Consanguinity, prevalence pattern of hereditary malformations, and genetic analyses of rare anomalies segregating in families from Southern Punjab, Pakistan

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This is to certify that this thesis entitled "Consanguinity, prevalence pattern of hereditary malformations, and genetic analyses of rare anomalies segregating in families from Southern Punjab, Pakistan" submitted by Ms. Hafiza Fizzah Riaz is accepted in its present form by the Department of Animal Sciences Quaid-i-Azam University Islamabad as satisfying the dissertation requirements for the degree of Doctor of Philosophy in Human Genetics.

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Declaration

I hereby declare that I have worked on my thesis "Consanguinity, prevalence pattern of hereditary malformations, and genetic analyses of rare anomalies segregating in families from Southern Punjab, Pakistan" independently and work presented here is original. This thesis has not been submitted in current or a similar form to any other university.

Hafiza Fizzah Riaz

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Thesis Abstract

Rahim Yar Khan District is a remote town of Southern Punjab Pakistan with inhabitants of mixed culture and origin. This multi-faceted study was designed in order to, 1): get an insight into the population structure of Rahim Yar Khan district through determining consanguinity and inbreeding coefficient; 2) to observe the prevalence pattern of hereditary and congenital anomalies commonly occurring in the population; 3) to report phenotypic variability in limb deficiency disorders; 4) and to molecularly characterize rare malformations segregating in extended families. First, I conducted an epidemiological survey to collect data about consanguinity prevalence and its relationship with biodemographic parameters. In a cross-sectional approach, first-hand data of 2174 females were obtained and bivariate and multivariate logistic regression analyses were performed. It was observed that Rahim Yar Khan District has a high prevalence of consanguinity, i.e., 58.5% with a cumulative coefficient of inbreeding IC-F=0.0355. Consanguinity showed a significant association with variables like rural origin, Saraiki language, nuclear household and illiteracy. In subjects with consanguineous unions, fertility and mean live births were higher contrasting to category of non-consanguineous unions, but no statistically significant differences were detected in consanguineous couples and non-consanguineous couples regarding child mortality and morbidity.

Secondly, a total of 231 independent cases of congenital and hereditary anomalies (HDs) were recruited from Rahim Yar Khan District. An estimated 62.8% cases were sporadic and 37.2% familial; and 82.7% isolated and 17.3% syndromic. HDs were categorized into 12 broad phenotypic categories. Neurological defects (n=65; proportion:0.2814; CI:0.2234-0.3394) topped the list of all anomalies followed by limb anomalies (n=58), musculo-skeletal defects (n=33), deaf/mute cases (n=31), and visual impairments (n=21).

Thirdly, eight independent cases with transverse limb defects (TLD) were recruited (case series 1), 7 of which were nonsyndromic and one was syndromic. The anomalies in these subjects exhibited as unilateral amputation through the palm, accompanied with the short or hypoplastic thumb, mild to moderate shortening of the affected limb, distorted palmer creases, and relatively unaffected contralateral limb or feet. Moreover, six independently recruited cases with thumb aplasia are reported (case series 2). All cases had isolated presentation and five subjects had sporadic occurrence. The involved arms of subjects showed the absolute absence of first digital rays, medial inclinations of middle and little fingers, narrowing of palms, absence of small carpals, and reduction in the normal size of zeugopod.

Cenani-Lenz syndactyly syndrome (CLSS) is a hereditary condition having phalangeal disorganization with a variable degree of oligodactyly/syndactyly features. Mutations in *LRP4* have been implicated in families with CLSS. Two independent Pakistani families with characteristic features of CLSS were recruited. In kindred 1 and 2, one and two affected individuals born to consanguineous couples were observed, respectively. Affected subjects in both families were presented with drastically reduced autopod and zeugopod with grossly disorganized skeletal elements, the features consistent with CLSS spoon-head type. Additionally, affected subjects presented certain anomalous facial features including hypertelorism, down-slanting palpebral fissures and enamel hypoplasia. Mutation analyses revealed a A>G base transition in exon 12 at position c.1820 in *LRP4* in the index patients in both families. The mutation segregation was concordant with the disease model in both

families. Our study provided a support to genotype-phenotype correlation as a missense mutation caused a relatively milder form of CLS.

DuPan syndrome, one of several chondrodysplasias, affect appendicular skeleton without causing any harm in the axial skeleton. It is caused by a member of the BMP family, *CDMP1* which regulates condensation and differentiation of mesenchymal tissues during skeletal development in embryonic growth. Another independent limb malformation, Brachydactyly type C (BDC) depict consistent clinical features like brachymesophalangy of second, third and fifth digits, with hyperphalangy of the second and third digits along with short proximal phalanges and reduced anterior metacarpal. An extended family with simultaneous segregation of DuPan syndrome and BDC in various loops of the pedigree was studied. These characteristic phenotypic entities were observed to show clear autosomal recessive and autosomal dominant inheritance patterns, respectively. Molecular genetic analyses of this family demonstrated that a novel mutation NM_000557(GDF5):c.404delC in *CDMP1* segregated with the DuPan syndrome and brachydactyly type C phenotypes in homozygous and heterozygous states, respectively.

Intellectual disability (ID) is characterized by reduced adaptive and cognitive functionality affecting 3% population worldwide. A large Pakistani family with multiple affected subjects exhibiting the symptoms of inherited ID was studied. Initially, SNP based genotyping was carried out with the help of a commercial service provider. Further, homozygosity mapping was used in order to detect regions of homozygosity shared among the patients. One patient from this family was selected for exome sequencing. Analyses of these data led to the exclusion of many of the previously known genes for ID. Nonsynonymous homozygous variants were identified in four genes which also fall within the homozygous intervals detected in SNP analyses. One of the identified genes has been implicated in autosomal recessive intellectual disability while the rest three are expressed in the brain. Identified variants the currently being tested through Sanger sequencing. In conclusion, this study presents interesting data regarding inbreeding coefficient and clinical and molecular characterization of hereditary anomalies of rare phenotypes.

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Hafiza Fizzah Riaz

List of Abbreviations

ACC	Aplasia cutis congenita
AER	Apical ectodermal ridge
ANOVA	Analysis of variance
AOS	Adams-Oliver syndrome
BDA2	Brachydactyly type A2
BDB	Brachydactyly type B
BDC	Brachydactyly type C
BMP	Bone morphogenetic protein
bp	Base pair
CDMP1	Cartilage-derived morphogenetic protein 1
CLSS	Cenani-Lenz syndactyly syndrome
CU	Consanguineous unions
DFC	Double first cousins
DPS	DuPan syndrome
DR	Distant relatives
EDTA	Ethylenediaminetetraacetic acid
FBD	Father's brother daughter
FC	First cousins
FCOR	First cousin once removed
GDF5	Growth differentiation factor 5
HDs	Hereditary and congenital disorders
IC-F	Inbreeding coefficient
ID	Intellectual disability
IQ	Intellectual quotient
LLD	Longitudinal limb defects
LRD	Limb reduction defects
LRP4	Low-Density Lipoprotein Receptor-Related Protein 4
MBD	Mother's brother daughter
MR	Mental retardation
NCU	Non-consanguineous unions
OMIM	Online Mendelian inheritance in Man
OR	Odds ratio
PCR	Polymerase chain reaction
PZ	Progress zone
RoH	Runs of homozygosity
SC	Second cousins
SCOR	Second cousin once removed
SNP	Single Nucleotide Polymorphism
TLD	Terminal transverse limb defects
ZPA	Zone of polarizing activity

1. General Introduction

Pakistani population is extremely diverse with a significant component of genetic admixture of Central Asian, West Asian and European migrants which infiltrated the indigenous South Asian gene pools in different waves (Qamar et al. 2002). The population of Pakistan can be organized in distinct ethnicities, tribes/subtribes, and caste-systems based on migratory history, linguistic, cultural, and geographical origin. Generally speaking, the Pakistani population comprises five major ethnic subgroups, i.e. these are the Sindhis, Punjabis, Pashtuns, Baluchis, and Muhajirs. These groups have unique identities in place of origin, language, culture and values, and health beliefs (Banuazizi, 1986). A characteristic feature of these populations is tribal and caste endogamy which has resulted in significant stratifications and homogeneity within ethnic groups (Hussain, 1999; Ahmad et al. 2016a).

Pakistan for various reasons is most suitable for the study of genetic disorders. Firstly, there are early marriages and large family size which enable the researchers to work out ratios and thus test their hypothesis regarding the mode of inheritance of the disease. The significance of large families multiplies many folds when we go for linkage analysis. Then, there are stable communities and lack of awareness regarding the hereditary anomalies. The communities, particularly in the rural areas are stable and the whole kindred can conveniently be found living together. Due to lack of awareness and absence of genetic counseling facilities, the communities showing genetic defects usually contain a large number of affected persons. For instance, Ahmad et al. (1987) studied a large inbred Pakistani family, living in a village in

District Shikarpur, Sind, in which 36 persons were found to be affected with an X-linked split-hand/split-foot anomaly.

As stated above, an important aspect of Pakistani society is the high rate of consanguinity. Almost 55% of the marriages are commenced within the tribe or same caste-system (Ahmad et al. 2016b). A marriage is said to be consanguineous if the spouses have at least one ancestor in common and an individual is said to be inbred if his/her parents are biologically related (Connor and Ferguson-Smith, 1987). The fundamental disadvantage of consanguinity is the emergence of rare recessive homozygous disorders which otherwise may not appear in an outbred population (Cavalli-Sforza and Bodmer, 1971). In Pakistan, the level of consanguinity remains high, thus enabling autosomal recessive defects to become homozygous and express themselves (Yaqoob, 1996).

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. The geneticists feel fascinated and overawed seeing genetic disorders making their appearance, in some cases with devastating effects, in kindreds generation after generation (Ahmad, 1998). In our society, the persons showing genetic defects are in general not treated with respect, sympathy, and consideration that they deserve. The children born with genetic defects are regarded as a source of stigma and shame and in many cases, the parents of the affected children develop a sense of guilt. All this results in social and psychological stress on the family. In many instances, the families come to a dead end when the medical treatment does not produce any results, as most of the genetic diseases are yet without a cure. As a last resort, the parents of the affected children pay visits to saints and shrines, praying and longing for a miraculous cure.

Monogenic disease or Mendelian disorders are individually rare but collectively they affect millions of people around the world (WHO, 2005). Studies have shown that an estimated 8 million infants are born annually with some kind of genetic or congenital disorder globally. Congenital anomalies and monogenic disorders have emerged as the common cause of mortality in the early years of life since the prevalence of infections and pathogenic borne diseases have declined in many populations across the globe.

An accurate diagnosis of Mendelian disorders is quite often challenging and is heavily dependent on detailed phenotypic assessment including clinical examination, radiological evaluation, biopsy results, karyotyping and candidate-gene approach along with some additional tests such as metabolic tests, blood serology and hormonal tests (Chong et al. 2015). In recent years, however, the advent in SNP-based genotyping methods, next-generation high throughput sequencing tools, and computational approaches have revolutionized the discovery of pathogenic variants underlying the monogenic disorders as well as multifactorial traits (Biesecker, 2010). The identification of causative mutations underlying the Mendelian disorders and monogenic traits has a great impact on the understanding of the molecular pathways, and biochemical and cellular networks which lead to the disease phenotype. Further, it is also beneficial for the identification of targets and novel routes for therapeutics (Bainbridge et al. 2011; Xue et al. 2015).

SNP-based genotyping methods have increasingly become part of gene mapping experiments. The declining cost of these tools, dense marker maps and comprehensive coverage of the whole genome make them a better choice compared to microsatellite markers. SNP-based genotyping methods are used not only in the mapping of monogenic disorders but also in the study of quantitative trait loci and

association studies for complex and multifactorial diseases. Likewise, exome sequencing is the targeted DNA sequencing method where the coding regions (i.e. exons and their boundaries) are sequenced. Due to the fact that the human exome comprises approximately ~1% of the genome, this method greatly reduces the cost of detecting variations in the coding regions as compared to far instance whole-genome sequencing.

The current research was intended to report various aspects of genetic anomalies prevalent in one of the reprehensive population of the Southern Punjab, Pakistan. Rahim Yar Khan district in Southen Punjab, which was primarily focused for data collection in this study, is a unique assemblage of both native and migratory populations. Within the context of consanguinity, this study reports families/subjects with rare and interesting hereditary phenotypes, to demonstrate their prevalence pattern, segregation, to characterize their phenotypes by conventional clinical methods, to identify the underlying mutation, and to carry out investigations with the help of modern tools like SNP-based genotyping and exome sequencing. The overall goal is to highlight various rare disorders prevalent in the target population and to employ the current research findings for the improvement of clinical diagnosis of genetic conditions by using molecular analysis and to provide genetic counseling to the patients and their families.

1.1 Overview of the current study: the scheme of study

This was a multi-faceted study in which I have used various levels of sampling, experimental and analytical approaches. Initially, I used an epidemiological approach to collect the data on married female subjects belonging to Rahim Yar Khan district of Southern Punjab, Pakistan, in order to infer the rate of inbreeding coefficient. After applying certain exclusion and inclusion criteria, first-hand data on

2174 subjects from independent families were collected and the prevalence of consanguinity and inbreeding coefficient were established for Rahim Yar Khan population. Here, I used random sampling and door-to-door surveys. Among those 2174 families, a total of 231 families were presented with individuals having certain kind of genetic and congenital anomalies. Hence, the second tire of data was collected in order to ascertain the pattern prevalence of genetic and congenital anomalies. In this respect, phenotypic data, pedigree structures, and familial and phenotypic heterogeneity were observed. In this cohort of 231 families, neurological disorders were in the highest proportion (n=65) followed by limb anomalies (n=58), and other malformations. Curiously, cases with congenital limb amputations were in high frequency. In the next level, I saturated the cases with rare anomalies, particularly with limb defects. In the meantime, I collected blood samples of families with rare phenotypes with the possibility to carry out molecular genetic analyses. Collectively, 18 cases/families were selected for detailed descriptive/molecular study. Fourteen independent cases of congenital limb amputations were collected and their detailed clinical, radiological and descriptive epidemiological study was conducted. These cases were further analyzed in two separate categories of transverse limb amputations and thumb aplasia. On the other hand, two families with Cenani-Lenz syndactyly syndrome and one family with DuPan syndrome were also ascertained. The phenotypes in these three families were segregating autosomal recessively. Mutation analyses for candidate genes in these families led to the detection of novel mutations in *LRP4* and *CDMP1*, respectively. Finally, in a large family with multiple affected subjects exhibiting the symptoms of intellectual disability, we generated SNP based genotyping data with the help of a commercial service provider. I used homozygosity mapping in order to detect regions of homozygosity shared among the affected

subjects. Further, one of the affected individuals in this family was subjected to exome sequencing. Analyses of these data led to the shortlisting of rare variants which are pathologically relevant to the phenotype and also fall in the homozygous intervals detected in the SNP scan. In conclusion, I used a multi-faceted approach in order to study various aspects of population genetics of Rahim Yar Khan District, and in the clinical and genetic elucidation of subjects/families with rare disorders. The overall scheme of study is depicted in Fig. 1.1.

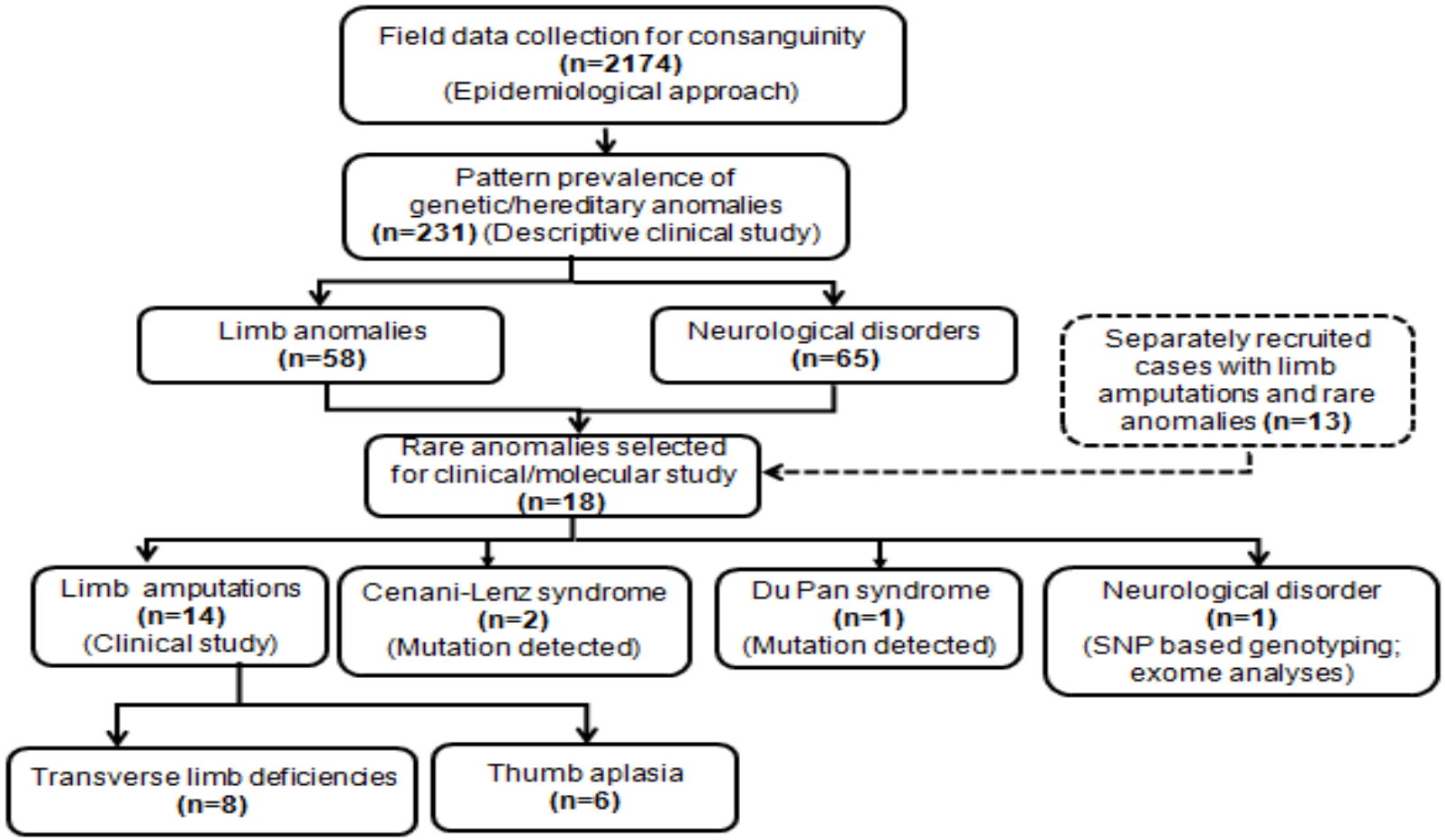

Figure 1.1. Flowchart of the study

2. Demographic pattern of consanguinity and its impact on health: a study in Rahim Yar Khan District of Southern Punjab, Pakistan

2.1 Abstract

Rahim Yar Khan (RYK) District is a remote town in Southern Punjab, Pakistan. It comprises a multi-ethnic assemblage of both ancient or native populations and migrated communities which settled here in various cascades. There is a lack of knowledge about the bio-demographic structure of different subpopulations residing in this region. To fill this gap and to get an insight of the pattern of endogamy we conducted a cross-sectional epidemiological study in RYK District and recruited 2174 Muslim married females by random sampling method. A comprehensive account of bio-demographic and socio-economic variables was taken and data on marital union types, level of consanguinity, and subject's fertility were acquired. Descriptive statistics followed by univariate and multivariate logistic regression approach was adopted and the inbreeding coefficient (IC-F) was estimated. The analyses of these data revealed that consanguineous unions (CU) accounted for 58.5% of the marriages, rendering IC-F = 0.0355. The prevalence of CU was observed to be significantly higher with respect to variables like the rural origin of the subject, Saraiki language, illiterate or having a religious/*Madarsa* education only, and belonging to the nuclear family type. The estimates of CU were witnessed to be higher in women whose spouses were engaged in manual jobs and had parental consanguinity. Further, the multivariate logistic regression demonstrated that socio-demographic variables such as illiteracy, Saraiki language, parental consanguinity, and reciprocal marriages, were

the significant predictors of CU. Analyses of the data also revealed that among the seven major marital union types first cousin unions were the most common type i.e. 52%; and among those parallel-cousin unions patrilineal marriages had a higher representation (54% and 57%, respectively), and father's brother's daughter type were more pronounced (31 %). We further show that subjects' fertility and mean number of live-births were significantly higher in the CU group compared to the non-consanguineous (NCU) sample. Nonetheless, women with CU had a significantly higher ratio of male offspring contrasting to the NCU group. There were however no statistically significant differences in the CU and NCU samples with respect to pre- or post-natal mortalities and child morbidities. The results of this study are not in agreement with a few of the populations previously studied in Pakistan and may clue toward a unique bio-demographic nature of this population. This study presents a comprehensive account of consanguinity and IC-F in RYK District and would be helpful in getting an insight into the structure of this population.

2.2 Introduction

2.2.1 Consanguinity: An area of interest for researchers

The application of autozygosity to map rare recessive diseases has enhanced the interest of researchers to develop a thorough understanding of the consanguinity dynamics. Moreover, the clinical relevance and widespread effect of consanguinity on shaping the genome-wide runs of homozygosity (RoH) provides a base for evaluating demographic and biological determinants of consanguinity (Woods et al. 2006; Overall, 2009).

2.2.2 Consanguineous communities

Consanguinity is a highly practiced social custom in many parts of the globe (Schulpen et al. 2006). Consanguineous unions (CU) are long established and respected social custom in most communities with more than 20-50% marriages of consanguineous type. These communities primarily belong to the developing world. Communities with a high choice of consanguinity constitute about twenty percent of the global population and are residing mainly in the region of Middle East, North Africa and South Asia (Tadmouri et al. 2009; Bittles and Black, 2010). The prevalence of CU across Asian and African countries varies greatly with predominance in Islamic countries. Other prompting features that induce differences in consanguinity prevalence include ethnic, religious and socio-cultural attributes (Bittles, 2001).

2.2.3 Demographic contributors of consanguinity

Demographic variables associated with the high rate of consanguinity are low literacy rate, low socioeconomic power and rural residence (Zaoui and Biemont,

2002; Bener and Hussain, 2006). The maternal education has a strong impact on the choice of consanguineous unions and shows a negative correlation with it (Nabulsi et al. 2003). Likewise, paternal literacy level and occupation levels are also associated with consanguinity: its low levels being linked to the high rate of consanguinity (Liascovich et al. 2001). Moreover, couples who contract consanguineous unions more frequently have an extended family structure and reside in smaller towns (Hussain and Bittles, 2000). Generally, CU are also associated with early age at marriage. Fuster and Colantonio (2004) found a correlation between CU and economic variables and congruence between second-cousin marriages and rural residence.

2.2.4 Consanguinity: a custom

Demographic factors are not the only reason that induces a trend of consanguinity in population rather sometimes it is performed only because it is a preferred choice either religiously or socially (Rajab and Patton, 2000). In various religious sects, the reason to practice CU arises from their faith (practiced as a religious custom). For example, certain Pakistani families like Syeds are not open to other castes for marital unions (Hussain, 1999). CU is also a socially preferred choice of the Muslim world (Inhorn et al. 2009). Moreover, families also prefer CU in order to maintain the integrity of their estates and strengthening family ties (Rao et al. 2009; Sandridge et al. 2010). The pattern of consanguinity varies from one population to another along with its associated variables. In geographically isolated populations of Croatian Islands, CU is preferred because of limited mate availability while in Tunisia, CU was prevalent because of cultural aspects. These two societies possessed different attributes and delineated unique prompting reasons for the prevailing high rate of consanguinity. In inbreeding studies in human populations, the association of

CU with literacy level and socioeconomic status ought to be considered, and the association will be extremely specific and strongly rely on the cultural context/social settings of each studied population (Kerkeni et al. 2006).

2.2.5 Consanguinity and health outcomes

A significant proportion of children born to consanguineous parents have congenital malformations. First generation kids of consanguineous couples are highly susceptible to a high risk of congenital anomalies and this risk increases further with each successive CU down the generation lane. Epidemiological data reveals that the incidence of reproductive losses (sterility, abortion, and stillbirth) is significantly influenced by consanguinity. It also gives rise to infant mortality, child mortality and congenital anomalies (Khayat and Saxena, 2000; Banerjee, 2002; Tamim et al. 2003). Events of mortality in perinatal, neonatal, post-neonatal, child, and prereproductive stage happen more frequently in consanguineous unions contrasting to non-consanguineous unions (Overall, 2009). The high frequency of consanguinity is thought to be the single imperative driver underlying such genetically-related outcomes in developing nations (Modell and Darr, 2002).

2.2.6 Consanguinity in Pakistan

With few exceptions, consanguinity is prevalent in Pakistan. Timeline analysis revealed no reduction in the popularity of consanguineous marriages in many parts of the country. If some people of society change their choices about consanguineous marriages and stop favoring them, some others enter into the division that is in favor of consanguinity (Hussain and Bittles, 1998). The comparative demographics of various levels of CU in many subpopulations of Pakistan have been recorded. The incidence of consanguinity in several parts of Pakistan has been measured like in

northern areas of Punjab, Balochistan, Sindh, southern Khyber Pakhtoonkhwa and Kashmir (Mian and Mushtaq, 1994; Qidwai et al. 2003; Hussain and Bittles, 2004; Jabeen and Malik, 2014a; Hina and Malik, 2015; Sthanadar et al. 2016). As a whole consanguinity prevalence reported from these studies is over 40% with first-cousin marriages being the commonest form of CU. In Pakistan, CU is marked as a basic cause/main determinant for the high prevalence of infant deaths while overlooking/neglecting other contributory demographic reasons prompting to early deaths in infants like socioeconomic, occupational and educational attainments of parents (Bittles, 2003; Hamamy et al. 2011).

District Rahim Yar Khan (RYK) is comparatively an underdeveloped area of the Punjab province located in its southern part. No previous research has investigated consanguinity and its prevalence in this region. The key goal of this study was to bridge this information gap and assess socio-demographic parameters of consanguinity in the sample population of District Rahim Yar Khan (RYK). Furthermore, research was conducted to provide a comprehensive account of the consanguineous union (CU) as well as nonconsanguineous unions (NCU) regarding variables like fertility rate in female respondents, the proportion of live births, antenatal and postnatal mortality, and child morbidity.

2.2.7 Consanguinity and infant mortality

Characteristics and marital practices of a family may affect its probability to have children and their survival beyond childhood. The preferred choice to marry within family affects its odds of child mortality (Bokhari et al. 2015). In fact, the high prevalence of CU, particularly first cousin unions, preceded high rate of autosomal recessive Mendelian disorders and hence, adversely affect the general health status of a community (Darr et al. 2016). The degree of relatedness is proportionally related to

the rate of birth defects (Denic and Nicholls, 2007). In a developed country like Britain, infant mortality is primarily related to smoking and socioeconomic deprivation, while in a developing country like Pakistan CU is considered as the main reason for the rise in infant mortality rates (Hussain et al. 2001). In a migrant population of Britain from Pakistan, the high raised infant mortality rate has been ascribed to the custom of consanguinity, which provides the basis for congenital anomalies to emerge (Pinto et al. 2006; Bittles and Small, 2016). First cousin unions raise a couple's risk to endure the demise of a child by 1.18 times. A modest decline in child mortality can be achieved by avoiding first cousin marriages (Shah et al. 1998; Hosseini-chavoshi et al. 2014).

2.2.8 Consanguinity and genetic counseling

Societies and communities where consanguineous marriage is customary have increased the prevalence of neonates born with severe recessive disorders. The custom of consanguineous marriages expands the possibilities for genetic counseling and require an interactive struggle to investigate medical outcomes of CU: i.e. to identify high-risk families and to advance their knowledge about risk possibility and carrier testing when feasible (Modell and Darr, 2002). A systematic and comprehensive public health program needs to be formulated in such communities to provide effective genetic counseling although social determinants of CU make it very challenging. Besides the appearance of affected children outside the consanguineous wedlock makes community members hesitant to associate consanguinity with genetic anomalies often (Fathzadeh et al. 2008). Information about the type, degree, and causes of consanguinity common in a society helps in effective genetic counseling, particularly in child well-being. Counseling should also be provided at the stage of preconception and premarital levels. Individuals sharing close wedlock should have

easy access to genetic counseling and family planning aids/services. Training of primary health care providers should also include education about related health risks (Hamamy, 2012). Along with proper genetic counseling comprehensive religious, governmental, and media-based intervention programs based upon the medical and epidemiological research, provide a platform for effective prevention of recessive disorders (Read and Donnai, 2012). When a child born to a consanguineous couple is diagnosed with an anomaly, a thorough and systematic investigation of the condition and related symptoms must be performed particularly focusing on autosomal recessive conditions in the diagnosis. Autosomal recessive conditions not necessarily and merely arise because of consanguineous marriage, but additionally, can arise from two different mutations by chance. Randomly selected people may carry these mutations but the chances of these people to produce an affected child are low although it does occur. This information can be very helpful for the parent to get over the feeling of guilt and shame they have that the health issues of their offspring are because of them or in dealing the misconception that these health issues are a result of a curse.

2.3 Subjects and Methods

2.3.1 Study population

District Rahim Yar Khan (RYK) is located in southern Punjab at its juncture with Sindh province. It occupies a geographical area of 11,880 square kilometers. The total population of this area is 4.7 million with the majority living in rural areas. The economy of the District is primarily agro-based and main products are cotton, wheat, sugarcane, mango, orange, and dates. There are four tehsils in the District namely, i.e. Liaquatpur, Khanpur, Rahim Yar Khan, and Sadiqabad (Fig. 2.1). Several caste systems are congregated in the study area and among them, predominant are Arain, Jutt, Gujjar, and Rajput, which migrated from subcontinent India in 1947. The indigenous castes include Syed, Wattoo, Daudpota, Joya, Balouch, and Pathan Muslims constitute the majority of the district population (96.7%) while Hindu community comprises second major religious population (1.8%). Based on linguistic divisions, Saraiki is more prevalent and spoken by 62.6% people, followed by Punjabi which is the linguistic identity of 27.3% population. Urdu speakers comprise only a small percentage of the total population, i.e. 2.9%. The literacy level of the area is very low as merely thirty-three percent of the area population is literate. Population has accessibility to sufficient primary medical and healthcare services but lacks proper secondary and tertiary health standards to enhance maternal, infant and young child health. Other opportunities as programs on immunization for early control of the disease and improving the health status of the community are also scarce. Nonavailability of these facilities is attributed to illiteracy, unawareness, and poor socio-economic status (MICS, 2009; PAP, 2014).

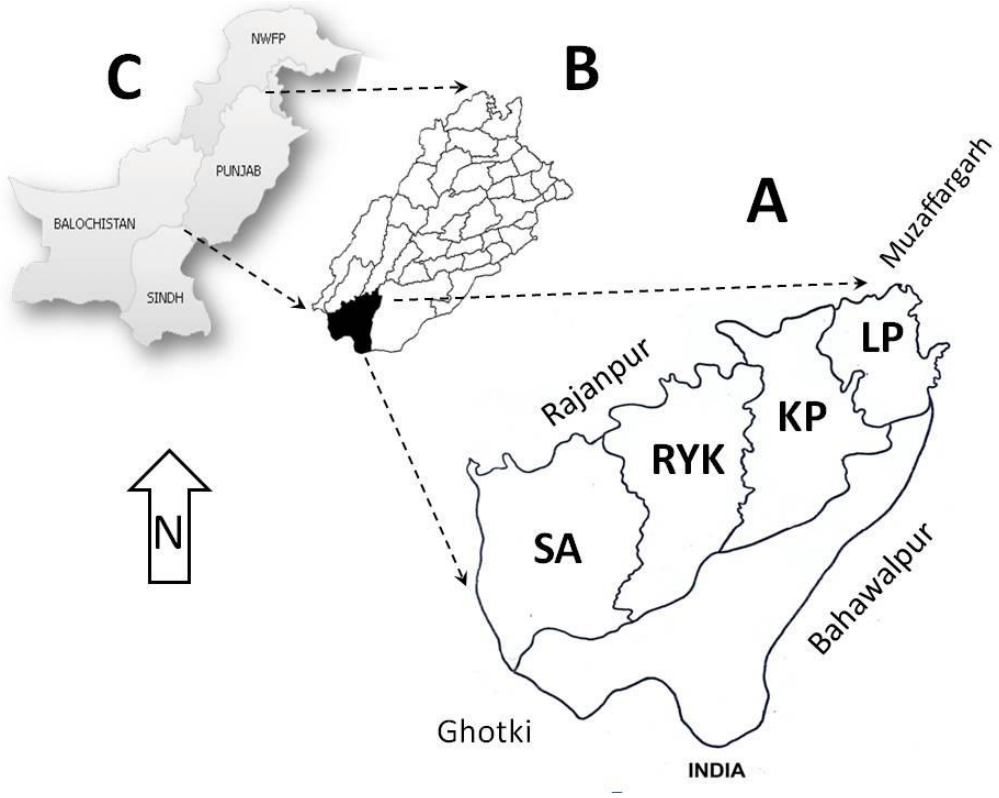

Figure 2.1. Map of District Rahim Yar Khan

(A): From South-to-North: Districts Sadiqabad (SA), RahimYarKhan (RYK), Khanpur, and Liaquatpur (LP), **(B):** superimposed on map of Punjab province **(C):** and Pakistan

2.3.2 Subject recruitment

Through a descriptive epidemiological approach, we collected the data from 35 sampling sites during the study period. Sampling sites were selected both from rural and urban territories of RYK district. A random sampling method was applied. Study participants were approached either at the residential, work or public places like hospitals and community centre. The survey team also comprised local members from the study areas, such as lady-health-visitor and volunteer resource persons with sufficient knowledge of the area. Married females with the permanent resident status of the study area were included in the study. Consent of study participants or their

guardians was gained on a nondisclosure agreement prior to the data acquisition. Moreover, they agreed to provide the complete information about the medical condition of the family. All the respondents were interviewed in person to collect the data. Generally, questions asked by the participants about the purpose and consequences of the study were answered appropriately before data acquisition. All the data were collected through a comprehensive questionnaire. Each household was represented by only one woman. Rahim Yar Khan District has an estimated 416,000 independent households (PESA, 2012). Sampled households represented 0.005 percent of the total housing units. Data were also collected on socio-economic and fertility variables. Hindu population of the area does not contract consanguineous unions, hence, their data were not included in further analysis.

2.3.3 Definitions

Two major categories of marriages were used: Consanguineous unions (CU) comprised marriages between relatives who are second cousin or closer than that and non-consanguineous unions (NCU) included marriages in which spouses are either unrelated or are related beyond the second cousin. The CU included four classes of marital unions as double first cousins (DFC) unions in which members of a pair have both sets of grandparents in common; first cousins (FC) unions in which spouses have closest common ancestor as paternal or maternal grandparents; first cousin once removed (FCOR) unions where marriage of a child with his parent's first cousin is performed; and second cousins (SC) unions in which members of a couple have great-grandparents in common. Second major category of marital unions named NCU included three classes of marital unions in which spouses were related as second cousin once removed (SCOR), distant relatives (DR) locally known as *Biradari* unions, or totally unrelated (PDHS, 2007).

With respect to geography, respondents were grouped into two categories as rural or urban. This division was done depending upon the union council record. Details were also taken regarding "marriage arrangements". Based upon this information marital unions were classified as 1: arranged marriages, 2: self-arranged marriages (love co-arranged marriages), and 3: reciprocal marriages. In "arranged marriages", seniors of the family selected partners of respondents. "Self-arranged" were those in which the respondents selected their spouses themselves while elders of the family allowed the marriage. "Reciprocal marriages" (locally known as *watta-satta*) were those in which mutual exchange of spouses between two families was arranged with the will of elders of both families. Three types of households were identified in the current sample and were designated as a nuclear family system, grandparent-and-one-couple (a modified nuclear family structure usually with one grandparent) or extended family system. Nuclear family systems constituted husband-wife living with their kids in the same household as a single family unit. In family systems called grandparent-and-one-couple (a type of nuclear family system) grandparents also live with a couple and its kids in the same household. The extended family system comprised of two or more couples with multiple overlapping generations living together in a single household. Categorical organization of occupational work identified in the current study sample was in accordance with standard occupational classes made and standardized in Pakistan Demographic and Health Survey (PDHS, 2007). The observed literacy level in this study was estimated as formal education and the total count of years of school attendance.

Statistical analyses were performed using GraphPad Prism (ver.5) and Microsoft Excel. The coefficient of inbreeding (IC-F) was calculated from the weighted proportion of each class of CU (Bittles, 2010). The chi-square ($\chi 2$) test was

used to determine a correlation between variables by categorical comparison and 95% confidence intervals were computed for total frequencies. ANOVA and T-test were applied to compare means of continuously distributed variables having homogeneity of variances. Multivariate logistic regression analyses were conducted to infer likely dependence of consanguinity on socio-demographic parameters. For interpretation of odds ratios (OR) in binary logistic regression analysis, the category of the respective socio-demographic parameter with a minimum rate of consanguinity was used as a reference.

2.4 Results

2.4.1 Sample characteristics

During the field survey, almost 2662 females of separate households were randomly approached while 2174 subjects (82 %) consented and joined in the study. Maximum numbers of representatives were from Sadiqabad (n=941) and a minimum number of individuals belonged to Liaquatpur (n=57). Age of the study subjects ranged between 15-90 years (with a mean 35.2 ± 13.5 years).

2.4.2 Types of marital unions and inbreeding coefficient (IC-F)

Majority of the marital unions were CU (n = 1,271; 58.5%) while 41.5% marriages were NCU (n = 903) (Table 2.1). Among all marital unions (CU+NCU) and CU, predominant were first cousin (FC) unions (n = 1129; 51.9%). In NCU distantly related (DR) was the prevalent marriage type (n = 824; 37.9%). The recorded coefficient of inbreeding (IC-F) in this studied population was 0.0355 (Table 2.2).

