CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Genetic Analysis of Hereditary Polydactyly in Pakistani Families

by

Muhammad Sajid Khan

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This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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CERTIFICATE OF APPROVAL

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List of Publications

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Abstract

In vertebrates, the development of limb is a field of active research in developmental as well as in evolutionary biology. It initiates during fourth week of gestation period in which several genes and molecular factors are involved via different biological pathways. Congenital limb abnormalities may occur if these signaling pathways are disturbed. These limb abnormalities may be present as an isolated entity of upper as well as lower limbs or may present in association with any other syndrome. Polydactyly is widely reported congenital hand abnormality. It has prevalence of 519/10000. Isolated form of polydactyly is categorized into postaxial, central and preaxial polydactyly. Postaxial polydactyly (PAPA) is classified into two main types i.e. type A and type B. Postaxial polydactyly type A has been further classified into seven types as PAPA1, PAPA2, PAPA3, PAPA4, PAPA5, PAPA6 and PAPA7. Postaxial type B is further classified into two types. Preaxial polydactyly has been classified into four types i.e. PPDI, PPDII, PPDIII and PPDIV. Polydactyly is frequently associated with syndactyly, a digit disorder in which adjacent fingers are webbed. This is due to failure of separation of digits during the period of development.

In current study, three families one with postaxial polydactyly and syndactyly of 4^{th} and 5^{th} fingers (A) and two (B, C) with preaxial polydactyly belonging to various areas of Pakistan were selected for linkage analysis. It indicated autosomal dominant mode of inheritance on basis of pedigree. Linkage analysis for already reported loci/genes of these families was done by homozygosity mapping having highly polymorphic microsatellite markers. The family A with postaxial polydactyly in addition to syndactyly of 4^{th} and 5^{th} finger was mapped to known loci and genes *GLI3*, *SHH*, *LMBR1* (*ZRS*), *ZNF141* and *IQCE*, *HOXD13*). Genotyping analysis showed that markers *D7S2541*, *D7S2548*, and *D7S691* showed some evidence of linkage to *GLI3* gene. Homozygosity mapping of family B showed no linkage with any of the known gene. Exclusion of linkage indicates that an unknown gene is responsible for preaxial polydactyly in this family. Family C with affected individuals having preaxial polydactyly was tested for linkage to several

genes as mentioned for family A. By genotyping analysis marker *D17S54* showed homozygous pattern for both affected members of the family despite absence of immediate consanguinity. The marker lies midway between *LMBRI* and *WDR60*. The presence of mild brachydactyly and mild short stature in one of the patients indicated the possible involvement of *WDR60* that causes short rib thoracic dysplasia. No other gene showed linkage to the phenotype.

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Abbreviations

PAP	Post-axial polydactyly
PPD	Pre-axial polydactyly
SNPs	Single nucleotide polymorphism
PCR	Polymerase chain reaction
SHH	Sonic hedgehog
CNV	Copy number variation
TPT-PS	Triphalangeal thumb-polysyndactyly syndrome
ZPA	Zone of polarizing activity
PPD	Pre axial polydactyly
PAPA	Post axial polydactyly type A
PAPB	Post axial polydactyly type B
STS	Short tandem sequences
WES	Whole exome sequencing
EDTA	Ethylene diamine tetraacetic acid
ZRS	ZPA regulatory sequence
OMIM	Online Mendelian Inheritance in Man
MIPOL	Mirror image polydactyly
TGFb	Transforming growth factor, beta-1
SDS	Sodium dodecyl sulphate
EtBr	Ethidium bromide
cM	Centimorgan

Chapter 1

Introduction

1.1 Introduction

Polydactyly is a congenital abnormality which is primarily indicated as superficial toe/fingers that is completely grown in addition to the bony component or an additional softhearted material with no bone component. This disorder is a hereditary limb abnormality having prevalence of 519/10 000 babies which are alive (Schwabe and Mundlos, 2004; Christensen, 2011; Malik, 2014). Association of the feet have been identified to be less frequent as compare to the upper limbs, right hand more familiar as compared to left hand and the right foot is less prevalent as compared to left foot (Malik *et al.,* 2014). It may be present in addition to other phenotypes called as syndromic or it may be present as isolated entity without having any abnormality beyond this disorder so called as non-syndromic polydactyly (Biesecker, 2011). The main classes of non- syndromic polydactyly are pre-axial polydactyl, mesoaxial polydactyl and post- axial polydactyly (Deng, 2014; Malik, 2014). Pre-axial polydactyly has been further classified into four types PPD1, PPD2, PPD3 and PPD4. On the other hand post-axial polydactyly further classified into type A and type B. Type A has seven types named as PAPA1, PAPA2, PAPA3, PAPA4, PAPA5, PAPA6 and PAPA7(Malik, 2013). These different types of non-syndromic polydactyly are caused by nine genes and about 10 loci reported up till now. These

reported genes are *GLI3*, *HOXD13*, *ZNF141*, *IQCE*, *MIPLO1*, *PITXI*, *ZRS/SHH*, *GJA1* and *FBLN1*. A locus has been reported for autosomal recessive PAP-type A5 and (Umm-e-Kalsoom, 2012a; Umm-e-Kalsoom, 2012b). Four PPD types were identified by Temtamy and McKusick as mentioned above.

1.1.1 Pre-Axial Polydactyly

1.1.1.1 Pre-Axial Type I

The type of polydactyly in which the components of biphalangeal thumb are repeated one or more times. It is reported in a study, hands are favorably involved as compare to feet and in case of hands mostly cases are bilateral. It is also reported that mostly right hand is involved as compare to left hand (Malik S. *et al.,* 2012). There is high prevalence of this type in males that are affected than females (Malik *et al.,* 2012; Orioli *et al.,* 1999).

In Pre-axial Type I, the appearance diversity is variable which includes recurrence of ends of bones of fingers also known as distal phalanx, the extra incompletely developed thumb and the ends of bones of fingers may show bifurcation and these end might also be broad, two extra phalanges may be present on the same incompletely developed thumb or there may be the repetition of the whole thumb (Temtamy *et al.,* 1978). The name of triplicated thumb may also be given to this type that results in total of seven digits (heptadactyly) (Zuidam *et al.,* 2008). It has also been reported that hereditary type I pre-axial polydactyly which is commonly segregates in a dominant way (autosomal) having decreases extent of expression (Castilla *et al.,*, 1978; Orioli *et al.,* 1999). It is a heterogeneous disorder. Variations in the gene sonic hedgehog are responsible to produce this type of abnormality (Wieczorek *et al.,* 2010; Lettice *et al.,* 2002).

This disorder of thumb polydactyly may possibly come with doubling of hallux in spite of this, hallux polydactyly also present as a separated element or a prevalent appearance in families (Castilla *et al.,* 1973). Hallux duplication is infrequent than polydactyly of thumb (0.025/1000 vs 0.154/1000 present in South American

country. Hallux doubling showed a meaningful overabundance in men, associated mostly the limb of right side. Mostly cases reported are those in which disorder is on one side only (Orioli *et al.,* 1999). Hallux polydactyly still has unknown molecular basis.

1.1.1.2 Pre-Axial Type II

It is a type of polydactyly which exhibits the existence of generally opposable triphalangeal thumb. It may be with or without extra duplication of thumb (Temtamy *et al.,* 1978). It is also known as triphalangeal thumb polydactyly (TPT). The disorder may possibly be present in lower appendages as well and opposable in most cases and exhibit toe doubling (Temtamy *et al.,* 1978). TPT is often bilateral and symmetrical (Swanson *et al.,* 1962). It has also been reported that this disorder segregates autosomal dominantly having incomplete penetrance (Temtamy *et al.,* 1978). This disorder type showed a reduced prevalence in women. Triphalangeal thumb polydactyly is a somewhat infrequent type. It has been observed that variations in *ZRS/SHH* locus on chromosome 7q36 are responsible to cause separate triphalangeal thumb polydactyly, many other abnormalities and several of the triphalangeal appendage anomalies (Lettice LA *et al.,* 2002). In spite of this, *ZRS/SHH* alterations are not responsible for whole polydactyly of lonely triphalangeal thumb relatives not various varieties of triphalangeal thumb polydactyly reflecting other processes may be the source of this type of pre-axial polydactyly.

1.1.1.3 Pre-Axial Type III

In this type of pre-axial polydactyly which is uncommon, there is doubling of index finger. It is considered as autosomal dominant. One or two triphalangeal digits take the place of thumb. In this type epiphysis has been shown for the metacarpal of the additional finger, so this unit become distinct than other types (Swanson *et al.,* 1962). Ist and 2nd toes may be affected. As it is considered a modified form

of thumb repetition but sometimes it is added in the type of polydactyly called as central polydactyly (Wood, 1970). The radial-sided doubling normally is smaller and the stage of repetition may possibly at the metacarpal or more distal. The deviation of the superfluous finger occur here radially or deviation of ulna occur in normal finger (Graham and Ress, 1998).

1.1.1.4 Pre-Axial Type IV

It is also called as polysyndactyly in which repetition of thumb occurs to some extent along with deviation of the phalanx in outward direction. The broadness in the thumb also occur. Mostly 3rd and 4th finger show the sign of syndactyly (Temtamy *et al.,* 1978). There is divergence of 1st metacarpal having abnormality in former toe. Digits of the whole foot may show syndactyly or some time 2nd and 3rd toe exhibit syndactyly. Pre-axial type 4 is different from synpolydactyly. In synpolydactyly extra finger exhibit syndactyly (Malik and Grzeschik, 2008). A word crossed polydactyly is normally in use on behalf of existence of PAPA and PPD in addition to variation in angle of the extra fingers present among the feet as well as hand. Cross polydactyly shows a form of polydactyly that is pre-axial in feet while post-axial in hands. In cross polydactyly type II post-axial polydactyly exist in feet along with pre-axial polydactyly present in hands (Temtamy *et al.,* 1978). It is reported that type 1 is allelic to PAP (A/B) and variation in *ZRS/SHH* and *GLI3* are directly involved in this disorder. (Radhakrishna *et al.,* 1999).

1.2 Post-Axial Polydactyly

The type of polydactyly in which one or more additional fibular or ulnar digits are present. There may be only a part of ulnar or digits exist. Particularly for the ulnar polydactyly, two different units A and B have been documented. Both these two types of post-axial polydactyly show marked differences of seriousness, heritage model as well as penetration assessments (Temtamy *et al.,* 1978).

1.2.1 PAPA

The kind of polydactyly which exhibit complete development of additional digit which is functional. The articulation of additional 5th digit or additional carpel (Temtamy *et al.,* 1978). 1 to 3 bony elements present in additional digit along with the nail which is formed completely. It indicates the mode of inheritance which is autosomal dominant and show 70% penetrance rate (Kucheria *et al.,* 1981).

