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Identification of Genes involved in Postaxial Polydactyly, Sample of FATA territory, Pakistan

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Postaxial Polydactyly (PAP) is characterized by fifth digit duplication in hands and/or feet. Two types of PAP including PAP-A, representing the development of well-formed extra digit, and PAP-B, representing the presence of rudimentary fifth digit, have been described. Both isolated and syndromic forms of PAP have been reported. Isolated forms of PAP usually segregate as an autosomal dominant trait and to date four loci have been identified. In the present study, we have described mapping of the first locus of autosomal recessive PAP type A on chromosome 13q13.3–13q21.2 in a consanguineous Pakistani family. In family with autosomal dominant forms of postaxial polydactyly most of the affected individuals exhibit features representing type A PAP. In the respected family mapped on chromosome 13q13.3–13q21.2, affected individuals showed additional feature of post axial polydactyly. Several locus of causative genes are involved in this disease. To check the specified locus, we use several microsatellite markers (D13S1246, D13S218, D13S1288, D13S1233, D13S263, D13S1312, D13S153, D13S328, D13S119, D13S632, D13S889, D4S90, D4S2936, D4S111, D4S43, D4S16114, D4S95, D4S127, D4S126, D4S179, D4S432, D4S2957, D4S431, D4S2366, D4S2935, D4S3007, D4S412, D4S3023, D4S2285). All the affected individuals showing no homozygosity. Genetic mapping of all the affected individuals showed that novel genes are responsible for the disease. In future we will work on and try to find out the causative genes.
Keywords: Genes, Postaxial Polydactyly, FATA, Pakistan

1. Introduction:

1.1. Skeleton

Genetic disorders affecting skeleton comprise a large group of clinically distinct and genetically heterogeneous conditions. Genetic studies of diseases that affect skeletal growth and development are providing invaluable insights into the roles not only of individual genes, but also of entire developmental pathways. Clinical manifestations range from neonatal lethality to only mild growth retardation. Although they are individually rare, but disorders of the skeleton are of clinical relevance because of their overall frequency. Their overlapping clinical features and diversity make these disorders often difficult to diagnose, and many attempts have been made to delineate single entities or groups of diseases to facilitate diagnosis. A sensitive method for analyzing structure–function relationships is provided by correlating clinical phenotypes with the identified molecular alterations. Disease condition may be due to different mutations in the same gene that result in a range of abnormalities, and disease 'families' are frequently caused by mutations in components of the same pathway or often one gene influence function of other gene in the same pathway so loss of one gene function may result number of abnormalities. Skeleton is derived from different embryonic lineages which proliferate and migrate to form distinct mesenchymal condensate at the site of future skeleton element. The embryonic cells within these mesenchymal condensations differentiate into chondrocytes, osteoblasts, and osteoclasts. Chondrocytes eventually differentiate into cartilage while osteoblasts and osteoclasts into bone. Subsequently, three-dimensional patterning and remodeling lead to the formation of craniofacial, axial and limb skeleton [1]. These processes of differentiation, development and organ formation are governed by various transcription factors, hormones, growth factors and their intercellular signals [1].

The morphology of the human hand is of extraordinary importance not only because it is an excellent tool to manipulate the environment but also because it is at the root of the evolutionary progress of our species. The design of

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the human limb follows the basic plan of the pentadactylies' tetrapod limb. Three segments can be readily differentiated: (i) the proximal segment called the stylopodium, which contains a single skeletal element (humerus/femur), (ii) the intermediate segment called the zeugopodium, which contains two skeletal elements (radius–ulna/ tibia–fibula), and (iii) the distal segment called the autopodium, containing the numerous skeletal elements of the hand or foot. Between the zeugopod and the autopod, another segment can be distinguished that corresponds to the carpus/tarsus and is called the mesopodium. In the autopodium, the digits are numbered according to their position, from anterior to posterior, so that the most anterior digit, the thumb in the human hand, is called digit 1 and the most posterior digit, the little finger, is called digit 5. What distinguishes the morphology of the human hand is the presence of an opposed thumb that makes it a magnificent multifunctional tool.

