Molecular Characterization of Inherited Skeletal Deformities in Pakistani Families

By

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PRAYER

In the name of ALLAH, The most beneficent, The most merciful.

Praise to Allah, Lord of worlds

The beneficent, The merciful

Master of the Day of Judgment

Thee we worship, Thee we ask for help

Show us the straight path

The path of those whom thou worth favored

Neither of who earn Thins anger

Nor of those who go astray

(Ameen)

Al-Quran
Surrah Al-Fatiha

Dedicated to

My Beloved Mother,
Brothers and Sisters
And to My caring Wife

Quaid-I-Azam University
Islamabad, Pakistan

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CERTIFICATE

This thesis submitted by Mr. **Ikram Ullah** is accepted in its present form by the Department of **Biotechnology**, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the degree of **Master of Philosophy** in **Biotechnology**.

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Ikram Ullah

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LIST OF ABBREVIATIONS

APS	Ammonium persulphate
ATPase	Adenosine triphosphatase
bp	base pairs
CLM	Congenital limb malformation
cM	Centimorgan
CTEV	Congenital talipes equinovarus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTCS	Dye terminator cycle sequencing
EDTA	Ethylenediamine tetra acetic acid
g	gram
GH	Growth hormone
GHR	Growth hormone receptor
GHRHR	Growth hormone releasing hormone receptor
HGMD	Human gene mutation database
IGHD	Isolated growth hormone defeciency
Kb	Kilobase
KDa	Kilo Dalton
mg	milligram
$MgCl_2$	Magnesiun chloride
ml	milliliter
mm	millimeter
mM	millimolar
µg	microgram
µL	mico liter
NaCl	Sodium chloride
NCBI	National center for biotechnology information
ng	nanogram
OD	Optical density
OMIM	Online mendelian inheritance in man
ORF	Open reading frame
p	Short arm of chromosome

PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
q	Long arm of the chromosome
rpm	revolution per minutes
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	N,N,N,N-Tetra methylethylene diamine
TEV	Talipes equinovarus
UCSC	University of California santa cruz
UTR	Untranslated regions
UV	Ultra violet
V/V	Volume/Volume
W/V	Weight/Volume

ABSTRACT

Genetic disorders of the skeleton are a large and heterogeneous group of diseases whose unifying features are malformation, disproportionate growth, and deformation of the skeleton or of individual bones or a group of bones. Clinical severity differs between individual, ranging from minor handicap to death in the neonatal period. Both genetic and environmental factors contribute to the etiology of skeletal disorders. The genetic factor is mostly due to single gene mutation. More than 6000 human genetic diseases are caused by a mutation in a single gene resulting in a disorder that can be inherited through generations. Over 300 genetic conditions involve problems with the growth and development of the skeleton. To date, no data is available about the genetic contribution to skeletal deformities in Pakistani population. The consanguineous marriage pattern has significant implication for increased rate of recessive genetic disorders. Autosomal recessive diseases are more prevalent in endogenous and isolated populations. Pakistan represents a true treasure for molecular dissection of genetic disorder because 60% marriages are consanguineous and out of those approximately 80% are between first cousins.

Present study was conducted to detect pathogenic variants in genes underlying skeletal deformities in Pakistani families. Three skeletal disorder families (SD-1, SD-14 and SD-26), segregating as autosomal recessive, were identified from Charsada District. Skeletal disorder family (SD-1) has the phenotype of club foot, characterized by medial rotation, inclined inward and pointed downward. Skeletal disorder-14 (SD-14) and skeletal disorder-26 (SD-26) families were dwarf, and was characterized by proportionate short strature. Genomic DNA was extracted using standard phenol/Chloroform extraction method. Conventional linkage analysis was performed using microsatellie markers to refine the critical region and to identify the disease causing gene. *WNT7A* gene located on chromosome 3p25, *PITX1* gene located on chromosome 5q31 and *Gli 3* gene on chromosome 7p13 was excluded by genotyping tightly linked microsatellite markers in skeletal disorder-1 (SD-1) family.

Skeletal disorder family (SD-14), was linked to microsatellite markers D17S944 (90.91cM), D17S1290 (87.75cM) and D17S792 (87.75cM) in the *GH1* gene interval. The exonic sequences of the candidate gene (*GH1*) were analyzed by direct sequencing, but no pathogenic mutation was detected. So it was concluded that the

mutation may be present in the promoter region of the *GH1* gene. The third skeletal disorder family (SD-26), showed linkage to microsatellite markers D7S2564 (42.92 cM), D7S2252 (50.85 cM) and D7S817 (50.85 cM), tightly linked to *GHRHR* gene located on chromosome 7p14. So it is suggested that *GHRHR* gene will be sequenced for the purpose to find any pathogenic variant involve in the disease.

Chapter No. 1

Introduction

1.1: BACKGROUND

More than 6000 human genetic diseases are caused by a mutation in a single gene resulting in a disorder that can be inherited through generations. Furthermore, genetic predisposition plays a major role in a growing group of common human diseases. Understanding the complex series of events leading from a gene mutation (genotype) to disease symptoms (phenotype) is both a challenging and an important task, as it may lead to the identification of novel therapeutic strategies for genetic diseases (Ballabio, 2009).

Two major approaches have been used to map genetic variants that influence disease risk: linkage analysis and association studies. Association studies look for a particular marker to be correlated with disease (or trait values) across a population rather than within families. Association studies, although potentially more powerful than linkage, but it require samples sizes of hundred to thousands of individuals (Risch, 1990; Carlson, 2004). In linkage analysis, a genome-wide set of a few hundred or a few thousand markers spaced millions of bases apart is typed in families with multiple affected relatives. Markers that segregate with disease (or the trait) in relatives more often than expected are used to localize the disease genes (Hirschhorn, 2005). This approach has the advantage of being an unbiased, comprehensive search across the genome for susceptibility alleles, and has been successfully applied to find the genes for many single gene disorders (Altmuller *et al.*, 2001).

Autosomal recessive diseases is more prevalent in endogenous and isolated populations (Friedman and Griffith, 2003) and Pakistan represents a true treasure for molecular dissection of genetic skeletal disorder because 60% marriages are consanguineous and out of those approximately 80% are between first cousins (Hussain and Bittles, 1998) as compared to other European and Arab countries. In Pakistan, there is a strong cultural preference for consanguineous marriage and two normal and two affected informative individual in a family is enough to localize the disease gene.

Genetic disorders of the skeleton are a large and heterogeneous group of diseases whose unifying features are malformation, disproportionate growth, and deformation of the skeleton or of individual bones or a group of bones. Clinical severity differs between individual, ranging from minor handicap to death in the neonatal period (Superti-Furga *et al.*, 2001).

Genetic studies of diseases are providing invaluable insights into the roles not only of individual genes, but also of entire developmental pathways. Different mutations in the same gene may result in a range of abnormalities (Zelzer and Olsen, 2003). Traditionally, skeletal disorders have been subdivided into dysostoses, defined as malformations of individual bones or groups of bones, and osteochondrodysplasias, defined as developmental disorders of chondro-osseous tissue (Kornak and Mundlos, 2003).

The elucidation of most protein coding genes, and development of new tools for the assessment of genomic variation and regulation, have greatly facilitated our ability to identify specific genes and gene variants involved in diverse human traits (Day *et al.*, 2009) Therefore identification of carriers, increasing awareness about the possible effects of consanguineous families, offering the genetic counseling and prenatal diagnosis to the families can help to reduce the incidence of hereditary Skeletal disorder in our population. Molecular characterization using linkage analysis for identification of critical region and DNA sequencing for identification of pathogenic variant is very helpful for constructing DNA mutation Database of Genetic diseases of Pakistani origin. This mutation database will be very helpful for the future Molecular biologist to identify novel therapeutic strategies for inherited skeletal diseases.

1.2: SKELETOGENESIS

The human skeleton contains 206 bones which vary in size from the almost microscopic ossicles of the inner ear to femora which may exceed 450 mm in length. This great variation in size is accomplished by similar variation in shape which makes the identification of individual bones relatively straight forward.