It has been reported that it exist as autosomal recessive type (OMIM-263450) (Briard and Kaplan, 1982). In an epidemiological study, enrolled 6586 people having PAP (A/ B). By complicated segregated analysis, these writers found large heritability assessment for this feature and a main recessive gene involvement. (Castilla *et al.,* 1998). It was reported that chromosome 13q13.3-q21.2 as well as 4p16.3 (*ZNF141*) had been linked to 2 types of recessive PAP respectively (Kalsoom *et al.,* 2012). On the basis of studies of mapping different types of PAP have been observed having phenotypes of A and B. These are PAPA-1, 2, 3, 4,5,6,7. It was reported that mutated *ZRS/SHH* as well as *GLI3* are responsible for PAPA-1 (Radhakrishna *et al.,* 1999). However on genetic bases the heterogeneity of both these types of polydactyly was not confirmed.

1.2.2 PAPB

It is one of the most frequent type of polydactyly in different inhabitants. There is incomplete development of additional digit. There may be an existence of small lump on ulnar feature with 5th finger or the length may lead up to four centimeter like a nubbin having an element of bone and normally nail may also be present (Castilla *et al.,* 1973). The site of articulation of this lump with the 5th finger show alteration. It was indicated that this type show more complication genetically. Reported penetrated rate of this type is 42% (Castilla *et al.,* 1973). The disturbance in hands and left hand is mostly reported (Malik, 2013). The genetics of PAP-A and PAP-B shows that there is heterogeneity and diversity in both these types. The reason behind that is their array of segregation is different and they

occur independently (Castilla *et al.,* 1973). Although it was reported occurrence of PAP-A and B in same person (Kucheria *et al.,* 1981).

1.3 Other Polydactylies

Beyond PPD and PAP phenotypes other phenotypes are also familiar which are different from both these types.

1.3.1 Mirror-Image Polydactyly

The type of polydactyly in which digits on the posterior side are duplicated. There is replacement of digits of anterior one by posterior one while the sequence is reversed. The sequence of additional fingers from index finger 5-4-3-2-3-4-5 while hallux or thumb is not present (Temtamy *et al.,* 1978). The pattern of segregation is autosomal dominant. Non syndromic cases are very infrequent, mostly it exist in addition with other abnormalities i.e. syndromic cases are frequent (OMIM-135750) where ulna and fibula are duplicated, thumb is not present while rest fingers are duplicated. Hands and feet tubular bones show repetition and on the basis of this various types of MIP are suggested. One of them is infrequent in which tibia is faulty in addition to pre-axial polydactyly. This type is indicated as autosomal dominant with different penetration and expression. The second type is that in which tibial development remain incomplete and mirror feet. The third type shows duplication in ulna fibula (Temtamy *et al.,* 1978). The region in chromosome 14q13 harboring *MIPOL1* mutated to cause a kind of mirror image polydactyly (Kondoh *et al.,* 2002). It was also reported that variation in *PITX1* is also responsible to cause mirror image polydactyly in feet (Klopocki *et al.,* 2012).

1.3.2 Haas Type Polydactyly

It is the type of polydactyly whole digits are merged showed a complete fusion. The syndactyly is present along with additional ray of digit. The additional ray may be post axial or pre axial (Haas SL 1940). The shape of hand looks like a cup because of presence of severe syndactyly. This form is regarded as syndactyly of type 4 (Malik S. 2012). It was reported that the variations in *ZRS/SHH* gene and *GLI3* gene are responsible for causing this type of polysyndactyly on the other hand this form of polydactyly is observed as heterogeneous genetically (Radhakrishna *et al.,* 1999).

1.3.3 Central Polydactyly

This type of polydactyly is mostly bilateral while abnormalities of various kinds are also observed. There is presence of lump of tissue in middle of upper limb in addition to syndactyly and nail are fused. Repetitions may considered as hidden but not all of its forms. Index digit is duplicated. As compare to index digit the repetition of ring digit is mostly observed (Temtamy *et al.,* 1978).

1.3.4 Dorsal and Palmer/Ventral Polydactyly

This type of polydactyly is very infrequent and uncommon in population. In this type additional raised near dorsal or ventral side of auto pod. There is partial development of digit ray or it shows complete development but nail is absent. The additional digit may be moveable as well as functional. It was reported that the basis of this moveable digit is present in hand at ventral or palmar side (Nair *et al.,* 2001). Similarly it was also reported in lower limb as well at dorsum site (Hussain *et al.,* 2007).

1.3.5 Syndactyly

It is the form of polydactyly some digits attain a design like a web. This is due to failure of separation during development of limb. Although the association between syndactyly and polydactyly is very close so that it become difficult to classify them. However it may be regarded as part of kind 4 of pre-axial polydactyly (Temtamy *et al.,* 1978). Its incidence rate is 3 to 10 in 10000 births. (Malik *et al.,* 2012). Mostly 1st to 2nd or 3rd to 5th digit may accompanied in triphalangeal type. I t was observed that extra toe in foot may fused with other digit in non-syndromic form of pre-axial polydactyly. It was reported that 4th to 5th digits of hands and feet are fused when PAP and PPD both are present along with syndactyly (Goldstein *et al.,* 1994). It is indicated that this abnormality is inherited recessively (Briard *et al.,* 1982). The hand looks like a shape of a cup in case of MIP when whole syndactyly is present (Temtamy *et al.,* 1978). There may be heterogeneity in the same person and he attain asymmetrical phenotypes hands and feet and right as well as left appendages. Syndactyly may be complete or incomplete, phalanges only or metatarsal and metacarpals are also involved (Malik *et al.,* 2012).

Various classes of syndactyly have been observed. These are syndactyly type 1 to IX on bases of involvement of digits/toe. The syndactyly type 1 is further classified into type 1-a, type 1-b, type 1-c and type 1-d (Malik *et al.,* 2012).

1.4 Limb Development

1.4.1 Digit Development

For learning organ development the limb has long considered as a model. A repeated set of fingers and toes shows distal feature of appendage of vertebrate. These form a consecutive pattern on the other hand there is variety in shape, size

and structure of these digits and toes. It was reported that the process of development of fingers and toe is different from the process to give them identification (Zwilling, 1964).

1.4.2 Digit Specification

The idea of positional information gives specificity of digit identification. Morphogens which are called variety of signals are involved in establishing the positional values. It was proposed that this information is interpreted by the tissues in the next step. Digits are formed as a result of these values (Saunders *et al.,* 1957). The conclusions of the experiments of inserting of cells at ZPA towards the anterior distal margin from the posterior one are according to the information provided by morphogens (Tickle *et al.,* 1975). This the way that hint to duplication of fingers and toes (Saunders and Gasseling, 1968). Zone of polarizing activity provide the strength of morphogens. In this view, the concentration of a morphogen produced by the ZPA. The digit primordia inferred this strength of morphogens in limb region as positional information help in digits identification. Although this morphogens exemplary gives only the morphology of digits and not position or spaces pattern between digits.

1.5 Molecular Pathways in Limb Development

In a publishing (Raspopovic*et al.,* 2014) molecules were reported which control the space pattern of establishing centers. The molecules that are associated with are *WNT* and *BMP*. *WNT* was observed to be associated with interdigit field and Bmp with digit field. The action of Wnt hinders the chondrification so that *SOX9* is not expressed. This help in specification of inter digit. On the other hand the action of *BMP* enhances chondrification so that *SOX9* expression is enhanced beyond the approach of *WNT* activity so specification of digits occur. The arrival of *BMP* and *WNT* suppressed by *SOX9* in digit. It becomes the origin of a model called as three-node Turing model. (Raspopovic *et al.,* 2014).

This model not properly explain the initiation of digit pattern so there is requirement of more study for understanding the mechanism of digit pattern initiation. The Wnt/ planer cells polarity and TGF-b are also involved in digit initiation pattern. Preventing of origination of extra PFR/DCs digit controllers during the expansion of auto pod is necessary. Wnt is possible nominee responsible to cause anti-chondrogenesis in appendages (Hartmann and Tabin, 2001; ten Berge *et al.,* 2008) and is known to be the part of turning like models suggested for specificity of digits by Raspopovic *et al.,* (2014). When the controlling hubs are created near the metacarpal origin the extra digit does not usually created. Due to the delivery of Bmp at abnormal place at the edge of developing digit, there may be the bifurcation of rays of the digits (Ganan *et al.,* 1996; Duprez *et al.,* 1996). If the controlling hub is not split one ray of cartilage may arranged proximally. On the other hand when the controlling hub is split results in bifurcation into more outlets. If the ectoderm is removed or TGF-b is applied, the ectopic digits which are completely independent could be provoked among endogenously ray of the digits (Hurle and Ganan, 1987; Ganan *et al.,* 1996; Macias *et al.,* 1993). It was suggested that this digit is started just like one particular pimple away from the origin having TGF-b and due to the distal development of auto pod this pimple lengthens to develop into a shape like digit (Lorda-Diez *et al.,* 2011). So that ectopic digit managing hubs are induced via these processes. Then these hubs perform as endogenous managing hubs in the course of development of hand plate. A cluster of cells of mesenchyme produced at the posterior margin of the limb bud called ZPA. This is the signaling hub are proposed to involved in digit development. Mirror image digit repetition may be result of polarizing actions of these cells. *SHH* is responsible to intervene its activity (Saunders *et al.,* 1968). The antagonistic factors are *SHH* and *GLI3*. These are responsible to control not only number of digit and identification of digits as well.

1.6 Purpose

More than a hundred genes have been discovered in human to be involved in polydactyly (Xiang *et al.*, 2016). Most of these genes show syndromic polydactyly. Very few genes are reported in non-syndromic polydactyly. The purpose of this study is to identify the genes in the selected families of Pakistani origin showing inherited non-syndromic polydactyly.

1.7 Objectives

- To perform genotyping of known genes of polydactyly.
- To check heterozygosity in family with dominant inheritance.
- To check homozygosity in family with consanguanity.

Chapter 2

Literature Review

2.1 Molecular Genetics of Polydactyly

2.1.1 PAPA1

Heterozygous mutation in *GLI3* gene was observed by Radhakrishna et al.,. (1997) in family of India having post axial polydactyly type A-1 and the linkage was found to chromosome 7p. A variation in *GLI3* gene in an individual having PAP- type B. The variation is heterozygous in nature and this variation is because of decay of nonsense mediated mRNA (Furniss et al., 2007). A family of Saudi Arabia having PAP. The patients of family have broadness in thumb and additionally syndactyly was also present in upper limbs as well as in lower limbs. A deletion of 2bp of *GLI3* gene was observed (Al-Qattan 2012).

2.1.2 PAPA2

A family having PAPA selected by Akarsu et al., (1997) for linkage analysis. The family showed a total of 18 informative meioses of which 11 were phase-known. He omitted association to 7p15-q11.23, where a form of PAPA (PAPA1; 174200) had been mapped in a large Indian kindred. They demonstrated linkage

to markers from 13q21-q32. An extreme lod score 2.34 at 7 cM was observed with *D13S1230*. They observed no recombination in affected members between centromeric D13S800 and telomeric *D13S154* in a 23-cM interval. One unaffected member of the family received the 'affected haplotype' from his affected parent. Coding this individual as of 'unknown' status, they obtained the highest lod score with *D13S1230* (maximum lod = 3.612 with no recombination).