1.2. Cartilage and bone

The vertebrate skeleton consists of two predominant tissue types: cartilage and bone. Although generally considered a vertebrate character, cartilage is found across a broad range of animal taxa, indicating a long and complex evolutionary history [2]. Cartilage differs from bone in several ways; cartilage has a lower metabolic rate, is mostly avascular, and contains different cellular and extracellular components that give it unique structural properties. Classically, true cartilage was defined by three criteria (1) it contains chondrocytes suspended in rigid matrix, (2) the matrix has a high content of collagen, and (3) the matrix is rich in acidic polysaccharides [3]. The proposal that the cartilage of some vertebrates, such as lampreys and hagfishes, is noncollagenous led to a revision of this definition to substitute ''fibrous proteins'' for ''collagen'' [4] however, recent work has shown that these jawless fishes also have collagen-based cartilage [5]. Such studies of cartilage in nontetrapod lineages have revealed that a deeply conserved genetic system underlies a diverse array of cartilage types. These discoveries have enhanced our understanding of the early evolution of cartilage and raised new questions about the homologies of animal connective tissues. Here, we review these advances in the context of skeletal developmental genetics and the evolutionary history of vertebrates and discuss how changes to developmental and genomic programs may have contributed to the origin of the vertebrate skeleton.

1.3. Morphogenesis of the digits

The digits are a novel evolutionary acquisition of the tetrapod limb [5, 6]. During embryonic development, they arise as single chondrogenic condensations that later segment and grow. In the rounded digital plate (autopodium), the digits soon become demarcated and form the digital rays that are separated by the flattened interdigital tissue. The fate of the cells in these two regions is very different; while the digits will form in the digital rays, the interdigital tissue will disappear through apoptosis. Interestingly, the cell death fate of the interdigit can be experimentally diverted to form a digit if the appropriate signals are provided [3, 7]. Indeed, the simple generation of a wound in the interdigital tissue is sufficient to activate the chondrogenic phenotype resulting in an extra digit (M.A.R., personal observations). The cellular and molecular mechanisms controlling the formation of the correct number of digital rays are not completely understood but correlate with the size of the digital plate and can be modified at relatively late stages. In the pentadactylies' limb, each digit presents a specific morphology according to its anterior–posterior position in the autopodium and is therefore distinguishable from the other digits. The chick is a widely used model for studying limb development and particularly useful for studies on digit identity, because in the leg, each digit has a different number of phalanges, which is therefore taken as diagnostic for the identity of the digit. Experimental studies with this model have demonstrated that the identity of the digits remains labile up to a relatively late stage in development [8]. At the time the phalangeal condensations of the digits are first being laid down, it is still possible to modify the identity of the digits by interfering with the signals emanating from the encompassing interdigit. BMP signaling, regulated by the transcription factors. Tbx2 and Tbx3, is a firm candidate for this function [9]. There is some controversy as to whether interdigital signaling acts directly upon the digital rudiments to control their identity or whether there is indirect effect acting through the AER to prolong its duration its and therefore the time for digit elongation [10].

1.4. Molecular and cellular bases of limb development

The first morphological evidence of a limb during embryonic development is the emergence of a bulge at the appropriate level in the lateral body wall. This bulge will rapidly form a bud consisting of mesenchymal cells of mesodermal origin that are covered by the ectoderm. The main source of the limb mesoderm is the somatopleura, but other contributions come from the migration of muscle precursors from the somites and from the progressive invasion of the limb bud by endothelial and nerve cells. This apparently simple bud will develop into a complete and patterned limb under the control of a few well-identified signaling centers. Signaling centers are specialized groups of cells that produce and secrete molecules to direct the developmental behavior of neighboring cells. The three main signaling centers identified in the growing limb bud are the apical ectodermal ridge (AER), the zone of polarizing activity (ZPA), and the non-ridge ectoderm, each

one being primarily responsible for directing growth and patterning along one of the three orthogonal axes [11].