Table 2.1. Tehsil wise distribution of marital classes

Tehsils	Consanguineous unions: No.(%)				Non-consanguineous unions: No.(%)			
	Double first cousin	First cousin	First-cousin-once-removed	Second cousin	Second-cousin-once-removed	Distantly related/*Biradari*	Non-related	All marriages
SadiqAbad	11 (1.2)	477 (50.7)	23 (2.4)	16 (1.7)	5 (0.5)	345 (36.7)	64 (6.8)	941
Rahim Yar Khan	7 (1.2)	343 (57.4)	24 (4.0)	3 (0.5)	2 (0.3)	214 (35.8)	5 (0.8)	598
KhanPur	7 (1.2)	287 (49.7)	28 (4.8)	6 (1.0)	1 (0.2)	247 (42.7)	2 (0.4)	578
LiaquatPur	2 (3.5)	22 (38.6)	15 (26.3)	0	0	18 (31.6)	0	57
Total	**27 (1.2)**	**1,129 (51.9)**	**90 (4.1)**	**25 (1.2)**	**8 (0.4)**	**824 (37.9)**	**71 (3.3)**	**2,174**

2.4.3 Geographic distribution of consanguinity and IC-F quantification

Liaquatpur tehsil outnumbered other tehsils of the district in the rate of CU (68.42%). IC-F of the Liaquatpur was 0.0367. However, district RKY was presented with the highest IC-F (0.0386) owing to the high prevalence of FC unions (Table 2.2; Fig. 2.2). Furthermore, the distribution of CU and NCU among the four tehsils varied significantly.

Table 2.2. Geographic distribution of consanguineous and all marital unions and IC-F

Geographic variable	**Consanguineous unions (n=1271; 58.5%)**		**All marital unions (n=2174; 100%)**		**Bivariate logistic regression OR**	**IC-F**
	No	%	No	%		
Tehsils*						
Rahim Yar Khan	377	63	598	27.5	1.34*	0.0386
SadiqAbad	527	56	941	43.3	1.00	0.0342
KhanPur	328	56.8	578	26.6	1.03	0.0342
LiaquatPur	39	68.4	57	2.6	1.70	0.0367
Total	1271	58.5	2174	100.0		0.0355
Rural-urban *						
Rural	844	60.7	1,390	63.9	1.29*	0.037
Urban	427	54.5	784	36.1	1.00	0.0327

*:Statistically significant distribution in Chi-test
OR: Odd Ratios; IC-F: Inbreeding coefficient F.

The overall prevalence of CU was high in respondents from rural areas (60.7%) compared to the urban respondents (54.5 %) and this difference in prevalence was statistically significant (Table 2.2).

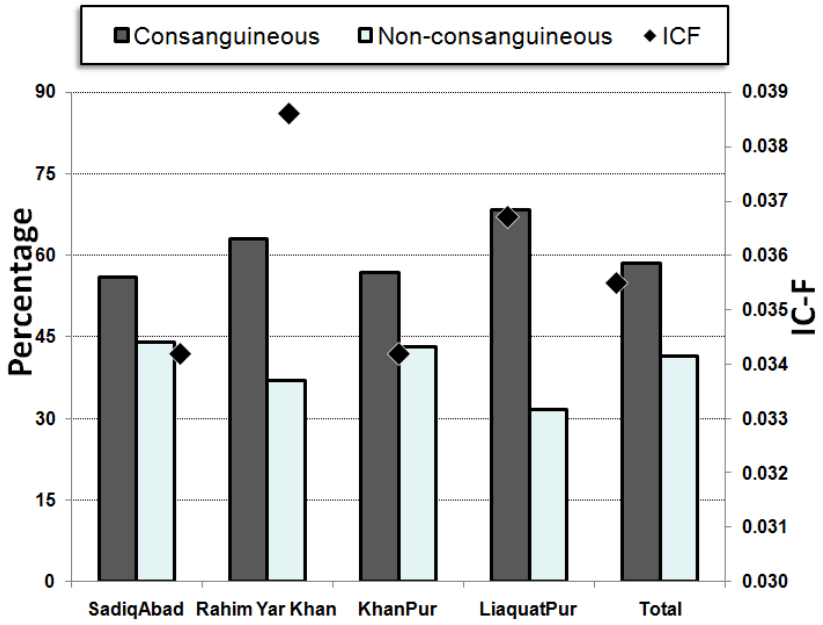

Figure 2.2. Bar-graph showing tehsil wise distribution of CU and NCU among the respondents of RYK District (along the left Y-axis) and coefficient of inbreeding (IC-F) (along with the right Y-axis) in black diamonds

2.4.4 Consanguinity in relation to the linguistic and ethnic identity of subjects

In our study, the prevalence of CU was also checked in linguistic divisions of subjects. It showed that CU were more frequent in Saraiki speakers (with 75.4%; IC-F = 0.0454) followed by Urdu speakers (46%). Punjabi speakers showed almost similar proportion of CU (45.2%) as Urdu speaking individuals (Table 2.3).

This study sample presented a remarkable ethnic heterogeneity. Female subjects recruited in this study belonged to almost 150 sub-castes which were grouped together as main castes. The frequency of consanguinity in households of the main castes (n > 42) was highly variable. Prevalence of CU was high in Saraiki speaking castes like Sheikh, Arain, Larr, and Khokhar, (ranged 80% to 91%) contrasting to Punjabi speaking castes like Rajput Mughal, Jut and Arain which ranged 39% to 54%

($p < 0.0001$) (Table 2.3). Bivariate logistic regression analyses showed a substantially high rate of consanguinity in most of the examined/studied castes than the reference caste-system.

Table 2.3. Distribution of consanguineous unions in female respondents regarding the linguistic and ethnic identity

Ethnic/Linguistic identity	**Consanguineous unions**		**All marital unions**		**Bivariate logistic regression OR**	**IC-F**
	No	%	No	%		
Native language* (n=2174)						
Saraiki	706	75.4	937	43.1	3.71*	0.0454
Punjabi	499	45.2	1,105	50.8	1.00	0.0278
Urdu	23	46	50	2.3	0.99	0.0275
Others	43	52.4	82	3.8	1.66	0.0309
Ethnicity (caste-group)* (n=1371) **						
Arain$	54	80.6	67	3.1	6.50*	0.0483
Larr$	37	84.1	44	2	8.27*	0.0526
Sheikh$	37	80.4	46	2.1	6.43*	0.0530
Khokhar$	41	91.1	45	2.1	16.04*	0.0535
Baloch$	50	58.1	86	4	2.17*	0.0338
Bhatti$	37	74	50	2.3	4.45*	0.0441
Malik$	67	77.9	86	4	5.52*	0.0458
Arain#	239	39	613	28.2	1.00	0.0239
Jut#	49	45	109	5	1.28	0.0288
Mughal#	22	51.2	43	2	1.64	0.0334
Rajput#	72	53.7	134	6.2	1.82*	0.0332
Malik#	28	58.3	48	2.2	2.19*	0.0332

#: First language *Punjabi* speaking; $: First language *Saraiki*; ^: First language *Pushto.*
*: Statistically significant distribution;
**: Ethnic/caste groups with more than 42 samples are shown here
OR: Odd Ratios; IC-F: Inbreeding coefficient F.

2.4.5 Consanguinity distribution by occupational and educational level of respondents

CU were more prevalent in female subjects married to men doing unskilled manual jobs (IC-F = 0.0406), followed by category of skilled manual jobs (IC-F = 0.0378). Respondents engaged in agriculture/farming also presented a higher rate of CU (IC-F = 0.0362). Conversely, the rate of CU was seen to be the lowest in females whose spouses were involved in either sales/business (IC-F = 0.0315) or jobs (IC-F = 0.0313) (Table 2.4).

The frequency of marital unions was evaluated with respect to literacy level of respondents. CU were predominantly higher in illiterate respondents (and in respondents with religious educational achievements) contrasting to the literate counterparts ($p < 0.0001$). Among the educated respondents, a negative association between level of education and the rate of CU was found. Higher IC-F values were seen in subjects with less educational achievements. (Chi-square for trend; $p = 0.1211$) (Table 2.4).

Table 2.4. Distribution of Consanguineous union regarding educational and occupational status

Geographic variable	**Consanguineous unions (n=1271; 58.5%)**		**All marital unions (n=2174; 100%)**		**Bivariate logistic regression OR**	**IC-F**
	No	%	No	%		
Spouse's occupation*						
Manual (Unskilled)	423	67.1	632	29.1	1.94*	0.0406
Manual (Skilled)	68	63	108	5	1.79*	0.0378
Agriculture/farming	288	59.9	481	22.1	1.45*	0.0362
Business/sales	217	50.7	428	19.7	1	0.0315
Office/service (Job)	239	51.7	462	21.3	1.04	0.0313
Late/deceased	24	52.2	46	2.1	-	0.0313
Unemployed	12	70.6	17	0.8	-	0.0368
Educational attainment*						
Illiterate (uneducated)	564	65.6	860	39.6	2.33*	0.0396
Religious education	399	63.4	629	28.9	2.12*	0.0383
Literate (Educated)	308	45	685	31.5	1	0.0278
Literacy level (years of school attendance)						
Up to 8 years	146	47.4	308	14.2	0.67	0.0301
9 to12 years	128	44.6	287	13.2	0.89	0.0265
Above 12 years	34	37.8	90	4.1	1	0.0236

*: Statistically significant distribution
OR: Odd Ratios; IC-F: Inbreeding coefficient F.

2.4.6 Age-wise distribution of consanguinity

Three age categories were made based on the age of respondents. Increased prevalence of CU was seen in the old age category of respondents as compared to the low age groups. A decreasing trend in consanguinity rate and IC-F was observed with decreasing age of respondents (Chi-square for trend; p = 0.0124) (Table 2.5).

Table 2.5. Age-wise distribution of marital unions in respondents

Geographic variable	**Consanguineous unions (n=1271; 58.5%)**		**All marital unions (n=2174; 100%)**		**Bivariate logistic regression OR**	**IC-F**
	No	%	No	%		
Age category (years)						
Up to 25	342	54.7	625	28.8	1	0.0328
26-50	781	59.4	1,315	60.5	0.75*	0.0363
Above 50	148	63.3	234	10.8	0.83	0.0379

*: Statistically significant distribution

2.4.7 Marriage arrangements and house-hold types

People of the district orchestrate their marriages differently. Regarding this classification of marriages, the frequency of CU was observed to be high in female respondents who solemnized self-arranged and reciprocal marriages (Table 2.6). Majority of our female subjects belonged to a nuclear family system and they showed a higher frequency of CU. A decline in the proportion of consanguinity was observed in subjects belonging to a family system comprising grandparent-and-one couple or extended family system (Table 2.6).

2.4.8 Subject consanguinity in relation to parental consanguinity

Regarding marriage type in parental generation, data were available for 1998 subjects. The proportion of CU and NCU in consanguineous subject's parental generation was found to be 48.5% and 51.5%, respectively. The coefficient of inbreeding (IC-F) for the parental generation was 0.0453. In the next generation, the

trend has shifted more towards CU both in the parental category of consanguineous unions and non-consanguineous unions. The trend of favoring CU in parental generation was prevailed or enhanced in the next generation. Parental generation with a higher rate of CU had a future generation with a high frequency of CU and parental generation with NCU also showed a tendency to favor CU more as compared to NCU in subsequent generation ($p < 0.0001$) (Table 2.6).

Table 2.6. Differential distribution of consanguineous unions regarding marriage arrangements, family type and parental consanguinity

Variable	Consanguineous unions (n=1271; 58.5%)		All marital unions (n=2174; 100%)		Bivariate logistic regression OR	IC-F
	No	%	No	%		
Marriage arrangement (n=2,117)*						
Arrange Marriage	736	50.2	1,466	69.2	1	0.0306
Self Arrange Marriage	27	79.4	34	1.6	3.83*	0.0455
Reciprocal /Exchange Marriage (*Watta-Satta*)	470	76.2	617	29.2	3.17*	0.0466
Family/house-hold type (n=2108)						
Nuclear Family	695	60.2	1,154	54.7	1.12	0.0367
Grandparent-and-one-couple (Advanced Nuclear)	292	57.5	508	24.1	1	0.0347
Extended Family	244	54.7	446	21.2	0.89	0.0337
Parent's marriage type (n=1998)*						
Consanguineous unions	725	74.7	970	48.5	4.05*	0.0453
Non-consanguineous unions	434	42.2	1028	51.5	1	0.0256

*: Statistically significant distribution.
OR: Odd Ratios; IC-F: Inbreeding coefficient F.

Most of the results obtained by the contingency tests were iterated through bivariate logistic regression (odd ratios: OR) (Tables 2.2-2.6). In bivariate logistic regression, all the socio-demographic parameters except caste of subjects were found to be significantly associated with consanguinity.

Multivariate analyses were performed on consanguinity regarding multiple socio-demographic parameters. Two models of multivariate logistic regression were used. In first model correlation of consanguinity with Saraiki language (OR = 2.01), illiteracy (OR = 1.31), religious education only (OR = 1.42), reciprocal marriage (OR = 1.82), and parental consanguinity (OR = 3.06) were tested while caste-system was not considered in this model (Table 2.7). In this first model of logistic regression involving multiple variables age category, tehsil, rural/urban origin, occupation of the husband, and family type were turned out to be insignificant. In the second model, caste-system was also considered along with other multiple variables and results showed that consanguinity was in positive association with variables like Khokhar caste (OR = 3.20), Saraiki language (OR = 1.93), illiteracy (OR = 1.41), reciprocal marriage (OR = 1.64), and parental consanguinity (OR = 2.87) (Table 2.7).

Table 2.7. Multivariate analysis of socio-demographic variables associated with CU

Variable	**OR**	**Std.Err.**	***P***	**95% CI**
First Model (Caste-system excluded)				
Mother tongue: Saraiki	2.01	0.26	<0.0001	1.55-2.60
Educational attainment				
Religious education	1.31	0.18	0.040	1.01-1.71
Illiterate	1.42	0.19	0.008	1.10-1.83
Marriage arrangement: Reciprocal	1.82	0.25	<0.0001	1.39-2.37
Parental union: Consanguineous	3.06	0.32	<0.0001	2.50-3.75
Second Model (Caste-system included)				
Caste-system: Khokhar	5.41	3.20	0.004	1.70-17.25
Mother tongue: Saraiki	1.93	0.61	0.038	1.04-3.59
Literacy: Illiterate	1.41	0.22	0.024	1.05-1.91
Marriage arrangement: Reciprocal	1.64	0.29	0.006	1.15-2.33
Parental union: Consanguineous	2.87	0.35	<0.0001	2.25-3.65

2.4.9 Distribution of first cousin unions

Data on types of first cousin unions were available for 1112 respondents (out of 1129 cases of FC unions). A relatively high proportion of FC unions was constituted by patrilineal unions (n=635; 57%). In total, parallel (patrilateral and matrilateral) cousin marriages (n=600; 54%) surpassed cross-cousin unions (patrilateral and matrilateral) (n=512; 46%). Highly frequent type of first cousin unions in the present data was father's brother's daughter (FBD) type (31%) while the least frequent type was mother's brother's daughter (MBD) unions (20 %). The distribution of different types of FC unions with respect to demographic variables is given in Table 2.8.

Table 2.8. Types and distribution of first cousin unions in RYK population

Variable	**First cousin unions**				**n**	**% in total marriages**
	FBD	**FSD**	**MBD**	**MSD**		
Tehsil						
SadiqAbad	166	120	95	93	474	50.4
Rahim Yar Khan	107	90	64	81	342	57.2
KhanPur	76	72	68	71	287	49.7
LiaquatPur	1	3	0	5	9	15.8
Total	**350**	**285**	**227**	**250**	**1112**	**51.1**
Rural/urban origin						
Rural	250	189	146	154	739	53.2
Urban	100	96	81	96	373	47.6
Native language*						
Punjabi	122	118	104	110	454	41.1
Saraiki	212	152	106	129	599	63.9
Educational attainment						
Illiterate	157	125	86	106	474	57.3
Religious education/ *Madarsa*	112	81	76	77	346	54.8
Literate	82	81	65	68	296	41.3

*: Distribution was statistically significant.

2.4.10 Fertility and live births

The proportion of ever-pregnant women in the present study data was 90.90% (n=1958). The mean pregnancies per women were calculated to be 3.94 ± 2.91 (Table 2.9). The fertility rate was higher among consanguineously married female subjects compared to non-consanguineously married females ($p = 0.007$). A total of 7503 babies were delivered alive, and calculated mean of live births/female subject was 3.48 ± 2.61. The proportion of live-born babies per female was higher in consanguineous respondents contrasting non-consanguineous subjects ($p = 0.0064$). Further, in consanguineous females, a greater number of male babies were born as compared to non-consanguineous subjects ($p = 0.0002$). However, both groups showed no discordance in the number of live-born female babies (Table 2.9; Fig. 2.3).

Table 2.9. Subject's fertility and live-births in consanguineous and non-consanguineous unions

Parameter	Consanguineous union	Non-consanguineous union	Total	p value
Average age of subjects	34.99±13.36	35.16±13.31	35.06±13.34	*t*: 0.670
Fertility				
Ever pregnant women (No.)	1,138	820	1,958	
Ever pregnant women (%)	91.9	89.6	90.9	
Total pregnancies (No.)	5058	3423	8481	
Fertility: pregnancy/women (mean±SD)	4.08±2.98	3.74±2.81	3.94±2.91	*t*: 0.007 *
Currently pregnant (No.)	84	66	150	
Currently pregnant (%)	6.78	7.21	6.96	
Live-births				
Total live-births (No.)	4,479	3,024	7,503	
Live-births/women (mean±SD)	3.62±2.68	3.30±2.50	3.48±2.61	*t*: 0.006 *
Live-birth: sons (No.)	2,350	1,505	3,855	
Live-birth: sons (mean±SD)	1.90±1.64	1.64±1.47	1.79±1.58	*t*: 0.0002 **
Live-birth: daughters (No.)	2,129	1,519	3,648	
Live-birth: daughters (mean±SD)	1.72±1.61	1.66±1.59	1.69±1.60	*t*: 0.406

*: Significant at p<0.01; Significant at p<0.001; **: Differences were highly highly significant (analyses were repeated with Mann-Whitney test and Welch's correction).

2.4.11 Prenatal mortality, postnatal mortality, and child morbidity

Among these 1958 female subjects, a total of 978 pregnancy losses/mortalities (prenatal, postnatal-within first year, and total) were observed. Female subjects who contracted consanguineous unions and those who contracted non-consanguineous marriages showed no significant differences in average mortalities. Similarly, the birth rate of child born with a congenital anomaly was the same in respondents belonging to both CU and NCU categories (Table 2.10; Fig. 2.3).

Table 2.10. Prenatal and postnatal mortality and child morbidity in the consanguineous and non-consanguineous unions

Variables	Consanguineous union	Non-consanguineous union	All	p value
Mortalities				
Data available on mothers (No.)	1,239	915	2,154	
Total mortalities (No.)	579	399	978	
Mortality/women (mean±SD)	0.47±1.11	0.44±1.17	0.45±1.14	*t*: 0.528
Prenatal mortality (No.)	316	249	565	
Prenatal mortality (mean±SD)	0.26±0.85	0.27±0.93	0.26±0.88	*t*: 0.658
Postnatal mortality (No.)	263	150	413	
Postnatal mortality (mean±SD)	0.21±0.73	0.16±0.74	0.19±0.73	*t*: 0.169
Child morbidity (No.)				
Mortality in sons	43	36	79	χ^2: 0.295; OR: 0.699 (CI: 0.357-1.37)
Mortality in daughters	41	24	65	
Total mortality	84	60	144	

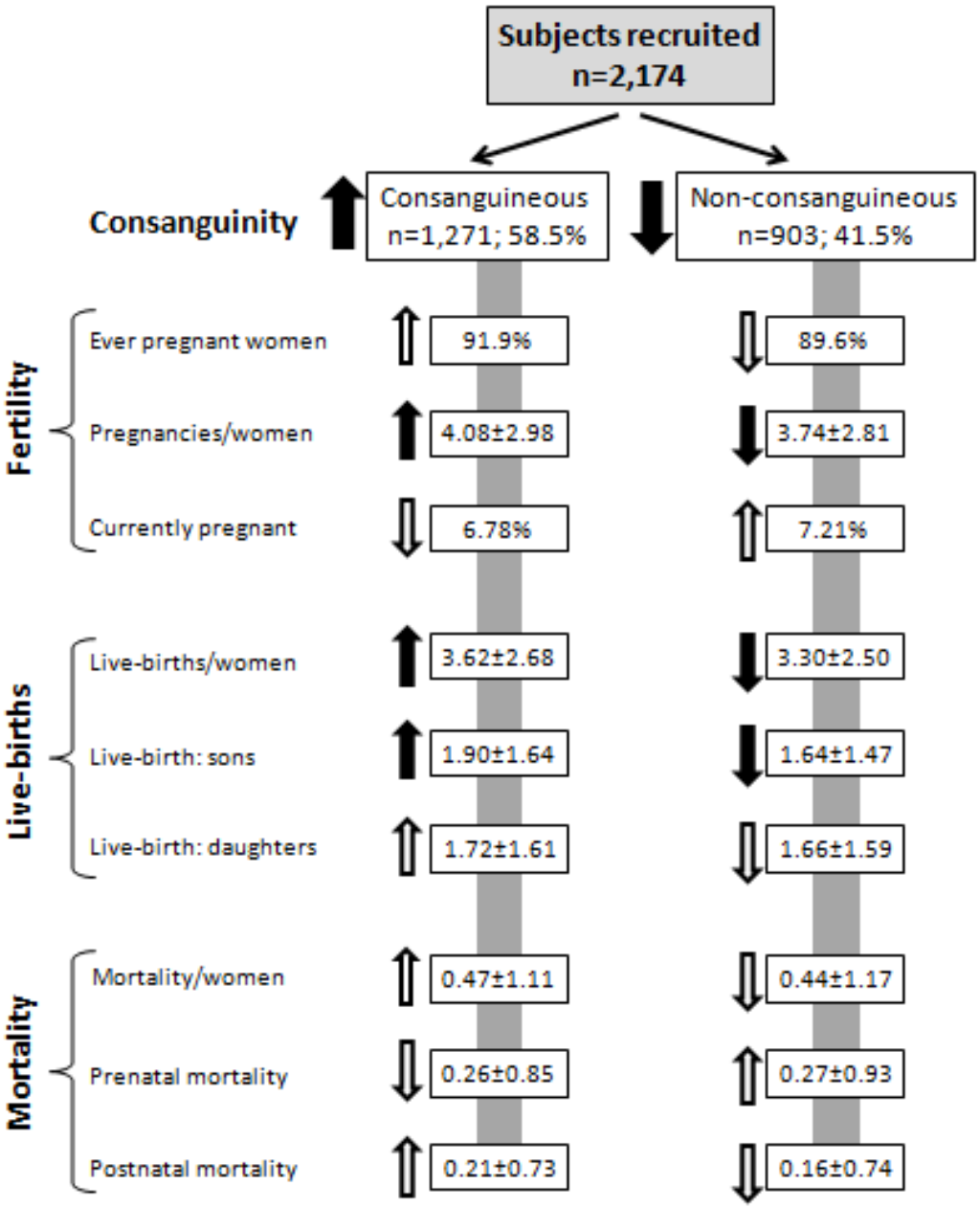

Figure 2.3. A schematic comparison between respondent of CU and NCU categories regarding health impacts (Fertility, Live births, and Mortality).

Up-ward Arrows depict higher and down-ward arrows lower estimates in the respective respondents. Filled arrows show significant distribution while empty arrows demonstrate non-significant distribution between samples of the CU and NCU categories.

2.5 Discussion

For the population of Rahim Yar Khan district, correlation of consanguinity with demographic variables was documented in this study. Among all tehsils of the studied district, the proportion of consanguinity in Liaquatpur tehsil (68.42 %) was high compared to other three tehsils while IC-F in the RYK tehsil (0.0386) was turned out to be higher despite its relative low rate of consanguinity (i.e. 63 %). However, respective estimate of RYK was most conspicuous, which had a relatively low estimate of CU than Liaquatpur. The plausible explanation of the observed discordance between the CU and IC-F could be the high rate of FC unions in the female individuals recruited from RYK tehsil (57.4 %) and high rate of FCOR unions and low prevalence of FC unions in female individuals sampled from the Liaquatpur tehsil (Table 2.1). Additionally, logistic regression (OR= 1.34; $p = 0.006$) analyses also iterated substantial higher rate of CU in the RYK tehsil. Rural community surpassed the urban community (OR 1.29; $p = 0.005$) in the prevalence of CU. In this aspect, this study results were concordant with previous studies (PDHS, 2007).

Among all types of consanguineous unions, FC unions were more prevalent in this study. Hamamy et al. (2005) also reported a high rate of FC unions in all types of consanguineous marriages. In this study, consanguinity was higher in the illiterate subjects (OR= 2.33; $p < 0.0001$). Previous studies also reported a high rate of CU in illiterate female individuals than literate females in some areas of Pakistan (Ahmad et al. 2016a). Interestingly, we also found a high rate of consanguinity in female individuals who had a religious education (OR= 2.12; $p < 0.0001$). The level of consanguinity in this group was almost equal to that in the illiterate category. The literacy rate in women of the current study was as low as 31.5%. Similar literacy level was reported in the census record of the district i.e. 32%. This proportion of female

literacy found in this study was in agreement with that reported in other studies and census record of the district (PSLM, 2009). However, this study found that 28.9% females of the area had attained religious education with no formal schooling. A significant proportion of Pakistani girls attain religious education either by attending religious institutes called Madarsa (mosque) or at a nearby place by the tutor. A number of factors are responsible for the low rate of literacy in the country particularly socio-economic issues including social restrictions, cultural norms, tribal culture, parental illiteracy, unawareness, gender inequality, poverty, lack of facilities, and institutional weaknesses. Moreover, the high growth rate of the Pakistani population, lack of appropriate sources, and inflexible attitudes toward education are the other main contributor of low literacy (L&NFBED, 2010). Hence, literacy exerts a strong impact on the prevalence of CU by means of several interplaying factors.

Economic status of the subjects has a conspicuous impact on consanguinity (Shamshad et al. 2014). In this study, the economic positions of respondents were not inferred directly. However, the occupational status of husbands was used as an indicator of the economic position of the respondents. Among occupational categorization, high rate of CU was seen respondents whose husbands were employed in manual jobs (skilled/unskilled) and agriculture/farming with low pay/income scale. Both the Pay/income scale and literacy rate in people engaged in manual jobs or agriculture farming were low. Moreover, these people normally belonged to rural communities. Conversely, lowest rates of CU were observed in respondents whose spouses were employed in ‘offices/services’ or engaged in ‘businesses/sales’. In these categories, economic conditions and educational level were better contrasting the aforementioned categories. However, in the multivariate logistic regression analyses, spouse’s profession turned out to be an insignificant contributor of consanguinity. In

present data marital unions were predominated by arranged marriages (98%): a type of marriage contraction where parents determine/select potential spouses of their kids as their prime responsibility. Reciprocal marriages involve arranged exchange of spouses between two groups, preferentially of the same lineage. Reciprocally arranged marriages are a traditional practice and have several social and financial advantages including protection of family property, maintenance of the family structure, low demand of dowry, and the ease to arrange a marriage (Saadat, 2007). Age gap of spouses is wide in arranged unions. In the current study data, there was a small presentation (n=34; 1.6 %) of self-arranged or arranged-love marriages. These unions are established by the parents, but the appropriate spouse is chosen by the subjects themselves normally from the relatives. The trend of self-arranged unions is expanding as time advances (Shaw, 2001).

The trend of the extended family system is quite prevalent among rural communities of Pakistan. In joint or extended family system practice of consanguinity is highly widespread. In contrast, this study found a high rate of CU among nuclear family structure. Multigenerational co-habitation in RYK is scarce possibly because couples do not stay with the parental couple and settle themselves in a separate house. This finding contradicts with the findings of other studies carried out in upper areas of Punjab where the extended family system (multigenerational co-habitation) is a common observation owing to several traditional factors and economic issues. A decreasing trend in an extended family system with increased urbanization and economic transformation has been articulated previously (Hussain and Bittles, 1998). No significant association was found between consanguinity and type of household (family system) in Multivariate regression analyses. Category of ‘parental marriage type’ was one of the studied variables which showed a statistically significant

association with consanguinity in female subjects. The coefficient of inbreeding (IC-F) in parents and subjects were 0.0453 and 0.0355 respectively. The organization of marriage types in subject's parents regarding subjects' marriage types showed that the type of parent's marital unions was an important predictor variable of subjects' marriage type i.e. high rate CU in parents led to a substantially higher ratio of CU in subsequent generation individuals, and NCU in parents led to a substantially higher proportion of NCU in the next generation, i.e. subjects. This correspondence in marriage pattern of two generations depicted a traditional and cultural influence. Joseph et al. (2015) reported a high rate of consanguinity in respondents with a positive history of prenatal consanguinity.

Several studies have reported a strong correlation between CU and live births. A previous meta-analysis approach incorporated data of thirty independent studies conducted in multiple communities and depicted a high birth rate in the offspring of CU parents contrasting NCU parents. Present study reiterated the findings of previous studies by showing a higher proportion of live births in CU category. A likely explanation for this phenomenon is the low age of marriage contraction, early pregnancy and prolonged reproductive period in CU (Bittles, 2010). Several confounding parameters are considered to influence fertility rate including socio-economic status, religious beliefs, artificial contraception, marriage duration, and area of residence (Bittles et al. 2002; Hussain and Bittles, 1999). The phenomenon of reproductive compensation, with immediate replacement of a nonsurviving child at an early age can be responsible for increased live birth in CU (Gyimah and Fernando, 2002). No significant difference occurred between the CU subjects and the NCU for female live birth but they existed for male live births in current data set.

Fareed et al. (2017) computed the contributory effect of CU on sex ratio in offspring, i.e. high level of consanguinity linked with high sex ratio. However, no significant link between consanguinity and sex ratio was found by Rao et al. (1984). Studies conducted on large data sets from Denmark and USA (comprising 0.82 and 1.67 million births, respectively) articulated that father's age strongly influences secondary sex ratio, however, maternal age does not exert an impact on it (Jacobsen et al. 1999). The contribution of several social variables in determining sex ratio in societies like early age of marriage, early conception after the marriage has been reported (Barber, 2001). CU and NCU categories showed no discordance with respect to the proportion of children born with congenital anomalies. These results are consistent with previous findings by El-Mouzan et al. (2008) but inconsistent with some other studies particularly the one carried out by Zlotogora (2005). In conclusion, more studies of this type are needed to assess the socio-demographics of consanguinity and its relevant health impacts in neighboring populations. Contrasting with a previous study present study quantified no significant differences in infant mortality in CU and NCU category subjects (Khoury and Massad, 2000). For assessment of prenatal losses in the current study, self-reported data were acquired so there could be an under-representation of the real number of cases. Self-reported data is primarily collected by recall information. In the process of recall information, the error is introduced by the cognitive ability of subjects and other demographic features like age, economic position, education, perception about the gravity of the issue, health and health care access of subject. Lastly, the impact of CU on adult morbidity/mortality needs to be addressed in RYK district. Recently, no association was detected between adult morbidity and consanguinity (Jabeen and Malik, 2014b).

2.6 Conclusions

This study demonstrated that CU were highly frequent in the RYK population, a previously unexplored area in Southern Punjab, Pakistan. There was a significant association between CU and socio-demographic variables like illiteracy, reciprocal marriages, Saraiki language and parental consanguinity. The mean fertility rate was also high among CU subjects. A higher number of mean live-births and a relative high proportion of male babies per subject were seen in CU group contrasting to the NCU group. It would be helpful to monitor the prevalence of congenital and hereditary genetic disorders, adult morbidity and their relationship with consanguinity in future studies.

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3. Pattern of hereditary and congenital disorders in the population of Rahim Yar Khan District, Southern Punjab, Pakistan.

3.1 Abstract

Hereditary and congenital disorders (HDs) are a significant cause of morbidity in Pakistan and have heavy bearings on the neonate, young and adult health. Epidemiological data on the pattern and distributions of HDs are vital for their management and therapeutics. Southern Punjab is an under-developed region in Pakistan and there is no systematic documentation of HDs prevalent in the area.

To draw an overall picture of HDs in the general population of District Rahim Yar Khan, a retrospective descriptive genetic epidemiological study was launched. Recruited individuals with certain types of HDs were physically examined with the help of local physicians and detailed phenotypic data were obtained. Extended pedigrees were generated to assess familial attributes of index subjects.

A total of 231 independent subjects/families exhibiting certain types of HDs were recruited. Among the index subjects (138Males, 86Females), 62.8% were sporadic and 37.2% familial; and 82.7% isolated and 17.3% syndromic. HDs were categorized into 12 broad phenotypic categories, of which five major entities were: neurological defects (n=65; proportion:0.2814; CI:0.2234-0.3394), limb anomalies (n=58), musculo-skeletal defects (n=33), deaf/mute cases (n=31), and visual impairments (n=21). Detailed phenotypic and descriptive genetic analyses of this cohort were performed, and distribution across major socio-demographic attributes of this population has been presented. A wide-range of HDs was witnessed in the study

population which have milder to severe nature. It is need of the hour to incorporate the molecular testing and genetic counseling for HDs into the health-care system and to embark wider public-health measures for their prevention and management in Pakistan.

3.2 Introduction

Hereditary and congenital disorders (HDs) are common among many populations and their prevalence varies across different sub-populations, linguistic and ethnic groups, and socioeconomic strata within the population. They cause lifelong illness (physical or mental) or early death (Lucas et al. 2003). HDs can be severe in nature when affecting vital organ-systems and causing major disabilities, i.e. malformations of the central nervous system, heart, kidneys, etc., or can be milder when they do not seriously affect the normal life functions of the subject, i.e. polydactyly, syndactyly, albinism, etc. HDs may occur as isolated abnormalities or as a part of a syndrome and involve multiple organ-systems (Temtamy and McKusick, 1978; Leppig et al. 1987). HDs may appear earlier in life as soon after birth or later in life. The estimated prevalence of HDs in developing countries is higher than that of developed countries.

HDs are an important cause of neonatal and infant morbidity and mortality (Movafagh et al. 2008). It has been estimated that approximately three million fetuses/infants are born each year with major congenital malformations, which accounted for nearly half a million deaths worldwide in 1997. The surveillance of live-births in large population-based studies has revealed that major HDs have an incidence of 2-3% (Rosano et al. 1987; ORS, 2011). HDs not only cause neonatal/child morbidity and mortality but also have a substantial impact on the young and adult health. For instance, it has been estimated that an approximately equal number of additional major HDs are diagnosed later in life.

In Pakistan, an estimated 6-10% of perinatal deaths are attributed to HD (Korejo et al. 2007). Studies have shown that 40-60% of HDs are of unknown etiology, 20% have hereditarty plus non-hereditary component, 8% are a result of

single gene mutations, 6% are due to chromosomal abnormalities, and 5% are due to maternal factors and pregnancy events (Kalter and Warkany. 1983). The range of socio-demographic factors associated with HDs varies in different populations. For instance, in the experience of Costa et al. (2006), low socio-economic status and low level of literacy were significantly associated with the incidence of HDs.

Studies have demonstrated that HDs most commonly involved brain (10/1,000), heart (8/1,000), kidneys (4/1,000), and limbs (2/1,000) (van Regemorter et al. 1984; Czeizel, 1997; Mattos et al. 1987). Khan et al. (2015) carried out a cross-sectional study at the Khyber teaching hospital in Peshawar. Among 1062 deliveries, 3% of newborns had certain types of HDs. Among the major anomalies, hydrocephalus (23%), anencephaly (13%), and spina bifida (10%) were prominent. Zahra et al. (2016) carried out an observational study on the epidemiology of HDs in Kurram Tribal Agency in northwest Pakistan, which is a population adversely affected by war and political unrest. A total of 246 independent families/individuals with HDs were recruited. In those data, neurological disorders were the most frequent, followed by musculoskeletal defects, limb anomalies, sensorineural/ear defects, ectodermal anomalies, congenital heart defects, and eye/visual impairments. Further, the sporadic occurrence of HDs was more frequent than familial occurrence, and isolated cases were more in high preponderance than syndromic.

In Pakistan, there is no systematic surveillance mechanism for the monitoring of HDs (Jabeen and Malik, 2014b). The national health surveillance system primarily addresses infectious, traumatic and common diseases, while HDs are not focused. In this context, it is indispensable to monitor the pattern of HDs across different geographic regions, ethnicities, and socioeconomic levels in order to determine their burdens on the society and to plan effective intervention approaches. Therefore, the

present study on HDs was conducted in the population of Rahim Yar Khan District, Southern Punjab, Pakistan.

3.3 Subjects and methods

3.3.1 Study population

Rahim Yar Khan (RYK) is a remote district in the extreme south of Punjab province (28.42°N;70.30°E). The District is bounded on the east by Bahawalpur District, on the north by Muzaffargarh District, on the west by Rajanpur District, and on the south by Ghotki District of Sindh province and Jaisalmer District of India (Fig. 2.1). It is comprised of four tehsils (i.e. Rahim Yar Khan, Khanpur, Liaquatpur and Sadiqabad), while there is a long desert strip of Cholistan which extends from north-east to south-east of the district (Fig. 2.1) (PCO, 1988; NRB, 2013).

3.3.2 Methodology and sample ascertainment

In order to get an insight into the common types of HDs prevalent in RYK District, a retrospective cross-sectional study was carried out. Subjects with a certain type of HDs were recruited from District Headquarters Hospitals in the four tehsils of District RYK. Cases were also ascertained through door-to-door surveys and by visiting public places and community centers. It was an observational study in which convenience sampling was conducted due to the availability of resource persons familiar with local languages/dialects, and traveling and logistics options. Only the subjects originating from the District RYK were included in this study and all the individuals who were not the regular members of the surveyed household were excluded. Respondents not consenting to provide complete information were also excluded. Informed consent was obtained from each subject or his/her parents prior to

the data acquisition. For each individual detailed data were obtained on basic demographic variables including personal information, ethnicity, parental consanguinity, family type, etc. All the subjects were physically examined with the help of the resident medical officers and local physicians. Clinical features were documented and photographs depicting the phenotype were taken. Index subjects belonging to the remote and rural areas were brought to the nearest district hospital for clinical evaluation. Pedigrees extending at least three generations were drawn in order to ascertain the familial segregation of malformations, generations with the disease, and the affected sibships. Multiple affected subjects in one family with a particular malformation were considered as one mutational event, and hence, only the index subject in each family was included in the analyses. This study was approved by the Ethical Review Committee of the Department of Animal Sciences, Quaid-i-Azam University Islamabad.