The recent identification of the genetic basis of hereditary skeletal disorders is providing important insights into the intricate processes of skeletal formation, growth, and homeostasis. The skeleton contains three specific cell types: chondrocytes of various size and shape in cartilage and osteoblasts and osteoclasts in bones. Whereas chondrocytes and osteoblasts are of mesenchymal origin, osteoclasts belong to the monocyte-macrophage cell lineage (Karsenty, 2008). Skeletal development starts with the formation of a pattern, a process during which the number, size, and shape of the individual skeletal elements are delineated. The vertebrate skeleton is formed by mesenchymal cells condensing into tissue elements outlining the pattern of future

bones (the patterning phase). This is followed by differentiation to cartilage cells (chondrocytes) or bone cells (osteoblasts) within the condensations as shown in Figure 1.1. The cells of the mesenchymal condensations will then differentiate into chondrocytes that form the template, or anlagen, of the future bone. Later on, through a process called endochondral ossification, this cartilaginous template, will eventually be replaced by bone. In the other cases cells of the mesenchymal condensations will bypass the cartilaginous intermediary step and will differentiate directly into osteoblasts. This process, called intramembranous ossification, occurs in a few skeletal elements, such as the lateral halves of the clavicles and parts of the skull (Hall, 1988; Karsenty and Wagner, 2002). Mutations in transcription factors such as HOX and PAX and members of the transforming growth factor-3 super family cause disorders associated with abnormal mesenchymal condensation, whereas defects in the transcription factor SOX-9 lead to abnormalities in chondrocytes differentiation. Abnormal growth plate function, resulting in dwarfism, is the consequence of mutations in receptors for fibroblast growth factors and parathyroid hormone-related peptide (Mundlos and Olsen, 1997). Multiple nuclear proteins contribute to the regulation of osteoblast differentiation and function. Some of them act throughout the skeleton, others act only in a subset of skeletal elements, and members of a third category modulate the activity of classical transcription factors. Remarkably, many molecular determinants of osteoblast differentiation and function were identified through human genetic studies as well as through mouse genetics and molecular studies. Runt-related 2 *(Runx2)* is the master gene of osteoblasts differentiation (Zelzer and Olsen, 2003). Growth hormone secreted by the pituitary gland and IGF1 produced by proliferating and hypertrophic chondrocytes, among many other tissues, act largely independently to control the rate of chondrocyte proliferation. Disorders affecting this pathway result in proportionate dwarfism without much skeletal dysplasia, suggesting that they are major regulators of linear bone growth and, thus, of body size. The vast majority of other short-stature disorders, however, result in disproportionate dwarfism, presumably because they affect not only proliferation but also differentiation of chondrocytes (Kornak and Mundlos 2003).

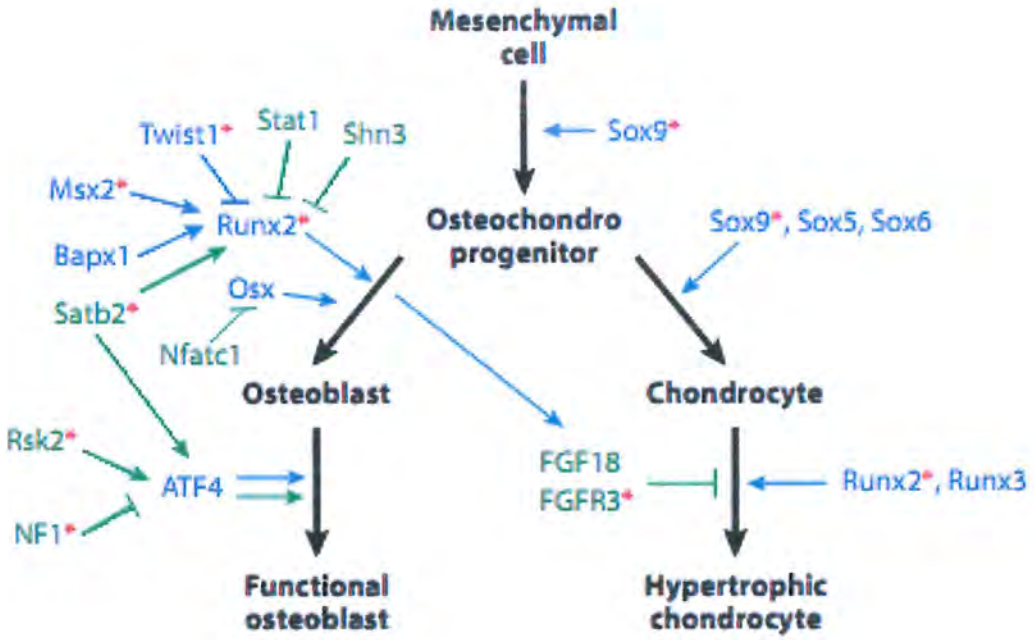


Figure 1.1: Schematic representation of the transcriptional control of cell differentiation along the chondrocyte and osteoblast lineages. Lines with arrowheads indicate a positive action and lines with bars indicate an inhibition. Regulation at the transcriptional level is shown in blue; regulation at the posttranscriptional level is shown in green. Red asterisks indicate genes whose mutation has been identified as disease-causing in humans (adapted from Karsenty, 2008).

1.3: DWARFISM

Short stature is a statistically defined height threshold. Some children with short stature are healthy, some have a medical condition known to be associated with short stature and in some, short stature is the result of an undiagnosed illness (Sandberg, 2000; Taback and Elliott, 2002).

Growth assessment requires accurate measurements of height and weight over time, the measurement of parental height, pubertal staging, and the selection of appropriate group reference standards (Tanner *et al.*, 1994; Goldbloom, 1997).

Dwarfism, was defined by Little People of America (LPA), is a condition with an adult height of 4 ft and 10 inches, or shorter as a result of a medical or genetic condition. However, Dwarf Athletic Association of America (DAAA) uses a criterion of 5 ft. or less. In general, dwarfs are at least three standard deviations below the mean height of the general population.

The definition of short stature has varied with time and place. On some growth charts, short stature is defined as a height of less than the fifth percentile for age. On others, it is defined as the lower limit of normal for height at the 3rd and even the 0.4th percentile for age (Freeman, 1995; Health Statistics, 2002).

1.3.1: Types of dwarfism

Over 300 genetic conditions involve problems with the growth and development of the skeleton that result in genetic conditions called short stature syndromes. Dysplasia' is a scientific word that means disordered growth and therefore skeletal dysplasia is the general term to describe conditions in which there is abnormal or disordered growth of the skeleton. About half of the known genetic conditions that involve the skeleton, are called skeletal dysplasias. Skeletal dysplasias are usually characterized by short stature that may be proportionate or disproportionate. In disproportionate short stature some parts of the body may grow at a normal rate while other parts have a reduced growth rate. Conditions in which there is a deficiency of growth hormone causing proportionate short stature are not classified as skeletal dysplasias. Since our concern here is with the proportionate cases of dwarfism that are due to the hormonal deficiency or it receptor will be discussed.

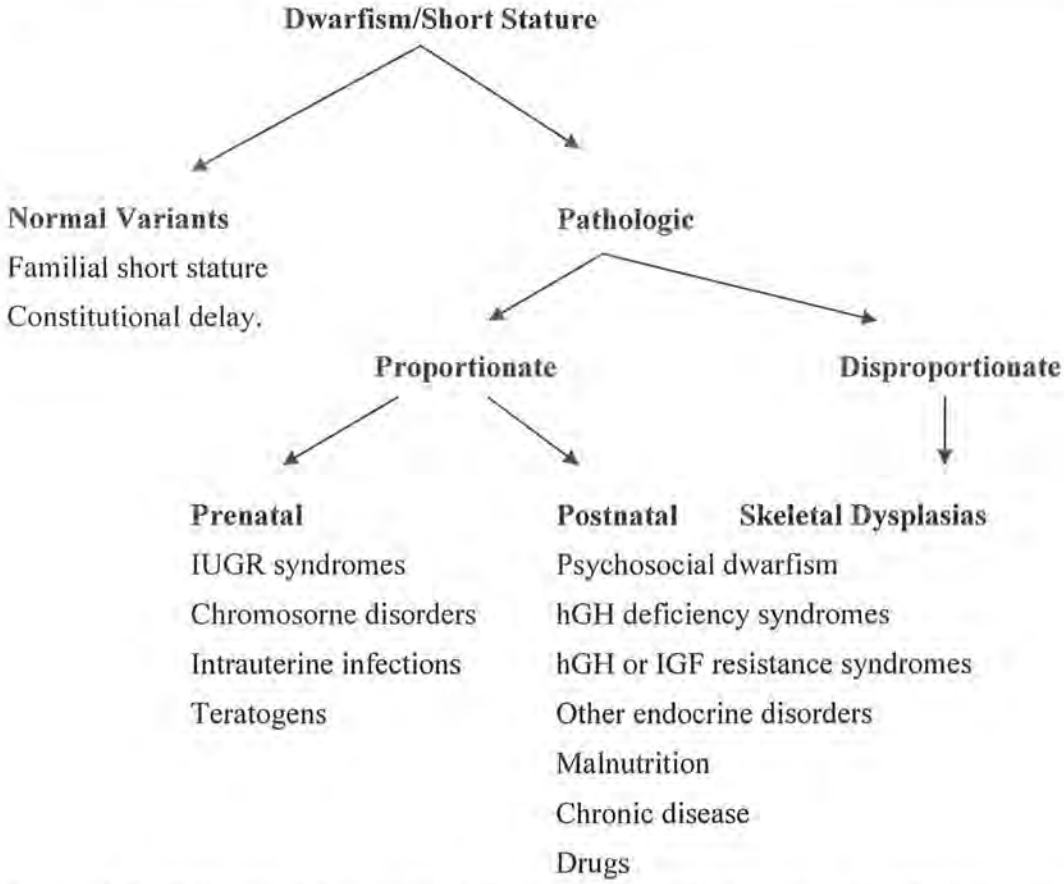


Figure 1.2: Differential diagnosis of short stature. hGH = human growth hormone, IGF= insulin like growth factor, IUGR = intrauterine growth retardation (adapted from Rimoin *et al.*, 1986)