1.5. The AER and fibroblast growth factors

Possibly, the main signaling center during limb development is the AER. The AER is the epithelial rim at the distal border of the limb. It forms a specialized structure – a pseudostratified epithelium in birds and a squamous poly stratified epithelium in the mouse that separates the dorsal from the ventral surfaces of the limb bud. The importance of this structure is clearly evidenced by the phenotype resulting when it is removed experimentally. Removal of the AER arrests further development and results in a proximo-distal transverse truncated limb (Saunders et al.,1948). Also, several mutations, spontaneous in chick and targeted in mice, are characterized by the inability to form an AER, and consequently, they are [12]. The earlier the stage at which the AER is lost, the more proximal the level of truncation. This phenotype was taken to indicate that the AER is necessary for the progressive specification of distal identities (fates) in the sub-AER mesoderm called the progress zone. The progress zone model assumes that the cells become progressively distalized in relation to the time that they spend in the progress zone under the influence of the AER [13]. However, the AER is also necessary early on for the survival, and later for the proliferation, of the subjacent mesoderm. Therefore, the cell death or reduced proliferation that follows its removal can also account for the truncated phenotype [14]. The action of the AER is mediated by several members of the family of fibroblast growth factors (Fgfs), five of them (Fgf8, Fgf4, Fgf2, Fgf9, and Fgf17) being secreted by the AER cells [15]. Of these, Fgf8is expressed by all AER cells, from their early specification to their disappearance at late limb development and is thus considered the marker of the AER. A source of FGF can replace all AER functions, and furthermore is sufficient to trigger the development of a complete extra supernumerary limb if applied to the flank (interlimb) region (Cohn et al.,1995). The induction of the AER results from a classical epithelial–mesenchymal interaction, whose molecular bases are currently being unraveled. It has recently been shown that the pre-limb ectoderm, in birds and mammals, requires Wnt signaling to form an AER [10, 11, 16]. In the absence of this Wnt signaling, the AER and, consequently, the limb fail to develop [17]. In the chick, earlyFgf10expression in the pre-limb mesoderm is responsible for the induction of Wnt signaling in the ectoderm. This Wnt expression is also regulated by bone morphogenetic protein (BMP) signaling originating in the ventral ectoderm [18]. Once the AER is formed, it also requires maintenance from the subjacent mesoderm.

1.6. Joint formation

Joint formation is another process crucial for patterning. It occurs in several stages [19], either at the boundary between two adjacent condensations or within a single condensation. For example, in the autopod, individual digital rays are divided by joints into hand or foot elements and the phalanges that comprise the digits. Therefore, proper positioning of joints within a condensation influences the number and size of skeletal elements. Joints become morphologically detectable when chondrocytes in the prospective joint-forming region become denser and flatten, and chondrogenic differentiation is inhibited. This creates an 'interzone', the site of the future cell death that creates the joint space. Two genes, Gdf5and Nog, which encode a BMP-related protein and BMP antagonist, respectively, are crucial in joint formation. Loss of Gdf5 function results in several skeletal abnormalities including absence of specific joints in the autopod [20]. Further analysis has suggested that Gdf5 has multiple roles in skeletal development, including restriction of joint development to the appropriate locations [21]. Loss of Nog function causes complete failure of joint formation in the autopod, at least in part via an effect on Gdf5 expression [22]. Ectopic expression of Wnt14in chicken limb buds induces morphological and molecular manifestations of early joint formation, including expression of Gdf5, suggesting that this member of the Wingless family of intercellular signaling molecules also has a role in normal joint formation [23]. These and related studies have provided a good start towards understanding the molecular mechanism of joint formation and how they are localized to specific sites.

1.7. Polydactyly

Malformations affecting the limbs and particularly the number of digits are the most frequent congenital malformation in human occurring in about one in 1000 neonates. The presence of supernumerary digits is termed as polydactyly whereas oligodactyly indicates severe underdevelopment or less than normal number of digits. Polydactyly, one of the most common and frequent congenital deformities, is inherited and often several members of a family are affected [24]. The condition may range from a small amount of additional soft tissue without osseous structure to a complete, fully developed extra digit or digits. The primary treatment is surgical removal. Polydactyly occurs both in a sporadic form and in a hereditary form [25]

Polydactyly usually involves the last digits of the hands/ or feet. Although some cases involve only a distal phalanx, other cases are more much complex, involving the entire digits, with duplication of nails, tendons, and vascular structure. polydactyly have great percentage in blacks than whites (3.6 to 13 cases per 1,000 live births versus 0.3 to 1.3 cases per 1,000 live births, respectively) [26].

1.8. Classification of Polydactyly

Temtamy and McKusick classified polydactyly by determining whether a case of polydactyly is isolated or a part of syndrome. Within these two categories, further classification of polydactyly is done upon the anatomic location of the duplicated digits, either as preaxial or postaxial. Nathan and Keniston noted that in sporadic cases, when both extremities express polydactyly, preaxial or postaxial classification is inadequate and difficult. This condition has been classified as crossed or mixed polydactyly. Crossed polydactyly, as in polydactylism in general, might be expected to occur as an isolated phenomenon or as a part of a syndrome. Venn-Watson described polydactyly of the foot that expressed both preaxial and postaxial forms, possibly indicating a variation of mixed polydactyly classification. Preaxial polydactyly of the hand is more common than postaxial polydactyly and postaxial polydactyly of the foot is more common than preaxial in white patients [27,28,29].

