Clinical and Molecular Genetic Study of Kindreds with Limbs and Neurological Anomalies

PhD Dissertation

By

Muhammad Afzal

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Human Genetics Lab, Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University, Islamabad, Pakistan

Clinical and Molecular Genetic Study of Kindreds with Limbs and Neurological Anomalies

PhD Dissertation

A dissertation submitted in partial fulfilment of requirements for degree of Doctorate of Philosophy in Human Genetics

Supervised by:

Dr. Sajid Malik

Conducted by

Muhammad Afzal

2020

Human Genetics Lab, Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University, Islamabad, Pakistan

Certificate of Approval

To Whom It May Concern

This is to certify that the research work presented in this thesis, entitled **"Clinical and Molecular Genetic Study of Kindreds with Limbs and Neurological Anomalies"** was conducted by Mr. Muhammad Afzal under the supervision of Dr. Sajid Malik. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Animal Sciences, faculty of biological sciences, Quaid-i-Azam University, Islamabad, Pakistan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Human Genetics.

Student Name: Muhammad Afzal Signature: _____

Examination Committee:

a) External Examiner 1: Name Signature:_____
(Designation & Office Address)

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Muhammad Afzal

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List of Abbreviation

Abbreviation	*Elaboration*
AER	Apical Ectodermal Ridge
BDB1	Brachydactyly type B1
BDB2	Brachydactyly type B2
CH	Congenital hypothyroidism
CLSS	Cenani-Lenz syndactyly syndrome
DGK	Dera Ghazi Khan
Dsh	Dishevelled phosphoproteins
EGF	Epidermal Growth Factor
FFU	Femur-fibula-ulna
Fzd	Frizzled as G-protein coupled receptors
ID	Intellectual disability
IFSSH	International Federation for Societies for Surgery of the Hands
IRB	Institutional Review Board
ISO	International Organization for Standardization
ISPO	International Society for Prosthetics and Orthotics
KPK	Khyber PakhtunKhwa
LDDs	Limbs deficiency defects
LDL	Low-density lipid
LDLR	Low-Density Lipoprotein Receptor
LMD	London Medical Database
LOD	Multipoint logarithm of odds
LRP4	Low-desnsity lipid Receptor Protein 4
MOI	Mode Of Inheritance
MR	Mental retardation
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
Ror	Tyrosine kinases like orphan receptor
ROR2	Receptor tyrosine kinase-like Orphan Receptor 2
SHH	Sonic HedgeHog
SOST	Sclerostin
THs	Thyroid hormones
TPO	Thyroid peroxidase
TPT	TriPhalangeal Thumb
UCSC	University of California Santa-Cruse, genome browser
Wnt	Wingless signaling pathways
YWTD	Domains of low-density lipid receptor protein
ZPA	Zone of Polarizing Activity
ZRS	ZPA Regulatory Sequence

General Summary

General summary

For various reasons, Pakistan is an ideal ground for the study of hereditary and congenital anomalies. These reasons include extended families, large sib-ships and inbred unions. Owing to its historical and geopolitical aspects, the Pakistani population is comprised of a unique combination of a large number of ethnic, linguistic and socio-demographic strata. To the interest of human biologists and geneticists, different ethnic groups exhibit a distinct pattern of hereditary and congenital anomalies likely due to their distinct genetic ancestry, consanguinity and population structure. Hence, hereditary and congenital anomalies are commonly observed in the clinical practice and one is surprised to see odd and anomalous phenotypic presentations. These facts give reasons to study the nature and patho-morphogenesis of the anomalies prevailing in our society. This study was aimed at describing the phenotypic and molecular genetic aspects of various rare, hereditary and congenital anomalies mostly related to limb morphology, among the Pakistani subjects/families.

Subjects/families with hereditary and congenital anomalies were recruited through field visits in various towns of Southern Punjab. Most of the subjects were ascertained with the help of local resource persons including para-medical staff, teachers and social workers. Subject/families were also recruited from district hospitals. Clinical data including photographs, radiographs, MRI, and laboratory investigations were obtained accordingly at the nearest tertiary care hospitals. Phenotypic characterization was carried out with the help of resident officers and specialized doctors at the tertiary care hospitals. Descriptive statistics were utilized for the analyses of data from large cohorts of subjects with similar phenotypic presentations. For molecular characterization, PCR based Sanger sequencing, homozygosity mapping through SNP-based linkage analysis, and exome sequencing were carried out. The

results obtained throughout the study period are described in the six chapters (first chapter presents a general introduction) of this dissertation.

In Chapter 2, the clinical and epidemiological aspects of 103 independent probands with polydactyly are presented. These probands exhibited different types of polydactylies which varied in the combination of involved limb, laterality and symmetry. In 67% of subjects, upper limbs were involved and 33% had lower limb involvement. The polydactylies were characterized as type I, II, and IV (3 preaxial polydactylies), type A and B (2 postaxial polydactylies. This is the largest cohort of polydactyly cases reported from Pakistan.

In Chapter 3, a molecular study of a Baloch tribe kindred with polydactyly is presented. A novel *ZRS* c.287C>A (chr7:156,584,283) mutation was observed that segregate with preaxial polydactyly type II or triphalangeal thumb polydactyly (TPT; OMIM 174500) in an extended Balochi tribe family. The phenotypic features of TPT were triphalangeal thumb with or without thumb polydactyly, bilateral small knob-like outgrowth on the little finger and clino-camptodactylous appearance of the involved digits, making it distinct from the reported typical TPT phenotypes. The inheritance pattern was autosomal dominant.

In Chapter 4, the clinical aspects of rare limb reduction defects are presented. The clinical evaluation of four patients who were recruited from various towns of Punjab was carried out. The recruited cases of limb reduction defects had sporadic and isolated phenotypes.

In Chapter 5, two unrelated families with Cenani-Lenz syndactyly, which is a rare and one of the most severe syndactyly types, are presented. Here, two mutations were identified by direct PCR based Sanger sequencing (c.316+1G>A and c.1151A>G) and found to segregate with the phenotypes. Both variants were predicted to be pathogenic by bioinformatics analyses.

In Chapter 6, a sporadic case of a male patient with brachydactyly type B1 is presented. The clinical symptoms in this patient were the congenital absence of 2nd phalanges with hypoplasia/absence of last terminal phalanx in all fingers/toes except thumb/big toe in all limbs. This phenotype was due to *de novo* heterozygous mutation c.2265 C > A$^{p.Y755*}$ in exon 9 of *ROR2*.

In Chapter 7, the case of a large family initially diagnosed with intellectual disability but later proved to be the case of hypothyroidism, is presented. The phenotype was quite diverse and puzzling and segregated in an autosomal recessive manner in the pedigree. SNP-based genotyping of this family lead to the identification of homozygous intervals common among the affected subjects, and a large number of intellectual disability-related genes were excluded. Whole exome sequencing led to the identification of genetic alteration c.719A>G in the *TPO* gene as a likely cause of autosomal recessive congenital hypothyroidism with intellectual disability in the family.

Overall, the study findings improve our understanding of clinical and molecular aspects of polydactyly, limb reduction defects, Cenani-Lenz syndactyly, brachydactyly, and congenital hypothyroidism with intellectual disability. This study will be beneficial for clinicians, researchers, and genetic counselor and government officials for implementing programs of genetic testing, counseling and management for hereditary anomalies.

Chapter 1

1: Introduction

Genetic disorders arise due to mutations in genes which disrupt the normal functioning of the respective proteins and lead to a medical condition (McClellan & King 2010; Korf *et al.* 2019). When these mutations are inherited generation after generation, they become part of the gene pool of the population. The genetic anomalies divided into single gene diseases, chromosomal anomalies, complicated disorders, and mitochondrial abnormalities. The single gene malformations occur primarily due to point mutations (Slayton & Kantaputra 2019).

Hereditary disorders are of great significance and deserve our special attention due to their impact on the lives of the affected persons, their families and the society at large. Geneticists feel fascinated and awed seeing genetic disorders making their appearance (Ahmad, 1998). In our society, persons showing genetic defects are, in general, not treated with respect, sympathy, and considerations that they deserve. The children born with genetic defects are regarded as a source of stigma and shame. All these result in social and psychological stress on the families.

For various reasons, Pakistan is an ideal ground for the study of hereditary and congenital anomalies. These reasons include extended families, large sibships and inbred unions. There are overlapping generations, marriages at young ages, and extensive pedigrees with infrequent transmissible disorders (Wahab & Ahmad 1996). One of the unique features of the Pakistani population is the high rate of consanguinity. Almost 55-63% of the 1st and 2nd cousins marriages occured within the tribe or same caste-system (Ahmad *et al.* 2016; Richard *et al.* 2019). The primary disadvantage of consanguinity is the appearance of rare recessive homozygous disorders which otherwise may not appear in an outbred population. Further, owing to its historical and geopolitical aspects, the Pakistani population comprised of a unique combination of many ethnic, linguistic and socio-demographic strata. To the interest

of human biologists and geneticists, different ethnic groups exhibit distinct patterns of hereditary and congenital anomalies likely due to their distinct genetic ancestry, consanguinity and population structure. Hence, hereditary and congenital anomalies are commonly observed in clinical practice. These facts give reasons to study the nature and patho-morphogenesis of the anomalies prevailing in our society. The research was aimed to describe the clinical and genetic molecular spectrum of rare, hereditary and congenital anomalies of skeletal and nervous system among the Pakistani subjects/families.

Different studies reported variations in the prevalence rate of congenital/hereditary anomalies in Pakistan. Congenital anomalies account for 40/1,000 prevalence rate in the Pakistani population and are major cause of stillbirth (Jehan *et al.* 2007). Congenital anomalies making the prevalence of 11.4/1,000 in 5,776 studied cases and are more commonly seen in the primiparas (Perveen & Tyyab 2007). In Sindh, the southern part of Pakistan, certain congenital anomalies like fronto-nasal bone, scalp defects, microcephaly and talipes were associated with failed misoprostol (thalidomide exposure in the first trimester of pregnancy) termination of pregnancy (Gul *et al.* 2012) . In Karachi, among the lower socio-economic groups, congenital malformations occur in 28/1,000 births and neural tube defects constituted 54.6% of the total malformations (Ayaz & Saleem 2010). Another descriptive study conducted at Liaquat University Hospital Jamshoro in Sindh showed that 16% of fatal deaths were due to congenital malformations (Khaskheli *et al.* 2007). Due to lack of social support system, subjects with disability are a major burden for the parents and family (Khaskheli *et al.* 2007).

Especially in interior and remote areas of Pakistan several factors complicate field data acquisition from human subjects. For instance, there is a lack of education and awareness, low accessibility of several areas due to inadequate transport facilities, and problems with communication due to local languages and dialects. Additionally, other factors

like low level of trust for the field surveyor/researcher, and poor rate of consent approval for participation, further add another tier to the problem. Furthermore, the unique socio-cultural and ethnic assemblage requires the involvement of District Health Officers, District Coordinator Officers, and Lady-Health-Visitors, to interact with the community. Hence, epidemiological studies may prove to be a daunting task in Pakistan.

The identification of Mendelian disorders by traditional diagnostic tests is not very promising in most cases. Consequently, to identify the causative factors, clinicians depend on multiple diagnostic assays which include morphological, radiographic, hormonal, metabolic, surgical, karyotyping and gene specific tests (Chong *et al.* 2015). Sanger sequencing has been a widely used approach to detect sequence variants underlying genetic disorders. However, this method cannot be utilized if there are many candidate genes or the large gene coding regions that making huge sequencing tasks difficult for resource compromised laboratories. For this reason, geneticists rely on positional cloning, linkage analyses and LOD score computations in order to reduce the number of loci to prioritize for further analyses. Genome-wide linkage scans or locus-specific linkage analyses allow the researchers to rule out several putative candidate loci. Over the years, microsatellite-based genome scans have been replaced by high-density SNP array genotyping methods. Recently, advancements in sequencing and computational techniques have boosted the identification of uncommon variants that cause genetic diseases (Biesecker 2010).

This thesis presents some of the results that were generated through a multi-faceted project that was conducted in the Southern Punjab, Pakistan, in the last few years. In order to gain insight into the commonly occurring hereditary and congenital anomalies prevalent in Southern Punjab, a population-based survey was launched. Subjects/families with certain types of hereditary and congenital anomalies were recruited through field visits in various towns of Southern Punjab. Priority was given to limb defects which were preferably non-

syndromic and segregated in families over several generations. Most of the subjects were ascertained with the help of local persons including para-medical staff, doctors, teachers and social workers.

Initially, among the collected subjects/families, polydactyly (additional fingers or toes) was the most prominent anomaly witnessed among the recruited families. Hence, a cohort of 103 independent subjects/families with isolated polydactyly was generated. Descriptive clinical and genetic study of this cohort was carried out. The second tier of data was assembled by recruiting rare congenital limb deficiency defects. In this context, limbs deficiency defects prevalent among the recruited subjects were evaluated and detailed clinical study was carried out. Further, large families which could yield useful genetic findings were prioritized and subjected to molecular analyses. In this regard, the Sanger sequencing was used to identify the genetic alterations in multigenerational family with the autosomal dominant triphalangeal polydactyly. Additionally, two unrelated families with Cenani-Lenz syndactyly were also resolved with the help of clinical and genetic analyses. Another sporadic male patient with brachydactyly type B1 was clinically and genetically analysed. Finally, a large family with intellectual disability and hypothyroidism was subjected to SNP-based genotyping and exome analyses.

Overall scheme of the study is depicted in Fig. 1.1. The scientific findings of this study would help to elucidate the genetic bases of hereditary and congenital anomalies prevalent in the Southern Punjab region of Pakistan. This study would also be beneficial for clinicians, researchers, genetic counselors and government officials for implementing programs of genetic testing, counseling and management for hereditary anomalies.

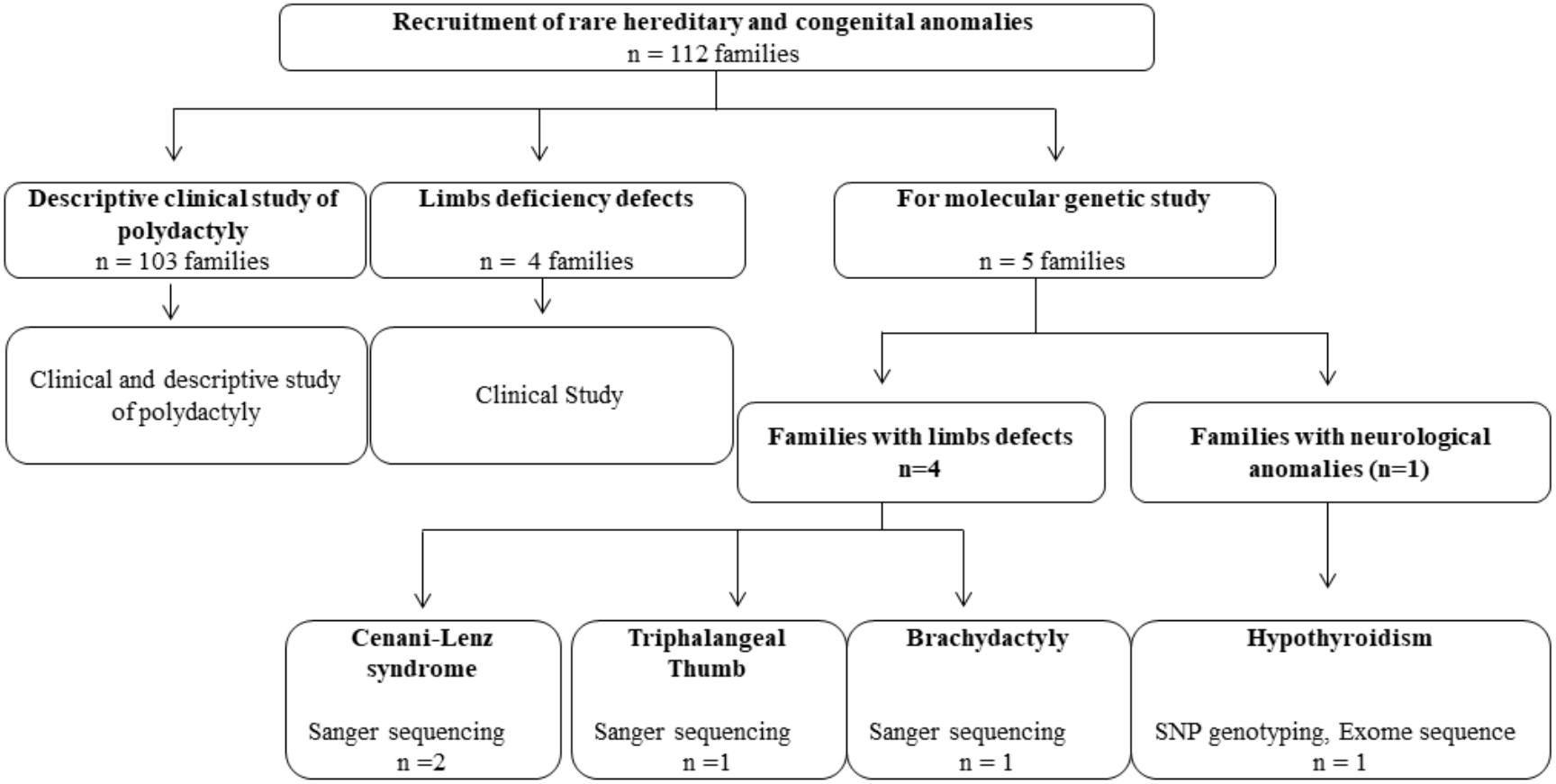


Fig. 1.1: Schematic representation of study design.

Chapter 2

2: Clinical and epidemiological study of 103 probands with polydactyly

2.1: Abstract

Polydactyly is the most prevalent congenital and hereditary digit anomaly with variable clinical expression. Its severity depends on the involvement of skeletal elements. It may be presented as isolated or syndromic and with dominant or recessive inheritance pattern. Polydactyly may also have polygenic or pleiotropic nature. The study was aimed to present clinical and epidemiological aspects of polydactyly in 103 probands. A prospective cross-sectional study was conducted in various geographic entities of Pakistan. The recruited 103 subjects had different types of polydactylies in 187 limbs. In the upper and lower limbs, polydactyly was found in 67% and 33% of the subjects, respectively. In aggregation, 44% of the subjects had preaxial polydactyly type I followed by 40% type A (postaxial polydactyly), 9% type B (postaxial polydactyly), and 4% type II, IV (preaxial polydactyly) each. There was substantial clinical heterogeneity in each polydactyly in terms of the involved limbs, laterality and symmetry. This study expands our understanding about the clinical and epidemiological aspects of polydactylies.

2.2: Introduction

The human skeleton of upper and lower limbs is built on a pentadactyly pattern and comprises bones of specific sizes and shapes (Anderson *et al.* 2012). When this arrangement of pentadactyly pattern is disrupted then it resulted in birth defects of hands and feet. Out of these defects, polydactyly, the presence of more than five digits, is the most prevalent congenital malformation of the limbs, with a ratio of 2/1000 live births (Sun *et al.* 2011).

The word polydactyly is a combination of two Greeks word "poly" means many, "daktylos" means fingers. Polydactyly is also known by polydactylism or hyperdactyly. It is more common than any other malformation of the limbs (Malik 2014). The anomaly may occur in isolated form or in association with other disorders. In either form, it shows both dominant and recessive inheritance patterns (Abbasi 2011).

2.2.1: Clinical spectrum of polydactyly

Polydactyly is heterogeneous in its morphological and anatomic expression. The severity in clinical representation of the polydactyly is dependent on the involvement of digit duplication and the number of skeletal elements which are involved in the duplication of the digits. For the duplication, the basic idea is a bifurcation of digital elements from the distal to the proximal parts along the longitudinal axis. This bifurcation may be of the digital phalanx, metacarpal/metatarsals, or carpals/tarsals of upper or lower limbs (Wassel 1969). Moreover, polydactyly has a dual nature in occurring as sporadic or familial cases. In sporadic cases, it affects one autopod of any limb whereas, in familial occurrence, polydactyly affects both autopods of upper or lower limbs but with similar phenotypic effect (Castilla *et al.* 1973; Biesecker 2002; Phadke & Sankar 2010). On the bases of pleiotropy, polydactyly may be of the rational type i.e with a known genetic mechanism or of mosaic type i.e without known genetic mechanism (Verma & El-Harouni 2015).

2.2.2: Classification, inheritance, and genetics of polydactylies

As the most commonly reported phenotype in the literature, polydactyly has wealth of data for its description, elaboration, and classification. There are different approaches that can be used for the grouping of polydactyly into different types. These include the arrangement and involvement of different skeletal elements, nature of occurrence and transmission of polydactyly and a genetic-developmental approach (Malik 2014).

Based on phylogeny and ontogeny the malformation of limbs has been divided into four groups (Lösch *et al.* 1984). These groups are:

- Group I: Lack of different parts formation
- Group II: Lack of separation of different parts
- Group III: Duplication of different parts
- Group IV: By birth constriction ring band syndromes

According to the above classification, polydactyly belongs to group III of limb malformation (Cheng *et al.* 1987). Broadly, on the bases of location, polydactylies in hands/feet can be of three types. These types are preaxial polydactyly, postaxial polydactyly, and mesoaxial polydactyly. Firstly, preaxial Polydactylies mean duplication of digits or digital parts on the thumb side. Secondly, postaxial polydactylies include duplication of digits or digital elements on the ulnar side. In last, mesoaxial polydactylies involve duplication along either side of central ray/digit.

Based on the above-mentioned classification approaches, initially, there were two international well-established systems for the classification of abnormalities of limbs extremities. Swanson's classification system (1976) classifies the malformation on the bases of clinical and anatomic features (Swanson 1976), while Temtamy and McKusick classification system (1969 and 1978) groups the malformations on the base of anatomic and

genetic background (Blauth & Olason 1988). Subsequently, different scientists have updated on the classification of the various polydactylies on the bases of newly discovered aspects and information to solve the hidden nature of polydactylies (Guo *et al.* 2013; Malik 2014; Lange & Müller 2017). Despite these advances, gaps in knowledge still exist. Hence, there is a need to improve our understanding of the classification of this genetic entity. Through literature surveys, it was found that at least 20 different types of polydactylies are listed in Online Mendelian Inheritance in Man (OMIM). Additionally, two extra phenotypes such as central polydactyly and dorso-ventral polydactyly are not allocated with OMIM number. The summary of different polydactylies (by considered locus or genes heterogeneity) is given in Figs. 2.1 and 2.2.

Classes of isolated polydactylies	*Sub-classes types*	*ID*	*OMIM*	*MOI*	*Loci/Genes*
Preaxial polydactylies →	Preaxial polydactyly type I	PPD1	174400	AD	7q36/*ZRS,SHH*
	Preaxial polydactyly type II	PPD2	174500	AD	7q36/*ZRS*
	Preaxial polydactyly type III	PPD3	174600	AD	-
	Preaxial polydactyly type IV	PPD4	174700	AD	7p14.1/*GLI3*
	Preaxial hallucal polydactyly	-	601759	-	-
Postaxial polydactylies →	Postaxial polydactyly type A1	PAPA1	174200	AD	7p14.1/*GLI3*
	Postaxial polydactyly type A2	PAPA2	602085	AD	13q21-q32
	Postaxial polydactyly type A3	PAPA3	607324	AD	19p13.2-p13.1
	Postaxial polydactyly type A4	PAPA4	608562	AD	7q22
	Postaxial polydactyly type A5	PAPA5	263450	AR	13q13.3-q21
	Postaxial polydactyly type A6	PAPA6	615226	AR	4p16.3/*ZNF141*
	Postaxial polydactyly type B	PAPB	607324	AD	19p13.2-13.1
Complex polydactylies →	Synpolydactyly	SPD1	186000	AD	2q31.1/*HOXD13*
		SPD2	608108	AD	22q13.31/*FBLN1*
		SPD3	610234	AD	14q11.2-q12
	Polysyndactyly Polysyndactyly cross	-	175690	AD	-
	Polysyndactyly type Hass	-	186200	AD	7q36.3/*LMBR1*
	Mirror image polydactyly	-	135750	AD	14q13/*MIPOL*
Genetically identified →	Central polydactylies	-	-	-	-
	Ventro-dorsal polydactylies	-	-	-	-

Fig. 2.1: Classification and genetics of different isolated polydactylies

Key: -, not identified; ID, phenotype identity; MOI, mode of inheritance.

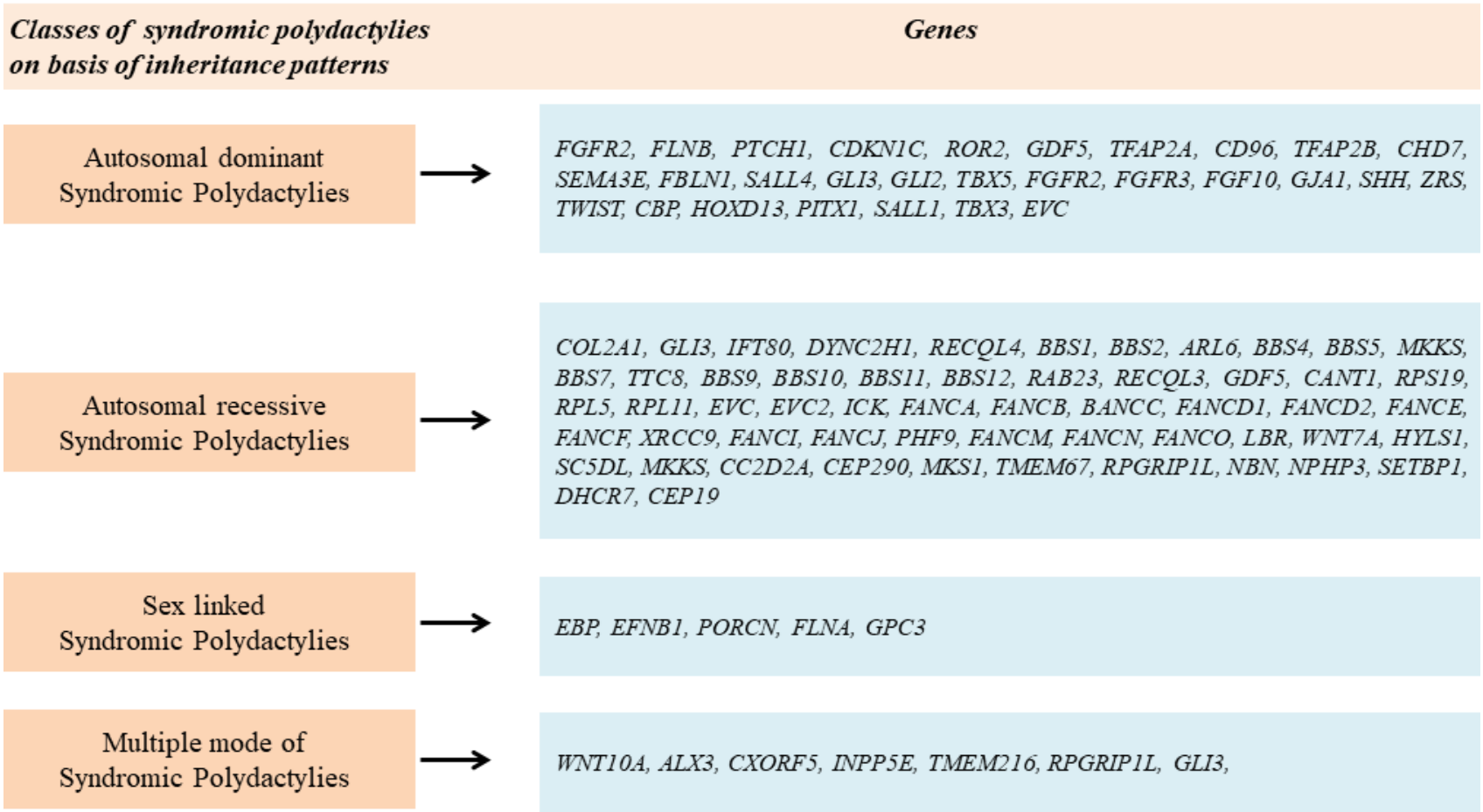


Fig. 2.2: Summary of genes that cause syndromic polydactylies with different inheritance patterns (Feb. 2019).

2.2.3: Polydactyly as a ciliopathy

The previous section showed that both the clinical expression and the genetic basis of different polydactylies are diverse and heterogeneous. However, there are unexplained aspects of the nosology of the clinical and molecular genetics of the polydactyly (Ahmed *et al.* 2017). Consequently, this study was designed to investigate the clinical aspects of polydactyly in 103 independent and unrelated probands recruited from Punjab, Pakistan.

2.3: Subjects and methods

A prospective and descriptive research was conducted over a period of three years, after approval from the Institutional Review Board (IRB) of the advanced studies. The subjects were recruited from the populations in the southern and northern parts of Punjab province, Pakistan, with all subjects being visited at their residences. The primary data were collected from different areas of selected regions through door-to-door surveys. A designated comprehensive questioner was used for a proper demographic and clinical study of polydactyly in this population. The major parts of questioner were demographic features, age, social status, marriages union types, family types and size, consanguinity, ethnicity, the area of residence, and different clinical parameters related to polydactyly. All the information and materials were collected after obtaining informed and written consent.

At the initial consultation, a preliminary diagnosis was made with the help of local resident doctors and senior researchers at the time of the survey. The diagnosis was subsequently rechecked by cross-referring with available clinical records at OMIM (https://www.omim.org/) and the London Medical Database (LMD; https://www.face2gene.com/lmd-library-london-medical-database-dysmorphology/). The diagnosis was also reconfirmed through consultation with orthopedic clinicians.

By use of designed study protocol, the families of 103 probands were investigated. All of these, polydactylies were found to be congenital anomalies either in sporadic or familial form. All the affected subjects were physically observed to document all clinical features of the anomaly. In the familial form, polydactyly exhibited both recessive and dominant inheritance patterns. Lastly, photographs and radiograms were taken to document polydactyly in the subjects.

2.3.1: Exclusion criteria

The syndromic forms of polydactyly were not included in the study because the aimed to describe isolated polydactylies. Moreover, in syndromic form polydactyly was associated as a secondary feature with other phenotypes such as brachydactyly, syndactyly, camptodactyly, and clinodactyly which have different genetic basis. Cases of mesoaxial polydactyly were not part of the study.

2.3.2: Statistical analysis

Tables showing percentage distribution and cross-tabulation of different variables were created by using Microsoft Excel. The statistical program GraphPad Prism was used to calculate chi-square test for different categorical variables with $p<0.05$ set to be valuable as statistical significance.

2.4: Results

In the sampled population, males represented 68% of the subjects, and females 32% (Table 2.1). The difference in the gender-wise distribution of polydactyly was statistically significant (P = 0.0001). Overall, there was almost equal representation of polydactyly among the included subjects from both studied areas, 49% were recruited from Southern Punjab whereas 51% were from Northern Punjab. In Northern Punjab, females were more (79%), whereas in Southern Punjab males were more (61%; Table 2.1).

With respect to their native language, the frequency distribution of subjects with polydactyly was unequal (P=0.0002). There was a high representation of Punjabi speaking subjects (45%) followed by 35% of Saraiki speaking, 11% of Urdu speaking, and 10% in other language groups (Table 2.1).

Table 2.1: Percent distribution of index subjects in samples population with respect to demographic features and gender

Variable	Male		Female		Total		P-value
	No.	%	No.	%	No.	%	
Region							
Northern Punjab	27	39	26	79	53	51	0.0001
Southern Punjab	43	61	7	21	50	49	
Rural/urban origin							
Rural	39	56	22	67	61	59	0.391
Urban	31	44	11	33	42	41	
Marital status							
Married	29	41	11	33	40	39	0.518
Single	41	59	22	67	63	61	
Age group (years)							
Up to 9	12	17	9	27	21	20	0.382
>9-19	15	21	7	21	22	21	
>19-29	15	21	7	21	22	21	
>29-39	11	16	7	21	18	17	
>39	17	24	3	9	20	19	
Language							
Punjabi	23	33	23	70	46	45	0.0002
Saraiki	34	49	2	6	36	35	
Urdu	8	11	3	9	11	11	
Others	5	7	5	15	10	10	
Total	**70**	**68**	**33**	**32**	**103**	**100**	

In Southern Punjab, representation of affected males with polydactyly was 86% than affected males of Northern Punjab and statistical value was significant P=0.0001). In contrast in Northern Punjab, the representation of affected females with polydactyly was 49% as compared to affected females from Southern Punjab (Table 2.2). There was a high representation of samples obtained from rural areas of Southern Punjab (70%), but urban representatives were higher in the sample obtained from Northern Punjab (Table 2.2).

In both geographic regions, the frequency distribution with respect to familial /sporadic nature of polydactyly was random and the Chi-square value was non-significant. In Northern Punjab, polydactyly of a sporadic nature was 62% as compared to 38% of familial polydactyly. Similarly, in Southern Punjab sporadic cases of polydactyly were 56% as compared to 44% of familial cases of polydactyly (Table 2.2).

Further, in Southern Punjab, the representation of male sporadic cases was 82% as compared to 18% of sporadic female cases. But in Northern Punjab, the proportion of females with sporadic polydactyly was 61% compared to 39% of males (Table 2.2). In familial cases, overall, there were 244 affected subjects in 42 families (41% of 103 families) with familial polydactyly. Among these, males were more common 63% (n=153/244) whereas affected females were 37% (n=91/244) (Table 2.2).

The distribution of parental consanguinity was random in familial cases and was statistically nonsignificant with percentage of 41% in the sampled population. Overall, within familial cases, parental consanguinity was 88% (Table 2.2). The extended family type was 64% and nuclear family type was 36%. Moreover, family type did not show any significant relation with any gender in familial cases in which the extended family type was ranged 61-67% (Table 2.2).

Overall, frequency distribution in 305 affected subjects from 103 total recruited families (including sporadic and familial cases) was random. Among these 305 affected individuals, affected males were 62% (n=189) and affected females were 38% (n=116) as given in Table 2.2.