A karyotyping was performed by (van der Zwaag et al., 2010) in an individual having PAP in upper limbs. The disorder was present in both of upper limbs. He indicated heterozygous condition. He found that chromosome 13 has de novo inverted repetition in its large arm.

2.1.3 PAPA3

A family in china having PAP studied by Zhao et al., (2002). The family showed autosomal dominant mode of inheritance. A negative lod score was observed by him with already known forms PAPA locus on 7p (PAPA1; 174200) plus 13q (PAPA2; 602085). But for STS on chromosome 19p a positive lod score was also observed. This was founded after performing genome screen. An extreme 5.86 lod score was observed with D19S221.

2.1.4 PAPA4

A Dutch family having 32 people along with 11 polydactyly patients was studied by Galjaard et al., (2003). The family has post-axial polydactyly with various types i.e. post-axial type A, type B or both in addition to syndactyly in some individuals. 4th locus was observed in this family. An association was observed between the polydactyly and markers on 7q chromosome after performing the whole genome analysis. Between *D7S1799* and *D7S500* a maximum lod score was observed i.e. 3.33.

2.1.5 PAPA5

Umm-e-Kalsoom et al., (2012) studied a family in Pakistan having PAP. The family have autosomal recessive mode of inheritance and have a consanguineousity as well. By utilizing highly polymorphic microsatellite markers by performing genome wide search. She observed association to markers on 7q. A highest lod score of 3.85 was observed with phenotype. Among markers *D13S1288* (32.39 cM) and *D13S632* (57.16 cM) on chromosome 13q with 17.87 cM critical field was demarcated by recombinations (NCBI36).

2.1.6 PAPA6

Kalsoom et al.,. (2013) studied a family in Pakistan with PAP-A. The family exhibited recessive mode of inheritance and have consanguineousity as well. Mutated *ZNF141* was found to be responsible by using a technique of WES. Genotyping was performed by using 26 markers of above mentioned gene. Linkage was found to be 6.52-Mb on 4p16.3-p16.2 chromosome. For markers *D4S412* highest 3.38 lod score was observed.

2.1.7 PAPA7

Umair et al., (2017) studied a family in Pakistan having PAP in foot. The family exhibited recessive mode and have consanguineousity as well Mutated *IQCE* gene was found to be responsible for this abnormality. Homozygous splice site variation in the *IQCE* (617631.0001) was observed after performing WES.

2.1.8 PPD1

Kelly (1982) identified a family with polydactyly. He found that thumb and toe was duplicated symmetrically. He observed that the abnormality was transmitted men to men over many generations. Graham et al.,. (1985, 1987) was studied

the pre-axial polydactyly. He proposed a polydactyly type in which thumb is incompletely developed. Mostly it may inherited with autosomal dominant mode of inheritance. As a French biologist (Fromont, 1895) defined this previously so they called this slight variation as Fromont anomaly.

Ray (1987) founded an Indian family with PPD. The family comprising 15 men and 5 women in several generations. Repetition of thumbs were not as much stable property. There was variability in expression and repetition of large toe in both feet exhibited condensed penetration. Orioli and Castilla (1999) performed a study based on epidemiology. They obtained a data from a Latin American department. They selected a total of 3,444,374 newborns from 1967 to 1995 in which 921 were born with 1st digit repetition. The incidence of non-syndromic cases was found to be 714 (2.08/ 10,000). These are further divided into 5 classes. Thumb repetition (568), hallux repetition (82), and polysyndactyly (37), TPT (24), Thumb/hallux repetition (4). Men were mostly affected, right limbs were more involved, and mostly cases were unilateral. It was also founded in this study that incidence was more in Bolivia 3.4 per ten thousand as compared to ten Latin states of America.

2.1.9 PPD2

The variations in *SHH* regulatory unit was responsible to cause PPD-2. This element was located on chromosome 7q36. Variations in ZRS which is present in between intron five of *LIMBR1* gene showed segregation with abnormality in families of PPD-2(605522.0002, 605522.0004, and 605522.0005) (Lettuce et al., 2003).

Four families were examined by Gurnett et al., (2007) having phenotypes of PPD-2 and TPT. The families were already studied by Dobbs et al., (2000). He pointed out two variations in three families (605522.0007 and 605522.0008, respectively) while 4th family had just TPT phenotype.

A large family having four generations in which nine individuals were involved in disorder of TPT plus syndactyly (Klopocki et al., (2008). He pointed out a heterozygous condition for identified 589-kb repetition.

Sun et al., (2008) studied five families having TPT PS and few individuals had syndactyly-4. He pointed out repetitions involves ZRS having a range of 130-397kb. He used polymerase chain reaction technique and identified a common coinciding section of 32757 base pair. This section possessed ZRS enhancer. It was found co-segregation of the repetitions with phenotypes in five family individuals. The normal individuals did not occupy these duplications.

Wang et al., (2007) examined any of these families. Point mutation was observed in *LIMBR1* gene and the location was intron five. The variation could denote polymorphism which was infrequent proposed by Sun et al., (2008).

Furniss et al., (2008) studied three families in England having TPT polydactyly in both hands and one of them had unilateral *PPD*. He pointed out variation in ZRS in *LIMBR1*. There was observed heterozygosity in mutation in all disturbed members. In England TPT was commonly caused by variation in 295T-C which was dominant mutation. Balci et al., (1999), Wieczorek et al., (2010) studied a family in Turkey having TPT-PS. Mutation in ZRS/ *LMBR1* was observed. The mutation was found in those individuals of the family which were abnormal while normal individuals did not had such mutation. The doubling of 276-kb was nominated arr7q36.3. Both of them explained that repetition region was impossible to be identified because of its repetitive nature.

Albuisson et al., (2011) studied two families in France having *PPD*-2 phenotype. He observed two dissimilar variations showing heterozygosity in ZRS area of *LIMBRI* (297G-A; 605522.0013 and 334T-G; 605522.0014). These were present in expected attachment locations for *SOX9* and *PAX3* which were transcription factors. The two families had the variations completely penetrated. It was observed that *SOX9* as well as *PAX3* have a role pattern of digit formation during the development of embryo in mouse. Therefore it was proposed that *SHH* might be regulated from ZRS by *SOX9* as well as *PAX3*.

Vander Meer et al., (2014) examined two families in Mexica having TPT as well as PPD phenotypes which were segregated in an autosomal dominant way. The abnormal members of these two families showed heterozygosity for variations in ZRS field of *LIMBR1* gene (297G-A; 605522.0013 and 334T-G; 605522.0014).

2.1.10 PPD3

Manoiloff, 1931 observed the transmission of abnormality in a Scipio kindred. He stated that the transmission might be for about 2000 years in this family (Swanson and Brown, 1962) stated that triphalangeal digits substituted the thumb. The feature of opposability could or could not be present (Manoiloff, 1931; James and Lamb, 1963) stated that PPD of first and second toes showed by lower limbs in few circumstances (Swanson and Brown, 1962) found that the extra digit metacarpal showed distal epiphysis.

2.1.11 PPD4

Radhakrishna et al., (1999) examined a family having PPD-4. He observed a frameshift insertion of one nucleotide. It was resulted in abnormal protein consisting of 1245 amino acids. Fujioka et al., (2005) studied a family having PPD-4. A nonsense variation was observed in *GLI3* (R290X; 165240.0014). which was heterozygous in nature. It was observed that left upper limb of the baby showed syndactyly of 3rd as well as 4th digits while dad possessed no irregularities with upper limbs. It indicated that different expressions might be observed between PPD cases with same variation in *GLI3*.

Chapter 3

Methodology

3.1 Study Subjects

Three families from different areas of Rawalpindi including family A with post-axial polydactyly along with syndactyly of 4^{th} and 5^{th} finger while family B and C both have pre-axial polydactyly were examined in the current study. Historical and clinical information of families was collected after visiting at their residencies. After getting the informed consent the blood samples of abnormal and unaffected members were drawn at local clinic. After the approval from the supervisor and higher authorities of CUST Islamabad Pakistan the study was performed.

3.2 Pedigree Sketch

Different questions were asked regarding the history of family and then pedigree was drew using method provided by Bennet et al., (1995). Circles are used to denote females while squares are used to denote the males in this pedigree. Filled circles and squares are used to represent abnormal males and females respectively. On the other hand unaffected males and females are represented by empty squares and circles respectively. Doubles lines are used to represent consanguineousity

in the families. Every generation is represented by roman numerals. Deceased members are denoted by crossed circles and squares.

3.3 Collection of Blood

Sterilized syringes were used to take the 5 ml blood samples of affected and normal individuals. The samples were then shifted to Vacutainers containing potassium EDTA. Then samples in the tubes were stored in human molecular genetics laboratory, Department of Biochemistry, CUST, Islamabad.

3.4 DNA Extraction

DNA was then extracted from the blood by the manual method named as Phenol-chloroform DNA extraction method (Sambrook et al., 1989).

- In the first step 750 microliter of blood and equal amount of solution A was added in an eppendorf tube (1.5 ml), and mixed gently by inverting the eppendorf tube 4 to 6 times. After mixing the blood with solution A, eppendorf tube was incubated for 20-30 minutes at room temperature.
- In second step, the eppendorf tube was spun for one minute at l3, 000 rpm (Revolutions per minutes) in a centrifuge (eppendorf Microfuge, 5415D; USA), after incubation at room temperature.
- Pellet was separated after the removal of supernatant present in 460ul of solution A
- Centrifugation of mixture was repeated for 60 seconds at 13000 rpm.
- Again pellet was separated after removal of supernatant and again it was suspended in 450 microliter of B solution, 10 microliter of proteinase K (10mg/ ml) and 15 ul of SDS (2W.4) and l0 microliter of proteinase K (10mg/ ml).

- Sample was placed for overnight incubation at 37^oC.
- Solution C and D having equimolar volume was mixed with sample coming day.
- Centrifugation of mixture was performed for 15 minutes at 13000 rpm after mixing
- Three different layers were appeared. Micropipette was used to separate the DNA which was present in top layer in eppendorf.
- Thereafter solution D was added about 0.5 ml to the new eppendorf tube containing DNA sample.
- The centrifugation was repeated for 15 minutes at 13000 rpm and top layer was separated containing DNA in new eppendorf tubes. The precipitation of DNA was done after mixing 66 microliter of three molar CH3COONa along with 455 microliter of cold isopropanol. DNA was precipitated after gentle inversion of tubes.
- To get the pellet of precipitated DNA again centrifugation was performed for 10 minutes at 13000 rpm, supernatant was discarded carefully.
- 200 μI of chilled 70% ethanol was added to the tubes having the DNA pellet followed by centrifugation for 7 minutes at 13000 rpm.
- Again carefully removal of ethanol was done so that DNA pellet was not disturbed. Vacuum concentrator was used to dry the pellet at 46^0C for 15 minutes.
- Finally DNA pellet was dissolved in 120-200 ul (depending on the quantity of pellet) of Tris-EDTA (TE) buffer by incubating whole night at 37^oC.
- DNA was stored at 4^0C after complete dissolution of DNA pellet in TE buffer after 24 hours.