1.3.2: Dwarfism due to growth hormone defeciency (GHD)

Human skeletal growth and final height attainment are a result of a multifactorial regulation involving systemic and local hormones, growth and nutritional factors, lifestyle and genetic factors. Heritability estimates (Chatterjee *et al.*, 1999) and genome-wide linkage analyses (Hirschhorn *et al.*, 2001) have shown that genetic factors play a major role in determining stature. Growth is an inherent property of life and is mainly the result of the action of growth hormone (GH). The human GH protein is composed of a 4-α helical bundle with two disulfide bridges. It contains two distinct sites that interact with the GH receptor (GHR). GH binding activates the GHR by causing receptor dimerization (Zhu *et al.*, 2001).

Growth hormone (GH) is essential for normal postnatal growth in humans as well as other mammalian species. Growth hormone (GH) is secreted by the somatotroph cells

of the anterior pituitary gland into the systemic circulation. Although its main function is to promote postnatal longitudinal growth, GH exerts numerous additional metabolic functions, such as (among others) regulation of lipid metabolism, bone apposition, skeletal and cardiac muscle mass growth, and arterial pressure (Salvatori, 2004). Secretion of GH from pituitary somatotroph is under the control of GH releasing hormone (GHRH) secreted from hypothalamus (Kamijo, 2004).

Dwarfism due to growth hormone (GH) insufficiency is an etiologically heterogenous group of disorders. Most are caused by deficient production of the hormone, while at least one type is caused by resistance to it (Adam *et al.*, 1981).

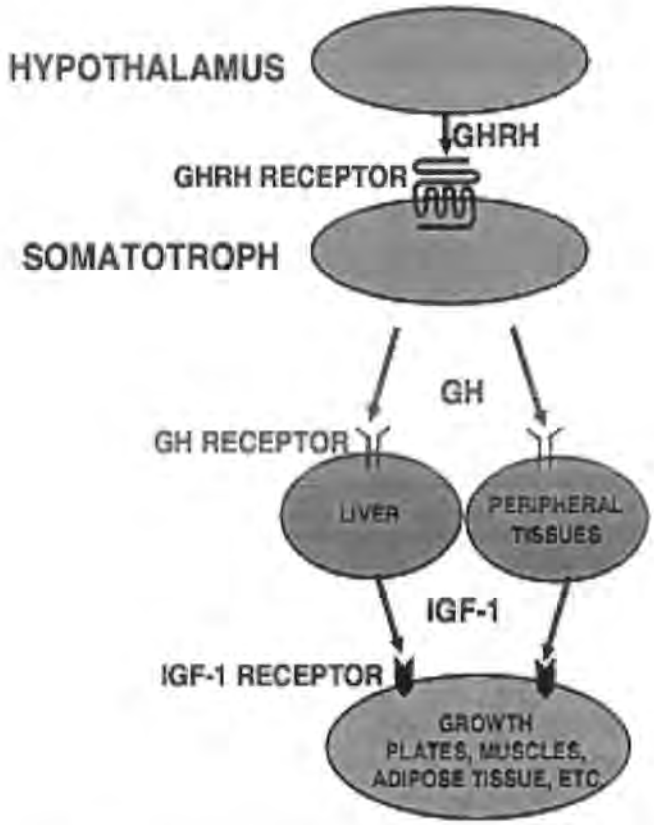


Figure 1.3: Schematic representation of the stimulatory control of GH secretion and action on peripheral organs. GH = growth hormone. GHRH = GH releasing hormone. IGF-1 = Insulin like growth factor 1. Both autosomal dominant and recessive as well as X-linked modes of inheritance have been observed in familial IGHD, suggesting that several different molecular mechanisms must account for GH deficiency (adapted from Salvatori, 2004)

1.3.3: Isolated GH deficiency (IGHD)

Hereditary Isolated Growth Hormone Deficiency (IGHD) is a heterogeneous disorder with different modes of inheritance. *GH1* gene is the main candidate gene for congenital IGHD in children. Familial IGHD due to *GH1* gene defects is associated with four hereditary forms. Two forms are inherited as autosomal recessive traits (IGHD IA, IGHD IB), one form is inherited as an autosomal dominant trait (IGHD II), and one form is inherited as an X-linked disorder (IGHD III) (Cogan *et al.*, 1993; Phillips and Cogan, 1994). Patients affected by IGHD IA lack detectable serum GH, and they often develop anti-GH antibodies after treatment with GH. Patients with type IB (the most frequent form) and type II have low, but detectable, serum GH levels and usually do not develop antibodies against exogenous GH. Type III patients have distinct clinical findings (sometimes associated with agammaglobulinemia) in different families (Cogan and Phillips, 1998; Salvatori *et al.*, 2001). The incidence of short stature due to isolated deficiency of growth hormone (IGHD) is estimated to be 1/3,480– 1/10,000 live births (Rimoin and Phillips, 1997; Salvatori *et al.*, 1999). The majority of cases with isolated GH deficiency (IGHD) are idiopathic. Monogenetic recessive inheritance of IGHD was shown to be caused by complete deletions of the *GH1* (IGHD IA) and, more recently, by nonsense mutations of the *GHRH* receptor gene (Binder *et al.*, 2001).

1.3.3.1: *GH1* gene

Growth hormone (GH) is synthesized by acidophilic or somatotropic cells of the anterior pituitary gland. Genetic causes of GH deficiency within the *GH1* gene have been established; however, they are rarely recognized and only sought in major GH deficiency states during childhood and in family studies (Mullis, 2005). The *GH1* gene (OMIM 139250) is located on chromosome 17q23, as part of a cluster of related genes, including two chorionic somatomammotropin genes (*CSH1* and *CSH2*), a chorionic somatomammotropin pseudogene (CSHP1), and a GH pseudogene (*GH2*) that is expressed exclusively in the placenta during the second half of pregnancy. There is a high degree of homology between these five genes, suggesting that they arose through a series of duplication events. The *GH1* gene consists of 5 exons and encodes for a 217- amino acid protein precursor of the mature GH. The nascent protein is processed by cleavage of a 26-amino acid leader sequence (Salvatori, 2004).

According to Human Gene Mutation Database (HGMD) 2010, total of 67 (86) mutation are reported in the *GH1* gene, including deletions, frame shifts, splice site, and nonsense mutations, which lead to decreased GH expression or action have been described (Rimoin and Phillips, 1997; Wagner *et al.*, 1998).

GH1 gene presents a complex map of single nucleotide polymorphisms (SNPs) in the entire promoter, coding and noncoding regions. Esteban *et al.* (2007) designed a systematic *GH1* gene analysis in a control population of 307 adults of both sexes with height normally distributed within normal range for the same population. Twenty-five SNPs presented a frequency over 1%: 11 in the promoter, three in the 5′UTR region, one in exon 1, three in intron 1, two in intron 2, two in exon 4 and three in intron 4.

Takahashi *et al.* (1996) reported the case of a boy with short stature and heterozygosity for a mutant *GH* gene (139250.0008). In this child, the GH not only could not activate the GH receptor (GHR; 600946) but also inhibited the action of wild type GH because of its greater affinity for GHR and GH-binding protein (GHBP), which is derived from the extracellular domain of the GHR. Thus, a dominant-negative effect was observed.

Millar *et al.* (2003) sought to identify subtle mutations in the *GH1* gene, which had been regarded as a comparatively rare cause of short stature, in 3 groups: 41 individuals selected for short stature, reduced height velocity, and bone age delay, 11 individuals with short stature and IGHD, and 154 controls. Heterozygous mutations were identified in all 3 groups but disproportionately in the individuals with short stature, both with and without IGHD.