3.3.3 Phenotypic characterization

HDs were categorized into twelve broad phenotypic groups (OMIM, 2017; Temtamy and McKusick, 1978). All the observed anomalies were further classified according to the criteria set by the International Classification of Diseases (ICD-10) and with respect to their closest definitions in the OMIM database (ICD, 2017). Subjects with the involvement of more than one organ-system were identified with respect to the more severe symptoms in the following order: neurological, neuro-muscular, musculo-skeletal, visual, deaf/mute, limb. Index subjects were further categorized with respect to gender, demographic variables, and the sporadic/familial nature of the malformation. Proportions (and 95% CI) of each category were estimated from the total cases. The true prevalence of HDs was not checked in the study population. Limb malformations were further characterized into well-

established phenotypic entities (Malik, 2014). Each entity was further classified with respect to the involvement of upper and/or lower limbs, laterality, symmetry, and axis of involvement. Descriptive summaries were generated and the departure from random distributions was established with Z-test, χ^2 test and Fisher's exact test statistics (at a cut off significance value of $p<0.05$) (Garstman, 2006).

3.4 Results

A total of 231 independent subjects from different families with a certain type of genetic malformations were ascertained. Among these index subjects, there were 138 males (59.7%) and 93 females (40.3%) (Table 3.1). Sporadic cases were in majority compared with the familial presentations (145 vs. 86). Most of the malformations had isolated appearances (n=191), while there were 40 syndromic cases.

All the HDs witnessed in the present cohort were categorized into 12 broad categories. Neurological defects/mental retardation had the highest representation (n=65; proportion: 0.2814; 95% CI: 0.2234-0.3394), followed by limb anomalies (n=58), musculo-skeletal defects (n=33), deaf/mute cases (n=31), and visual impairments (n=21) (Table 3.1).

All the major categories were further resolved according to their nearest definitions in OMIM and ICD-10 databases and hence, at least 48 minor categories could be identified. Table 3.2 presents all the major and minor categories with their proportions, 95% CI, OMIM entries codes and ICD-10 database identifiers (Table 3.2).

Table 3.1. Major categories of genetic disorders, their proportions, 95% CI, and distribution across various attributes

Malformation	All cases	Proportion	95% CI	Gender		Familial nature		Isolated/syndromic nature	
				Male	Female	Familial	Sporadic	Isolated	Syndromic
Neurological/mental retardation	**65**	0.2814	0.2234-0.3394	40	25	23	42	41	24
Limb anomalies	**58**	0.2511	0.1952-0.3070	34	24	13	45	54	4
Musculo-skeletal defects	**33**	0.1429	0.0977-0.1880	20	13	16	17	26	7
Deaf/mute	**31**	0.1342	0.0902-0.1782	21	10	11	20	31	0
Visual impairments	**21**	0.0909	0.0538-0.1280	14	7	13	8	17	4
Thalassemia	**6**	0.0260	0.0055-0.0465	3	3	1	5	6	0
Ectodermal defects	**6**	0.0260	0.0055-0.0465	1	5	5	1	6	0
Growth retardation	**3**	0.0130	-0.0016-0.0276	1	2	2	1	2	1
Lung/pulmonary anomalies	**3**	0.0130	-0.0016-0.0276	1	2	2	1	3	0
Uro-genital defects	**3**	0.0130	-0.0016-0.0276	2	1	0	3	3	0
Cleft lip/palate	**1**	0.0043	-0.0041-0.0128	0	1	0	1	1	0
Lymphedema	**1**	0.0043	-0.0041-0.0128	1	0	0	1	1	0
Total	**231**	**1.0000**	**1**	**138**	**93**	**86**	**145**	191	40

Table 3.2. Major and minor categories of congenital/hereditary malformations observed in the studied population

Malformation (Major/minor)	No.	Proportion	95% CI	OMIM*	ICD-10*
Neurological/mental retardation	**65**	0.2814	0.2234-0.3394		
Mentally retarded	40	0.1732	0.1244-0.2220	300243	F03
Epilepsy	7	0.0303	0.0082-0.0524	607208	G40
Microcephaly	6	0.0260	0.0055-0.0465	251200	Q02
Cerebral palsy (unspecified)	5	0.0216	0.0029-0.0404	605388	G80.9
Congenital hydrocephalus	3	0.0130	-0.0016-0.0276	236600	Q03.9
DOWN syndrome	2	0.0087	-0.0033-0.0206	190685	Q90
Spina bifida	2	0.0087	-0.0033-0.0206	182940	Q05, Q76.0
Limb anomalies	**58**	0.2511	0.1952-0.3070		
Talipes	17	0.0736	0.0399-0.1073	119800	Q66.89, Q66.0
Polydactyly	16	0.0693	0.0365-0.1020	603596	Q69.9, Q69
Brachydactyly	8	0.0346	0.0111-0.0582	112500	Q68.1
Syndactyly	5	0.0216	0.0029-0.0404	609815	Q70.9
Amputations/deficiency	3	0.0130	-0.0016-0.0276	217100	Q73.0, Q72.0
Split hand/split foot anomaly	3	0.0130	-0.0016-0.0276	183600	Q72.7
Camptodactyly	2	0.0087	-0.0033-0.0206	114200	
Leg length discrepancy	2	0.0087	-0.0033-0.0206		Q72.9
Arthrogryposis	2	0.0087	-0.0033-0.0206	108110	Q74.3
Musculo-skeletal defects	**33**	0.1429	0.0977-0.1880		
Muscular dystrophy	12	0.0519	0.0233-0.0806	310200	G71.0
Dwarfisms	7	0.0303	0.0082-0.0524	100800	Q77.4
Ataxia telangiectasia	4	0.0173	0.0005-0.0341	208900	G11.3
Congenital scoliosis	3	0.0130	-0.0016-0.0276	181800	Q76.3
Hunch back	1	0.0043	-0.0041-0.0128	114300	
Congenital absence of both arms	1	0.0043	-0.0041-0.0128		Q71.2
Arthritis	1	0.0043	-0.0041-0.0128	180300	M06.9
Osteogenesis imperfecta	1	0.0043	-0.0041-0.0128	166200	Q78.0
Du Pan syndrome	1	0.0043	-0.0041-0.0128	228900	
Congenital short arm	1	0.0043	-0.0041-0.0128		Q71.8
Spinal muscular atrophy	1	0.0043	-0.0041-0.0128	253300	G12.1
Deaf/mute	**31**	0.1342	0.0902-0.1782		
Mute only	12	0.0519	0.0233-0.0806		R47.0
Deaf-mutism	10	0.0433	0.0170-0.0695	304400	H91.3
Deaf (partial)	8	0.0346	0.0111-0.0582		H91.9
Microtia, external absent	1	0.0043	-0.0041-0.0128	600674	Q17.2
Visual impairments	**21**	0.0909	0.0538-0.1280		
Blindness	5	0.0216	0.0029-0.0404	613216	H54.1

High myopia	4	0.0173	0.0005-0.0341	160700	H52.1
Squint eye	4	0.0173	0.0005-0.0341	231000	Q10
Nystagmus, eye ball problem	3	0.0130	-0.0016-0.0276	164100	H55
Night blindness	3	0.0130	-0.0016-0.0276	247270	H53.6
Usher syndrome	2	0.0087	-0.0033-0.0206	276901	H35.5
Thalassemia (Beta-Thalassemia)	6	0.0260	0.0055-0.0465	613985	D56.1
Ectodermal defects	**6**	0.0260	0.0055-0.0465		
Alopecia universalis	2	0.0087	-0.0033-0.0206	203655	L63.1
Ichthyosis	2	0.0087	-0.0033-0.0206	242500	Q80.4
Dental abnormality/ tooth decay	1	0.0043	-0.0041-0.0128		K02
Ectodermal dysplasia, anhydrous	1	0.0043	-0.0041-0.0128	224900	Q82.4
Growth retardation (childish look)	**3**	0.0130	-0.0016-0.0276	612938	Z00.70
Lung/pulmonary anomalies: asthma	3	0.0130	-0.0016-0.0276	600807	
Uro-genital defects: congenital	**3**	0.0130	-0.0016-0.0276	143400	Q62.39
Cleft lip/palate	1	0.0043	-0.0041-0.0128	119530	Q35
Lymphedema	**1**	0.0043	-0.0041-0.0128	153100	Q82.0

*: Online Mendelian Inheritance in Man, and International Classification of Disease–10 database identifier/Entrez number; CI: confidence interval.

These five categories alone constituted 90% of the total subjects, while 10% subjects exhibited seven other malformation types. Among the five major categories, neurological/mental retardations, limb anomalies, and deaf/mute cases mainly had sporadic presentations, while visual impairments were primarily familial. Among the familial cases (n=86), the affected index subjects were mostly males (55Males, 31Females). In all the familial cases, a total of 292 affected subjects were witnessed (166Males, 126Females) (Table 3.3). Pedigree analyses of the familial cases revealed that the malformations mostly segregated in just one generation (n=50), followed by segregation in two and three generations (25 and 10, respectively). Likewise, the number of affected sibships in the pedigrees were mostly single, followed by two, three, four and five sibships (n=42, 23, 13, 4 and 4, respectively) (Table 3.3).

Table 3.3. Familial cases (n=86): total affecteds, number of disease segregating generations and affected sibships in pedigrees

Malformation	Total affected			Disease segregating generations…				Number of disease affected sibships				
	Male	**Female**	**Both**	**I**	**II**	**III**	**IV**	**1**	**2**	**3**	**4**	**5**
Neurological/mental retardation	38	34	72	14	7	2	0	10	9	2	1	1
Limb anomalies	24	12	36	5	7	1	0	4	5	4	0	0
Musculo-skeletal defects	33	26	59	12	2	2	0	12	1	2	0	1
Deaf/mute	20	18	38	6	2	3	0	5	2	3	0	1
Visual impairments	23	12	35	10	3	0	0	9	3	1	0	0
Thalassemia	1	2	3	1	0	0	0	0	1	0	0	0
Ectodermal defects	11	14	25	1	2	1	1	1	2	0	2	0
Growth retardation	9	7	16	1	0	1	0	1	0	0	0	1
Lung/pulmonary anomalies	7	1	8	0	2	0	0	0	0	1	1	0
Total	**166**	**126**	**292**	**50**	**25**	**10**	**1**	**42**	**23**	**13**	**4**	**4**

3.4.1 Limb malformations

Limb malformations comprised one of the largest group of genetic disorders encountered in the present cohort; there were a total of 72 limb malformations (58 isolated, 14 in associations/syndromic) which could be further classified into nine distinct entities, i.e. clubfoot (n=24), polydactyly (n=20), brachydactyly (n=9), syndactyly (n=8), amputations (n=3), split-hand/foot (n=3), camptodactyly (n=2), leg length discrepancy (n=2), and oligodactyly (n=1) (Table 3.4). There were 52 cases with sporadic presentations while 20 were familial. In the familial cases alone, there were a total of 63 affected individuals. In all the ascertainment categories, the affected males were higher in number than the affected females.

Table 3.4. Distribution of index subjects with limb malformations (n=72) with respect to gender in sporadic and familial presentations

Limb defect	**Index subjects (n=72)**			**Sporadic cases (n=52)**		**Affected family members in 20 families (n=63)**		
	M	**F**	**B**	**M**	**F**	**M**	**F**	**B**
Clubfoot	16	8	**24**	10	7	12	5	17
Polydactyly	11	9	**20**	9	7	13	8	21
Brachydactyly	4	5	**9**	3	2	3	7	10
Syndactyly	5	3	**8**	3	2	3	4	7
Amputations	3	0	**3**	3	0	0	0	0
Split hand/foot	2	1	**3**	1	1	3	0	3
Camptodactyly	2	0	**2**	1	1	4	1	5
Leg length discrepancy	1	1	**2**	1	0	0	0	0
Oligodactyly	1	0	**1**	1	0	0	0	0
Total	**45**	**27**	**72**	**32**	**20**	**38**	**25**	**63**

M: Male; F: Female; B: Both

To appreciate the pattern of affected limbs detailed analyses of cases with limb malformations were carried out. Among the index 72 subjects, a total of 127 limbs were involved, and the involvement of lower limbs was observed to be double than the upper limbs (86 vs. 41) (Table 3.5). The pattern of affected limbs and the combination of involved limbs in distinct limb entities is depicted in Table 3.4. With respect to the literality, the limb defects commonly had bilateral involvement (n=45), of which 30 had symmetrical presentations (Table 3.6). Bilateral involvement was more frequent in the familial cases compared to the sporadic (80% vs. 55.77%).

Table 3.5. Pattern of affected limbs and the combination of involved limbs in subjects with limb malformations

Limb defects	**No. of cases (n=72)**	**Total affected limbs (n=127)**	**Upper limb (n=41)**		**Lower limb (n=86)**		**No. of cases with involvement of ….**			**No. of limbs involved (in 72 cases)**			
			RA	**LA**	**RL**	**LL**	**Arms only**	**Legs only**	**Both**	**Any 1**	**Any 2**	**Any 3**	**All 4**
Clubfoot	**24**	43	0	0	21	22	0	24	0	5	19	0	0
Polydactyly	**20**	36	12	13	6	5	17	6	3	8	9	2	1
Brachydactyly	**9**	13	0	1	8	4	1	8	0	5	4	0	0
Syndactyly	**8**	17	2	2	6	7	2	7	1	1	6	0	1
Amputations	**3**	6	2	2	1	1	3	1	1	2	0	0	1
Split hand/foot	**3**	6	3	1	1	1	3	1	1	2	0	0	1
Camptodactyly	**2**	3	1	2	0	0	2	0	0	1	1	0	0
Leg length discrepancy	**2**	2	0	0	0	2	0	2	0	2	0	0	0
Oligodactyly	**1**	1	0	0	1	0	0	1	0	1	0	0	0
Total	**72**	**127**	**20**	**21**	**44**	**42**	**28**	**50**	**6**	**27**	**39**	**2**	**4**

RA:Right arm; LA:Left arm; RL:Right leg; LL:Left leg

Table 3.6. Laterality and symmetry in limb malformations

Limb defects	**Index cases (n=72)**			**Sporadic cases (n=52)**			**Familial cases (n=20)**			**Total cases**
	Unilateral	**Bilateral**	**Symm.***	**Unilateral**	**Bilateral**	**Symm.***	**Unilateral**	**Bilateral**	**Symm.***	
Clubfoot	5	19	12	3	14	9	2	5	3	**24**
Polydactyly	8	12	7	8	8	5	0	4	2	**20**
Brachydactyly	4	5	5	3	2	2	1	3	3	**9**
Syndactyly	2	6	5	1	4	4	1	2	1	**8**
Amputations	2	1	0	2	1	0	0	0	0	**3**
Split hand/foot	2	1	0	2	0	0	0	1	0	**3**
Camptodactyly	1	1	1	1	0	0	0	1	1	**2**
Leg length discrepancy	2	0	0	2	0	0	0	0	0	**2**
Oligodactyly	1	0	0	1	0	0	0	0	0	**1**
Total	**27**	**45**	**30**	**23**	**29**	**20**	**4**	**16**	**10**	**72**

*: Symmetrical presentations among the bilateral cases.

There were a total of 40 malformations with syndromic presentations. Among the five major types of HDs, deaf/mute cases always had isolated appearance (n=31). Syndromic cases were analyzed in order to identify the common associations. There was the highest representation of neurological/mental retardation cases among the syndromic malformations (n=24; 60%), followed by musculo-skeletal defects (n=7) (Table 3.7). Most common associations were deaf/mute (n=16), followed by limb anomalies (n=15).

In the total sample, 162 subjects belonged to tehsil Sadiqabad, 36 from Khanpur, 29 from Rahim Yar Khan, and 4 from tehsil Liaquatpur (Table 3.8). Detailed distribution of index subjects and familial/sporadic cases is across the key demographic variable is presented in Table 3.8. Distribution was observed to be statistically significant in variables like tehsil, origin, age, mother tongue, occupation, and literacy.

Table 3.7. Associated malformations in syndromic cases (n=40)

Major presentation	No. of cases	Associations								
		Deaf/mute	Limb anomalies	Musculo-skeletal defects	Visual impairments	Ectodermal defects	Obesity	Oro-facial defects	Gastro-intestinal anomaly	Total
Neurological/mental retardation	**24**	9	7	9	4	1	0	0	1	31
Musculo-skeletal defects	**7**	4	3	0	0	0	0	0	0	7
Visual impairments	**4**	2	2	0	0	0	1	0	0	5
Limb anomalies	**4**	0	2	0	0	1	0	1	0	4
Growth retardation	**1**	1	1	0	0	0	0	0	0	2
All cases	**40**	**16**	**15**	**9**	**4**	**2**	**1**	**1**	**1**	**(49)**

Table 3.8. Demographic distribution of 231 index subjects with genetic malformations (with respect to gender and familial/sporadic nature)

Demographic variable	Index subject		Familial attributes		Total
	Male	Female	Familial	Sporadic	
Tehsil/region (n=231)					
SadiqAbad	101	61	57	15	162
KhanPur	19	17	12	24	36
Rahim Yar Khan	16	13	14	15	29
LiaquatPur	2	2	3	1	4
Total	**138**	**93**	**86**	**145**	**231**
	χ^2=1.59; p=0.6612;NS		χ^2=23.87;p<0.0001;S**		
Origin (n=231)					
Rural	11	68	71	17	178
Urban	28	25	15	38	53
	χ^2=23.07;p<0.0001;S**		χ^2=38.15;p<0.0001;S**		
Age range (yrs; n=153)					
≤ 9	77	48	37	88	125
10-19	26	24	19	31	5
20-29	17	1	15	12	27
30-39	9	3	5	7	12
≥ 40	9	8	1	7	17
	χ^2=11.64;p=0.0202;S**		χ^2=8.86;p=0.065;NS		
Mother tongue					
Saraiki	73	41	4	74	114
Punjabi	48	44	36	56	92
Karnali	8	1	6	3	9
Urdu	5	1	1	5	6
Others	4	6	3	7	10
	χ^2=9.25;p=0.055;NS		χ^2=34.29;p<0.0001;S**		
Religion					
Muslim	135	91	85	141	226
Hindu	3	2	1	4	5
	χ^2=0.0001;p=0.99;NS		χ^2=0.65;p=0.42;NS		
Occupation (age ≥16 yrs; n=66)*					
Self-employed (farming, livestock, handicraft)	9	11	7	13	20
Business/shopkeeper	13	2	3	12	15
Students/housewives	8	14	5	17	22
Others	7	2	3	6	9
	χ^2=14.90;p=0.002;S**		χ^2=1.40;p=0.706;NS		
Literacy level (age ≥6 yrs; n=126)*					
Illiterate	11	18	12	17	29
Literate (all)	71	26	19	78	97
	χ^2=12.22;p=0.0005;S**		χ^2=5.72;p=0.0168;S**		

Caste /ethnicity (n=231)					
Araien	42	31	25	48	73
Trali	16	5	12	9	21
Rajput	8	10	9	9	18
Chaudhry	9	5	4	10	14
Mehr	6	8	3	11	14
Malik	6	4	5	5	10
Others	51	30	28	53	81
	χ^2=6.39;p=0.38;NS		χ^2=7.97;p=0.24;NS		
Parental marriage type					
Consanguineous	79	55	48	86	134
Non-consanguineous	59	38	38	59	97
	χ^2=0.082;p=0.77;NS		χ^2=0.271;p=0.60;NS		
Family type/structure (n=231)					
Nuclear	86	61	56	91	147
More than one couple	19	13	10	22	32
Extended family	33	19	20	32	52
	χ^2=0.395;p=0.82;NS		χ^2=0.57;p=0.75;NS		

*: Data of only these subjects was available.
**: Statistically significant.

3.5 Discussion

Studies on the pattern of HDs in the young and adult Pakistani population strata have been largely ignored (Jabeen and Malik, 2014b; Riaz and Malik, 2018). Majority of the population-based studies on HDs have been carried out in the hospital-settings on neonates. In a study in Liaquat National Hospital, Karachi, the incidence of anomalies in live-birth deliveries was 15.8 /1,000 (Shamim et al. 2010). Another hospital based study witnessed a rate of 11.4/1,000 in total births (Parveen and Tayab, 2007). On the other hand, a higher rate of 16% was observed in stillbirths in a university hospital in Sindh (Khaskheli et al. 2007).

The pattern of malformations has been quite characteristics in different studies. In the present study, neurological/mental retardations were observed to have the highest prevalence. A number of studies have also witnessed CNS malformations as the most common group (Parveen and Tayab, 2007). Several other studies reported a higher incidence of musculo-skeletal anomalies (Golalipour et al. 2005). Musculo-skeletal anomalies were the third most prevalent disorders in our present study. In the experience of Shamim et al. (2010), the most common anomalies in the neonates were gastro-intestinal tract (GIT) defects. In our cohort, there was no case of GIT malformation.

Hereditary/congenital limb defects (CLDs) were observed to be the second most common malformations in the present cohort. Limb malformations were further categorized into nine types. Clubfoot was the most frequent observation among the limb anomalies, followed by polydactyly. In various Pakistani population-based studies, polydactyly has been shown to be the most frequent digit defect among the CLDs (Malik et al. 2014; Riaz and Malik, 2018). Polydactyly was also the most common presentation in CLDs among the subjects recruited from the Chitrali

population of Pakistan (Ullah et al. 2015). On the other hand, club-thumb and brachydactyly were observed to be most common in married females from Bhimber Kashmir, Pakistan, and they had estimated prevalence of 10.4/1,000 and 6.93/1,000, respectively (Jabeen and Malik, 2014b). CLDs were also a most prevalent group of malformations observed in a married females sample from Southern Punjab, Pakistan, and polydactyly was observed to be the most common observation (Riaz and Mailk, 2018). In a nationwide survey of infants in Korea, among all anomalies, anomalies of the cardiovascular, musculoskeletal and gastrointestinal system were more prevalent and in decreasing order of frequency (Jung et al. 1999).

Majority of the recruited subjects (82.2%) in the present cohort belonged to age ≤ 9 years. HDs generally have congenital presentations or they appear in the early neonatal period. This observation is consistent with Czeizel (1997) who found that of all congenital malformations diagnosed by the end of the first year of life: nearly 60% are identified in the first month and about 80% by the end of 3 months.

Whereas a detailed phenotypic and descriptive genetic study of the recruited subjects was carried out, the present study, however, did not involve any invasive histopathological and molecular laboratory investigations, i.e. chromosomal and FISH analyses, neuroimaging, cardiograms, ultrasound, MRI, and biopsies. For a more refined clinical characterization of HDs, it would be worthwhile to launch a molecular genetics study of the present cohort.

Data on the prevalence of HDs are readily practical in health care planning and in defining gene pool characteristics, environmental effects, and recurrence risks. Surveillance between different geographic regions, ethnic groups, and socioeconomic levels is justified on the basis of variations and in determining the burden on the family and the society. Surveillance data can be helpful in diagnosis and management

and in evaluating the impact of genetic counseling and prenatal diagnosis (Chung and Myrianthopoulos, 1987). There have been so far no countrywide public health measures for the prevention and management of HDs in Pakistan. Therefore, it is need of the hour to incorporate the molecular testing and genetic counseling for HDs into the tertiary health care medical institutes.

3.6 Conclusion

A wide-range of hereditary and congenital anomalies were observed in the population of Rahim Yar Khan. Among those anomalies, neurological defects, limb anomalies, musculo-skeletal defects, deaf/mute cases, and visual impairments, were prominent. It is need of the hour to incorporate the molecular diagnostics and genetic counseling into the health-care system and to launch measures for their prevention and management.

Publication: The results of this study are in the publication process:

Riaz HF, Mannan S, Malik S. Spectrum of genetic disorders in the population of District Rahim Yar Khan, Southern Punjab, Pakistan. Submitted in Asian Biomed.

4. Phenotypic manifestation of congenital limb reduction deformities in subjects recruited from various localities of Pakistan: clinical and molecular study

4.1 Abstract

Limb reduction defects (LRD) are presented with transverse limb defects or longitudinal limb defects (LLD). They are rare, occasionally sporadic and asymmetrical. Eight cases of transverse limb defects (case series 1) and six cases of longitudinal limb defects (case series 2) were studied.

Case series 1: Terminal transverse limb defects (TLD) are very rare congenital limb disorders affecting the appendages in a transverse orientation. The most common clinical depictions are unilateral, asymmetrical and nonsyndromic forms. Both in syndromic and nonsyndromic forms, there is high clinical heterogeneity. The data on the prevalence and clinical manifestations of TLD is not available for Pakistani population. Eight independent cases with TLD were recruited, 7 of which were nonsyndromic and one was syndromic. The anomalies in these subjects exhibited as unilateral amputation through the palm, accompanied with the short or hypoplastic thumb, mild to moderate shortening of the affected limb, distorted palmer creases, and relatively unaffected contralateral limb or feet. The study provides a comparative account of clinical features of TLD in the Pakistani subjects and draws attention toward the elucidation of genetic causes of these anomalies.

Case series 2: Thumb aplasia is a type of longitudinal limb defects (LLD). Herein, six independently recruited cases with clinical features of thumb aplasia as an

essentially limb-specific phenotype are reported. Phenotype in all cases of thumb aplasia was isolated. Five of six subjects presented sporadic occurrence of the anomaly with no familial occurrence of limb or any other anomaly. The involved arms of subjects showed the absolute absence of first digital rays (shortening of the overall length of the first ray), medial inclinations of middle and little fingers, narrowing of palms, absence of small wrist bones (carpal bones), and reduction in the normal size of zeugopod (forearm bones). Three of the six subjects had parental consanguinity. This isolated form of the condition has not been attributed to any of the gene(s) so far. Investigation of cases with similar clinical characteristics could be helpful to better understand the underlying genetic and molecular basis of the disease.

4.2 Introduction

4.2.1 Characteristics, prevalence and types of limb reduction defects (LRD)

Limb reduction defects (LRD) are the type of defects in which either a part of or entire limb fails to develop normally. LRD affect 2-7/10,000 live births (Ephraim et al. 2003). Upper appendicular elements are more often affected than the lower ones. In the United States, upper limb reductions affect four children every 10,000 while lower limb reductions affect 2 every 10,000 also showing a co-occurrence sometimes (Canfield et al. 2006). Left-sided limb reduction defects show slightly high preponderance than right-sided limb reduction defects (Vasluian et al. 2013). Transverse limb deficiency (TLD) is a subcategory of limb reduction defects as categorized in International Society for Prosthetics and Orthotics (ISPO) classification system (Gold et al. 2011). It is manifested as the disruption in the formation of limb structures through the transverse axis, i.e. across the long axis producing a residual limb (Phadke et al. 2006). TLD can be proximal, intercalary and distal/terminal in their location. Amputations below the elbow are more common (Kozin, 2003). TLDs are less common than the longitudinal limb deficiencies (LLD) which cause malformations along the long axis, i.e. of radial, tibial or fibular and phalangeal bones (Wilcox et al. 2015). A limb reduction defect in which congenital reduction of thumb occurs is known as Thumb aplasia. In thumb aplasia first, digital ray and its components are malformed/not formed altogether.

4.2.2 Clinical Spectrum of transverse limb defects and thumb aplasia

Phenotypic heterogeneity is evident in TLD and the malformation ranges from mild to severe types. The spectrum of TLD extends from unilateral slightly reduced

terminal phalanges (autopod reduction) to the absence of entire limbs, i.e. tetramelic deficiency in transverse orientation (through the stylopod, i.e. humerus/femur). Other intermediate clinical grades may also occur, for instance, aphalangia, adactylia, acheiria, achiropodia and amelia. This disrupted growth/ maldevelopment usually produces malformation of some or all below wrist structural elements (Drapkin et al. 2003; Yiltok et al. 2008).

Bilateral transverse limb defects are extremely rare. Leftness of congenital upper limb deficiencies is more frequently observed feature. Typically, the transverse deficiencies are asymmetric and non-syndromic, yet specific patterns of deficiencies are reported to be a part of certain syndromes (Agarwal et al. 2015; Nayar et al. 2017). Patients with the isolated case may lead normal lives than those with a syndromic disease appearance. Serious TLD of all limbs may occur with congenital heart malformations (Digilio et al. 2015). Another syndromic form of TLD is Adams-Oliver syndrome (AOS; OMIM 100300): a combined clinical manifestation of terminal limbs malformations and scalp defects alike those characteristic of aplasia cutis congenita (ACC; OMIM 107600, 207700; Bacchelli et al. 2001; Sankhyan et al. 2006). AOS is variably expressed and shows low penetrance (Papadopoulou et al. 2008).

Thumb aplasia depicts clinical heterogeneity (OMIM 188100). The level of affection ranges from a slight shortening of the thumb size (thumb hypoplasia) to no thumb at all (Thumb aplasia) with some intermediate grades between these extreme ends (Riley and Burgess, 2009). It is further categorized into five types with increasing severity of thumb defect from one end/type to the other end/type (Blauth and Schneider-Sickert, 1981; James et al. 1996). Complete retardation/loss of the first finger i.e. thumb, classified as type V, is a rare anomaly. It affects 0.25/10,000 births

(McGuirk et al. 2001). Males are more affected by this anomaly than females. This condition is bilateral in about 2/3 of the cases (Castriota-Scanderbeg and Dallapicolla, 2005).

Thumb agenesis appears both as an isolated situation and syndromic forms. Majority of the isolated thumb aplasia cases are sporadic. In familial appearances, it shows the autosomal dominant mode of inheritance particularly when the disease is syndromic (OMIM, 2014; Temtamy and McKusick, 1978). In the majority of the syndromic appearances, it co-occurs with radial deficiency of varying degrees Additionally, it accompanies VACTERL association (OMIM 192350), thrombocytopenia-absent radius syndrome (OMIM 274000), Holt-Oram syndrome (OMIM 142900), Seckel syndrome (OMIM 210600), phocomelia, Duane-radial ray syndrome (OMIM 607323), and Fanconi anemia (OMIM 227650). Cardiac manifestation is very common in syndromic forms of thumb aplasia. Skeletal, renal, ocular and hematological organs are also affected in the majority of its syndromic conditions (Manske and Goldfarb, 2009; Riley, 2008; Elmakky et al., 2015).

4.2.3 Adams-Oliver syndrome: a syndromic presentation of TLD

AOS is a phenotypically heterogeneous and very rare disorder (Madan et al. 2015). AOS consists of terminal TLD as a cardinal clinical presentation in patients ranging from mild-to-severe as distal tapering to the complete loss of the hand or foot. In AOS, lower limbs (78%) are more frequently involved compared to the upper limbs (59%) (Seo et al. 2010). Syndactyly, oligodactyly, and camptodactyly are often accompanied with AOS. Proximal parts of autopod/entire limb are less commonly involved (Kocer et al. 2001; Yagci-Kupeli et al. 2011). Contrastingly, longitudinal reduction abnormalities as hypoplastic radius or fibula are not observed in AOS (Isrie

et al. 2014). AOS in a majority of the patients occurs sporadically while in a few cases it shows familial occurrence, therefore, possessing a recurrence risk not greater than that of the general population for subsequent pregnancies (Narang et al. 2008; Mendiratta et al. 2017). In AOS, limb anomalies can be unilateral or bilateral including a higher frequency of bilateral abnormalities.

Aplasia cutis congenita (ACC) in association with congenital scalp defect, is the second extensively seen phenotype depicted in AOS malformations (Verdyck et al. 2003). ACC's phenotypic occurrence is also variable ranging from isolated clinical anomaly to complex presentation involving other congenital disorders. ACC lesions are usually in the midline of scalp vertex, but sometimes symptoms may include lesions on the appendages and abdomen. Occasionally, these lesions may appear like a healed scar at the time of birth. In AOS, smaller ACC lesions usually involve merely the skin and are cured within months, however; larger lesions more often involve the skull and probably the dura, and are highly prone to complications like infection, hemorrhage, or thrombosis, and may lead to mortality and morbidity (Udaykumaran et al. 2013). In ACC, skin membrane by being thin and clear allows visibility of covered structures through it. Other major anomalies most frequently involve cardiovascular malformations/dysfunction, brain anomalies, and rarely renal, liver, orofacial defects and eye defects in addition to the typical combination of TLD (Major and minor criteria for the diagnosis have been proposed. The combination of two major anomalies confirms the presence of AOS whereas co-appearance of a major and a minor anomaly provides a suggestive indication of the disease. AOS may or may not affect the lifespan of the bearer (Snape et al. 2009; Peralta-Calvo et al. 2012).

4.2.4 Differential diagnosis of Adams-Oliver syndrome

Spectral overlap of Adams-Oliver syndrome with other well-characterized malformations makes its diagnosis challenging and pose a great difficulty in diagnosis, classification, genetic counseling and risk approximation. It shares a wide array of phenotypic manifestations with aplasia cutis congenita (ACC; OMIM 107600), Poland syndrome (OMIM 173800) and cutis marmorata telangiectatica congenital (CTCC) (OMIM 219250). Therefore, phenotypic manifestations of the disease should be carefully examined (Lehman et al. 2016).

4.2.5 Pathogenesis of TLD

Disruption of the vascular system causing developmental arrest has an important effect in the isolated or syndromic case of TLD (Piazza et al. 2004; Renfree and Dell, 2016). Most cases of limb defects arise due to the vascular insult occurring in a vulnerable period of embryonic life, i.e. 4-6 week of gestation when the development of limb begins (Saeed et al. 2011). Rarely, a traumatic pressure during early pregnancy also causes the condition. Prenatal testing techniques like chorionic villus sampling also increase the incidence of such disorders (Golden et al. 2003). The most popular etiologic concept for unilateral deficiencies is that of abnormal pressure exerted during embryonic development by inflammation, or by decrease or absence of amniotic fluid, or by amniotic adhesions. A proposed mechanism explains that amniotic band sequence (OMIM 217100) is a non-Mendelian disorder in which congenital reduction anomalies of the limbs arise in conjugation with constriction ring bands by strands at the amniotic surface or opening (Pedersen and Thomsen, 2001).

The occurrence of dilated veins in the scalp of patients and vascular changes in the skin of AOS patients like CTCC (cutis marmorata telangiectatica congenital;

OMIM 219250) hint that situation may be the result of vascular disruption/interruption (Pereira-Da-Silva et al. 2000; Frantz et al. 2015). Co-occurrence of Poland anomaly with AOS also hints toward it being a vascular disease (Al-Qattan, 2001).

Early onset embryonic disruption is a suggestive underlying mechanism of AOS (Becker et al. 2002). Limb development is completed in the 8th week of gestation starting by the 4th week. A damage by an external agent capable to cause a gene alteration or developmental arrest happens within 4-6 weeks after conception, at a very early time when even women are unaware of their own pregnancy (Loder, 2004). The interaction of upper ectodermal cell layer (called AER; apical ectodermal ridge) with the underlying mesoderm derives limb development in proximo-distal (i.e. from humerus/femur-hand/foot) direction (Niswander, 2003). They are ascribed to a number of factors like embryologic differences, hormonal impact on development, vascular irregularities, and/or sporadic genetic interruptions (Modrcin and McLaughlin, 2013, Ordal et al. 2016).

People with congenital limb reduction defects need proper counseling regarding their problem and an effective intervention strategy to improve the status of life. Physical therapy is used to improve the reduced limb's functioning. Upper limbs extremities function in a complicated manner each working relatively independently of the opposite one. In upper extremities prosthesis are used to improve cosmetic appearance and to enhance reduced function. In lower extremities functioning is improved by the use of prostheses to enhance walking, standing or running ability (Kozin, 2004; Hermansson et al. 2005).

4.2.6 Genetic factors underlying TLD and thumb aplasia

The genetic basis and molecular pathogenesis of isolated cases of this disorder are not yet clearly understood. Taking genetic attributes into consideration, less is known about isolated TLD. Limb reduction defects exhibit inconsistent inheritance patterns (Shaheen et al. 2011). Symmetrical TLD affecting the four limbs or bilateral upper or lower limbs may suggest a genetic predisposition. Malformed AER may give a rise to transverse limb defects in embryonic stages. Several signaling mechanisms are entailed in the TLDs like Rho and Rac signaling involved in Adams-Oliver syndrome (OMIM 100300). However, abnormal bone morphogenetic protein (*BMPs*) pathway and *NOTCH1* mutation have also been reported in patients with the autosomal dominant inheritance of disease (Stittrich et al. 2014). Autosomal dominant forms of AOS have been found to be caused by the mutations in *DLL4*, *ARHGAP31*, and *RBPJ* genes (Meester et al. 2015). Mutations in the *DOCK6* (OMIM 614194) and *EOGT* gene have been linked with a recessive form of AOS (OMIM 614789) (Sukalo et al. 2015; Shaheen et al. 2013). The identification of genetic factors underlying TLD may lead to improved risk estimation, better genetic counseling of affected subjects/families, and further understanding of limb morphogenesis. Candidate genes for selected types of limb reduction defects are listed below (Table 4.1).

Table 4.1. Candidate genes for some limb reduction defects

Limb reduction defects	**Candidate genes**
Terminal limb deficiency	*LMBR1-SHH*
Adams-Oliver syndrome	*EOGT, ARHGAP31, RBPJ, DLL4, DOCK6*
Acheiropody	*C7orf2*
Split hand and foot malformation	*TP63, WNT10B, FBXW4, DLX4, DLX5, DSS1*
Holt-Oram syndrome	*TBX5*
Fibrodysplasia Ossificans Progressiva (FOP) Terminal transverse limb defects	*ACVR1, G328E*
Moebius syndrome	*REV3L, PLXND1*
Oromandibular limb hypogenesis syndrome	*MSX2*

Mutations in various genes like *SF3B4*, *FANCE*, *ATR* and *WNT7A* have been implicated in acrofacial dysostosis-1, Fanconi anemia, Seckel syndrome-1, and fibular aplasia with poly-,syn-,oligodactyly, respectively, suggesting their possibel involvement in thumb pathomorphogenesis (OMIM, 2014).

