analyzed the frequency of various types of congenital limb malformation and gene expression profile of human limb bud from aborted fetus. Mutational spectrum of $GLI3$, $HOXD13$, $GJA1$ and $TP63$ gene in polydactyly, syndactyly and ectrodactyly cases were reported which identified 8 pathogenic mutations (5 in $GLI3$, 2 in $HOXD13$ and 1 in $TP63$). Case control associated study did not reveal any significant association of two studied SNPs with congenital limb malformation in Indian population. Cytogenetic analysis in split hand/foot malformation (SHFM3) revealed gain of 4 Mb region on 10q24.31 in all affected members. This region contains 4 genes $LBX1$, $BTRC$, $POLL$ and $FBXW4$. Analysis revealed that $BTRC$ and $FBXW4$ might have role in SHFM3 manifestation in the family. A novel variant of frontonasal dysplasia spectrum was reported with an intermittent deletion of 125 Kb region on 1p13.3 chromosomal region containing two genes $SLC25A24$ and $NBPF4$. Analysis revealed that $NBPF4$ gene might be associated with clinical manifestation in the patient.