Table 2.2: Percentages of polydactyly in index subjects with respect to various epidemiological area of origin

Variable	Northern Punjab		Southern Punjab		Total		P-value
	No.	%	No.	%	No.	%	
Gender							
Male	27	51	43	86	70	68	0.0001
Female	26	49	7	14	33	32	
Total	**53**	**51**	**50**	**49**	**103**	**100**	
Rural/urban origin							
Rural	26	49	35	70	61	59	0.045
Urban	27	51	15	30	42	41	
Familial/sporadic							
Familial	20	38	22	44	42	41	0.552
Sporadic	33	62	28	56	61	59	
Total affected family members in sporadic cases (n=61)							
Male	13	39	23	82	36	59	0.002
Female	20	61	5	18	25	41	
Total affected family members in familial cases (n=42)							
Male	58	64	95	62	153	63	0.891
Female	33	36	58	38	91	37	
Parental consanguinity in familial cases (n= 42)							
Yes	20	87	16	84	36	88	0.689
No	3	13	3	16	6	12	
Family type in familial cases (n=42)							0.922
Extended	16	67	11	61	27	64	
Nuclear	8	33	7	39	15	36	
Total	**24**	**100**	**18**	**100**	**42**	**41 of 103**	
Total affected family members in all cases (n=103)							
Male	71	57	118	65	189	62	0.187
Female	53	43	63	35	116	38	
Total	**124**	**41**	**181**	**59**	**305**		

On the bases of clinical evaluation of all cases (n=103), polydactyly was divided into five types including postaxial polydactyly A, postaxial B, preaxial type I, II, and IV polydactylies. The frequency distribution of different polydactylies was not statistically significant with respect to gender. Overall there was a high frequency of preaxial polydactyly (51%; n=53) and similar frequency of postaxial polydactyly (49%). Similarly, in each sub-type of polydactyly, preaxial type I was 44%, followed by frequency of postaxial type A (40%), postaxial polydactyly type B (9%), and 3.5% for each preaxial type II and IV (Table 2.3).

Table 2.3: Frequency distribution of different polydactylies with respect to gender

Polydactyly types	**Gender**				**Total**		**P-value**
	Male		**Female**				
	No.	**%**	**No.**	**%**	**No.**	**%**	
Type A postaxial	29	41	12	36	41	40	
Type B postaxial	4	6	5	15	9	9	
Type I preaxial	29	41	16	48	45	44	0.168
Type II preaxial	4	6	0	0	4	3.5	
Type IV preaxial	4	6	0	0	4	3.5	
Sub-total	**70**	**68**	**33**	**32**	**103**	**100**	

The frequency distribution of different types of polydactyly was non-random in cases of familial and sporadic polydactylies (P=0.0052). This proved, the high prevalence of sporadic preaxial polydactyly as compared to high prevalence rate of postaxial type A in familial form (Table 2.4).

Table 2.4: Frequency of different polydactylies with respect of occurrence of familial or sporadic nature

Polydactyly types	**Familial/sporadic nature**				**Total**		**P-value**
	Familial		**Sporadic**				
	No.	**%**	**No.**	**%**	**No.**	**%**	
Type A postaxial	21	50	20	33	41	40	
Type B postaxial	3	7	6	10	9	9	
Type I preaxial	11	26	34	56	45	44	0.0052
Type II preaxial	3	7	1	2	4	4	
Type IV preaxial	4	10	0	0	4	4	
Sub-total	42	41	61	59	103	100	

Among 103 individuals, polydactyly was found in 412 limbs of all probands. From the frequency distribution table it was inferred that preaxial polydactyly type I was associated with upper right hands, and postaxial polydactyly was strongly associated with left upper hands (42%), right lower feet (73%), and to left lower feet (75%) and it was statistically significant (P= 0.0002; Table 2.5). Collectively, among 187 affected limbs out of 412 limbs of all probands, the frequency of postaxial polydactyly was high about 49% than any other type of polydactyly and at gross limbs level, the frequency of polydactyly was 67% in upper limbs and 33% in lower limbs. Moreover, the frequency of polydactyly in upper right hands was 37%, in left upper hands was 29%, in left lower feet was 17% and in lower right feet was 16% (Table 2.5).

Table: 2.5: Spectrum of polydactylies in 187 affected limbs among 103 index subjects

Polydactyly types	**Upper Limbs;** n=125 (67%)				**Lower Limbs; n=**62 (33%)				**Total**		**P-Value**
	Right hand		**Left hand**		**Right foot**		**Left foot**				
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	
Type A postaxial	22	31	23	42	22	73	24	75	91	49	
Type B postaxial	7	10	6	11	1	3	1	3	15	8	
Type I preaxial	33	47	18	33	2	7	2	6	55	29	0.0002
Type II preaxial	4	6	4	7	1	3	1	3	10	5	
Type IV preaxial	4	6	4	7	4	13	4	13	16	9	
Total	70	37	55	29	30	16	32	17	187	100	

2.5: Discussion

This study presented variable clinical and epidemiological aspects of isolated polydactyly in a cohort of 103 probands from two regions of Punjab, Pakistan. At present, there are different genetic and nongenetic factors that cause variable clinical and molecular heterogeneity in reappearance of polydactylies. Though, little is well-known about the innate factors that cause the additional digits in the limbs. Therefore, this cohort could be used as a source for molecular studies to find new mutations/genes and may prove to be helpful to understand the etiology of polydactyly.

This study focused the demographic and phenotypic features of polydactyly. There was a high frequency of male subjects with polydactyly (68%) compared to females (32%), and the differences in the dissemination were statistically significant across the populations of South and North parts of Punjab. This difference in frequency was might be due to high ratio of male population from south part whereas female population participation ratio was high from north part of the province and it was statistically significant (P=0.0001). The inequality in the male-female ratio may be due to differences in culture of the two populations because in Southern part the male dominance is more as compared to female. Moreover, the inhabitants of the Southern part were reluctant in the provision of information about affected females in order to preserve their family's secrecy and also to avoid the other social issues. In Northern part, people were more educated and there was less dominancy of males, therefore they (especially females) were not reluctant to participate in the study

Another significant factor was the high frequency of sporadic polydactyly in the male probands, (Table 2.2). Again, there was high proportion of males with sporadic polydactyly from Southern Punjab and vice versa from Northern Punjab. Similarly, in the case of familial polydactylies analysis indicated high representation of males as compared to females (Table

2.2). Collectively, the study revealed two types of polydactyly, radial side (preaxial polydactyly) and ulnar side (postaxial polydactyly). There was no report of mesoaxial polydactyly. Overall, the frequency of preaxial polydactyly was high with 51% and postaxial polydactyly was 49% in the studied population.

In sporadic and familial cases, preaxial type I was 56%, whereas type A (postaxial polydactyly) was 50%, respectively. This inequality was significant (P= 0.0052) (Table 2.4). Additionally, comparison of different types of polydactyly according to the involvement of limbs and laterality of body proportions was remarkable. In this regard, postaxial type-A polydactyly was more prevalent (49%) compared to preaxial type I (29%). Similarly, postaxial polydactyly was strongly associated with lower limbs, whereas the preaxial type I of polydactyly had low involvement of lower limbs (Table 2.5). Additionally, postaxial type-A polydactyly appeared in the upper left side in 42% cases whereas preaxial polydactyly was only limited to the upper right limb (47%).

In the past, hospital-based studies relied on newborn screening to estimate the prevalence of polydactyly instead of door-to-door surveys in the general population and leaving its prevalence unknown in many human populations (Robert *et al.* 2018). Hence the epidemiological studies may be helpful in the prevention of hereditary polydactylies through genetic counseling that requires information about its etiology, prenatal detection and risk factors estimation for later pregnancies (Parikh *et al.* 2018). There are many unsolved facts about polydactyly in the populations, with the previous studies not representing the whole population (Malik *et al.* 2014).

The high prevalence of preaxial polydactyly in the Pakistani population is in concordance with the high rate of preaxial polydactyly in the native population of the Amerindian and united states (Castilla *et al.* 1997). The high occurrence preaxial polydactyly

high occurrence in the studied Pakistani cohort is inconsistent with high occurrence of postaxial polydactyly in population of Khyber PakhtunKhwa (Ullah *et al.* 2015) and South American population (Castilla *et al.* 1997). A similar contradiction was also found within the African population (Lopez-Camelo & Orioli 1996). Moreover, the high rate of affected males, sporadic cases, and upper limb involvement in the studied cohort are similar findings in Pakistani populations (Ullah *et al.* 2015).

In conclusion, the study results added valuable information about the current clinical spectrum of polydactyly with the presented data being useful for understanding the comparative distribution of different polydactylies. Moreover, the phenotypic presentation, expressivity, and inheritance of these cases allow the researchers/clinicians to register all clinical aspects of the anomaly in epidemiological surveys (Baas *et al.* 2018). Further, as an indicator of birth defects, the epidemiological information on polydactyly would also be helpful in exploring the prevalence of polydactyly-associated congenital anomalies in Pakistani populations. At the end, such analysis indicates different etiological factors of sporadic and familial polydactyly.

Results of this study were published in Clin Genet:

Malik S, Ullah S, Afzal M, Lal K, Haque S (2014). Clinical and descriptive genetic study of polydactyly: a Pakistani experience of 313 cases. Clin Genet; 85: 482-486.

Chapter 3

3: A novel mutation in *ZRS* locus c.287C>A segregates with triphalangeal thumb polydactyly phenotype with a high degree of intrafamilial variability

3.1: Abstract

Type II, preaxial polydactyly (OMIM 174500) is a rare autosomal dominant Mendelian phenotype of human limb malformation. The cardinal phenotypic features include triphalangeal thumb, preaxial polydactyly, and digit fusion with high interfamilial/intrafamilial variability. This may be syndromic or non-syndromic. The isolated form may be opposable or non-opposable on the bases of the functionality of triphalangism. All forms of triphalangeal thumb (TPT) caused by different genetic changes in *ZRS* that is found on 7q36.3. The study is aimed to report a mutation in *ZRS* region in large Balochi tribe family. The phenotype was fairly unusual due unfamiliar clinical symptoms like bilateral small knob-like outgrowth on the little finger and TPT with or without thumb polydactyly and clino-camptodactylous appearance of the involved digits, in addition to the characteristic TPT features. Through direct Sanger sequencing, a novel ZRS c.287C>A (chr7:156,584,283) mutation was detected in this family which segregated with the phenotype. This study expands our understanding regarding the clinical and molecular aspects of TPT.

3.2: Introduction

Birth defects of the first digital ray include the malformation of the thumb and first metacarpal. The hereditary and congenital defects pertaining to the thumb are represented by preaxial polydactyly, megalodactyly, broad thumb, short thumb, and aplasia of thumb (Poznanski *et al.* 1971). In type I thumb polydactyly (MIM 174400), there is a replication of one or more digital elements of the biphalangeal thumb. It is a relatively common polydactyly type. In type II preaxial polydactyly, there is triphalangeal thumb (TPT) polydactyly. It occurs with a prevalence rate of 0.08-1.4/1000 live births in different populations (Temtamy & McKusick 1978; Malik 2014; Goldfarb *et al.* 2015). As part of radial polydactyly, the TPT is an unusual malformation in which thumb has three phalanges instead of two phalanges and morphologically looks like a finger (Baas *et al.* 2017). It was first reported in the literature in 1559 by Columbi (Kalikian, 1974). This malformation can occur in both isolated and syndromic forms. Additionally, in isolated forms, it is further classified as opposable and non-opposable types in both hands and feet (Qazi & Kassner 1988).

For classification purposes, the International Federation for Societies for Surgery of the Hands (IFSSH) put TPT in group III (polydactyly) of hand congenital malformation. Historically, there are different systems for the classification of congenital TPT. These systems classify TPT on the bases of the shape or size of the extra phalanx with some consideration of tissue and muscle consideration (Zuidam *et al.* 2008).

In 1907, Hilgenreiner (Hilgenreiner 1907) classified TPT into four different types:

1. Incomplete hyperphalangism with small underdeveloped triangular extra phalanx;
2. Partially completed hyperphalangism with more developed extra phalanx as compared to the first type;

3. Complete hyperphalangism with a thumb like appearance; and
4. Complete hyperphalangism without thumb resemblance.

This system of classification had limitations due to the use of word 'incomplete' and the difficulty in measuring the extent of hyperphalangism. Later, the above-mentioned limitations were removed by the addition of the words brachymesophalangy (rudimentary extra phalanx), and dolichophalangy (finger-like thumb without opposition) by Cocchi in 1952 (Schinz *et al.* 1953) and in 1962 by Swanson (Swanson & Brown 1962).

For the development of easy and widely used classification system, Wassel (1969) divided the congenital defects of the thumb into 7 classes (type I-VII) on the bases of the duplication (Cheng *et al.* 1984). In this classification system, all possible cases of TPT were included in type VII (Table 3.1). Although, Wessel's classification is more practical and enough to classify all simple cases of preaxial polydactyly. Yet, it can't classify complex phenotypes of thumb polydactyly. To overcome this, Miura and Wood independently proposed a modified and updated classification (Miura 1976; Wood 1978) (Table 3.1).

Table 3.1: Modified classification system of Wassel (1969) by Muira and Wood (1978)

Classification types	**Features**	**References**
Type I	Bifid distal phalanx	Wassel, 1969
Type II	Duplicated distal phalanx	Wassel, 1969
Type III	Bifid proximal phalanx	Wassel, 1969
Type IV	Duplicated proximal phalanx	Wassel, 1969
Sub-type IV A	Partial and completely duplicated proximal phalanx contained triphalangeal components	Wood, 1978
Sub-type IV B	Triphalangeal components on radial side	Wood, 1978
Sub-type IV C	Triphalangeal component on ulnar side	Miura, 1976
Type V	Bifid metacarpal	Wassel, 1969
Type VI	Duplicated metacarpal	Wassel, 1969
Type VII	Triphalangism	Wassel, 1969
Sub-type VII A	Triphalangeal ray emerge at level of metacarpal on ulnar side	Wood, 1978
Sub-type VII B	Both radial and ulnar side had triphalangeal components	Wood, 1978
Sub-type VII C	Triphalangeal of radial side only	Wood, 1978
Sub-type VII D	Triplication on either side without triphalangism	Wood, 1978

Later in 1987, Buck-Gramcko presented an entirely new classification system and divided the TPT into six different types on the bases of extra phalanx treatment, different position of epiphyses in 1st metacarpals, and intrinsic musculature (Buck-Gramcko 1987). The identified classes in this system are shown in Table 3.2.

Table 3.2: Buck-Gramcko (1987) classification of triphalangism

TPT type	***Morphological and clinical symptoms***
Type I	Rudimentary triphalangism with slightly deviated unsegmented long epiphyses of the distal phalanx
Type II	Triphalangism of wedge-like middle phalanx with ulnar deviation and normal thumb anatomy
Type III	Triphalangism with trapezoid of middle phalanx (long thumb with short middle phalanx which is neither triangular nor rectangular
Type IV	Triphalangism with long quadrangular central phalanx, finger-like appearance and little musculature
Type V	Hypoplastic TPT with unstable rudimentary tendon and syndactyly with the index finger
Type VI	TPT with preaxial polydactyly either on the radial or ulnar side of triphalangeal thumb with a possible combination of middle phalanx and triplication

However, this system was difficult to follow and not frequently used. All above mentioned six types of TPT were placed into opposable (type I-III), and non-opposable (type IV-VI) groups on the bases of the functionality of the thumb with the extra phalanx.

From the above discussion it is evident that all systems have certain degree of limitations and raised the need for a new or revised classification system. To solve the problem of unclassified cases of preaxial polydactyly, Ziudam (2008) proposed a new and modified classification system, which is known as the Rotterdam's classification system. Building on previous classifications, they proposed eight classes (type I-VIII) for classification of thumb ray polydactylies including the triphalangeal thumb. They added the type VII and type VIII on the bases of partial and complete duplication of carpals. The summary of the classification is given in Fig. 3.1.

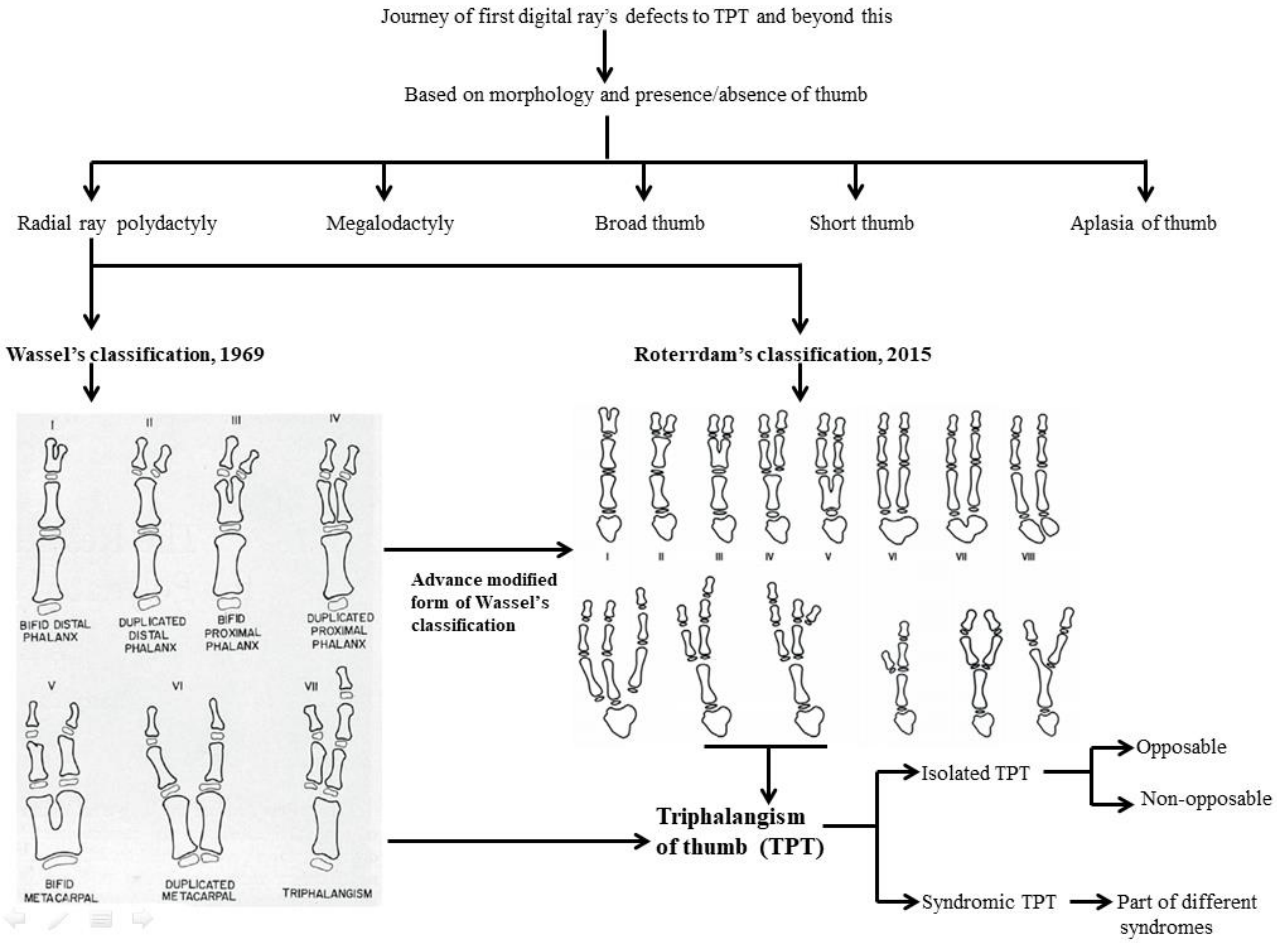


Fig. 3.1: The summary of triphalangeal thumb classification from 1969-2008.

TPT cases that segregate in various families have been linked to mutations in the ZRS (ZPA Regulatory Sequence) locus. The cis-acting ZRS is conserved intronic region in the *LMBRI* gene on chromosome 7q36. The sonic hedgehog (SHH) has a strong expression in the zone of polarizing activity (ZPA) of the growing limb bud (Lettice *et al.* 2003; Gurnett *et al.* 2007).

With the inclusion of this reported mutation, at least 15 variants in the ZRS cause different types of Mendelian phenotypes, which include preaxial polydactyly type II, Werner mesomelic syndrome, and acheiropody (MIM 200500). All of the ZRS mutations can be grouped into following different types: i) single nucleotide base change throughout the ZRS

region such mutations causing preaxial polydactyly; ii) single base change at one specific position in ZRS such alterations cause Werner mesomelic syndrome; iii) ZRS duplications which cause polysyndactyly; and iv) ZRS based insertion causing preaxial polydactyly (Table 3.3).

Table 3.3: The summary of 15 reported mutations in the ZRS region

Mutation type	***Variant***	***Major phenotype***	***Clinical features***	***Reference***
Random point mutation that occur throughout the ZRS region	pZRS156585476G>C	TPT with/without preaxial polydactyly (174500)	TPT-polysyndactyly syndrome	Potuijt et al. 2018
	ZRS105 C > G		Triphalangeal thumb, and preaxial polydactyly	Bass et al. 2017
	ZRS 428T>A		Bilateral thumb duplication, unilateral TPT	Wu et al. 2016
	ZRS406 A > G		Triphalangeal thumb, and preaxial polydactyly	Zhao et al. 2016
	ZRS402 C > T		Triphalangeal thumb, and preaxial polydactyly	VanderMeer et al. 2014
	ZRS406 A > G		Tibial hypoplasia, polydactyly and triphalangeal first fingers	Norbnop et al. 2014
	ZRS287 C > A*		Triphalangeal thumb, preaxial polydactyly, postaxial polydactyly, small knob-like protuberance, syndactyly	VanderMeer et al. 2012
	ZRS619 C > T		Preaxial polydactyly, triphalangeal thumb, radial deficiency	Al-Qattan et al. 2012
	ZRS295T>C		Triphalangeal thumb, and preaxial polydactyly	Furniss et al. 2008
	ZRS621 C > G		Triphalangeal thumb, and preaxial polydactyly	Gurnett et al. 2007
	ZRS739 A > G		Triphalangeal thumb, and preaxial polydactyly	Gurnett et al. 2007
Insertion mutation	ZRS603ins13		Preaxial polydactyly with triphalangeal thumb	Laurell et al. 2012
Localized point mutation that occurs to the specific position in the ZRS region	ZRS404 G > T	Werner mesomelia syndrome (188770)	Small malformed radius and ulna, short forearm, abnormal wrist with short disorganized carpals, 5-7 fingers, malformed thumb, TPT with or without radial polydactyly, lower limbs deficiencies, clubfoot, syndactyly in hands and feet, feet with abnormal nails	Wieczorek et al. 2010; Girisha et al. 2014
	ZRS404 G > A			
	ZRS404 G > C			

*this study

In this study, we describe, a Balochi tribal family that originated in Southern Punjab, Pakistan, presented with TPT. The main features in the family were a bilateral triphalangeal thumb, preaxial polydactyly, postaxial polydactyly, and syndactyly. Detailed clinical study of this family was conducted. Mutation analyses in the family led to the discovery of a novel variant c.287C>A in the *ZRS* locus that is linked to TPT.

3.3: Subjects and Methods

The presented family belongs to the Baloch tribe and live in the Koh Salman mountain range in the Dera Ghazi Khan (DGK) district of Southern Punjab, Pakistan. The studied tribe migrated to the agricultural rural area of Rajan Pur district from where the family was located with the help of local persons. The elders of the family were illiterate and had no idea about hereditary anomalies and genetic study. Therefore, all family members were verbally informed (in the local Saraiki language) about the purpose of the study with help of local assistants. All information and biological material were obtained after taking the verbal consent from the family guardian/elders.

Most of the family members were physiologically examined for the morphological and clinical aspects of the malformation. Photographs and X-rays films were taken from eight subjects (7 affected and 1 unaffected) and peripheral blood samples were obtained from 12 subjects (7 affected and 5 unaffected) for the molecular genetic part of the study. All the information and material were collected during multiple visits.

Genomic DNA was extracted from the blood lymphocytes using a modifying the standard phenol-chloroform method (Sambrook and Russel, 2001). Briefly, the blood samples were taken in sterile EDTA tubes. About 0.5 ml of blood sample was taken in Eppendorf tubes to which 1 ml cell lysis buffer was mixed and kept at 4°C for 15 minutes (min). Tubes were centrifuged at 4,500 revolutions per minute (rpm) at 4°C. The supernatant was discarded, and the pellet was re-suspended in 1 ml of cell lysis buffer. It was centrifugation at 4°C for 15 min. The supernatant was discarded, and the pellet was again suspended in 1 ml nucleus lysis buffer. In the reaction mixture, 15 µl of Proteinase K and 20 µl of 10% SDS were added in order to digest the proteins. The reaction was kept at 37°C overnight. On the next day, samples were treated with solution C and solution D and mixed

by vigorous shaking, respectively. Centrifugation was done at 4500 rpm for 12 min at 4°C to separate the precipitated proteins in the form of a pellet. The supernatant was poured into a fresh Falcon tube, and two volumes of chilled ethanol were added. The floating white thread of DNA was fished out and transferred to a new tube. The dry DNA pellet was dissolved in an appropriate volume of Tris-EDTA (TE) buffer.

3.3.1: Polymerase chain reaction (PCR) amplification and Sanger sequencing

After a detailed literature survey and owing to a close similarity of the phenotype segregating in this family with preaxial polydactyly type II (or TPT), primers were designed for PCR amplification to cover the *ZRS* region (Table 3.4). The amplification was carried out in 20-25 µl volume with 50 ng genomic DNA as template, 2 µl 10X PCR buffer, 0.6 µl dNTP mix (10 mM), 0.5 µl primer (10 pMol/µl),0.6 µl$MgCl_2$ (50 mM), 0.2 µl *Taq* DNA polymerase (Rapidozym, Germany). Amplifications were performed at 55°C on Applied Biosystems MiniAmp Thermal Cycler.

Table 3.4: List of primers used for PCR amplification of ZRS region

Region	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
ZRS_1	TTTCAAATGCTCACTTTACATGG	TTTTATGACCAGATGACTTTTTCC
ZRS_2	AGGCTGGACTTCCTACTCACTCT	GAATAAAAATGTCAGGAGGAAAAA
pZRS_1	AAATTTTACATAACAATCATATGGAG	AAGCAGCTAACTTTTATCTTGGAA
pZRS_2	AGGTGACAGCAAAATAATCTAAA	TGCTGAAGTGATACTGAAGAGAGG

Sequencing of amplicons was carried out by Quintara Biosciences (Albany CA USA) according to set procedure and the analyses of the sequence traces were done by Sequencher software (Gene Codes Corporation, Ann, MI USA). The University of California Santa-Cruse (UCSC) Genome Browser (GRCh37/hg19) Assembly was taken as references. The detected variants were scrutinized through the online databases: 1000 Genome Project

(http://www.1000genomes.or), dbSNP (single nucleotide polymorphism database) (build 135; http://www.ncbi.nlm.nih.gov/projects/SNP), and ExAC (Exome aggregation consortium) Browser (http://exac.broadinstitute.org/).

3.4: Results

3.4.1: Clinical report

The five generations pedigree of a Baloch tribe family was constructed. Pedigree evaluation indicated that almost all marriages were consanguineous at first cousin level, showing their strong tribal inclinations of inbreeding (Fig. 3.2). The phenotype likely appeared *de novo* in subject 305 and segregated in the next two consecutive generations (IV-V). Moreover, the descendants of the affected subject were the product of consangenious unions (Fig. 3.2).

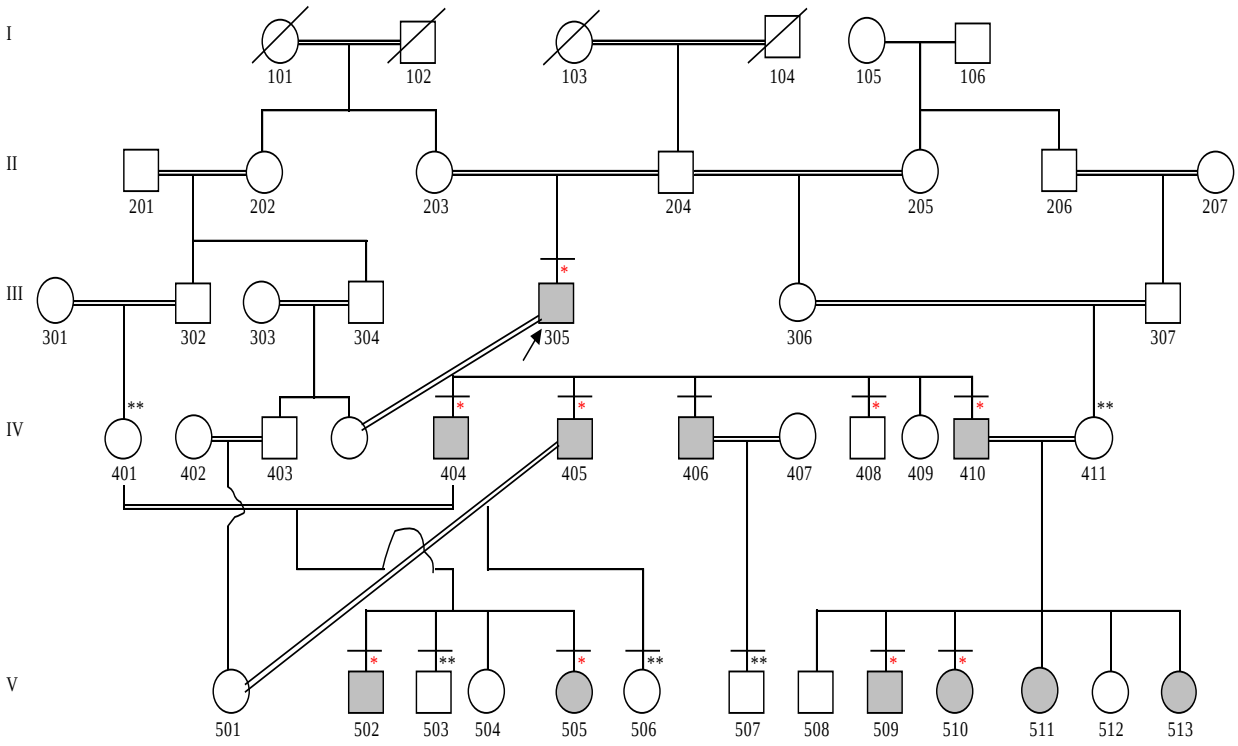


Fig. 3.2: Pedigree showing transmission of TPT in Bloch tribe family

*, subjects selected for physical and clinical/radiological examination, and blood sampled; **, person only blood sampled; and horizontal bar above the symbol indicates that the subject was physically observed.

The phenotype in this family was manifested with major and minor symptoms with involvement of hands and feet. The summary of the clinical findings in eight affected subjects presented in Table 3.5 and Fig. 3.3.

The most striking features of the phenotype in this family were bilateral triphalangeal thumb, preaxial polydactyly and knob-like small outgrowth at distal phalanx of little fingers in all affected subjects (Fig. 3.3; Table 3.5). The minor features were ulnar inclination of TPT, camptodactyly of TPT, syndactyly (Fig. 3.3; 410c, 502d) and postaxial polydactyly type B of hands, camptodactyly of 3^{rd} and 4^{th} finger (Fig. 3.3; 410c), and bifid big toes with inside tibial inclination (Fig. 3.3; 405b), and syndactyly in feet (Table 3.5).

Table 3.5: Summary of clinical features in the examined affected subjects in the family

Variable / Pedigree ID	305	404	405	410	502	505	509	510	Concordance
Gender/age	M/65	M/25	M/20	M/22	M/6	F/3	M/6	M/5	
Major features									
Triphalangeal first finger	B	B	B	B	B	B	B	B	8/8
Knob-like structure on distal digit of fifth finger	B	B	B	B	B	-	B	-	6/8
Angular pre-axial polydactyly in hands	-	R	R	R	L	R	-	-	5/8
Clinodactyly of TPT	-	-	L	R	-	-	R	-	3/8
Camptodactyly of TPT	-	-	R	L	-	-	L	-	3/8
Pre-axial polydactyly in hands	B	R	R	R	B	B	-	-	3/8
Syndactyly in hands	-	-	-	B, 4/5	-	-	B, 4/5	-	3/8
Triphalangeal finger is curved	-	B	-	-	B	B	-	-	3/8
Minor features									
Camptodactyly of 4^{th} finger	-	-	B	B	-	-	-	-	2/8
Post-axial polydactyly in hand	-	-	-	-	-	B	-	-	1/8
Bifid big toes/ preaxial polydactyly	-	B	-	-	R	-	-	-	1/8
Camptodactyly of 3rd finger in hand	-	-	-	L	-	-	-	-	1/8
Syndactyly in feet	-	-	-	-	-	R,4/5	-	-	1/8

B, bilateral; L, left; R, right; 4/5, 4^{th} and 5^{th} digits; -, feature absent

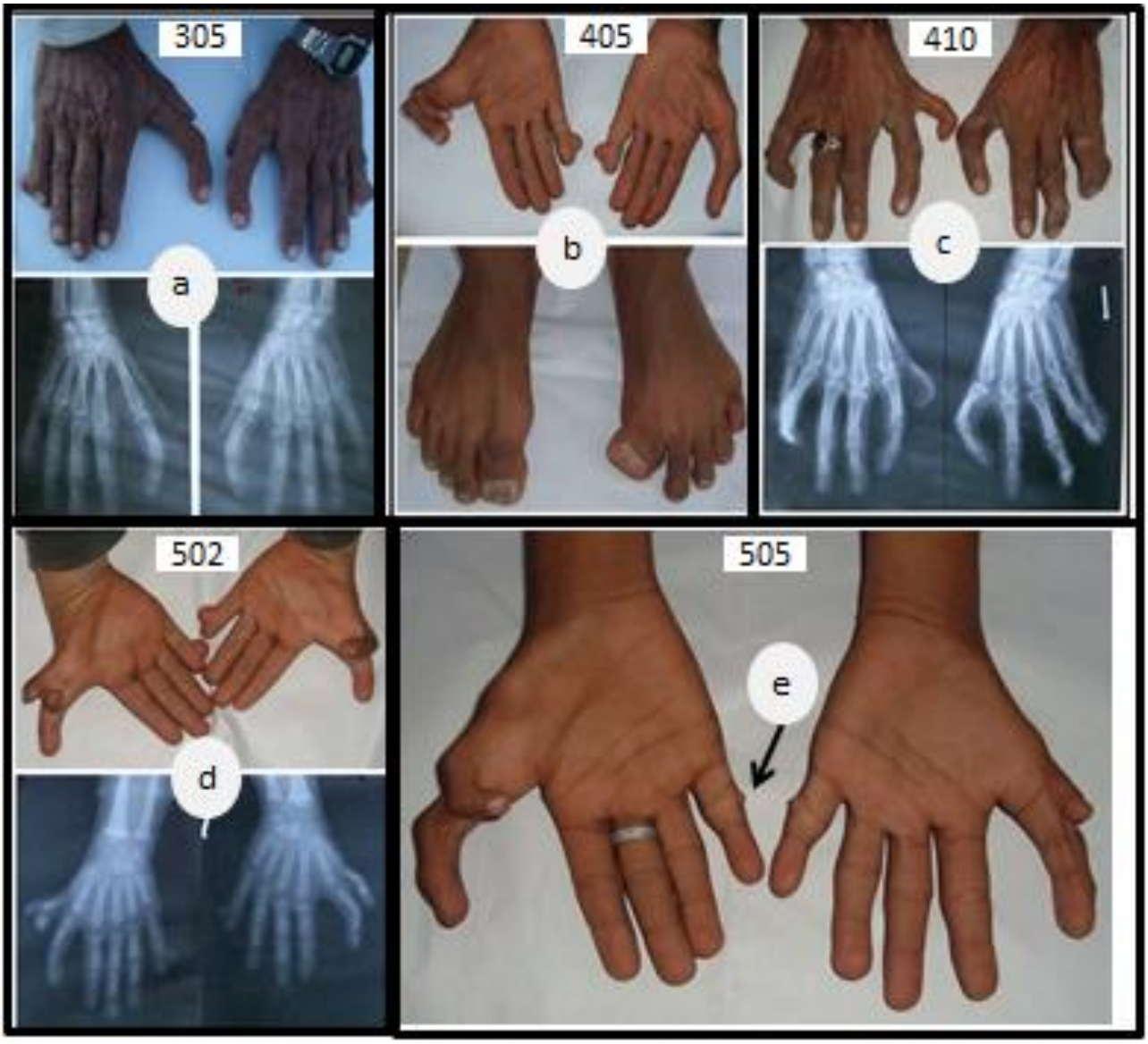


Fig. 3.3: Morphological and phenotypic features of affected subjects.