The data on the prevalence and clinical manifestations of limb dysmorphologies/anomalies is largely scarce in Pakistan (Assir and Waseem, 2012; Malik and Afzal, 2013b). In this study, we present seven cases of isolated congenital TLD, one case of syndromic TLD and six different cases with thumb agenesis. All of the cases showed sporadic appearance. Further, the genetic basis and molecular pathogenesis of these disorders are also not yet clearly understood. The current study was meant to draw attention to the importance of studying the characteristics/dynamics of the deformity and search for etiologic factors to provide baseline data for efficient diagnostic and therapeutic modalities.

4.3 Subjects and methods

Fourteen subjects (10M, 4F) with limb reduction deficiencies (LRD) were recruited during the study period from different localities of Pakistan. Eight subjects possessed clinical appearance like transverse limb defects (TLD) and six subjects had clinical phenotype like thumb aplasia. In eight subjects with TLD, seven individuals (5M, 2F) had isolated TLD and one male infant subject had syndromic TLD (Tables 4.1, 4.2). Six subjects (4M, 2F) had thumb deficiencies (Tables 4.3, 4.4). All the 6 cases showed isolated forms of thumb agenesis. Clinical assessments of the cases were carried out with the help of specialized doctors and orthopedics. Clinical categorization of TLD subjects was based on Blauth and Gekeler (1971) classification system and clinical classification of the cases with thumb agenesis was in accordance with the revised scheme of hypoplastic thumb by Blauth and Schneider-Sickert (1981) and James et al. (1996). Ethical clearance of the study was provided by the Ethical Review Board of the Quaid-i-Azam University, Islamabad. Physical examination to check the integrity of vital organs was done in addition to the clinical assessment of limbs. Physical and anthropometric measurements were acquired accordingly. Photographs of all subjects and radiographs of selected cases were obtained. Prior to the data acquisition, a formal consent of subjects or their guardians was acquired. All the collected data was in accordance with the declaration Helsinki-II. A comprehensive description of socio-demographic and biological parameters was also taken. Three generational pedigrees were drawn for each subject to ascertain the mode of inheritance. Information regarding inheritance of any other congenital anomaly in the family was also obtained. Data about types of parental marital unions and inbreeding coefficient (IC-F) was measured in order to account for a likely recessive

inheritance. Photographic images of all thumb aplasia patients's affected hands were taken while radiographs of two patient's hands were obtained.

In seven cases, TLD of hand was essentially unilateral and there was no previous familial occurrence of appendicular or any further congenital defect. However, one case depicted the syndromic appearance of TLD. Transverse amputations of different grades were present in 8 study subjects resulting in absence of four digits, mostly postaxial (Table 4.1). The subjects also showed a length discrepancy in the amputated arm as compared to the opposite arm. Moreover, they exhibited an abnormality of palmer creases. In six of the eight subjects, the feet showed no sign of anomaly. Intellectual ability of all seven subjects was quite normal and no other deformity was noted in the detailed physical evaluation. The patients experienced great limitation in their daily life activities.

4.3.1 Case series 1: Transverse limb defects

The data on socio-demographic and biological attributes of the recruited cases including family history of any congenital anomaly, parental consanguinity, sibship composition, and phenotype were observed (summarized in Tables 4.2, 4.3).

Table 4.2. Demographic features, parental consanguinity and family attributes of recruited cases with TLD (case series 1).

Cases	Gender	Age(yrs)	Geographic origin	Language/Caste	Parental consanguinity	Paternal and maternal age at subject's birth (year)	Subject's parity	Subject's normal sibs (Brother:Sister)
I	Male	1	S.Punjab	--	First cousin	24/19	5 of 5	0:5
II	Male	7	S.Punjab	Punjabi /Arain	Distantly related	40/38	4 of 4	0:1
III	Male	20	I.Sindh	Saraiki/Lashari	First cousin	20/18	1 of 5	1:3
IV	Female	8	U.Punjab	Saraiki /Laang	Distantly related	29/22	1 of 3	1:1
V	Female	16	S.KPK	Pushto/Pathan	Non-related	27/23	1 of 7	4:2
VI	Male	12	N.KPK	Shauteye/Khowar	Non-related	29/28	3 of 4	1:2
VII	Male	8	N.KPK	Pushto/Swati	Non-related	37/30	7 of 9	3:5
VIII	Male	6	S.Punjab	--	Non-related	34/29	1 of 3	0:2

I: Interior; N: North; S: South; U: Upper

Table 4.3. Phenotypic characteristics of recruited cases with transverse hand amputation (isolated/syndromic) (case series 1).

Cases	Amputation axis	Affected hand	Fingers	Thumb	Affected arm size	Contralateral arm	Others	Isolated/ syndromic
I	Palm, median	Right	Bead-like 5th finger with short nail	Rudimentary	Moderately reduced	Unaffected	one chondrogenic island	Isolated
II	Palm, median	Left	Bead-like remnants of fingers 2-5	Distal hypoplasia, short nail	Moderately reduced	Mild shortening of zeugopod and stylopod	Carpals absent; hypoplastic metacarpals	Isolated
III	Palm, proximal	Right	Digits 2-5 absent	Short, distal symphlangism	Shortly reduced	Medial inclination of 2nd digit; crowding of carpals	Fused carpals; metacarpals 2-4 not visible; reduced metacarpal 5	Isolated
IV	Palm, median	Right	Bead-like remnants of fingers 2-5	Distal hypoplasia	Moderately reduced	Left thumb with extra palmer creases	--	Isolated
V	Palm, median	Left	Digits 2-5 absent	Not affected	Shortly reduced	Unaffected	Swelling on left throat	Isolated
VI	Palm, proximal	Left	Digits 1-4 absent, 5th digit present	Missing	Moderately reduced	Unaffected	--	Isolated
VII	Phalanges, proximal	Left	All fingers affected	Distal hypoplasia, short nail	Unremarkable	Unaffected	--	Isolated
VIII	Phalanges, proximal	Both	Short and irregularly amputated fingers, R: 2nd-4th L:5th	short with tapering ends	Unremarkable	Unaffected	Disorganized dermatoglyphics, hypoplastic carpals, toe symbrachydactyly, aplastic/hypoplastic proximal and distal phalanges, hypoplastic tarsals/metatarsals, defected scalp	Syndromic

L: Left; R: right

4.3.2 Clinical and radiographic study

The phenotypic features observed among the recruited cases are presented below:

4.3.2.1 Case I

The subject is a male neonate, born to a consanguineously married couple. Inbreeding coefficient was F = 0.0625. The subject had congenital transverse deficiency across the palm of right hand lacking all digits. The overall length of the right arm was reduced (Fig. 4.1A, 4.1B). Right hand of the neonate was amputated and lacked all the digits with a small and solitary globule-like nubbin on the radial side. The nubbin had a misshaped convex nail and was appended to the hand by a skin-stalk. In addition, a rudimentary 5th finger was appended to the posterio-distal aspect of the amputated hand (Fig. 4.1B). The affected arm had normal movement of wrist and elbow joints. Palmer ridges were almost completely absent and the digital triradius pattern was also not recognized (Fig. 4.1.B).

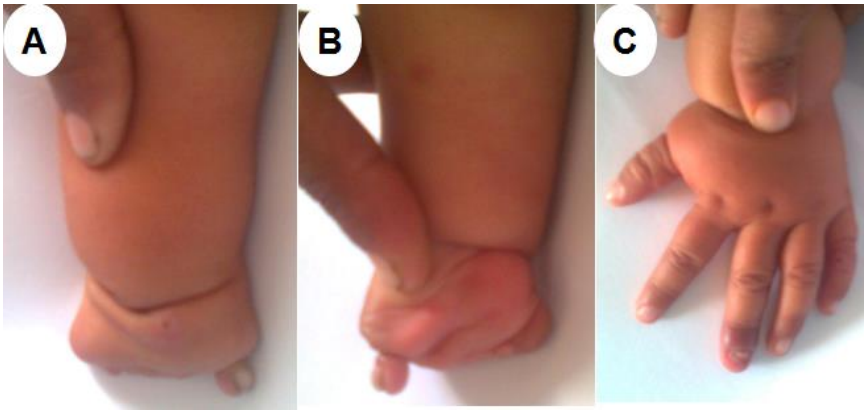

Figure 4.1. Illustration of upper limbs of case I.

(A): Dorsal view of the right hand showing terminal transverse reduction and a solo nubbin, **(B):** Ventral view of the right hand presenting vestigial 5th digit at the distal post of the truncated palm, **(C):** Dorsal view of the left hand. A nail bed with a newly emerging nail is visible in third digit.

Roentgenographs of the amputated arm showed slightly reduced humerus with hypoplastic phalanges (Fig.4.2A; Table 4.3). The radius and ulna were dysplastic and closely aligned. In the middle arm, ulna was shortly reduced. Radius and ulna showed hypoplastic distal ends while no sign of hypoplasticity or any other anomaly were noted in their proximal ends and they were articulated normally (Fig.4.2A). Carpals and metacarpals were present in the form of a solitary and immature chondrogenic island. All structural elements of left arm appeared unaffected without showing any sign of stunted growth (Fig.4.2B). Roentgenographs showed two carpals and five metacarpals. Phalangeal growth was also normal (Fig.4.2B). No sign of any other skeletal deformity, orofacial manifestation or neurological disorder was observed. He showed normal developmental milestones. All other individuals of the kindred were otherwise normal with no sign of limb defects or any inherited disorder.

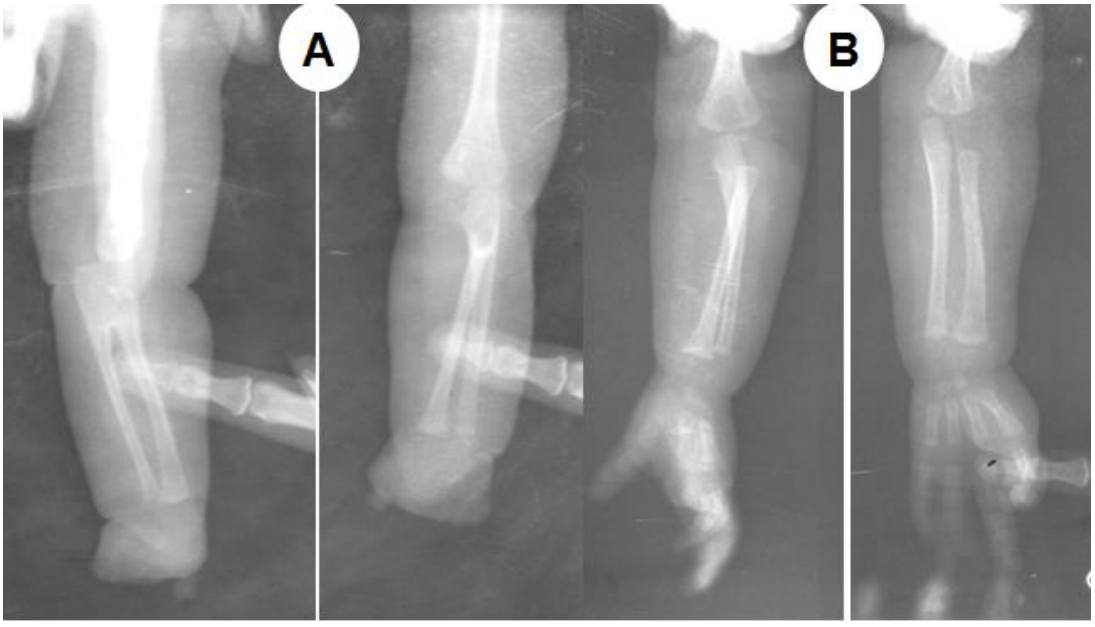

Figure 4.2. Radiographic illustration of upper limbs of case I
(A): Right arm, **(B):** Left arm.

4.3.2.2 Case II

The individual was youngest than his siblings. His parents were distantly related and his mother was two years younger than her husband. It was reported that her first pregnancy had three years delay and her first two daughters died within six months after birth. The male patient was examined to have TLD through his left palm. Tiny bead-like remnants were present at his 2-5 digits and his palmer creases were distorted (Fig. 4.3A, B).

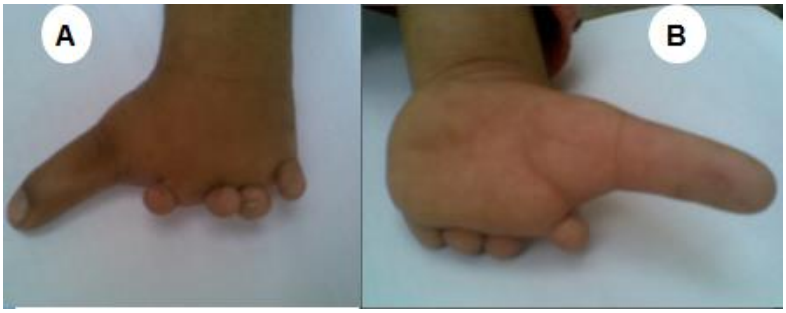

Figure 4.3. Phenotypic illustration of the left hand in case II
(A): Dorsal view, **(B):** Ventral view.

Roentgenographs of the right hand depicted carpals aplasia/hypoplasia, metacarpals aplasia, phalangeal hypoplasia at the distal post, and slight reduction of the size of radius/ulna (Fig. 4.4).

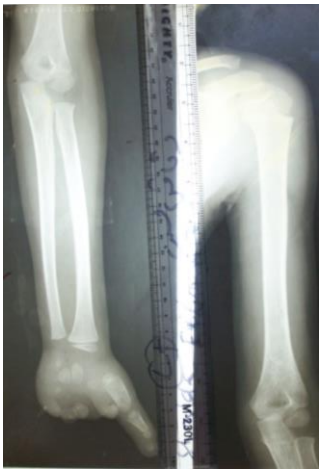

Figure 4.4. Roentgenographic presentation in case II.

4.3.2.3 Case III

The individual was at the fifth number in parity order. His parents were first cousins. This male subject had transverse reduction defect of the upper right limb. Hand of this limb was amputated through the palm. In the affected hand thumb was short (Fig. 4.5A). Roentgenography revealed loss of several carpals and metacarpals; a little osseous peg supplanted the fifth metacarpal, and only present first digital ray depicted distal symphalangism (Fig. 4.5B). Radiographs of the contralateral hand revealed inclination of the index finger of intermediate intensity. In the left hand, wrist carpals were crowded like a mass of bones.

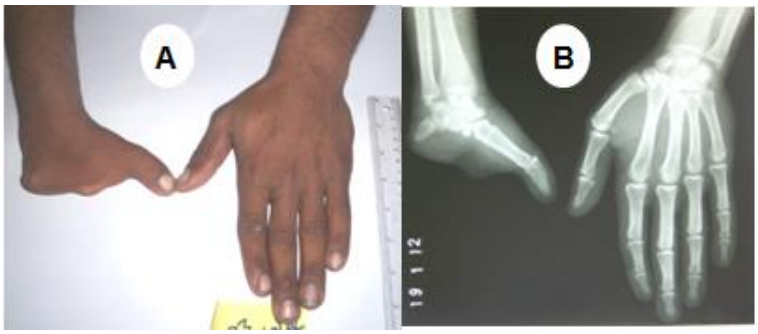

Figure 4.5. (A): Clinical photographs and **(B):** roentgenographs of hands of case III.

The radiographic study showed the bilateral involvement of feet with characteristically hypertrophic first digital ray accompanied by hallux valgus (Fig.4.6).

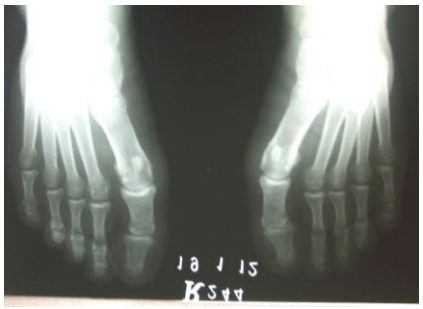

Figure 4.6. Radiographs of feet of case III.

4.3.2.4 Case IV

The female subject was elder in a sibship of three. Her parents were distantly related. Right hand showed TLD with four fingers which looked like nubbins. The subject had a hypoplastic thumb with tapering end. Dermatoglyphics were also distorted (Fig.4.7A, 4.7B).

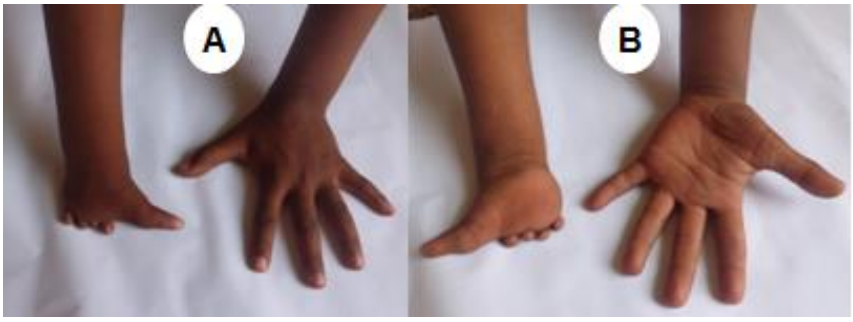

Figure 4.7. Phenotypic illustration in case IV
(A): Dorsal view of hands, **(B):** Ventral view of hands.

4.3.2.5 Case V

The female subject was the first child born to the nonrelated parents. The subject showed TLD of the left hand along the middle axis of palm. Fingers 2-5 were totally absent. There was a normally formed thumb (Fig.4.8).

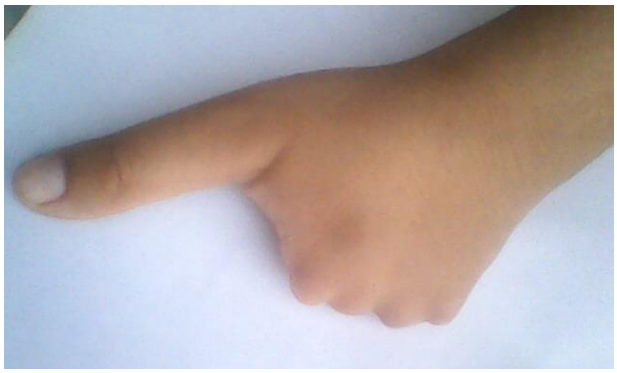

Figure 4.8. Phenotypic illustration in case V

4.3.2.6 Case VI

The male patient was the third offspring of nonrelated parents. There was TLD of his left hand affecting it through the proximal plan of palm. Digits one through four were entirely absent and only digit 5 was present (Fig. 4.9). There was a noticeable reduction in total length of the left arm.

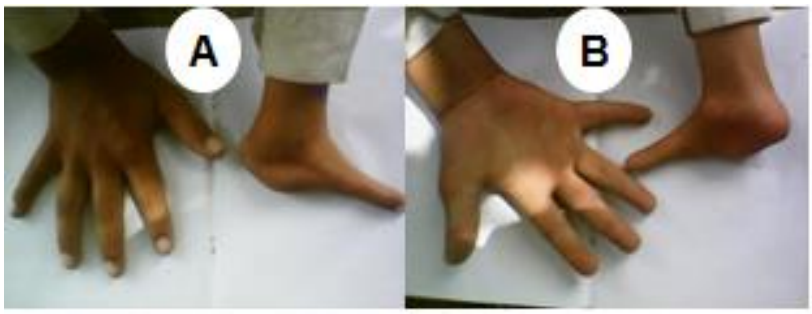

Figure 4.9. Phenotypic illustration of hands in case VI.

4.3.2.7 Case VII

The male individual was at 7th position in a sibship of nine. There was TLD through the left hand (Fig. 4.10). Digits from second to fifth were affected at their proximal posts. The patient had a negative family history. Thumb had a distal deficiency.

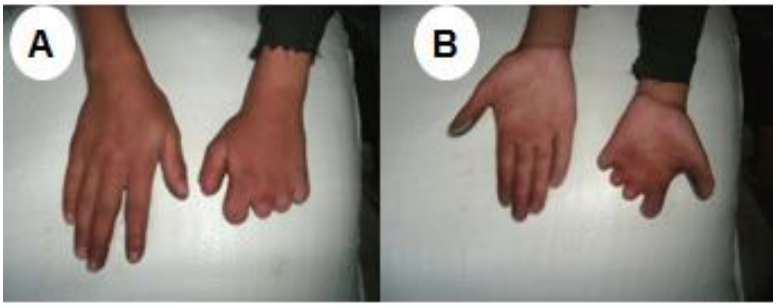

Figure 4.10. Phenotypic illustration of amputated hands in case VII.
(A): Dorsal view, **(B):** Ventral view.

4.3.2.8 Case VIII

Subject was elder of two other apparently normal sibs. His prenatal history had been unremarkable and delivery was uncomplicated. The patient showed bilateral terminal reduction defect of digits (Fig. 4.11A,B). In his right hand, fingers 2-4 were tiny and showed an irregular pattern of amputation affecting the mentioned phalanges in the transverse plane. In the left hand, amputation affected only little finger along proximal inter-phalangeal (PIP) joint. There was a bilateral shortening of thumbs heights with tapering ends. In the right-hand dermal ridge pattern abnormalities like triradii disorganization was depicted in deficient people while in contralateral hand, palmer creases were not markedly formed. In addition, the fingers 2-4 showed three interphalangeal creases between disproportionate phalangeal segments (Fig. 4.11B). Palmar surface of the right thumb revealed two interphalangeal creases while left thumb revealed only one.

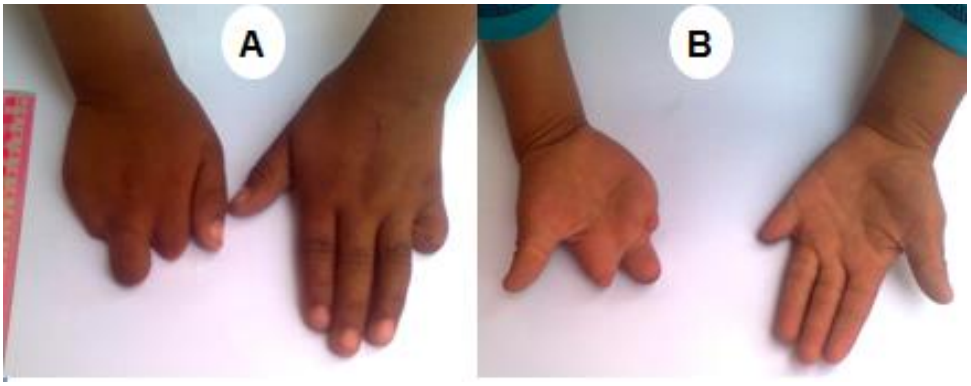

Figure 4.11. Phenotype in case VIII
(A): Dorsal view of hands, **(B):** Ventral viewof hands.

Radiographs revealed several abnormalities of his upper right autopod. Radiographic images/plates of his right hand revealed that there were only two wrist bones, i.e. carpals. These carpal bones were hypoplastic and malformed (Fig. 4.12). Hand plate had five metacarpals. Four metacarpals (1-4) were normal while the fifth

showed underdeveloped epiphysis. There was thinning and hypoplasticity of the fourth digital ray. The second digit was tapering at its terminal edge and its middle and terminal phalangeal bones were grossly absent/dysplastic. Digit four and five showed similar manifestation. In third digit distal phalanx was completely lost. Roentgenographs of his left hand depicted that the first metacarpal had a thick and broad structure while the second metacarpal had a hypertrophic proximal base (Fig. 4.12). Proximal phalanx of the digit 5 was hypoplastic while total dysplasia of middle and terminal phalanges was monitored. Moreover, all digits had typical hypoplastic distal phalanges.

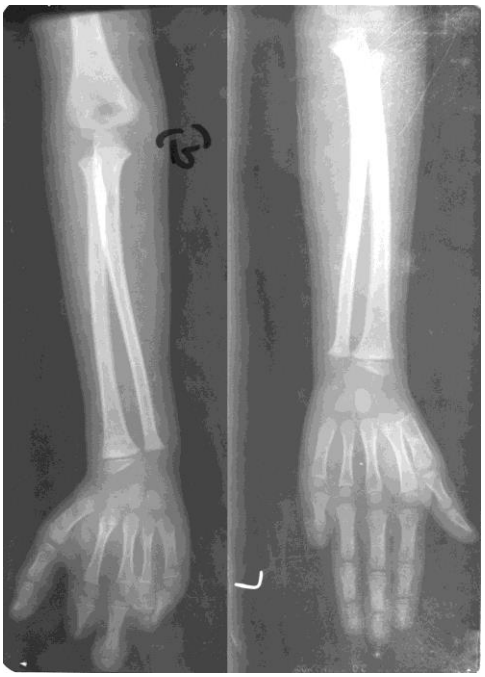

Figure 4.12. Roentgenographs of forearms of case VIII,
Autopods with dysplastic carpals and amputated phalanges; Zeugopods with hypoplastic radio-ulnar distal terminals and immature epiphyses.

Bilateral symbrachydactyly of toes with a variation in expression was noted in the feet. In both feet, varus deviation of halluces was evident. In his right foot, second through fifth toes were cutaneously webbed together while in his left counterpart,

second and third toes were partially fused and this webbing was partial (Fig. 4.13). His toes were short particularly those involved in webbing. They were totally devoid of nails. There was severe bilateral hypoplasia/absence of distal and proximal phalangeal components of the toes (Fig. 4.13). There were widely spaced hypoplastic tarsals. Unusual hypertrophy of first digital rays was evident bilaterally. Radiographs of the dorsal view of his feet revealed bilateral overriding and crowding of metatarsals 2-5. (Fig.4.13).

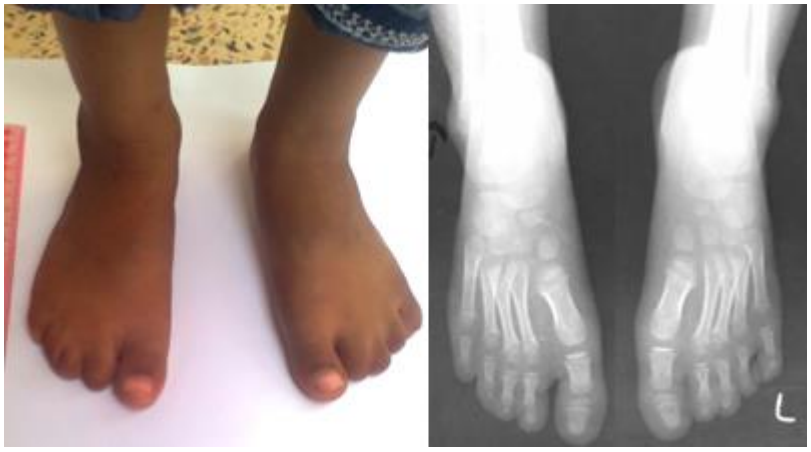

Figure 4.13. Phenotypic and radiographic illustration of feet in case VIII

Additionally, the physical evaluation revealed that the patient had scalp deformity. Skin patch in the mid-scalp area was alopecic, atrophic and was unable to perspire due to the presence of a lesion in antero-posterior and lateral dimensions of 1.2cm and 5cm, respectively. The lesion scar characteristically had a heterogeneous and rough surface (Fig. 4.14).

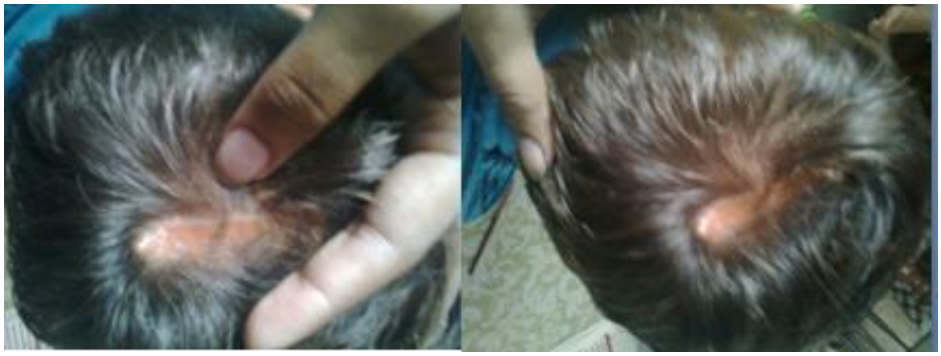

Figure 4.14. Photographs of mid-scalp hairless skin patch

It was reported that at birth the child was presented with a congenital open wound in the atrophic area. The scalp scar healed progressively and was replaced by a raised lymphoma. This lymphoma was removed by surgical procedure after three years leaving a postoperative wound which was completely healed and the healing process was unremarkable. There were no other systemic manifestations, i.e. orofacial, neurological, skeletal and internal organs. The patient is otherwise healthy and attained his developmental milestones in normal time. No other congenital malformation was witnessed in the family. Further clinical detail of case VIII is enlisted in Table 4.4. Differential diagnosis of AOS is presented in Table 4.5.

Table 4.4. Anthropometric and radiological measurements of subject VIII*

Anthropometric attributes	***Measurement (cm)***	
Stature	103.8	
Sitting height	54	
Head circumference	49	
Neck circumference	28.5	
Arm span	99.4	
Leg length	53	
Radiological attributes	*Right arm*	*Left arm*
Arm length	39	42.8
Humerus-distal head circumference	3.4	3.5
Middle arm (Zeugopod)	16	16.5
Radius	13.5	13.8
Radius-distal head circumference	1.8 (1.5)	1.9 (1.5)
Ulna	14.8	15
Ulna-distal head circumference	0.9	1.0
Radius/Ulna space	0.7	
Hand (Autopod)	9.4	11.7
Palm	6.0	6.5
Wrist (width)	4.2	4.3
Carpal island 1	1.2	1.1
Carpal island 2	0.9	1.0
Metacarpal I	2.7	2.9
Metacarpal II	4.1	4.9
Metacarpal III	3.8	3.9
Metacarpal IV	3.5	3.5
Metacarpal V	3.3	3.2
Digit 1	3.8	3.8
Digit 2	2.2	5.7
Digit 3	3.5	6.4
Digit 4	1.5	6.0
Digit 5	2.0	2.5

* Data was collected at age of six years.

Table 4.5. Differential diagnosis of Adams-Oliver syndrome.

	Malformation (OMIM; Inheritance; Locus/Gene)					
Clinical features	***Adams-Oliver syndrome***	***Poland syndrome telangiectatica congenita***	***Scalp defects & postaxial polydactyly***	***Aplasia cutis congenita***	***Cutis marmorata congenita with epibulbar dermoids***	***Aplasia cutis***
	100300; AD,AR; 3q13;ARHGAP31	173800; AD	181250; AD	600268;AD	219250;AD	107600; AD
Limb anomalies						
Autopod (hand/foot) anomalies	CS	CS				
Terminal transverse limb defects	CS					
Symbrachydactyly	CS	CS				
Polydactyly			CS			
Skin anomalies						
Aplasia cutis congenita	CS			CS		CS
Scalp defects	CS		CS			CS
Livedo reticularis					CS	
Vascular defects						
Cutis marmorata telangiectatica	OS					
Telangiectases				CS	CS	
Other signs						
Pulmonary disorders	OS					
Cardiac malformations	OS					
Eye problems/glaucoma				CS	OS	
Facial problems	OS			OS		
Pectoralis muscle defects		CS				

CF: Cardinal symptom; OS: Occasional symptom;
AD: Autosomal dominant; AR: Autosomal recessive.

4.3.3 Case series 2: Thumb aplasia

The socio-demographic and biological attributes and phenotypic variability of cases with thumb is presented in Tables 4.6 and 4.7.

Table 4.6. Socio-demographic and biological attributes of patients with thumb aplasia (case series 2).

Cases	Gender	Age (Yrs)	Geographic origin	Rural/ Urban	Language/Caste	House-hold type	Parental Consanguinity	Paternal and maternal age at subject's birth (years)	Subject's parity	Subject's normal sibs (B:S)	Family history of limb defect
IX	F	45	S.Punjab	R	Punjabi/Arain	Nuclear	First cousin	23/20	3 of 6	3:02	No
X	M	6	I.Sindh	R	Marwari/Hindu/Bheel	Nuclear	Non-related	34/30	7 of 8	3:05	No
XI	M	6	S.KPK	U	Pashto/Pathan	Extended	Non-related	26/23	2 of 3	2:00	No
XII	F	3	S.KPK	U	Pashto/Pathan	Extended	First cousin	27/22	1 of 2	1:00	Yes
XIII	M	7	N.KPK	R	Pashto/Yousafzai	Extended	Non-related	35/32	4 of 5	2:02	No
XIV	M	26	S.KPK	R	Pashto/Mughal	Nuclear	First cousin	39/35	4 of 6	3:02	No

I: Interior; N: North; S: South; R: rural; U: urban

Table 4.7. Phenotypic presentation of thumb aplasia in the recruited subjects IX-XIV (case series 2)

	Phenotype				
Cases	**Absent thumb**	**Index finger, medial inclination**	**Other digits**	**Palm, thin/reduced**	**Arm, reduced/short**
IX	Both	R+, L++	L. clinodactyly of 5th digit	R+, L+	R+, L+
X	Both	R+, L+		L+	R+, L+
XI	Left			L+	L+
XII	Left		5th digit small	L+	L++
XIII	Right; left mild hypoplastic	R++	5th clinodactyly	R+	R++
XIV	Left	L+	5th digits small	L+	L+

+: Features present; ++: Severe phenotype; R: Right limb; L: Left limb

4.3.4 Clinical and radiographic study

The phenotypic features observed among the recruited cases are presented below:

4.3.4.1 Case IX

A 45 year old female subject was originated in the rural area. Her parents were consanguineously married (coefficient of inbreeding, F=0.0625), to each other and had five other healthy kids. The subject was non-consanguineously married (F≤ 0.0156), (Table 4.6). She had five unaffected kids. Bilateral and symmetrical thumb aplasia was seen in her upper autopods. (Fig. 4.15A). Her second digits showed medial deviation. Her left hand showed clinodactyly. Additionally, she had slightly short arm's length (Table 4.7). The subject was a house wife and was well-adopted to perform her daily household work. The roentgenographic study revealed a complete absence of first digital ray along with its components bilaterally (Fig. 4.15B). The right hand showed general crowding of carpal bones; absence of trapezoid and hypoplasia of trapezium. Radiographic images revealed that scaphoid and trapezoid bones were absent in her left hand and trapezium was dysplastic (Fig. 4.15B). Along the ulnar-axis, pisiform was not seen. Distal heads of radius and ulna were hypoplastic (Fig. 4.15C).

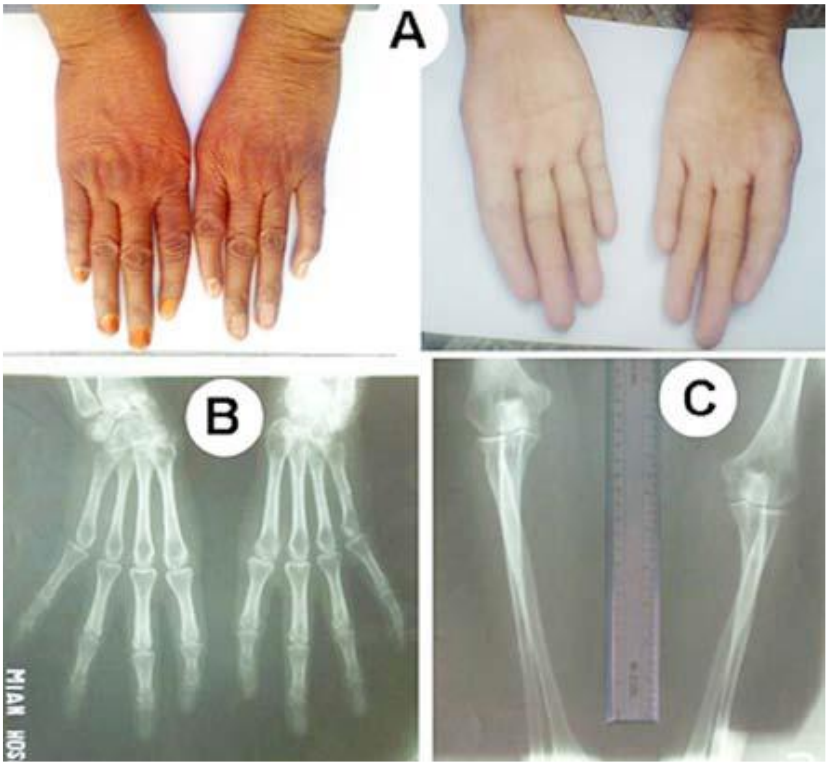

Figure 4.15. Phenotypic and radiographic illustration of hands in case IX
(A): Dorsal and ventral view of hands, **(B):** Roentgenographs of hands, **(C):** Roentgenographs of zeugopods.

4.3.4.2 Case X

A 6 years old boy had origin from a rural area of interior Sindh (Table 4.5). He was observed to have bilateral agenesis of thumbs and a slight reduction in length of arms (Table 4.7; Fig. 4.16D).

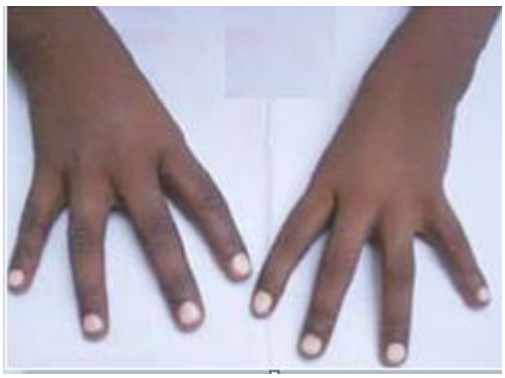

Figure 4.16. Phenotypic illustration of hands in case X

4.3.4.3 Case XI

A 6 years old boy belonged to South KhyberPakhtunkhwa (KPK) was observed to have unilateral aplasia of left thumb (Fig. 4.17). The second digit of his left hand was mild and medially deviated. Moreover, his arm length was also reduced (Table 4.7).