Dennison *et al.* (2004) examined associations between common SNPs in the *GH1* gene and weight in infancy, adult bone mass and bone loss rates, and circulating GH profiles. The authors concluded that common diversity in the *GH1* region predisposes to osteoporosis via effects on the level of GH expression.

Millar *et al.* (2008) reported significant *GH1* gene variants among Europeans. GH variants occurs not only in individuals with idiopathic growth hormone (GH) deficiency, but also fairly frequently in the general population. They have screened 163 individuals from Benin, West Africa for mutations and polymorphisms in their GH1 genes. A total of 37 different sequence variants were identified in the *GH1* gene region, 24 of which occurred with a frequency of >1%. Although four of these

variants were novel missense substitutions (Ala13Val, Arg19His, Phe25Tyr and Ser95Arg), none of these had any measurable effect on either GH function or secretion in vitro.

1.3.3.2: The GH receptor (GHR)

The cDNA for the human GH receptor encodes a 638 amino acid protein with single extracellular, transmembrane and cytoplasmic domains. A single GH molecule binds to two molecules of the GH receptor Figure 1.4. GH binding to the GH receptor–GH receptor complex is thought to be sequential (Herrington and Carter-Su, 2001). The initial step is high-affinity binding of GH to one GH receptor. A different face of GH then contacts the second GH receptor, stabilizing the GH receptor dimmer (Wells *et al.*, 1996). GH binding to a dimer of GH receptors is thought to be an initial and crucial event in GH signaling. The biological effects of growth hormone (GH) are initiated by its binding to the GH receptor (GHR) followed by association and activation of the tyrosine kinase JAK2 (Billestrup *et al.*, 1995). Laron syndrome (OMIM 245590) (Laron *et al.*, 1966), in its most severe form is transmitted as an autosomal recessive trait resulting from mutations in the GH receptor gene (*GHR*) (Baumann, 2002). Molecular defects have been identified in all *GHR*-coding exons, except exon 3, a sequence that encodes part of the extracellular domain of the receptor. In humans, GHR transcripts exist in two isoforms differing by the retention (GHRfl) or exclusion (GHRd3) of this particular exon (Pantel *et al.*, 2003).

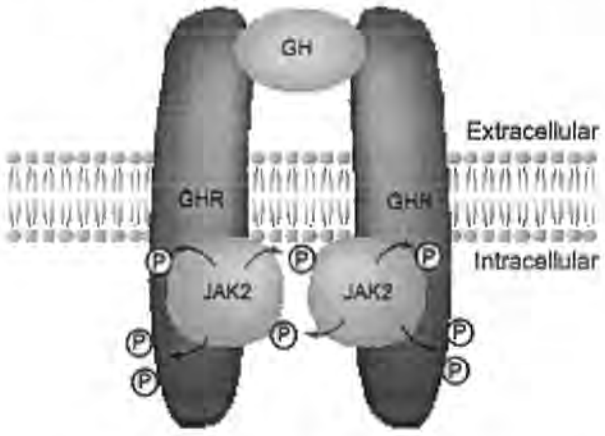


Figure1.4: Model of GH activation of JAK2 tyrosine kinase. GH binding to two GH receptors increases the affinity of each receptor for JAK2 (adapted from Herrington and Carter-Su, 2001).

1.3.3.3: *GHRHR* gene

Growth hormone-releasing hormone (GHRH; 139190), acting through the GHRH receptor (GHRHR; OMIM 139191), plays a pivotal role in the regulation of GH synthesis and secretion in the pituitary. The GHRHR is a member of a large family of heptahelical transmembrane receptors that couple to G proteins upon receptor activation (GPCR's). The GHRHR consists of a N-terminal extracellular domain, 7 hydrophobic transmembrane domains linked by 3 extra-and 3 intra-cellular loops, and a C-terminal intracellular domain. The native receptor contains 423 amino acids, but a post-translational cleavage removes the first 22 amino acids of the extracellular domain (DeAlmeida, 1998). The *GHRHR* gene is expressed primarily in the pituitary somatotroph and is essential for GHRH stimulated secretion of GH (Gaylinn, 1993).

In humans, the *GHRHR* gene is located on chromosome 7p14. The gene consists of 13 exons and 12 introns; in humans its coding region spans about 15.5 kb (Maheshwari and Banmann, 1997). The mature GHRHR is a 401-residue, single-chain protein with a large (108-amino acid) extracellular domain and a 42-amino acid cytoplasmic tail, one potential N-linked glycosylation site in the extracellular domain, and seven extracellular cysteines (the 22-amino acid signal peptide is not included in the numbering). The GHRHR is expressed primarily in the anterior pituitary, with lesser expression levels in many other tissues (Gonzalez-Crespo and Boronat, 1991; Mayo, 1996). The GHRHR signals principally through the G protein-cyclic adenosine monophosphate (cAMP) pathway, but also has effects on calcium channel fluxes. Activation of the cAMP pathway in the pituitary effects not only GH release, but also stimulates GH synthesis and somatotroph proliferation (Baumann, 1999). Mutations in the GHRHR are being recognized with increasing frequency in patients with familial IGHD. So far, one nonsense, five missense, two splice mutations, two small deletions (one of them with possible dominant negative effect), and a promoter mutation have been reported (Cogan and Phillips, 1998; Salvatori *et al.*, 2002). In most families, parents of affected subjects are either consanguineous or are connected by common ancestry even when they live in different geographical areas (Wajnrajch *et al.*, 1999).

Salvatori *et al.* (2002) analyzed peripheral genomic DNA of a family with two sibs affected by IGHD IB for mutations in the *GHRHR* gene. The *GHRHR* was analyzed

by direct sequencing of the 13 exons, intron–exon boundaries, and of the proximal 327 bp of the promoter region. Both subjects were compound heterozygotes for two previously undescribed mutations in the *GHRHR* that are predicted to cause complete lack of functional GHRHR protein: a nonsense mutation in codon 43 (Q43X), and a splice mutation at the beginning of intron 3 (IVS3 + 1G-A), and suggested that *GHRHR* mutations need to be considered in IGHD IB patients even in the absence of parental consanguinity.

In at least 2 members of a consanguineous family with profound growth hormone deficiency, Wajnrajch *et al.* (1996) demonstrated a nonsense mutation in the human *GHRHR* gene. The phenotype in these Indian muslims kindred was comparable to that in the 'little' mouse. The authors pointed out those other members of the G protein-coupled receptor superfamily are subject to mutations that can cause an increase in ligand-mediated signaling or constitutive receptor activation and resulted in hyperfunction of target cells.

1.4: TALIPES EQUINOVARUS (TEV)

Foot is complex skeleton, consisting of 26 to 28 bones. The foot can be divided into three anatomic regions: the hindfoot or rearfoot (talus and calcaneus); the midfoot (navicular Bone, cuboid bone, and three cuneiform bones); and the forefoot (metatarsals and phalanges) (Gore and Spencer, 2004).

Congenital talipes equinovarus (CTEV), often known as 'club-foot' is a common but little studied developmental disorder of the lower limb. It is defined as fixation of the foot in adduction, in supination and in varus, i.e. inclined inwards, axially rotated outwards and pointing downwards. Clubfoot is a complex, multifactorial deformity with genetic and intrauterine factors. One popular theory postulates that a clubfoot is a result of intrauterine maldevelopment of the talus that leads to adduction and plantarflexion of the foot (Rodgveller, 1984). Clubfoot occurs in one to two per 1,000 live births; however, the incidence is higher in Hispanics and lower in Asians (Rodgveller, 1992).