1.8.1. Syndromic polydactyly

London Dysmorphology Database lists 221 syndromes with polydactyly and 120 syndromes with oligodactyly. The commonly seen syndromes with digit anomalies are Greig syndrome, Bardet Biedl syndrome, Cornelia de Lange syndrome, ectrodactyly – ectodermal dysplasia – clefting syndrome (EEC), oro-facio-digital syndromes and short rib polydactyly syndromes. Many of these syndromes have some common features. Syndromes of polydactyly associated with midline malformation include Pallister Hall syndrome, acrocallosal syndrome, orofaciodigital syndromes, hydrolethalus syndrome, pseudotrisomy syndrome and short rib polydactyly syndromes. Cases with features overlapping with two or more syndromes have been reported. The clinical similarity indicates possibility of a common causative gene or genes involved in a common pathway.

1.9. Non-Syndromic Polydactyly

1.9.1. Postaxial Polydactyly

Postaxial polydactyly (PAP) is a frequent congenital malformation characterized by presence of extra digit on the ulnar or fibular edge of the extremities in hands and /or feet. Mode of inheritance is dominant, recessive or as part of syndrome [30] or with other defects of the feet and hands [31], or it may occur as a single birth defect. Partial cutaneous syndactyly of the 4th and 5th digit is often seen in combination with post axial polydactyly (PAP). It can be further subdivided into type "A" and "B". Unlike pre-axial polydactyly, which is distinctly more common in patients of European ancestry, post-axial polydactyly is more common in patients of African descent. The incidence is reported as 1 in 1,339 live births in Caucasians, compared with 1 in 143 live births in Africans and African Americans. The presence of post-axial polydactyly in patients of African ancestry is typically isolated, whereas in Caucasians it is often associated with an underlying syndrome [32]. Inheritance is most often autosomal dominant with incomplete penetrance; however, recessive inheritance has been reported, particularly in association with syndromic presentations [33]. Several isolated genetic defects have been definitively associated with ulnar polydactyly, including a frameshift mutation of the Gli3 gene on chromosome 7, and linkage to loci on the long arm of chromosome 13 and the short arm of chromosome 19 [34].

1.9.2. Postaxial Polydactyly Type A/B

Postaxial polydactyly Type "A" (PAP-A) is autosomal dominant disorder with a presentation of 5th digits duplication which is usually functional that articulates with the fifth or an extra metacarpal while postaxial polydactyly Type "B" (PAP-B) is autosomal recessive which is dominantly inherited disorder characterized by presence of vestigial or rudimentary extra fifth digit and is usually represented by an extra skin tag. Both types of postaxial polydactyly may be taken as different entities [35] or they may coexist in the same kindred. To date, four loci of PAP has been mapped on chromosome 7p15-q11 (Radhakrishna et al., 1997) caused by mutation in the GLI3 gene designated as PAPA1 (including PAP-A/B in one patient) , 13q21–q32 [35] (with PAP-A only) designated as PAPA2, 19p13.1-13.2 [36] (with PAP-A/B) designated as PAPA3 and 7q21-q34 [37] designated as PAPA4 in an Indian family, Turkish family, large Chinese kindred and large Dutch family respectively. Recently it has been proved that beside other mutations postaxial polydactyly could also be a result of inversion of chromosome 7 (p15.2q36.3). The result of inversion was not disruption of candidate gene instead separation of the HOXA cluster from a gene desert containing several conserved noncoding elements took place, that may cause disruption of a cis-regulatory circuit of the HOXA cluster and the underlying cause of the phenotype in patient [38].

1.9.3. Preaxial Polydactyly (PPD)

Preaxial refers to the tibial or radial side of a line bisecting the second ray or long finger, respectively. Initially there were four types of Preaxial polydactyly recognized by Temtamy and McKusick (1969). Remarkable feature of PPD-1 is thumb and/or big toe polydactyly with duplication of one or more of the skeletal components of a biphalangeal thumb, PPD-2 is polydactyly of a triphalangeal thumb, and that of PPD-3 is polydactyly of the index finger and type IV polydactyly (PPD-IV) is recognized by biphalangeal thumb duplication associated with mesoaxial syndactyly and PAP while several unclassified PPD phenotypes have also been described. The locus of PPD types II and III have been mapped to 7q36 [39] and mutations in intron 5 of LMBR1

gene were associated with PPD- II. Simultaneously, TPT-PS syndrome which is a complex form of preaxial polydactyly was also found to be linked to the same region on chromosome 7q36 [40], this provides evidence that same genetic defect could cause different types of PPD.