3.4.2: Molecular study

DNA sequencing led to the identification of a novel heterozygous single base pair substitution mutation C>A in *ZRS* at position 287 nucleotide (chr7:156,584,283; hg19) (Fig. 3.4). This variant segregated in all affected subjects of the family, was absent in the normal subjects.

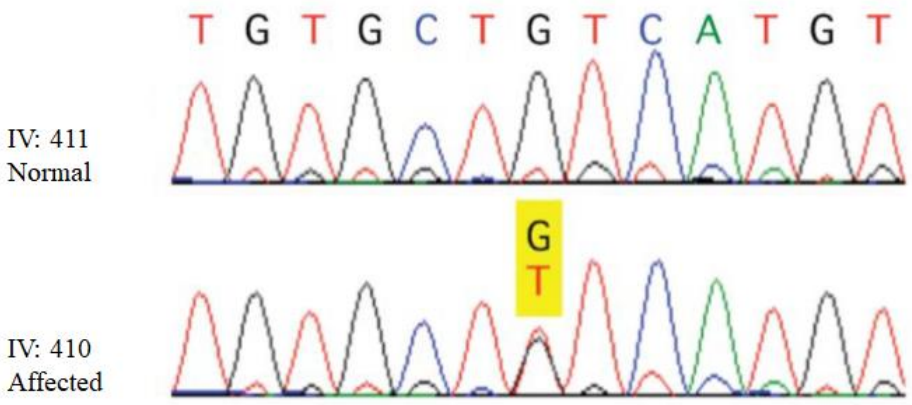


Fig 3.4: Chromatogram of the *ZRS* region showing the mutation in the affected subject

Thus, this mutation was likely responsible for the phenotype in the presented family, and the mutation was segregated in three consecutive generations. This variant was not reported in the online databases like dbSNP (build 135; http://www.ncbi.nlm.nih.gov/projects/SNP), 1000 Genome Project (http://www.1000genomes.or), and ExAC Browser (http://exac.broadinstitute.org/). Moreover, this mutation was not found in the 42 unrelated individuals including 19 unrelated Pakistani subjects who were sequenced simultaneously.

3.5: Discussion

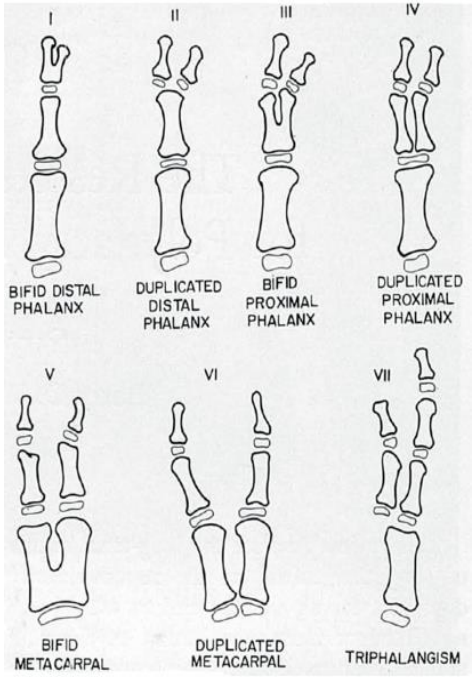
Herein, the study reported the clinical and molecular study of an extended family with TPT which segregated in a Baloch tribe family. The major clinical features were a triphalangeal thumb, preaxial polydactyly, small knob-like protuberance at the distal end of small fingers, postaxial polydactyly, and syndactyly in the affected members of this family. All these features were likely to the due heterozygous mutation at c.287C>A in ZRS. This mutation completely segregated in all affected subjects but was not found in the normal subjects of the family.

The described family showed great intra-familial phenotypic and clinical variability regarding the severity and the involvement of limbs. This phenotype was concordant with previously reported phenotypes caused by mutations in ZRS (Gurnett *et al.* 2007; Furniss *et al.* 2008; Zhao *et al.* 2016b). In contrary to this, the clinical features are not concordant with the following previous reported studies (Wieczorek *et al.* 2010; Al-Qattan *et al.* 2012; Girisha *et al.* 2014).

For the anatomical classification of the phenotype, the six hands X-rays of three patients were evaluated through the systems proposed by Wassel (1969), Wood (1978), and Zuidam et al. (2008), as shown in Table 3.6. The findings showed that most of the clinical variants evident in the family could be described with these systems. An attempt was also made to classify the phenotypic and anatomical variability in the family according to classifications proposed by Wood (1978), and the modified classification system of Wassel and Rotterdam's classification system (Fig. 3.5).

Table 3.6: Comparison of anatomical features of TPT in presented family with Wassel's and Rotterdam's classification

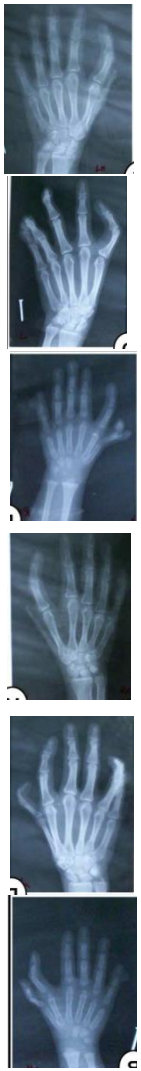
Wassel's classification, 1969



Comments on Wassel's classification

This was initial widely used classification system to classify the radial polydactyly. But this system did not classify all the cases of preaxial polydactyly. So its limitations removed by the Roterrdam's classification system.

Presented family comparison, 2018



A: 1. Triphalangism with ulnar deviation
2. Duplicated 1st metacarpal with single phalanx

B: 1. Triphalangism with ulnar deviation
2. Cutaneous fusion pf 4/5 fingers

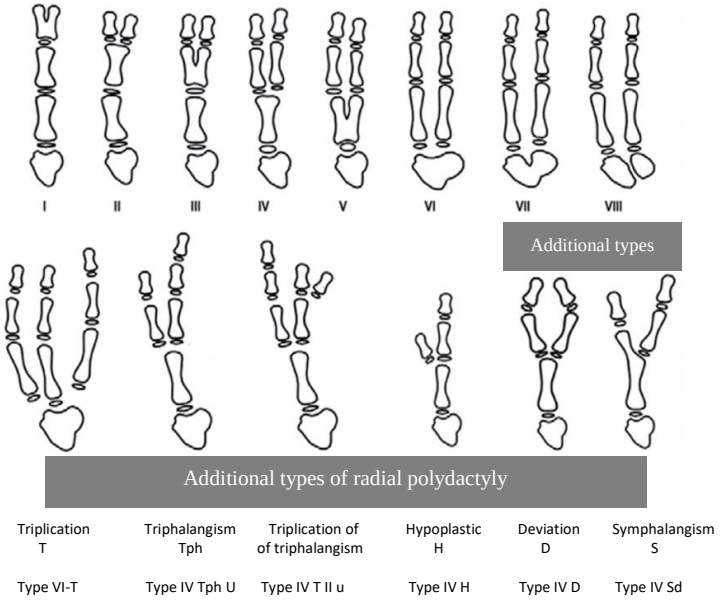
C: 1. Triphalangism with ulnar deviation
2. Duplicated of 1st metacarpal with normal ist phalanx, but distal phalanx is duplicated

D: 1.Triphalangism ulnar deviation
2. Duplication of 1st metacarpal with single phalanx

E: 1. Triphalangism with ulnar deviation
2. Duplicated 1st metacarpal with two phalanges and varus inclination
3. Osseous fusion of 4/5 fingers

F: 1. Triphalangism with ulnar deviation
2. Duplicated 1st metacarpal with two phalanx

Rotterdam's classification, 2008



Comments on Roterrdam's Classification

This is the new adopted classification system for the classification of triphalangeal thumb with preaxial polydactyly because such cases mostly not classified by the Wassel's classification.

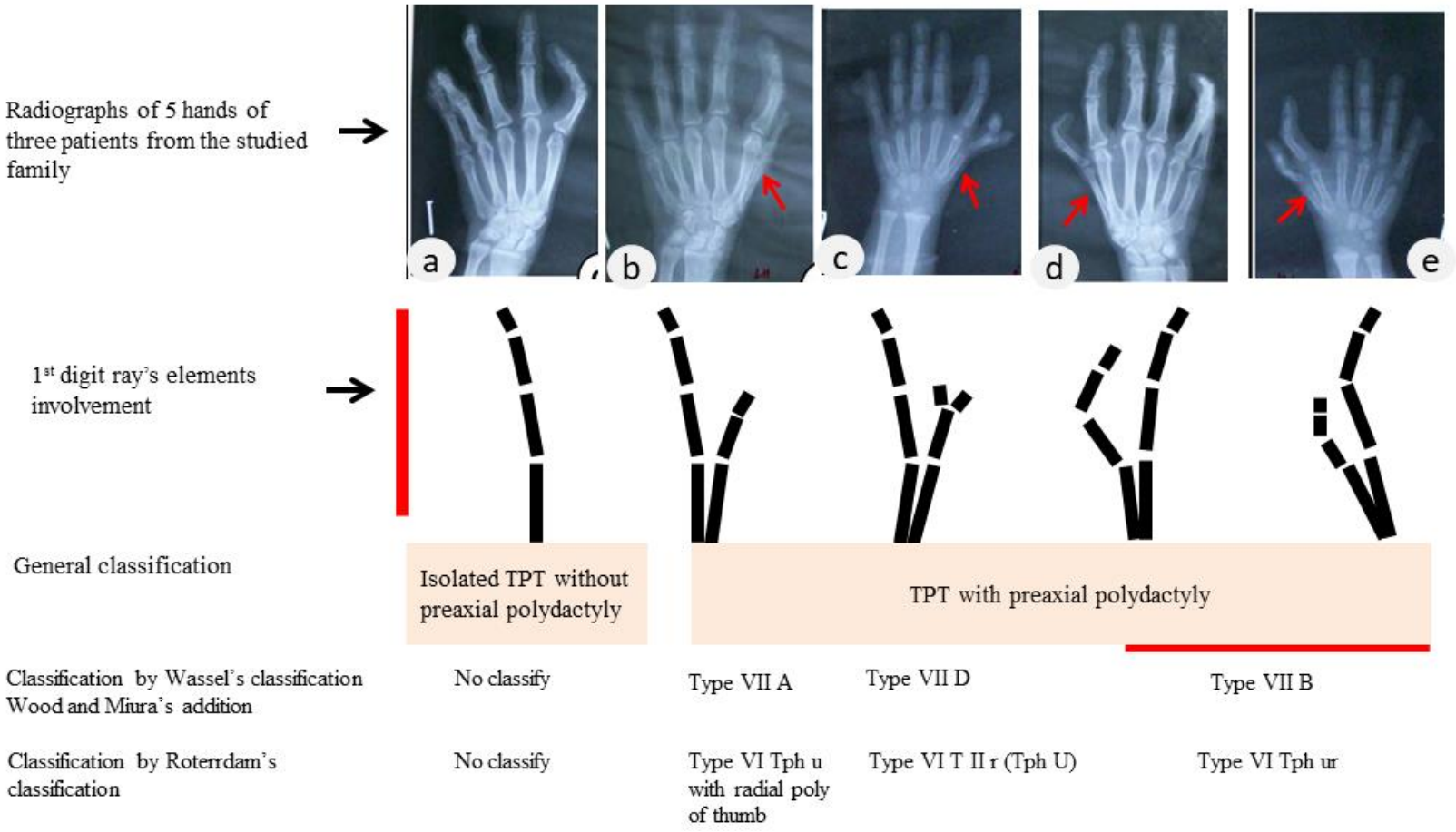


Fig. 3.5: Anatomical classification of observed phenotype in family

Five hands' radiographs of three affected subjects from the family can be classified by both well-documented systems of classification (Wood, 1978; Zuidam, 2008). Therefore, the cases of triphalangism can be classified by both systems. Rotterdam's system is more explanatory and descriptive. However, both systems cannot classify the cases of isolated TPT because, in these systems, TPT with polydactyly is classified on the bases of duplication of phalanxes or 1st metacarpal and carpal.

Compared to the other reported cases of TPT, the phenotype in this family was striking and unique because point mutations in the *ZRS* generally cause penetrant and inconsistent features rather consistent phenotypes across the affected family members (Semerci *et al.* 2009; Farooq *et al.* 2010; Laurell *et al.* 2012). However, there are reported exceptions, such as, the cases of reduced penetrance and phenotypic variability (Lettice *et al.* 2003; Gurnett *et al.* 2007). The studied family showed wide clinical variability across the affected family members and, as such, greatly expanded the phenotypic spectrum of anomalies linked with *ZRS* mutations. The underlying mechanism through which *ZRS* mutations cause disruption in limb developmental pathways and results in limb anomalies remains to be more fully elucidated. It is quite likely that syndactyly and post-axial polydactyly variants observed in the present kindred may reveal that the position of mutation within the *ZRS* is functionally distinct from already reported *ZRS* mutations. Functional studies are required to more completely answer this question.

In conclusion, the research findings expand the clinical and molecular aspects of TPT phenotype. The results will also prove helpful for clinicians and researcher in the field of limb dysmorphology, clinical pediatrics, and genetic counseling.

Results of this study were published in Am J Med Genet:

VanderMeer JE, Afzal M, Alyas S, Haque S, Ahituv N, Malik S (2012). A novel ZRS mutation in a Balochi tribal family with triphalangeal thumb, pre-axial polydactyly post-axial polydactyly, syndactyly. Am J Med Genet A; 158A (8): 2031–2035.

Chapter 4

4: Clinical study of longitudinal and transverse deficiencies of upper limbs in four unrelated sporadic cases

4.1: Abstract

Limb deficiency defects (LDDs) are very rare and occur with a prevalence rate of 8 in 10000 live births in the general population. LDDs have been divided into deficiencies of longitudinal and transverse axis, complete absence of limbs, mixed deficiency disorders, deficiencies of central rays, and others that do not fall in the above-mentioned groups. The longitudinal defects result due to aplasia/hypoplasia of skeletal elements from the proximal to distal axis whereas the terminal deficiency disorders are defects of distal segments of the limbs with almost normal proximal parts. In this study, four independent subjects were reported from the rural area of Southern Punjab, Pakistan. The subjects were thoroughly scrutinized and photographs were taken in addition to radiographs in order to observe the clinical and phenotypic variability. The drafted cases of limb reduction defects had sporadic and isolated presentations ranged from mild to severe disability. The consistent phenotypic pattern of LDDs may demonstrate disruption of common specific factors/pathways during embryonic stages.

4.2: Introduction

Congenital anomaly involves any functional, physical, or bio-molecular deficiencies that may progress in the embryo from conception until birth, whether it is identified at the time of delivery or not (Shawky & Sadik 2011). Congenital aberrations are long-lasting changes with variable superficial consequences due to defective development in body structures during prenatal life (Jones 1988). Such disabilities cause lifelong physical, mental, visual, and auditory disabilities to its carrier, and also have negative impact on the economic status of their families, and communities if these left untreated (Christianson *et al.* 2006).

The etiology of limb malformation includes defective patterning, and establishment, elongation, and segmentation of different skeletal elements of arms and legs (Stricker & Mundlos 2011). It means that are complex pathologies behind different clinical features of the limb malformations. These anomalies are due to i) lack/incomplete formation with limb deficiencies; ii) absence of differentiation with syndactylies, symphalangism, carpal combinations, and synostosis; iii) duplication with polydactylies; and iv) shortening with brachydactylies (Aucourt *et al.* 2012).

Limb bud development takes place within the first month of pregnancy after fertilization. The upper limbs develop first then the lower limbs at the 4th week of pregnancy. For the development of both limbs, the underlying genetic factors are identical. This indicates a striking similarity in the morphogenesis and phenotypes of the affected limbs. Usually, limb formation occurs along the three axes. For instance, the proximal to distal axis development is under the control of the apical ectodermal ridge (AER) (Cohn & Bright 1999), the posterior to interior axis development is regulated by the zone of polarizing activity (ZPA) (Riddle *et al.* 1995), and dorsal to ventral axis development is influenced by different protein factors (Tabin 1995). These entire axes are interlinked, and there is three-dimensional developmental

plane of the limb (Lewandoski *et al.* 2000). Any insult of these axes leads to different kind of limb congenital deficiencies, e.g. insult of AER leads to longitudinal deficiencies at the proximal end due to early disruption of AER or distal end transverse reduction defects due to late disruption of AER (van der Hoeven *et al.* 1994). Similarly, increased apoptosis in the mesodermal area of the developing limb leads to errors in chondrification or ossification resulting in the lack of or malformed bones as in longitudinal deficiencies defects (Sun *et al.* 2002).

The errors in genetic pathways occur during pregnancy and appear at birth which is a highly unpleasant event for parents and relatives. The parental response and expression on the birth of a baby with certain limb malformations or any structural defects are gloomy. Most often, the parents became upset and many questions came to their mind. Why did this happen to us? Did we do something wrong? Is it our fault? What can be done about this? What about the future of child? Finally, the relatives and friends became shocked by the birth of an abnormal child (Singh & Gupta 2009).

4.2.1: Limb reduction defects:

Limb reduction defects are handicapped conditions that affect both the health and the wellbeing of the patients (Ephraim *et al.* 2003). Among all the congenital defects of limb, the deficiency defects of limbs are the most severe congenital limbs reductions which are due to extrinsic and intrinsic causes. The extrinsic causes include thalidomide effects, whereas the intrinsic factors are gene or chromosomal mutations (Ordal *et al.* 2016).

Limbs deficiencies occur with a prevalence rate of 0.0008 among the general population (Browne *et al.* 2012). Different classification systems are used to classify and describe the limb deficiency defects in an accurate and logical way. For this purpose, Frantz and O'Rahilly took an initial step and classified limb deficiencies into two groups, i.e.,

terminal and intercalary with subgrouping as a transverse and longitudinal deficiency (Frantz & O'rahilly 1961). Burtch (1966) revised the classification system of Frantz and O'Rahilly for more comprehensive and detailed description of LDDs (Burtch 1966). Further, in 1975, Kay proposed the approved terminology for the classification of limb reduction defects and this system was published by the members of International Society for Prosthetics and Orthotics (ISPO) (Kay & Tasman-Jones 1975). For more precision and accuracy, the International Organization for Standardization (ISO) in 1989 presented a method for classifying the limb deficiencies at birth and this was accepted by 168 participating members of different nations. Later, in 1991, this system was again modified by Day. James et al. (1999) presented the global classification system to classify the radial longitudinal deficiencies (James *et al.* 1999). All of these systems were based on a small number of newborns that were reported in the same single hospital. Therefore, an expanded form of all these systems was presented by Gold et al. (2011). This expanded modified anatomical classification system included all kinds of long bones deficiencies, with the absence of any finger or toe with their specific population prevalence rate being noted (Gold *et al.* 2011).

Based on above mentioned classification systems, Bedard et al. (2015) classified the limb deficiencies as transverse and longitudinal reductions, amelia, intercalary, split hand foot malformation, complex defects, and other type of limbs deficiency given in Table 4.1 (Bedard *et al.* 2015).

Table 4.1: The types and summary of clinical features for different limbs deficiency disorders

Types of LDDs classes	***Clinical features***
Longitudinal	Absence/underdeveloped parts of mesopodal or autopodal regions
Longitudinal Preaxial	i. Aplastic/hypoplastic radius/tibia ii. Absence/hypoplasia of radius/tibia, thumb/hallux, and digit 2 iii. Aplasia/hypoplasia of thumb/hallux and digit 2 iv. Hypoplasia/aplasia of thumb/hallux alone
Longitudinal postaxial	i. Absent/reduced ulna/fibula with digit 5 ii. Aplasia/hypoplasia of ulna/fibula with digit 4 and 5 iii. Absence/reduction of digit 5 only.
Longitudinal central	Hypoplasia/aplasia of central digits in different combinations like absence/reduction of 2^{nd} digit to 4^{th} digit, 3^{rd} and 4^{th} digits, $2^{nd}/3^{rd}$ digits, digits 2, 3, or 4 alone.
Longitudinal mixed	Aplasia/hypoplasia of different axes at the same time e.g. absent 1^{st} and 5^{th} digits together.
Transverse	Hypoplasia/aplasia of terminal segments of upper or lower limbs but with normal proximal structures of all limbs.
Intercalary	Aplasia/hypoplasia of proximal structures of limbs with normal extremities.
Split hand/foot malformation	Aplasia/hypoplasia of central axis with the involvement of metacarpals and digits with the normal lateral axis
Amelia	Complete absence of arm/limb
Complex deficiencies	Occurrence of different types of limb reductions likes intercalary and longitudinal reduction together.
Others	Limb reduction other that above classification

Ulnar deficiency is a rare and isolated type of postaxial longitudinal reduction defect of the forearm with the complete or partial absence of ulna with compromised extension/flexion at elbow joint as primary feature. The secondary features include variable ulnar length (in case of the incomplete condition), reduced number of digits, and muscular atrophy and relaxation of ligament (Frantz and O'Rahilly, 1971). It occurs with prevalence rate in the range of 0.000006 - 0.00001 with 3:2 male-female ratio among 150000 – 100000 live births in general population, and in 70% of the reported cases it affects the right side of the body (Agarwal *et al.* 2017). In 1986, Miller et al. classified the ulnar deficiencies into four classes, i.e., Type A-D, based on the degree of ulnar deficiency. In previously reported cases, the anomaly was described as unilateral and sporadic nature, and its similar unique presentation in different cases indicated that there should be a common genetic factor behind it (Miller *et al.* 1986).

Terminal transverse limb defects mean the absence or hypoplasia of distal structures of limbs with more or less normal proximal structures (Temtamy & McKusick 1978). They are a rare congenital deficiency which manifests itself as an abrupt truncation through the transverse axis of limb and produces an amputation-like stump. The most common level of truncation is the forearm followed by the amputations at the wrist (Neumann *et al.* 1998). The transverse deficiency could be the proximal transverse deficiency of the humerus with the absence of all distal skeletal elements that are distal to it; mesopodal transverse deficiency with absent distal elements; and distal terminal deficiency of autopodal elements with reduced/absent fingers (Burtch 1966). The genetic basis of isolated terminal limb deficiencies is not well understood due to the rare occurrence of familial cases and a limited number of subjects available for molecular analyses.

Molecular embryology of limb indicates that the development of the limb is a complex and complicated process that needs the normal working of a large number of genes.

Therefore, the etiology of limb deficiency defects is not fully described. However, the similar presentation of LDDs indicates that there might be a similar genetic program for both upper and lower limb deficiency or it may be different for upper limb reduction disorders than the lower limb deficiencies (Barham & Clarke 2008).

Due to their congenital nature, the limb deficiency defects can be easily recognized at birth. Yet, little information is available due to the lack a surveillance system in Pakistan to monitor the epidemiology of congenital anomalies. The objective of this part of the thesis was to describe the clinical findings of limb deficiency defects in four unrelated male sporadic cases from the population of Southern Punjab, Pakistan. Among the reported patients, postaxial longitudinal deficiency was found in the three affected subjects and transverse deficiency reported in remaining subject. The ulnar longitudinal deficiency was presented as the mild, moderate, and severe type with congenital absence of ulna in both right and left arms, whereas terminal transverse deficiency included the absence of all metacarpals and digital phalanges of the left hand that contained only five fingers protuberance.

4.3: Subjects and Methods

All of index subjects with certain types of congenital limb deficiencies were recruited from three different districts (Muzaffargarh, Dera Ghazi Khan, and Layyah). Three of the subjects were adult males (Subject A, B, and D) whereas subject C was a male boy. All are sporadic cases. For phenotypic characterization, all of subjects were photographed at their residences. For the clinical findings, all of subjects were radiographed at tehsil headquarter hospitals of their respective residential places and the evaluations were performed with the help of specialized radiologists/doctors. For detailed medical and family history, the parents were interviewed by the medical superintendent. To gain information about the pregnancy and birth events, mothers were also interviewed with the help of Lady Health Visitors (LHV). All information and data were collected after the informed and written consent from each subject.

4.4: Results

4.4.1: Subject A: Longitudinal deficiency of ulna with absence of postaxial fingers

Subject A was a 45-year-old man and had eight normal sibs (5 brothers, 3 sisters). He had a marriage with his female cousin in the family and had 6 normal offspring (2 sons, 4 daughters). Reportedly, his father dealt in leather processing. Additionally, the family had superstitions about the occurrence of the limb anomaly due to moon eclipse.

Phenotypically, he was observed to have a shortened right forearm that had a oligodactylous hand with two 1st and 2nd fingers that were malformed and hypoplasia of the nails. The affected arm was partially functional (Fig. 4.1 A, C, D). The palm was reduced in size with atypical flexion and transverse crease (Fig. 4.1D).

In order to confirm the type of longitudinal deficiency, both upper limbs of the subject were X-rayed along the AP view. The obtained X-ray films confirmed the post-axial longitudinal deficiency of ulna and each ulna was short, hypoplastic, and terminated with hypoplastic distal end (Fig. 4.1B, E). Each radius was short, thin, hypoplastic, and ends of radius bent towards the missing end of the ulna. The autopodal region was malformed with only two abnormal carpals which are followed by two short and stubby metacarpals (Fig. 4.1B). The first digit had two fused phalanges and the second finger contained three abnormally formed small phalanxes. The distal phalanx was hypoplastic in both fingers (Fig. 4.1E).

The left hand was normal with a single transverse palmar crease. In the radiographs, no skeletal element was found missing except for crowding of carpals (Fig. 4.1B, E). The patient did not report any sign of functional complication of the left arm functionality. Moreover, the lower limbs and other body parts were found, phenotypically, normal and there weren’t any Orio-facial abnormality.

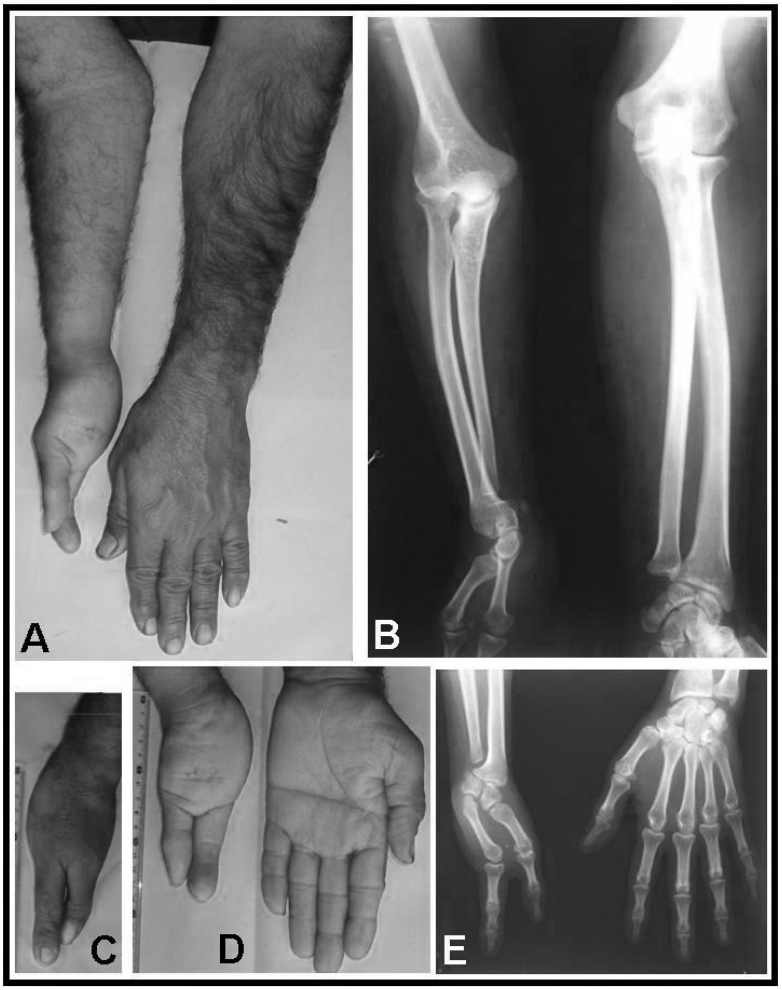


Fig. 4.1: The photographic and radiographic views of the phenotype in subject A

4.4.2: Subject B: Sever longitudinal deficiency of ulna with absent of certain fingers

Subject B was 25-year old male who had a sib-ship of two brothers and three sisters of unaffected status. His morphological assessment indicated that he had a short right arm with reduced middle and distal segments (Fig. 4.2A, C). The hand was small with a narrow palm harboring two widely spaced digits. Both fingers were short and functional.

The X-rays demonstrated a drastically reduced ulna, and a shortened radius with varus curvature and dislocation at the elbow joint (Fig. 4.2D). The autopodal region of the affected right arm was malformed with reduced and malformed carpals, two short metacarpals, and two digits. Both digits had a hypoplastic distal phalanx. The X-ray films of pectoral girdle indicated compromised shoulder joint (Fig. 4.2E, F). The left arm was unremarkable normal except for clinodactyly of the 5^{th} finger (Fig. 4.2A, B).

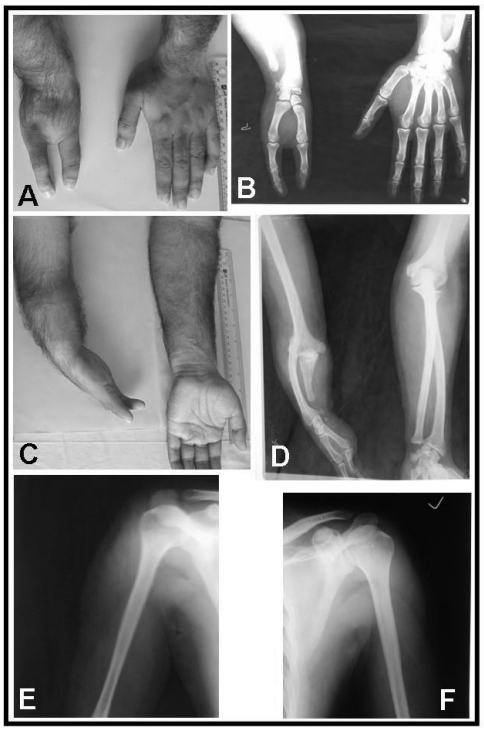


Fig. 4.2: Photographs and radiographs of upper limbs of subject B

4.4.3: Subject C: Complete longitudinal deficiency of ulna in left arm

Subject C was a 12-year-old boy, who was recruited from the rural area of Layyah district. The child was the product of an inbreed union between first-cousins. The parents reported a normal delivery without any unpleasant incidents and the delivery was carried at the home with the help of a traditional labor-attendant.

The phenotypic evaluation of the subject was carried out at the family residence. The child had an affected left arm and normal right arm. The affected arm had weak/thin stylopod with normal length, short meso-autopodal segments (Fig. 4.3A). The hand was oligodactylous with three functional fingers (3^{rd}, 4^{th}, and 5^{th} finger) with two interphalangeal creases but the 1^{st} and 2^{nd} finger were absent. The palm of the hand was reduced in size with missing creases (Fig. 4.3 A, B). Moreover, the fifth finger was underdeveloped and indicated volar inclination (Fig. 4.3C), while movement at the elbow joint was restricted and hindered the performance of daily life activities.