Solution Name	Chemical used	Concentration
Solution A	Sucrose	320mM
	Magnesium chloride	5Mm
	Tris (pH 7.5)	10Mm
	Triton-X100	1% (v/v)
Solution B	Tris (pH 7.5)	10mM
	EDTA (pH8.8)	2MmM
	Sodium chloride	400mM
Solution C	Saturated phenol	
Solution D	Isoamyl alcohol	1 volume
	Chloroform	24 volume
10X TBE	Tris	890mM
	Boric Acid	250mM
	EDTA	pH 8.3
SDS 20%	SDS, Water	10 gm, 50ml
Bromophenol Blue	Sucrose	40 gm
	Bromophenol	0.25 gm

FIGURE 3.1: List of Solutions

3.5 Agarose Gel Electrophoresis

To analyze the magnitude and quality of obtained DNA, 1% agarose gel was used. It was prepared (100 ml) by addition of 1 gram agarose into flask. Then 100 milliliter of IX TBE (0.90 molar Tris, 0.026 molar Borate, and 0.033 molar EDTA) buffer was added. For proper miscibility the mixture containing agarose was heated in oven for about one or two minutes. After fully dissolution of agarose 2-6 microliter of EtBr (0.5 g/ml) was poured into the solution and shacked. Then it was shifted to gel tank and solution was left at 26^0C for an hour to solidify. Before further process combs were separated to load the sample in the well. Then 2 microliter of DNA after mixing with the dye was introduced into the well. The dye used for this purpose was bromophenol blue 0.26% along with 39% sucrose. Then Electrophoresis was done four half an hour at a voltage of 140 volts carefully in IX TBE buffer. After that gel was visualized in UV illuminator to analyze the DNA and the image was got with the help of camera EDAS 280 (Kodak, USA).

3.6 Homozygosity Mapping

The family A with post axial polydactyly plus syndactyly while family B and C both having pre axial polydactyly were undergo homozygosity mapping for loci which were already known by using polymorphic microsatellite markers. The distance of these loci were determined with the help of UCSC genome browser followed by finding the genetic distance of markers nearby these loci.

Many markers of distinct cM were utilized to map any locus. The purpose of this mapping was to point out the heterozygous nature in unaffected members and homozygous nature in abnormal members. When these markers were linked with a specific locus in all abnormal individuals, the same pattern were observed by descendant in abnormal members at a specific locus and more likely there will be disturbance in gene occupy that specific locus. To find out the mutation the abnormal gene will be suggested to perform sequencing. For homozygosity mapping the STS markers utilized are noted in Figure. 3.2.

3.7 PCR

The STS markers used for mapping were undergo amplification by PCR. PCR tubes of about 200 microliter were used to prepare the PCR mixture. The whole volume of this blend was about 26 microliter comprising the following ingredients i.e. 1.5μl of 25 mM MgCl2, 0.5μl of forward and reverse primers, 2.5μl l0X buffer (750 mM Tris HCl pH 8.8, 200 mM (NH4)2so4, 0.1 % Tween 20), 0.5 l of 10 mM dNTPs, 0.2μl of Taq polymerase, 17.5- μl of PCR water, 2μl (20 ng/μl) DNA dilution. For shaking and mixing of the whole material, it was rotated for I minute at 4500 rpm. After that PCR tubes containing mixture were shifted to thermocycler.

Thermocycler possessed the following conditions

1. DNA denaturation at 95^0C for 480 seconds.

2. Amplification having forty cycles and every cycle comprising of following sub stages.

   (a) DNA denaturation (increased product) at 95^0C for 60 seconds

   (b) Primers annealing designed for amplification of STS markers to their corresponding DNA bp at 54-65^0C for 60 seconds,

   (c) Elongation of complementary DNA strands by Taq DNA polymerase at 72^0C for I minute.

3. Residual partial corresponding DNA filaments undergo last elongation by Taq DNA polymerase at 72^0C for about 400-600 seconds.

3.8 Polyacrylamide Gel Electrophoresis

8% polyacrylamide gel was used to analyze the amplified DNA for genotyping of banding pattern. To prepare the gel for 1 plate having a volume of about 50 ml in a cylinder the following ingredients were used i.e. 13.6 ml of 30 % acryl amide solution (N, N Methylene-bisacrylamide plus 28: l ratio of acryl amide), 5-6 ml of 10X Tris-Borate-EDTA. Then distilled water was poured in solution in 500 ml measuring cylinder to make the whole volume about 50ml. Then 10% APS (Ammonium per sulphate) of about 355 microliter was added along with TEMED and shacked the whole mixture for proper mixing.

The two glass plates were assembled together using metal clips and the distance between plates were 1.7 mm. Several plates were prepared according to the requirement and then the mixture was added in between the plates. Then combs were introduced in between plates in gel to create the wells and then left the solution for 1 hour at room temperature to polymerize acrylamide. After that, for electrophoresis these plates were hanged into gel tanks which were filled with buffer IX-TBE. Then 5 ul loading dye (0.25% bromo- phenol blue with 40% sucrose) and the amplified DNA were mixed with each other. The dye gave blue

colour to the mixture. After that mixture was added into wells and left the solution for 2.5 hours at 140 volts for electrophoresis. At the end of 2.5 hours the gels were separated from the tanks and stained with ethidium bromide (10mg/ml). To visualize these gels UV transilluminator was used and camera was used to take the images.

No.	Genes/ Loci	Cytogenetic location	STS Markers	cM
1	*GLI3*	7p14.1	D7S2541	60.9
			D7S2454	62.11
			D7S2548	62.57
			D7S691	62.99
			D7S2428	64.26
			D7S667	65.75
			D7S2427	66.58

2.	*SHH/ZRS*	7q36	D7S598 D7S550 D7S104 D7S468 D7S559 D7S2423 D7S54 D7S22	178.43 180.67 182.84 182.84 183.93 185.38 186.09 186.09
3.	*ZNF141*	4p16.3	D4S3360 D4S90 D4S2936 D4S111 D4S43 D4S3038	0 0 0.61 0.97 2.86 0.97
4.	*IQCE*		D7S2474 D7S1532 D7S2484 D7S531	1.94 3.12 5.35 5.81
5.	PAPA2& PAPA5	13q21-32 & 13q13.3-21	D13S269 D13S1492 D13S233 D13S889 D13S137 D13S 1318	69.23 55.56 56.13 58.51 54.74 64.58

			D13S 803	55.64
6.	PAPA3& PAPA4	19p13.1-13.2& 7q21-q34	D19S403 D19S566 D19S410 D19S915 D19S1171 D19S547 D19S581 D7S2480 D7S496 D7S509 D19S916 D19S905 D19S550 D19S596 D19S865	29.1 45.27 41.41 44.26. 40.3 36.58 31.21 64.26 117.99 143.97 27.32 2298 79.74 78.7 28.23
7.	*HOXD13*	2q31.1	D2S2314 D2S138 D2S148	185.61 186.92 186.92
8.	*FBLN1*	22q13.31	D22S1153 D22S1160 D22S928 D22S1170	63.22 63.55 61.24 68.37

FIGURE 3.2: List of Highly Polymorphic Microsatellite Markers

Chapter 4

Results

4.1 Description of families

4.1.1 Family A

Family A is native of Rawalpindi, province of Punjab, Pakistan. The information regarding family history and pedigree sketch was taken from family members after their consent. Affected members were present in many generations and each affected member had an affected parent. It indicated an autosomal dominant mode of inheritance. Due to the presence of consanguinity in the family, autosomal recessive mode cannot be ruled out. Clinical examination of affected members showed post-axial polydactyly of both hands and feet. Syndactyly of 4^{th} and 5^{th} finger was present in both hands. The pedigree sketch (Figure 4.1) showed five generations, consisting of twenty one members with six affected members in second, third, fourth and fifth generations. Blood samples were collected in a local medical clinic from normal individuals and affected individuals.

Six genes *GLI3*, *SHH*, *LMBR1* (*ZRS*), *ZNF141*, *HOXD13* and *IQCE* were checked for their possible involvement in the present case of polydactyly. Highly polymorphic microsatellite markers (Figure 3.2) close to each genes were used in genotyping. Markers *D7S2541*, *D7S2548* and *D7S691* showed linkage to *GLI3* gene. No

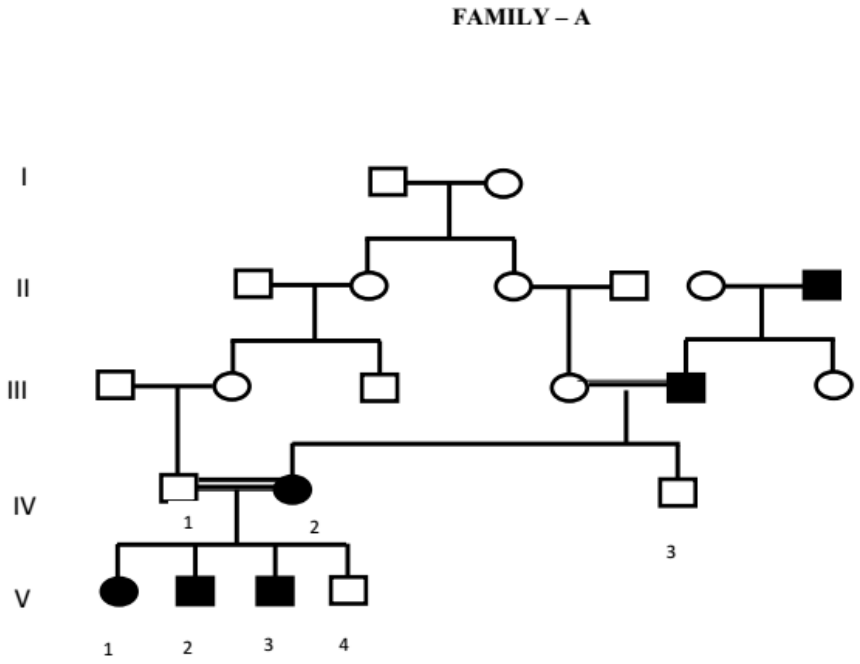


FIGURE 4.1: Pedigree design of family A with hereditary Post-axial polydactyly. Circles and squares represent females and males respectively, A filled symbol represents affected individual while unfilled symbol represents unaffected individual. Consanguineous marriages indicated by double lines. The number of generations indicates by Roman numerals.