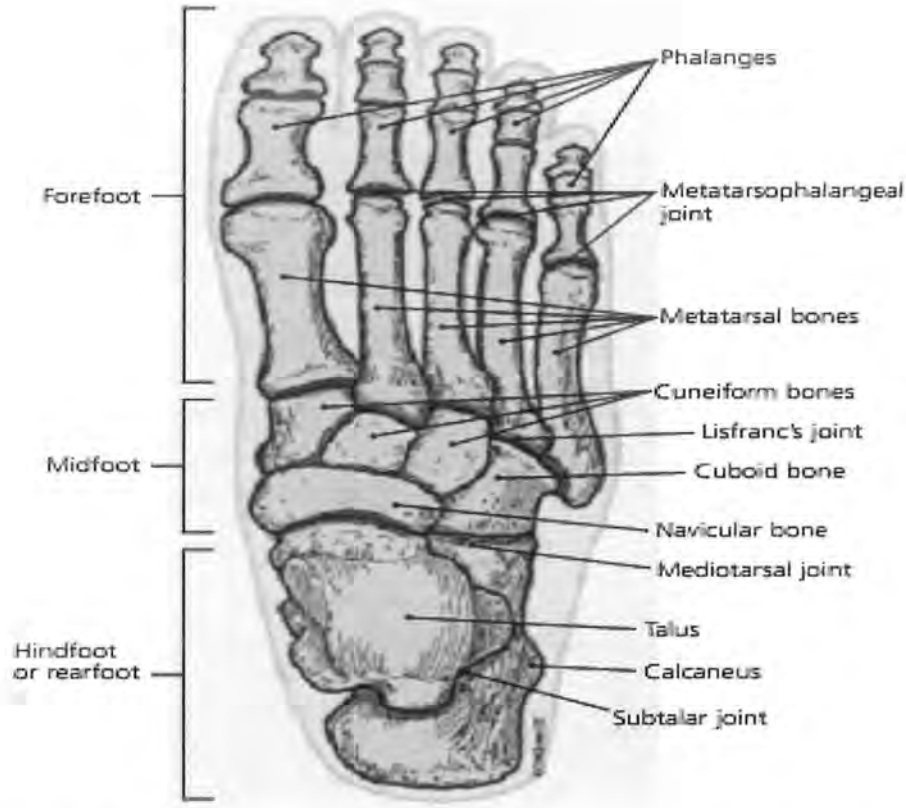


Figure 1.5: Bone structure and divisions of the adult foot (adapted from Gore and Spencer, 2004).

The foot appears smaller, with a flexible, softer heel because of the hypoplastic calcaneus. The medial border of the foot is concave with a deep medial skin furrow, and the lateral border is highly convex. The heel is usually small and is internally rotated, making the soles of the feet face each other in cases of bilateral deformities (Gore and Spencer, 2004). Most cases of ITEV are bilateral; in unilateral cases, the right side is affected more often than the left (Moorthi *et al.*, 2005).

Figure 1.6: Bilateral talipes equinovarus (adapted from Miedzybrodzka, 2003).

The average birth prevalence of ITEV is 1 per 1000 live births (Jones, 1997; Gurnett, *et al.*, 2008). However, the birth prevalence of ITEV varies among different populations, ranging from 0.39-7 per 1000 live births, with the highest rate in the Hawaiian and Maori populations (Beals, 1978; Cartlidge, 1984; Bonafé *et al.*, 2002).

Table 1.1: Birth prevalence of idiopathic congenital Talipes equinovarus in different populations (Miedzybrodzka, 2003).

Population	**ICTEV birth prevalence**
UK (Wynne-Davis, 1964)	1.2:1000
Scotland (Barker and MacNicol, 2001)	2:1000
Maori (Brougham, 1988)	6-7: 1000
Hawaii (Chapman *et al.*, 2000)	6-7: 1000
Tonga (Chapman *et al.*, 2000)	6-7:1000
China	0.3:1000

1.5: MOLECULAR GENETICS OF TALIPES EQUINOVARUS (TEV)

Genetic factors make an important contribution to the aetiology of congenital Talipes equinovarus (CTEV), the most common developmental disorder of the lower limb. Epidemiological studies implicate multifactorial inheritance. Pedigree analyses have suggested a major role of a single gene, with both variably penetrant autosomal dominant and recessive patterns (Wang, 1988; Liu *et al.*, 2008). Multiple cases of ITEV may be seen in a family, with an inheritance pattern more complex than a simple Mendelian disorder. Twin studies have showed that the concordance for ITEV is higher for mono- zygotic twins at 32.5%, compared with 2.9% in dizygotic twins. This finding points to genetic factors being more important than environment, because twins share the same intrauterine environment (Barker *et al.*, 2003).

Over all, little is known about the pathogenesis of human ICTEV. Many candidate genes for this disorder have been proposed because the molecular and cellular components of vertebrate limb bud development are well known (Cao *et al.*, 2009). Second, genetic mutations causing congenital limb malformation (CLM) have been identified in model organisms such as the mouse, and the orthologous gene in humans has been screened for mutations in patients with a similar phenotype (Lettice *et al.*, 2003).

Previous investigations into the genetics of human congenital limb malformation (CLM) have taken two approaches. First, positional candidate methods have been used to identify mutated genes in affected families following linkage analysis, or in individuals harbouring chromosome abnormalities (Vortkam *et al.*, 1991; Muragaki *et al.*, 1996; Furniss *et al.*, 2009).

The interaction of multiple genes and environmental factors complicates the detection of genetic variation underlying complex disorders. Some cases may be caused by a major gene with minor effects from other genes and the environment, or may result from an environmental effect with little genetic contribution (Ester *et al.*, 2007). Because of these different possible etiologies, identification of the ITEV genes has been challenging. Chapman *et al.* (2000) conducted a study on 287 New Zealand Maori and Pacific club foot families. The data were analyzed by complex segregation analysis and predicted that the best genetic model for club foot in this population is a

single dominant gene with a penetrance of 33% and a predicted gene frequency of 0.9%.

A recent report suggested homozygosity for the R279W mutation in the sulphate transporter gene (*DTDST*) (*SLC26A2*) as the aetiology of ITEV in two sibships (Huber *et al.*, 2001). As part of ITEV linkage study, Bonafe and coworkers, (2002) have tested for linkage and association to the *DTDST* gene and the R279W mutation. Bonafé and coworkers, 2002 genotyped one hundred and twenty-five ITEV probands and their Parents for D5S1507 and 155 for D5S1469. Linkage results for D5S1469 showed a slightly significant p value. None of the known pathogenic mutations were found in the DNA from 10 ITEV probands. Sequencing of the whole coding region excluded the presence of any new, previously unknown mutations.

Recently, a deletion in the chromosomal region 2q31-33 was found to be associated with clubfoot. Microsatellite markers spanning the region were genotyped by Heck *et al.* (2005) in 57 multiplex ITEV families and 83 simplex trios. Family-based analysis revealed that two microsatellite markers, GATA149B10 and D2S1371, were associated with ITEV in the simplex trios. The 6cM region between the two markers contained the candidate genes *CASP8, CASP10,* and *CFLAR.* These genes encode proteins that are regulators of apoptosis, which is important during growth and development. Genotyping of SNPs throughout the genes in this sample of ITEV families has revealed positive linkage with association to the major allele of a variant in *CASP10* in simplex ITEV white and Hispanic trios and concluded that this region strongly implicate in the pathogenesis of ITEV.

For our study we investigated previously several chromosomal deletion regions identified in patients with talipes equinovarus as these regions may harbor genes contributing to idiopathic Talipes equinovarus (Brewer *et al.*, 1998; Ester *et al.*, 2007), and chose candidate genes in which mutations causing human ITEV had been described previously: *PITX1, GLI3* and *WNT7A.*

1.5.1: *WNT7A* gene

The *Wnt* gene family consists of structurally related genes encoding secreted signaling molecules that have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis. *WNT7A* gene (OMIM 601570) is located on chromosome 3p25, up to date total 2 (3)

mutation are reported in the human gene mutation database (HGMD), 2010. *Wnt* genes encode a family of highly conserved cysteine rich glycoproteins that play an important role in the normal developmental processes during embryogenesis (McMahon *et al.*, 1992; Peifer and Polakis, 2000). *WNT7A* is known to be involved in limb development (Woods *et al.*, 2006). Thus it is an excellent candidate gene for CTEV.

In 2005, Dietz and co-workers reported a genome wide scan for linkage in a four generation CTEV family, comprising 13 individuals with clubfoot and 41 unaffected members (Dietz *et al.*, 2005). The highest LOD score of 2.18 was obtained for markers on chromosome 3, close to the *WNT7A* gene.

In 2008, Liu and co workers investigated the role of *WNT7A* using a family-based linkage approach in a large series of European multi-case CTEV families. Ninety-one CTEV families, comprising 476 individuals of whom 211 were affected, were genotyped. But no significant evidence for linkage was observed using either parametric or non-parametric models, suggesting that the *WNT7A* gene is unlikely to be a major contributor to the aetiology of familial CTEV (Liu *et al.*, 2008).

Woods *et al.* (2006) found homozygous missense mutations in the dorso ventral patterning gene *WNT7A* and confirmed their functional significance in retroviral-mediated transfection of chicken mesenchyme cell cultures and developing limbs. They suggest that a partial loss of *WNT7A* function causes Fuhrmann syndrome (a phenotype similar to mouse *Wnt7a* knockout), illustrating the specific and conserved importance of *WNT7A* in multiple aspects of vertebrate limb development.