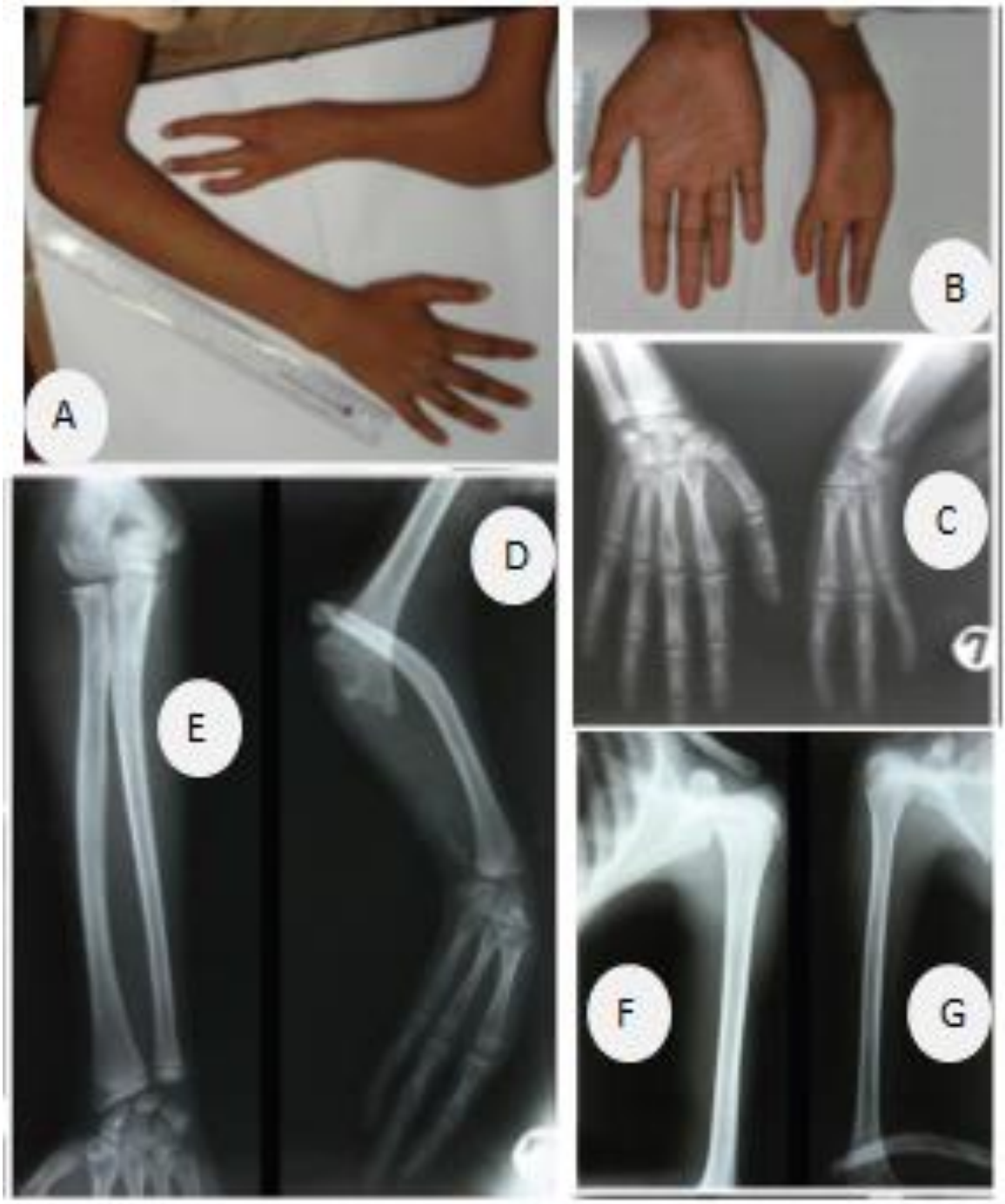


Fig. 4.3: The phenotypic and radiographic features of ulnar aplasia in the male child

Radiographic films indicated the complete absence of the ulna, and the radius was malformed. Due to the absence of the ulna, a compensatory effect was developed by the inward curvature of the radius with varus cavity of about 20^{o} (Fig. 4.3D). Both the proximal and distal ends of the radius were abnormal (Fig. 4.3D). Similarly, the humerus was short and acalcified (Fig. 4.3G) with a loose connection at the shoulder and elbow joints and could not support the middle and terminal part of the limb.

The radiographic films of the autopodal region of the affected left arm revealed four carpals that were crowded and malformed (Fig. 4.3C), and three normal sized metacarpals. Additionally, the 1^{st} and 2^{nd} digits were absent at the preaxial side, the 3^{rd} digit showed volar inclination at central axes, and there was a partial cutaneous fusion b/w 4^{th} and 5^{th} digits at the post axial side in addition to clinodactyly of the 5^{th} digit (Fig. 4.3E, C).

The right arm was normal except for crowding of carpals as indicated in the X-ray (Fig. 4.3A, E). The patient's parents could not recall any drug exposure during the pregnancy. The subject has fully adapted to this limb deficiency and can well manage his daily-life activities.

4.4.4: Subject D: Terminal transverse deficiency of upper limb

This morphologically studied and observed subject had an isolated terminal transverse deficiency at level of the metacarpals to beyond this on the left affected hand that abruptly ended with beads like fingers offcuts and a severely condensed palm. Instead of normal fingers, the five beads like fingers marks (through cutaneous bridges) were found at the distal rim of the abnormally developed palm. The underdeveloped nubbins had nails at the dorsal side and pre/post axial nubbins were larger than the mesoaxial nubbins (Fig. 4.4A, B). The amputated hand had compromised extension and flexion.

The radiographic films of the left arm showed an underdeveloped radius and ulna with hypoplasia of their heads at distal end (Fig. 4.4C, D), with carpals, metacarpals, and the proximal/middle phalanges being completely missing. Each nubbin had a single hypoplastic bony element (Fig. 4.4C, D). In contrast to this, the humerus was unaffected with normal movement at shoulder and elbow joints (Fig. 4.4E, F).

Additionally, the right arm and all segments of the lower limbs were found to be unaffected after phenotypic and radiographic assessment of the subject. The family could not recall any kind taratogen exposure that occurred during the pregnancy.

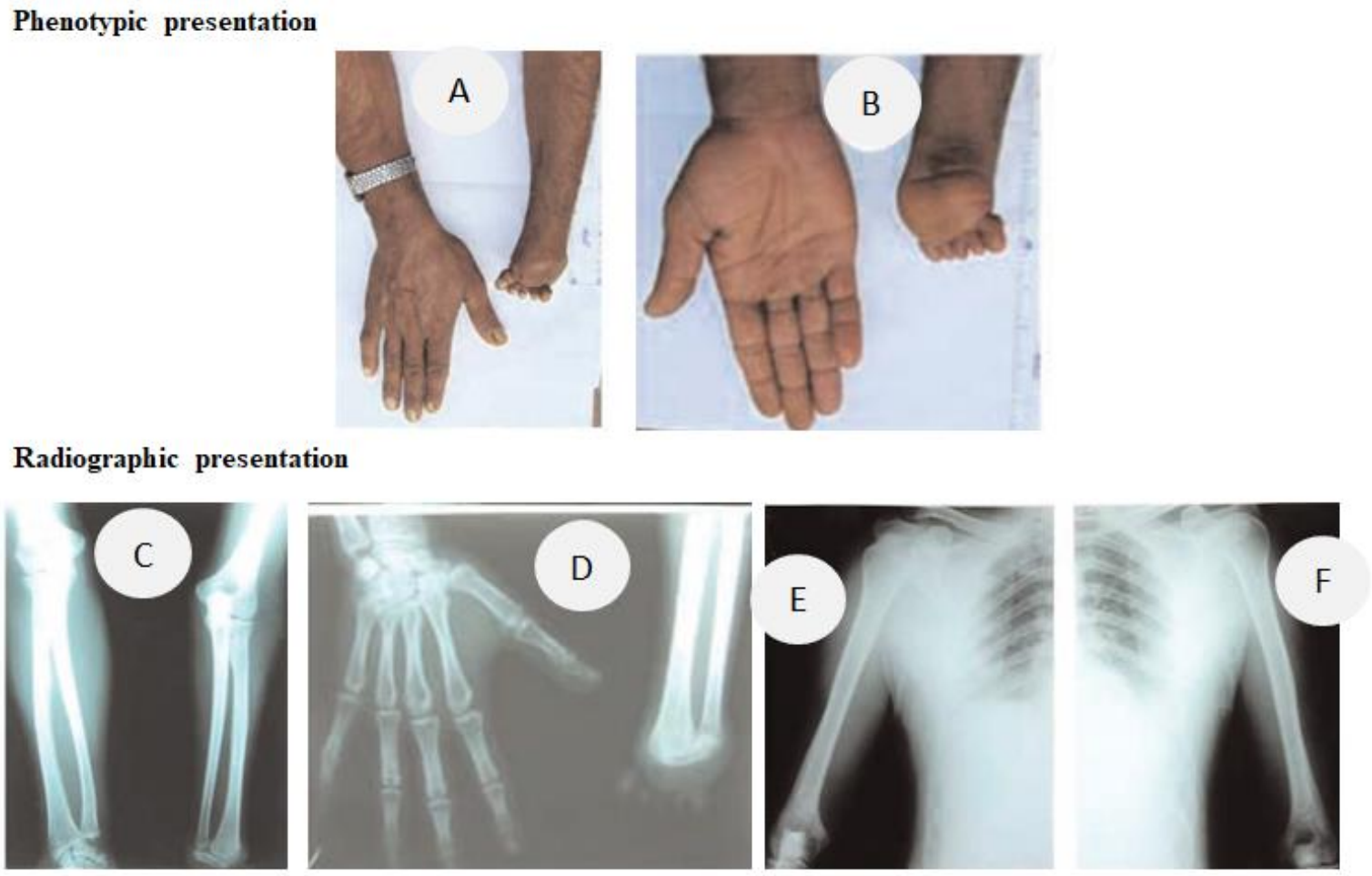


Fig. 4.4: The phenotypic and radiographic characterization of subject D

4.5: Discussion

Herein, four unrelated sporadic cases, who had congenital limb reduction defects have been described. Out of these affected subjects, three (A, B, and C) had mild, moderate, and severe types of unilateral isolated longitudinal deficiencies of ulna of upper limbs on the base of hypoplasia/aplasia of the ulna, while remaining the 4th patient had isolated unilateral congenital terminal transverse deficiency of metacarpals, digital elements with severely reduced palm that had nubbin like fingers marks in the left arm, the site where deficiency appeared.

In the medical literature, there are some reports of limb deficiencies defects from the population of Pakistan (Riaz *et al.* 2016). In the reported subjects the ulnar longitudinal reduction affected both the right arm of the body as in subject A, and B, and the left arm of the body in subject C. however, its phenotypic expression was considerably different in each subject because it had a less severe effect on the right arm than the left arm, as found in the patients, but in contrast to its variable expression, its isolated and unilateral nature with oligodactylous hands indicated the common features. These features clearly separated this phenotype from the other well-described phenotypes include femur-fibula-ulna (FFU) complex (OMIM-228200), ulnar hypoplasia with lobster-claw deformity of feet (OMIM-314360), and Schinzel syndrome (OMIM-181450) of the upper limb deficiencies due to their syndromic form (OMIM, 2018).

In general, the longitudinal deficiencies of upper limbs are associated with the fusion of the radio-humural joints with limited motion of the limb as in FFU complex (OMIM). However, these features were not found in the four subjects (Frantz and O'Rahilly, 1971). In the presented cases, there was no fusion of radio-humeral joint. Subject A has normal movement at the elbow joint while subject B and C had restricted extension. Radioulnar

fusion was also not witnessed in any subject. On the anatomic bases due to hypoplasia, or incomplete absence of ulna, and or complete absence of ulna it belongs to type A and Type D according to the previously described classification system (Swanson *et al.* 1984; Ogino *et al.* 1994). Moreover, the ulnar deficiency of the left arm in these subjects is an additional finding.

Longitudinal deficiencies have a severe impact on the physical, psychological, and daily routine lives of the affected individuals, and molecular etiology of such deficiencies is poorly understood. Mostly, sporadic, unilateral, and isolated post axial limbs deficiencies are due to thalidomide effects as a non-genetic cause. However, consistent similar phenotypic expression patterns in many sporadic cases (Gold *et al.* 2011) suggest a common underlying genetic cause(s) or pathway that is affected.

In addition to longitudinal deficiencies of upper limbs, the incidence of terminal deficiencies is lacking in many populations, and terminal reduction defects are reported with an occurrence rate of 0.00019 among 10000 live births (McGuirk *et al.* 2001). The clinical features of terminal deficiency in Subject D are like the patients reported by Neumann et al. (1998), since in all those cases the phenotype only affected the distal parts of upper limbs, but few cases had a terminal reduction of lower limbs. In contrast to this, a previous study has inconsistent features as compared to the studied subjects (Drapkin *et al.* 2003). However, currently there is a need for molecular diagnostic tests that could prove that subjects with similar clinical manifestations have the same genetic etiology.

In the light of these cases, it is more about important to know the limb developmental plan and provide models for identification of morphogenetic factors that maintain the development of limb along the different anterior-posterior and proximo-distal axis of developing limbs bud which regulates the digit numbers, identity, and length of the growing

bud, respectively (Grzeschik 2004). However, the number of digits is dependent upon the number of cells and width of bud in the growing limb field, particularly the length of the AER (Towers & Tickle 2009). Moreover, limb reduction defects are due to the arrest of SHH signaling along the anterio-posterior axis (Scherz *et al.* 2007).

In conclusion, the partial or complete loss of the SHH pathway in conjunction with the loss of molecular players that maintain the integrity of AER may be a possible cause leading to the terminal longitudinal limb defects. Therefore, further molecular studies are needed on a large number of sporadic cases with consistent similar phenotypic expression to identify the molecular genetic causes.

The results of this study were published as;

Afzal M, Malik S, (2014). Longitudinal deficiency of upper limb: similar case presentation of two subjects with unilateral ulnar hemimelia, carpal and metacarpal deficiency, and oligodactyly. Asian Biomed; 8(4): 569-575.

Malik S, Afzal M (2013). Congenital terminal transverse deformity of upper limb: clinical and radiological in a sporadic care. JCPSP; 23(3): 219-220.

Malik S, Afzal M (2013). Ulnar aplasia, dysplastic radius and preaxial oligodactyly: Rare longitudinal limb defect in a sporadic male child. J Res Med Sci 18: 818-21.

Chapter 5

5. Clinical and molecular study of two independent families with Cenani-Lenz syndactyly syndrome: elucidation of phenotypic variability and identification of novel mutations

5.1: Abstract

Cenani-Lenz syndactyly syndrome (CLSS; MIM-212780) is a rare, hereditary, and heterogenetic limb anomaly featured by complete bony fusion of all fingers and toes. There is a bizarre arrangement of phalangeal elements and severe shortening of the radius and ulna. The common associations of this recessively segregating anomaly are oro-facial defects, scoliosis, renal hypoplasia, hearing impairment, and genital anomalies which are considered as secondary features. Herein, we present two independent consanguineous families (A and B) that exhibited features of CLSS, with certain associated other symptoms. The typical features of CLSS include the complete syndactyly of fingers and certain toes, disorganization of fingers, oligo-syndactyly of toes and shortening of limbs. The affected subjects additionally presented with short stature, frontal bossing, cleft lip and hypoplastic pectoral girdle, and kidney agenesis. In Family A, a novel splice site mutation (c.316+1G>A) identified in the *LRP4* gene. The identified mutation segregated with the phenotype in a 5^{th} generation in pedigree. This mutation is predicted to add 29 non-native amino acids (amino acid due transcription of intronic sequences) with a premature termination, resulting in approximately 90% reduction of the wild-type transcript. In Family B, a mutation c.1151A>G (p.Y384C) was detected in the exon 10 of the *LRP4* gene. These findings not only further expand the phenotypic variability of CLSS but also support the notion that truncated and loss-of-function mutations in *LRP4* lead to severe CLSS symptoms.

5.2: Introduction

The word ‘syndactyly’ is the combination of two Greek words, i.e., syn meaning "together/fused/webbing" and dactyly meaning "finger/digits". It is an inherited condition and presented as a heterogenous limb malformation that shows a partial or complete fusion of fingers and toes of hands and feet, respectively, and also shows the fusion of internal anatomic structures of distal extremities of all limbs (Temtamy & McKusick 1978). This fusion may be cutaneous or osseous, mostly in the autopodal region of both upper and lower limbs. The syndactyly’s variable morphological and clinical expression of syndactyly indicates that it may be unilateral/bilateral, simple/complex, and syndromic/nonsyndromic. Moreover, in its syndromic form, syndactyly is the part of about 300 different diseases and whereas nonsyndromic form of syndactyly is classified into nine different classes, type I-IX (Malik 2012). For syndactylies, at least 11 loci and 8 genes have been implicated. Among these, type VII, Cenani-Lenz Syndactyly Syndrome (CLSS) is the most complex and very rare form of syndactyly.

CLSS is a very rare hereditary limb anomaly (Cenani & Lenz 1967). Its major symptoms of this autosomal recessive malformation included complete fusion of all fingers, synostosis of short radius and ulna, and disorganization and undifferentiated phalangeal parts with bilateral symmetric expression pattern, although the feet are less severely affected (Temtamy & McKusick 1978; Harpf *et al.* 2005).

The morphological features of CLSS are divided into consistent or inconsistent features. The consistent primary features include the total union of all the fingers resulting in the mitten appearance of hands, disorganization/fusion of phalanges, metacarpals and carpals, radio-ulnar synostosis, and lack of digital rays. The inconsistent secondary characters may involve appendicular features (brachymesomelia, metatarsal synostosis, thumb absence,),

ectodermal findings (teeth irregularities, nail unity, hearing loss,), craniofacial dysmorphism (prominent frontal head, depressed nasal bridge, down-slanting palpebral fissure, hypertelorism,, thoracic/spine deformities (scoliosis), cleft palate, genital malformation, hyperthyroidism, hip dislocation and ectopic/hypoplastic kidneys (Cenani & Lenz 1967; Seven *et al.* 2000; Bacchelli *et al.* 2001; Temtamy *et al.* 2003; Malik *et al.* 2004; Jarbhou *et al.* 2008; Li *et al.* 2010; Khan *et al.* 2013; Lindy *et al.* 2014; Afzal *et al.* 2017).

Harpf et al. classified CLSS into two non-syndromic anatomic classes that included CLSS-I (classical spoon hand type) and CLSS-II (oligodactylous type), based on reported morphological and anatomic features (Harpf *et al.* 2005). Later, it was suggested that CLSS was a syndromic form of syndactyly (Jarbhou, Hamamy, Al-Hadidy, & Ajlouni, 2008). In 2012, this classification was revised (Malik 2012).

At least 35 families with CLSS have reported so far (as of 1938-2015); of these, the clinical features of 20 families (CL1-CL20) are presented in Table 5.1.

Table 5.1: The clinical characterization of CLSS in the families

Fam. ID	***Pheno-type***	***Family origin***	***Inbreed unions***	***No. of patients***	***MOI***	***Clinical symptoms***	***References***
CL1	Oligo. type	Pakistan	+	1	AR	Bilateral typical hands syndactyly, radio-ulnar synostosis, malformed metacarpals and phalanges, syndactyly in feet, nail hypoplasia, renal hypoplasia, prominent forehead, hypertelorism, micro-retrognathia, abnormal teeth	Bacchelli et al. 2001
CL2	Spoon hand	Turkey	+	1	AR	Unilateral hand syndactyly, malformed/disorganized metacarpals & phalanges, feet syndactyly, bilateral broad hallux valgus, nails aplasia	Seven et al. 2000
CL3	Oligo. type	Egypt	+	3	AR	Bilateral hands syndactyly, radio-ulnar synostosis, malformed metacarpals & phalanges, feet syndactyly, malformed meta-tarsals and phalanges, nails aplasia, prominent forehead, hypertelorism, micro-retrognathia, teeth anomalies, nails hypoplasia, developmental delay	Temtamy et al. 2003
CL4	Spoon hand	Egypt	+	3	AR	Bilateral hands syndactyly, short forearms, radio-ulnar synostosis, malformed/disorganized metacarpals & phalanges, feet syndactyly, malformed metatarsals &phalanges, prominent forehead, hypertelorism, down-slanting palpebral fissures, microretrognathia, teeth abnormalities, nails aplasia	Li et al. 2010
CL5	Oligo. type	Turkey	+	1	AR	Bilateral hands syndactyly, oligodactylus hands, short forearms, radio-ulnar synostosis, malformed/disorganized metacarpals & phalanges, feet syndactyly, malformed metatarsals & phalanges, nails were dysplastic, scoliosis, hearing loss	Elcioglu et al. 1997
CL6	Oligo. type	Turkey	+	2	AR	Bilateral hands typical syndactyly, radio-ulnar synostosis, malformed metacarpals & phalanges, feet syndactyly, nails hypoplasia, renal abnormalities, teeth abnormalities	Percin and Percin, 2003

CL7	Oligo. type	Pakistan	+	1	AR	Hands were syndactylous, malformed/disorganized metacarpals, feet's syndactyly, malformed metatarsals & phalanges, hypoplasia of nails, prominent forehead, hypertelorism, teeth abnormalities	Harpf et al. 2005
CL8	Spoon hand	Jordan	+	6	AR	Bilateral hand's syndactyly, malformed/disorganized metacarpals & phalanges, feet's syndactyly, malformed metatarsals & phalanges, nail's hypoplasia, renal agenesis, prominent forehead, low set ears, high arched narrow palate, short-beaked nose, hypothyroidism, retrognathia, congenital hips dislocation	Jarbhou et al. 2008
CL9	Spoon hand	Turkey	+	2	AR	Bilateral syndactyly of hands, malformed/disorganized metacarpals & phalanges, syndactyly of feet, nail's hypoplasia, renal agenesis, prominent forehead, hypertelorism, teeth abnormalities	Li et al. 2010
CL10	Spoon hand	Turkey	+	2	AR	Prominent forehead, malformed/disorganized metacarpals & phalanges, syndactyly of feet, renal agenesis, developmental delay	Li et al. 2010
CL11	Spoon hand	Turkey	-	1	AR	Bilateral syndactyly of hands, short forearms, malformed/disorganized metacarpals & phalanges, syndactyly of feet, malformed metatarsals & phalanges, nail's hypoplasia, renal agenesis, prominent forehead, hypertelorism, teeth malformation	Li et al. 2010
CL12	Spoon hand	Egypt	+	1	AR	Bilateral syndactyly of hands, radio-ulnar synostosis, malformed metacarpals & phalanges, syndactyly of feet, nail's aplasia, prominent forehead, hypertelorism, down-slanting palpebral fissures, micro-retrognathia, teeth malformation, pectus excavatum	Li et al. 2010
CL 13, 14	Spoon hand	?	?	1, 2	AR	?	Li et al. 2010

CL15	Spoon-Oligo. Type	Pakistan	+	9	AR	Syndactyly of hands, oligodactylous hands, syndactyly of feet, oligodactylous of feet, malformed metatarsals & phalanges, renal agenesis	Khan et al. 2013
CL16	Oligo. Type	Iranian	+	1	AR	Oligodactylous hands and feet, complete syndactyly of fingers and toes, short forearms, radio-ulnar synostosis, malformed/disorganized metacarpals & phalanges, abnormal elbow joints, malformed hemurii, malformed metatarsals & phalanges, short/agenesis of tibia-fibula, abnormal knee joints, pelvic girdles malformation, renal agenesis, prominent forehead, down-slanting palpebral fissures.	Kareminejad et al. 2013
CL17	Oligo. Type	?	?	2	AR	Oligodactylous hands, short forearms, syndactyly of feet, renal hypoplasia, micro-retrognathia, nasal malformation, phocomelia, skin edema, collapsed cranium, ventricular septal defects, ribs and spine deformities, small thorax, hypo-plastic lungs, renal and uterine agenesis.	Lindy et al. 2014
CL 18	?	Saud-i-Arabia	+	4	AR	Syndactyly of hands, broad forehead, hypertelorism, depressed nasal bridge, protruding large ears, teeth malformation, scoliosis.	Patel et al. 2015
CL 19	Spoon & Oligo. Type	Pakistan	+	3	AR	Spoon-like appearance of hands, short forearms, radio-ulnar synostosis, malformed/disorganized metacarpals & phalanges, malformed elbow joints, malformed humurii, deformed shoulder joints, clavicle malposition, syndactyly of feet, malformed metatarsals & phalanges, knee joint hypo-plastic, prominent forehead, high hairs line, depressed nasal bridge, hypo-plastic nose, and others.	Present study, 2018
CL 20	Spoon hand	Pakistan	+	2	AR	Syndactyly of hands, spoon-likethe appearance of hands, radio-ulnar synostosis, malformed/disorganized metacarpals & phalanges, syndactyly of feet, protruding large ears.	Present study, 2018

+, feature present; ?, not reported; AD, autosomal dominant; AR, autosomal recessive; MOI, mode of inheritance; oligo., oligodactylous type

At the molecular level, alterations in the *LRP4* gene causes CLSS. The *LRP4* gene (MIM: 604270) is located on chromosome 11p11.2-q13.1 and the absence of mutations in the *LRP4* gene in two CLSS families suggested the existence of genetic heterogeneity (Li *et al.* 2010). In 2015, this view is supported when a mutation was found in the adenomatous polyposis coli (APC) gene on 5q22.2 as the second locus for CLSS (Patel *et al.* 2015).

LRP4 is a member of Low-Density Lipoprotein Receptor (LDLR) subfamilies which contain many conserved secreted glycoproteins that act as co-receptors. Structurally, *LRP4* has different extracellular domains. These extracellular domains included eight N-terminus repeats, one module of epidermal growth factor (EGF), and four YWTD (domains of low-density lipid receptor protein) motifs with beta-propeller domains. As a co-receptor, *LRP4* (along with its domains) is involved in the maintenance of Wnt-β-catenin signaling pathway in bone metabolism (Thompson & Monga 2007). In this pathway, the β-catenin regulates the gene expression in the nucleus by interacting with various transcription factors through the involvement of transmembrane proteins complex of Wnt (Wingless signaling pathways), Fzd (Frizzled as G-protein coupled receptors), low density receptor proteins (LRPs; LRP5 and LRP6), Dsh (Dishevelled phosphoproteins) and Axin (APC gene), as this protein complex regulates the concentration of β-catenin. *LRP4* has the opposite effect to the β-catenin (Johnson & Rajamannan 2006; Bonewald & Johnson 2008; Wang *et al.* 2014). The Wnt's ligand interaction with the transmembrane receptors activates the β-Catenin pathway that regulates the intracellular signals trafficking during cells polarization and migration in different developmental pathways (Johnson *et al.* 2004).

The understanding of this signaling nosology is crucial for understanding the biology of human disease (Rey & Ellies 2010). Signals changes in the Wnt pathway cause developmental/skeletal disorders. Mutations in the pathway result in various kinds of diseases like limb anomalies (syndactyly and polydactyly), sclerosteosis and myasthenic syndrome,

and other disorders. However, the exact pathways that underwent alteration due to these mutation remains to be elucidated. This elucidation should be supportive for the identification of new disease loci and establish genotypic-phenotypic correlation for these diseases (Johnson & Rajamannan 2006; Choi *et al.* 2009).

Overall, seventeen mutations were identified from 2010-2018 after DNA sequencing of 20 different families from different ethnic populations, along with the inclusion of the two reported families in this study. Out of these mutations, mutation 3 and 5 showed a common founder effect in families from Egypt (CL 3-4) and Turkey (CL 5, 9, 10), respectively (Tables 5.1; 5.2). All these mutations were found in either coding or non-coding regions of two genes. The sixteen mutations were identified in *LRP4* and one mutation was found in the coding region of *APC*. The summary of identified mutations is given in Table 5.2.

Table 5.2: The summary of reported mutations in *LRP4* gene

Mut. No.	***Family ID***	***Mutation analyses***					***References***
		Gene region	***Mutations type***	***Allelic combination***	***Nucleotides change***	***Protein changes***	
Mut. 1	CL1	Intron 6	Splicing	?	c.547 + 1G>A	-	
Mut. 2	CL2	Exon 5	Missense	Homo	c.479G>A	p.C160Y	
Mut. 3	CL3	Exon 4	Missense	Homo	c.409G>A	p.D137N	
	CL12	Exon 4	Missense	Homo	c.409G>A	p.D137N	
Mut. 4	CL4	Exon 12	Missense	Homo	c.1417C>T	p.L473F	
Mut. 5	CL5	Exon 13	Missense	Homo	c.1585G>A	p.D529N	
	CL9	Exon 13	Missense	Homo	c.1585G>A	p.D529N	Li et al. 2010
	CL10	Exon 13	Missense	Homo	c.1585G>A	p.D529N	
Mut. 6	CL6	Exon 12	Missense	Homo	c.1345G>A	p.D449N	
Mut. 7	CL7	Exon 11	Missense	Homo	c.1382A>C	p.T461P	
Mut. 8	CL8	Exon 22	Missense	Homo	c.3049T>C	p.C1017R	
Mut. 9&10	CL11		Splicing	?	c.200-9G>A, c.4959G>C	-	
	CL13	Mutation not identified					Li et al. 2010
	CL14	Mutation not identified					
Mut. 11	CL15	Exon 21	Missense	Homo	c.2858T > C	p.L953P	Khan et al. 2013
Mut. 12	CL16	Exon 3	Termination	Homo	c.289G>T	p.E97X	Kareminejad et al. 2013
Mut. 13&14	CL17	Exon 17 Exon 22	Termination Frameshift	?	c.2401A>T, c.3062delC	p.K801X, p.S1020Qfs*27	Lindy et al. 2014
Mut. 15	CL18	Exon 5	Deletion	?	c.423-5_423-3delAAT	p.R141SfsTer8	Patel et al. 2015
Mut. 16	CL19	Intron 3	Splicing	Homo	c.316+1G>A	29 non-native additional A.A.	Present study, 2018
Mut. 17	CL20	Exon 10	Termination	Homo	c.1151A>G	p.Y384C	Present study, 2018

Mut, Mutation; Homo, Homozygous

In the study, the genetic analysis of two families with CLSS revealed two mutations. One was a splice-site mutation c.316+1G>A in intron 3 of *LRP4* in the family A with a severe form of CLSS with oro-facial and skeletal anomalies in addition to the kidney agenesis. The other was c.1151A>G (p.Y384C) in exon 10 of the *LRP4* gene in the two patients of the family B which had bilateral fused unrecognized fingers that gave mitten-like appearance of hands, disorganized carpals/metacarpals, radii-ulnae synostosis without shortening, and renal agenesis, and facial dysmorphic symptoms.

5.3: Subjects and Methods

5.3.1: Family recruitment and assessment

Two independent families A and B were recruited from rural the areas of two different provinces of Pakistan. Both provinces are separated by the Indus River as a partial barrier. Family A was living in Thal, the desert of the central Punjab, district Bakhar, whereas Family B was living in the arid and dry environment of the Koh Sulaman mountainous range of Khyber PakhtunKhwa, province. The pedigrees of the families were constructed by interviewing elders and all information was crossed checked with other relatives of the respective families. Both families were ascertained at their residences.

The available affected and unaffected family members were physically evaluated with the help of residential doctors at the local hospitals. Photographs, X-ray films, and ultrasound of the affected organs of the affected subjects in both families were obtained, and all available normal sib and parents were also examined. Blood samples from the available affected and unaffected subjects were also drawn. All the information and materials were obtained after informed written consent according to the Helsinki II declaration, and the study was approved by the ethical review committee of Quaid-i-Azam University.

5.3.2: Molecular methods

5.3.2.1: DNA extraction

Genomic DNA was extracted according to the standard phenol-chloroform method. For this purpose, the whole peripheral blood samples were taken in sterile EDTA tubes. From the stored blood samples, I took 0.5 ml of blood each sample was taken separately in Eppendorf tubes to which 1 ml cell lysis buffer was mixed and kept at 4°C for 15 minutes (min). Tubes were centrifuged at 4,500 revolutions per minute (rpm) at 4°C. The supernatant was discarded, and the pellet was re-suspended in 1 ml of cell lysis buffer. It was

centrifugation at 4°C for 15 min. The supernatant was discarded, and the pellet was again suspended in 1 ml nucleus lysis buffer. In the reaction mixture, 15 µl of Proteinase K and 20 µl of 10% SDS were added in order to digest the proteins. The reaction was kept at 37°C overnight. On the next day, samples were treated with solution C and solution D and mixed by vigorous shaking, respectively. Centrifugation was done at 4500 rpm for 12 min at 4°C to separate the precipitated proteins in the form of a pellet. The supernatant was poured into a fresh Falcon tube, and two volumes of chilled ethanol were added. The floating white thread of DNA was fished out and transferred to a new tube. The dry DNA pellet was dissolved in an appropriate volume of TE buffer.

5.3.2.2: PCR and Sanger sequencing

The exonic regions and the exon/intron boundaries of *LRP4* were PCR amplified and then subjected to Sanger sequencing. The PCR reaction was performed in 20 µl volume with 50 ng genomic DNA as template, 2 µl 10× PCR buffer, 0.6 µl dNTP mix (10 mM), 0.5 µl primer (10 pMol/µl),0.6 µl$MgCl_2$ (50 mM), 0.2 µl *Taq* polymerase (Rapidozym, Germany). Primers sequences and their polymerizing conditions are presented in Table 5.3.

The purified PCR products were subjected to cycle sequencing using BigDye v3.1 (Applied Biosystems) sequencing kit. The sequences were record by capillary ABI3730 sequencer (Applied Biosystems). The sequencing annotation were processed by DNA-STAR software (DNA-Star). For this study, the UCSC Genome Browser (GRCh37/hg19) Assembly was taken as references. The list of primers used is given in Table 5.3.