1.9.4. Syndactyly

Webbing of the digits or syndactyly is a heterogeneous hereditary condition of webbed finger and toes. It is caused by failure of the usual longitudinal interdigital necrosis that normally separates the fingers during the sixth to eight weeks of intrauterine life [41]. To date, nine non syndromic types have been identified except Cenani-Lenz (type VII) and Malik-Percin (type XI) both of which segregate in an autosomal recessive fashion [42], all other show autosomal dominant mode of inheritance.

1.9.4.1. Types of Syndactyly

Generally, there are five types of syndactyly termed as Zygodactyly (I), Synpolydactyly (II), Ring-small Syndactyly (III), Haas-type Syndactyly (IV) and complex type of syndactyly (V) recognized by Temtamy and McKusick (1978). All isolated types of syndactyly have autosomal dominant mode of inherence. Characteristic feature of Zygodactyly or type I syndactyly is webbing between the long and ring fingers, its locus mapped to 2q34-q36 (Bosse et al., 2000).

In Synpolydactyly or Syndactyly type II Disease condition is characterized by the presence of soft tissue syndactyly between 3/4 fingers and 4/5 toes, with partial or complete digit duplication within the syndactylous web [43], showing incomplete penetrance (a normal phenotype in some mutation carriers) and variable expressivity (different degrees of phenotypic severity in affected individuals). To date, three loci have been mapped on chromosome 2q31, 22q13.31 and 14q11.2-q12, and have been designated as SPD1, SPD2 and SPD3 respectively [43]. Causative gene for SPD1 and SPD2 (a complex type of synpolydactyly) were identified as HOXD13 [40, 44, 45,46] respectively. SPD3 is central in hands and post-axial in feet segregating in large Pakistani kindred, causative gene yet not known. Ring-small Syndactyly (type III) is usually bilateral, and occasionally the distal phalanges are fused. There may be only a rudimentary middle phalanx in the small finger and mapped on chromosome 6q21-q23.2. Causative gene was also identified as (GJA1) [47].

Type IV Haas-type Syndactyly is described by Haas in 1940 in the American Journal of Surgery and is characterized by complete webbing of all digits, occasionally a sixth metacarpal and phalanges may be included in the cup-shaped hand. Finally Type V syndactyly is very complex type having both the long and ring fingers and second and third toes are syndactylized. The fourth and fifth metacarpals and metatarsals may be fused [48]

1.9.4.2. Etiology of polydactyly

As indicated above, a polydactylous limb implies interference with normal development. Because the control of digit morphogenesis is complex and remains labile for a considerable time, the possibilities for interference are numerous. Despite the enormous advance in our understanding of the processes that control digit formation, it is not always possible to provide a reasonable explanation for each of the multitude of outcomes observed in human limb pathology. The number of digital rays that develop in the autopodium depends on the amount of tissue available. Thus, the control of the size of the limb bud is of decisive importance. Three factors strongly influence the size of the limb bud: (i) the number of progenitors that start up the bud, (ii) the rate of proliferation, and (iii) the amount of cell death. A deregulation of these factors results in a subsequent modification in the pattern of the autopodium and the number of digits

1.9.4.3. Involvement of Fgf signaling

FGF signaling from the AER appears to function at a very early stage in limb development to ensure that enough progenitor cells are available to form the normal limb skeleton [49]. This conclusion is reached because the doubleFgf8; Fgf4 knockout limbs are smaller than normal from their earliest emergence. Furthermore, the increased cell death observed on these double mutant embryos at proximal level contributes to their marked reduction in size by 10.5 days after coitum and results in the total absence of skeletal elements. In the case of the conditional single Fgf8 knockout, the limb is hypo plastic at the three proximo-distal levels with the absence of the first digit [50] and abnormal areas of cell death. This indicates that enough FGF signaling is required for the appropriate survival of the cells and consequently to produce a normal digit number [51]. Conversely, mutations that cause increased Fgf signaling from the ectoderm result in polydactyly. For example, double ridge is a transgene-induced mutation in the mouse characterized by thickening of the AER and increased Fgf signaling in the subjacent mesoderm that results in forelimb ulnar polydactyly [52]. The transgene insertion in double ridge causes a notable decrease in the expression of Dkk1, a secreted Wnt antagonist that modulates Wnt signaling in the ectoderm and that has also been shown to mediate BMP control of apoptosis [52]. Likewise, other mutations that cause expansion of the AER, such as the genetic disruption ofEn-1that interferes with the normal formation and maturation of the AER, result in polydactyly [53]. An increase in FGF signaling, rather than changes in the morphology of the AER, is responsible for the increased