1.5.2: *PITX1* gene

Limb patterning and growth are regulated through a complex network of transcription factor and expression signaling-molecule (Tickle, 2003; Zelzer and Olsen, 2003). Two transcription-factor genes, *Pitx1* (OMIM 602149) and *Tbx4* (OMIM 601719), are expressed predominantly in the developing hind limb. Gurnett and her colleagues analyzed the DNA of 35 extended family members of an infant male patient severely affected in the family, had clubfoot in both feet. Through the genome-wide study, Gurnett and her colleagues found a region on chromosome 5 that was common to all family members affected. From there, they identified a single missense nucleotide change c.388G/A that results in the substitution of lysine for glutamic acid (E130K) in

a gene critical for early development of lower limbs called *PITX1*. The *PITX1*, E130K mutation was found in all affected family members and in three carriers who showed no clinical symptoms (Gurnett *et al.*, 2008).

1.5.3: *Gli 3* gene

The *Gli3* gene (OMIM 165240), Gene map locus: 7p13 encodes a member of the zinc finger gene family related to Kruppel, a gene that is known to regulate development in Drosophila (Wild *et al.*, 1997). The *Gli3* gene is expressed as an 8.5-kb mRNA in tissues such as testis, myometrium, placenta, and lung, and the protein product (relative molecular mass, 190,000) shows sequence-specific DNA binding (Ruppert *et al.*, 1988; Ruppert *et al.*, 1990). The gene encodes a predicted 1,596-amino acid polypeptide. The mammalian *Gli* gene family encodes zinc finger transcription factors and plays a role in developmental regulation and human diseases (Li *et al.*, 2004). One member of this family, *Gli3,* was identified as a candidate gene for ICTEV. The changes in *Gli3* gene expression may be caused by changes in some transcription factors that regulate the *Gli3* gene, ultimately leading to the development of ICTEV (Cao *et al.*, 2009). Wild *et al.* (1997) stated that the *Gli3* gene contains 15 exons spanning 240 kb. The zinc finger domain is contained within 4 exons (10 through 13). Exon 1 is untranslated.

1.6: OBJECTIVES OF THE PRESENT STUDY

The present study was conducted to detect pathogenic variants in genes underlying inherited skeletal malformation in Pakistani families. Disease families were identified and linkage analysis was performed to known genes using microsatellites markers. The candidate gene responsible for the disease was identified and then sequenced for the detection of possible pathogenic variations.

Chapter No. 2

Materials and Methods

2.1: ENROLMENT OF FAMILIES

Families were selected according to the information provided. Families with three or more affected individuals with the disease in two or more loops were preferred for the study. If a family had other relatives affected with the disease were also included in the study depending on their willingness and availability. Informed consent was obtained from the participant in the study. Detailed history was taken from each family to minimize the presence of other abnormalities and environmental causes. Pedigree of the enrolled families were drawn using excel work sheet.

2.2: PEDIGREE DRAWING

A pedigree is a chart that depicts family relationships and pattern of inheritance for particular traits. It is the most important step while studying genetic disorders. For researchers, families are tools, and the bigger the family the better- the more children in a generation, the easier it is to discern mode of inheritance. For genetic inferences an extensive pedigree was constructed for each family by the standard methods described by Bennett *et al.* (1995). A pedigree consists of shapes connected by lines. Vertical lines represent generations; horizontal lines that connect two shapes at their center depict parents; shapes connected by vertical lines joined horizontally represent sibling. Squares indicate males; circles, females, and diamonds, individuals of unspecified sex. Roman numerals designate generation while the individual in a generation were designated by Arabic numerals or by names. Double lines in the pedigree represent the consanguineous marriages. Colored or shaded shapes indicate individuals who express the trait under study, unshaded shapes represent normal individuals and half filled shapes represent known carriers (Lewis, 2005).

2.3: COLLECTION OF BLOOD

Families were first briefed about the research program in simple words. A detailed pedigree was drawn by asking relevant questions about individual relation, medical history or any other important information about the disease. Informed written consent was taken from all adults. For children consent was taken from their parents. Pedigree number and complete name was written on consent form before obtaining the signature and or thumbprint. Same pedigree number and name was written on the side and lid of 50 ml tube containing EDTA as anticoagulant. Five to ten milliliter blood was drawn by an experience technician into a 50 ml tube containing 400 µl

(0.5M) EDTA. Tube was inverted many times for complete mixing of anticoagulant to prevent blood clotting.

2.4: STORAGE OF BLOOD SAMPLES

Blood samples were stored in an ice box during sampling. After reaching the laboratory a list of all samples was prepared. This includes pedigree number, individuals name and the amount of blood collected. The samples were then stored at -20°C or -70°C depending when the DNA extraction was planned.

2.5: GENOMIC DNA EXTRACTION

Genomic DNA was extracted by using the standard protocol. 750 µl of blood was taken in the eppendorf tube, 750 µl of solution A was added to it. Mixed it by inverting the tube several time and then keep at room temperature for 5-10 minutes. Centrifuged it at 13,000 rpm for 1 min. The supernatant was discarded and the nuclear pellet was re-suspended in 400 µl of solution A. Again centrifuged at 13,000 rpm for 1 min, discarded the supernatant and re-suspend the nuclear pellet in 400 µl of solution B (Appendix 1), 12 µl of 20% SDS and 5 µl of Protenase K. Incubate at 65°C for three hours or 37°C overnight.

Then 500 µl of a fresh mixture of equal volume of solution C (Phenol) and solution D (chloroform: isoamylalcohol) were mixed and centrifuged for 10 min at 13,000 rpm. Aqueous phase (upper phase) was collected in a new tube. 500 µl of solution D was added and centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube. Precipitated the DNA by adding 55 µl of sodium acetate (3M, pH 6) and equal volume (500 µl) of isopropanol or 2 volume of ethanol and the tube was invert several times to precipitate the DNA.

The tube was centrifuged for 10 min at 13,000 rpm, and the supernatant was discarded. The pellet were washed with 70% chilled 200 µl ethanol and centrifuged at 7000 rpm for 7 min, and the ethanol was discarded.

The DNA pellet was dried for half an hour by keeping the tube at 37°C for 30 min or dried it by using concentrator for 10 min at 45°C. The precipitated, DNA was dissolved in 200 µl of Tris EDTA buffer. Quality of DNA was checked by running 1-2 µl DNA on 1.0 % Agarose gel in 1X TBE buffer with comparison to a known standard DNA dilution run on the same gel.

2.6: Composition of solutions

Solution A

0.32 M sucrose

5 mM $Mgcl_2$

1% (v/v) Triton X-100

Solution B

10 mM Tris (pH 7.5)

400 mM NaCl

2 mM EDTA (pH 8.0)

Solution C

400 µl Phenol

10 mM Tris

Solution D

Chloroform 24 volumes

Isoamyl alcohol 1 volume

DNA Dissloving Buffer

10 mM Tris (pH 8.0)

1 mM EDTA

2.7: PCR AMPLIFICATION OF MICROSATELLITE MARKERS

For haplotype analysis microsatellite markers were used. For PCR amplification of microsatellite 25µl reaction volume was used. PCR reaction were performed with 50ng of template DNA in 25µl reaction containing 2.5 µl PCR buffer, 1.5 µl $MgCl_2$, 0.5 µl dNTPs, 0.3 µl each of the forward and reverse primer, 0.2U of Taq polymerase and the remaining of deionised water. The template DNA in the mixture was denatured at 95°C and amplified using Biometra and Corbett thermal cyclers at annealing temperature of 57°C and extension at 72°C. The entire markers were dinucleotide repeats and were chosen from the Marshfield human genetic Maps/UCSC genome browser.

2.8: POLYACRYLAMIDE GEL ELECTROPHORESIS

The amplified PCR products were resolved on 8% non denaturing polyacrylamide gel (13.5 ml (29 g acrylamide, 1 g N, N Methylene-bis-acrylamide), 5 ml of 10X TBE, 350 µl of 10 % APS, 20 µl of TEMED and 31.13 ml Distilled Water). The gel was prepared in a flask and poured into the gel plate. Combs were inserted and the gel was allowed to polymerize at room temperature. PCR amplified product were mixed with loading dye (0.25% bromophenol blue and 40% sucrose) and loaded into the wells. Electrophoresis was performed (using a gel tank of Whatman Biometra Germany) at 110 volts for 2-3 hours depending on the product size. The gel was stained in ethedium bromide solution (100 mg/ml) and visualized on UV transilluminator. Photograph was taken by using digital camera DC 290, Kodak USA.