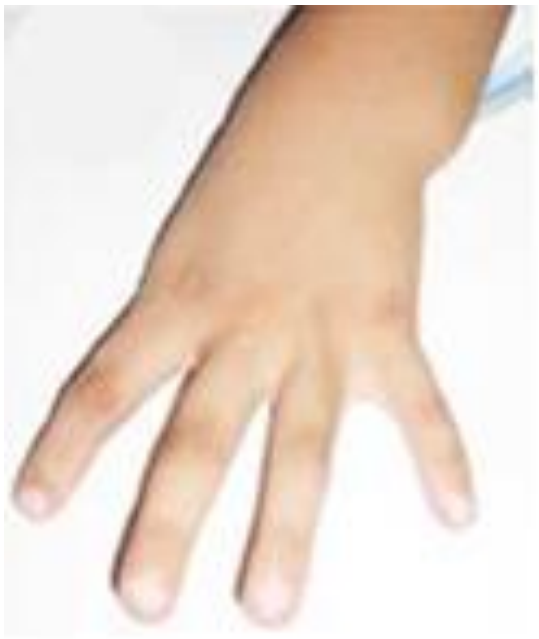

Figure 4.17. Phenotypic illustration in case XI

4.3.4.4 Case XII

A three years girl was noticed to have unilateral agenesis of her left thumb (Fig. 4.18B). She was born to a consanguineous couple (F= 0.0625) (Table 4.6). There was a history of other limb anomalies in her family. One of her maternal aunt had 2nd to 3rd toes syndactyly and a fourth-degree relative was told to had polydactyly (postaxial type B in hands only) (Fig. 4.18). There was no sign of any other anomaly in the subject.

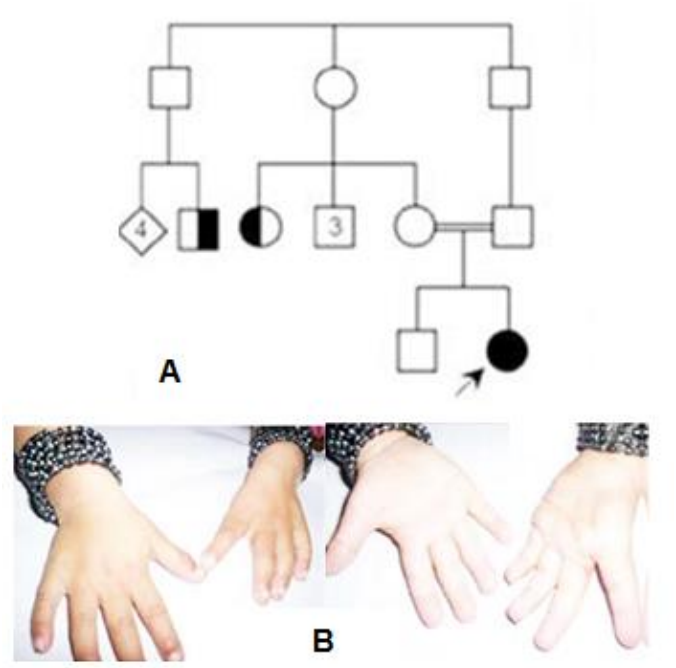

Figure 4.18. Phenotypic illustration in case XII
(A): Pedigree of subject XII denoted by a black filled circle in generation III (III-2). Family members II-5 and II-6 had polydactyly and syndactyly, respectively, **(B):** Hands photographs showing thumb aplasia

4.3.4.5 Case XIII

A seven years old boy from North KPK had unilateral thumb aplasia of the right hand. A slight reduction in the length of the right arm was observed with short zeugopod and restricted movements at the elbow joint on ipsilateral side (Fig. 4.19) (Table 4.7). The second finger was medially inclined and 5th finger had clinodactyly. Thumb on the contralateral hand, displayed minor hypoplasia (Fig. 4.19C).

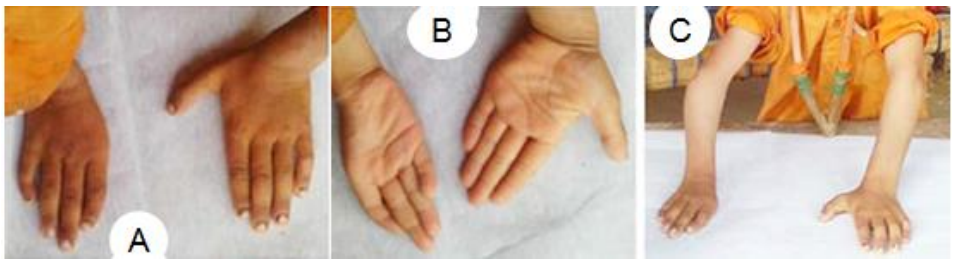

Figure 4.19. Phenotypic illustration of upper limbs in case XIII

4.3.4.6 Case XIV

Physical examination of a 26 years old male subject revealed complete thumb agenesis of his left hand (Fig. 4.20). The affected autopod had a shortened palm and overall length of the affected arm was also reduced (Fig. 4.20; Table 4.7). The male patient also showed/demonstrated limited movement (extension/flexion) at elbow and wrist joint He was engaged in a technical job and was trained to manage his occupational duties mainly with the right hand. Radiographic images of his affected autopod revealed that trapezoid bone was absent and scaphoid bone was hypoplastic. His trapezium was fused with capitate, and lunate with triquertal (Fig. 4.20B). Hypoplasia of radial and ulnar bones was seen in addition to the posteriorly dislocated proximal head of radius (Fig. 4.20C).

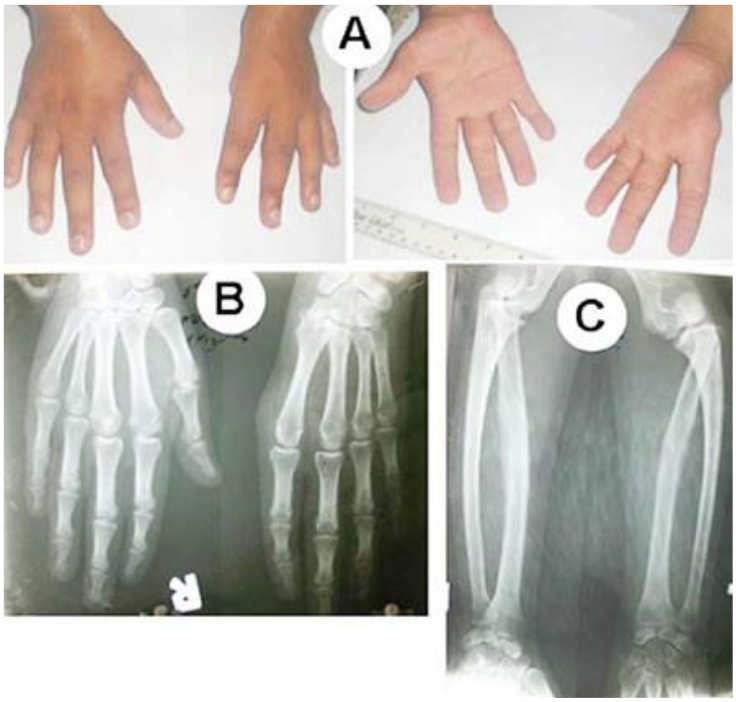

Figure 4.20. Phenotypic and radiographic presentation in case XIV.
(A): Dorsal and ventral view of hands **(B):** Radiographs of hands, **(C):** Radiographs of forearms.

4.4 Discussion

TLD is also delineated as symbrachydactyly. Amputation of the forearm in transverse orientation represents proximal symbrachydactyly (Oberg et al. 2010; Tonkin et al. 2013). The seven subjects presented here with the isolated manifestation of terminal transverse deficiencies shared clinical features/characteristics with each other. Moreover, their characteristics were also consistent with the monodactylous type of symbrachydactyly. The definitive characteristics of this type are the absence of the digits altogether including parts of the metacarpals. Usually, thumbs are spared in this anomaly. We noticed preaxial amputation involving the thumb in one of our cases and only the 5th finger was spared.

In the clinical spectrum of symbrachydactyly, a highly consistent feature is that fingers are represented by tiny nubbins lacking any phalangeal bones. Interestingly, in 2 patients recruited in this study residual digits were also represented by vestigial nubbins at the distal post of an amputated hand. Furthermore, this finding was supported by Kallemeier et al. (2007) who found that almost seventy percent of all sampled subjects with upper limb reduction defect showed nubbin like digits at the distal post of their amputated hands. Most of the reported transverse limb deficiency defects occur unilaterally. In symbrachydactyly patients recruited by Ekblom et al. (2010), the left hand showed a high propensity as compared to the right one. In our recruited cases, the left hand was also proportionally more involved than right hand in the disease and this finding was consistent with the previous study. This finding was further supported by Goodell et al. (2017) who also observed unilateral symmetry of symbrachydactyly with a preponderance of left side limbs. In other studies, unilateral cases were also extensively seen. For example, Ylimainen et al. (2010) also described

that the majority of the limb reduction defects are transverse and have more tendency of being unilateral.

Transverse amputations usually appear as isolated defects and their etiological causes are not known, i.e. no specific gene/locus has been found to cause these defects. The defect in our seven cases also has isolated and unilateral occurrence. The isolated and unilateral occurrence of symbrachydactyly in the majority of patients pinpoints that defects are causally nongenetic. However syndromic appearance (co-occurrence of this deformity with other defects) likes in AOS and ADAM complex may suggest a contribution of some genetic aetiological factors. On the other hand, several aetiologies have been considered for syndromic limb amputation like symbrachydactyly apart from the prenatal vascular disruption. The transverse failure of the mesenchymal cells of growing limb bud may also produce a phenotype characterized by the terminal or intercalated deficiency by causing developmental arrest in limb structures particularly the distal ones (Yu and Ornitz, 2008; Tayel et al. 2005; Bisneto, 2012).

We can rule out constriction band syndrome in our cases which generally co-occurs with circumferential indention rings around the limb usually the fingers and from minor to major acrosyndactyly of distal parts as described by Knight et al. (2012). AOS exhibits genetic heterogeneity. Previous literature has described a few genes that caused various syndromic forms of TLD. For instance, several genes have been found responsible for Charcot-Marie-tooth disease (OMIM 600882), peripheral sensory neuropathy and ulcero-mutilating neuropathy plus transverse limb deficiencies coexist in this deformity. Likewise, autosomal dominant acheiropodia (OMIM 200500) characterized by transverse tetramelic amputations extending to the humerus/ femur. *LMBR1* gene located at 7q36 has been considered responsible for

this deformity. Further, the primary heritable basis of various syndromic anomalies with transverse limb defects as the main variants are not known. For Instance, aphalangia with syndactyly and duplication of metatarsal IV (OMIM 600384), microgastria-limb reduction defects (OMIM 156810). AOS exhibits variable modes of inheritance with a predominance of the autosomal dominant mode of inheritance (McGoey and Lacassie, 2008). The familial appearance of the syndrome is infrequent with sporadic inclination. Autosomal dominant forms of AOS have been found to be caused by the mutations in *DLL4*, *RBPJ* and *ARHGAP31*genes located at 3q13, 4p15.2 and 15q15.1, respectively (Southgate et al. 2011; Isrie et al. 2014; Meester et al. 2015). Homozygous or compound heterozygous mutations in the *DOCK6* gene (614194) at 19p13has been linked with a recessive form of AOS (614789). Additionally, AOS patients with definitive characteristics and recessive mode of inheritance showed a homozygous mutation in *EOGT* at *3p14* chromosomal position (Cohen et al. 2014; Sukalo et al. 2015). However, a strong evidence of further genetic heterogeneity arises due to the lack of *ARHGAP31* mutation in AOS patients with the sporadic/ familial occurrence. As apical ectodermal ridge is the main driver of limb development in proximo-distal orientation and hence the genes expressing in this region are also the proposed candidates of terminal transverse limb reduction defect (Niswander, 2003). The clinical presentation in case VIII is consistent with definitive/typical AOS manifestations i.e. aplasia cutis congenital plus transverse limb defects co-occurred with no other systemic manifestations. Limb defects are primary /definitive clinical feature present in 84% of AOS patients (OMIM, 2014). In AOS, limb reduction anomalies are characteristically/typically asymmetrical and generally bilateral. Lower limbs are extensively involved than their superior counterparts. Aplasia cutis congenita is the second extensively observed anomaly in almost three

quarters/ three fourth of AOS patients. One-fifth of all AOS patients depict congenital cardiac malformations (Al-Sanna'a, 2000). Infrequently associated anomalies of AOS include vascular, pulmonary, orofacial defects and skin defects (Table 4.6) (OMIM, 2014). Additionally, AOS diagnosis is not easy due to its spectral overlap with other well-characterized malformation, i.e. Poland anomaly. Two isolated kindreds evaluated by Der Kaloustian et al. (1991) revealed clinical characteristics of both disorders, i.e. AOS and the Poland anomaly.

Numerous cases predominantly severe ones are possibly hard to manage. Hence, to lower the prevalence and etiology of transverse limb defects and to reduce the loss caused by them to the people effective health care strategies are required. For instance, in an epidemiological study authors found the correlation between the genes implicated in metabolic pathways of folate and methionine and isolated limb deficiency deformities. Increased folic acid intake before conception may lower the risk of having an anomalous fetus with congenital limb reductions (Cleves et al. 2011). Identification of effective interventions for congenital malformations of the limbs including transverse anomalies needs further research.

Transverse deficiencies exert a remarkable impact on the patients and families. Social and psychological impact of transverse deficiencies and absence anomalies on patients is far-reaching and variedly devastating. They make their patients functioning very challenging (Lal et al. 2014). Baseline data is needed to put an intervention in place for the prevention of a diseased condition. There is a scarcity of epidemiological, prevalence and etiological information on AOS which is vital for effective genetic counseling and risk estimation (Malik and Afzal, 2013a). There is a great need for further research which addresses these issues in Pakistan.

We presented six independently recruited subjects with absent thumbs. The observed phenotypes in subjects IX-XIV were consistent with hypoplastic thumb "type-V" by Blauth and Schneider-Sickert (1981) and James et al. (1996). Two subjects (I, II), had a bilateral absence of thumbs and four subjects (III-VI) showed unilateral agenesis of thumbs. Interestingly, in the subjects with unilateral thumb aplasia, there was a high preponderance of left hand involvement (n=3/4). Subject V with unilateral thumb aplasia also depicted minor hypoplasia of contralateral thumb. Other manifestations in the affected autopods apart from thumb agenesis were clinodactyly or shortening of 5th digits, short and narrow palms and reduced arm lengths. All the patients were of normal intellect and there was no involvement of any other organ-systems. All cases except subject IV showed negative family histories of thumb aplasia/hypoplasia. Patient IV, however, had a previous family history of polydactyly and syndactyly in two different sibships. The clinical band/continuum of LRDs in the Pakistani population has not been studied well and only a few cases have been reported (Riaz and Malik, 2011). Malik and Jabeen (2011) reported a sporadic case of right hand thumb aplasia. Additionally, he had unilateral zygodactyly of his left foot, a lean body, and low body weight. Another male patient, a child of a consanguineous couple showed complete agenesis of thumb and index finger in left hand (Malik and Afzal, 2013a). The subject had additional symptoms as ulnar deficiency, shortening of the affected arm, reduced zeugopod and autopod, and severe flexion contracture at the elbow joint. In the current study, isolated thumb deficiency has been observed in all cases. Thumb is vital for proper hand performance that is imperative for independent routine activities. Some other symptoms co appears with thumb aplasia like reduction of hand musculature, narrowing of palm and shortening of the arm/hand's normal size, which exert a negative impact on hand functioning and

may cause psychological stress on child/patient. Thus, it is the prime responsibility of the parents to assist their kids to get rid of their feelings of grief and guilt of being afflicted with the anomalous condition (Flatt, 2002). In this study, it was seen that the patients had adopted their situations accordingly. For example, case IX was well-trained to perform almost all of her routine house-hold work. However, she had difficulty in carrying heavy articles and fast-grasping of objects. Subjects having thumb aplasia develop the habit to grasp objects between their index and middle fingers. In case of unilateral thumb aplasia, the subjects rely more on their contralateral normal hand. The molecular etiology of thumb hypoplasia/ aplasia remains less described (Riaz et al. 2014). Non-syndromic thumb aplasia has not been attributed to any particular gene. However, some syndromic types of thumb aplasia have been linked to several genes. Holt-Oram syndrome (OMIM 142900) is characterized by thumb agenesis along with radial deficiency and cardiac defects. Mutated *TBX5* is responsible for a type of Holt-Oram syndrome. Fanconi anemia (OMIM 227650) is a rare anomaly with characteristic symptoms of radial deficiency, leukemia anomalous kidneys, and skin. Almost thirteen different genes have been linked to this disease (Duaneradial ray syndrome (OMIM 607323) share phenotypic spectrum with Holt-Oram syndrome in addition to some characteristic differentiating features like defects of eyes, ears, and kidneys. Some mutated forms of *SALL4* gene have been linked with it (OMIM, 2014; Temtamy and Mckusick, 1978). Thumb is a vital digit of hand required for its functioning of hand. For the management of thumb agenesis, the index finger is pollicized to reconstruct a thumb by index finger. A person having a pollicized index finger is able to perform his daily life functions (Tay et al. 2006). Pakistani patients usually cannot access a surgical expert for a better opinion to deal with their anomalous conditions. Most often the patients have low

socio-economic status and are unable to bear the enormous expense of surgery. The recruited cases had, otherwise no major restriction in their day-to-day activities, however, pollicisation procedures could be highly useful for their occupational lives. Thumb agenesis is rare, and most of its nonsyndromic cases have sporadic appearances, thus limiting the number of patients who are available for molecular analyses. Therefore, collection of such cases is imperative for successful genetic and molecular investigations. Recent advances in technology like comparative genomic hybridization, SNP arrays, and whole-exome analyses could help in the identification of causative mutations in sporadic cases.

4.5 Conclusion

There is a paucity of knowledge regarding transverse limb defects in Pakistani subjects and their prevalence and clinical aspects have not been studied in Pakistan. To fill this gap we recruited 8 independent cases of TLD from different localities of Pakistan and presented their phenotypic manifestation in both syndromic and isolated forms. Both forms of the limb amputations depicted phenotypic heterogeneity. This study highlights the importance to monitor the prevalence of these anomalies in the country with high consanguinity rate and to elucidate genes responsible for such disorders.

Thumb aplasia affects the normal working of the anomalous hand. Index finger pollicization should be used to effectively restore the performance of the anomalous hands and quality of life of the patients. This isolated form of the condition has not been attributed to any of the genes. Investigation of sociodemographic and molecular aspects of cases with similar clinical characteristics could be helpful to better understand the genetic and molecular basis of the disease.

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Riaz HF, Lal K, Ullah S, Bhatti NA, Ullah W, Malik S. 2016. Phenotypic manifestation of congenital transverse amputation of autopod in Pakistani subjects. Pak J Med Sci 32(2):519-522.

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5. Novel mutation in *LRP4* underlies Cenani-Lenz syndactyly, spoon-head type, with a distinctive combination of facial features in two independent families

5.1 Abstract

Cenani-Lenz syndactyly syndrome (CLSS) is a hereditary condition having phalangeal disorganization with a variable degree of oligodactyly/syndactyly features. It is one of the limb anomalies with characteristic unabridged synostoses of metacarpals, carpals, phalangeal elements with varying levels of disorganization, reduced number of phalanges/digital reduction, radioulnar synostoses and more or less same exposition in lower limbs. In addition, characteristic craniofacial features and renal agenesis are also observed in CLSS patients. Mutations in *LRP4* have been implicated in families with CLSS. Here, we present two independent Pakistani families with characteristic features of CLSS. In kindred 1 and 2, one and two affected individuals born to consanguineous couples were observed, respectively. Affected subjects in both families were presented with drastically reduced autopod and zeugopod with grossly disorganized skeletal elements, the features consistent with CLSS spoon-head type. Further, there were certain anomalous facial features including hypertelorism, down-slanting palpebral fissures, and enamel hypoplasia; however, there were no renal agenesis. Mutation analyses revealed a A>G base transition in exon 12 at position c.1820 in *LRP4* in the index patients in both families. The mutation was concordant with the disease model in both families. The low-density lipoprotein receptor-related protein 4 (LRP4), centrally involved in several

signaling cascades, has a potential role in the development of distal limbs. Any abruption in signaling cascades defining the development of limb extremities appears in different forms of distal limb maladies. Our study provided a support to genotype-phenotype correlation as a missense mutation caused a relatively mild type of CLSS.

5.2 Introduction

5.2.1 Features of Cenani-Lenz Syndactyly

Cenani-Lenz syndactyly syndrome (CLSS; OMIM 212780), also known as syndactyly type VII (SD7), is an autosomal recessive limb anomaly described as complete and complex syndactyly of digits in hands while feet are sometimes less severely affected This condition arises due to failure in the separation of singular digital extremities. Cenani-Lenz syndactyly is a rare anomaly affecting 1 out of 100,000 individuals (Temtamy et al. 2003; Malik, 2012; Hettiaracchchi et al. 2018).

CLSS represents a distinct pattern of digit fusion where all digits are intimately fused. The fusion is at the level of metacarpals/metatarsals and usually, no digits are recognizable. It has a set of unique clinical features that make it distinct from other limb morphopathies along with a bunch of extremely variable characteristics. Two clinically variable subgroups of CLSS have been delineated: [1] spoon-head type also known as classical type, characterized by completely syndactylous hands, and [2] oligodactyly type also referred to as a nonclassical type, with partial Syndactyly. Classical type is referred as spoon-head type syndactyly because the appearance of hand resembles the head of a spoon due to the extreme and disorganized syndactylism of distal bone elements of the hand. However, nonclassical type possesses some degree of digital individualization and partial syndactyly of fingers and toes. Nonclassical type of CLSS is more frequently observed (Li et al. 2010; Harpf et al. 2005).

5.2.2 Classical or nonclassical forms of CLSS

CLS either in its classical or nonclassical form appears both isolated or coupled with malformations other than limb anomalies (Harpf et al. 2005). In isolated cases, it comes along with typical malformations of distal limbs like shortening of the forearm bones marked with extensive shortening and fusion of radius and ulna, carpal and metacarpal synostosis with disorganization, abnormal dermatoglyphics of hand, abnormal and disorganized phalanges with synostosis, cutaneous ossification or symphalangism, and reduction in the number of phalanges or toes (Seven et al. 2000). Among these hallmark features of CLSS, the constant attributes are carpal, metacarpal and phalangeal synostosis, with a reduction in the total count of digital rays with extreme disorganization.

Inter- and intra-familial expression variability of the consistent features is also noticed as among different individuals of the same family and those recruited from different kindreds. Every patient has his own unique clinical features that need a tailored approach. Moreover, a few inconsistent features of CLSS include radio-ulnar fusion, middle arm/forearm shortness, metatarsal fusion, dislocated radial head and reduction of metatarsal rays (Harpf et al. 2005). In a rare case of CLSS, limbs were more severely affected involving hypoplasticity of humerii and tibiae with aplastic fibulae (Kariminejad et al. 2013). The extent of forearm shortness varies from case to case. In some cases, it is totally absent. Nails mostly are multipartite. The condition affects the lower extremities less strikingly: in the majority of cases, toes syndactyly, metatarsal synostoses or the reduced sum of rays is noticed. Clinical representations of the tibia or fibula are very rare and have not been seen in all cases except one (Nezarati and McLeod, 2002).

Syndromic CLSS shows conjugation with several anomalies like vertebral/hemivertebral defects, tooth anomalies, renal hypoplasia or agenesis and craniofacial abnormalities. This combination of anomalies is highly variable (Seven et al. 2000; Bacchelli et al. 2001). Almost all cases of renal aplasia/hypoplasia have been noted in oligodactyly type of syndactyly (Harpf et al. 2005). Renal aplasia has been observed as an associated attribute in more than 50% of CLSS cases (Li et al. 2010). Mild facial dysmorphia (ptosis, high-arched palate, frontal bossing, hypertelorism, saddle nose, downward slanting palpebral fissures, short nose, malar hypoplasia short and deep philtrum groove) are observed as additional features. Various other minor anomalies were found associated with CLSS, including hypothyroidism, laryngomalacia and congenital dislocation of the hips (Jarbhou et al. 2008). Its association with genitourinary malformations has also been observed. Some other rare associated features of CLSS may include mixed type hearing loss, cleft lip, congenital cataract, and supernumerary nipples. Cognition is normally developed. Recently short stature has also been observed in CLSS patients (Li et al. 2010; Lindy et al. 2014; Afzal et al. 2017).

5.2.3 Genetics of CLSS

CLSS being genetically heterogeneous has more than one genetic causes. One of the responsible genes of the anomaly is Low-Density Lipoprotein Receptor-Related Protein 4 (*LRP4*) and it is located on chromosome 11p11.2-13.1. The majority of mutations observed in CLSS patients are splice site and missense, eventually causing syndactyly, oligodactyly, and renal defects (Li et al. 2010). Moreover, a homozygous nonsense mutation has also been linked to the dysmorphic condition in patients with typical CLSS symptoms (Kariminejad et al. 2013). Additionally, compound heterozygosity for two truncating mutations in *LRP4*, causing the prenatal lethal

clinical manifestation of CLSS like mesomelic limb reductions, oligosyndactyly and genitourinary malformation were found to be associated with this anomaly (Lindy et al. 2014). However, all patients with this syndrome do not always harbor *LRP4* mutations, hinting the involvement of one new locus at least.

Interestingly, two new clinically delineated entities within the Cenani-Lenz oligosyndactyly spectrum demonstrated different genetic causes not previously described. Genomic alterations of the long arm of chromosome 15q13.3-q14 at *GREM1-FMN1* locus have been linked to the oligosyndactylous types of CLSS. Syndromic Cenani-Lenz phenotype is due to a homozygous genomic deletion of the *FORMIN1* (*FMN1*) gene and autosomal dominant, non-syndromic CLSS like oligosyndactyly is a result of tandem duplication of *GREM1-FMN1* locus. In addition to *LRP4* and *GREM1-FMN1*, molecular characterization of a new CLSS family of Saudi origin with characteristic CLSS manifestations plus prominent scoliosis revealed that the disease was associated with a mutation in *APC* (Gu et al. 2008; Dimitrov et al. 2010).

5.2.4 Low-Density Lipoprotein Receptor-Related Protein 4 (LRP4)

Human *LRP4* comprises 38 exons and spans over 61,906 bp. It codes a receptor protein comprising 1905 amino acids, which acts as a transmembrane receptor (Tian et al. 2006; Weatherbee et al. 2006; Ohkawara et al. 2014). It belongs to the low-density lipoprotein receptor family (LDLR; OMIM 606945), which consists of many evolutionarily conserved transmembrane proteins (Herz and Bock, 2002). LRP4 has a complex big extracellular region, transmembrane domain, and a short intracellular C terminal region (Barik et al. 2014). The extracellular domain in LRP4 is implicated in the modulation of several signaling pathways. Typically, LRP4 is considered to act as a cell surface receptor that endocytoses extracellular ligands by

binding and then internalizes them where they are chopped/disintegrated by the lysosomes for degeneration.

Clinically, *LRP4* is involved in limb, kidney, bone and neuromuscular junction (NMJ) fabrication and stabilization by being implicated in Wnt/β-catenin signaling LRP4 regulates synapse formation by simultaneously stimulating both postsynaptic differentiation and presynaptic differentiation at NMJ (Karner et al. 2010; Li et al. 2010; Yumoto et al. 2012).

Most mutations in *LRP4* gene are a loss of function mutations as a result of which Wnt signaling is activated excessively. Abnormal limb development resulting from the excessive Wnt signaling cause syndactyly, synostoses and renal agenesis in Cenani-Lenz syndactyly. Interestingly, in CLSS patients the part of the *LRP4* coding center of the 3rd β-propeller domain harbor most of the genetic alteration. Majority of the causative mutations in CLSS are missense mutations. A homozygous mutation involving a c.1585G>A transition in exon 13 of the *LRP4* gene, causing p.Asp529Asn (D529N) substitution was identified in three isolated consanguineous families of Turkish descent with characteristics of Cenani-Lenz syndactyly syndrome. Another homozygous mutation involving c.409G>A transition in exon 4 of the *LRP4* gene, resulting in an p.Asp137Asn (D137N) substitution was observed in 2 unrelated consanguineous Egyptian kindreds with Cenani-Lenz syndactyly syndrome. In a female patient with characteristic CLSS features, descended from a consanguineous Pakistani family another homozygous mutation, i.e. c.547+1G>A transition in intron 6 of the *LRP4* gene was considered the cause of the anomaly. These authors also identified a homozygous mutation in of exon 5 of the *LRP4* gene at locus c.479G>A, resulted in a p.Cys160Tyr (C160Y) substitution in a male patient with Cenani-Lenz syndactyly syndrome from a consanguineous Turkish family. In a female patient and

her niece with Cenani-Lenz syndactyly syndrome from a consanguineous family of Turkish descent, homozygous c.1345G>A transition in exon 12 of the *LRP4* gene was observed, leading to the substitution of p.Asp449Asn (D449N). In a female patient with Cenani-Lenz syndactyly from a consanguineous Jordanian family, homozygosity was observed for c.1382A>C transversion in exon 12 of the *LRP4* gene, resulting in a p.Thr461Pro (T461P) substitution in a highly conserved region was discerned. In a female fetus with Cenani-Lenz syndactyly syndrome from a consanguineous Turkish family, compound heterozygosity for a G-to-A transition in intron 2 (200-9G>A) of the *LRP4* gene, resulting in a 7-bp insertion causing premature termination, with c.4959G>C transversion in exon 34 of the *LRP4* gene, resulting in skipping of exon 34 and premature termination was pinpointed Disease-linked mutations were not found in their respective controls used in all above-mentioned studies. However, it was studied that all above-mentioned mutations perturb efficient transport of LRP4 receptor to the plasma membrane and subsequently mutant LRP4 loses its antagonistic effect on LRP6 mediated activation of WNT (OMIM 164820)/beta-catenin (OMIM 116806) signaling. (Li et al. 2010).

In all the above-reported cases of CLSS, the disease was caused by a splice site or missense mutation. In such mutations, functional ability of protein might decline. In another study, the disease was caused by a homozygous nonsense mutation. A homozygous stop mutation produces more adverse effects on the phenotype of the bearer by absolute loss of LRP4 activity causing a fatal damage in embryological development and hence, giving a more severe phenotype. This work further extended the clinical range of the CLSS adding hypoplasia of humerii and tibiae at its severe end (Kariminejad et al. 2013). As seen above, in most of the diseased cases, patients having a different country of origin carry different mutations

in the same causative genes. A novel splicing mutation in upstream of exon 5 of APC results into the introduction of the premature stop codon and generating a truncated transcript which lacks canonical acceptor site and as a result of which WNT/beta-catenin is upregulated giving the same phenotypic outcome prompted by *LRP4* mutation (Patel et al. 2015).

5.2.5 Genotype-phenotype correlation

Several attempts have been made to develop a correlation between CLSS genotype and phenotype but strong evidence supporting the genotype-phenotype correlation is still lacking. For CLSS, genotype-phenotype correlation is difficult to establish, and it is further complicated by the fact that widely different phenotypes observed in patients share similar mutation. The genotype-phenotype correlation in CLSS is a challenge by reason of phenotypic as well as causative mutations variability. Every patient has his own unique clinical features that need a tailored approach. Despite considerable investigational efforts, no method to find a direct link between genotype and phenotype has been successfully determined.

The mutated *LRP4* gene produces a wide spectrum of defects. Sometimes the clinical subdivision based on phenotypic appearance does not withstand the clinical or molecular reality. Therefore, the mutation type does not always tell about the associated severity of the clinical features, possibly because of the other influences, such as stochastic elements, epigenetic events, or decreased penetration of the deleted genes. However, due to a heterogeneous group of mutations associated with two distinct types of Cenani-Lenz syndactyly, it is supposed that there is a particular genotype-phenotype correlation. Moreover, a case of CLSS with a homozygous truncating mutation associated with a severe phenotype also provided some data about genotype-phenotype correlation (Kariminejad et al. 2013).

5.2.6 Limb development

Limb development is a very complex phenomenon and controlled by several regulatory processes. *Shh*, FGFs, and their downstream effectors cooperate to regulate limb development and coordinate the expression patterns of the genes, especially the *Hox* family (Tickle, 2000). The dorsal ectoderm produces the growth factor *Wnt*7, a *Wnt* family member, necessary for dorsalization (Parr and McMahon, 1995). Days from 24 to 36 after fertilization are considered the most critical period of limb development (Sant, 2002). Complex interactions between a multitude of morphogens, their modifier genes and/or far-reaching regulatory elements, have been implicated in limb development (e.g. ZRS) further complicate the disease pathomorphology (Elliott et al. 2009). Blocking of cellular processes involved in apoptosis may account for syndactyly or webbing of the fingers or toes (Rayan and Upton. 2014).

5.2.7 Differential diagnosis of CLSS

Differential diagnosis of CLSS faces various challenges due to overlapping phenotypes in the wide clinical spectrum. Typing of CLSS is more complicated due to the genetic heterogeneity. Moreover, diagnosis of CLSS is complicated because of inheritance pattern, incomplete penetrance and diminutive count of CLSS linked families. For instance, intrafamilial and intraindividual phenotypic variation may bring about its overlap with the phenotypic representation of other diseases. Sometimes the disease expression is variable to the extent that makes it confusingly similar to split-hand and foot malformation (SHFM; OMIM 183600). Moreover, CLSS also encounters some daunting diagnostic tasks because it shares the phenotypic presentation with Kabuki syndrome (OMIM 147920). CLSS patients represent phalangeal disorganization with some degree of syndactyly/oligodactyly as core features of the anomaly. Although rarely found and described CLSS patients may

have midfacial dysmorphism with large, prominent ears. Clinical features of Kabuki syndrome also include facial dysmorphia like large, conspicuous ears; scanty, angled and medially flared eyebrows, wide palpebral fissures, long eyelashes and oligodontia with flat head 'screwdriver-shaped' incisors, in addition to persistent finger and thumb-tip pads. Therefore, CLSS patients need a careful evaluation and description to figure out if there is any peculiar associated facial phenotype as well (Elliott et al. 2004). Antenatal ultrasonography also assists clinical diagnoses of the condition.

Syndactyly in Apert syndrome is complete like in CLSS and is often referred to as the mitten hand. The feet are also affected and there is coronal suture synostosis (Stephenson et al. 2017). Correct diagnosis of CLSS cannot be carried out without radiological examination that is the ultimate technique to detect bone defects.

Furthermore, defects of limb extremities like CLSS are often alleged as stigmatizing as they cause potential emotional and social distress because of physical appearance, but adequate and timely surgical and conservative management is effective in reversing the compromised phenotype, improving the cosmetic appearance and restoring the normal physiology. The condition is considered unimportant in toes but challenging in fingers and requires a surgical intervention to make them resume normal functionality by separating joined fingers of the hands (Moore et al. 2015). Corrective surgery is the only hope to create a “normal” hand. The task of CLSS type identification specifically becomes difficult when only a few individuals are affected by the condition.

The current study aims to expand our knowledge about the genetic and developmental causes of CLSS. This study will look at the genetic attributes of Cenani-Lenz syndactyly in two independent Pakistani families of Sindhi origin.

5.3 Subjects and Methods

5.3.1 Family ascertainment

Two unrelated families were ascertained from different towns of interior Sindh, Southern Pakistan. Formal informed consent was obtained from the patients and elderly family guardians prior to the data collection. The families were briefed about the study purpose and objectives. There were one and two affected subjects in Kindred 1 and 2, respectively. Affected subjects together with eight and seven unaffected individuals in Kindred 1 and 2, respectively, were physically examined with the help of local physicians. Photographs and radiographs depicting the phenotype were obtained and blood samples of the available affected and unaffected subjects were taken for molecular analyses. The study was approved by the Ethical Review Committee of Quaid-i-Azam University, Islamabad and all the material were acquired according to the Helsinki II declaration.

5.3.2 Pedigree analysis and clinical characteristics

5.3.2.1 Kindred 1

In Kindred 1, the pedigree consists of four generations with a single consanguineous loop in the third generation (Fig. 5.1). The affected subject IV-1 was a male born to this consanguineous couple. The affected subject was the first child born to this couple. Reportedly, his pregnancy events had been unremarkable. Both parents of the affected person were physically unaffected. The affected subject had six unaffected younger sibs. No other family member showed any unusual symptom related to limb or any other organ-symptom.

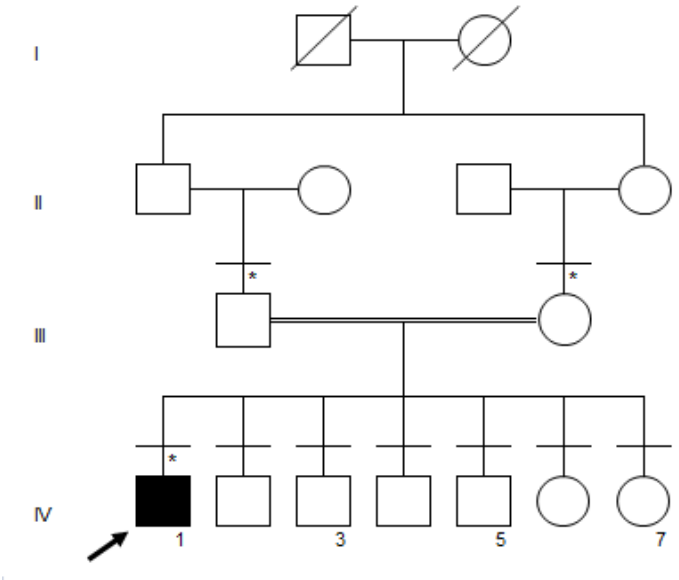

Figure 5.1. Pedigree of Kindred 1 with CLSS phenotype.
A horizontal line above the symbol indicates that physical examination was performed. Asterisk depicts availability of DNA for genetic study.

The phenotype of affected subject IV-1 is characterized by typical features of Cenani-Lenz syndactyly like fused radius and ulna with extreme shortening, synostosis of the metacarpals, and disorganized and completely fused appearance of digits giving the hand a spoon head like appearance (Fig. 5.2).