proliferation and cell survival of the underlying mesenchyme, which leads to the syndactyly and polydactyly phenotype. Recently, the genetic modifications performed to elevate Fgf signaling through the introduction of a hypermorphic mutant form of the receptor 1 of FGF (FGFR1) [54] lead to the appearance of radial polydactyly by a mechanism involving extended AER function and reduced cell death. Interestingly, the topographic restriction of polydactyly to digit 1 suggests a specific FGFR1-dependent regulation of the pool of cells destined to give rise to this specific digit.

2. Materials And Methods

For the study, presented here, one family with postaxial polydactyly belonging to Mohallah Munshian Bannu city district Bannu Khyber pakhtoonkhwa Pakistan. The family was visited at their places of residence to construct pedigree and collect other relevant information. Informed consent for the study including presentation of the photographs was obtained only from affected members. Information regarding medical history of each family was collected.

2.1. Pedigree Construction

For genetic implication, an extensive pedigree was constructed for the family by the standard methods. The pattern of inheritance of the disease was deduced by observing the mode of segregation or transmission within family. The exact genetic relationships for all the affected individuals were obtained through interviews of elders and relatives of the families. Males were symbolized by squares and females by circles. The normal individuals were designated with unfilled symbols while the affected individuals by filled symbols. A number enclosed within a symbol indicates the number of sibs. Crossed circles or squares represent the deceased individuals. Each generation was indicated by Roman numeral (I, II, III) while individuals within a generation were denoted by Arabic numerals(1,2,3). Marriages within the family were shown by double lines between the partners.

2.2. Blood Sampling

Blood samples from both affected and normal members of the family was collected in 10 ml vacutainer tubes (BD vacutainer K2 EDTA 18 mg) using 5 ml (BD 0.60 mm X 25 mm, 23 G X 1 TW) and 10 ml syringes (BD 0.8 mm X 38 mm 21 G X 11/2 TW). Blood samples from children (below 2 years) were collected using special vacutainer (BD Vacutainer® safety-LokTM blood collection set). The blood samples collected were stored at 4°C.

2.3. Genomic DNA Extraction and Purification

1. Genomic DNA was extracted from venous blood samples using the standard phenol/chloroform method [55]. 750 µl of human blood was taken in a micro-centrifuge tube along with 750 µl of solution A, mixed by inverting the tube 8-10 times and kept at room temperature for 20-30 minutes.
2. The tube was centrifuged at 13,000 rpm for 1 minute and supernatant was discarded. The nuclear pellet was resuspended in 400 µl of solution A.
3. The tube was again centrifuged for 1 minute at 13,000 rpm and after discarding the supernatant the nuclear pellet was resuspended in 400 µl solution B, 12 µl of 20% SDS and 10 µl of proteinase K (10 mg/ml stock).
4. The sample was incubated at 65oC for 3 hours in water bath or at 37oC overnight.
5.On the following day when the pellet was completely digested, 500 µl of fresh mixture of equal volume of solution C and solution D was added in the sample, mixed and centrifuge for 10 minutes at 13,000 rpm.
6. The upper aqueous layer was collected in a new tube and equal quantity (500 µl) of solution D was added and centrifuged at 13,000 rpm for 10 minutes.
7. Transfer the upper aqueous layer to a new tube and DNA was precipitated by adding 55 µl of sodium acetate (3M, pH 6) and equal volume (500 µl) of iso-propanol or 2 volumes of ethanol (stored at -20 ˚C).
8. Tube was inverted several times to precipitate the DNA and centrifuged at 13,000 rpm for 10 minutes. The supernatant was then carefully discarded without disturbing the DNA pellet.
9. To the DNA pellet obtained, 200 µl of 70% ethanol (stored at -20 ˚C) was added and centrifuged for 7 minutes at 13,000 rpm.
10. The ethanol was then discarded, and the DNA dried by keeping the tubes for 10 minutes at 45oC in a vacuum concentrator 5301 (Eppendorf, Germany) [55].
11. The precipitated DNA was then dissolved in 150-200 µl of Tris-EDTA (TE) buffer. The samples were then kept in an incubator for a day to ensure complete suspension of the DNA in the buffer.