2.9: HAPLOTYPE ANALYSIS

A haplotype represents an individual's chromosome segment, i.e. a set of genotyped alleles arranged according to the cM distance along a chromosome. Alleles were arranged in a way that confirms the inheritance pattern of segregating disease. If three polymorphic fully informative markers located in the linkage interval of a locus did not show homozygosity among the affected of a family, the locus was considered as unlinked. Linkage to a particular locus was confirmed when homozygous data of affected members correlate with the disease pattern in the family tree.

2.10: PCR AMPLIFICATION OF THE CANDIDATE GENE

For the amplification of the candidate gene primer were designed for each exon in the intronic sequence of the gene. Short and very close exons in the genomic DNA sequence was amplified by a single primer set. The primer were designed either manually or using the online version of Primer3. The Maximum length of the primer was kept 20 bases with a GC content of 50-60%. Each exon was amplified using two primers; a forward primer and a reverse primer in the 5 to 3 direction. For each amplification, 50 µl reaction mix (5 µl PCR buffer, 4 µl $MgCl_2$, 1 µl dNTPs, 2.5 µl (each) Primers, 1 µl DNA, 0 .6 µl Taq polymerase and 34.4 µl PCR H_2O) were used. PCR amplification was performed at annealing temperatures calculated from the GC content of the primers. The PCR reaction was programmed as; initial 5 minutes denaturation of template DNA at 95°C, followed by 35 cycles of 95°C for 1 min, 57°C for 1min, and 72°C for 1-2 minutes and final extension at 72°C for 10 min.

2.11: PURIFICATION OF PCR AMPLIFIED PRODUCT

The amplified PCR products were purified using PureLink™ PCR Purification Kit of Invitrogen. Amplified exon from 1st PCR and 200 µl of H1 binding solution were added to Label eppendorf tube, Vortex and short spin. Pour the solution from eppendorf tube into label column. Centrifugation was performed at 13000 rpm for 1 minute. The through flow was discarded from collection tubes and again placed column in it.

Then 500 µl of (washing buffer) H2-binding solution were added to removes dNTPs and protein from the sample. Centrifuge for 1 minute at 13000 rpm. The flow through were discarded from collection tube. Columns were placed in newly labeled eppendorf tube and 25 -30 µl Tris-EDTA (TE) (65°C) was poured. It was kept for 5 minutes at room temperature and then centrifuge at 13000 rpm for 5 minutes and then checked at 2% agarose gel.

2.12: SEQUENCING PCR

The purified product was amplified using 10 µl cycle sequencing reaction using Big Dye Terminator Cycle Sequencing Kit v 3.1 (1 µl Buffer, 3 µl DTCS, 1 µl Forward/Reverse primer, 2 µl Purified template and 4 µl PCR H_2O) and were purified for BACKMAN sequence analyzer.

Table 2.1: Primers and PCR conditions required for the amplification of the GH1 gene

Exon	Primers	Sequence	Product size	Annealing temperature
1+2	GH_1 × 1-2F	CACAAGAGACCAGCTCAAGG	652bp	57C°
	GH_1 × 1-2R	ACTCAGCGTGTGCTCATCTG		
3+4	GH_1 × 3-4F	TGCAGGCAGATGAGCACACG	588bp	59C°
	GH_1 × 3-4R	TTCTCTTGGGTCAGGGCCTG		
5	GH_1 × 5F	CTCCAGGCCTTTCTCTACAC	542bp	57C°
	GH_1 × 5R	ACTGCACTCCAGCTTGGTTC		

2.13: PURIFICATION OF SEQUENCING PRODUCT

Fresh stock solution of 1.0 µl of 3M Na-acetate (pH 5.2), 1.0 µl of 100mM Na-EDTA (pH 8.0) and 0.5 µl of 20 mg/ml glycogen (PureLink™ PCR Purification Kit of Invitrogen) was prepared and short spin. Eppendorf tubes were labeled for respective

exon. The stock solution and amplified product were mixed. 70 µl of 100% chilled ethanol were added. Centrifugation was performed at 13000 rpm for 20 minutes. Supernatant were removed with pipette carefully. 150 µl of 70% chilled ethanol were added, and centrifuged at 13000 rpm for 15 min. Supernatant were removed, dry the pellet in the concentrator for 10 min. Re-suspend the pellet in 30 µl of SLS (sample loading solution), short spin at 8000 rpm for 30 second. One drop of mineral oil was added for sequencing through BECKMAN sequencer.

2.14: SEQUENCE ALIGNMENT

For identification of mutation, control gene sequence was obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) database and was aligned with the gene sequence of affected and normal individual using BioEdit Sequence Alignment Editer Version 6.0.7.

Table 2.2: Microsatellite markers used for genotyping of candidate genes for Dwarfism and Talipes equinovarus.

Disease	Gene	Locus	cM	Markers
Dwarfism	GH1	17q24.2	87.75	D17S792
			87.75	D17S1290
			90.91	D17S944
			95.46	D17S1821
			97.52	D17S2193
			97.67	D17S789
			101.06	D17S2059
			104.45	D17S1351
Dwarfism	GHRHR	7p14	42.92	D7S2556
			42.92	D7S2440
			42.92	D7S2564
			48.65	D7S632
			49.27	D7S526
			50.85	D7S2252
			50.85	D7S817
			53.72	D7S2250
Talipes equinovarus	PITX1	5q31.1	129.92	D5S2098
			135.40	D5S642
			136.27	D5S2110
			136.27	D5S2057
			137.30	D5S2002
			138.54	D5S2117
			139.55	D5S2056
			141.18	D5S816
Talipes equinovarus	WNT7A	3p25	28.75	D3S3611
			31.81	D3S2403
			32.54	D3S651
			33.11	D3S1516
			33.42	D3S1110
			33.73	D3S3608
			33.73	D3S2385
			33.73	D3S3602
			33.73	D3S1252
			34.27	D3S1554
			36.91	D3S2431
			38.17	D3S2338
			43.77	D3S3038
Talipes equinovarus	GLI3	7p13	60.90	D7S2541
			64.26	D7S2428
			71.09	D7S1818

Chapter No. 3

Results

Over 300 genetic conditions involve problems with the growth and development of the skeleton that result in genetic conditions called short stature syndromes. So, on the basis of genetic linkage study some candidate gene intervals were tested for either linkage or exclusion prior to sequencing for pathogenic mutations. Three families, with skeletal disorders, segregating as autosomal recessive were enrolled from Charsada District of KPK, Pakistan. Family member were interviewed to rule out environmental causes of the disease. One of these families SD-1 has club foot and two, SD-14 and SD-26 was dwarf. The DNA samples of these families were screened for linkage to known loci using informative STS markers within the candidate linkage interval. The gene responsible for the disease in SD-14 was then sequenced to find any pathogenic mutation.

3.1: FAMILY SD-1

3.1.1: ENROLMENT

This family was enrolled from Charsadda. It is a four generations consanguineous family with multiple loops having seven affected individuals including five female and two male in 4th generation of the pedigree loops indicating as an autosomal recessive segregation as shown in the Figure 3.1. The age of the affected individuals ranges from seven months to 25 years. Blood was taken only from four family members, two affected sisters (IV-9 and IV-11) (aging three and seven years respectively) and their parents (III-7 and III-8). The phenotype of the disease in the family was club foot, characterized by inclined inwards, axially rotated outwards and pointing downwards. It was found that the disease is segregating as non syndromic.

3.1.2: HAPLOTYPE ANALYSIS

Genetic disorders of the skeleton are large and involve many candidate genes that produce a significant number of affected individuals. Therefore, we have genotyped known genes involve in *Talipes equinovarus* (club foot) in the family SD-1. These includes *WNT7A* (OMIM 601570), *PITX1* (OMIM 602149), and *Gli3* (OMIM 165240) gene locus.

WNT7A: located on chromosome 3p25. Eight microsatellite markers D3S3611 (28.75cM), D3S2403 (31.81cM), D3S651 (32.54cM), D3S1516 (33.11cM), D3S1110 (33.42cM), D3S3608 (33.73cM), D3S2385 (33.73cM), D3S3602 (33.73cM),

D3S1252 (33.73cM), D3S1554 (34.27cM), D3S2431 (36.91cM), D3S2338 (38.17cM) and D3S3038 (43.77cM) were used, of which (D3S2385) is intragenic. The marker D3S2403 is 700 kb 5' to the start of the *WNT7A* gene while D3S1252 located 20 kb from the 3' end of the gene. The only marker D3S1252 linked the disease to the *WNT7A* gene locus.

PITX1: *PITX1* is located on chromosome 5q31. DNA of two normal (parents) and two affected (children) individuals were analyzed using polymorphic microsatellite markers D5S816 (141.18cM), D5S2056 (139.55cM), D5S2117 (138.54cM), D5S2002 (137.30cM), D5S2057 (136.27cM), D5S2110 (136.27cM), D5S642 (135.40cM) and D5S2098 (129.92cM) flanking the region.