Table 5.3: Primers utilized for sequencing the coding region of *LRP4*

Exon	Name	Sequence (5'-3')	Length [bp]	Tm[°C]	Fragment-length in bp
1	LRP 1for	GCT CTG GCA GCA CTG GAG	18	61	586
	LRP 1rev	TCT TCG CAC GCA TTC ATT C	19	54	
2	LRP 2for	GTG ACT CTC CAG GTG ACA GTG	21	62	389
	LRP 2rev	CAA GCT TGC AAA AGA CCA AC	20	55	
3	LRP 3for	TGT ACT CCA ACC TGG GTG AC	20	59	403
	LRP 3rev	TTC GAG AGG AAG TGA AAG GAC	21	58	
4-5	LRP 4-5for	GGG CAG CAC TGG AGC TAC	18	60	846
	LRP 4-5rev	TGC TCC CTC CCT AGC TCA G	19	61	
6-7	LRP 6-7for	TAT AAG GGC TGT CCC AGA TG	20	57	754
	LRP 6-7rev	TCC ACC CAC CTT GGT CTC	18	58	
8	LRP 8for	GTG ATC CCG GTG TCA AGA G	19	59	381
	LRP 8rev	AAG GCC GCA GGT CAA TG	17	55	
9-10	LRP 9-10for	CAT TCA GCC AGC CCT GTC	18	58	755
	LRP 9-10rev	GAA GGA TGA TCC CCA CCT C	19	59	
11-12	LRP 11-12for	CCC ACT AGG CCT GGA AAG	18	58	917
	LRP 11-12rev	TCC TGC TGC CTA AGG TTT G	19	57	
13	LRP 13for	CTA GCT CAC AGA GTT GTG ATG C	22	60	499
	LRP 13rev	TAC TGA CCT CAG GTG ATC TGC	21	60	
14-15	LRP 14-15for	AAA TTC ATT CTG TGG ACC AAA C	22	55	805
	LRP 14-15rev	AGT GCC CTG CTG GAT TTC	18	56	
16	LRP 16for	TGT GCC GGG TAC TCA CTG	18	58	406
	LRP 16rev	GGC TCC CTG AGG AAT GTG	18	58	
17-18	LRP 17-18for	ACC AGA TCC CAG GAA GTG TG	20	59	698
	LRP 17-18rev	CTC CGG CTT CTG ACC TAC C	19	61	
19	LRP 19for	GCC CCT ACT CTG TGC TCT G	19	61	341
	LRP 19rev	AGC TGC TCT TGC TTC CTT G	19	57	
20	LRP 20for	CTG GGC TGG ACA CTG ATG	18	58	500
	LRP 20rev	CAG CCT CAG AGA AAC AGC AC	20	59	
21-22	LRP 21-22for	AGA TTG ATA GAG CAA GGC TCA G	22	58	698
	LRP 21-22rev	GCC TCT AGA AGC AGC AGG AC	20	61	
23	LRP 23for	GCT AGA AAC TAG CAG GGA CAG G	22	62	364
	LRP 23rev	GGG AAT GGG GAA CAA ACA C	19	57	
24-25	LRP 24-25for	TGA TAT TTC TGC GTT TTC CTT G	22	55	629
	LRP 24-25rev	CAG GGA GGT CAC CTT GTT TC	20	59	
26-27	LRP 26-27for	GTG GCC CTT ATG AAG GTT G	19	57	825
	LRP 26-27rev	TGG AAG GGA GCT TAA ACA GG	20	57	
28	LRP 28for	CAG GCA CTG AAT CCT GTC AC	20	59	499
	LRP 28rev	GTG GTC AGA ACA CAA CCT CAC	21	60	
29-30	LRP 29-30for	TCC CTG TGG AGC TTA CAG TTC	21	60	725
	LRP 29-30rev	TGA TTC CTC TTC CCC ACA TAC	21	58	
31	LRP 31for	TCA TCG AGG GTG CTT CTT G	19	57	357
	LRP 31rev	ATG AGC TGC AAT AGC ACG TC	20	57	
32-33	LRP 32-33for	TGT TGA TGC ACA GAA ATG AGG	21	56	830
	LRP 32-33rev	AAA ACC CAA GCA GAC TGC TAC	21	58	

34	LRP 34for	AGA AGG GCT ACT AAT GAA GGT G	22	58	395
	LRP 34rev	AGG GAC TGG TAG CTC CTG AC	20	61	
35-36	LRP 35-36for	CTC CAT GCT TCT GAT GTC TTC	21	58	1038
	LRP 35-36rev	GGT TTC CAC CCT TTC CTT C	19	57	
37	LRP 37for	AGG GCA TGA GTA CCA GGA AG	20	59	439
	LRP 37rev	CTT CCC AGA AAC CCA AAT TAC	21	56	
38	LRP 38for	TTT GTG CGC TTC ACT CCT AC	20	57	661
	LRP 38rev	CCA GGT CTA AAT TCT CGT GAT G	22	58	

5.4: Results

5.4.1: Family A: Clinical report

The pedigree expanded over five generations, and three of five siblings were the products of consanguineous marriage. The affected children had characteristic features of CLSS that segregated in the fifth generation (Fig. 5.1). The typical primary features of CLSS were associated with secondary features like oro-facial malformations, underdeveloped shoulder joint and mild short stature. The clinical features of the affected family members are summarized in Table 5.4.

5.4.1.1: Primary features

The major limbs morphological characters were a bilateral total fusion of fingers and shortened autopods, zeugopods and stylopods of the upper limbs (Fig.5.2: A, B, C). The feet were bilaterally oligodactylous and reduced in size (Fig. 5.2: D, E, F), and a unilateral pre-axial cutaneous fusion of toes was also found (Fig. 5.2: D).

5.4.1.2: Secondary findings

The affected subjects had oro-facial features such as a prominent forehead, deformed ear, and up-slanting/short nose with depressed nasal bridge (Fig. 5.2: G, H, I). They also had frontal bossing and increased occipito-frontal circumference (OFC) (Table 5.4). One of the subjects (502) presented with operated cleft-lip that resulted in mal-aligned frontal teeth (Fig. 5.2; G). A mild depression on the nasal apex of one of the subjects (504) was noteworthy (Fig. 5.2: H).

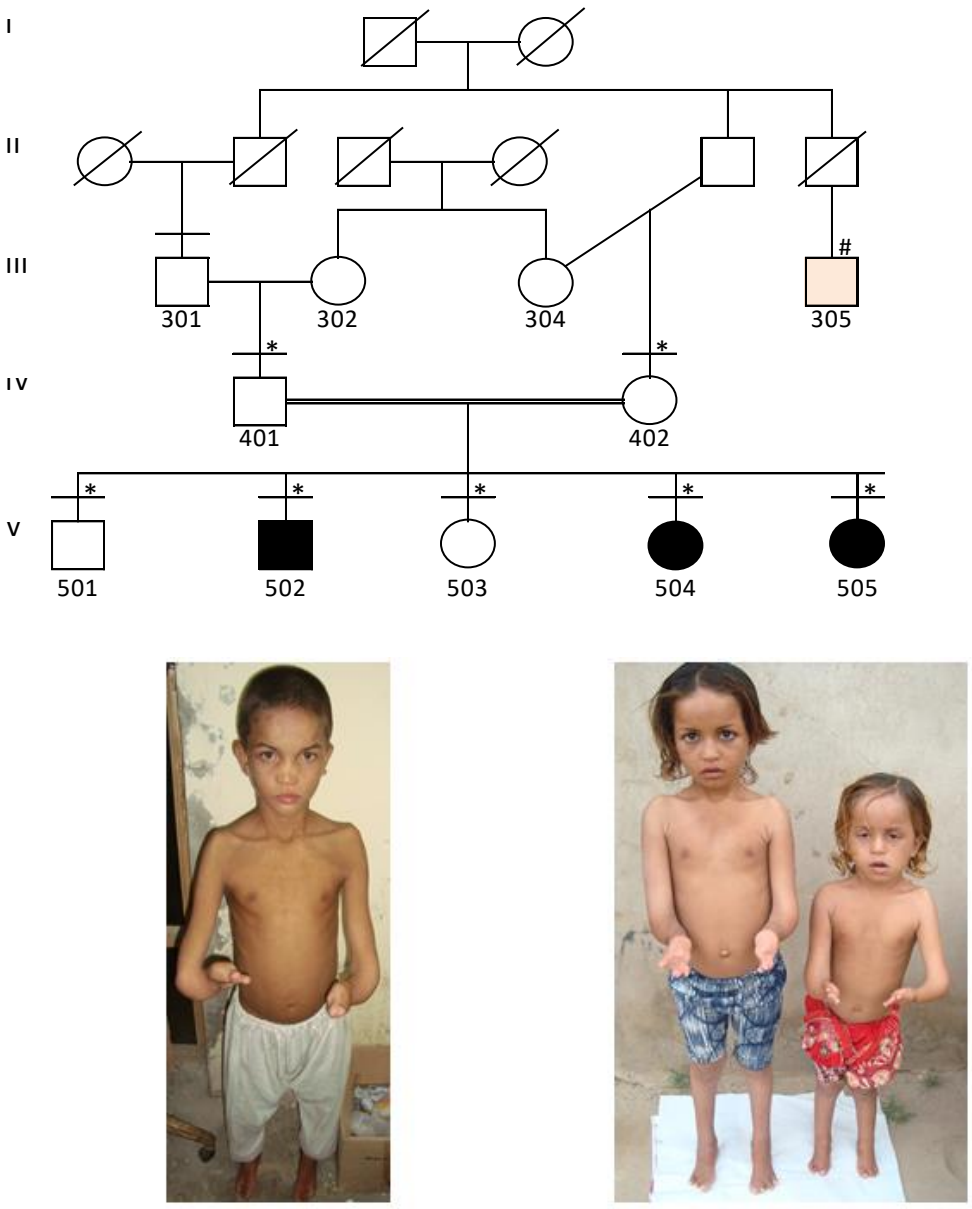


Fig. 5.1: Pedigree and affected subjects of Family A

*, Subjects underwent molecular investigations after blood sampling; horizontal line above the symbol shows subjects physically examined; #, Cleft lip/palate.

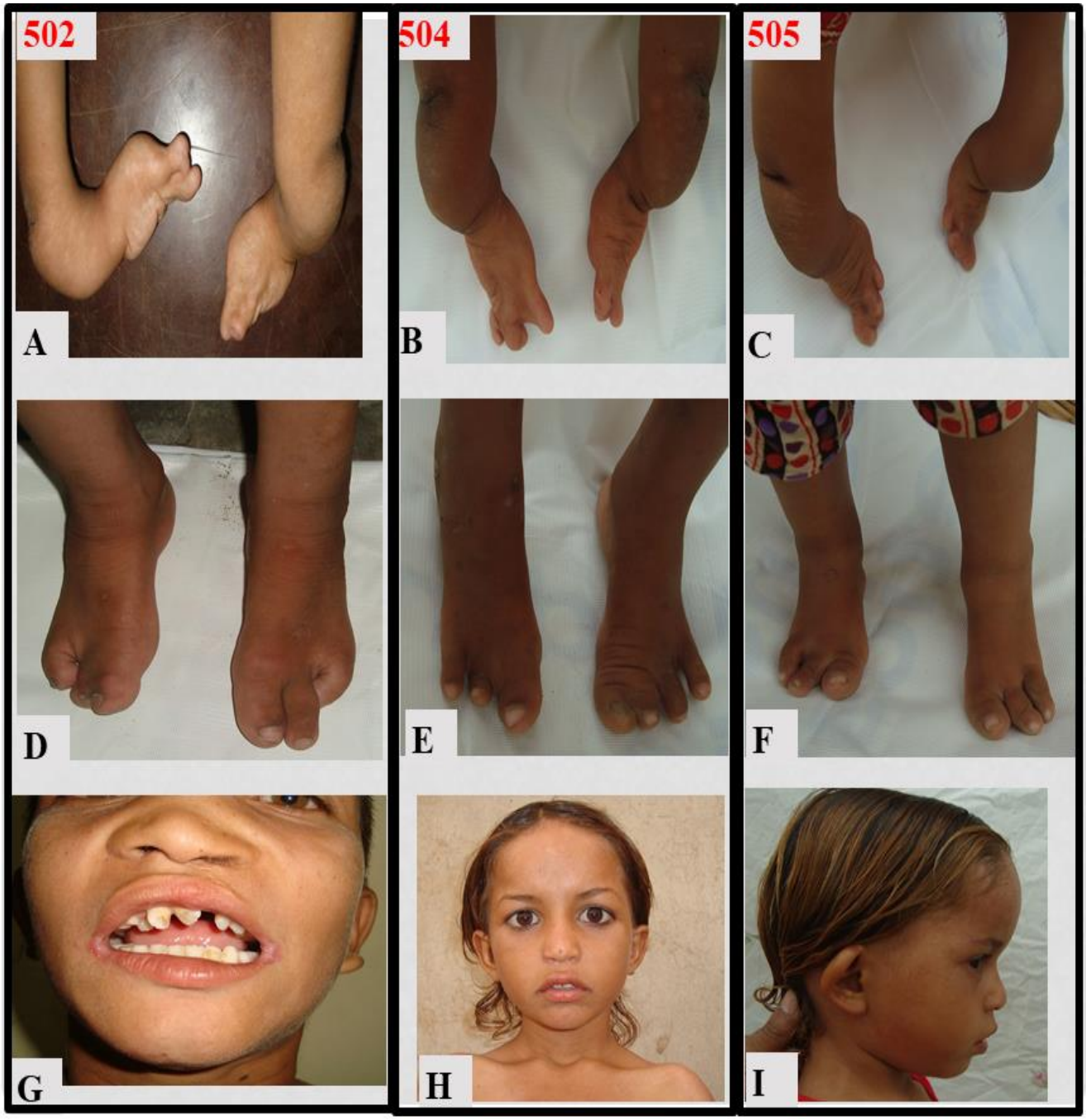


Fig. 5.2: Morphological presentation of the affected subjects

5.4.1.3: Radiographic findings

Among the three affected subjects, two (502, and 505) individuals underwent radiographic study. The radiographs of their upper forearm revealed short osseous fused radii-ulnae, and dysplastic or unidentified carpals, metacarpals and phalanges in hands (Fig. 5.3 J, K).

Anterio-posterior radiographs of the feet showed missing and irregularly organized tarsals and meta-tarsals, whereas the first digital rays appeared hypertrophic (Fig. 5.3 L, M). Similarly, the radiographs of the lower limbs of both patients showed a hypoplastic ankle and knee joints, and fibulae were of reduced size (Fig. 5.3 N, O). The malformed knee and ankle joints made walking difficulties (subject 505) (Fig. 5.3 N). The abnormality in the lower limbs also resulted in mildly short stature.

The chest radiograph with shoulder joints showed short slender humeri with hypoplastic distal heads resulted in compromised extension and flexion movement of elbow joints. Additionally, shoulder joints were malformed because the chest radiographs also indicated delayed bone maturation in the pectoral girdles. Dysplastic proximal heads of humeri were with malformed scapulae and dislocated clavicles (Fig. 5.3 P, Q). The affected individuals were living a rather handicapped and compromised life because they were unable to lift heavy objects, and, they have mild thoracic scoliosis (Fig. 5.3 P, Q).

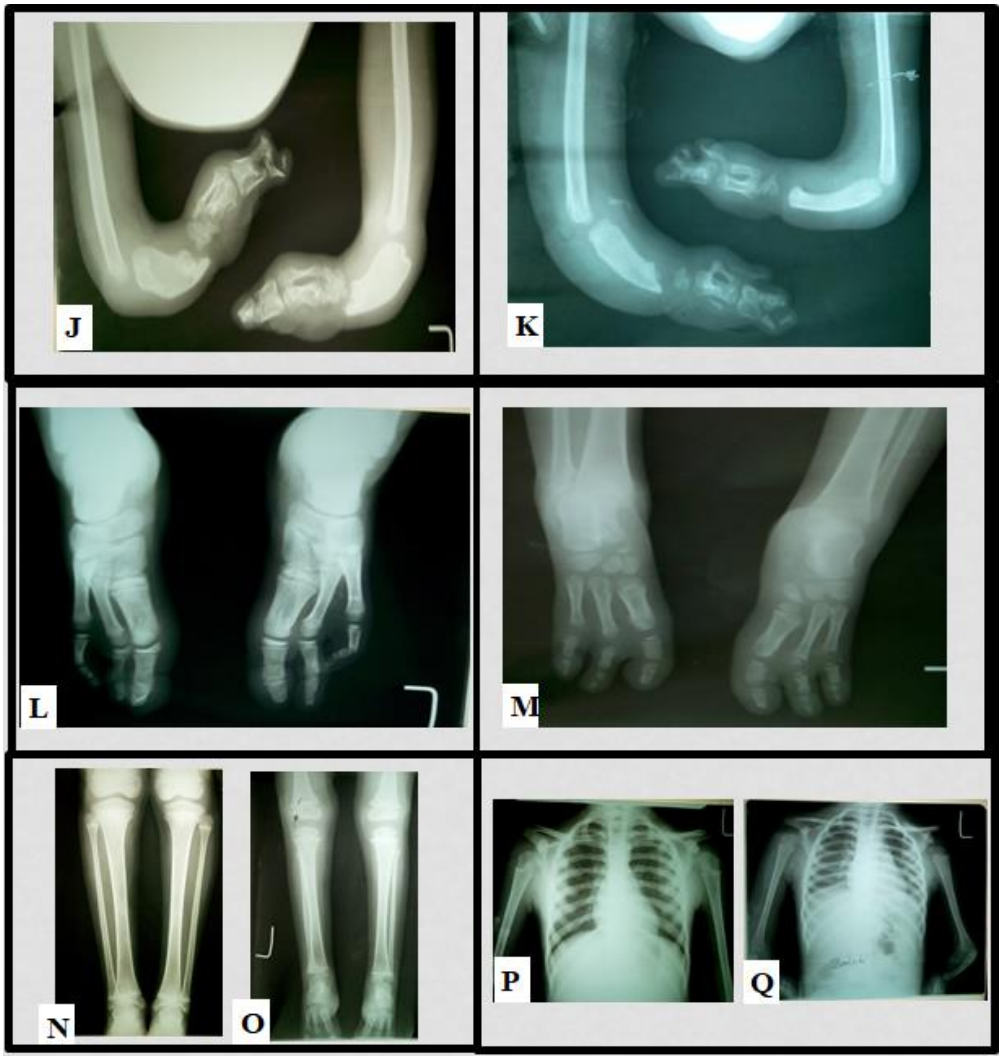


Fig. 5.3: Representation of internal anatomical structures of thoracic and appendicular skeleton through radiographs of affected individuals 502 (J,L,N,O) and 505 (K,M,P,Q)

Table 5.4: Morphological and radiographic findings of the affected individuals in family A

Variables	**502**	**504**	**505**
Gender, age (years)	M, 12	F, 9	F, 6
Upper limbs			
Unrecognizable fingers	+	+	+
Shortening of forearms	+	+	+
Fusion of carpals/metacarpals	+	NA	+
Fusion of radius-ulna	+	NA	+
Hypoplastic elbow joint	+	NA	+
Hypoplastic shoulder joint	+	+	+
Prominent clavicle	+	+	+
Lower limbs			
Short feet	+	+	+
Oligodactylous feet	3 toes, bilaterally	3 toes in right, 4 toes in left	3 toes bilaterally
Fusion of tarsals/metatarsals	+	+	+
Hypoplastic knee joints	–	NA	+
Walking difficulty	–	–	+
Facial and other features			
Prominent forehead/frontal bossing	+	+	+
Deformed ear (pinna)	+, left	–	+, right
Up-slanting/short nose	+	+	+
Depressed nasal bridge	+	–	+
Small depression on nasal tip	–	+	–
Cleft lip and hypoplastic frontal teeth	+	–	–
Thoracic scoliosis	+	NA	+
Standing height (cm (centile))	112 (<1)	99 (<1)	80 (<1)
OFC (cm)	51.5	49.0	46.5

+, feature present; –, feature absent; NA, not ascertained;

Reportedly, one of the subjects in the family (302) had cleft lip only without any limb phenotype. The affected subjects had normal vision, hearing, and speech. They were attending conventional schools without any sign of intellectual disability and, were adapted to perform daily tasks independently.

5.4.1.4: Ultra-sonography

In order to evaluate the vital organs, all patients underwent ultrasonography. The ultrasonographic examination revealed congenital agenesis of the left kidney in two patients (504, and 505); although, the urinary bladder was unremarkable in its positions and length. Multiple foci were found in both kidneys of a single patient (504). The other abdominal organs like the liver, gallbladder, spleen, and pancreas were found to be normal. The sonographic measures and findings of kidneys are shown in Table 5.5.

Table 5.5: Summary of sonographic measurements in the patients of Family A

Variable		**502**	**504**	**505**
Right kidney (mm)	Length	78.7	73	87.3
	Width	31.4	2	33.4
Left Kidney (mm)	Length	65	NPV	NPV
	Width	32.8		
Multiple foci		-	+	-

+, present; -, not present; NPV, not properly visualized

5.4.1.5: Molecular study

In the first round of analyses, Sanger sequencing was carried out for the index subject. This led to the detection of a splice variant (c.316+1G>A) in intron 3 (ENST00000378623). This variant was found in a homozygous state in the index subject and in other two affected siblings (Fig. 5.4), while all other unaffected subjects harbored the putative mutation in a heterozygous state (Fig. 5.4).

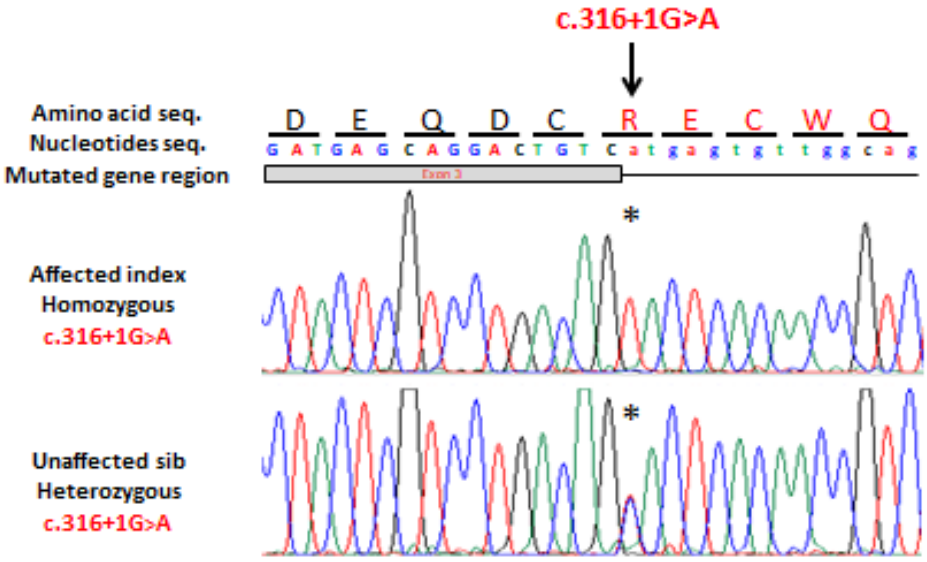


Fig. 5.4: The chromatograms showing the splicing mutation in intron 3 of the LRP4 gene. The upper panel shows the exon/intron boundary with the putative sequence variant. Middle panel shows the detected variant in the homozygous state in the index patient and the lower panel shows the variant in the heterozygous state in one of the unaffected sibs.

The detected intronic mutation resulted in the addition of 29 non-native amino acids in the protein leading to an early stop codon and truncated protein (Fig. 5.5). This variant was bioinformatically proved to be damaging (online tools NetGene2, MutationTester, NNSPLICE), and is presented in the general population with a very low heterozygote frequency of approximately 1/20,000 (ExAC database). Therefore, the variant is likely to be causative for the observed phenotype in this family.

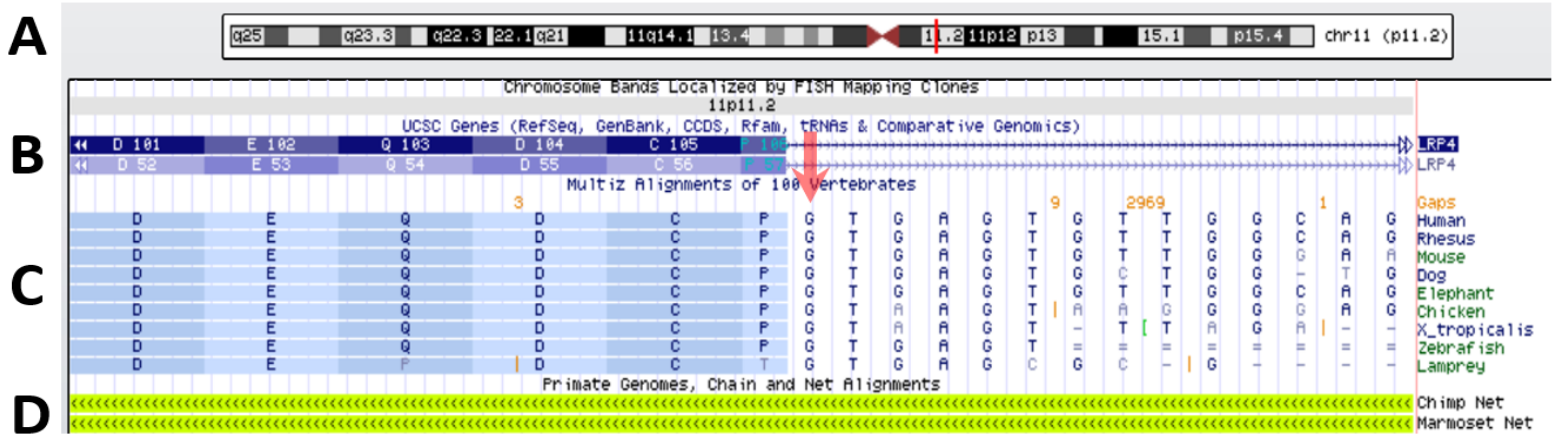


Fig. 5.5: Screenshot of UCSC genome browser showing the exon-3/intron-3 boundary of LRP4 and the detected variant

A. Chromosome showing the position of LRP4 at 11p11.2. B. Exon/intron boundary. C. Comparative protein sequence alignment showing complete conservation in diverse vertebrate species at the variant position (Red arrow; Multiz Alignment of 1000 vertebrates Track). D. The primate genome, chain and net alignment track.

5.4.2: Family B: Clinical report

Family B originates from a rural area of the Southern part of Khyber PakhtunKhwa (KPK), Pakistan. The pedigree of the family was constructed with the help of the family elders, and the information was cross-checked by interviewing the different family relatives.

The pedigree spanned four generations and disease appeared in the last generation that comprised 3 sibs (two male and one female) whose parents had a first consain marriage (Fig. 5.6). Out of the three sibs two (male, 402; and female, 403) had CLSS with primary features like bilateral fused unrecognized fingers, mitten-like appearance of the hands, irregular carpals/metacarpals, radii-ulnae synostosis, and kidney agenesis. The secondary features were abnormal elbow joint, malformed pectoral girdle, up-slanting nose, down-slanting eyes, large protruding ears, and a large narrow face (Fig. 5.7; Table 5.6).

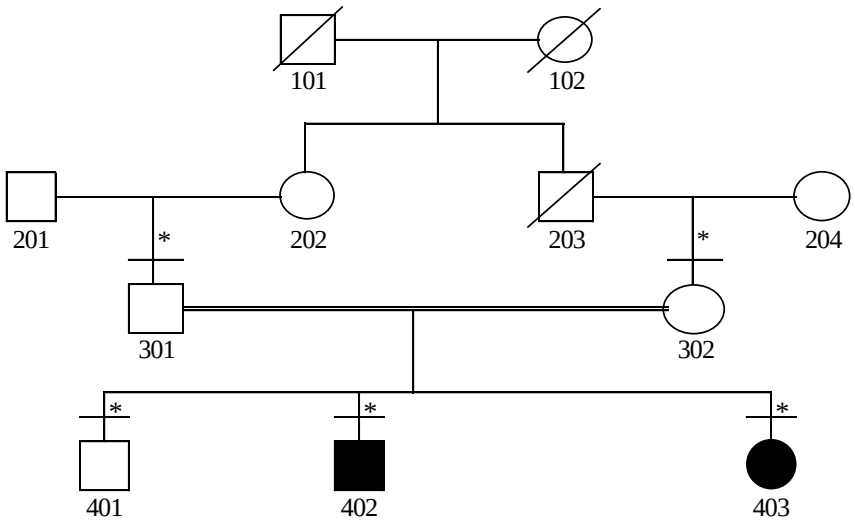


Fig. 5.6: Pedigree of family B with CLSS

Individuals with asterisk and horizontal bar were clinically and physically examined and available for molecular analyses, respectively.

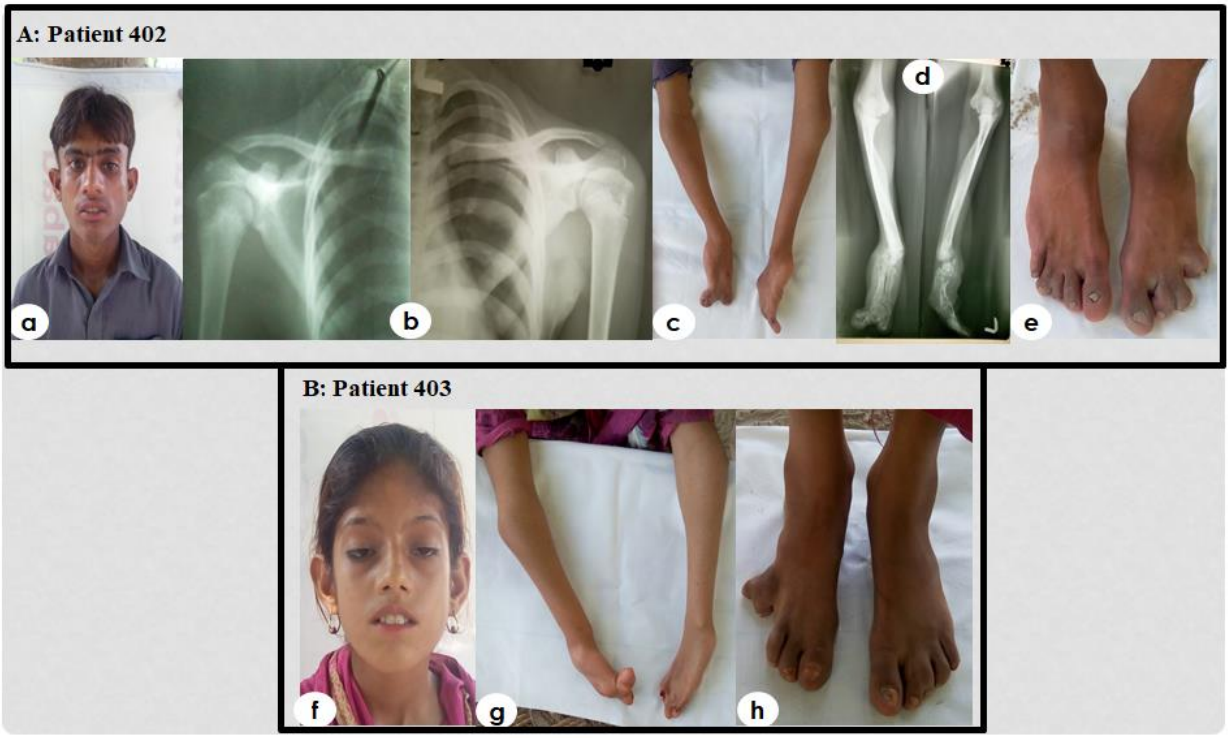


Fig. 5.7: Photographs and radiographs of affected subjects in Family B

A: Morphological and radiographic features of 402; B: Facial appearance and affected limbs of 403.

The clinical description of the family is based on the morphological, radiographic, morphometric measurements, and ultrasonography of the two affected individuals who were examined with the help of local physicians.

5.4.2.1: Patient 402

The index patient 402 is a 14-year-old boy. The physical examination showed characteristic features of CLSS. In the upper limbs, the autopod regions gave the mitten-like appearance with unrecognizable fingers (Fig. 5.7). Mesopodal segments were thin and disproportionate in length. In the lower limbs, all segments (stylopods, mesopods, and autopods) were normally developed except for the bilateral cutaneous fusions of toes. In the right foot, there was a fusion of 3-4 toes, and hypoplasia of the fifth toe. In the left foot, there

was fusion of 2-3 toes and hypoplasia of postaxial toes. Moreover, the nails were hypoplastic in the feet (Fig. 5.7).

The radiographic examination yielded defective radiographs that depicted undifferentiated disorganized carpals/metacarpals/phalanges in the autopods, bony fusion of elongated radius and ulna in both mesopodal segments with dysplastic epiphyses of proximal radio-ulnar and distal humeri ends at elbow joints, and pectoral girdles were also malformed in both stylopods (Fig. 5.7; Table 5.6). In addition to this, orio-facial examination showed up-slanting nose, down-slanting eyes, and ears were large protruding with a large narrow face (Fig 5.7; Table 5.6).

5.4.2.2: Patient 403

Patient 403 is a 12-year old girl with parity order of 3/3. She presented with same morphological features of upper and lower limbs as index subject (402) except for reduced/missing metatarsals rays of the fused 4/5 toes in right foot, and all segments were normal in the left foot with just ¾ fused toes (Fig. 5.7; Table 5.7). Morphometric measurements indicated a relatively short appendicular skeleton in both patients (Table 5.7).

Table 5.6: Summary of clinical features of CLSS in patients of Family B

Variable	***402***	***403***
Gender, age (years)	M, 14	F, 12
Upper appendicular skeleton		
Bilateral total fusion of all fingers	+	+
Hands Mitten-like appearance	+	+
Radio-Ulnar synostosis	+	NI
Acromesomelic forearms	-	-
Carpals/Metacarpals synostosis	+	NI
Un-recognized fingers	+	+
Irregular carpals/metacarpals	+	+
Absence of digital rays	+	+
Abnormal elbow joints	+	NI
Malformed pectoral girdles	+	NI
Lower appendicular skeletons		
Bilateral oligodactylous feet	-	-
Bilateral cutaneous toes syndactyly	+	+
Secondary features		
Up-slanting nose	+	+
Down slanting eyes	+	+
Large protruding ears	+	+
Face large narrow	+	+

+, feature present; -, feature absent; NI, not investigated

5.4.2.3: Ultrasonography

In order to extend the clinical spectrum of the phenotype and to observe internal vital organs and to confirm the kidney agenesis in the family, we conducted ultra-sonographic study of both patients. This study revealed that the right kidneys were normal with 9.1 cm length and 4.5 cm width, and 9.1 cm length and 4.2 cm width in 402 and 403, respectively, whereas, the left kidney was normal in patient 402 with 8.5 cm length and 2.3 cm width but it was not properly visualized in the lumbar region or in pelvis or on its opposite side in patient 403 (Table 5.7).

Table 5.7: Anthropometric measurements of subjects 402 and 403

Variable (cm)	***402***	***403***
Height	175.3 (<70)	139.7 (<3)
Head circumference	52.1	52.1
Humerus length	33.0	30.5
Leg length	104.1	81.3
Arm length	61.0	50.8
Femur length	52.1	39.4
Forearm length	30.5	26.7
Mesopod length	17.8	16.5
Renal Morphometry		
Right kidney	+	+
Length	9.1	9.1
Width	4.2	4.5
Left kidney	+	NPV
Length	8.5	-
Width	2.3	-

+, present; -, not present, NPV, not properly visualized

Kidney agenesis was found in only one affected Subject 403. Apart from kidney agenesis, all other internal vital organs like liver, liver parenchyma, bile canaliculi, gallbladder, urinary bladder, spleen, and pancreas were found to be normal. No ascites, para-aortic lymph adenopathy, abdominal/pelvic mass were seen in either patients.