2.4. Agarose Gel Electrophoresis

Extracted DNA was analyzed on 1% agarose gel prepared by melting 0.5 g of agarose in 50 ml 1 X Tris-Borate-EDTA (TBE) in a microwave oven for 1-2 minutes. 5 µl (10 mg/ml) of ethidium bromide was added to the gel to stain the DNA. The DNA was mixed with loading dye (bromophenol blue) and loaded into the wells of the agarose gel. The electrophoresis was performed at 120 volts for 30-40 minutes. Then the DNA was visualized under UV transilluminator, and results were recorded by using a gel documentation system.

2.4.1. Dilution of DNA

Genomic DNA was quantified by taking optical density

Chemicals	**Stock Concentration**	**Amount Used**	**Final Concentration**
PCR Buffer	10 X	2.5 µl	1 X
MgCl2	25 mM	1.5 µl	1.5 mM
dNTPs	10 mM each dNTP	0.5 µl	0.2 mM each dNTP
Microsatellite marker (R)	20 ng/µl	0.3 µl	0.24 ng/µl
Microsatellite marker (F)	20 ng/µl	0.3 µl	0.24 ng/µl
Taq	0.5 U/µl	0.3 µl	0.006 U/µl

Table 2.2: Composition of Solutions Used

Solutions	Compositions
	0.32 M Sucrose
Solution A	10 mM Tris (pH 7.5)
	5 mM MgCl
	2
	1 % (v/v) Triton X-100
Solution B	10 mM Tris (pH 7.5)
	400 mM NaCl2 mM EDTA (pH 8.0)
Solution C	400 µl Phenol
Solution D	Chloroform 24 volumes
	Isoamyl alcohol 1 volume
Gel Loading Dye	40 g Sucrose
(Bromophenol Blue)	0.25 g Bromophenol Blue
10 X TBE Buffer	0.89 M Tris
	0.025 M Boric Acid
	EDTA 0.5 M (pH 8.3)
30% Acrylamide-Bis	290 g Acrylamide
acrylamide solution	10 g N-N MethyleneBisacrylamide
8 % Polyacrylamide Gel	5 ml 10 X TBE Buffer
	13.5 ml 30% acrylamide solution
	400 µl 10% Ammonium
	persulphate (APS)
	25 µl TEMED (Tetra
	Methyl Ethylene

(OD) at 260 nm wavelength and subsequently diluted to 40-50 ng/µl by using GeneRay UV-Photometer (Biometra®,

Composition of Solutions

Germany) for polymerase chain reaction (PCR) using PCR water, according to the amount of DNA extracted.

Generally, dilutions were made in 10 µl stock and 90 µl PCR water.

2.4.2. Linkage Analysis and Genotyping

Polymerase chain reaction (PCR) amplification of microsatellite markers was carried out in 0.2 ml tubes (Axygen, USA) according to a standard procedure in a total volume of 25 µl containing 1 µl DNA dilution, 0.3 µl of each forward and reverse microsatellite marker (20 ng/µl), 2.5 µl 10X PCR buffer (200 mM (NH4)2SO4, 750 mM of Tris-HCl pH 8.8, 0.1 % Tween 20), 1.5 µl 25 mM MgCl2, 0.5 µl 10 mM dNTPs and 0.2 µl of 0.5 unit Taq DNA Polymerase (MBI-Fermatas, England) in 18.7 µl PCR water. The reaction products were centrifuged for 30 seconds at 8,000 rpm for thorough mixing. Reactions were performed by means of T3 thermocyclers [56].

PCR was carried out with the following thermal cycling conditions: an initial denaturation of template DNA at 95oC for 4 minutes, followed by 40 cycles of amplification each consisting of 3 steps: denaturation of DNA into single strand at 95˚C for one minute, annealing or hybridization of microsatellite markers to their complementary sequences on either side of target sequence at 54-59˚C for one minute, and 72˚C for one minute for extension of complementary DNA strands from each primer. This was finally followed by a final extension at 72˚C for ten minutes.

The four families with alopecia were tested for linkage by using microsatellite markers tightly linked to different loci, associated with various forms of hair loss. Table 2.1 summarizes microsatellite markers located in the region of known alopecia loci, which were used as first pass analysis for the genetic linkage in the families.