There were short arms, with middle arm and autopod grossly reduced in size. Mesomelic shortness of arms was obvious. No digits in the hands was recognizable. Bilateral nail aplasia was also observed in both hands (Fig. 5.2A). The articulation of radius and ulna at elbow joint appeared anomalous. In the roentgenograms, there was characteristic radio-ulnar fusion represented by a thick bone with dysmorphic proximal and distal heads. There was dislocation of proximal heads of radius and ulna at the elbow joint. In the autopod, carpals fusion was evident, metacarpals were not recognizable and were amalgamated into an osseous mass. No phalangeal elements were recognizable. Both forelimbs were symmetrically affected.

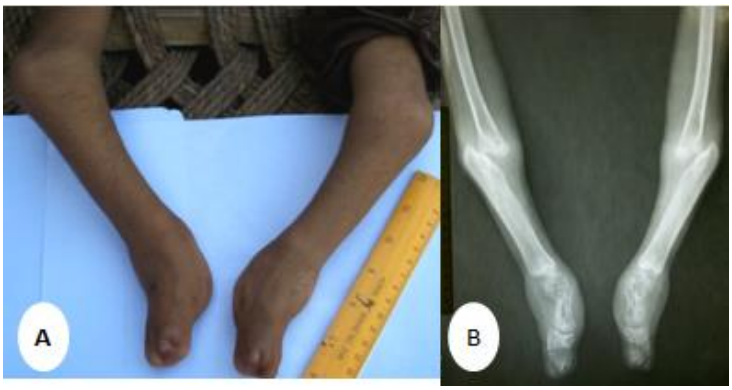

Figure 5.2. Phenotypic and roentgenographic appearance of upper limbs of subject IV-1 (index person).
(A): Photographs showing short arms and hand with highly unrecognizable fingers, **(B):** Radiographs showing mesomelic shortening, dislocation at the elbow joint, radio-ulnar fusion and synostoses of hand bones (carpals, metacarpals, and phalanges).

The feet were less severely affected (Fig. 5.3A). The right foot exhibited cutaneous synostosis of second-third toes and fourth-fifth toes. Left foot showed a fusion of second through the fourth toe. The nail was absent on the third toe. The fifth toe was separate, bigger in size and appeared splayed out of the foot plane. Left foot showed a fusion of 4^{th} and 5^{th} metatarsal resulting in a single wide bone. No pain was associated with the condition. Facial features of the patient were remarkable as he had downward slanting eyes, degenerative incisors, and prominent ears (Fig. 5.3B). The patient had normal intellect. Scoliosis of the thoracic vertebral column was also evident in radiographs of the chest (Fig. 5.3C).

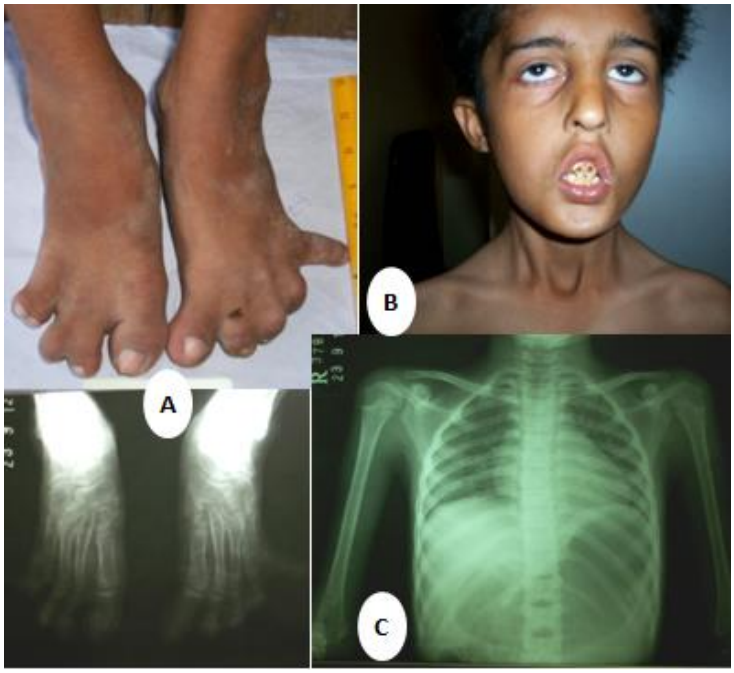

Figure 5.3. Phenotypic illustration of subject IV-1 with CLSS phenotype
(A): Photographs and radiographs of feet of subjects IV-1 depicting the cutaneous fusion of toes, hypertrophic first metatarsals, fusion of metatarsals and reduction in their total count, **(B):** Facial appearance with downward slanting eyes, degenerative incisors, and prominent ear, **(C):** X-ray of chest showing mild scoliosis in the thoracic region.

5.3.2.2 Kindred 2

Pedigree of Kindred 2 consists of four generations. Index person IV-3 was at the third number in sibship. She had two sisters and four brothers. Index person and one of his brothers IV-6 showed features of Cenani-Lenz syndactyly. Parents of the affected subjects had a cross-cousin union. (Fig. 5.4). Their mother was clinically and physically normal while father III-2 exhibited camptodactyly of the fifth finger of his right hand. There is no prior history of any limb or other congenital disease in the family.

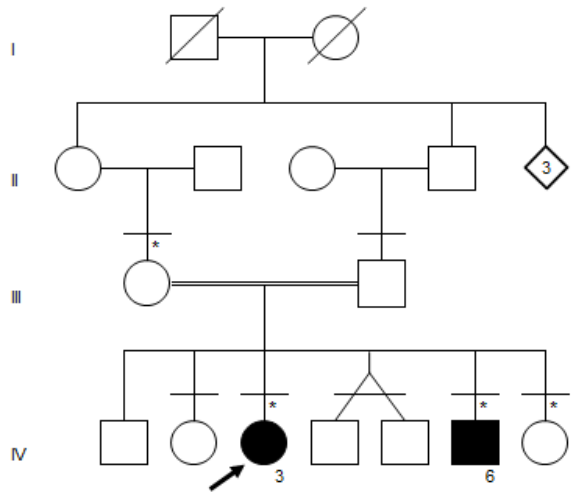

Figure 5.4. Pedigree of Kindred 2 with CLSS phenotype.
A horizontal line above the symbol indicates that physical examination was performed. Asterisk depicts availability of DNA for genetic study.

The affected subjects had partial to complete syndactyly of hands and feet. In the index subject (IV-3), both hands had a Mitten-like appearance. A single underdeveloped and anomalous nail was present in each hand. Radius and ulna showed complete fusion resulting into a single thick bone. The articulation of the radius and ulna at the elbow joint was not normal (Fig 5.5A). Radiological study revealed that propositus had typical features of Cenani-Lenz Syndactyly as there was shortening in the overall length of the arm mainly due to the mesomelic shortening of long bones and radioulnar synostoses. There were indistinguishable elements in the autopods like fused carpals and metacarpals, synostosed phalangeal elements with reduced number (Fig. 5.5A).

Lower limbs of the proband showed mild manifestation. The right foot of the subject had four toes showing cutaneous fusion of second and third toes. Nails of fused toes were seen to be underdeveloped. The left foot showed a fusion of first with the second toes, and reduced number of toes was observed in right foot. The third toe had a rudimentary nail. Both right and left foot radiograph showed a reduced number

of metatarsals and phalanges with variable toes involvement in soft tissue Syndactyly (Fig. 5.5B). Scoliosis was also evident in the radiographs (Fig. 5.5C).

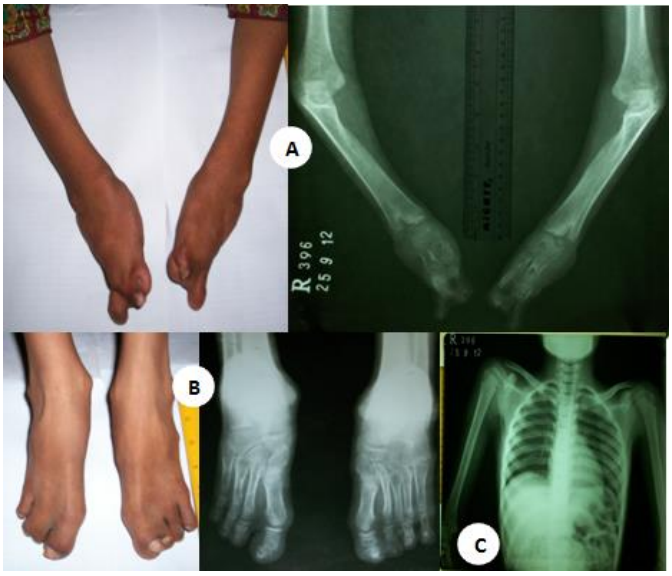

Figure 5.5. Photographs and radiographs of proband IV-3

(A): Photographic illustration of the upper limbs of patient shows a reduction in length of forearm bones with a Mitten-like appearance of the hands having unrecognizable fingers, Corresponding radiographic illustration shows bilateral radio-ulnar articulation and a mild dislocation of these bones at the elbow joint, striking amalgamation of carpals and metacarpals, **(B):** Photographic illustration of the feet of the patient depict cutaneous fusion of toes, corresponding radiographs depict hypertrophic first metatarsals, reduced number of metatarsals in right foot and fusion of tarsals/metatarsals, **(C):** Chest X-ray showing mild scoliosis.

Affected sib IV-6 manifested characteristics of CLSS syndrome. He had bilateral radioulnar synostosis, complete synostoses, and disorganization of metacarpals and phalanges, and dislocated radial head (Fig. 5.6A). The radiographs of subject IV-6 revealed characteristic limb phenotype with gross synostotic disorganization of limb elements like severe bilateral fusion of radius and ulna culminating into a single wide bone with the subluxated proximal end, syndactyly of phalanges, and synostoses of phalangeal elements of both hands into a spoon-like shape. There was an extensive fusion of metacarpals with reduction. Phalange bone reduction in the number and disorganized fusion was also a marked symptom noticed.

The right foot had cutaneous synostosis between the second and third phalanges with broadening of second toe and shortening of the fourth toe. The left foot had cutaneous synostosis between the second through fourth toes (Fig. 5.6B). There were four metatarsals evident in the footplate. Feet had extensive cutaneous fusion. First metatarsal was hypertrophic and the fourth metatarsal was broad and brief/short. The right foot had syndactyly between the second and third toe not involving bone. Inward bending of the fourth toe was also noticed. The left foot had synostosis between the second, and third toe. Mild scoliosis was also evident.

He had characteristic dysmorphic facial features, i.e. broad and prominent forehead, hypertelorism, flattened nasal bridge, downward slanting palpebral fissures, short nose, short philtrum eminence, enlarged ear, and flattening/hypoplasia of malar bones (Fig. 5.6C). Intellectual development is within the normal range.

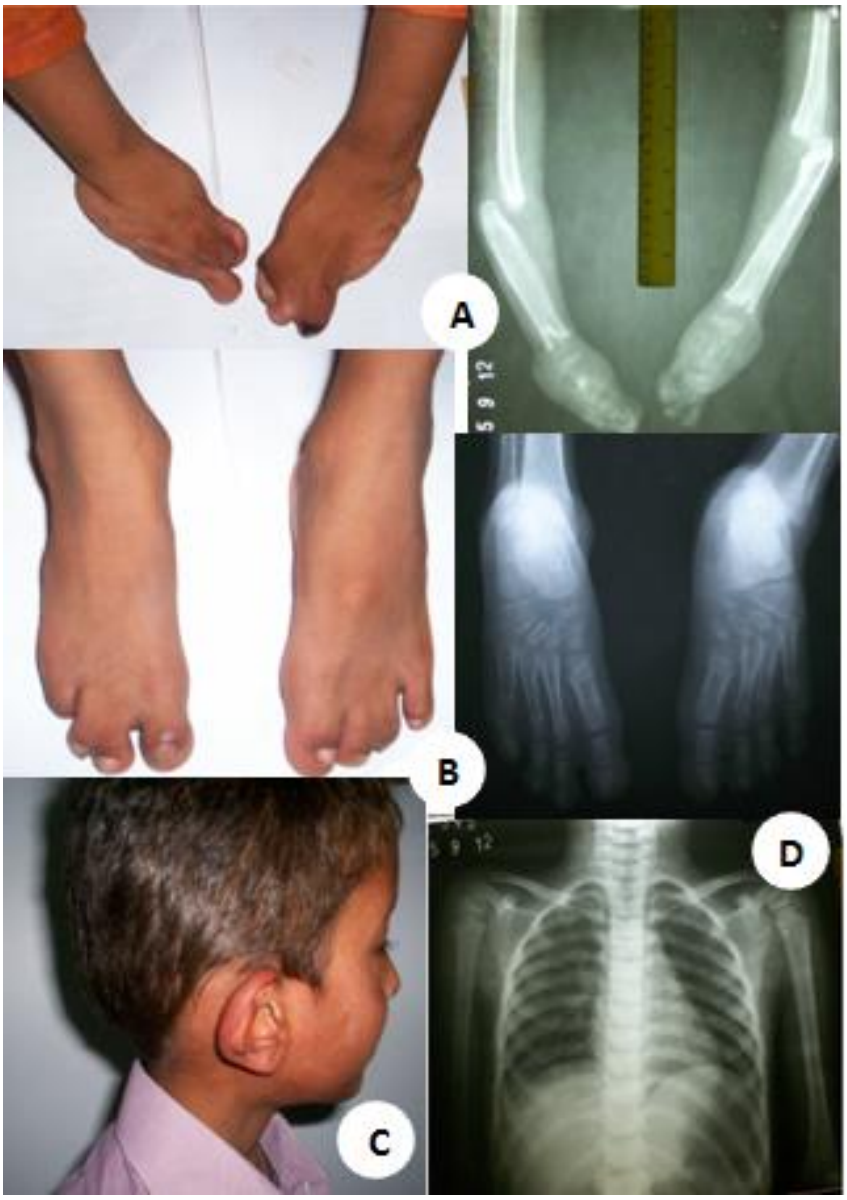

Figure 5.6. Clinical manifestation of subject IV-6

(A): Photographs of upper limbs depict shortening of arms and rosebud like hands, corresponding radiographs reveal dislocated radial head, radio-ulnar fusion with reduction in size, unrecognizable phalanges, completely coalesced carpals and metacarpals, **(B):** Photographs of the feet of the patient showing cutaneously fused toes, corresponding radiographs reveal hypertrophic first metatarsals, reduced number of tarsals/metatarsals and fusion of metatarsals, **(C):** Deformed pinna, **(D):** Chest X-ray showing mild scoliosis and low bone age.

Father of the affected children (III-2) depicted camptodactyly of fingers without any other dysmorphism (Fig. 5.7).

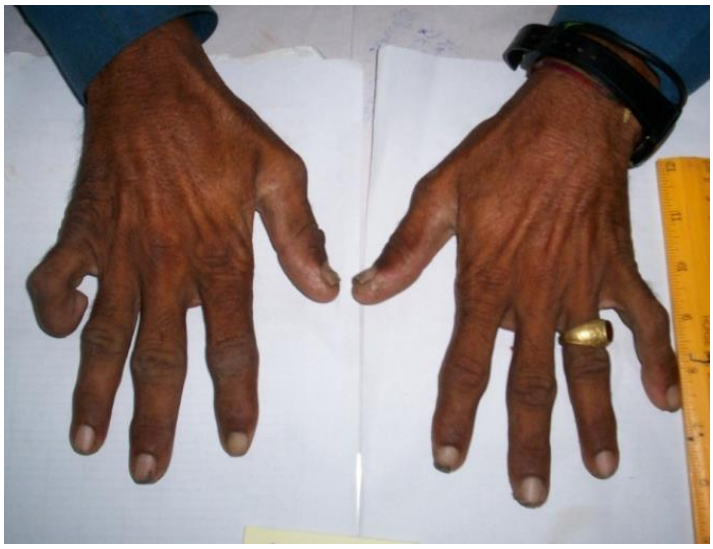

Figure 5.7. Hands of proband's Father (III-02) showing asymmetric camptodactyly of 5th fingers.

Phenotypes in Kindred 1 and 2 are summarized in Table 5.1.

Table 5.1. Clinical features of the affected subjects in Kindred 1 and 2

	Kindred / Individuals			
	1	**2**	**2**	**2**
Features	IV-01	III-02	IV-03	IV-06
Gender	M	F	F	M
Laterality	Bilateral	Bilateral	Bilateral	Bilateral
Upper Limbs				
Shortened zeugopod	+	−	+	+
Fused Radius and Ulna	+	−	+	+
Dislocated radial head	+	−	+	+
Hypoplastic humerii	−	−	−	−
Fused metacarpals	+	−	+	+
Elbow joint Anomalous	+	−	+	+
Disorganized/missing metacarpals	+	−	+	+
Absent phalanges	+	−	+	+
Fused digits	+	−	+	+
Deformed digits	+	+	+	+
Nail aplasia	+	−	+	+
Lower Limbs				
Hypoplastic tibiae	−	−	−	−
Tibia-Fibula synostoses	−	−	−	−
Fused metatarsals	+	−	+	+
Disorganized/missing metatarsals or phalanges	+	−	+	+
Fused toes	+	−	+	+
Nail aplasia	?	−	+	?
Renal defects				
Renal agenesis	−	−	−	−
Renal ectopia	−	−	−	−
Facial anomalies	+	−	+	+
Prominent forehead	−	−	−	−
Hypertelorism	+	−	?	?
Downward slanting palpebral fissures	+	−	?	?
Enamel hypoplasia	+	−	?	?
Skeletal abnormalities				
Scoliosis	+	−	?	+
dislocation of hip bones	−	−	−	−
Eye defects				
Myopia	−	−	−	−
Others				
Hypothyroidism	−	−	−	−
Laryngomalacia	−	−	−	−
Genital abnormality	−	−	−	−
Developmental delay	−	−	−	−

+: Feature present; −:Feature absent; ?: Not clear

5.3.3 Genetic methods

Blood samples of three and four subjects in Kindreds 1 and 2, respectively, were available for molecular analyses. All blood samples were collected in separately labeled EDTA vacutainers. Genomic DNA was extracted from the peripheral blood lymphocytes using standard phenol-chloroform protocol. The extracted DNA was stored at -20˚C until use. The quality of extracted DNA was assessed by using agarose gel electrophoresis.

Owing to the phenotypic similarity of subjects in both families with CLSS direct Sanger sequencing was used to screen for variants in the coding sequence of *LRP4*. Before performing Sanger sequencing, DNA was amplified by PCR (Primers sequences in Table 3.2). PCR procedure was as follow: denaturation at 95°C for 5 min, then 35 cycles of denaturing at 95°C for 30s, annealing at 52-63°C for 30s and extension at 72°C for 5 min, and finally incubation for 10 min at 72°C. The PCR products were purified. Mutation screening of all coding and exon-intron junctions of *LRP4* gene was executed by using ABI PRISM Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and separated on an ABI 377 automated sequencer/DNA analyzer (Applied Biosystems). When a DNA variant was detected, DNA from the stock was amplified by PCR and sequencing was repeated. The effect of the missense variant was predicted using the PolyPhen-2 software http://genetics.bwh.harvard.edu/pph2/). The *LRP4* mutation described is based on sequences from NM_ 002334.3.

Table 5.2. 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

The affected subjects in both families had characteristic symptoms of Cenani-Lenz syndactyly syndrome and had parental consanguinity. Hence, the index subjects in both families were initially subjected to Sanger Sequencing of the coding regions of *LRP4*.

In kindred 1, a homozygous A>G base transition in exon 12 at position c.1820 of the *LRP4* was detected in the index case. The variant was further validated in all the available family members, and it appeared in homozygous state among the affected and heterozygous state in unaffected subjects. Hence, the mutation cosegregated in the family and was not found in the normal members of the family. The variant is predicted to lead the cysteine substitution at place of tyrosine (Y607C) (Fig. 3.7). The detected novel variant was not found in the general population (ExAC and 1000 Genome database). Further it was bioinformatically predicted to be disease-causing by using online tools such as NetGene2, and MutationTester.

In second pedigree, two patients of Cenani-Lenz Syndactyly also demonstrated homozygosity of 1820 A-G mutation in exon 12 of the *LRP4* gene. Unaffected subjects of the family did not show the mutation. Mutant LRP4 has residual functionality. The observed mutation in *LRP4* is predicted to abolish its antagonistic effect on *WNT* signaling and hence impairs normal process of digit separation in the limbs.

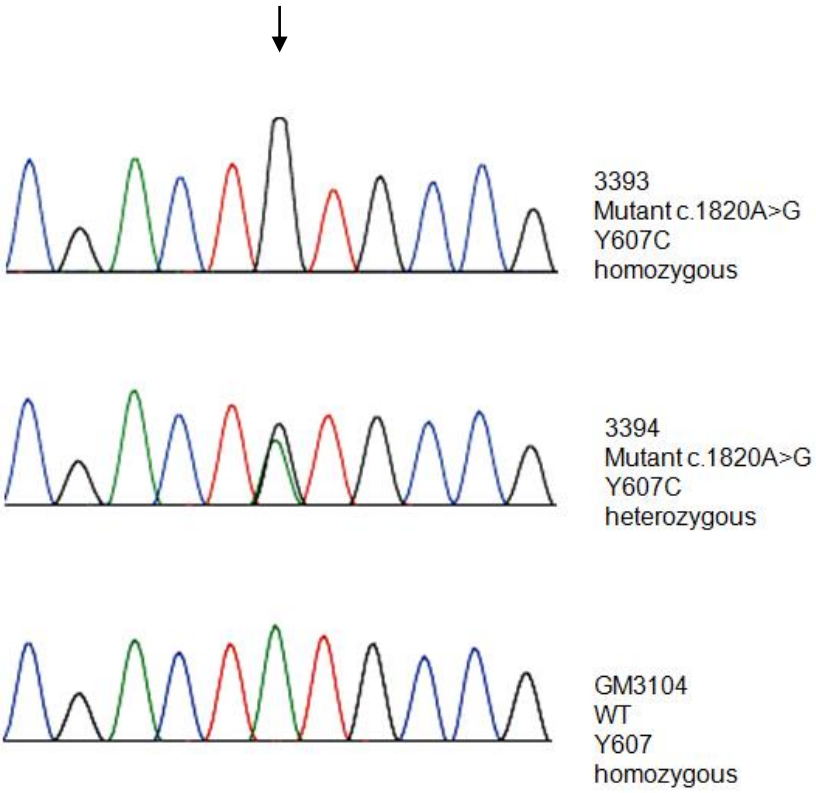

Figure 5.8. Electropherogram showing c.1820A>G transition in *LRP4*.
Homozygous mutant (upper panel) is compared with the heterozygous carrier (middle) and wild-type sequences (bottom).

5.5 Discussion

In this work, we found *LRP4* mutation in clinically diagnosed CLSS patients. Association of CLSS with *LRP4* mutations has previously been studied. These findings support our findings. *LRP4* mutations perturb the normal development of the limbs (Li et al. 2010). The protein product of *LRP4* gene is a transmembrane protein receptor belonging to LDL receptor family. Lrp4 is primarily involved in skeletal formation (Styrkarsdottir et al. 2008). It dictates limb development in dorso-ventral orientation (BioGraph). It is an important regulator of Wnt signaling pathway that is critical to limb development. Wnt signaling directs a number of physiological processes like cell proliferation and cell fate determination. LRP4 acts as an inhibitor of Wnt signaling. It facilitates SOST dependent inhibition of Wnt signaling. It also inhibits LRP5/LRP6 mediated activation of WNT/β-catenin signaling/signal transduction (Xiong et al. 2015; Leupin et al. 2011). In the course of limb development, the attenuated function of LRP4 further impairs Wnt and β-catenin signaling. Disrupted Wnt/β-catenin influences several developmental cascades giving rise to various maladies mostly those characterized by impaired bone (Clevers and Nusse, 2012). LRP4 mutations are implicated in sclerosteosis 2 (OMIM 614305), and congenital myasthenic syndrome 17 (OMIM 616304) (Ohkawara et al. 2014, Leupin et al. 2011). In total eight mutations of all the reported CLSS linked mutations of LRP4 are missense (transitions/transversions) associated with mild phenotypes with minimal involvement of the ulnae and radii. A few splice site mutations have also been reported to cause variable CLSS manifestations from mild to severe depending on the degree of alterations caused in the protein (Li et al. 2010; Afzal et al. 2017; Khan et al. 2013). Nonsense mutations are associated with severe phenotypic manifestation due to abrupt truncation of the coded protein (Kariminejad et al. 2013).

Mutations that cause early truncation of the protein produce a severe phenotype. Two splice site mutations in compound heterozygous condition caused a severe affection in a fetus leading to medical abortion. In this case, severe truncation of the LRP4 protein was considered responsible for the fetal demise (Li et al. 2010). Lindy et al. (2014) reported two compound heterozygous truncating mutations in two fetal sibs. These truncating mutations caused the prenatal death of the fetuses. Recently, Afzal et al. (2017) reported splice site mutation in individuals at the severe end of the CLSS phenotype. In these patients, severe phenotypic manifestation appeared due to the truncation of the protein caused by the mutation. We identified two families with characteristic CLSS phenotype representing the mild form of CLSS. Patients presented several typical features of CLSS. Hearing loss, high arched palate, genitor-urinary malformations, pulmonary morphopathies, cataract, supernumerary nipples and other skeletal abnormalities were not among the clinical findings. However, scoliosis was seen in affected subjects of both families. We found a previously unreported homozygous (c1820 A>G) missense mutation cosegregating in the CLSS patients of two studied families. Khan et al. (2013) reported a Pakistani family with the mild presentation of CLSS specific limb anomalies. Affected individuals of the family were found to have missense mutations of *LRP4* gene. Our findings are in agreement with the previous findings that missense mutations usually cause a milder form of the CLSS. Further, functional assays were not performed to characterize the mutation. Computational studies confirmed that missense mutation at the location of c.1820 altered the amino acid 607 of lrp4 that altered its expression and produced a variant phenotype of CLSS. Bioinformatical tools were used to predict the functionality of the mutated variant. These tools predicted mutated variant to be disease-causing with partial loss of function The mutated lrp4 proteins are predicted

to lack antagonizing activity and unable to maintain a normal level of wnt/b catenin signal transduction. Lrp4 harboring missense mutations do not integrate properly in the plasma membrane. CLSS phenotype in our families may also be due to the mislocalized integration of LRP4 but functional studies are required to elucidate the consequences of these mutations. Hence, this study provided an additional support to the idea of genotype to phenotype correlation in CLSS. The previous study reported that lrp4 harbors most of the missense mutations in the extracellular domain. LRP4 mutations in the extracellular region render adverse effects on its physiological activities (Li et al. 2010). Presence of a mutation in the extracellular region of Lrp4 in current study adds evidence to this notion.

It is pertinent to mention that the currently studied families have different surnames and have apparently no kinship. There is an overlap of certain clinical feature in both families but also have non-overlapping symptoms. The presence of the same mutation in two completely isolated families may suggest a common founder mutation in Pakistani Cenani-Lenz subjects of Sindhi origin.

5.6 Conclusion

The phenotype observed in the current study is within the wide range of the phenotypic spectrum of CLSS. Our finding supported previous findings that a missense mutation in the extracellular domain causes a milder form of CLSS with no occurrence of renal agenesis. This type of clinical presentation arises due to altered expression of the lrp4 protein. Functional studies are required to clearly elucidate the underlying mechanism through which such phenotypes appear. Moreover, genetic counseling is important by reason of the probability of further generations affected with this syndrome and an early and effective prenatal determination of the diseased condition and intervention can avert the complication related to the syndrome.

Publication: We are currently screening two more families with Cenani-Lenz syndactyly and the combined results are likely to be submitted for publication under the title:

Riaz et al. Novel mutation in *LRP4* underlies Cenani-Lenz syndactyly, spoon-head type, with a distinctive combination of facial features in Pakistani families.

6. Clinical and genetic characterization of a family with brachydactyly type C and DuPan syndrome

6.1 Abstract

DuPan syndrome, one of several chondrodysplasias, affect appendicular skeleton without causing any harm in the axial skeleton. It is caused by a member of the BMP family, *CDMP1* which regulates condensation and differentiation of mesenchymal tissues during skeletal development in embryonic growth. Mutations in *CDMP1* disrupt its potential to promote chondrogenesis and bone growth and the abnormal phenotype is manifested as malformed skeletal elements particularly of the limbs. Brachydactyly type C depict consistent clinical features like brachymesophalangy (shortening) of second, third and fifth digits, with hyperphalangy of the second and third digits along with short proximal phalanges and reduced anterior metacarpal. Chondrodysplasias like Grebe syndrome, Hunter Thompson type, DuPan syndrome and brachydactyly type C (BDC) are caused by mutations in *CDMP1* and exhibit overlapping phenotypic spectrum. In the current study, we present an extended family with simultaneous segregation of DuPan syndrome and BDC in various loops of the pedigree. These characteristic phenotypic entities were observed to show clear autosomal recessive and autosomal dominant inheritance patterns, respectively. Molecular genetic analyses of this family demonstrated that a novel mutation NM_000557(GDF5):c.404delC in *CDMP1* segregated with the DuPan syndrome and brachydactyly type C phenotypes in homozygous and heterozygous states, respectively. This is a unique family giving a molecular clue that DuPan syndrome and BDC have the same genetic etiology.

6.2 Introduction

6.2.1 Phenotypic spectrum of Brachydactyly type C (BDC)

Brachydactyly type C is a rare congenital defect with brachymesophalangy (shortening) of second, third and fifth digits, with hyperphalangy of the second and third digits along with short proximal phalanges and anterior metacarpal subjected to pruning. In type C brachydactyly (BDC) the fourth digit is usually spared and longest of all digits. Other occasional features include hypoplasia/shortening of the metacarpals, ulnar deflection, and symphalangism. The feet are either normal or show ordinary brachydactyly. Rarely, talipes equinovalgus or equinovarus may appear as an associated feature (Savarirayan et al. 2003). It is transmitted in an autosomal dominant, semi-dominant and autosomal recessive pattern. It shows intra- and interfamilial variation, with variable expressivity and lack of penetrance (Schwabe et al. 2004; Seo et al. 2013).

Further, BDC shares the phenotypic spectrum with brachydactyly types BDA2 and BDB but differs from them in its genetic cause. Disproportionate digit shortening, phalangeal hypersegmentation, and cropping of first metacarpals differentiate BDC from BDA2 and BDB types (Galjaard et al. 2001).

6.2.2 Phenotypic manifestation of DuPan syndrome

DuPan syndrome (DPS; OMIM 228900) also termed as fibular hypoplasia and complex brachydactyly is a very rare limb malformation characterized by the anomalous development of appendicular skeleton: aplasia or hypoplasia of appendicular skeletal elements, while axial skeleton and skull remain unaffected. There is, however, no vertebral abnormality (Faiyaz-Ul-Haque et al. 2002). Normally,

DuPan syndrome affects the distal portions of the limb extremities: absent or undersized fibulae with severe acromesomelic limb dwarfism and toes which are smaller and non-functional (Ahmad et al. 1990). Anomalous signs of fibular bones are usually present in both legs, i.e. the condition is bilateral. Other associated skeletal malformations include disrupted patella and ankle joint. Hands of the patients also depict clinical features like complex brachydactyly, reduced hand size, malaligned carpals, short metacarpals (mostly metacarpal I) and phalangeal hypoplasia, particularly of middle and proximal phalanges. Feet deformities include talipes equinovalgus, rounded toes, deformed tarsal bones, short metatarsals (metatarsal I) and aplastic or hypoplastic phalanges of the toes with hypoplastic-absent toenails. Thumbs are intensely malformed. At the proximal end tibiofibular articulation shows instability and at the distal end, tibial displacement is manifested (Kohn et al. 1989; Douzgou et al. 2008; Faiyaz-Ul-Haque et al. 2002). The facial features and intelligence are normal (Szczaluba et al. 2005).

DPS shares phenotypic spectrum with acromesomelic dysplasia Hunter Thompson type (AMDH; OMIM 201250) and the acromesomelic dysplasia Grebe type (AMDG; OMIM 200700). In DPS, stature is much shorter with complex brachydactyly and hypo-/aplasia of fibular bones. Large joint dislocation plus common carpo-tarsal fusion are the features that make DPC distinct from Hunter Thompon type while the stature length is mildly reduced and tubular bones are slightly affected in DPS compared to Grebe type. These conditions have been studied in several populations. DPS and brachydactyly type C (BDC) also have some features in common notably brachydactyly, phalangeal hypoplasticity, shortening of metacarpals and clubfoot (OMIM, 2017).

6.2.3 Signaling pathways implicated in appendicular development

The development of appendicular skeleton initiates as limb buds grow from the lateral plate mesoderm, as a consequence of the progression of interactive communications of the later with the overlying ectoderm. Initially, limb bud comprises undifferentiated cells. A three-dimensional coordinate control system specifies location and specialization of each progenitor cell accordingly amid limb development (Ornitz and Marie, 2015; Provot et al. 2013). It consists of the proximodistal, anteroposterior and dorsoventral axes. Every axis is further instructed by a corresponding specified set of signaling agents generated by a distinct cellular mass called signaling center. Three signaling centers dictate growth of the appendages: the apical ectodermal ridge (AER), regulates proximodistal pattern during appendicular outgrowth; ectoderm located on the superior sides of the bud, influences the patterning of the limb along the dorsoventral axis; and the zone of polarizing activity modulates the growth along the anterioposterior axis. These signaling centers generate many signaling agents which have been distinguished and characterized (Capdevila and Belmonte, 2001). The AER is an anatomical structure situated at the limb bud tip and comprises a dense population of ectodermal cells. The mesenchyme just below the AER is termed as Progress Zone (PZ). Cells of the mesenchymal tissue of the growing limb bud stimulate overlying ectoderm (via *FGF10*) to differentiate into a signaling center, i.e. AER that sends back signals, i.e. *FGF8* and forms a positive feedback loop. Fibroblast growth factors (FGFs) signaling is forwarded through the FGF receptors, presented by the underlying mesenchyme. Many diverse FGFs expressed and released by the AER, commence and control appendage protrusion. FGF signaling system controls development at later stages, by controlling skeletogenesis and growth (Ornitz and Marie, 2015; Ota et al. 2007).

Wnt/B catenin signaling help FGF signaling to maintain proximo-distal (PD) plasticity (Provot et al. 2013).

A candidate of the Wnt family of secreted glycoproteins, *Wnt7a* is pivotal for dorsoventral patterning. A dorsoventral gradient of this is required for patterning in this axis. Wnt7a, while produced by the dorsal ectoderm of the limb bud, causes *Lmx1b* (LIM homeobox-containing transcription protein, 1 beta) to express, a dorsalizing transcription factor in the mesenchyme which is related to the LIM homeodomain protein family while Engrailed 1 inhibits its expression on the ventral side. It helps in determining the cell fate (Niswander, 2003).

The ZPA is a nested set of mesenchymal cells (MCs) positioned just below the AER at the caudal boundary of the limb bud. It is the major signaling center important for the anteroposterior patterning. Sonic hedgehog (Shh) is the main mediator expressed in a gradient in A-P region with a higher concentration on the posterior end (Tickle and Towers, 2017; Chiang et al. 1996). *Shh* is a morphogen that is auto-processed after release and then doubly modified structurally by the addition of a palmitate to a cysteine at the N terminus and a cholesterin molecule to the carboxyl terminus to make it biologically active (Ingham and McMahon, 2001). Much of the intracellular signaling cascade initiated by hedgehog, is communicated by transcription factors of Gli family, particularly Gli3. Shh, influences the zinc finger protein Gli3 to convert into Gli3A (the activated form) to regulate Shh mediated cascades, whereas, otherwise, higher levels of Gli3R (the repressor form) downregulates Shh forming a negative feedback loop. Gli3 expression creates an anteroposterior gradient in the limb bud, in accordance with Shh levels, that is important for limb patterning (Littingtung et al. 2002; Te-Welscher et al. 2002). Limb skeletogenesis like numerous other developmental processes requires a concentration

gradient of signaling molecules and compartmentalization among functionally variable cells to direct differentiation and morphogenesis (Donahue, 2000; Lecanda et al. 2000).

During the development of elemental skeleton of the limbs, *HOX* genes show distinctive stage-dependent expression patterns. *HOX* genes are a family of 39 related transcription factor genes, organized into four overlapping clusters (*HOXA-D*). They code for homeodomain-containing proteins that function as patterning morphogens being instrumental in cellular proliferation and differentiation. *Hox* genes are expressed in anterioposterior symmetry. Higher number *HOX* genes are tuned to express amid late development and more in distal and posterior limb parts than lower number genes. *Hox* genes influence patterning along the anteroposterior and proximodistal axis. A chromosomal region located upstream of *hoxd13* govern repression of *hoxd* cluster (Kmita et al. 2002; Snajdr et al. 2010). In general, FGF or Shh pathways make basic patterning signals that lie upstream of *HOX* genes. The skeleton of vertebrate limbs comprises an array of elements, parted by joints in between them.

During development, pattern formation ensures delineation of the cartilaginous template. After determination of the pattern, cells aggregate at the locales of future skeletogenesis and increase cellular density that initiates pre-cartilaginous condensation. This condensation anticipates the shape and size of the future skeletal structures (Otto et al. 1997; DeLise and Tuan, 2002). It relies on cell-cell adhesion, to aid recruitment of chondrogenic MCs to the central region of the appendicular bone, and results in enhanced cell-cell interaction via gap junctions to organize the nascent (Zhang et al. 2002).

CDMP1 (Cartilage-derived morphogenetic protein 1 or GDF5; growth and differentiation factor 5) is involved in boosting up condensation of mesenchymal cells and differentiation of chondrocyte (Tsumaki et al. 1999). Possible candidates upstream of *CDMP1* are *Hox* genes, which are anticipated to regulate the positional information of skeletal elements. Condensed cells are capable to differentiate into two cell types. In areas of endochondral bone formation, they directly convert into cartilage-forming chondrocytes and in areas of membraneous bone formation, bone matrix forming osteoblasts are derived from them (Otto et al. 1997). Cartilage is further replaced by mineralized bone. Primarily, the cartilaginous skeletal elements are formed in the limb bud while the stem cells of the mesenchyme condense into chondrocytes that ultimately undergo the process of differentiation and give rise to a cartilaginous template of the respective elements, linked at joints. In skeletal development, a single condensation can give rise to more than one bone or cartilage elements. This is especially obvious in the appendages. Prechondrogenic condensations that specify the formation of the digital rays are spatially continuous initially but segment into individual skeletal elements eventually due to the degeneration of interdigital mesenchyme. *Wnt14a*, a representative of the Wnt family is involved in joint induction (Hartmann and Tabin, 2001). Mutations of *Hoxa* and *Hoxd* genes causes carpal joints coalition.