5.4.2.4: Molecular analyses

In order to screen for mutation, *LRP4* was PCR amplified from exons 1-38 and subsequently sequenced in both directions. In the index (402) and the other affected sibling (403), a splice-donor variant c.1151A>G (p.Tyr384Cys) in exon 10 of the *LRP4* gene was detected (ENST00000378623) in a homozygous state. Among the normal subjects, the variant appeared in a heterozygous state (Fig. 5.8).

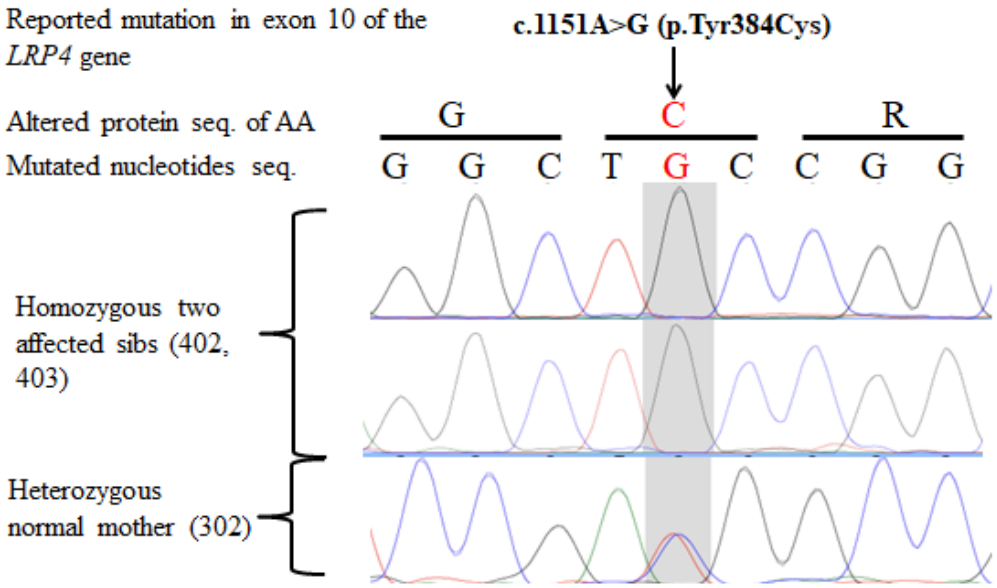


Fig. 5.8: Electropherogram showing the detected variant in homozygous state in the affected and in heterozygous state in the unaffected subjects of Family B.

The detected novel variant is predicted to be disease-causing but is not found in the general population according to the 1000 Genomes and ExAC databases. The affected amino

acid is evolutionarily conserved in all species (Fig. 5.9). Therefore, it is highly recommended that this newly found variant is causative for the observed phenotype.

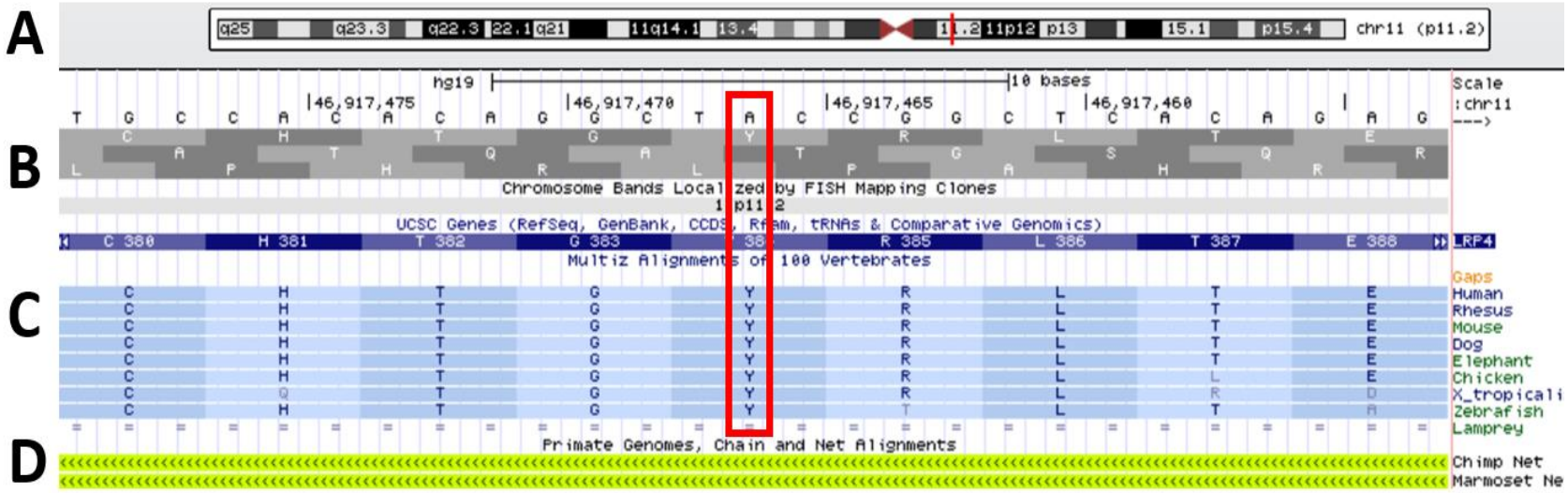


Fig. 5.9: Screenshot of UCSC genome browser showing part of exon-10 of LRP4

A. Chromosome showing the position of *LRP4* at 11p11.2, B. Exon 10 and the detected variant (red box), C. Comparative protein sequence alignment showing complete conservation in diverse vertebrate species at the variant position (Multiz Alignment of 1000 vertebrates Track). D. The primate genome, chain and net alignment track.

The clinical features of the studied families were compared with the reported CLSS families. It was noted that Family A was presented the most severe form of CLSS of the all of reported families as shown in Table 5.8.

Table 5.8: Comparison of clinical variability in the presented families with selected reported families with CLSS

Variable	*(Kariminejad et al. 2013)*	*(Harpf et al. 2005)*	*(Khan et al. 2013)*	*Present Family A*	*Present Family B*
Family origin	Iranian	?	Pakistani	Pakistani	Pakistani
No. of affected subjects	1	1	9	3	2
Parental consanguinity	+	+	+	+	+
Inheritance pattern	AR	AR	AR	AR	AR
Radiographic findings					
Radio-ulnar synostosis	+	+	-	+	+
Carpal/metacarpal/phalanges disorganization/synostosis	+	+	-	+	+
Abnormal elbow joints	+	-	-	+	-
Humerii heads abnormal	+	-	-	+	-
Shoulder joints deformed	-	-	-	+	-
Clavicle malpositioned	-	-	-	+	-
Tarsals/metatarsals/phalanges disorganization/synostosis	+	+	+	+	-
Malformed metatarsal and phalanges	+	-	-	+	
Hypoplastic knee joints	+	-	-	+	-
Kidney/renal hypoplasia/agenesis	+	-	+	+	+
Morphological Appearance					
Total syndactyly of all finger	+	+	-	+	+
Spoon hand type appearance	+	+	-	+	+
Shortness of forearm	+	+	-	+	-
Syndactyly of toes	+	+	+	+	+
Oligodactyly of feet	+	+	+	+	-
Preaxial cutaneous webbing of toes	-	-	-	+	-
High broad prominent fore head	+	-	-	+	-
Forehead frontal bossing	-	-	-	+	-
High hairs line	-	-	-	+	-
Depressed nasal bridge/tip	+	-	-	+	-
Hypoplastic teeth	-	-	-	+	-
Cleft lip	-	-	-	+	-
Out-inward bending of ears	-	-	-	+	-

+, feature present; -, feature absent; ?, not reported; AR, autosomal recessive

5.5: DISCUSSION

LRP4 encodes the Low-Density Lipoprotein Receptor-Related Protein 4. *LRP4* is a mediator of sclerostin (SOST) dependent regulation of bone formation and inhibition of Wnt signaling (Xiong *et al.* 2015). Since *LRP4* antagonizes the LRP5/LRP6 activation of WNT/β-catenin signaling (Leupin *et al.* 2011). Since *LRP4* regulates the Wnt signaling by decreasing the availability B-catenin, and understanding the role of Wnt/B-catenin is important for defining disease mechanism (Asai *et al.* 2014) in the development of limbs, bone, kidney, teeth and neuromuscular junctions. Mice with hypomorphic *LRP4* receptors showed abnormal development of the hind and forelimbs, with incomplete penetrant bone, kidney and teeth abnormalities, whereas complete null *LRP4* mice are not viable due to the failure of neuromuscular junctions to form (Ohazama *et al.* 2010).

The *LRP4* functions as a cell surface endocytic receptor that bind and internalizes extracellular ligands for degradation by lysosomes (Karner *et al.* 2010). It has been suggested that, due to the loss of *LRP4* function, excessive Wnt and β-catenin signaling leads to limb bud development. Besides CLSS, *LRP4* is also known to cause congenital myasthenic syndrome 17 (MIM-616304) and sclerosteosis 2 (MIM-614305).

Most of the reported mutations in *LRP4* are splice site or missense mutations and do not result in premature truncation or frameshift (Li *et al.* 2010). These mutations have been implicated in rather milder CLSS symptoms with minimal involvement of the ulnae and radii. This study described two families (A and B) with a severe CLSS phenotype.

In family A, the identified novel splice variant in *LRP4* (c.316+1G>A) segregated with the phenotype. This missense variant adds 29 non-native amino acids with a pre-mature stop-codon. This early truncated protein is 90% shorter than the wild-type

transcript. It is one of the shortest transcripts for *LRP4* in the reported literature except for one case (Kariminejad *et al.* 2013) caused by the c.289G>T mutation that resulted in premature termination (p.E97X). The early truncated protein would have only had the low-density lipid (LDL) repeats and lacked the different important binding motifs in *LRP4*. The patient reported by Kriminejad et al. (2014) also showed a severe CLSS phenotype, i.e., oligodactyly in hands and feet, hypoplastic radius-ulna and tibia-fibula, renal hypoplasia, hearing impairment, high arched palate, hypoplasia of tooth enamel and supernumerary nipples. In Family A, certain features like high arched palate, hearing loss, hypoplasia of tooth enamel and supernumerary nipples were not evident. On the other hand, short stature, scoliosis, hypoplasia of the pectoral girdle and cleft-lip were present. Recently, compound heterozygous truncating mutations in *LRP4* have been implicated in a lethal phenotype of CLSS (Lindy *et al.* 2014). Two sibling fetuses reported by Linddy et al. (2014) had marked oligosyndactyly, limb shortening, genitourinary anomalies, rib anomalies and hypoplasia of kidneys. In summary, the observations of these independent families suggest that early truncation of *LRP4* leads to severe CLSS phenotypes. In all three cases of Family A, the truncated proteins are likely to be truncated/absent or pathogenic, while, in the case of non-sense mediated decay, it would result in a complete lack of functional protein.

In Family B, another variant c.1151A>G (p.Tyr384Cys) in exon 10 of the *LRP4* gene was detected. This variant caused CLSS in two patients who had bilateral fused unrecognized fingers that gave mitten-like appearance of hands, irregular carpals/metacarpals, radii-ulnae synostosis, and renal agenesis, up-slanting nose, down-slanting eyes, large protruding ears, and large narrow face. These phenotypes of the affected subjects were almost similar with the clinical features of CLSS in the family A. Although in Family A, the phenotype was more severe likely due to the early truncated protein. This view was also supported by Li et al. (2010) who reported similar findings in a

Turkish family in which CLSS segregated as seen in our presented family. Based on our findings it could be hypothesize that CLSS is an unexpected autosomal recessive form of the gene which can cause disease in all its possible genetic forms. For example, *APC* homozygous state resulted in lethality, heterozygous state in cancer, and homozygous recessive state in CLSS (Linday et al. 2015). Similarly, *LRP4* works in same way as of APC gene. Overall, our findings enhance understanding about CLSS and will be helpful in developing the genotypic and phenotypic correlation.

In conclusion, the present cases add support to the notion that truncated *LRP4* mutations lead to more severe CLSS expression characterized by limb shortening and malformation in several other organ-systems, compared with the splice site and missense mutations that have rather milder symptoms. These findings, thus, support the genotype-phenotype correlations suggested in previous reports.

The results of this study were published in Eur J Med Genet as;

Afzal M, Zaman Q, Kornak U, Mundlos S, Malik S, Flöttmann R. (2017). Novel splice mutation in LRP4 causes severe type of Cenani-Lenz syndactyly syndrome with oro-facial and skeletal symptoms. Eur J Med Genet; 60(8): 421-425.

Chapter 6

6: Brachydactyly type B1 with a heterozygous de novo mutation in *ROR2*

6.1: Abstract

Brachydactyly type B1 (BDB1, MIM: 113000) is characterized by aplasia/dysplasia of the terminal phalanges of fingers/toes from 2-5 of both upper and lower limbs with the absence or underdeveloped nails. There are certain variable and inconsistent features which include distal symphalangism of digits and osseous/cutaneous syndactyly. All these primary consistent and secondary features have been implicated due to heterozygous mutations that fall quite often in exon 8 or exon 9 or intron 8 of the *ROR2* gene. Herein, we report a heterozygous *de novo* mutation c.2265C>A, p.Y755* (NM_004560 (ROR2_v001): c.2265C>A) in exon 9 of *ROR2* gene in a male Pakistani patient with BDB1. The subject had characteristic BDB1 features in addition to certain unusual findings like underdeveloped epiphyses of long bones, dislocated proximal ends of radii and ulnae of upper limbs, and bell-shaped rib cage and milder involvement of feet. The research findings will help us to understand the expanded phenotypic spectrum of BDB1. The study suggested direct screening of the candidate gene for molecular identification of brachydactyly.

6.2: Introduction

Brachydactyly is a condition with the shortening of digits due to abnormal development of the phalanges or their respective phalangeal rays of both in the distal segments of the upper and lower extremities. It is a heterogeneous group of diseases with high clinical variability which may be complete, symmetric and/or asymmetric in its expression (Temtamy & Aglan 2008; Wang *et al.* 2018). In early history, brachydactyly was the first reported Mendelian heritable phenotype in medical human genetics before Kellie in 1808 or after the publication of Mendel's laws (Farabee 1905; Marshall 1929; Bilginturan *et al.* 1973). Phalangeal brachydactylies are more common than those forms which are due to the shortening of metacarpals, especially of the ring finger. On the other hand, brachydactylies are less common than polydactyly, polydactyly, syndactyly, and ectrodactyly (Feil & Katz 1924).

Initially, brachydactyly was classified into three classes. These included (i) shortening of digits, (ii) ankylosis, and (iii) partial loss of bones (Feil & Katz 1924). Later, the classification of brachydactylies was reviewed and these were grouped into nonsyndromic and syndromic forms. The isolated brachydactylies were subsequently classified in the current classification scheme from A-E with inclusion of their submultiple types based on different segmental involvement of upper and lower limbs, as described in Table 6.1 (Bell 1951; Temtamy & McKusick 1978; Fitch *et al.* 1979).

Table 6.1: Phenotypic description, OMIM identifier and gene/locus of well-characterized brachydactyly

Brachydactyly Types	***OMIM***	***Inheritance***	***Genes***	***Locus***	***Phenotypic features***	***References***
Brachydactyly Type A					Middle phalanges shortened	
BDA1	112500	AD	*IHH* *BDA1B*	2q35-q35 5p13.3-p13.2	**All fingers & big toes involvement:** Variable shortening of middle phalanges of all digits, occasional fusion of terminal phalanges, shortened proximal phalanges of thumbs and big toes.	(Gao *et al.* 2001; Kirkpatrick *et al.* 2003)
BDA2	112600	AD	*BMPR1B* *GDF5*	4q21-q20 20q11.2	**Index fingers & little fingers involvement:** Middle phalanx of index finger/little finger hypoplastic/aplastic, a triangular middle phalanx of index fingers/second toes, radially curved index finger.	(Lehmann *et al.* 2003; Kjaer *et al.* 2006)
BDA3	112700	?	*?*	?	**Little fingers involvement:** middle phalanx shortened, radial deflection of distal phalanx, clinodactyly, single flection crease.	
BDA4	112800	?	*HOXD13*	2q31-q32	**Hands: Brachymesophalangy:** 2nd, 5th, & 4th fingers: Abnormal shaped middle phalanx, distal phalanx radial deflection, mild radial curvature of 4th/5th fingers. **Feet:** Absent middle phalanges of lateral four toes, talipes, short stature.	(Zhao *et al.* 2007)
BDA5	112900	?	*?*	?	**Only thumbs involvement:** Absent middle phalanges, duplicated terminal phalanx, nail aplasia.	

Brachydactyly Type B	113000				**Index to little fingers involvement**: Absent/hypoplastic terminal parts, fingernails completely absent, duplication/flattening or splitting of distal phalanges, ulnar side is more affected, symmetric presentation, syndactyly/symphalanges **Feet:** Less affected.	(Schwabe *et al.* 2000) (Lehmann *et al.* 2007)
BDB 1		AD	*ROR2*	9q22		
BDB 2		AD	*NOG*	4q23-q24		
Brachydactyly Type C	113100	AD	*CDMP1*	20q11.2	**Hands:** Brachmesophalangy of the index, middle, and little fingers, hyperphalangy of the index and middle finger, shortened 1st metacarpal, longest ring finger, ulnar deflection of proximal phalanx of the index finger, shortened metacarpals and synphalangism, short hands. **Feet:** Feet mostly normal or ordinary brachydactyly.	(Polinkovsky *et al.* 1997)
Brachydactyly Type D	113200	AD	*HOXD13*	2q31-q32	**Only thumbs involved:** Only shortened distal phalanx of thumb either unilateral/bilateral, variable shortening of thumbs, broad base of distal phalanx of thumbs.	(Johnson *et al.* 2003)
Brachydactyly Type E	113300	AD	*HOXD13*	2q31-q32	**Metacarpals:** Variable shortening of metacarpals with an almost normal length of phalanges, **Metatarsals:** short meta-tarsals, fused metatarsal epiphyses, short terminal phalanges. **Others:** Hyper flexibility of hands joints, high axial tri-radius, short stature.	(Johnson *et al.* 2003)

?, unknown.

In the literature, different synonyms were used for brachydactyly type B1 (BDB1) like symbrachydactyly (Mackinder 1857), and apical dystrophy (MacArthur & McCullough 1932). The typical features of brachydactyly B included shortening of the distal phalanges from 2-5 fingers with aplastic nails. These two features are the diagnostic characters of type B1 brachydactyly (Gong *et al.* 1999). Among all the brachydactylies, brachydactyly B is the most severe condition (Dong *et al.* 2015). It is further divided into two subclasses which are BDB1 and brachydactyly type B2 (BDB2) after the discovery of mutation in *ROR2* and *NOG* genes, respectively.

The primary features of BDB1 include short middle phalanges with rudimentary distal phalanges in all digits, with the involvement of both hands and feet, deformed thumbs and big toes, symphalangism, and 2/3 toes syndactyly (Mackinder 1857). The phenotypic spectrum of the malformation was expanded due to the inclusion of facial features like the prominent nose, high nasal bridge, and high arched palate (Hamamy *et al.* 2006). In addition to primary features, the most severe form of BDB1 has flat and broad thumbs, and bilateral syndactyly of hands/feet, underdeveloped 3rd toes, bifid thumb at the distal phalanges, hypoplastic distal phalanges of 2 and 4 fingers, distal symphalangism of finger 2 and 3, absent distal phalanges of finger 5, and bi-phalangeal toes (Lv *et al.* 2009).

Due to its wide clinical variability, the following four classes for the quantification of the BDB1 phenotype have been proposed. They include (i) mild (hypoplastic distal phalanges and nails present with frequent symphalangism), (ii) moderate (aplasia of distal phalanges and nails in only one/two fingers), (iii) moderately severe (absent distal phalanges and nails in three fingers), and (iv) severe (absence of distal phalanges with aplasia of the nails in fingers 2-5) (Schwabe et al. 2000).

The molecular etiology of BDB1 was revealed when this phenotype was localized to chromosomal segment 9q33-q34 (Gong et al. 1999). In the same year, a refined phenotypic and genotypic classification showed that the disease has genetic heterogeneity due to the involvement of another locus at 9q22 (Oldridge *et al.* 1999). Subsequently, the secondly reported locus, receptor tyrosine kinase like orphan receptor 2 (ROR2) was found as the responsible molecular cause of the BDB1.

ROR2 has a pleiotropic effect because it causes two phenotypes. In homozygous form, it causes autosomal recessive Robinow syndrome (OMIM: 268310) whereas in the heterozygous form, it causes autosomal dominant brachydactyly type B I (OMIM: 113000). Therefore, the occurrence of both autosomal dominant and recessive diseases confirms the allelic heterogeneity of *ROR2* gene. Twelve different ROR2 mutations (1 homozygous and 11 heterozygous) have been identified and all the mutations show complete penetrance. Most of the heterozygous mutations in exons 8 and 9 of *ROR2* are truncating (Oldridge et al. 2000; Schwabe et al 2000; Hamamy et al. 2006; Lv et al. 2009; Kjaer et al. 2009; Dong et al. 2015). However, it is not yet known whether a mutation in this gene causes autosomal recessive brachydactyly type B 1 or not. Similarly, it is also questionable, how and why consanguineous heterozygote carriers of *ROR2* (either parents or patients) remain unaffected by BDB1 (Kjaer et al. 2009).

Herein, a Pakistani patient with typical primary and atypical secondary features of brachydactyly type B1 is reported. These features included congenital absence of 2nd phalanges with hypoplasia/absence of last terminal phalanx in all fingers/toes except the thumb/big toe in all limbs. The molecular diagnosis of this case led to the discovery of the heterozygous *de novo* mutation (c. 2265C>A, p. Y755*) in exon 9 of *ROR2*.

6.3: Subjects and methods

The study was conducted with an inbreed family that was recruited from the rural area of Bhakhar district of Punjab, Pakistan. The study was conducted after verbal and written informed consent from every participant, and the study design was approved from the ethical review board of Quaid-i-Azam University.

Initially, in order to collect the clinical record of the phenotype, the parents of the subject were asked about prenatal and postnatal events during the pregnancy. A detailed systematic head to toe physical examination was carried out with the help of local physicians. Limbs were photographed and examined for detailed phenotypic characterization of the deformity. Additional anthropometric measurements were taken to investigate the proportionality of different body parts. The measured morphometric features were head and neck circumference, chest size with or without expansion, each arm length, arms span, each leg length, and standing height. In addition, craniofacial features were also investigated.

The clinical and laboratory examination was conducted at the nearest local clinical laboratory. The upper appendicular skeleton, feet of lower limbs, and ribs cage were radiographed to get fine X-rays for a comprehensive anatomical description of the phenotype. Peripheral blood samples were taken from the patient and his normal sibs and parents for molecular study. The mutation was found according to the method as described by Schwabe et al. (2000).

6.4: Results

6.4.1: Subject history

The study result was based on the investigations on the single sporadic Pakistani patient who was 19-year old male borne to a Saraiki speaking consanguineous family; and the patient was ascertained at his native town. The pregnancy and delivery events were reportedly as unremarkable. The parents of the index subject were phenotypically normal and did not report the occurrence of the phenotype in any other member of the family or relative.

6.4.2: Physical examination

The subject had a shortening of 2-5 fingers due to terminal phalangeal aplasia, aplastic nails, absence of inter-phalangeal creases on affected fingers of hands, malformed thumbs which were radially inclined, feet were normal except short 4/5 toes, smooth bell-shaped chest cavity. There was no evidence of other structural defects (Fig. 1; Table 6.2)

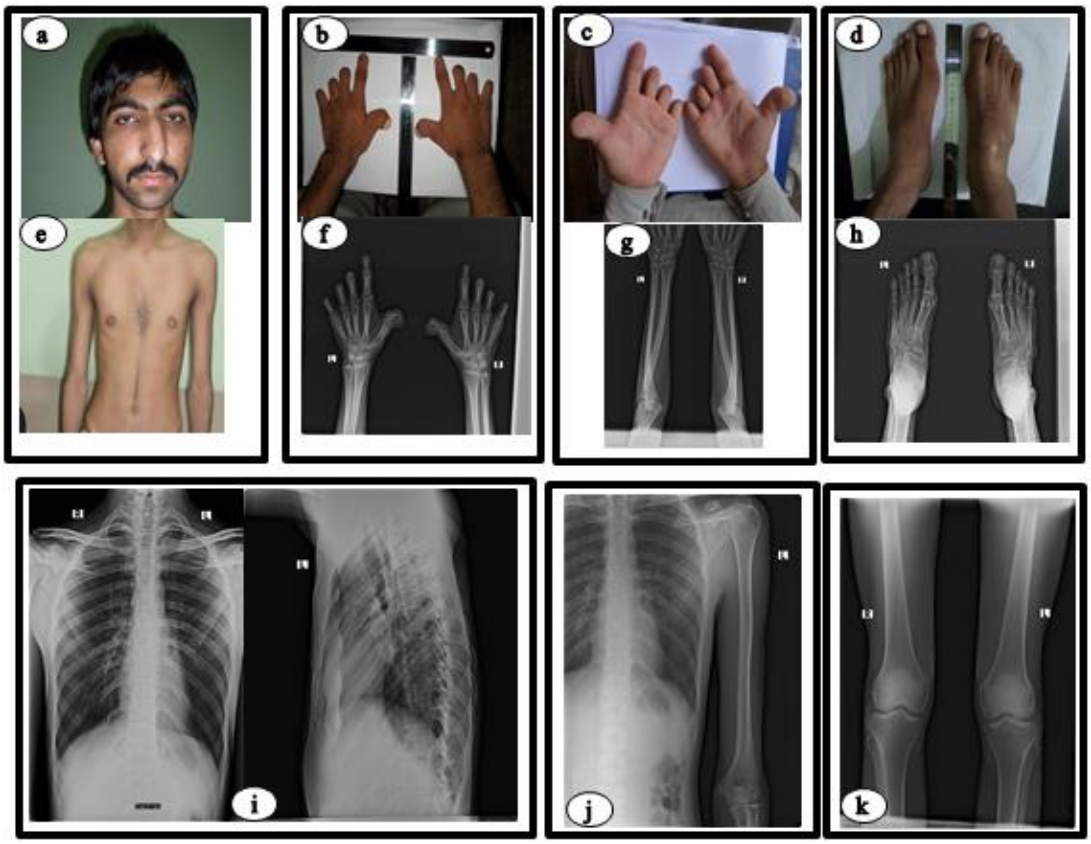


Fig. 6.1: The photographs and radiographs of the subject with type B1 brachydactyly

6.4.3: Radiographic examination

Radiographs evaluation showed aplasia of the terminal phalanges of digits from 2-5 (Fig. 6.1: b, c), malformed wrist joint (Fig. 6.1: f, g), dysplastic epiphyses of the upper long bones, malformed and dislocated radii-ulnae at proximal ends, and the lack of bone mineralization in upper arms (Fig. 6.1: g, j, k), with underdeveloped terminal phalanges in the toes 2-5 in feet (Fig. 6.1: h). The chest was smooth bell-shaped (ceiliocardinal dysostoses) with a hyperplastic diaphragm (Fig. 6.1: i), while the rest of the body skeleton was found unremarkable. The subject had normal intellectual development. Hence, the situation was consistent with the bilateral symmetric presentation of BDB1 without the occurrence of any other abnormality of other body parts. The morphological and clinical findings are summarized in Table 6.2; and Fig. 6.1.

Table 6.2: Phenotypic and clinical characterization of Brachydactyly type B 1 the subject

Primary Findings	***Bilateral***	***Symmetrical***
All short fingers	+	+
Dysplastic thumbs	+	+
Radial inclination of thumb	+	+
Rounded finger tips	+	+
Nail aplasia	+	+
Minute feet involvement	+	+
Dysplastic distal phalanges in thumbs	+	+
Radial inclination of thumb's phalanges	+	+
Absent 2nd distal phalanges in 2-5 fingers	+	+
Dysplastic/rudimentary last distal phalanges in 2-3 fingers	+	+
Absent last distal phalanges in 4-5 finger	+	+
Secondary Findings		
Broad distal end of radius	+	+
Dislocated proximal ends of radii and ulnae	+	+
Lack of mineralization in autopodial region	+	+
Distal ends of humerous broad and thick	+	+
Lack of mineralization in feet's autopodial regions	+	+
Big toe metatarsal and phalanges thick	+	+
Thin medial shaft of all the phalanges in toes 2-5	+	+
Rudimentary distal phalanges in toes 2-5	+	+
Bell-shaped cylindrical chest cavity	-	-

+, Present; -, present but non-specific

6.4.4: Molecular report

The exon 8 and exon 9 of the known candidate *ROR2* gene was Sanger sequenced using PCR based direct sequencing. The sequence data revealed a heterozygous *de novo* mutation (c.2265C>A, p. Y755*) in exon 9 at (rs94486579) at the distal end of the gene. The sequencing was performed with the collaboration of Prof. Dr. Stefan Mundlos, Institut für Medizinische Genetik, Charité, Universitätsmedizin Berlin, Germany.

6.5: Discussion

Herein, a mutation in the *ROR2* gene from a Pakistani subject with brachydactyly is reported. This mutation was identified in a male patient that had BDB1 which was determined through clinical and radiographic inspection of the hands and feet of the patient. The patient had bilateral symmetric dysplastic/aplastic terminal phalanges from 2-5 with aplasia of the nails in fingers of hands, and there was little involvement of feet. Review of the literature showed that there are at least 12 different mutations in *ROR2* gene associated with BDB1 (Table 6.3). Through this study, a mutation (c.2265C>A, p. Y755*) in exon 9 of *ROR2* gene was found. The mutation was already reported by others (Oldridge *et al.* 2000; Hamamy *et al.* 2006; Hellani *et al.* 2009; Li *et al.* 2011).

Receptor tyrosine kinases like orphan receptor (RoR) are pleiotropic and are involved in a variety of developmental process during the formation of various tissues and organs. The RoR signaling pathways are required for skeleton formation and neurogenesis by the Wnt signaling pathway during the different developmental processes. However, we have little knowledge about role that RoR signaling plays in these processes (Petrova *et al.* 2014). For example, in the human, disruption of ROR signaling is responsible for Robinow syndrome and BDB1. Hence, the future researches include identification of components which regulate RoR signaling, and better understanding may help us to reveals the functions of these pleiotropic receptors.

Table 6.3: Mutation spectrum in *ROR2* implicated in BDB1

Mutation	***Types***	***Allele status***	***Gene Position***	***Nucleotides change***	***Amino acid change***	***Mutational site at protein level***	***References***
1	Transition missense	Het.	Exon 9	c.2246G>A	p.W749X	Distal: After TK	(Oldridge *et al.* 2000)
2	Frame shift deletion	Het.	Exon 9	c.2249delG	p.G750fxX23	Distal: After TK	(Oldridge *et al.* 2000)
3	Missense	Het.	Exon 9	c.2265C>A	p.Y755X	Distal: After TK	(Oldridge *et al.* 2000)
4	Frame shift deletion	Homo.	Exon 8	c.1321_1325del(CGGCG)	p.R441fsX15	Proximal: Before TK	(Schwabe *et al.* 2000)
5	Splice site deletion with insertion	Het.	Intron 8	c.IVS8+3 >+5del3ins19	p.A463fsX64		
6	Insertion	Het.	Exon 9	c.1398–1399insA	p.Q467fsX57	Proximal: Before TK	(Schwabe *et al.* 2000)
7	Missense	Het.	Exon 9	c.2278C>T	p.Q760X	Distal: After TK	(Schwabe *et al.* 2000)
8	Missense	Het.	Exon 9	c.2247G>A	p.W749X	Distal: After TK	(Bacchelli *et al.* 2003)

9	Insertion	Het.	Exon 9	c.1266–1267insC	p.L456PfsX2	Proximal: Before TK	(Kjaer *et al.* 2009)
10	Frame shift deletion	Het.	Exon 9	c.2243delC	p.W749fsX24	Distal: After TK	(Lv *et al.* 2009)
11	Nonsense, frame shift deletion	Het.	Exon 9	c.1396-1398delAA	p.H523X	Proximal: Before TK	(Huang *et al.* 2014)
12	Missense	Het.	Exon 9	c.2273C >A	p.S758X	Distal: After TK	(Dong *et al.* 2015)
13	De-novo nonsense	Het.	Exon 9	c. 2265C>A	p. Y755X	Distal: After TK	**Present study**

At the protein level, all identified heterozygous mutations were identified after the tyrosine kinase domain in cytoplasmic region and led to protein truncation (Table 6.3) due to terminations in the C terminal region of the protein (Afzal & Jeffery 2003).

Four reports mentioned the same transversion substitution at c.2265C>A at the DNA level and these findings also proved the study result. In our patient the phenotypic expression overlapped with the features reported by Oldridge et al. (2000), Hammamy et al. (2006); Hellani et al. (2009) and Li et al. (2011). In summary, based on the phenotypic presentation of BDB1, direct Sanger sequencing is the best method for the characterization of *ROR2*. Our findings expanded understanding to explain the phenotypic spectrum of BDB1.

Chapter 7

7: Homozygous mutation c.A719G in *TPO* gene causes intellectual disability and congenital hypothyroidism in Pakistani kindred

7.1: Abstract

Intellectual disability (ID) involves compromised intellectual, learning, and behavioral capabilities with reduced psychomotor and cognitive skills. It may be preventable or non-preventable in nature. One of the preventable and treatable causes of ID includes congenital hypothyroidism (CH), an inherited congenital endocrine disorder due to thyroid-dysgenesis or thyroid hormonodysgenesis, which may be due to mutations in thyroid peroxidase (*TPO*) gene. The *TPO* is an important bio-catalytic enzyme that is involved in the production of thyroid hormones and is located on the membranes of thyroid follicles. CH, a type of metabolic disease, is characterized by high production of thyroid stimulating hormone (TSH), low quantities of T3 and T4 hormones, and hypertrophic thyroid glands in association with reduced motor and cognitive skills. In this study, a large family afflicted with ID was recruited. Differential diagnosis and detailed clinical and hormonal assessment led to the diagnosis of CH for the kindred. Through SNP-based linkage analyses, homozygosity mapping led to the identification of homozygous regions segregating among the affected subjects. Exome sequencing of one of the affected subjects revealed a homozygous mutation (c.719A>G) in the *TPO* gene, which falls in one of the identified homozygous regions. Through Sanger sequencing, this mutation was later determined to segregate with the phenotype in the whole family. Thus, this mutation caused autosomal recessive ID and congenital hypothyroidism due thyroid dyshormonogenesis in the recruited kindred.