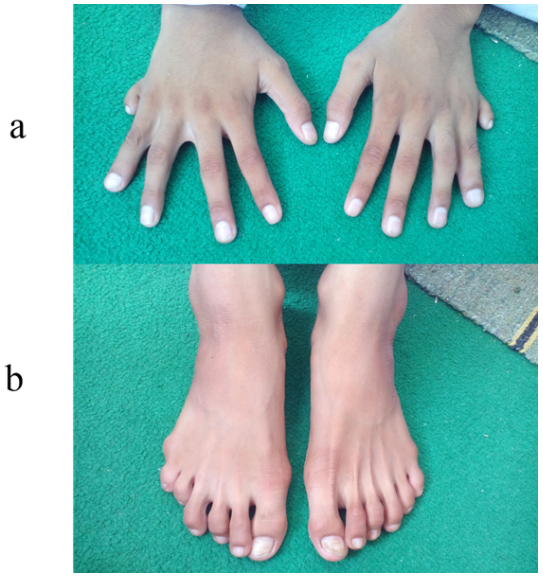


FIGURE 4.2: Photograph of member V-2 of family A: (a) showing post-axial polydactyly in both hands with syndactyly of digits 3 and 4; (b) showing post-axial polydactyly of both feet.

other gene showed linkage to the phenotype. Four loci of post-axial polydactyly were also checked by STS Genotyping (Figure 3.2. No linkage was found with any locus.

4.1.2 Family B

Family B is native of Rawalpindi, province of Punjab, Pakistan. The information regarding family history and pedigree sketch was taken from family members after their consent. Affected members were present in many generations and each affected member had an affected parent. It indicated an autosomal dominant mode of inheritance. Due to the presence of consanguinity in the family, autosomal recessive mode cannot be ruled out. Clinical examination of affected members showed pre-axial polydactyly present in one hand. The pedigree sketch (Figure 4.3) showed four generations, consisting of thirteen members with three affected members. Blood samples were collected in a local medical clinic from normal individuals and affected individuals.

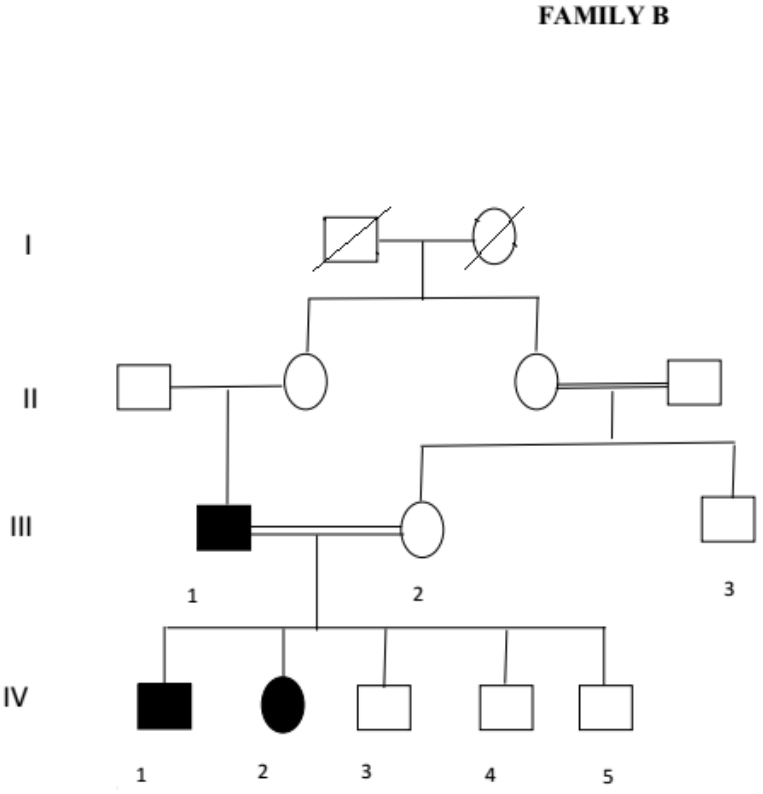


FIGURE 4.3: Pedigree design of family B with hereditary Pre-axial polydactyly. Circles and squares represent females and males respectively. A filled symbol represents affected individual while unfilled symbol represents unaffected individual. Consanguineous marriages indicated by double lines . The number of generations indicates by Roman numerals .

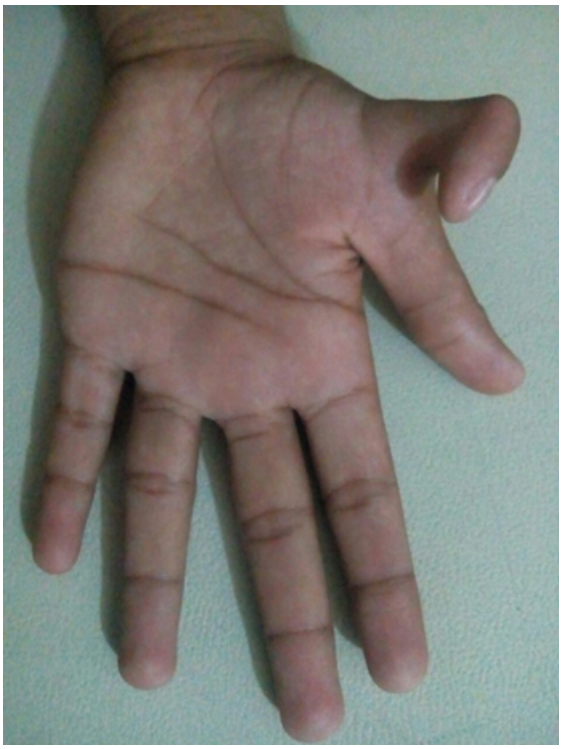


FIGURE 4.4: Photograph of member IV-1 of family B, showing pre axial polydactyly in one hand only.

Four genes *GLI3*, *SHH*, *ZNF141* and *IQCE* were checked for their possible involvement in the present case of polydactyly. Highly polymorphic microsatellite markers (table 3.2) close to each genes were used in genotyping. Results analysis showed no linkage of family B to any of the genes suggesting the involvement of a novel gene yet to be discovered as responsible for Pre-axial polydactyly in this family. Four loci of post-axial polydactyly were also checked by STS genotyping (table 3.2). No linkage was found with any locus.

4.1.3 Family C

Family C belongs to district Rawalpindi, Punjab province, Pakistan. The pedigree was constructed through discussion after willingness of family. Consanguinity was not observed in respective family after pedigree construction. It indicated an autosomal dominant mode of inheritance. Family history showed congenital Pre-axial polydactyly without any other malformation in the body. The pedigree (Figure 4.5) constructed have three generations, comprising of fifteen members

with three affected members in second and third generations. Blood samples were collected from four members including two normal and two affected individuals in a local medical clinic. Clinical observation revealed that the congenital Polydactyly was not caused by any environmental factor.

Four genes, *GLI3*, *SHH*, *ZNF141* and *IQCE* were checked for their possible involvement in the present case of polydactyly. Highly polymorphic microsatellite markers (Figure 3.2) close to each genes were used in genotyping. The marker D17S54 shows homozygous pattern for both affected members of the family. It lies midway between *LMBRI* and *WDR60*. The presence of brachydactyly and mild short stature in one of the patients indicate the possible involvement of *WDR60* that cause short rib thoracic dysplasia. No other gene showed linkage to the phenotype. Four loci of post-axial polydactyly were also checked by STS genotyping (Figure 3.2) No linkage was found with any locus.

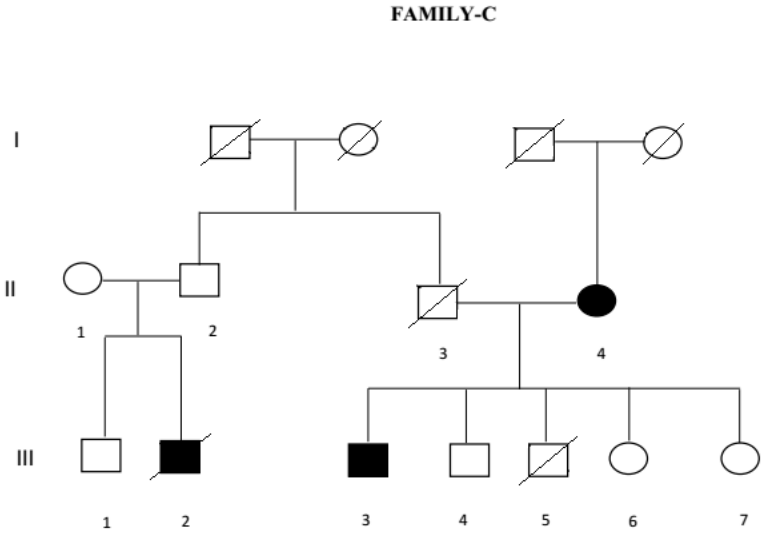


FIGURE 4.5: Pedigree design of family C with hereditary Pre-axial polydactyly. Circles and squares represent females and males respectively. A filled symbol represents affected individual while unfilled symbol represents unaffected individual. Consanguineous marriages indicated by double lines . The number of generations indicates by Roman numerals

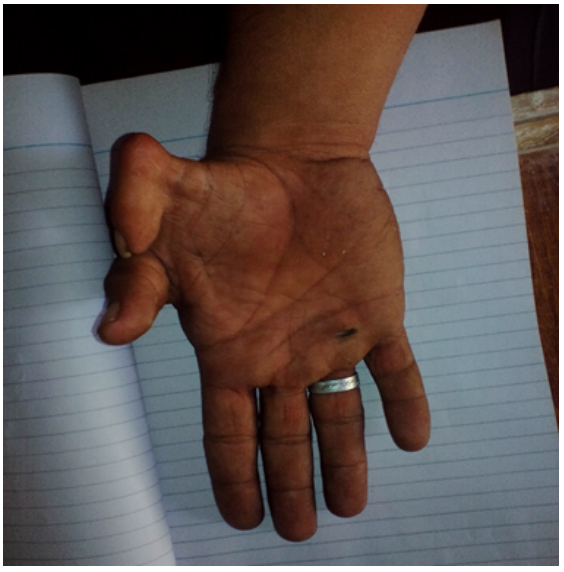


FIGURE 4.6: Photograph of member III-3 of family C, showing pre axial polydactyly in one hand only.