2.4.3. Polyacrylamide Gel Electrophoresis

The amplified PCR products of the microsatellite markers used in exclusion mapping were resolved on 8% non-denaturing polyacrylamide gel (PAGE). Reagents were mixed in a 250 ml conical flask, and the solution was poured between two glass plates held apart by spacers of 1.5 mm thickness. After inserting the comb, gel was allowed to polymerize for 45 minutes at room temperature. Amplified products were mixed with 5-7 µl loading dye containing 0.25% bromophenol blue prepared in 40% sucrose solution and loaded into the wells. Electrophoresis was performed in a vertical gel tank of Whatman Biometra (Biometra, Germany) at 100 volts (30 mA) electric current for 120-150 minutes depending upon the size of amplified length. The gel was stained with ethidium bromide solution (0.5 µg/ml final concentration) and visualized on UV transilluminator (Biometra, Germany) and gel was photographed by using Digital camera DC 290 (Kodak, USA).

2.4.4. Mutation Detection

To search for mutations in LIPH and LPAR6 genes, their respective exons were PCR amplified from genomic DNA using primers shown in Table 2.2 and Table 2.3, respectively.

Table 2.1: Microsatellite markers used to test linkage to known genes/loci involved in alopecies.

Table 2.1: Microsatellite markers used to test linkage to known genes/loci involved in alopecies.

S. No	Candidate Genes/Loci	Chromosomal Location	Microsatellite Markers*	Genetics Location (cM)*
1		13q13.3	D13S1246	25.41
			D13S218	38.60
			D13S1288	39.29
			D13S1233	42.64
			D13S263	43.02
			D13S1312	48.03
			D13S153	52.00
			D13S328	50.18
			D13S119	55.92
			D13S632	57.16
			D13S889	58.51
2		4p16.1	D4S90	0.0
			D4S2936	0.6
			D4S111	0.9
			D4S43	2.86
			D4S1614	2.86
			D4S95	3.6
			D4S127	3.6
			D4S126	3.6
			D4S179	3.99
			D4S432	5.7
			D4S2957	5.7
			D4S431	12.14
			D4S2366	12.57
			D4S2935	13.21
			D4S3007	13.21
			D4S412	3.6
			D4S3023	6.45
			D4S2285	7.97

* Average-sex distance in cM (Centimorgan) according to Rutgers combined linkage-physical human genome map (Kong et al., 2004).

Discussion

In the present study, we have presented a consanguineous Pakistani family segregating autosomal recessive postaxial

polydactyly Type "A" (PAP-A). All two affected individuals of the family presented sixth well-formed, functional digits in both hands and feet. The extra digits on the hands were amputated before the family was located and studied. In addition to the presence of a sixth digit in hands and feet, partially duplicated fork-shaped fifth metatarsals of feet and hands were observed in the affected individuals (II-1, II-2). In family with autosomal recessive forms of postaxial polydactyly most of the affected individuals exhibit features representing type A PAP [56, 57, 58, 59]. In the respected family mapped on chromosome 13q13.3–13q21.2, affected individuals showed additional feature of post axial polydactyly.

Genetic mapping established linkage of the family, presented here, on chromosome 13q13.3–q21.2. Several locus of causative genes are involved in this disease. To check the specified locus we use several microsatellite markers (D13S1246, D13S218, D13S1288, D13S1233, D13S263, D13S1312, D13S153, D13S328, D13S119, D13S632, D13S889, D4S90, D4S2936, D4S111, D4S43, D4S16114, D4S95, D4S127, D4S126, D4S179, D4S432, D4S2957, D4S431, D4S2366, D4S2935, D4S3007, D4S412, D4S3023, D4S2285) .All the affected individuals showing no homozygosity. Genetic mapping of all the affected individuals showed that novel genes are responsible for the disease. In future we will work on and try to find out the causative genes. Several studies described syndromic forms of polydactyly involving variable 13q segmental duplications, which include chromosomal segments 13q14, 13q21, 13q22, 13q31 and 13q32 [54, 60, 51, 61, 62, 63]. Although these chromosomal regions were not precisely mapped, still it is likely that some of these regions coincide with the region mapped in the present andthe study reported by [64]. Recently, Van der Zwaag et al. (2010) described a patient, born to non-consanguineous parents, with multiple clinical features including postaxial polydactyly type A of hands preauricular tag, anterior placed anus, a broad nasal bridge, tele canthus and frontal bossing[65].

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