7.2: Introduction

Intellectual disability (ID) or mental retardation (MR) is an umbrella of genetic disorders involving compromised intellect and learning with reduced psychomotor and cognitive skills (Inlow & Restifo 2004). It may be syndromic or non-syndromic. Its isolated form may be preventable and non-preventable. Preventable ID may be considered as an extra-thyroidal congenital anomaly (Gkini *et al.* 2016) because central nervous system development is dependent on the normal working of maternal and foetal thyroid glands (Pasquali *et al.* 2015). The irregularities in the concentration of thyroid hormones resulted in neurological, psychiatric and behavioural disorders (Ahmed 2018a). The psychiatric and cognitive symptoms of treatable ID included mood instability, psychosis, anergia, hypersomnia, depression, sleep and appetite irregularities, hyperactivity, noise irritating behaviour, and delayed motor skills (Goh *et al.* 2014; Noda 2015; Ahmed 2018a, b).

Treatable cause of MR includes congenital hypothyroidism (CH) which is a common endocrine neonatal metabolic disorder with declined growth and intellectual impairment (Liu *et al.* 2018). It results from insufficient thyroid hormones production by inadequate thyroid gland differentiation (thyroid dysgenesis) and irregular thyroid hormones synthesis (thyroid dyshormonogenesis) (Rastogi & LaFranchi 2010; Chen & Qin 2018). The low level of thyroid hormones (THs) resulted in the impairment of neurotransmission in different brain regions, especially the hippocampus, delayed proliferation and migration of neurogenic cells, reduced myelination of axons, and altered structure of dendrites (Park *et al.* 2017). The cognitive and motor complications (Targovnik *et al.* 2011) and untreated CH leads to severe form of permanent mental retardation and developmental delay (Löf *et al.* 2016).

CH is very rare disease that occurs with a prevalence rate of 1/3000-4000 live births (Makretskaya *et al.* 2018). The primary clinical manifestations include a high level of thyroid stimulating hormone (TSH), low/high level of thyroid 3 (T3), and thyroid 4 (T4) with hypoplasia/hypertrophy of thyroid glands in association with goiter. Secondary features include poor feeding, jaundice, umbilical hernia, and rough dry skin (Chang *et al.* 2012). The proper regulation of the aforementioned hormones is vital for proper neuro-cognitive development (Cherella & Wassner 2017). The serious life affecting effects of the disease can be minimized or prevented if it is diagnosed or treated in its early stages (Avbelj *et al.* 2007). In early steps, the complete diagnosis of CH includes clinical examination, biochemical tests, ultrasonography, a radio-iodine test, and a perchlorate discharge test (Jones & Rose 2018).

Hypothyroidism, presenting as a neonatal congenital endocrine disorder, is mainly due to iodine deficiency and different gene mutations. There are more than 600 genomic alterations which have been recorded in the Clinical Var database (Long *et al.* 2018). Among these, 80-85% gene mutations caused thyroid dysgenesis in the form of agenesis, hypoplasia, and or ectopy of thyroid gland and the remaining 10-15% of gene mutations caused thyroid dyshormonogenesis (Cherella & Wassner 2017).

Abnormal thyroid hormone synthesis resulted in bi-lobed thyroid gland, cervical hypertrophy of thyroid gland, high level of TSH, and the low thyroid hormones (T3 and T4) (Fu *et al.* 2016). This abnormal hormonal synthesis is caused by mutations in genes *DUOX2* (thyroid oxidase 2) (Grasberger 2010), *DUOXA2* (dual-oxidase mutation factor 2) (Grasberger 2010), *TG* (thyroglobulin gene) (Grasberger 2010), *SLC5A5* (solute carrier family 5 member 5), *SLC26A4* (solute carrier family 26 member 4) (Bizhanova & Kopp

2010), and *IYD* (iodotyrosine deiodinase) (Moreno & Visser 2010), and *TPO* (thyroid peroxidase) (Ris-Stalpers & Bikker 2010).

TPO is a glycosylated thyroid specific haemoprotein which is found in the form of a dimer with each dimer containing 933 amino acid residues. It contains four domains including peroxidase, extracellular, transmembrane helix, and C-terminal intracellular with all four domains being involved in the regulation of enzymatic pathways during thyroid-genesis or thyroid-hormono-genesis (Park & Chatterjee 2005). Therefore, its deficiency results in autosomal recessive congenital hypothyroidism as thyroid dysmorphogenesis 2A (TDH2A; MIM: 274500). The deficiency is due to homozygous or compound heterozygous mutations in the *TPO* gene (OMIM: 606765), which contains 17 exons and is located on 2p25 (Endo *et al.* 1995).

A high prevalence of consanguineous marriages results in the increased incidence of genetic diseases cause high mortality, morbidity, and intellectual disability, especially in Muslims world (Najmabadi *et al.* 2011; Harripaul *et al.* 2018; Younis *et al.* 2018). In the Pakistani population, the consanguinity rate is as high as 63% (Jabeen & Malik 2014). About 1% of the global population is affected with intellectual disability due to mutations in an estimated 2500 autosomal genes, and many of them remain without a molecular diagnosis (Harripaul *et al.* 2018). The poor clinical/molecular diagnosis and insufficient management/genetic counselling of ID are due to its complex and heterogeneous nature, which poses lifelong problems and care for both the patients and parents ((Iqbal *et al.* 2012; Blencowe *et al.* 2018).

Here, we reported a large family afflicted with ID has been reported, in which a homozygous mutation c.719A>G in *TPO* gene was observed to segregate with the

phenotype. This mutation caused autosomal recessive CH with an insufficient quantity of thyroid enzymes due to the thyroid dyshormonogenesis.

7.3: SUBJECTS AND METHODS

The family was recruited from the rural area of the Thal, the desert of Pakistan. A pedigree of the family was constructed with the help of the elders, and the information was cross-checked by interviewing relatives in different loops of the pedigree in multiple visits (Fig. 7.1). All the demographic information and clinical data were obtained after informed consent according to the Helsinki II declaration. The study was approved by the ethical review committee of Quaid-i-Azam University.

For the phenotypic description, the morphological and behavioral examination of the patients was carried out at their residences and photographs of them were taken there. The family was initially diagnosed with mental retardation and moderate/severe cognitive impairment. However, the diagnosis of CH was reached after detailed clinical, biochemical and hormonal profiling. Accordingly, a hormonal study (TSH, total T3 and T4) of five patients (303, 304, 307, 310, 314, and 401) was conducted. Moreover, the free level of thyroid hormones T3 and T4 was also determined in three patients (304, 307, and 310). Ultrasonography for the gross anatomic structure of the thyroid gland was performed for all the available patients (303, 304, 307, 310, 314, and 401). To observe growth patterns and aging, X-rays of hands of three index subjects (304, 307, and 310) were obtained. All the radiological examinations were conducted with the help of local physicians at private clinics in Southern Punjab. Radiographs were also taken. The thyroid function test was available only for one affected subject (303). For the molecular study, peripheral blood samples of six patients and nine unaffected subjects were obtained.

7.3.1: Whole-Genome SNP Genotyping and Exome Sequencing

Whole-genome SNP genotyping was carried out on three affected and two unaffected subjects using Illumina Human OmniExpress-24 BeadChip. Linkage analyses were performed through GeneHunter and Allegro, as implemented on the easyLINKAGE platform (v5.08), assuming autosomal recessive inheritance, a disease allele frequency of 0.001, and 99% penetrance. Multipoint logarithm of odds (LOD) scores were calculated using marker window sizes of 50 and 70. Haplotypes were constructed to investigate whether homozygosity was shared among the affected subjects. Exome sequencing was performed for one affected individual using the Agilent SureSelect Target Enrichment System and the Illumina HiSeq2000 platform. Rare (frequency <0.01) and novel variants in regions with LOD scores ≥2.5, with alternate depths >0.30, and possibly affecting protein function were considered. Sanger sequencing was carried out with the help of a commercial service provider (as described in Chapter 3), to validate the identified mutation and its segregation in the family.

7.4: Results

7.4.1: Pedigree

The pedigree comprised four generations and the disease segregated in the last two generations. There were seven affected subjects (303, 304, 307, 310, 312, 314, and 401). Among them, there were four affected males and three affected females. All patients were the products of inbred unions (first cousin) and all the affected subjects had normal parents (Fig. 7.1).

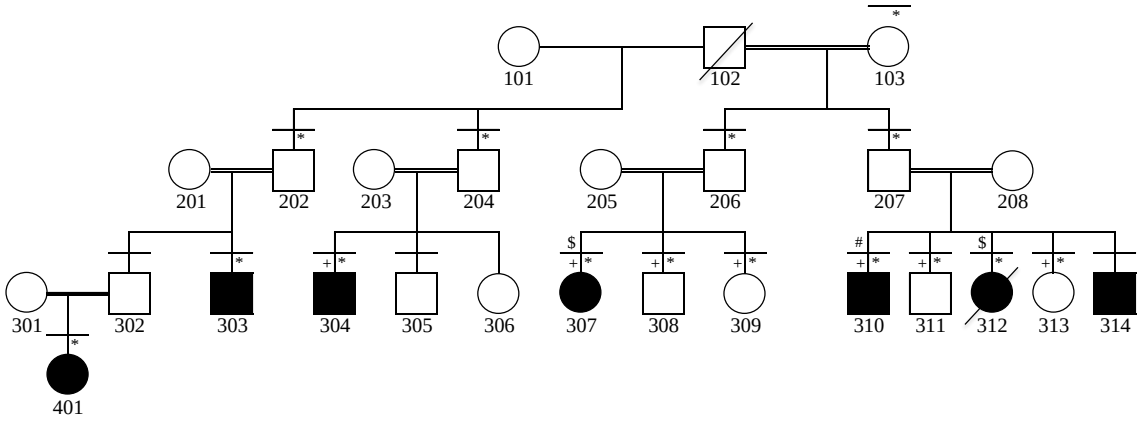


Fig. 7.1: Pedigree of the family with intellectual disability and hypothyroidism

Black shaded symbols depict affected subjects with congenital hypothyroidism; symbols with oblique line depict deceased subjects; horizontal line above the symbols show subjects that were physically examined; $, affected individuals with intellectual disability; *, subjects participated in the molecular study; +, SNP genotyping; #, Exome sequencing

7.4.2: Physical examination

Initially, in 2010, the family was recruited as a case study of hereditary mental retardation type. Physical examination of the patients showed delayed developmental milestones and behavioural anomalies. The delayed developmental milestones were

presented with late crawling and walking and delayed speech and hearing. The patients exhibited moderate to severe intellectual disability with reduced motor and cognitive skills. The patients also had short stature, enuresis and encopresis. The behavioural signs included hyperactivity, self-stimulation, and attention deficit, sleep disturbance, thumb sucking, and hand biting. The detailed features of these patients are given in Table 7.1.

Table 7.1: Developmental and behavioural features of the affected subjects

Features	303	304	307	310	312	314	401
Developmental milestones							
Developmental/growth delay	−	−	++	++	++	++	−
Crawling late	−	−	++	−	++	+	−
Walking late	−	−	++	−	++	+	−
Speech delay	−	+	++	++	++	++	+
Hearing late	−	−	++	−	++	−	−
Sequent eyes	−	−	−	+	−	+	−
Goiter	+	+	−	−	−	−	+
Short stature	−	−	++	−	++	−	−
Enuresis	−	−	++	−	++	−	−
Encopresis	−	−	++	−	++	−	−
Mental retardation	−	−	Severe	mild/ slow learner	Severe	mild/ slow learner	−
Behavioral traits							
Hyperactivity	−	−	++	+	+	+	−
Aggressive and intrusive behavior	+	+	++	++	+	+	+
Self-stimulating	−	−	++	−	++	−	−
Attention deficit	−	−	++	+	++	−	−
Head banging, tantrums, self-Mutilation	−	−	++	−	−	−	−
Bipolar episodes	−	−	+	−	−	−	−
Sleep disturbances	−	−	+	−	−	−	−
Sensitive to food and taste	−	−	+	−	−	−	−
No sense of clothes	−	−	++	−	−	−	−
Touch sensitive	−	−	++	+	−	−	−
Thumb sucking	−	−	++	−	−	−	−
Hand biting	−	−	++	−	+	−	−
Reduced motor and cognitive skills	−	−	++	+	++	+	−

+, feature present; ++, severe phenotype, −, feature absent

7.4.3: Clinical examination

In the follow-up visits, hormonal assays suggested hypothyroidism. Hormonal profiling including TSH, T3, and T4 showed abnormal ranges in these patients (Table 7.2). Further chemical tests, morphological study of thyroid glands by ultrasound, bone development study by X-rays were consistent with hypothyroidism. The patients (i.e., 303 and 314) were reported to have taken thyroxine tablets since an early age. The patients who used thyroxine tablets had improved clinical features compared to those patients who did not use thyroxine (304, 307, 310, and 401).

Ultrasonography of the thyroid gland, in the affected subjects, showed hypertrophy of thyroid gland with spongy appearance and mass/cystic formation. Additionally, the hypertrophy of the thyroid gland increased with the passage of time. Thus, abnormal thyroid function test, inconsistence goiter appearance, dependence on thyroxin tablets since early infancy were strongly suggestive of CH. Detail of the tests of each patient are shown in Table 7.2.

Table 7.2: Thyroid hormones profiling and thyroid ultrasonography

Patient	Age (yrs) at evaluation	Hormone	Units	Normal ranges	Results	Remarks	Thyroid gland ultrasonography
303	19 (1st time)	T3	nmol/L	1-3.2	3.3	High	Increased uptake of radiotracer in both enlarged lobes and correlate thyroid function test with multinodular goiter
		T4	nmol/L	59-141	51	Low	
		TSH	nmol/L	0.4-4.7	31.6	High	
	20 (2nd time)	T3	µlU/L	Vitros= 0.40-4.70	2.2	Normal	
		T4	µlU/L	Vitros= 59-141	Vitros= 135	Normal	
		TSH	µlU/L	Vitros= 0.40-4.70	Vitros= 0.1	Low	
304	18	Free T3	pg/ml	1.71 - 3.71	2.62	Normal	Hypertrophic with spongy appearance and cystic mass that obstruct the lateral blood flow
		Free T4	ng/dl	0.7 - 1.48	0.54	Low	
		Total T3	ng/dl	80 - 210	98.4	Normal	
		Total T4	µg/dl	4.6 - 10.5	3.7	Low	
		TSH	µlU/ml	0.35 - 4.95	>100.0	High	
307	15	Free T3	pg/ml	1.71 - 3.71	<1.00	Low	Hypertrophic with spongy appearance and cystic mass
		Free T4	ng/dl	0.7 - 1.48	<0.40	Low	
		Total T3	ng/dl	82 – 218	<25	Low	
		Total T4	µg/dl	5.6 - 11.7	1.6	Low	
		TSH	µlU/ml	0.51 - 5.27	>100.0	High	
310	9	Free T3	pg/ml	1.71` - 3.71	3.31	Normal	Hypertrophic with spongy appearance
		Free T4	ng/dl	0.7 - 1.48	1.15	Normal	
		Total T3	ng/dl	94 – 241	106.9	Normal	
		Total T4	µg/dl	6.4 - 13.3	8.4	Normal	
		TSH	µlU/ml	0.55 - 5.46	>100.0	High	
314	1 (1st time)	T3	nmol/L	1.7-3.07	0.3	Low	Hypertrophic with spongy appearance and cystic mass
		T4	nmol/L	7.8-16.5	0.49	Low	
		TSH	nmol/L	0.6-6.3	99	High	
	6 (2nd time)	T3	ng/dl	93-215	27.9	Low	
		T4	ng/dl	5.5-15.8	1.3	Low	
		TSH	µlU/ml	0.55-5.46	101	High	
401	7	Free T3	pg/ml	2.1-4.40	1.6	Normal	Appeared normal
		Free T4	ng/dl	0.8-2	0.82	Normal	
		Total T3	ng/dl	94 – 241	69.67	Low	
		Total T4	ng/dl	6.4 - 13.3	7.9	Normal	
		TSH	µlU/ml	0.7-6.4	>100.0	High	

7.4.3.1: Subjects 303 and 304

Patients 303 and 304 had nearly similar presentations in their physical examination. Thyroid scanning of subjects 303 was performed 20 minutes after injecting 185 MBq of 99mTcO intravenously. The scan showed heterogeneously and increased radiotracer uptake in both lobes of the enlarged thyroid gland and multinodular goiter correlated with thyroid function test. Patient 304 was a 18-year male whose ultrasonography showed the enlarged right lobe of the thyroid gland with multiple masses, with the largest one having measures about 1.8 cm ×1.1 cm and a cyst of about 1 cm × 0.9 cm in it. The left lobe had measures of about 7.1 cm × 4.2 cm and has a mass of about 2.6 cm × 1.4 cm. The patient was dependent on thyroxin and used it since the age of 1.5 years. Over the years, the dosage was increasing and now he took as many as six tablets per day to live a physiologically active life. Moreover, thyroxin withdrawal symptoms included neck swelling (Fig. 7.2; subject 304) and lethargic conditions. Patient 304 was also subjected to radiographic examination. The radiographic analysis of his upper limbs showed abnormal hypophyses and elbow and wrist joints with delayed bone age, lack of long bone ossification, hypoplastic elbow joints, dysplastic humeri distal heads, and hypoplastic carpal/metacarpals (Fig. 7.3; subject 304). Most of the typical structural and behavioural features were absent in this subject, although he had speech delay and aggressive behaviour.

Fig. 7.2: Phenotypic features in affected subjects

304: Goiter; 310: Slow learner, developmental delay, and sequent eyes; and 401: Abdominal swelling, developmental delay. **Note:** Photos were presented after permission from the family elders.

7.4.3.2: Subject 307

Patient 307 was a 15-year old girl and was the most severely affected subject among all patients. She was severely mentally retarded with behavioural symptoms like hyperactivity, drooling, self-talkative nature, hand biting, enuresis, encopresis, no sense of clothing, self-stimulating aggressive and an intrusive nature, head banging, sleep disturbance, attention deficit, sensitive to food and taste, touch sensitive, and thumb sucking. She also had a developmental delay in crawling, walking, speech and hearing.

The ultrasonography of her left thyroid lobe demonstrated that there was an echogenic mass of about 11.6 mm × 13.7 mm which was not compromising the blood flow in the associated vessels, and with the right lobe was being normal. She was currently using thyroxin tablets which improved her clinical conditions. Her thyroxin withdrawal symptoms were neck swelling, lethargy, fever, and irritation. Radiographic findings were abnormal epiphyses and malformed elbow and wrist joints with delayed bone age, lack of

long bones ossification, hypoplastic elbow joints, dysplastic humeri distal heads, and hypoplastic carpal/metacarpals (Fig. 7.3).

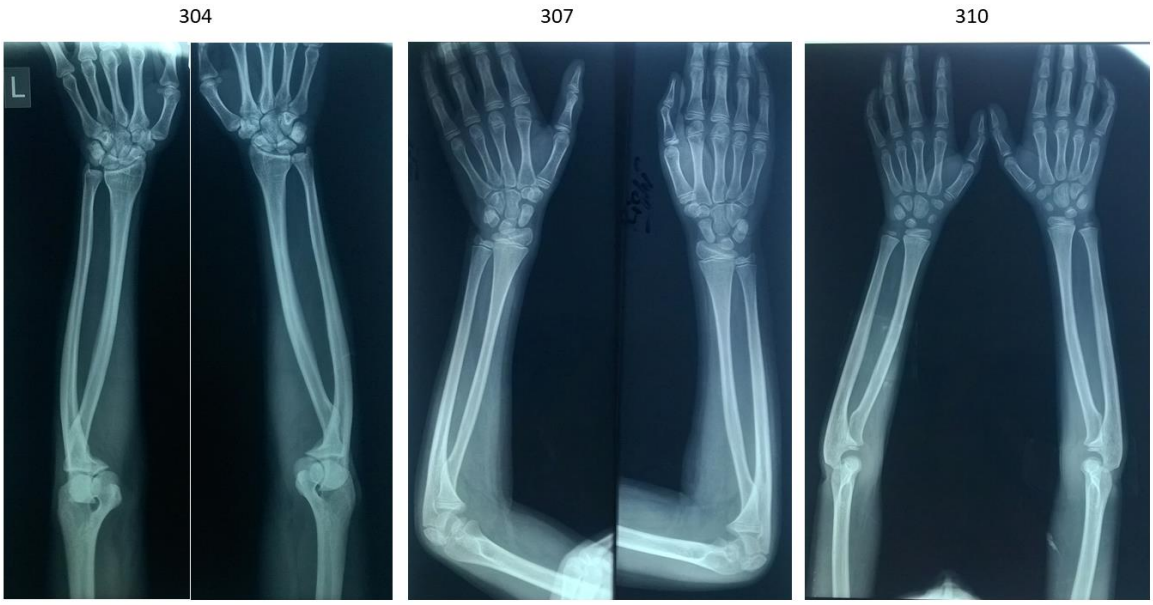


Fig. 7.3: Radiographic features in affected subjects 304, 310, and 401.

There was delayed bone age, lack of ossification at long bones, hypoplastic elbow joints, and dysplastic distal head of humeri, and hypoplastic carpals and metacarpals.

7.4.3.3: Patient 310 and 314

Patient 310 and 314 were male siblings who were 12 and 9-years old, respectively. They had a relatively normal life and were dependent on thyroxin tablets and its dosage increasing with the passage of time. The physical examination of both patients showed that they had a similar presentation of the phenotype. The craniofacial and musculoskeletal symptoms were not found except for sequent eyes (Fig. 7.2). Reportedly, the patients frequently fell when walking or running. Thyroxin tablet withdrawal symptoms included lethargy, unbalanced body posture, swelling on neck, fever, irritation, and oversleeping. Out of these two sibs, patient 310 underwent a sonographic evaluation that indicated the mild hypertrophy of the right lobe. However, no mass/cyst was observed, and blood flow

was normal in the associated vessels. His radiographic examination showed delayed bone age, lack of long bones ossification, hypoplastic elbow joints, dysplastic humeri distal heads, and hypoplastic carpal/metacarpals (Fig. 7.3).

7.4.3.4: Patient 401

Patient 401 was a 7-year old female and her ultrasonic examination showed normal thyroid gland, although she showed a wide range of thyroxin withdrawal features like neck swelling, lethargy, abdomen swelling (Fig. 7.2), tongue thickness, fever and irritation. Her developmental milestones were unremarkable, although her speech was delayed. Similarly, the behavioural attitude was very normal except the hyperactivity.

Table 7.3: Summary of thyroid gland ultrasonography and hand radiography

Subjects	**303**	**304**	**307**	**310**	**314**	**401**
Age at examination	23	20	15	12	9	7
Clinical features						
Thyroid gland ultrasonography						
Thyroid gland hypertrophy	+	+	+	+	+	−
Spongy appearance of thyroid gland	?	+	+	+	+	−
Cyst/abnormal tissue mass presence	+	+	+	−	+	−
Abnormal blood flow associated vessels	?	+	−	−	−	−
Using Thyroxin tablet since ages (in yrs.)	0.5	1.5	14	1.5	1.5	1.5
Dependent on Thyroxin tablet	++	++	++	++	+	+
Thyroxin tablets withdrawal symptoms						
Swelling of abdomen	+	+	?	?	?	+
Laziness/lethargy	+	+	+	+	+	+
Neck swelling/conditional goiter	+	+	+	+	+	+
Tongue thickness	−	−	−	−	−	+
Fever	+	+	+	+	+	+
Irritation/uneasiness	+	+	+	+	+	+
Upper limb radiographic findings						
Delayed bones age	?	+	+	+	?	?
Lack of long bones ossification	?	+	+	+	?	?
Hypoplastic elbow joints	?	+	+	+	?	?
Dysplastic humeri distal heads	?	+	+	+	?	?
Underdeveloped wrist joint	?	+	+	+	?	?
Hypoplastic carpals/metacarpals	?	+	+	+	?	?
Radio-ulna malformed widely spaced	?	+	+	+	?	?

+, feature present; ++, severe phenotype; −, feature absent; ?, not ascertained

7.4.4: Linkage analyses and homozygosity mapping

There were 26 autosomal intervals with a LOD score of ≥1.4 (Table 7.4). All of those regions were physically inspected for homozygosity through MS Excel. Regions yielding low LOD scores were excluded. Hence, there were nine regions having a size of >3Mb and LOD score >3.5. Those regions were further closely scrutinized for the presence of genes that might be relevant to the phenotype. For this purpose, all of the regions were checked through Morbid Map (https://www.omim.org/search/advanced/geneMap) and GeneDistiller (www.genedistiller.org) online resources (Table 7.5; 7.6).

Table 7.4: Summary of linkage analyses and homozygosity mapping in Family 7

Chromosome	Start position	End position	Size(Mb)	Lod score	Remarks
2	18674	3433368	34.1	3.55	Ok
3	40535809	40885879	3.5	3.55	Ok
4	136764416	137408872	6.4	3.55	Ok
7	16731253	17522503	7.9	3.55	Ok
8	124068128	124411678	3.4	3.54	Ok
11	105503658	105923373	4.2	3.55	Ok
14	87518037	88063317	5.5	3.55	Ok
15	86720146	87119340	4.0	3.55	Ok
17	17970229	18855611	8.9	3.55	Ok
12	33985843	39229699	52.4	3.55	Not homozygous
6	143007165	143077232	0.7	3.25	Small region
21	30890894	31195150	3.0	3.18	Low lod score
18	51584229	52528849	9.4	3.03	Not homozygous
10	32610703	33277782	6.7	2.72	Not homozygous
1	34525105	39054557	45.3	2.68	No homozygosity at that region
13	89230871	95000735	57.7	2.24	Not homozygous
5	166778493	174638216	78.6	1.65	Low lod score
19	37325826	38510256	11.8	1.57	Low lod score
9	138447855	141066491	26.2	1.55	Heterozygous for one of the affecteds
11	120628012	121201899	5.7	1.49	Low lod score
16	84005551	84634435	6.3	1.40	Low lod score

Table 7.5: Results of MorbidMap and GeneDistiller for the nine candidate regions prioritized homozygosity mapping

Start	**Stop**	**MIM Number**	**GeneID / Disease**
#Chromosome: 2			
2p25	2p22	602134	2112 Tremor, hereditary essential, 2
2p25	2p24	607329	387575 Hypertension, essential, susceptibility to, 3, 145500
2p25	2p25	609402	780908 Preeclampsia/eclampsia 2
2p25.3	2p25.3	603658	6201 Diamond-Blackfan anemia 8, 612563
2p25.3	**2p25.3**	**606765**	**7173 Thyroid dyshormonogenesis 2A, 274500**
#Chromosome: 3			
3p	3p	607135	261727 Creatinine clearance QTL
3p25	3p22	607893	574048 Ovarian cancer, susceptibility to
3p24	3p21	609954	100188800 Asperger syndrome susceptibility 4
3p24	3p21.2	609649	94014 Trichilemmal cyst 1
3p24	3p22	608088	378888 Neuropathy, hereditary sensory, type IB
3p23	3p21	182280	7864 Small-cell cancer of lung
3p22	3p21	610019	780918 Cataract, autosomal recessive congenital 2
3p22.2	3p21.32	255160	619511 Myopathy, hyaline body
3p22.1	3p22.1	610819	54977 Anemia, sideroblastic, pyridoxine-refractory, autosomal recessive, 205950
3p22.1	3p22.1	116806	1499 Colorectal cancer; Hepatoblastoma; Pilomatricoma, 132600; Ovarian cancer, 167000; Hepatocellular carcinoma, 114550
#Chromosome: 4			
4	4	151450	7895 Neutropenia, neonatal alloimmune
4p16	4q34	603783	100462721 Intelligence QTL1
4q	4q	601454	7889 Psoriasis susceptibility 3
4q	4q	603664	50979 Mental health wellness-2
4q	4q	610430	100188811 Macroglobulinemia, Waldenstrom, susceptibility to, 2
4q21	4q31	608371	474387 Orofacial cleft 4
4q24	4q28	613340	100415904 Epilepsy, hot water, 2
4q28	4q31	111800	6420 Blood group, Stoltzfus system
#Chromosome: 7			
7p22	7p14	614021	100653371 Ventricular tachycardia, catecholaminergic polymorphic, 3
7p21.3	7p15.1	607454	170545 Spinocerebellar ataxia 21
7p21	7p15	153880	1541 Macular dystrophy, dominant cystoid
7p21.2	7p14.3	613576	100505394 Ectodermal dysplasia-syndactyly syndrome 2
7p21.1	7p21.1	601622	7291 Saethre-Chotzen syndrome, 101400; Saethre-Chotzen syndrome with eyelid anomalies, 101400; Craniosynostosis, type 1, 123100
#Chromosome: 8			
8	8	608392	378427 Autoimmune disease, susceptibility to, 3
8q	8q	606789	171515 Fetal hemoglobin quantitative trait locus 4
8q	8q	600668	882 Chondrocalcinosis with early-onset osteoarthritis
8q23	8q24	140300	140805 Hashimoto thyroiditis

8q23	8q24	611376	100126595 Mungen syndrome
8q24	8q24	611469	100188841 Colorectal cancer, susceptibility to, 2
8q24	8q24	612113	100188883 Bone mineral density QTL 10
8q24	8q24	600669	1957 Epilepsy, idiopathic generalized, susceptibility to, 1
8q24	8q24	600131	50966 Epilepsy, childhood absence, 1
8q24	8q24	601068	50968 Epilepsy, myoclonic, benign adult familial
8q24	8q24	611100	100188834 Prostate cancer, hereditary, 10
8q24	8q24	610649	100188815 Bone size quantitative trait locus 3
8q24.13	8q24.22	612448	100233147 Age-related hearing impairment 1
8q24.13	8q24.13	610657	9897 Spastic paraplegia-8, 603563
8q24.13	8q24.13	603046	11236 Renal cell carcinoma, 144700
#Chromosome: 12			
12p13.2	12q24.1	601458	3378 Inflammatory bowel disease 2
12p13.2	12p11.23	610143	692220 Deafness, autosomal recessive 62
12p12.3	12p12.3	110600	420 Blood group, Dombrock
12p12.3	12p12.3	154870	4256 Keutel syndrome, 245150; Natural teeth remaining intact
12p12.3	12p12.3	601190	5149 Retinal cone dystrophy 3, 610024
#Chromosome: 14			
14	14	608251	404684 Phobia, specific
14q	14q	138800	4333 Goiter, multinodular, 1
14q	14q	213600	23706 Basal ganglia calcification, idiopathic
14q24.3	14q31	601208	3410 Diabetes mellitus, insulin-dependent, 11
14q31	14q31	275000	100312954 Graves disease, susceptibility to, 1
14q31.3	14q31.3	606890	2581 Krabbe disease, 245200
14q31.3	14q31.3	608132	123016 Bardet-Biedl syndrome 8, 209900; Retinitis pigmentosa 51, 613464
14q31.3	14q31.3	609868	55812 Leber congenital amaurosis 3, 604232; Retinitis pigmentosa, juvenile, autosomal recessive, 268000
#Chromosome: 15			
15q	15q	214900	84565 Cholestasis-lymphedema syndrome
15q	15q	604329	50986 Hypertension, essential, susceptibility to, 2, 145500
15q23	15q26.3	607248	338030 Glioma susceptibility 4
15q24	15q25	612274	100190786 Ciliary dyskinesia, primary, 8
15q25	15q26	606451	23719 Deafness, autosomal dominant 30
15q25.1	15q26.1	607728	353147 Porokeratosis, disseminated superficial actinic, 2
15q25.3	15q26.2	608691	431709 Major depressive disorder 2, 608516
15q25.3	15q26.1	609893	780915 Hypothyroidism, congenital, nongoitrous, 3
15q25.3	15q25.3	613624	9640 Spinocerebellar ataxia, autosomal recessive 5, 606937
15q26	15qter	612626	100271921 Chromosome 15q26-qter deletion syndrome
15q26	15q26	600318	3402 Diabetes mellitus, insulin-dependent, 3
15q26.1	15qter	166800	5012 Otosclerosis 1
15q26.1	15q26.1	174763	5428 Progressive external ophthalmoplegia, autosomal recessive, 258450; Progressive external ophthalmoplegia, autosomal dominant, 157640; Mitochondrial DNA depletion syndrome 4B (MNGIE type), 613662; Mitochondrial DNA depletion syndrome 4A (Alpers type), 203700; Mitochondrial recessive ataxia syndrome (includes SANDO and SCAE), 607459

15q26.1	15q26.1	180090	6017 Fundus albipunctatus, 136880; Retinitis punctata albescens, 136880; Newfoundland rod-cone dystrophy, 607476; Bothnia retinal dystrophy, 607475
15q26.1	15q26.1	142340	1732 Hernia, congenital diaphragmatic 1
15q26.1	15q26.1	155760	176 Spondyloepiphyseal dysplasia, Kimberley type, 608361; Spondyloepimetaphyseal dysplasia, aggrecan type, 612813; Osteochondritis dissecans, short stature, and early-onset osteoarthritis, 165800
15q26.1	15q26.1	604610	641 Bloom syndrome, 210900
15q26.1	15q26.1	605195	145873 Spondylocostal dysostosis, autosomal recessive 2, 608681
15q26.1	15q26.1	147650	3418 D-2-hydrosyglutaric aciduria 2, 613657
15q26.1	15q26.1	170290	5346 Lipodystrophy, familial partial, type 4, 613877
15q26.1	15q26.1	608552	26276 Arthrogryposis, renal dysfunction, and cholestasis 1, 208085
15q26.1	15q26.1	607131	260403 Macrocephaly with multiple epiphyseal dysplasia and distinctive facies
15q26.1	15q26.1	611254	374654 Hydrolethalus syndrome 2, 614120; Acrocallosal syndrome, 200990
15q26.1	15q26.1	611360	55215 Fanconi anemia, complementation group I, 609053

Table 7.6: Genes falling in the candidate intervals on chromosome 2 and 12

Start	Stop	Symbol	Strand	Interval
#Chromosome: 2				
38814	47042	*FAM110C*	-	28813..37041
218136	264820	*SH3YL1*	-	208135..254819
264869	278283	*ACP1*	+	254868..268282
279561	289002	*FAM150B*	-	269560..279001
290342	292920	*LOC101927262*	+	280341..282919
490771	492687	*LOC727944*	-	480770..482686
495265	496631	*LOC100996637*	+	485264..486630
545804	546667	*LOC101927362*	+	535803..536666
667793	677439	*TMEM18*	-	657792..667438
779837	864112	*LINC01115*	-	769836..854111
895902	901136	*LOC101060385*	-	885901..891135
945320	947797	*LOC101060391*	-	935319..937796
946554	1371401	*SNTG2*	+	936553..1361400
1407131	1417213	*LOC101927426*	+	1397130..1407212
1417233	**1547445**	***TPO***	**+**	**1407232..1537444**
1635659	1748291	*PXDN*	-	1625658..1738290
1708632	1712533	*LOC101927466*	-	1698631..1702532
1792885	2335147	*MYT1L*	-	1782884..2325146
1828217	1833705	*LOC101927493*	+	1818216..1823704
2323004	2330880	*MYT1L-AS1*	+	2313003..2320879
2874330	2875003	*LOC101927534*	-	2864329..2865002
2898820	3129798	*LINC01250*	-	2888819..3119797
3192741	3381653	*TSSC1*	-	3182740..3371652
3383446	3483342	*TRAPPC12*	+	3373445..3473341
#Chromosome: 12				
18233803	18243139	*RERGL*	-	10993926..11003262
18395912	18801352	*PIK3C2G*	+	11156035..11561475
18511377	18511901	*NDFIP1P1*	-	11271500..11272024
18646009	18646719	*ZKSCAN7P1*	+	11406132..11406842
18836110	18891020	*PLCZ1*	-	11596233..11651143
18845685	18847229	*PSMC1P9*	+	11605808..11607352

* Homo sapiens Genome (Annotation Release 105)

The homozygous region on chromosome 2 emerged as the second largest interval and it harbored loci associated with thyroid dyshormonogenesis, which could be a good candidate for the phenotype in the family.