4.2: Genotyping Results of Family A

***GLI3 gene* (Family A)**

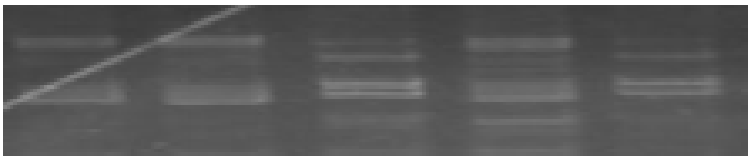
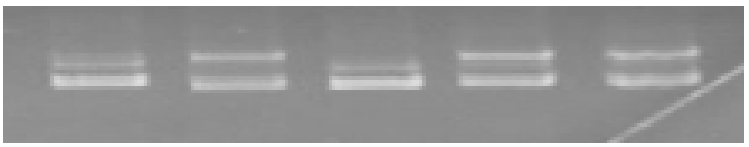
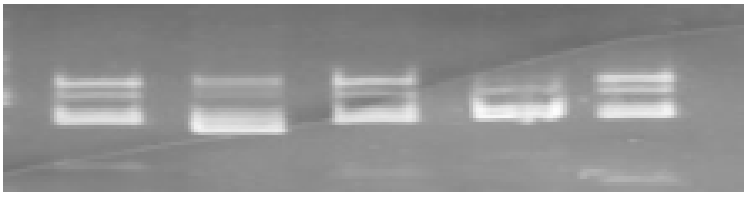
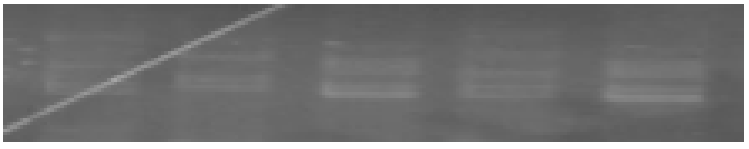
#	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D7S2541	60.9	
2	D7S2454	62.11	
3	D7S2548	62.57	
4	D7S691	62.99	
5	D7S2428	64.26	
6	D7S667	65.75	
7	D7S2427	66.58	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.7: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *GLI3* gene at chromosome 7p14.1 Numbers indicates the family members of the pedigree.

***SHH/ZRS* gene (Family A)**

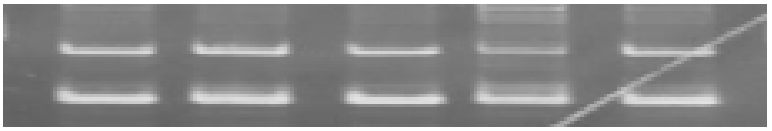
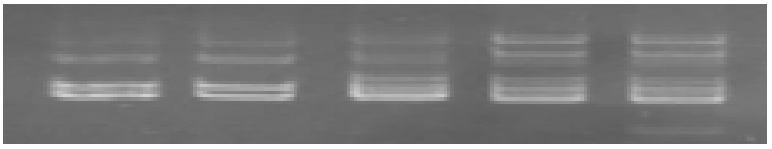
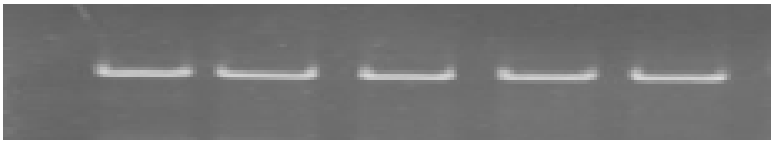
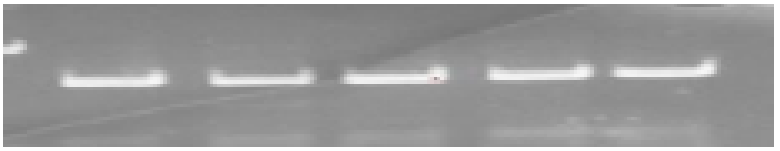
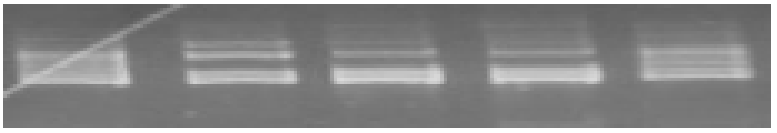
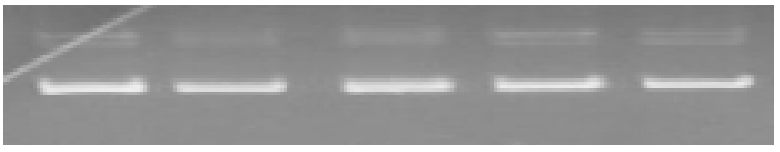
#	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D7S598	178.43	
2	D7S550	180.67	
3	D7S104	182.84	
4	D7S468	182.84	
5	D7S559	183.93	
6	D7S2423	185.38	
7	D7S54	186.09	
8	D7S22	186.09	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.8: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *SHH/ZRS* gene at chromosome 7q36. Numbers indicates the family members of the pedigree

***ZNF141* gene (Family A)**

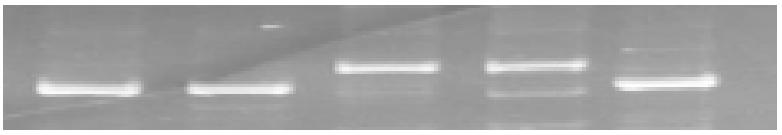
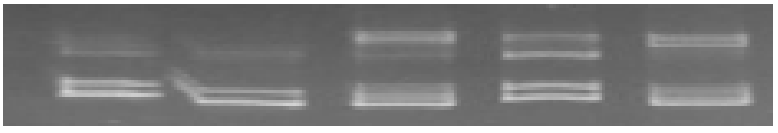
#	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D4S3360	0	
2	D4S90	0	
3	D4S2936	0.61	
4	D4S111	0.97	
5	D4S43	2.86	
6	D4S3038	0.97	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.9: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *ZNF141* gene at chromosome 4pl6.3. Numbers indicates the family members of the pedigree

***IQCE* gene (Family A)**

#	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D7S2474	1.94	
2	D7S1532	3.12	
4	D7S2484	5.35	
5	D7S531	5.81	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.10: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *IQCE* gene at chromosome 7p22.3. Numbers indicates the family members of the pedigree

***FBLN1* Gene (Family A)**

#	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D22S1153	63.22	
2	D22S1160	63.55	
3	D22S928	61.24	
4	D22S1170	68.37	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.11: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *FBLNI* gene at chromosome 22q13.31. Numbers indicates the family members of the pedigree

***HOXD13* Gene (Family A)**

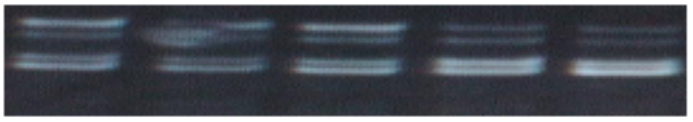
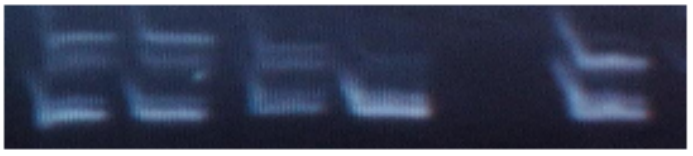
	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D2S2314	185.61	
2	D2S138	186.92	
3	D2S138	186.92	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.12: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *HOXD13* gene at chromosome 7p22.3. Numbers indicates the family members of the pedigree.

PAPA2 and PAPA5 Loci (Family A)

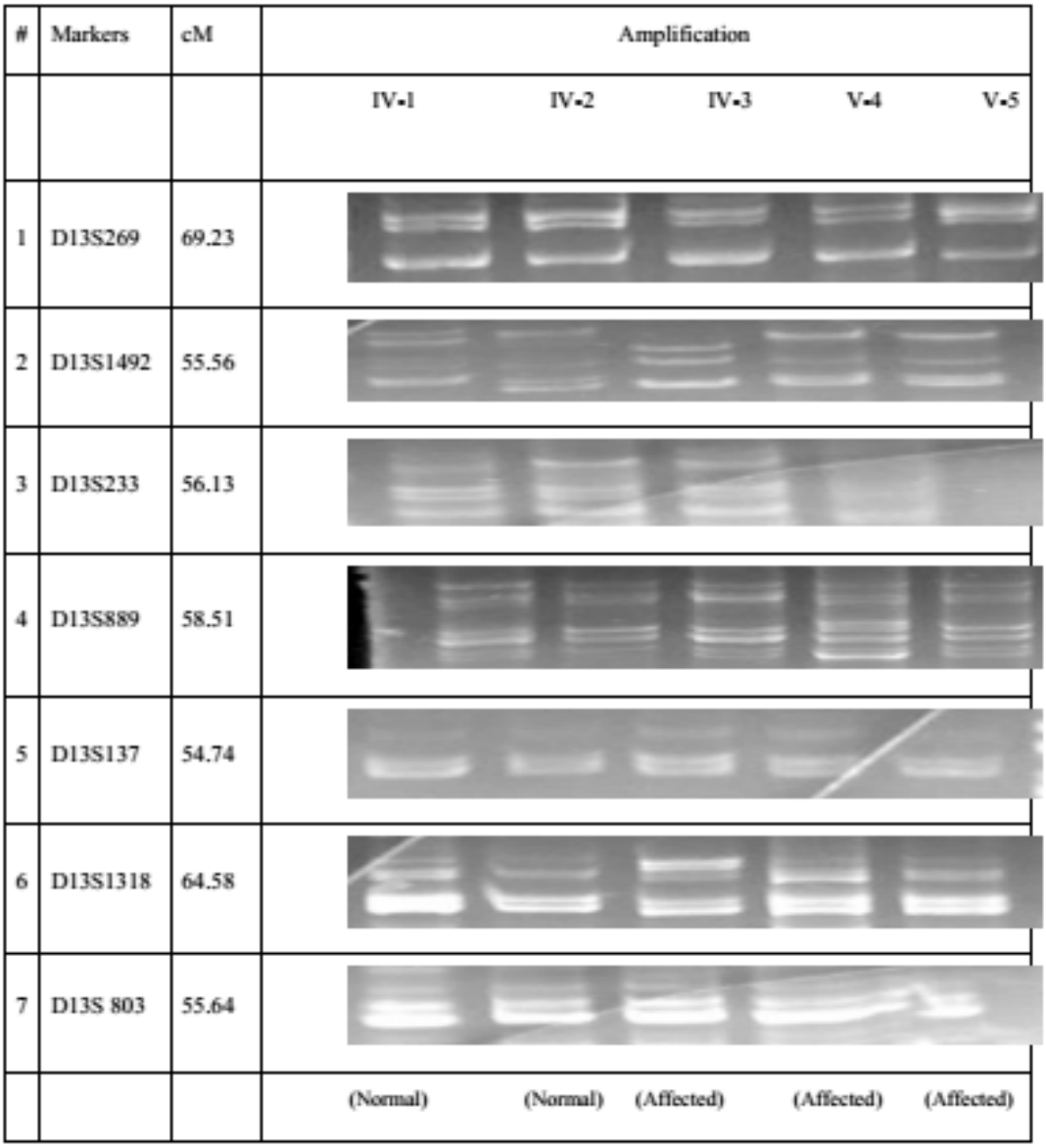


FIGURE 4.13: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *PAPA2* and *PAPA5* gene at chromosome 13q21-32 & 13q13.3-21. Numbers indicates the family members of the pedigree.