6.2.4 *CDMP1* and its role in skeletal development

Cartilage-derived morphogenetic protein 1 (*CDMP1*) is crucially required for embryonic connective tissue development and skeletal development, chondrogenesis, primary bone growth, longitudinal bone growth and joint formation/bone articulation (Tsumaki et al. 1999). This gene codifies a member protein of the bone morphogenetic protein (BMP) family. This proteins family mainly promotes the

adhesion, aggregation, and differentiation of mesenchymal chondrocytes at the early stage and significantly promotes the maturation and hypertrophy of chondrocytes in the late stage, with specific capacity to form chondrocytes induced ectopically. BMPs are also required for maintenance of the adult skeleton. BMPs are functionally diverse growth factors within the transforming growth factor β (TGF-β) superfamily. In the course of cartilage development, CDMP1 also known as GDF (growth and differentiation factor) take positive or negative regulatory route to govern chondrogenic differentiation (Storm and Kingsley, 1999).

The human *CDMP1* has a murine homologous counterpart *Gdf5*. During embryonic limb bud development, *CDMP1* is expressed throughout the pre-cartilaginous tissue, cartilaginous core, cartilage, joint interspace and growing long bones (Faiyaz-UL-Haque et al. 2002). Its expression is directly associated with the position, number and timing of developing joints. Its expression at the position of prospective articular spaces proposes that it is crucial in confining the development of joints to the suitable sites (Francis-West et al. 1999; Storm and Kingsley, 1996).

6.2.5 *CDMP1* associated skeletal disorders

Mutation in *CDMP1* causes DuPan syndrome (DPS) which is an autosomal recessive disorder with a very rare occurrence. There is a broad continuum of diseases caused by *CDMP1* mutations. The same gene is responsible for autosomal recessive chondrodysplasia, Grebe type (OMIM 200700), autosomal recessive acromesomelic dysplasia, Hunter-Thompson type (OMIM 201250) and autosomal dominant and semidominant brachydactyly type C (Table 6.1). All the morphopathies caused by the *CDMP1* mutation share certain phenotypic features (Thomas et al. 1997; Faiyaz-Ul-Haque et al. 2002; Basit et al. 2008; Yang et al. 2008).

Table 6.1. Phenotypic spectrum of *CDMP1* mutations

Phenotype	OMIM	Inheritance
Acromesomelic dysplasia, Hunter-Thompson type (AMDH)	201250	AR
Brachydactyly, type A2 (BDA2)	112600	AD
Brachydactyly, type C (BDC)	113100	AD
Brachydactyly, type A1, C (BDA1)	615072	AR, AD
DuPan Syndrome	228900	AR
Chondrodysplasia, Grebe Type (AMDG)	200700	AR
Multiple synostoses syndrome 2	610017	AD
Osteoarthritis-5	612400	
Symphanlangism, proximal, 1B	615298	

AD: Autosomal dominant; AR: Autosomal recessive

In these malformations, affected individuals show homozygosity or compound heterozygosity for inactivating mutations. These mutations reside in the greatly conserved mature region of CDMP1. These mutations restrict the chondrogenic property of CDMP1 and hence disrupts the CDMP1-mediated signaling. Misexpression of CDMP1 affect size and shape of developing skeletal structures in two ways: i.e. in early stages of development it alters cellular adhesion while changes cellular proliferation rate at later developmental stages (Francis-West et al. 1999). Acromesomelic chondrodysplasia associated with genital disorders/sex developmental disorders has a different genetic cause, i.e. a frameshift mutation in *BMPR1B* (Demirhan et al. 2005). Phenotypic similarity of the disorder caused by the *CDMP1* and *BMPR1B* because both genes are involved in the same developmental process.

Likewise, the phenotypic similarity of BDC with BDA2 and BDB arises because their causative genes are part of the single NOG-CDMP1-BMPR1B signaling pathway required for the regulation of chondrogenesis. Hence, these defects are caused by a disturbance in chondrification and ossification. Alteration of the different signaling molecules involved in the same pathway gives rise to a particular disease depending upon the mode of action of the specific mutations.

6.2.6 Structural features of *CDMP1*

CDMP1 gene contains 2 exons. Pre-propeptide of human CDMP1 constitutes 501 amino acids. It contains a glycosylation site at the N terminus and a potential polybasic cleavage site at the C terminus. The processed CDMP1 polypeptide is 13.6 kD comprising a total of 120 amino acids. Proteolytic processing at a characteristic site marked by arg-X-X-arg produces bioactive mature protein. N-glycosylation site is retained in this mature protein with 10 conserved cysteine residues. Human CDMP1 prepeptide shares 91% identity with its murine counterpart while mature proteins of both have only one different amino acid. CDMP1 is expressed as a dimer formed by a single interchain disulfide bond (Hotten et al. 1996). Mature protein has 7 cysteine residues which are highly conserved and one of them is involved in forming the intermolecular disulfide bond. Substitution of a serine in place any single unit of all the conserved TGF-β1 cysteine residues makes them biologically redundant. Site-directed mutagenesis revealed that a conserved pattern of cysteine is vital for the efficient functioning of the TGF-β superfamily. For instance, in Hunter-Thompson type chondrodysplasia, mutations are known that disrupt the 7 cysteine's highly conserved pattern and give rise to a protein product that has only initial 62 amino acids of the total amino acids count of the protein in-frame. Forty-three amino acids are not in the frame and do not share identity to the normal protein. This mutation occurs at locus 1475 inside the mature region (Thomas et al. 1996).

In the present study, we have recruited a family demonstrating a unique combination of DuPan syndrome and brachydactyly type C. Detailed clinical characteristics of both phenotypes are presented in this study. DNA sequencing led to the identification of a novel mutation in *CDMP1* gene.

6.3 Subjects and Methods

6.3.1 Family Ascertainment

An extensive family with several subjects showing shortening of certain fingers and toes and mesomelic shortening of limbs was investigated. These symptoms were characterized as brachydactyly type C and DuPan syndrome, respectively. All the available affected and unaffected subjects underwent clinical examination. Anthropometric measurements were taken and data were recorded on a standard proforma. Blood samples were obtained from all available subjects in EDTA tubes. All participating subjects consented to provide on a voluntary basis. Ethical approval of the study was acquired from the board of ethical review committee of Quaid-i-Azam University. The study protocol was in adherence to the Helsinki declaration.

6.3.2 Pedigree Analysis

An extensive pedigree chart of the family was drawn by having a detailed dialogue with family members and the elders. All acquired data were checked and cross-checked by interrogating different family members from various loops of the pedigree. There were sixteen marriages in the pedigree out of which at least six were consanguineous (Fig. 6.1). The family comprised five generations containing sixty-three family members. Forty-two family members were physically examined. A total of 7 family members were photographed while radiographs of four individuals were available.

In total, seven family members (three deceased) had a severe type of dwarfism, characteristic of DuPan syndrome (four males and three females) (Fig.6.1). Additionally, ten males and eight females had shortening of certain fingers and toes

without obvious dwarfism, the features concordant with brachydactyly type C (BDC). The pedigree suggested that the BDC phenotype segregated as a dominant entity while DuPan syndrome appeared as an autosomal recessive trait (Fig. 6.1).

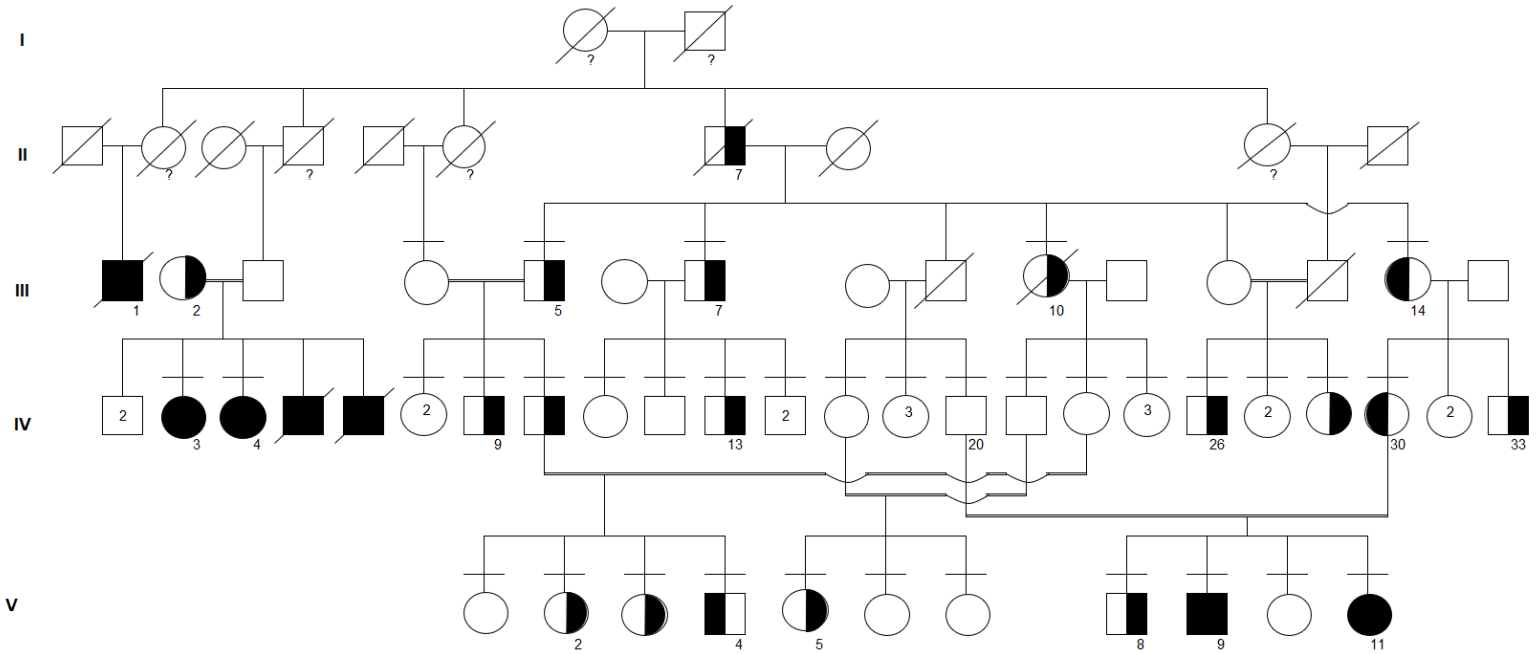

Figure 6.1. Pedigree of Family with brachydactyly and DuPan syndrome.
Filled and half-filled symbols represent subjects with DuPan syndrome and brachydactyly, respectively.

6.3.3 Clinical and radiological study

6.3.3.1 Brachydactyly phenotype

The malformation was segregating in generations II, III, IV, and V. The index male sibling (IV-13) of the proband and his father (III-7) had brachydactyly with a previous family history of brachydactyly.

Brachydactyly of both upper and lower limbs was observed in IV-13. The index and middle fingers were short, bilaterally. Fourth finger was slightly reduced in length in his left hand, however, it appeared normal in his right hand. Bilateral shortening of the thumbs was also noticed. In the feet, there was a reduction in the size of 4^{th} toes. Roentgenographs revealed shortening of second and third digits of both hands due to the ill grown middle phalanges while second digits had hypersegmented proximal phalanges with triangular shape proximal epiphysis of middle phalanges. Fourth digits were least affected and longest of all digits as their phalangeal bones had a subtle reduction. Middle phalangeal bones in all toes were either completely missing or fused with distal counterparts (Fig. 6.2). The subject had attained a normal height; however, he had curvature in elbow joint giving a clue to ulnar dislocation.

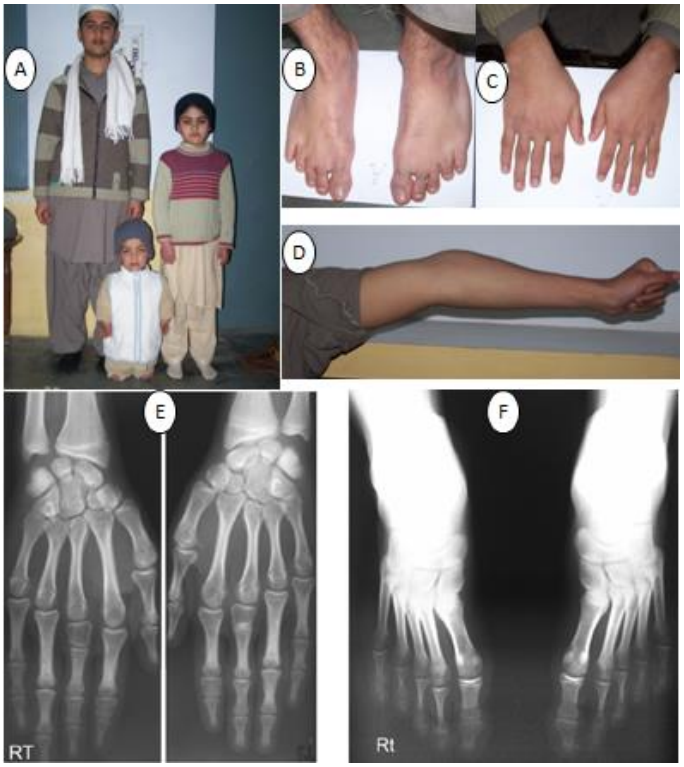

Figure 6.2. Phenotypic illustration of subjects IV-13.
(A): Subject IV-13 standing back left, V-8 back right, V-9 front, **(B):** Feet of IV-13 showing brachydactyly in toes, **(C):** IV-13 brachydactyly in hands, **(D):** arm demonstrating dysplastic elbow joint, **(E,F):** Radiographs of hands and feet of IV-13 showing reduction in the length of 2^{nd} and 3^{rd} phalangeal rays.

Subject III-7 (proband's father) had characteristic features of BDC with markedly reduced index and middle fingers in both hands and bilateral clinodactylous appearance of fourth fingers (Fig. 6.3). The fourth finger was less involved compared to the other digits. Fifth fingers were also short, bilaterally. The thumbs were least affected. Radiographs revealed a complete absence of middle phalangeal bones of second, third and fifth digits. Second and third digits had greatly shortened proximal and terminal phalanx. Fourth digits of both hands had three phalangeal segments but middle and distal phalanx were highly hypoplastic. First and 3-5 metacarpals were greatly hypoplastic. Radiographs of feet showed short and broad tarsals and metatarsals, and hypoplastic or dysplastic proximal and distal phalanges (Fig. 6.3).

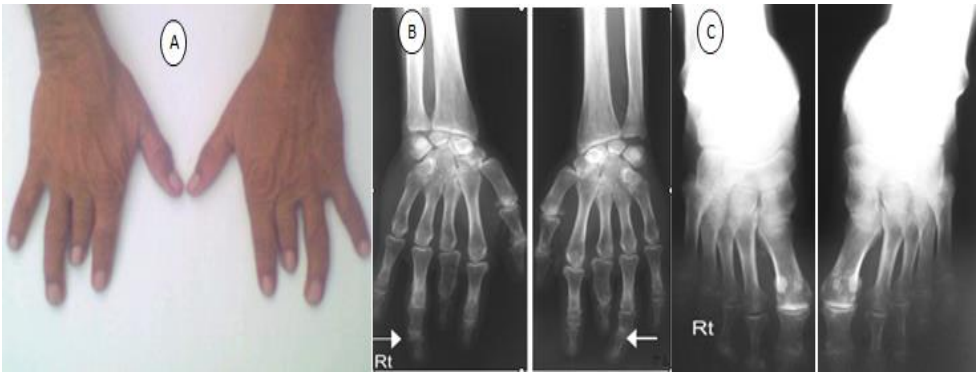

Figure 6.3. Upper and lower autopods of subject III-7.

(A): Photographs of hands showing brachydactyly type C. The fingers are disproportionately short, bilaterally; middle fingers are more severely reduced, while fourth fingers show clinodactylous inclinations; second and fifth fingers did not attain their full length, **(B):** Radiographs of hands showing hypoplastic phalanges. Most fingers had only two phalanges and hypoplastic terminal phalangeal bones. There is crowding of carpals in both hands, **(C):** The tarsals and metatarsals appear short and stubby with hypoplastic distal pahalanx.

Subject V-4 also showed characteristic BDC features. Radiographs of her autopods were taken at the age of 6 years. The carpal bones appeared ill grown and they were represented by only two bones. Metacarpal and phalangeal bones were hypoplastic. Distal and middle phalanges were short in almost all digits. In the feet, all toes had hypoplastic distal phalanges (Fig. 6.4).

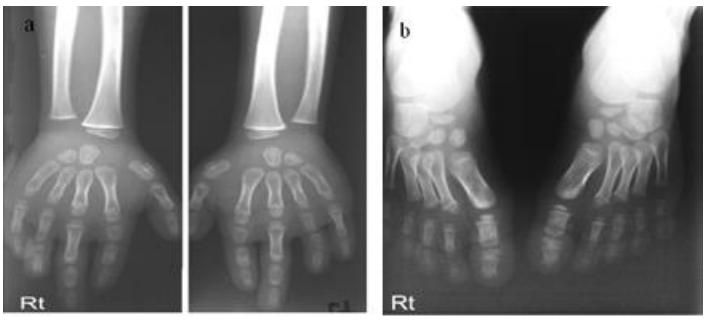

Figure 6.4. Radiographs of hands and feet of subject V-4 (age 6).

(A): hands showing reduction in number of carpal bones, only two carpal ossification centers are visible. Metacarpals and phalanges are hypoplastic and immature. Shortening of phalangeal elements is evident in all fingers, particularly the middle and distal phalanges are dysplastic, **(B):** In the feet,

tarsals are crowded, first tarsal rays are hypertrophic and all the toes show hypoplastic distal phalanges.

Hands and feet of the subject IV-30 (first cousin of the proband) had brachydactyly features. An overall reduction in digits one through five was observed in upper and lower autopods. Third digits in both hands showed camptodactyly (Fig. 6.5). She had normal height and no conspicuous anomaly of joints was evident. Her mother III-14 also exhibited features of BDC, i.e. feet showed remarkable brachydactyly of 2-5 digits while 4^{th} digit was remarkably reduced and slightly overriding the fifth toe. Second and third toes were cutaneously fused. The hands of the subject appeared normal, but she possessed slightly hypoplastic toenails. She was consanguineously married to a first cousin (cross-cousin in maternal lineage). Her partner had no apparent feature of the disease under study. Three of her four offspring had segregated the malformation. Subject V-8 was the elder son of the subject IV-30 and he had characteristic brachydactyly type C (Fig. 6.5). He had no other associated anomaly just like her mother while subjects V-9 and V-11 were severely affected. Subject V-8 exhibited a reduced length of third digits, however, other digits except for digits 4 were mildly reduced in length. Fourth and fifth digits of both hands were inclined radially while seconds digits had ulnar deviation. Radiographic examination revealed that affected individuals have phenotypic variability but the overall phenotype was concordant with BDC (Fig. 6.5).

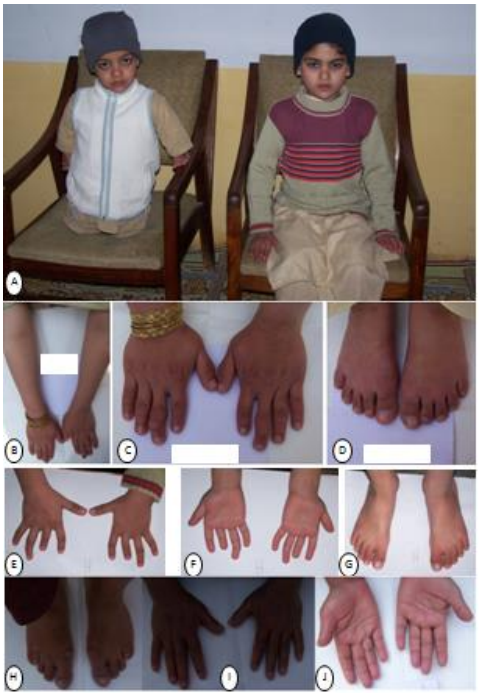

Figure 6.5. Clinical manifestation of four subjects of the family

(A): Photographs of subject V-9 (left) and V-8 (right), **(B-D):** Photographs of hands and feet of IV-30 depicting reduction of distal extremities, **(E-G):** photographs of hands and feet of V-8 depicting brachydactyly, **(H-J):** photographs of the hands and feet of III-14 showing brachydactyly.

6.3.3.2 DuPan syndrome phenotype

There were seven affected subjects with this phenotype. Affected subjects V-9 and V-11 showed a severe form of dwarfism in which middle and distal segments of the limbs were remarkably reduced in size with loss of autopodal elements. In both upper and lower limbs, mesomelic reduction was evident. The affected subjects had normal intelligence, speech, hearing, vision, and social behavior. The axial skeleton and cranium were unaffected and nail growth was normal.

Radiographic study of subject V-9 revealed that in upper limbs there were bilaterally short humerii while radius and ulna were drastically reduced in length. Carpal, metacarpal and phalangeal bones were completely lacking or aplastic. The

fingers and toes appeared as rudimentary nubbins. No bony element was found in autopods of subject V-9. In the legs, the femur was greatly reduced and no remnant of tibio-fibular bones were observed (Fig. 6.6).

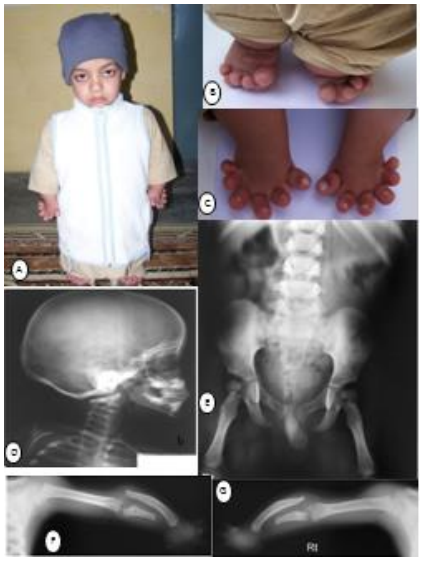

Figure 6.6. Phenotypic illustration of subject V-9.

(A): Disproportionately reduced stature, **(B,C):** Hands and feet photographs showing bead like fingers and polydactyly in hands with deformed zeugopod, **(D):** Radiographs subject V-9 showing unremarkable skull bones, heads are dysplastic and lack maturity, **(E):** lumbo-sacral region and pelvic girdle, **(F,G):** shortening of long bones, bilaterally. In both arms, the humerii, radii and ulnae are depicting retarded growth. In the autopods, there is absence of certain carpals and phalangeal elements.

6.3.4 Molecular methods

6.3.4.1 DNA extraction

Genomic DNA was extracted from peripheral blood samples by using the standard phenol-chloroform extraction method and diluted to 50ng/µl for the amplification reaction (Tables 6.2, 6.3).

For DNA extractions, the following procedure was done for cell lyses on day 1. About 500µl of blood was taken in Eppendorf tube and an equal amount of solution A was added. The reaction was kept at room temperature for 20-30 minutes and during this time they were inverted 4-6 times. The reaction was centrifuged at 4500 rpm for 5 min. The supernatant was removed and the pellet was suspended in 400µl of solution A. The pellet was dislodged. Centrifugation was repeated and the supernatant was discarded and the pellet was dislodged by tapping the Eppendorf. The pellet was suspended in 400 µl solution B, 20 µl SDS and 15 µl proteinase K. The reaction was left overnight in an incubator at 37°C.

On the second day, 500 µl of a fresh mixture of an equal volume of solution C (phenol and tris in 4:1) and D (chloroform and isoamyl alcohol in 24:1) was added and tubes were centrifuged for 15 min at 4500 rpm. After centrifugation, the supernatant was taken in a new tube and mixed with 500µl of solution D. Centrifugation was repeated. The supernatant was obtained in 15ml falcon and mixed with 55 µl of sodium acetate (3M, pH 6) and 3 volumes of cold absolute ethanol. Falcon tubes were inverted many times to precipitate the DNA. Precipitated DNA was fished out and transferred to a new tube after washing with 70% ethanol. About 100-200µl of TE buffer was added to dissolve the DNA.

6.3.4.2 Polymerase chain reaction

The amplification of primers was performed according to standard procedure in a 25 µl master mix, containing 50ng genomic DNA, 1 µl of each primer, 0.5 µl deoxyribonucleosides triphosphates (dNTPs), 0.125 unit of *Taq* DNA Polymerase, 2.5 µl of PCR buffer. The thermal cycling conditions used were as follows:

Table 6.2. Thermal cycling conditions of PCR

Step	Temperature	Duration	Cycle
Denaturation	94	5min	1
Denaturation	94	25sec	28-35
Annealing	52-60	25sec	
Extension	72	30sec	
Final Extension	72	10min	1

6.3.4.3 Solutions and Buffers

Table 6.3. Composition of solutions used in DNA extraction

Solutions	*Composition*	*Trademark*
Solution A	i. 0.32 M Sucrose	Daejung
	ii. 10mM Tris	Invitrogen
	iii. 5mM $Mgcl_2$	Riedel-dë Haen
Solution B	i. 10mM Tris	DAEJUNG
	ii. 400mM NaCl	Scharlau
	iii. 2mM EDTA	AnalaR
Solution C	i. Phenol (4 Volume)	Sigma-Aldrich
	ii. 10mM Tris (1 Volume)	Daejung
Solution D	i. Chloroform (24 Volume)	Sigma-Aldrich
	ii. Isoamyl alcohol (1 Volume)	Sigma-Aldrich
70% Ethanol	i. Ethanol	Sigma-Aldrich
	ii. Distilled water	-
Tris EDTA Buffer	i. Tris	Invitrogen
	ii. EDTA	AnalaR
	iii. Distilled water	-

Table 6.4. Solutions used in PCR

PCR Solutions	*Components*	*Volume/Reaction*
Master mix	i. Reaction Buffer (10X $(NH4)_2SO4$)	2.5 µl
	ii. 25mM $Mgcl_2$	2 µl
	iii. dNTPs	1 µl
	iv. Primers	1 µl
	v. *Taq* polymerase	0.11 µl
	vi. PCR water	15 µl
Genomic DNA	-	1 µl
Final Volume	-	22.6 µl

6.3.5 Mutation detection

To hunt for an underlying causative mutation, PCR was performed to amplify the *CDMP1* gene from genomic DNA by using primers designed from intronic regions of the gene (Table 6.5). Amplification products were purified with a PCR purification kit (Qiagen). The sequencing PCR was performed through ABI PRISM Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA) and separated on an ABI 377 automated sequencer/DNA analyzer (Applied Biosystems). The effect of the detected variant was predicted using the online computational tools like SIFT, MutationTaster, and PolyPhen-2. The *CDMP1/GDF5* mutation described is based on sequences from NM_ 000557(GDF5). Five primer pairs were used in order to cover the two exons of *CDMP1* (Table 6.5).

Table 6.5. Primers utilized in sequencing

Exon	Name	Sequence (5'-3')	Length [bp]	TM [°C]	Fragment-length in bp
1	1Afor	GCAGACTTCAAGAGTCTCAGAC	18	60	433
	1Arev	CTGACACAGCCCAAGAAGGAT	19	56	
1	1Bfor	CACCAATGCCAATGCCAGGG	21	61	786
	1Brev	GGCATCTGCATGAATGGAGGG	20	57	
2	2Afor	GAATGGGGCAGAGGTGAAAG	20	61	416
	2Arev	CGAAACTTTAAGAACTCGGCCC	21	60	
2	2Bfor	GCTGGGAGGTGTTCGACATCT	20	60	249
	2Brev	GGACGATAAGACCGTGTATGAGTACC	21	61	
2	2Cfor	TAATGAGATTAAGGCCCGCTCTG	20	59	514
	2Crev	GCACTCCTGGAATCACAGAGG	21	60	

6.4 Results

6.4.1 Clinical findings

Clinical inspection of subjects with DuPan syndrome depicted that anomaly was restricted to limbs. The distal portion of limbs was more severely affected contrasting to proximal regions. In the stylopods, the reduction was less while the more remarkable reduction was noticed in zeugopods and autopods. Fingers were either reduced in size or turned into globular or bead-like appendages. Carpal and metacarpals also showed hypoplasia. Fibulae were reduced in length. Tibial growth was also malformed. Here was a proximo-distal gradient of severity.

6.4.2 Mutation Detection

DNA sequencing revealed a deletion mutation at position 404 (from the start codon), NM_000557(GDF5):c.404delC (hg19; chr21: 34,025,305). This mutation was predicted to cause a frameshift at 135 amino acid and resulted in a premature stop codon at 145 aa. The length of the wild type protein is 501 amino acids.

After an initial sequencing run with the index and control persons, all available family subjects were subjected to mutation screening. The sequence analyses of trace reads demonstrated that this novel variant/mutation was segregating with the phenotypes in the whole family. All individuals with BDC were observed to be heterozygous for the detected variant (Fig. 6.7). Two individuals with a severe phenotypic representation, i.e. DuPan syndrome, turned up to be the bearer of homozygous mutation. Healthy participants were lacking the variant. In the course of sequence analyses of subjects with DuPan syndrome, we consumed all the DNA, and hence, high quality trace reads with homozygous mutation could not be generated. We

are currently re-contacting the family for fresh samples/or saliva from the subjects with DuPan syndrome in order to repeat the sequencing reactions.

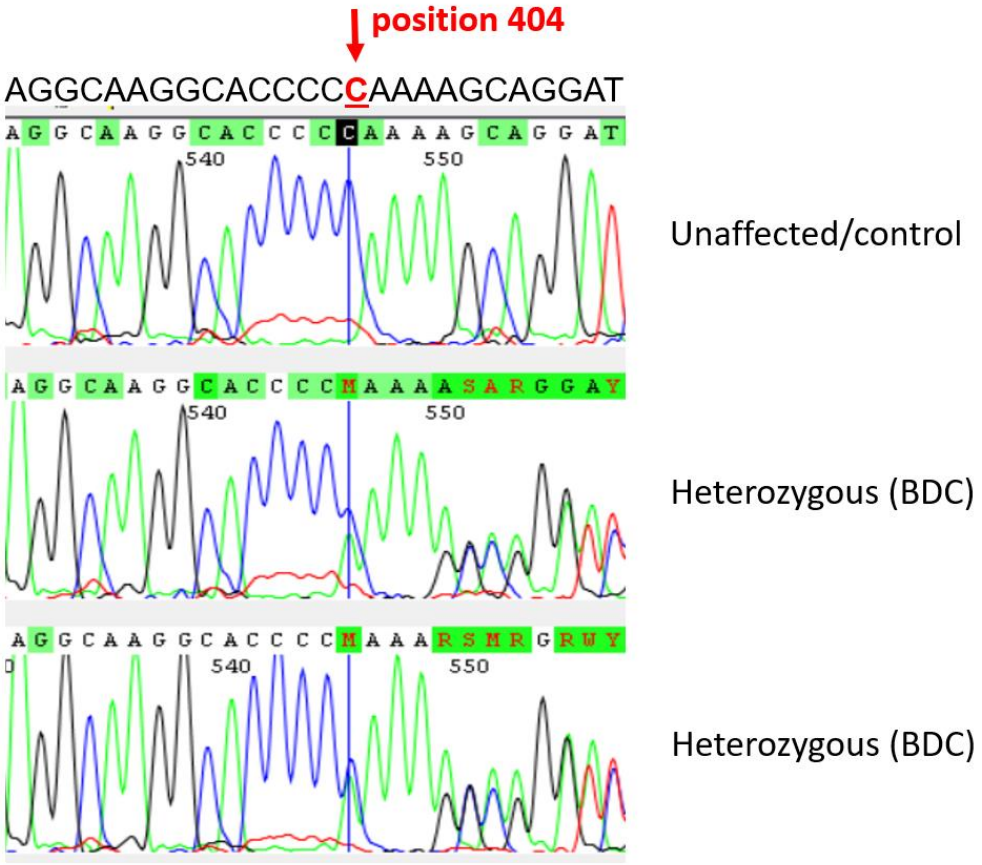

Figure 6.7. Electropherogram showing the deletion mutation c.404delC in *CDMP1*. The upper panel is for unaffected subject and the lower two panels are for BDC subjects. Homozygous sequence is not shown due to poor quality.

6.5 Discussion

A characteristic phenotype observed in current family i.e. DuPan syndrome showed autosomal recessive pattern of inheritance. This is consistent with earlier studies. Like the case reported by Faiyaz-Ul-Haque et al. (2002) from Pakistan also had autosomal recessive mode of inheritance. Heterozygous and compound heterozygous mutations have also been studied to be involved in fibular hypoplasia and complex brachydactyly (Szczaluba et al. 2005; Douzgou et al. 2008).

In the current family, cardinal features of brachydactyly were observed as short first metacarpals, hyperphalangism and disproportionate shortening of all digits with remarkable aplasia/hypoplasia of middle and proximal phalanges. Savarirayan et al. (2003) also reported similar phenotype in a family and classified it as brachydactyly type C. Our family is unique because it depicted brachydactyly type C in addition to DuPan syndrome apparently segregating in a dominant pattern with incomplete penetrance and variable expressivity. Autosomal dominant mode of inheritance is reported for BDC (Everman et al. 2002). Debeer et al. (2001) reported skipping of generations in a family with a similar phenotype. Schwabe et al. (2004) reported an autosomal recessive pattern of inheritance in a family with characteristic BDC. We observed great phenotypic variability in BDC phenotype among different individuals. This is concordant with Galjaard et al. (2001) who also reported clinical variation among different affected individuals of the same family. The great clinical variability in mutation carriers is probably due to different genetic mutations involved or different effect caused by the neighboring genes.

In the present family, we detected a novel heterozygous deletion mutation in subjects with BDC phenotype. It is reasonable to assume that the individuals with DuPan syndrome would be homozygous for that particular mutation because both

diseases are the part of the phenotypic spectrum of *CDMP1*. A plausible explanation could be that DuPan syndrome arises due to the twofold effect of the mutation in a homozygous state in CDMP1 that caused BDC in the heterozygous state. Homozygosity for a missense L441P mutation is implicated in complex brachydactyly. Affected individuals homozygous for a 1322T-C transition in the coding region of the *CDMP1* gene, were anticipated to result in a leu-pro substitution (L441P) at 441 amino acid of the protein (Faiyaz-Ul-Haque et al. 2002). Moreover, a diverse group of heterozygous mutations of CDMP1 gene results in a truncated non-functional protein which gives a clue that haploinsufficiency of *CDMP1* gene is involved in a number of defects like brachydactyly C (Everman et al. 2002).

Limb development proceeds in three main axes i.e. proximal-distal, dorsal-ventral, and anterior-posterior. This development is mediated through interactive communication among a number of signaling molecules (Tabin and Wolpert, 2007). In Du Pan syndrome distal structural elements (carpals, metacarpals, and phalanges) are more severely affected than the proximal (humerus and femur) and medial (radius, ulna, tibia and fibula) counterparts. Its mean that these disorders arise due to interrupted signaling that mediates development in proximo-distal axis. Several signaling molecules are intricated in these signaling cascades. This idea was confirmed by the identification of *CDMP1* mutations in patients affected with these diseases suggesting that *CDMP1* is crucial for limb development in proximo-distal orientation (Thomas et al. 2006). It is also proposed that there is great concordance between the proximo-distal gradient of affection severity and relative expression of *CDMP1* (Thomas et al. 1997).

6.6 Conclusion

In conclusion, we present a unique family in which two distinct phenotypes, BDC and DuPan syndrome segregated simultaneously, and were likely to be caused by a heterozygous and homozygous mutation, respectively. We intend to generate high-quality electropherograms for the homozygous subjects in order to report these findings as a research paper.

7. Clinical and molecular investigations of a family with intellectual disability

7.1 Abstract

Intellectual disability (ID) is characterized by reduced adaptive and cognitive functionality. About 3 % of the population is affected by this condition worldwide. A large Pakistani family with multiple affected subjects exhibiting the symptoms of inherited ID was studied. Initially, SNP based genotyping was carried out with the help of a commercial service provider. Further, homozygosity mapping was used in order to detect regions of homozygosity shared among the patients. One patient from of this family was selected for exome sequencing. Analyses of these data led to the shortlisting of rare variants which are pathologically relevant to the phenotype and also fall in the homozygous intervals detected in the SNP scan. Nonsynonymous homozygous variants are identified in four genes which are related to neurological functions. One of the identified genes has been implicated in autosomal recessive ID while the rest of the three are expressed in the brain. Identified variants are currently being scrutinized through Sanger sequencing. This knowledge will be helpful in understanding the disease pathogenicity at the molecular level and would be useful for the genetic counseling of the family.

7.2 Introduction

Intellectual disability (ID) or mental retardation (MR) is defined as a mental ailment in which an individual fails to develop a sufficient cognitive and adaptive level. In diagnostic terms, an individual with an intellectual quotient (IQ) below 70 is declared as mentally handicapped (Winnepenninckx et al. 2003).

On the basis of IQ level, ID is further categorized as mild (IQlevel50-69), moderate (IQ level 35-49), severe (IQ level 20-34), and profound (IQ<20) (Ropers and Hamel, 2005; Ropers, 2010). Mild ID is 85% prevalent, moderate is 10%, severe 3-4%, and profound ID is diagnosed in 1-2% patients of ID (Ludi et al. 2012). The term ID can be dichotomized as syndromic and non-syndromic ID. In syndromic form, ID patients have other abnormalities in an association like behavioral and skeletal abnormalities, facial deformities and metabolic issues whereas no abnormalities are found in association with ID in non-syndromic type (Rafiq et al. 2011).