In the exome data, variants were filtered through a standard scheme. Variants having an allele frequency of >0.001 in any of the publicly available databases or heterozygous, or read quality <30 were eliminated. Variants, with a very low allele frequency, that were homozygous and causing potentially pathogenic effect were retained. In the second round, those variants that fell in the homozygous regions identified through SNP homozygosity mapping were retained. This scheme led to the identification of homozygous nonsynonymous variant c.719A>G in exon 6 of *TPO* gene as a candidate variant. Later, for mutation validation, we performed Sanger sequencing on DNA and found a transition mutation in which adenine was replaced by guanine in exon 6 of *TPO* (Fig. 7.4).

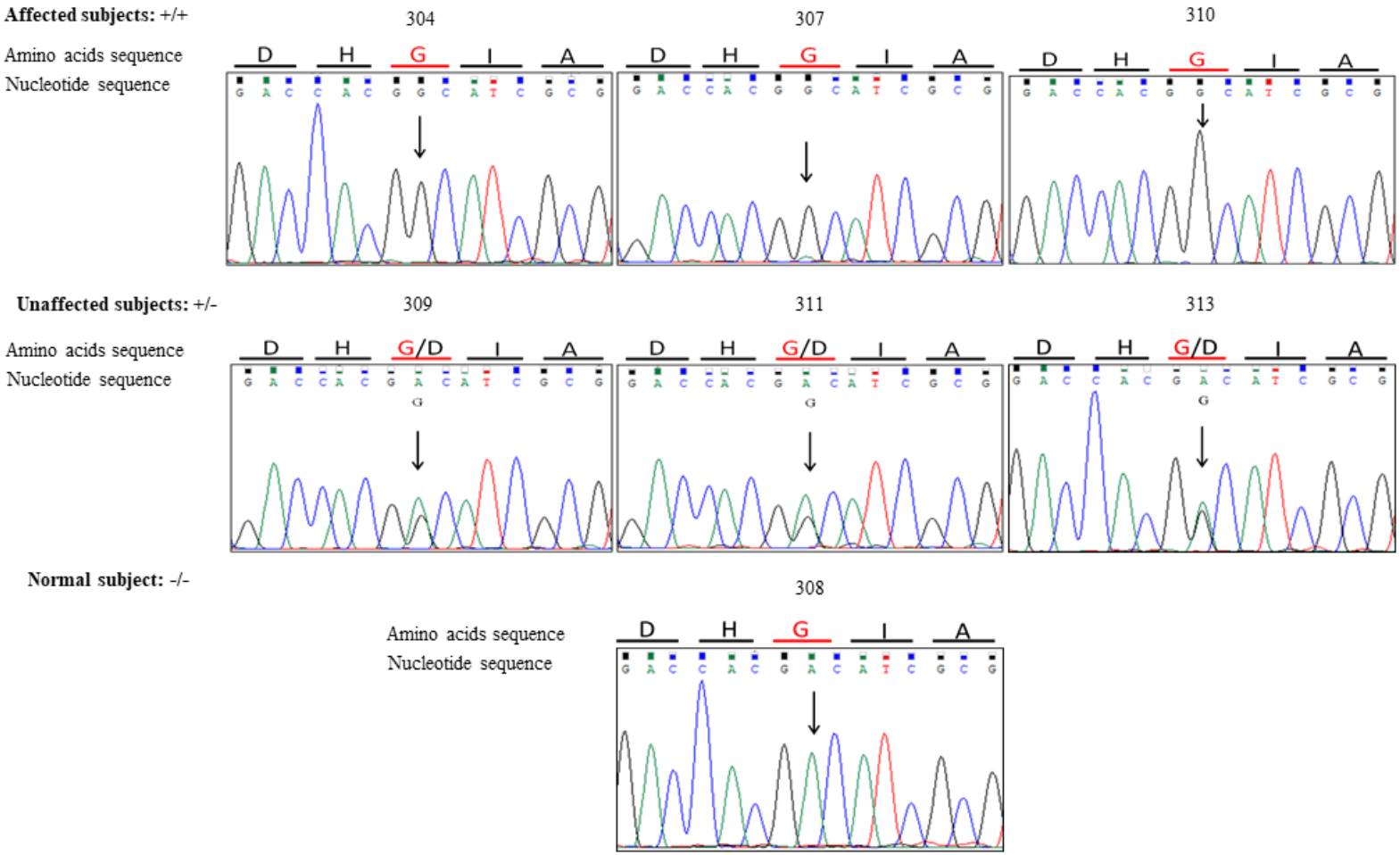


Fig. 7.4: Chromatograms showing the variant in homozygous state (upper panel), in heterozygous state (middle panel) and the normal variant (lower panel)

7.5: Discussion

CH is a treatable cause of preventable ID. Therefore newborn screening to prevent mental retardation (MR) at an early stage of life is necessary before the onset of any significant abnormality in the children (Zhao *et al.* 2016a). Moreover, another cause that affects the fetal cognitive development is maternal hypothyroidism which is most prevalent cause of treatable CH. During pregnancy, normal level of THs are necessary for proper development of the fetal central nervous system and thyroid gland to avoid the preventable MR (Ahmed 2015, 2018b; Ying *et al.* 2018). Similarly in accordance with worldwide strategies to detect CH in neonates at birth, it is not possible do so until 3 months of neonatal age due to residual THs functions and trans-placental passage of maternal THs that offer temporary protection against the setting of mental retardation (Ford & LaFranchi 2014). For these reasons, public health programs need to educate parents and health care providers about the early diagnosis and treatment of CH (Nasheeda *et al.* 2018).

About 70% of the Pakistani population faces the problem of iodine deficiency disorders (Malik & Butt 2008), and there are few studies that describe the high prevalence rate (1/1000) of CH in Pakistan (Lakhani *et al.* 1989). In addition, more studies are required for the molecular screening of CH in the general population (Alam Khan *et al.* 2002).

Herein this study reported large kindred of autosomal recessive CH due to a homozygous mutation in the *TPO* gene. The identified mutation is likely to cause a high level of TSH with a low level of thyroid hormones (T3 and T4), conditional goiter (due to lack of thyroxin), and cognitive and motor impairments in patients. The same clinical findings were reported due to an intragenic deletion of five exons (11-15) in *TPO* in another Pakistani family that was originated from the same region of the Punjab province (Iqbal *et al.* 2012). The compromised intellectuality due to mental retardation in the affected subjects is

supported by Harripaul et al. (2018), who reported three mutations in the TPO gene in families with autosomal recessive IDs (Harripaul *et al.* 2018). Similar findings were also reported in the previously conducted studies in Pakistan (Attaullah *et al.* 2016; Cielonko *et al.* 2016). The patients were dependent on thyroxin to live physiologically active lives and dosages of thyroxin were increased with the passage of time. Therefore, thyroxin dosage ranged from 1- 6 tablet per-day since early ages. Hence, the patients reported thyroxin tablets withdrawal symptoms that included neck swelling (conditional goiter), lethargy, abdomen swelling, tongue thickness, oversleeping, and fever.

On the bases of ultrasonography and biochemical tests, the obtained clinical investigations in the family showed that the hypertrophy of the thyroid gland and low levels of thyroid hormones increased with the passage of times for the affected subjects. Moreover, two patients (307 and 312) in the family did not get the treatment at early stages of their life and became victims of permanent mental retardation. Such findings were also reported in German-Thai patient (Park & Chatterjee 2005; Sriphrapradang *et al.* 2016).

At the molecular level, this study detected the 2nd novel mutation c.719A>G (p.D240G) in exon 6 of *TPO*, and the genetic change was found by both NGS and traditional Sanger sequencing. These findings were supported by another compound heterozygous mutation c.614G>A (p.R175Q) that was reported in the exon 6 of *TPO* gene in two Japanese siblings with CH (Kotani *et al.* 2004).

This is the fourth reported mutation in the *TPO* gene in Pakistani population, with previous mutations being were reported in earlier studies (Iqbal *et al.* 2012; Harripaul *et al.* 2018). By contrast, eleven autosomal recessive Pakistani families were screened for mutational analysis but mutations were found in the *TSHR* gene (14q31.1) that caused congenital hypothyroidism non-goitrous 1 (Ahlbom *et al.* 1997). In support of our findings,

the reported mutation was found at nucleotide position at 1457481 (c.719) in exon 6. There is no available record of the mutation/variant in ExAC Browser Beta for this position (http://exac.broadinstitute.org/gene/ENSG00000115705). Additionally, the identified mutation was a nonsynonymous missense mutation (p.D240G) in which aspartic acid was replaced by glycine at amino acid level. A similar mutation was found in exon 12 (p.D680G) of the gene from the African population.

The *TPO* gene product metabolizes hydrogen peroxide which is produced during synthesis of thyroid hormones, and the products of this metabolism are involved in the organification of the thyroid gland (Zheng *et al.* 2017). Thyroid hormones synthesis involves complex polygenic pathways that are regulated by different genes, and most of these pathways became dysregulated by mutations in the TPO gene (Hashemipour *et al.* 2012).

While more than 100 mutations have been reported in the *TPO* gene, and their genotypic-phenotypic correlation is still unclear (Fu *et al.* 2016). The study findings provide supporting information for the establishment of the phenotype-genotype correlation by taking account the spectrum of mutations in genes related to CH. However, we were unable to detect maternal hypothyroidism and also did not detect parathyroid hormones in the patients as hypothyroidism, which is also associated with the insufficient amount of parathyroid hormones (Mantovani *et al.* 2017).

In conclusion, the results of this study indicate that early treatment can prevent the chances of ID in patients with CH and improve mitigate their symptoms. Additionally, improved understanding of abnormal thyroid gland functions during early infancy will help us to control mental retardation in the general population.

Chapter 8

8: References

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List of Publications

1. Afzal M, Zaman Q, Kornak U, Mundlos S, Malik S, Flöttmann R. (2017). Novel splice mutation in LRP4 causes severe type of Cenani-Lenz syndactyly syndrome with oro-facial and skeletal symptoms. Eur J Med Genet; 60(8): 421-425.

2. Malik S, Ullah S, Afzal M, Lal K, Haque S (2014). Clinial and descriptive genetic study of polydactyly: a Pakistani experience of 313 cases. Clinc Genet; 85: 482-486.

3. Afzal M, Malik S, (2014). Longitudinal deficiency of upper limb: similar case presentation of two subjects with unilateral ulnar hemimelia, carpal and metacarpal deficiency, and oligodactyly. Asian Biomed; 8(4): 569-575.

4. Malik s, Afzal M (2013). Congenital terminal transverse deformity of upper limb: clinical and radiological in a sporadic care. JCPSP; 23(3): 219-220.

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Novel splice mutation in *LRP4* causes severe type of Cenani-Lenz syndactyly syndrome with oro-facial and skeletal symptoms

Muhammad Afzal [a], Qamar Zaman [a], Uwe Kornak [b, c, d], Stefan Mundlos [b, c, d], Sajid Malik [a, *], Ricarda Flöttmann [b, **]

[a] Human Genetics Program, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[b] Institut Fuer Medizinische Genetik und Humangenetik, Charité-Universitaetsmedizin Berlin, Berlin, Germany
[c] Max-Planck-Institut Fuer Molekulare Genetik, FG Development & Disease, Berlin, Germany
[d] Berlin-Brandenburg Center for Regenerative Therapies, Charité-Universitaetsmedizin Berlin, Berlin, Germany

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ABSTRACT

Cenani-Lenz syndactyly syndrome (CLSS; MIM-212780) is a rare autosomal recessive limb malformation characterized by complete osseous fusion of all fingers and toes, disorganization of phalangeal elements and severe shortening of the radius and ulna. It is occasionally associated with renal hypoplasia, oro-facial defects, scoliosis of the thoracic spine, hearing loss, and genital anomalies. Here we describe a consanguineous Pakistani kindred with a severe form of CLSS characterized by complete syndactyly and disorganization of fingers, oligo-syndactyly of toes, shortening of limbs, frontal bossing, and hypoplasia/agenesis of left kidney. The affected individuals were additionally presented with short stature, cleft-lip and hypoplastic shoulder joint with restricted upper limb movement. A novel splice variant in *LRP4* (c.316+1G > A) segregated with the phenotype in a five generations family. The mutation is predicted to add 29 non-native amino acids with a premature termination, resulting in approximately 90% length reduction of the wild-type transcript. These findings not only further expand the phenotypic variability of CLSS but also indicate that early truncated and loss-of-function mutations in LRP4 lead to a more severe CLSS phenotype.

1. Introduction

Cenani-Lenz syndactyly syndrome (CLSS; MIM-212780) is a rare autosomal recessive limb malformation initially described by Turkish and German colleagues Cenani and Lenz (1967). It is characterized by complete osseous fusion of all fingers and toes, shortening of the radius and ulna with fusion, and disorganization of phalangeal development (Temtamy and McKusick, 1978). The feet are less severely affected. The phenotype is usually bilateral and symmetrical. Of all the syndactylies known it is one of the most severe types (Harpf et al., 2005; Malik, 2012).

Seven et al. (2000) reported a patient who, in addition to the typical CLSS symptoms, also exhibited scoliosis of the thoracic spine and mixed hearing loss. Temtamy et al. (2003) reported two probands with CLSS and mild facial dysmorphism. In one of the families a similarly affected sibling with genital anomalies and cleft palate was reported. Jarbhou et al. (2008) described a patient with CLSS with facial dysmorphism, hypoplasia of the kidney, hyperthyroidism, laryngomalacia, and congenital dislocation of hips; thus further expanding the phenotypic manifestation of CLSS.

Homozygous or compound heterozygous mutations in *LRP4* at chromosome 11p11.2 have been implicated in the majority of cases with CLSS (Li et al., 2010). In 3 unrelated consanguineous Turkish families, CLSS segregated with a homozygous 1585G-A transition in exon 13 of *LRP4*. In 2 unrelated consanguineous Egyptian families, a 409G-A transition in exon 4 was detected. Other cases with the recessive type of CLSS were observed with homozygosity for 547+1G-A transition in intron 6, a 479G-A in exon 5, 1345G-A transition in exon 12, and 1382A-C transversion in exon 12 of the *LRP4* (Li et al., 2010). CLSS phenotypes with dominant and recessive inheritance patterns have been shown to be caused by genomic rearrangements of *GREM1-FMN1* locus and mutation in *APC*, respectively (Dimitrov et al., 2010; Patel et al., 2015).

Herein, we report a novel splice-site mutation c.316+1G > A in

* Corresponding author.
** Corresponding author.
E-mail addresses: malik@qau.edu.pk (S. Malik), ricarda.floettmann@charite.de (R. Flöttmann).

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Short Report

Clinical and descriptive genetic study of polydactyly: a Pakistani experience of 313 cases

Malik S., Saifullah , Afzal M., Lal K., Haque S. Clinical and descriptive genetic study of polydactyly: a Pakistani experience of 313 cases. Clin Genet 2014: 85: 482–486. © John Wiley & Sons A/S. Published by John Wiley & Sons Ltd, 2013

Polydactyly, a common hereditary condition with additional digits in hands and/or feet, is a very attractive model to appreciate clinical and genetic heterogeneity. In order to get an insight into its phenotypic manifestations, we ascertained a cohort of 313 independent families with polydactyly from Pakistan; 35% cases turned out to be familial while 65% were sporadic. In majority of the index cases, polydactyly was presented as an isolated digit defect. Preaxial polydactyly types were 48.24% and postaxial were 51.8%. Familial polydactylies mainly had bilateral and symmetrical presentations, whereas sporadic cases were mostly unilateral and less often symmetrical. In the 313 index subjects a total of 508 limbs with additional digits were recorded. Variable expression was evident as the involvement of upper limbs was more common than the lower, right hand than the left, and left foot than the right. The present cohort establishes interesting epidemiological attributes of polydactyly in the Pakistani population and highlights its extraordinary clinical heterogeneity. Molecular analyses of this cohort are anticipated to elucidate novel genetic factors involved in the origin of additional digits in the growing limb and may provide clues to the role of stochastic factors in the etiology of phenotypic variability in polydactyly.

Conflict of interest

The authors declare that they have no conflict of interest.

S. Malik, S. Ullah, M. Afzal, K. Lal and S. Haque

Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan

Key words: descriptive epidemiology–genetic epidemiology – Pakistani population – phenotypic variability – polydactyly

Corresponding author: Dr Sajid Malik, Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University, 45320 Islamabad, Pakistan.
Tel.: +92 51 9064 3158;
fax: +92 51 260 1176;
e-mail: malik@qau.edu.pk

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Polydactyly, a non-traumatic hereditary condition with additional digits in hands and/or feet, is one of the most common congenital malformations (1). In terms of clinical variability, polydactyly is one of the most heterogeneous types of digit anomaly (2). A number of polydactylous conditions have been identified which differ on the basis of, first, presence in the hands and/or in feet; second, location of additional digit on the preaxial, mesoaxial or postaxial axes of the autopod; third, the extent of digit duplication either partial or complete; fourth, presence or absence of bony elements within the superfluous digit; fifth, sporadic or hereditary nature; and sixth, segregation patterns within the families. According to the Temtamy and McKusick classification (2), there are about 10 well-characterized non-syndromic polydactylous entities, in addition to approximately 300 syndromes in which polydactyly coexists (3). Milder polydactylies are less conspicuous and may be overlooked. However, as an indicator of concomitant dysmorphology syndrome, its presence in a neonate warrants a more comprehensive clinical examination.

Castilla et al. conducted detailed epidemiological and genetic studies of polydactyly and remarked considerable variations in its prevalence, phenotype, expressivity, transmission, and associated anomalies, suggesting a high order of heterogeneity in polydactyly (4). Medical literature is devoid of such data for the Asian

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Clinical report

Longitudinal deficiency of upper limb: similar case presentation of two subjects with unilateral ulnar hemimelia, carpal and metacarpal deficiency, and severe oligodactyly

Muhammad Afzal, Sajid Malik
Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University, 45320 Islamabad, Pakistan

Background: Longitudinal deficiency of upper limbs with oligodactyly is a very rare congenital malformation. It manifests itself as preaxial or postaxial hypoplasia/aplasia of long bones accompanied by reduction of palm and phalanges.
Objective: To report two cases with essentially similar phenotypic presentation characterized by unilateral mesomelic shortening of limb, ulnar hypoplasia, and severe deficiency of skeletal elements of hand that were found in unrelated individuals.
Methods: Review of clinical and family history, phenotypic examination, physical and radiological investigations, and literature review.
Results: In both individuals, the right arm was short, the size of the middle arm and hand being dramatically reduced in size, and the hand comprising only two functional digits. Roentgenograms revealed hemimelia/dysmelia of the ulna, hypoplasia of radius, dysplastic distal radial head, and several missing carpals. Only two phalangeal rays were witnessed in the hand. Radiographic measurements showed a normal contralateral arm and lower limbs, and no other associated symptoms. These phenotypes were classified as type I and type D according to the schemes proposed by Swanson et al., and Ogino and Kato, respectively. Both individuals were the product of third degree consanguineous unions ($F = 0.0625$).
Conclusion: Consistent phenotypic pattern of longitudinal limb anomalies evident in two independent subjects suggest a common underlying genetic etiology. There is currently no known genetic factor to allow molecular testing and risk estimation for family members. Isolated limb anomalies may provide important clues to understand pathomorphogenetic mechanisms that lead to the disruption of normal limb development.

Keywords: Finger reduction, longitudinal limb deficiency, oligodactyly, Pakistani subjects, ulnar hemimelia

Ulnar hemimelia is a postaxial longitudinal deficiency of the upper limb wherein the ulna is completely or partially absent. The elbow joint may be in extension or in acute flexion [1]. If the deficiency is incomplete, the ulnar remnant may vary in length and contour. The number digits of the hand may be reduced. At the shoulder girdle, one may observe considerable muscular atrophy and ligamentous relaxation [1].

Longitudinal postaxial defects are very rare (prevalence of 8/10,000) [2]. The majority of the cases reported in medical literature are unilateral and sporadic. Similar clinical presentation in several instances may necessitate an underlying genetic nature of these defects. In this clinical report, we present two cases in unrelated individuals depicting remarkably similar phenotypes, i.e., unilateral longitudinal deficiency of right arm, ulnar hemimelia, deficiency of carpal and metacarpal, and severe oligodactyly.

Correspondence to: Dr. Sajid Malik, Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University, 45320 Islamabad, Pakistan. E-mail: malik@qau.edu.pk

Case studies

Two unrelated individuals come from Southern Punjab, Pakistan. Both individuals were male and belonged to a *Baloch* tribe, and were engaged in manual jobs. Family histories were devoid of any hereditary anomaly, and reportedly the pregnancies and birth events had been unremarkable. In both cases,

CASE REPORT

Ulnar aplasia, dysplastic radius and preaxial oligodactyly: Rare longitudinal limb defect in a sporadic male child

Sajid Malik, Muhammad Afzal
Human Genetics Program, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Islamabad, Pakistan

Ulnar hypoplasia is a rare longitudinal limb deficiency in which the ulna shows various degrees of deficiency. The condition is normally associated with radial defects, and in severe cases there is a reduction of postaxial/ulnar digits. Ulnar deficiency is an integral part of several syndromic malformations like Weyer's oligodactyly syndrome, limb/pelvis hypoplasia/aplasia syndrome, and ulnar-mammary syndrome. Here, we report an isolated unilateral ulnar deficiency in a boy who was a product of a consanguineous marriage. The subject demonstrated mesomelic shortening of the left arm with reduced zeugopod and autopod, and preaxial absence of two fingers. Additional findings in the affected limb were severe flexion contracture at the elbow joint, reduced and narrow palm, hypoplastic digits, and clinodactyly. Roentgenographic study revealed rudimentary ulna, dysplastic and posteriorly dislocated radius, crowding of carpals, and complete absence of digit rays of the thumb and index finger. Despite this anomaly, the subject could manage his daily life activities well. We present detailed clinical features and differential diagnosis of this rare limb malformation.

Key words: Finger reduction, longitudinal defect, limb deficiency, oligodactyly, Pakistani subject, radial dysplasia, ulnar aplasia

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INTRODUCTION

Ulnar hypoplasia/aplasia is a longitudinal deficiency at the posterior axis of the upper limb wherein the ulna is partially or completely absent. If the deficiency is incomplete, the ulnar remnant may vary in length and contour. The elbow joint may be in extension or in acute flexion.[1,2] At the shoulder girdle, one may observe considerable muscular atrophy, ligamentous relaxation, and a deep web in the axilla.[1,2] This condition may accompany hypoplasia of the radius. O'Rahilly proposed an anatomical classification of limb anomalies and expanded the concept of intercalary deficiencies.[2] Additionally, oligodactyly is a common presentation and the number of absent digits varies greatly. Depending upon the severity of longitudinal deficiency at the ulnar or radial ray in the zeugopod, the postaxial or preaxial fingers could be omitted, respectively. However, ulnar deficiency with the absence of preaxial digits is extremely rare and has not been much appreciated.[3] In this communication, we present a male child, a product of a consanguineous marriage, with a rare association of ulnar hypoplasia and the absence of two preaxial fingers, the remaining digits depicting postaxial/posterior identities.

CASE REPORT

The subject, a 12-year-old school-going boy, originates from a rural area of Southern Punjab, Pakistan. His parents were first cousins (inbreeding coefficient, $F = 0.0625$), and he had four normal siblings (3 brothers, 1 sister). The maternal and paternal ages were 28 and 33 years, respectively, at the time of his birth. The pregnancy had been uneventful and the birth was at home, in the presence of a traditional birth attendant. The study was approved by the institutional review committee and all the information was obtained according to the Helsinki II declaration. The initial ascertainment and detailed clinical examinations were carried out in several visits during 2009-2010.

The subject had normal developmental landmarks, dentition, and intelligence quotient (IQ). He was observed to have an isolated limb anomaly. Upon physical examination, he had a standing height of 146 cm, sitting height 71 cm, arm span 130 cm, head circumference 53 cm, neck circumference 29 cm, and chest 68 cm. There was no family history of any limb or other anomaly.

Address for correspondence: Dr. Sajid Malik, Human Genetics Program, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, 45320 Islamabad, Pakistan. E-mail: malik@qau.edu.pk
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CASE REPORT

Congenital Terminal Transverse Deformity of Upper Limb: Clinical and Radiological Findings in a Sporadic Care

Sajid Malik and Muhammad Afzal

Abstract

Congenital transverse limb anomalies are rare, which affect upper and/or lower limbs and may accompany several syndromic malformations. We present a sporadic male subject with congenital, unilateral transverse arrest of the left hand. The affected arm was observed to be short with reduced zeugopod and truncated palm. Fingers were represented by five bead-like nubbins. Roentgenographic examination revealed short radius and ulna with hypoplastic distal heads, absent carpals/metacarpals, and a hypoplastic bony island in each nubbin. Consanguinity was denied, and the subject had no symptoms in the orofacial, neurological and skeletal systems. Detailed clinical data with literature survey is presented.

Key words: *Transverse limb defect. Congenital hand amputations. Finger reduction. Finger nubbins. Pakistani subject.*

INTRODUCTION

Temtamy and McKusick defined terminal transverse limb defects as absence or hypoplasia of distal structures of limbs with more or less normal proximal structures.[1] It is a rare, congenital deficiency which manifests itself as an abrupt truncation through the transverse axis of limb and produces an amputation like stump.[2] The truncation through the hand may involve only certain phalanges, only the digits or the full hand. The malformation may be unilateral or bilateral and may affect upper limbs and/or lower limbs. Most of the cases reported are sporadic and isolated, however, the condition may appear as a part of syndrome.[3] The genetic bases of isolated terminal limb deficiencies are not well understood.

Here, we present a sporadic male subject with an isolated, congenital, unilateral terminal reduction deformity of the left hand.

CASE REPORT

The index subject, a 47 years old male, belonged to a rural area of the Southern-Punjab. He is self-employed as a kitchen chef and has adequately adapted to perform his routine activities. The subject is the third in a sibship of 6 individuals (3 brothers, 3 sisters) and has 5 normal children (3 boys, 2 girls). Parental consanguinity was denied.

The left arm of the subject was short and lacked a proper formed hand, the forearm abruptly ending in a stump (Figures 1A and 1B). The hand was amputated through the palm. Five bead-like finger remnants were attached through cutaneous bridges to the distal rim of reduced palm. They appeared as non-functional, under-developed nubbins and harbored nails at their dorsum (Figures 1A and 1B). The amputated autopod had compromised extension and flexion movements.

In the X-ray, left radius and ulna were observed to be short (Figures 1C and 1D, Table I). Their distal heads gave evidence of hypoplasia. Particularly, the radius was thin and its distal extremity revealed retarded growth and decalcification. A bony synostosis of dysplastic epiphyses covered the distal heads of radius and

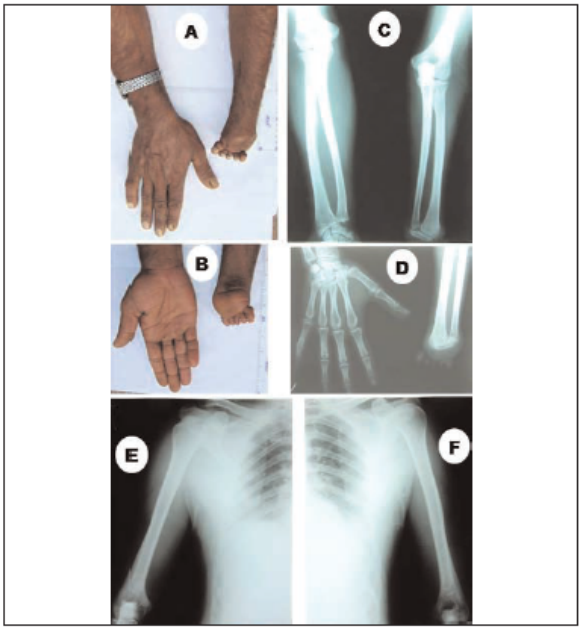

Figure 1: Limb phenotype in the subject. A-F: Photographs and radiograph of upper limbs.

Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University, Islamabad.

Correspondence: Dr. Sajid Malik, G-II/2, House No. 677, Street 61, Islamabad.
E-mail: malik@qau.edu.pk

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CLINICAL REPORT

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A Novel ZRS Mutation in a Balochi Tribal Family With Triphalangeal Thumb, Pre-Axial Polydactyly, Post-Axial Polydactyly, and Syndactyly

Julia E. VanderMeer,[1,2†] Muhammad Afzal,[3†] Saadia Alyas,[3] Sayedul Haque,[3] Nadav Ahituv,[1,2*] and Sajid Malik[3**]

[1]Department of Bioengineering and Therapeutic Sciences, University of California San Francisco, San Francisco, California

[2]Institute for Human Genetics, University of California San Francisco, San Francisco, California

[3]Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University Islamabad, Islamabad, Pakistan

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Limb malformations are one of the most common types of human congenital malformations. Mutations in the ZRS enhancer of Sonic Hedgehog are thought to be responsible for pre-axial polydactyly in multiple independent families. Here, we describe a large Balochi tribal family from Southern Punjab, Pakistan, with a variable set of limb malformations and a novel ZRS mutation. The family has a limb phenotype characterized by triphalangeal thumb, pre-axial polydactyly, and post-axial polydactyly. There is also a high degree of phenotypic heterogeneity with less common clinical findings in the affected family members that include osseous syndactyly of forth–fifth fingers, clinodactyly, hypoplasia of mesoaxial fingers, and bifid halluces. The presentation in most of the affected patients was bilateral and symmetrical. A heterozygous C>A mutation at position 287 of the ZRS enhancer (chr7:156,584,283; hg19) was detected in all affected subjects and is absent from four unaffected family members, 42 unrelated samples, and multiple databases of human variation. Combined, these results identify a novel ZRS287 C>A mutation which leads to a variable spectrum of limb phenotypes. © 2012 Wiley Periodicals, Inc.

Key words: polydactyly; triphalangeal thumb; syndactyly; ZRS

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INTRODUCTION

Pre-axial polydactyly is caused by disruptions to the developmental patterning of the limb along the anterior–posterior (AP; thumb to pinky) axis that lead to changes in digit number and identity. The AP axis is specified by a small population of cells in the posterior limb bud that form the zone of polarizing activity (ZPA). These cells express the gene Sonic Hedgehog (*SHH*; OMIM *600725) which defines the posterior side of the limb. The expression of *SHH* in cells of the ZPA is controlled by a long range *cis*-regulatory enhancer called the ZPA regulatory sequence (ZRS; OMIM *605522). The ZRS is located nearly 1 megabase away from *SHH*, within intron 5 of the limb region 1 homolog (*LMBR1*; OMIM *605522) gene. This enhancer is required for *Shh* expression in the limb and is highly conserved from humans to fish [Lettice et al., 2003; Sagai et al., 2005]. Mutations in the ZRS have been shown to cause pre-axial polydactyly in many animals including mice, dogs, cats, chickens, and humans [reviewed in VanderMeer and Ahituv, 2011].

In humans, 13 different point mutations and 10 duplications involving the ZRS have been shown to cause human limb malformations [VanderMeer and Ahituv, 2011]. Large duplications that encompass the ZRS and its surrounding sequence usually cause complex Haas-type polysyndactyly (webbing between digits and the presence of extra digits) and point mutations in the ZRS have

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†Julia E VanderMeer and Muhammad Afzal contributed equally to this work.
*Correspondence to:
Nadav Ahituv, 513 Parnassus Ave, HSE 901H, Box 0794, San Francisco, CA 94143. E-mail: nadav.ahituv@ucsf.edu
**Correspondence to:
Sajid Malik, Human Genetics Program, Department of Animal Sciences, Quaid-i-Azam University Islamabad, 45320 Islamabad, Pakistan.
E-mail: malik@qau.edu.pk
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