PAPA3 and PAPA4 Loci (Family A)

#	Markers	cM	Amplification
			IV-1 IV-2 IV-3 V-4 V-5
1	D19S403	29.1	
2	D19S566	45.27	
3	D19S410	41.41	
5	D1S91171	40.3	
7	D19S547	36.58	
9	D7S2480	64.26	
10	D7S496	117.99	
11	D19S916	27.32	
12	D19S905	22.98	
13	D19S550	79.74	
14	D19S596	78.7	
15	D19S865	28.23	
			(Normal) (Normal) (Affected) (Affected) (Affected)

FIGURE 4.14: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *PAPA3* and *PAPA4* gene at chromosome 19p13.1-13.2. & 7q21-34. Numbers indicates the family members of the pedigree

4.3: Genotyping Results of Family B

***GLI3* Gene (Family B)**

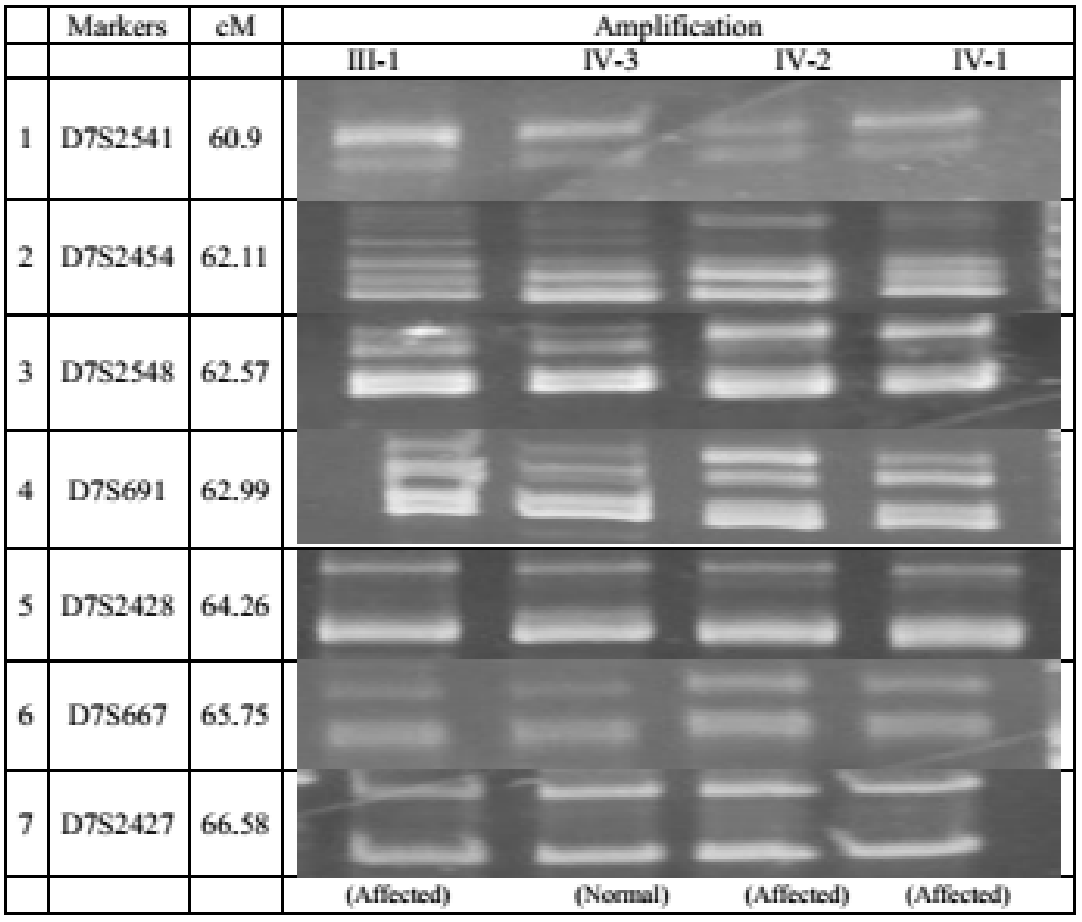


FIGURE 4.15: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *GLI3* gene at chromosome 7p14.1 Numbers indicates the family members of the pedigree.

***SHH/ZRS* Gene (Family B)**

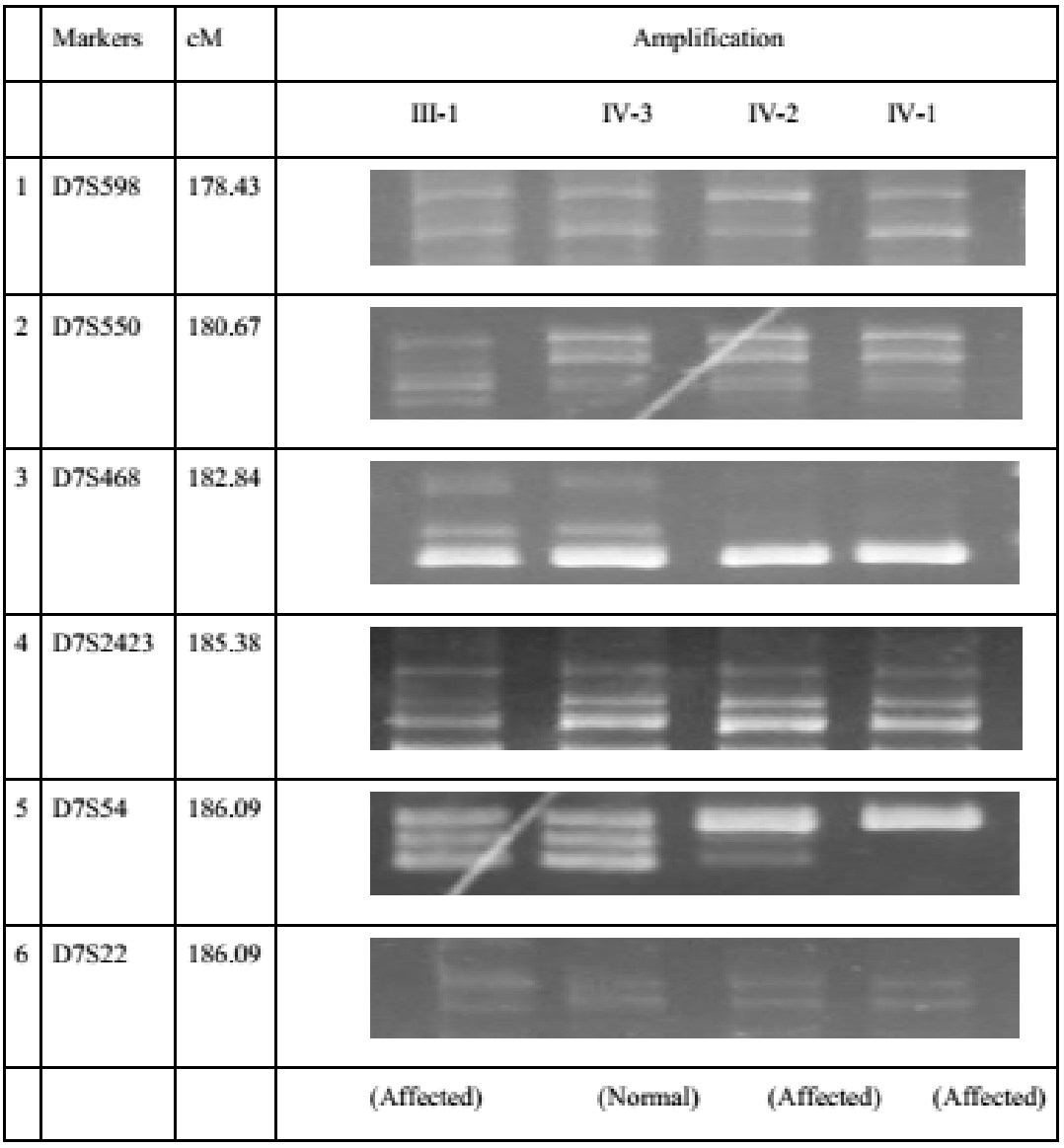


FIGURE 4.16: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *SHH/ZRS* gene at chromosome 7q36. Numbers indicates the family members of the pedigree.

***ZNF141* Gene (Family B)**

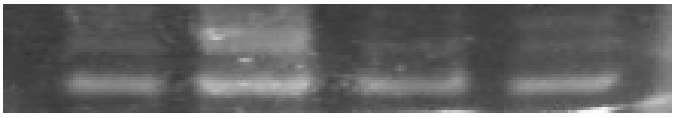
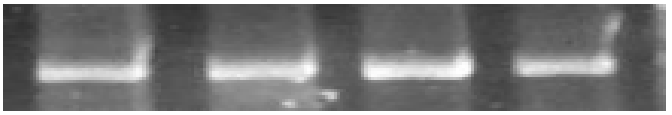
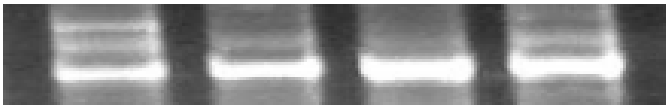
#	Markers	cM	Amplification
			III-1 IV-3 IV-2 IV-1
1	D4S3360	0	
2	D4S90	0	
3	D4S2936	0.61	
4	D4S111	0.97	
5	D4S43	2.86	
6	D4S3038	0.97	
			(Affected) (Normal) (Affected) (Affected)

FIGURE 4.17: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *ZNF141* gene at chromosome 4pl6.3. Numbers indicates the family members of the pedigree.

***IQCE* Gene (Family B)**

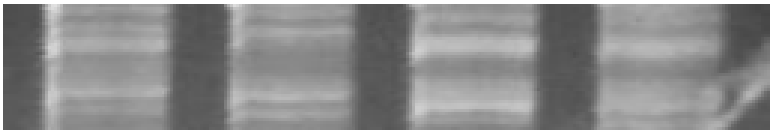
#	Markers	cM	Amplification
			III-1 IV-3 IV-2 IV-1
1	D7S2474	1.94	
2	D7S1532	3.12	
3	D7S616	4.79	
4	D7S2484	5.35	
5	D7S531	81	
			(Affected) (Normal) (Affected) (Affected)

FIGURE 4.18: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *IQCE* gene at chromosome 7p22.3 Numbers indicates the family members of the pedigree

PAPA 2 and PAPA5 Loci (Family B)

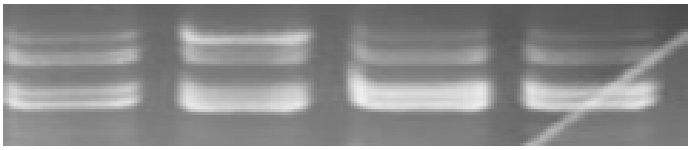
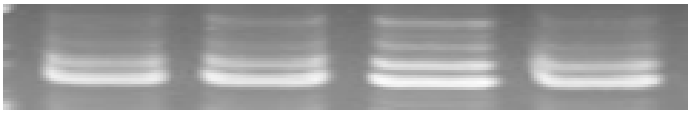
#	Markers	cM	Amplification			
			III-1	IV-3	IV-2	IV-1
1	D13S 269	69.23				
2	D13S 1492	55.56				
3	D13S 233	56.13				
4	D13S 889	58.51				
5	D13S 137	54.74				
6	D13S 1318	64.58				
7	D13S 803	55.64				
			(Affected)	(Normal)	(Affected)	(Affected)