On the basis of its etiology ID can be broadly classified as environmental and genetic. Environmental causes include a variety of reasons like brain infections and injuries, prenatal, perinatal complications, psychological trauma, and drug abuse. Genetic reasons involve an abnormality in DNA at variable levels (chromosome, chromatid, gene). These causes range from chromosomal aberrations encompasses monogenic autosomal/X-linked dominant or recessive mutations, polygenic to mitochondrial mutations. Genetic MR may constitute inherited or non-inherited MR (Winnepenninckx et al. 2003). The impact of inherited MR is quite larger than environmental and non-inherited genetic MR.

It has been estimated that 1-3% of the human population suffers from mental ailment ranging from mild psychological problems to severe learning and adaptive

disabilities (Croen et al. 2001; APA, 2000). Further, an estimated 20% of cases with ID are attributed to genetic reasons; the prevalence, however, varies from country to country.

According to the World Health Organization, Pakistan bears the largest ID population (WHO, 2011). An estimated 7.8% Pakistan population suffers from a certain type of ID and neurological problems. There is no systematic classification available for this segment of society. The characterization of the mentally retarded population based upon the etiological, phenotypic, and severity indices has not been established. Among the subjects with intellectual disability, the contribution of biologic and genetic etiology remains unexplored.

According to the Pakistan census report 1998, 6.9% of the total population falls in a separate category from ID, i.e. insane. As in Pakistan, clinical diagnosis for the majority of ID is not available, so 6.9% of population announced as “insane” may also include late-onset mental retardations, e.g. Huntington or genetic IDs with unknown etiology, other than drug abuse, psychological and emotional traumatized insane. The Capital city of Islamabad bears the largest ID population in the country, i.e. 8.1%. Prevalence of ID in the provincial areas is also alarming, with a larger number of ID in urban area in correspondence to the larger population living in urban areas (PCO, 1998).

More than 800 genes have been found accountable for syndromic and non-syndromic forms of ID, with the difference in the functioning of their protein products. These 800 genes comprise 18% of all (4500) disease-linked genes listed by the OMIM database (Chiurazzi and Pirozzi, 2016). About 40 genes have been identified so far, involved in the pathogenesis of nonsyndromic autosomal recessive ID (Musante and Roper, 2014).

Genes mutated in individuals with ID functions in methylation, proteolysis, metabolism of carbohydrates and lipids, signaling cascades, neural morphogenesis, ionic balance, ubiquitination of other molecular components, DNA damage response, modifications of tRNA through enzymatic activity and in degradation of mRNA. Common cellular processes affected by dysfunctioning of these protein products are neuronal development, migration of neurons, synaptic functions and transcriptional and translational changes (Khan et al. 2016).

Mutated signaling transduction pathways are significantly involved in causing ID. Mutations of RAS-MAPK signaling cascade (involved in x growth regulation and embryonic development) is responsible for certain intellectual disabilities known as rasopathies (Schubbert et al. 2007). Rho GTPase cascade is another pathway affected in ID. This pathway contains Rho GTPases, which function as molecular switches in the development of dendritic spines. Dendritic spines are important for the process of learning and memory. In different types of non-syndromic intellectual disability, mutations in regulators and effectors of RhoGTPase are reported (Ba et al. 2013). Mutations affecting transcriptional regulation and remodeling of chromatin are also elucidated for certain ID-linked syndromes e.g. Kleefstra syndrome (Kleefstra et al. 2012) and Coffin-siris syndrome (Santen et al. 2012). Some of the genetically characterized intellectual disability types are enlisted in Table 7.1.

Table 7.1. Genetically characterized ID types*

OMIM	Malformation	Inheritance	Locus	Gene
612337	MR, autosomal dominant 22	AD	1q44	*ZBTB18*
614607	MR, autosomal dominant 14	AD	1p36.11	*ARID1A*
616364	MR, autosomal dominant 37	AD	1q21.3	*POGZ*
615074	MR, autosomal dominant 18	AD	1q21.3	*GATAD2B*
616973	MR, autosomal dominant 42	AD	1p36.33	*GNB1*
156200	MR, autosomal dominant 1	AD	2q23.1	*MBD5*
616887	MR, autosomal recessive, 52	AR	2q11.2	*LMAN2L*
614255	MR, autosomal dominant 9	AD	2q37.3	*KIF1A*
617752	MR, autosomal dominant 49	AD	2q36.3	*TRIP12*
615075	MR, autosomal dominant 19	AD	3p22.1	*CTNNB1*
206700	Aniridia, cerebellar ataxia and MR	AD/AR	3p26.1	*ITPR1*
604717	MR, autosomal recessive 2	AR	3 p26.2	*CRBN*
617333	Intellectual developmental disorder with dysmorphic facies and ptosis	AD	3p25.3	*BRPF1*
615761	MR, autosomal dominant 23	AD	3p25.3	*SETD5*
616944	MR, autosomal dominant 41	AD	3q26.32	*TBL1XR1*
616355	MR, autosomal dominant 35	AD	6p21.1	*PPP2R5D*
616977	MR, autosomal dominant 43	AD	6q24.2	*HIVEP2*
612621	MR, autosomal dominant 5	AD	6p21.32	*SYNGAP1*
615834	MR, autosomal dominant 26	AD	7 q11.22	*KIAA0442*
613192	MR, autosomal recessive 13	AR	8q24.3	*TRAPPC9*
617051	MR, autosomal recessive 55	AR	11q24.2	*PUS3*
613970	MR, autosomal dominant 6	AD	12p13.1	*GRIN2B*
244450	Blepharophimosis-Ptosis-ID Syndrome	AR	12q24.11	*UBE3B*
616789	MR and distinctive facial features with or without cardiac defects	AD	12q24.21	*MED13L*
218340	MR with or without craniofacial dysmorphism	AR	12p13.31	*C12orf57*
616579	MR, autosomal dominant 40	AD	13q34	*CHAMP1*
615502	MR, autosomal dominant 21	AD	16q22.1	*CTCF*
616260	Overgrowth, macrocephaly, and ID syndrome	AD	18q12.1	*RNF125*
615873	MR, autosomal dominant 28	AD	20q13.13	*ADNP*
616330	Myasthenic syndrome, congenital, 18, with ID and ataxia	AD	20p12.2	*SNAP25*
614608	MR, autosomal dominant 15	AD	22q11.23	*SMARCB1*

300352	MR, X-Linked, With creatine transport deficiency	XLR	Xq28	*SLC6A8*
309548	MR, X-linked, FRAXE type	XLR	Xq28	*AFF2*
300088	Epilepsy, Female restricted, with MR	XL	Xq22.1	*PCDH19*
300143	MR, X-Linked 21	XLR	Xp21.3-p21.2	*IL1RAPL1*
300967	MR, X-Linked, Syndromic, Mircsof - Langouet type	XL	Xq13.1	*NONO*
300966	MR, X-linked, syndromic 33	XLR	Xq13.1	*TAF1*
300986	MR, X-linked, syndromic, Bain type	XLD	Xq22.1	*HNRNPH2*
300983	MR, X-linked 104	XLR	Xp22.2	*FRMPD4*
300438	MR with choriothetosis and abnormal behavior	XLD	Xp11.22	*HSD17B10*
309530	MR, X-linked 1/78	XLD	Xp11.22	*IQSEC2*

AD: Autosomal dominant; AR: Autosomal recessive; XLD; X-linked dominant; XLR: X-linked recessive
*: Selected list of well-characterized ID types from OMIM

7.3 Subjects and Methods

7.3.1 Pedigree characteristics

A highly inbred family presented with several subjects afflicted with moderate to severe ID was recruited from Southern Punjab, Pakistan. The pedigree of four generations was constructed by interviewing the family elders. The information was checked and rechecked by consulting the relatives in various loops of the pedigree (Fig. 7.1). The phenotypic data on affected subjects were obtained on a structured proforma. The clinical assessment was done with the help of local clinicians and consultant neurologists. In this family, eight individuals (4 Males and 4 Females) were afflicted with ID. They segregated in two independent loops and were the products of consanguineous couples, hence, an autosomal recessive inheritance was most likely. Clinical evaluation of five affected family members is given in Table 7.2.

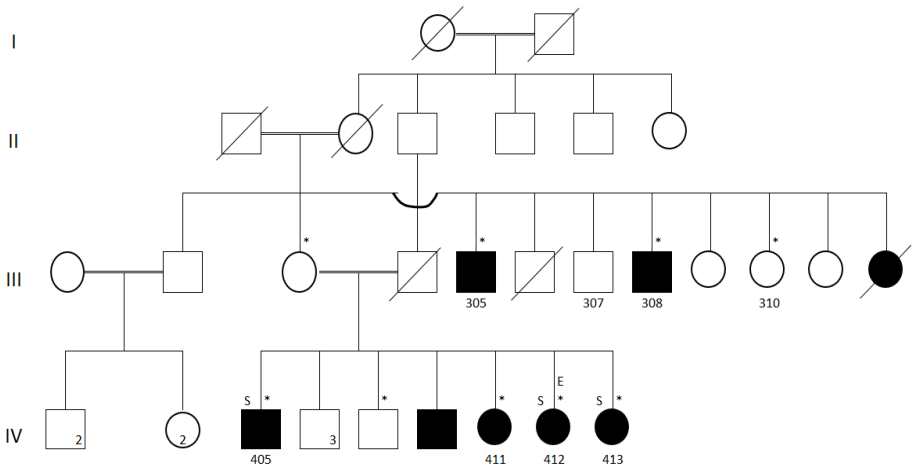

Figure 7.1. Pedigree of family with intellectual disability.
An asterisk above the symbol shows the subjects who were physically examined. S shows the subjects who were SNP genotyped and E show the subject who underwent exome analyses.

7.3.2 Phenotypic and clinical examination

There were certain common features evident in most of the examined affected subjects. For instance, the physical evaluation showed the structural anomalies like prominent jaws, squint eyes, mild-craniofacial anomalies, mild scoliosis and flat foot. Behavioral symptoms included mood instability, self-mutilation, recurrent aggressive behavior, hyperactivity, hunger-based aggression, crying, jumping or biting others and crowd sensitivity, and isolated and secluded behavior. None of the affected had any formal schooling; they were unable to carry out any manual work even under supervision. They had poor learning skills and poor concept of money. Reportedly, the subjects were also afflicted with developmental delay, and had late walking and running, late toileting, and digestive problems. The clinical features of affected subjects have been presented in Table 7.2.

We searched in the OMIM database (https://www.omim.org) by entering these symptoms but could not find any specific ID type. There were a large number of well-characterized disorders the symptoms of which overlapped with the condition segregating in the present family.

Table 7.2. Clinical description of affected subjects in family with intellectual disability

Variable	**305**	**308**	**405**	**412**	**413**	**Concordance**
Gender	M	M	F	M	F	3M:2F
Age	38	35	19	27	23	-
Prominent jaw	+	+	+	+	+	5/5
Forehead protruding	+	+	+	-	+	4/3
Squint eyes	+	+	-	+	+	4/5
Flat feet	+	+	+	+	+	5/5
Scoliosis	+	+	+	+	+	5/5
Walking late	+	+	+	+	+	5/5
Toileting late	+	+	+	+	+	5/5
Digestive problems	+	+	+	+	-	4/5
Behavioral symptoms						
Psychosis in childhood	+	+	+	+	+	5/5
Mood instability	+	+	+	+	+	5/5
Episodic recurrent aggression	+	+	+	+	+	5/5
Hyperactive	+	+	+	+	+	5/5
Fear based crying	+	+	+	+	+	5/5
Hand biting	+	+	+	+	+	5/5
Learning late	+	+	+	+	+	5/5
Concept of money	-	-	+	-	+	2/5
Social responsibility	+	-	+	-	+	3/5
Sense of self respect	+	+	+	+	+	5/5
Self-health caring	+	+	-	-	-	2/5
Poor concept of safety	+	+	-	-	-	2/5

7.3.3 Genetic analysis

Whole-genome SNP genotyping data were generated with the help of a commercial service provider. For genotyping, equal quantities of DNA of three patients (405, 412, and 413) was pooled. Pre-processed exome sequencing data file with the annotated list of variants were obtained for one patient (412).

Briefly, SNP genotyping was carried out by Illumina Human Omniexpress-24 BeadChip. SNP genotyping data were generated for three individuals depicted by “S” over shapes in pedigree (Fig. 7.1).

7.4 Results

7.4.1 Identification of homozygous intervals in whole-genome SNP data

SNP genotype data were scrutinized for the identification of homozygous intervals on each chromosome. For this purpose, the data were imported in Excel and the genotypes with heterozygous and homozygous status were highlighted in different colors. The genotypes were visually inspected and large intervals with homozygosity were marked. The homozygous regions having a length of ≥0.4Mb were selected. Chromosomes and size of homozygous regions were sorted in ascending order by using filters in Excel. These intervals were categorized on the basis of size and recorded chromosome-wise in Table 7.3 (Fig. 7.2).

Table 7.3. Summary table of identified homozygous intervals in whole-genome SNP data

Ch.	**No of homozygous intervals with size in Mb**							
	0.4-0.49	0.5-0.59	0.6-0.69	0.7-0.79	0.8-0.89	0.9-0.99	≥1	Total
1	2	6	3	3	2	1	2	19
2	6	5	2	5	-	-	1	19
3	9	2	2	3	1	-	3	20
4	4	3	3	1	-	-	1	12
5	5	4	2	-	1	-	1	13
6	5	4	2	1	1	-	1	14
7	5	1	2	2	2	-	4	16
8	1	2	3	2	1	-	1	10
9	3	2	2	-	-	-	1	8
10	2	5	1	2	-	-	3	13
11	4	1	3	-	-	-	1	9
12	1	-	3	1	1	1	2	9
13	4	1	2	-	-	-	-	7
14	4	-	-	-	-	2	1	7
15	-	1	1	1	-	-	2	5
16	2	3	-	-	1	-	1	7
17	2	1	4	1	-	-	1	9
18	1	1		1	-	2	1	6
19	2	2	-	-	-	-	-	4
20	1	1	-	1	1	-	2	6
21	-	-	-	-	-	-	1	1
22	1	1		-	-	1	-	3
X	11	6	6	1	3	-	7	34
Y	-	-	1	-	-	-	2	3
Total no.	**75**	**52**	**42**	**25**	**14**	**7**	**39**	**254**
%age	**29.53**	**20.47**	**16.54**	**9.84**	**5.51**	**2.76**	**15.35**	**100**

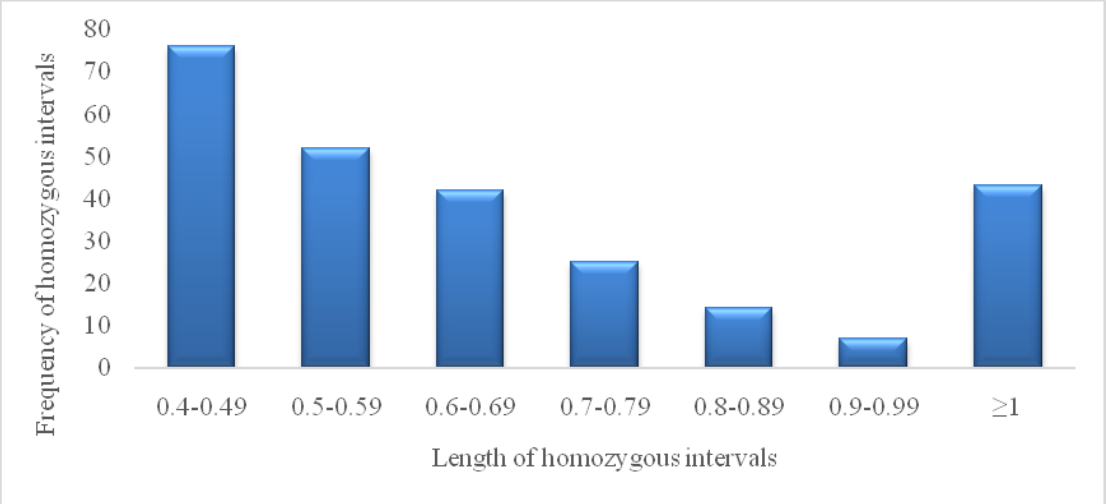

Figure 7. 2. Length-wise frequency of homozygous intervals of size ≥0.4 Mb.

Bar chart indicates that homozygous regions having a length of 0.4-0.49, 0.5-0.59 and ≥ 1Mb are more frequent in the genome.

7.4.2 Selection of homozygous intervals ≥0.8 Mb in whole-genome SNP data

For further analysis, regions having a length of ≥0.8 Mb were selected because they are larger in size and that could not be simply due to inbreeding, and there could be high likelihood to find the defective gene(s) in these regions. The boundary SNPs and their corresponding nucleotide positions of these intervals were obtained. In this way, each chromosome was analyzed from upper telomere to lower telomere. Finally, the information or intervals was tabulated chromosome-wise (Table 7.4).

Table 7.4. Identification and categorization of homozygous intervals ≥0.8 Mb from whole-genome SNP data

S. No	Chr. No	Start SNP	End SNP	Start bp	End bp	Diff (Size) Mb	Size (Mb)
1	1	rs4477212	rs35940137	82,154	940,203	858,049	0.86
2	1	rs9726225	rs4661500	12,880,356	13,743,099	862,743	0.86
3	1	rs506375	rs2815424	92,598,258	93,553,223	954,965	0.95
4	1	rs11249342	rs3010980	121,239,763	144,994,694	23,754,931	23.75
5	1	rs2999613	rs6685305	147,820,342	149,046,978	1,226,636	1.23
6	2	rs4442999	rs7604922	89,427,986	95,675,477	6,247,491	6.25
7	3	rs3804800	rs17041276	3,127,519	4,755,070	1,627,551	1.63
8	3	rs9830067	rs2670003	4,756,814	9,087,967	4,331,153	4.33
9	3	rs6775579	rs34791294	43,994,802	44,872,450	877,648	0.88
10	3	rs9714342	rs12631264	90,428,286	93,546,445	3,118,159	3.12
11	4	rs3761731	rs11722603	48,832,968	52,969,732	4,136,764	4.14
12	5	rs4318778	rs1898630	12,049,381	12,948,221	898,840	0.90
13	5	rs28703202	rs36065930	68,826,788	70,307,386	1,480,598	1.48
14	6	rs1361504	rs1321517	50,258,198	51,757,497	1,499,299	1.50
15	6	rs228420	rs17064526	134,887,047	135,773,225	886,178	0.89
16	7	rs12668378	rs12672832	54,009,270	54,905,511	896,241	0.90
17	7	rs2091228	rs13307167	64,409,809	65,466,565	1,056,756	1.06
18	7	rs7790351	rs7798412	71,936,690	72,972,329	1,035,639	1.04
19	7	rs757575	rs711302	73,961,074	75,155,747	1,194,673	1.19
20	7	rs258989	rs17158744	110,543,241	111,353,361	810,120	0.81
21	7	rs2699717	rs3898475	118,468,611	119,659,037	1,190,426	1.19
22	8	rs10087517	rs16938118	48,639,976	49,514,893	874,917	0.87
23	8	rs11984923	rs12545003	49,549,094	51,328,497	1,779,403	1.78
24	9	rs7853023	rs11141466	38,772,575	71,019,573	32,246,998	32.25
25	10	rs7090625	rs12242310	23,424,277	24,490,252	1,065,975	1.07
26	10	rs11599019	rs3740072	100,080,631	101,577,123	1,496,492	1.50
27	10	rs2002042	rs10883931	101,587,931	105,623,955	4,036,024	4.04
28	11	rs7479178	rs28826070	51,566,909	54,801,549	3,234,640	3.23
29	12	rs296759	rs2359006	50,360,461	51,314,760	954,299	0.95

30	12	rs1245652	rs9971828	62,600,764	66,658,101	4,057,337	4.06
31	12	rs1688545	rs1438997	89,708,714	90,567,752	859,038	0.86
32	12	rs337656	rs12424560	92,684,351	96,543,225	3,858,874	3.86
33	14	rs28848003	rs8004819	19,280,733	20,263,425	982,692	0.98
34	14	rs12893709	rs1205083	71,348,847	72,289,633	940,786	0.94
35	14	rs28725385	rs10133227	105,933,094	107,222,493	1,289,399	1.29
36	15	rs12905389	rs7167893	20,071,673	22,427,966	2,356,293	2.36
37	15	rs4779695	rs2338834	30,307,205	31,337,725	1,030,520	1.03
38	16	rs929207	rs4967739	31,827,215	47,060,239	15,233,024	15.23
39	16	rs4625742	rs16945716	47,089,357	47,980,267	890,910	0.89
40	17	rs7209871	rs12453728	22,217,680	25,384,867	3,167,187	3.17
41	18	rs8088010	rs8094619	14,994,970	18,568,966	3,573,996	3.57
42	18	rs1899058	rs2614999	41,343,118	42,289,601	946,483	0.95
43	18	rs4890544	rs16978548	43,045,153	43,946,837	901,684	0.90
44	20	rs11699316	rs6138795	25,199,992	26,078,211	878,219	0.88
45	20	rs860980	rs6087217	26,226,068	29,552,644	3,326,576	3.33
46	20	rs293712	rs17091794	31,940,540	33,026,003	1,085,463	1.09
47	21	rs28971224	rs11702489	10,827,533	15,052,812	4,225,279	4.23
48	22	rs12157537	rs2096537	16,114,244	17,094,749	980,505	0.98
49	X	rs12009355	rs5979479	10,842,553	11,866,932	1,024,379	1.02
50	X	rs6654060	rs7473613	19,262,785	20,313,678	1,050,893	1.05
51	X	rs6633357	rs3788756	20,474,300	21,762,921	1,288,621	1.29
52	X	rs968608	rs6651773	36,821,756	37,643,668	821,912	0.82
53	X	rs1021160	rs1409117	51,031,584	53,302,495	2,270,911	2.27
54	X	rs3005547	rs12559374	58,374,872	61,940,828	3,565,956	3.57
55	X	rs5964688	rs1386582	62,964,723	63,984,508	1,019,785	1.02
56	X	rs10482091	rs7877310	64,025,794	66,664,802	2,639,008	2.64
57	X	rs2498867	rs4501730	98,223,360	99,092,511	869,151	0.87
58	X	rs5987042	rs659139	154,066,677	154,898,561	831,884	0.83
59	Y	rs2534636	rs35797492	2,657,176	5,336,063	2,678,887	2.68
60	Y	rs2558271	rs9786224	6,082,610	28,817,458	22,734,848	22.73

7.4.3 Identification of phenotypically relevant genes that fall in homozygous intervals by GeneDistiller (http://www.genedistiller.org)

Selected homozygous intervals were further scrutinized by GeneDistiller (http://www.genedistiller.org/) and Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) to find out candidate genes of the phenotypically related syndrome. First, homozygous intervals were analyzed by using the UCSC Human genome browser (https://genome.ucsc.edu/) to identify their position on respective chromosomes and to visualize that they do not fall in telomeric or centromeric regions. In GeneDistiller, only chromosome number and starting and ending nucleotide positions of linkage interval were entered without changing all other pre-defined parameters. Homozygous intervals were analyzed thoroughly by using selected search terms ‘intellectual disability, mental retardation, cognitive impairment, developmental delay, brain, neuro developmental disorders etc. Regions harboring disease-related genes (having an expression in the brain) were written in the separate table along with respective genes and their expression (Table 7.5).

Table 7.5. Identification of phenotypically relevant genes in homozygous intervals through GeneDistiller

Chr. No	Start bp	End bp	Size (Diff)	Size (Mb)	Total genes	UCSC Location	Candidate Genes	Expression/ Phenotype
1	82,154	940,203	858,049	0.86	35	1p36.33	*KLHL17*	Brain development
1	92,598,258	93,553,223	954,965	0.95	18	1p22.1	*BTBD8*	Brain development
							FAM69A	Expression in brain
1	147,820,342	149,046,978	1,226,636	1.23	36	1q21.2	*PPIAL4A*	Expression in brain
							PPIAL4E	Expression in brain
3	3,127,519	4,755,070	1,627,551	1.63	11	3p26.2-3p26.1	*CRBN*	Autosomal recessive non-syndromic intellectual disability (MRT2)
							SUMF1	Developmental regression, Cognitive impairment, Intellectual disability
							TRNT1	Global developmental delay
							ITPR1	Motor delay, Cognitive impairment
3	4,756,814	9,087,967	4,331,153	4.33	23	3p26.1-3p25.3	*SRGAP3*	Severe mental retardation
5	68,826,788	70,307,386	1,480,598	1.48	34	5q13.2	*OCLN*	Intellectual disability profound, Microcephaly
6	50,258,198	51,757,497	1,499,299	1.50	10	6p12.3	*TFAP2B*	Intellectual disability, Cognitive impairment
6	134,887,047	135,773,225	886,178	0.89	10	6q23.2-6q23.3	*AHI1*	Intellectual disability, Cognitive impairment, Motor delay
7	64,409,809	65,466,565	1,056,756	1.06	28	7q11.21	*GUSB*	Intellectual disability, Neurodegeneration, Macrocephaly
7	71,936,690	72,972,329	1,035,639	1.04	23	7q11.22-7q11.23	*FZD9*	Brain development, William`s syndrome
7	73,961,074	75,155,747	1,194,673	1.19	31	7q11.23	*GTF2I*	William`s syndrome
7	110,543,241	111,353,361	810,120	0.81	3	7q31.1	*IMMP2L*	Tourette syndrome, Neurodevelopmental disorders
8	48,639,976	49,514,893	874,917	0.87	9	8q11.21	*PRKDC*	Brain atrophy, Microcephaly

							MCM4	Microcephaly, Postnatal growth retardation
8	49,549,094	51,328,497	1,779,403	1.78	8	8q11.21	*SNAI2*	Microcephaly, Cognitive impairment
9	38772575	71019573	32246998	32.25	222	9p13.1-9q21.11	*SPATA31A1*	Expression in brain
							CBWD3	Expression in brain
							PGM5	Expression in brain
10	23,424,277	24,490,252	1,065,975	1.07	8	10p12.2	*PTF1A*	Microcephaly, Triangular face
10	100,080,631	101,577,123	1.496,492	1.50	20	10q24.2	*COX15*	Microcephaly, Intellectual disability
10	101,587,931	105,623,955	4,036,024	4.04	96	10q24.2-10q24.33	*PITX3*	Cataract and associated mental retardation
							CWF19L1	Intellectual disability, Global developmental delay
							CNNM2	Intellectual disability, Microcephaly,
							NT5C2	Intellectual disability, Motor delay
							CHUK	Microcephaly
							SUFU	Cognitive impairment
							PSD	Expression in brain
12	50,360,461	51,314,760	954,299	0.95	18	12q13.12	*COX14*	Intellectual disability, Global developmental delay
							DIP2B	Intellectual disability
							ASIC1	Expression in brain
12	62,600,764	66,658,101	4,057,337	4.06	55	12q14.1-12q14.3	*TMEM5*	Microcephaly, Cognitive impairment
							LEMD3	Microcephaly, Cognitive impairment, Neurological speech impairment
							HMGA2	Cognitive impairment, Neurological speech impairment
12	92,684,351	96,543,225	3,858,874	3.86	58	12q22-12q23.1	*CRADD*	Autosomal recessive non-syndromic intellectual disability
							CEP83	Intellectual disability
							NDUFA12	Intellectual disability, Global developmental delay
16	31,827,215	47,060,239	15,2330,24	15.23	116	16p11.2-16q12.1	*GPT2*	Microcephaly, Intellectual disability, Global developmental delay

							ORC6	Microcephaly, Cognitive impairment, Delayed skeletal maturation
18	41,343,118	42,289,601	946,483	0.95	6	18q12.3	*SETBP1*	Mental retardation
18	43,045,153	43,946,837	901,684	0.90	12	18q12.3-18q21.1	*EPG5*	Microcephaly, Cognitive impairment
							ATP5A1	Microcephaly
20	25,199,992	26,078,211	878,219	0.88	18	20p11.21-20p11.1	*PYGB*	Expression in brain
20	31,940,540	33,026,003	1,085,463	1.09	27	20q11.21-20q11.22	*AHCY*	Intellectual disability, Global developmental delay
							ITCH	Global developmental delay
							CDK5RAP1	Brain development
21	10,827,533	15,052,812	4,225,279	4.23	25	21p11.2-21q11.2	*TPTE*	Expression in brain
X	10,842,553	11,866,932	1,024,379	1.02	9	Xp22.2	*HCCS*	Intellectual disability, Progressive microcephaly
							MID1	Global developmental delay
X	19,262,785	20,313,678	1,050,893	1.05	11	Xp22.13-Xp22.12	*RPS6KA3*	Intellectual disability
							PDHA1	Microcephaly, Global developmental delay
X	51,031,584	53,302,495	2,270,911	2.27	54	Xp11.22	*KDM5C*	X -linked mental retardation, Microcephaly
							IQSEC2	X-linked mental retardation, Microcephaly
							GPR173	Receptor expressed in brain
X	62,964,723	63,984,508	1,019,785	1.02	14	Xq11.1-Xq11.2	*ARHGEF9*	Syndromic X-linked mental retardation with epilepsy
							AMER1	Mild intellectual disability
X	64,025,794	66,664,802	2,639,008	2.64	25	Xq11.2-Xq12	*ZC4H2*	Mild intellectual disability, Global developmental delay
X	154,066,677	154,898,561	831,884	0.83		Xq28	*RAB39B*	X-linked mental retardation, Global developmental delay
							CLIC2	X-linked syndromic mental retardation, Global developmental delay
							TMLHE	Intellectual disability

The above-mentioned genes/phenotypes related to intellectual disability were identified in selected homozygous intervals. These analyses led to the compilation of a large number of syndromes and malformations with ID. In a second round, we again searched these entities for symptoms shared with our family. However, a straightforward indication was not found due to the extreme clinical heterogeneity and differential diagnosis.

7.4.3 Exome analysis

The generated exome data were imported in Microsoft Excel for analysis. Exome file was analyzed to identify the frequency of different exonic variants in both homozygous and heterozygous state and mentioned in Table 7.6.

Table 7.6. Frequency of different exonic variants in exome data

S.no	Mutation types	Homozygous	Heterozygous	Total variant
1	Frameshift deletion	33	84	117
2	Frameshift insertion	33	60	93
3	Non frameshift deletion	54	162	216
4	Non frameshift insertion	56	129	185
5	Non synonymous SNV	4083	7048	11131
6	Stop gain	21	75	96
7	Stop loss	3	10	13
8	Synonymous SNV	4484	7287	11771
9	Unknown	221	343	564
10	**Total Exonic variants**	**8988**	**15198**	**24186**

7.4.4 Identification of candidate variants in exome data

In the exome data, different filters were applied to find candidate variants. By using cut off allele frequency less than 0.005 as stated in different databases (1000Genome [http://www.internationalgenome.org/], ExAC[http://exac.broadinstitute.org/] and ESP650 [Exome Variant Server; http://evs.gs.washington.edu/EVS/]), homozygous variants were identified in the exome file. Bioinformatics algorithms SIFT and Polyphen 2 was employed to check the prediction of amino acid substitutions. Those variants were selected which had PASS status in quality control. The synonymous variants were also filtered as they do not change in amino acid. All identified variants were tabulated and respective genes were then checked through GeneDistiller (http://www.genedistiller.org/) and Expression Atlas (https://www.ebi.ac.uk/gxa/home) to check their expression in brain. Corresponding reference positions of candidate genes having an expression in the brain were checked in whole-genome SNP data file. Finally, we identified four genes (*SPATA31A3*, *CBWD3*, *CRADD*, and *TPTE*) falling in homozygous intervals of greater than the size of 1 Mb (Table 7.7). There is the likelihood of causative mutation in one of these identified genes.

Table 7.7. Identified candidate variants and corresponding genes detected in exome data

Chr. no	Locus	Ref Position	Genes	Variant	Start position	End Position	Size (Mb)
9	9p13.1	40703280	*SPATA31A3*	G > C	38772575	71019573	32.25
9	9q21.1	70863777	*CBWD3*	G > T	38772575	71019573	32.25
12	12q22	94072552	*CRADD*	T > G	92684351	96543225	3.86
21	21p11.1	10920098	*TPTE*	T > C	10827533	15052812	4.23

SPATA31A3, CBWD, and *TPTE are* expressed in various regions of the brain. Mutation in *CRADD* is already reported for autosomal recessive, mental retardation 34 in OMIM database. The encoded protein product of *CRADD* gene functions as adaptor protein needed for apoptosis pathway mediated by caspase-2 (CASP 2). *CRADD* plays role in neocortex development and intellectual functions. Mutated *CRADD* leads to an impaired apoptotic pathway which then causes cortical abnormalities (Di Donato et al. 2016).

The validation of these variants is required through Sanger sequencing. Currently, I am carrying out primer designing and Sanger sequencing for these variants in order to check their segregation with the disease in the pedigree by employing the whole family panel.

7.5 Discussion

In this study, an intellectual disability family concordant with an autosomal recessive mode of inheritance was ascertained from Southern Punjab of Pakistan. Clinical description of affected subjects showed consistent features of some structural deformities, behavioral abnormalities, and learning disabilities. Apart from these, some inconsistent features were also evident. Some features were shared in other ID types reported in OMIM.

For this family, whole-genome SNP genotyping and exome sequencing data were generated, in an effort to find candidate locus and variants involved in the pathogenesis of the disease. With the advent of high throughput methods, the pace of disease gene identification has been improved. Whole-genome SNP genotyping data is used to construct shared disease-causing haplotypes among affected individuals (Jiang et al. 2009). Exome sequencing is an effective approach for finding pathogenic variants. The exome is quite helpful for finding *de novo* mutations (Carr et al. 2013). These techniques are very effective regarding ease of interpretation, bioinformatics tools, and cost and time (Rabbani et al. 2014; Morin and McCarthy, 2007; Harripaul et al. 2017). So, for identifications of disease-causing locus and pathogenic variants these techniques are preferred choice.

In this family, to find the candidate regions associated with the disease, SNP genotyping was performed for three affected individuals by pooling their DNA and homozygosity observed in the data were attributed to all three affected subjects. Whole-genome SNP genotyping was performed by using Illumina Human Omniexpress-24 BeadChip. For analysis, SNP genotyping data were imported in excel and heterozygous genotypes were highlighted. Imported data were examined

carefully to identify homozygous intervals of ≥0.4 Mb. Chromosomes were inspected carefully from the upper end to lower end to find homozygosity. We identified a total 254 homozygous intervals of ≥0.4Mb in SNP data. These selected regions were then categorized on the basis of size. Frequency of these intervals were**:** 0.4-0.49 (29.53 %), 0.5-0.59 (20.47 %), 0.6-0.69 (16.54 %), 0.7-0.79 (9.84 %), 0.8-0.89 (5.51 %), 0.9-0.99 (2.76 %), ≥ 1 Mb (15.35 %).

For further analysis homozygous regions ≥0.8 Mb were selected. Likelihood of finding defective genes were higher in these regions because of their size and more chances of meiotic recombination's which can play role in disease pathophysiology. There were 60 homozygous intervals in genome meeting this criterion. The cytogenetic position of these regions was identified through UCSC genome browser (https://genome.ucsc.edu). To identify phenotype relevant genes, selected intervals were then searched in GeneDistiller by using predefined parameters. In GeneDistiller, by using selected search terms those genes were identified in homozygous intervals which were phenotypically relevant and have an expression in brain.

Thirty-three homozygous intervals were identified having phenotype relevant genes. Genes present within these homozygous intervals and playing role in intellectual disability and associated phenotypes were *CRBN, SUMF1, OCLN, TFAP2B, AHI1, GUSB, COX15, PITX3, CWF19L1, CNNM2, NT5C2, COX14, DIP2B, CRADD, CEP83, NDUFA12, GPT2,* and *AHCY*. Apart from these genes, genes falling in homozygous intervals of chromosome X were *HCCS, RPS6KA3, KDM5C, QSEC2, ARHGEF9, AMER1, ZC4H2, RAB39B, CLIC2, TMLHE*.

Exome sequencing is an effective approach for finding deleterious variants (Carr et al. 2013). For identification of causative variants implicated in this disease, exome sequencing was performed for one of the affectedsubjects. Firstly, the

frequency of different exonic variants was calculated. In the exome file, the estimated number of total exonic variants were 24186. Out of these 24186, 2988 were homozygous and rest heterozygous.

To analyze exome data and identify causative variants different filters were applied. In exome data file, we identified homozygous nonsynonymous variants in four genes (*SPATA31A3, CBWD3, CRADD,* and *TPTE*). These variants were selected by using frequency less than 0.05 reported in different databases (1000G, ExAC, ESP 650) and filtering variants based on predictionof amino acid substitutions reported in bioinformatics algorithms SIFT and Polyphen. Variants identified in these genes are: G>C for *SPATA31A3,* G > T for *CBWD3,* T > G for *CRADD* and T > C *TPTE*. *SPATA31A3, CBWD3,* and *TPTE* have their expression in brain identified through Gene Distiller (http://www.genedistiller.org). The frequency of these identified variants is not reported in 1000G, ExAC and ESP 650 except one (T > G for *CRADD*) whose frequency stated in ExAC as 0.000008.

Another candidate *TPTE* is found close to the centromeric region on chromosome 21 (Chen et al. 1999). Mutation in *CRADD* is reported for ID, autosomal recessive, with variant lissencephaly in OMIM database (OMIM 614499). By using whole exome sequencing, recessive mutations for intellectual disability associated with lissencephaly have been reported in *CRADD* in four distinct families of different ethnic origins (Di Donato et al. 2016). Recently, a study conducted in Pakistani consanguineous families identified 30 novel candidate genes implicated in intellectual disability through exome sequencing data (Riazuddin et al. 2017).

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7.6 Conclusion

In conclusion, the major finding of this study was the exclusion of several of the previously known loci for ID. We identified a total of 33 homozygous intervals in whole-genome SNP data which have phenotypically relevant genes. Then by exome analysis, nonsynonymous homozygous variants are identified in four genes (*SPATA31A3*, *CBWD3*, *CRADD*, and *TPTE)*. One of the identified genes is already reported for autosomal recessive ID while the rest of the three have an expression in the brain. Identified variants can be subjected to Sanger sequencing for identification of a causative mutation in future.

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Online Resources Utilized

OMIM	
SIFT	
Polyphen-2	
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Gene Distiller	
MutationTaster	
Genome data Viewer	
NNSplice	
NetGene2 Server	

List of Publications

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