FIGURE 4.19: : Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *PAPA2* and *PAPA5* at chromosome 13q21-32 & 13q13.1-21 Numbers indicates the family members of the pedigree

PAPA3 and PAPA4 Loci (Family B)

#	Markers	cM	Amplification
			III-1 IV-3 IV-2 IV-1
1	D19S403	29.1	
2	D19S566	45.27	
3	D19S410	41.41	
4	D1S91171	40.3	
5	D19S547	36.58	
6	D7S496	117.99	
7	D7S509	143.97	
8	D19S916	27.32	
9	D19S905	22.98	
10	D19S596	78.7	
11	D19S865	28.23	
			(Affected) (Normal) (Affected) (Affected)

FIGURE 4.20: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *PAPA3* and *PAPA4* at chromosome Numbe7q21-34. Numbers indicates the family members of the pedigree

4.4: Genotyping Results of Family C

***GLI3* Gene (Family C)**

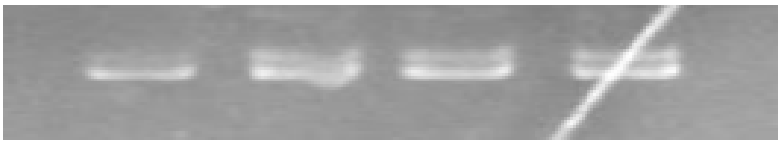
#	Markers	cM	Amplification			
			III-6	III-4	III-3	II-4
1	D7S2454	62.11				
2	D7S2548	62.57				
3	D7S2428	64.26				
4	D7S667	65.75				
			(Normal)	(Normal)	(Affected)	(Affected)

FIGURE 4.21: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *GLI3* gene at chromosome 7p14.1 Numbers indicates the family members of the pedigree.

***SHH/ZRS* Gene (Family C)**

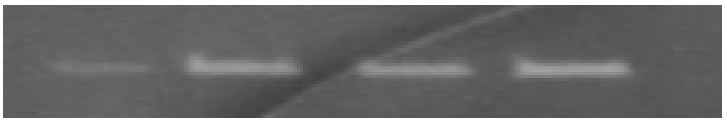
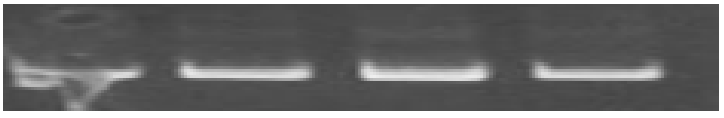
#	Markers	cM	Amplification
			III-6 III-4 III-3 II-4
1	D7S598	178.43	
2	D7S104	182.84	
3	D7S468	182.84	
4	D7S559	183.93	
5	D7S2423	185.38	
6	D7S54	186.09	
7	D7S22	186.09	
			(Normal) (Normal) (Affected) (Affected)

FIGURE 4.22: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *SHH/ ZRS* gene at chromosome 7q36. Numbers indicates the family members of the pedigree

***ZNF141* Gene (Family C)**

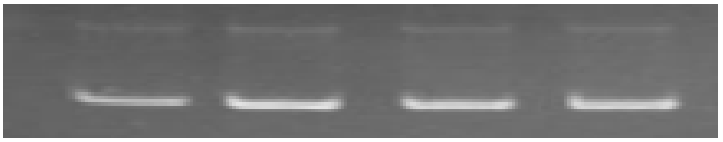
#	Markers	cM	Amplification			
			III-6	III-4	III-3	II-4
1	D4S90	0				
2	D4S2936	0.61				
3	D4S111	0.97				
4	D4S43	2.86				
5	D4S3038	0.97				
			(Normal)	(Normal)	(Affected)	(Affected)

FIGURE 4.23: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *ZNF141* gene at chromosome 4pl6.3. Numbers indicates the family members of the pedigree.

***IQCE* Gene (Family C)**

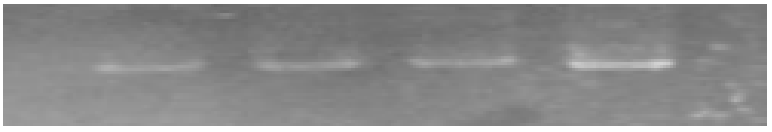
#	Markers	cM	Amplification
			III-6 III-4 III-3 II-4
1	D7S2474	1.94	
2	D7S1532	3.12	
3	D7S616	4.79	
4	D7S2484	5.35	
5	D7S531	5.81	
			(Normal) (Normal) (Affected) (Affected)

FIGURE 4.24: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *IQCE* gene at chromosome 7q22.3. Numbers indicates the family members of the pedigree.

PAPA2 and PAPA5 Loci (Family C)

#	Markers	cM	Amplification			
			III-6	III-4	III-3	II-4
1	D13S 269	69.23				
2	D13S 1492	55.56				
3	D13S 233	56.13				
4	D13S 889	58.51				
5	D13S 137	54.74				
6	D13S 1318	64.58				
7	D13S 803	55.64				
			(Normal)	(Normal)	(Affected)	(Affected)

FIGURE 4.25: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *PAPA2* and *PAPA5* at chromosome 13q21-32 & 13q13.3-21 Numbers indicates the family members of the pedigree

PAPA 3 and PAPA4 Loci (Family C)

#	Marker	cM	Amplification
			III-6 III-4 III-3 II-4
1	D19S403	29.1	
2	D19S566	45.27	
3	D19S915	44.26	
4	D1S91171	40.3	
5	D19S547	36.58	
6	D19S581	31.21	
7	D19S916	27.32	
8	D19S905	22.98	
9	D19S865	28.23	
			(Normal) (Normal) (Affected) (Affected)

FIGURE 4.26: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *PAPA3* and *PAPA4* gene at chromosome 19p13.1-13. & 7q21-34. Numbers indicates the family members of the pedigree.

Chapter 5

Conclusion and Future work

5.1 Conclusion and Future work

The development of digits and limb is of great consequence biologically. Polydactyly is an inborn physical anomaly in humans, dogs, and cats. It is characterized by additional fingers or toes. Polydactyly is present in association with other phenotypes (syndromic polydactyly). It is also found as separate element without any other abnormality (non-syndromic) Polydactyly is a hereditary limb abnormality having prevalence of 519/10 000 deliveries. (Schwabe and Mundlos, 2004; Christensen, 2011; Malik, 2014). Polydactyly can occur in any digit and described as pre-axial, post-axial and central (Bev Guo et al, 2013). Duplication of the thumb is the most common reported type of polydactyly, known as pre-axial polydactyly (PPD). Post axial polydactyly (PAP) is featured by duplication of the fifth finger/toe on side of hands and Feet (Buck-Gramcko, 1998). Pre-axial polydactyly has incidence as high as one in three thousands births. On the basis of further classification pre-axial polydactyly is divided into four types. The duplication of an osteal component of biphalangeal thumb is classified as pre-axial polydactyly (PPDl), Triphalangeal opposable thumb classified as pre-axial polydaetyly 2 (PPD2 in which thumb is present with or without duplication. The replacement

of thumb by triphalangeal digit is termed as pre-axial polydactyly 3 (PPD3) Special type of PPD is Pre-axial polydactyly 4 (PPD4) characterized by a duplication of hallux also called as hallux polydactyly (Schwabe and Mundlos, .2.004; Orioli and Castillal *et al.,* 1999). Non Syndromic forms of polydactyly usually segregate as an autosomal dominant trait. To date three nonsyndromic autosomal dominant loci for PPD have been reported including PPD2 and ZRS on chromosome 7q.36 (Tsukurov et al; 1994, Lettice et al; 200.2), PPD4 on chromosome 7pl.4J (Reynolds et al, 1994) and Hallux type on chromosome 2q31.1- 31.2 (Tsai et al. 2009). For PAP four non-syndromic autosomal dominant loci have been reported which include PAPA2, PAPA3, PAPA4, and PAPA5 which were mapped on chromosomes 13q21-q32, l9q13.1-13.2, 7q21-q34 and on l3q13.3-2l.2(Akarsu et al., 1997) (Zhao et al, 2002), (Umm-e-Kalsoom et al,2012) (Galjaard et al, 2003) respectively. In the present study, three consanguineous families one with post-axial polydactyly in addition with syndactyly of 4^{th} and 5^{th} fingers while two families with pre-axial polydactyly collected from different regions of Pakistan were subjected for linkage analysis. The family history and pedigree drawing showed that disorder was present at birth without any environmental cause and with autosomal dominant mode of inheritance. Linkage analysis was performed by homozygosity mapping using highly polymorphic microsatellite markers. Three families (A, B and C) were mapped to test the linkage to known loci and genes of non-syndromic form of Polydactyly. The family A with post-axial polydactyly in addition with syndactyly of 3rd and 4th fingers was mapped to known loci and genes *GLI3*, *SHH*, *LMBR1 (ZRS)*, *ZNF141* and (*IQCE, HOXD13).* Genotyping analysis showed that markers D7S2541, D7S2548, and D7S691 showed linkage to *GLI3* gene. No other gene showed linkage to the phenotype. Four loci of post-axial polydactyly were also checked by STS genotyping (Figure 3.2). No linkage was found with any locus. Homozygosity mapping of family B showed no linkage with any of the known gene. Exclusion of linkage indicates that an unknown gene is responsible for Pre axial polydactyly in this family. Family C with affected individuals having PPD was tested for linkage to several genes mentioned in above in family A. By genotyping analysis marker D17S54 shows homozygous pattern for both affected

members of the family. It lies midway between *LMBRI* and *WDR60*. The presence of brachydactyly and mild short stature in one of the patients indicate the possible involvement of *WDR60* that cause short rib thoracic dysplasia. No other gene showed linkage to the phenotype. Four loci of post-axial polydactyly were also checked by STS genotyping (Figure 3.2). No linkage was found with any locus.

In conclusion, three Pakistani families one with post-axial polydactyly in addition with syndactyly of 4^{th} and 5^{th} digit and two with pre-axial polydactyly were investigated at clinical and molecular level. Family A and C showed linkage to *GLI3* gene and *WDR60* genes respectively while family B did not show any linkage to known genes. Exclusion of linkage in this family suggest that novel gene for polydactyly may be involved in this family. The family A and family C should further processed for sequencing to identify the causable mutation in *GLI3* gene and *WDR60* gene while the family B should further processed for SNP microarray and whole exome sequencing for the identification of novel gene responsible for pathogenic phenotype in this family.

The aim of the study was to categorize and identify certain genetic predispositions involved in the demonstration of polydactyly, The current linkage and association study Vims for identifying the locus harboring the disease causing gene resulting in particular phenotypic consequences and in the gene based diagnosis of the disorder. The study would challenge to detect novel genes involved in particular diseases by whole genome scan. The study would help in improved genetic understanding of this rare autosomal dominant disorder and its fundamental molecular mechanisms. In addition, it would be helpful in the diagnosis, treatment and management of polydactyly.

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