Gli 3 gene is located on chromosome 7p13. Three microsatellite markers D7S2541 (60.90cM), D7S2428 (64.26cM) and D7S1818 (71.09cM) were used to find out significant linkage of the loci, but none of the three loci as shown in Figure 3.1, 3.2, and 3.3 showed linkage to the markers used. Therefore, these loci were excluded from further processing for mutation detection in this family.

3.2: FAMILY SD-14

3.2.1: ENROLMENT

The family SD-14 is four generation highly inbred family containing seven affected members in three different loops as shown in Figure 3.4. Among these three were female and four were male patient. Five of the affected patients were in the 3rd generation and two were in the 4th generation of the pedigree. Growth is an inherent property of life and is mainly the result of the action of growth hormone (GH). The family SD-14 has proportionate short stature in which all the body parts are short. The affected individuals did not have any other problem.

3.2.2: HAPLOTYPE ANALYSIS

After screening, the family showed linkage to the markers flanking the growth hormone (*GH 1*) gene. To define the boundaries of critical region at 17q24.2, a set of eight STS markers, D17S1351 (104.45cM), D17S2059 (101.06cM), D17S789 (97.67cM), D17S2193 (97.52cM), D17S1821 (95.46cM), D17S944 (90.91cM), D17S1290 (87.75cM) and D17S792 (87.75cM) were typed in the family. From the haplotype analysis it was found that all the affected individuals were homozygous and

both parents and the unaffected offspring were heterozygous for haplotype of markers D17S944 (90.91cM), D17S1290 (87.75cM) and D17S792 (87.75cM) as shown in Fig: 3.5, 3.6 and 3.7. The haplotype analysis showed a linkage interval of 16.70cM, delineated by markers, D17S1351 (104.45cM), which lies above the gene and D17S792 (87.75cM), locating below the gene. The linked markers D17S1290 (87.75cM) and D17S792 (87.75cM) were at a distance of 3.73cM and 6.89cM each from the gene location.

3.2.3: SEQUENCING THE CANDIDATE GENE

The gene encoding the disease has been fully characterized. It is located at the growth hormone locus on chromosome 17q24.2 and extends approximately 1636 bp DNA. It contains 5 exons separated by 4 introns and produces an mRNA transcript of 537 bp that encodes a precursor protein of 191 amino acids. The gene was sequenced using a set of primers designed manually. The DNA sequence was compared with the normal sequence taken from human gene mutation database, but we could not find any mutation in the DNA sequence of affected offspring. So it was concluded that the mutation may be present in the promoter region of the gene.

3.3: FAMILY SD-26

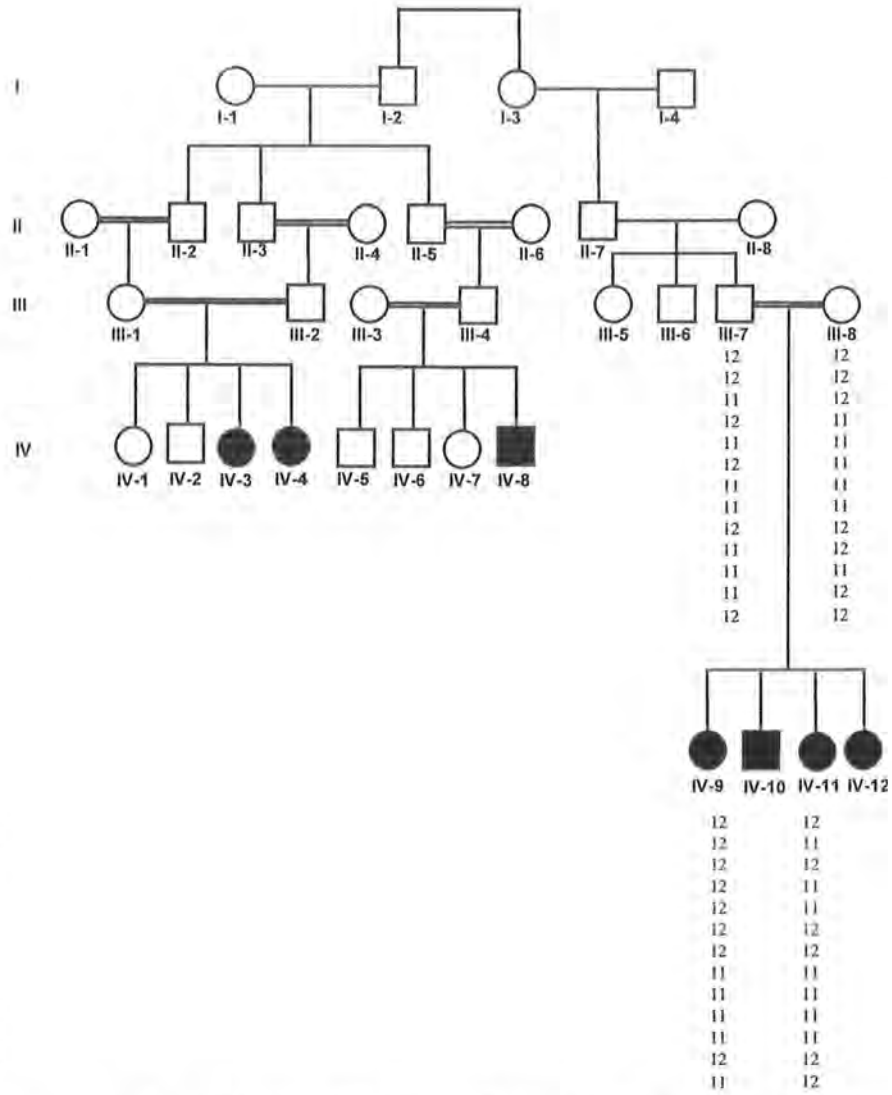
3.3.1: ENROLMENT

The family SD-26 was enrolled from Mardan District. It is much extended four generation family, consists of 52 members. The total numbers of affected individual is 12, of which seven are affected male (III-6, III-10, III-16, III-19, IV-5, IV-6 and IV-7) and five are affected female (III-15, III-18, III-20, IV-1 and IV-19). The affected female III-18 is from another family as shown in Figure: 3.8. The phenotype of affected represents proportionate dwarfism. No other abnormalities were associated with the disease.

3.3.2: HAPLOTYPE ANALYSIS

Based on the phenotypes, genotype mapping was performed for *GHRHR* locus using eight microsatellite STS markers, (D7S2556 (42.92 cM0, D7S2440 (42.92 cM), D7S2564 (42.92 cM), D7S632 (48.65cM) D7S526 (49.27 cM), D7S2252 (50.85 cM), D7S817 (50.85 cM) and D7S2250 (53.72 cM). The *GHRHR* locus is located on chromosome 7p14 (chr7: 31,003,636-31,019,141) on 50.51cM region. Homozygosity

mapping showed that the three markers D7S2564 (42.92 cM), D7S2252 (50.85 cM), D7S817 (50.85 cM) linked the locus that are lye 7.59 cM upstream of the gene locus and 0.34 each downstream of the locus respectively. Construction of parental haplotypes, illustrated in Figure 3.8, indicate that D7S2564, D7S2252 and D7S817 were homozygous for the 'disease' alleles in all affected siblings.



Markers	cM
D3S3611	28.75cM
D3S2403	31.81cM
D3S651	32.54cM
D3S1516	33.11cM
D3S1110	33.42cM
D3S3608	33.73cM
D3S2385	33.73cM
D3S3602	33.73cM
D3S1252	33.73cM
D3S1554	34.27cM
D3S2431	36.91cM
D3S2338	38.17cM
D3S3038	43.77cM

III-7	III-8
12	12
12	12
11	12
12	11
11	11
12	11
11	11
11	11
12	12
11	12
11	11
11	12
12	12

Markers	cM	IV-9	IV-10	IV-11	IV-12
D3S3611	28.75cM	12		12	
D3S2403	31.81cM	12		11	
D3S651	32.54cM	12		12	
D3S1516	33.11cM	12		11	
D3S1110	33.42cM	12		11	
D3S3608	33.73cM	12		12	
D3S2385	33.73cM	12		12	
D3S3602	33.73cM	11		11	
D3S1252	33.73cM	11		11	
D3S1554	34.27cM	11		11	
D3S2431	36.91cM	11		11	
D3S2338	38.17cM	12		12	
D3S3038	43.77cM	11		12	

Figure 3.1: Pedigree drawing of family SD-1. Haplotype for the closely linked markers to WNT7A gene locus in Club foot family.

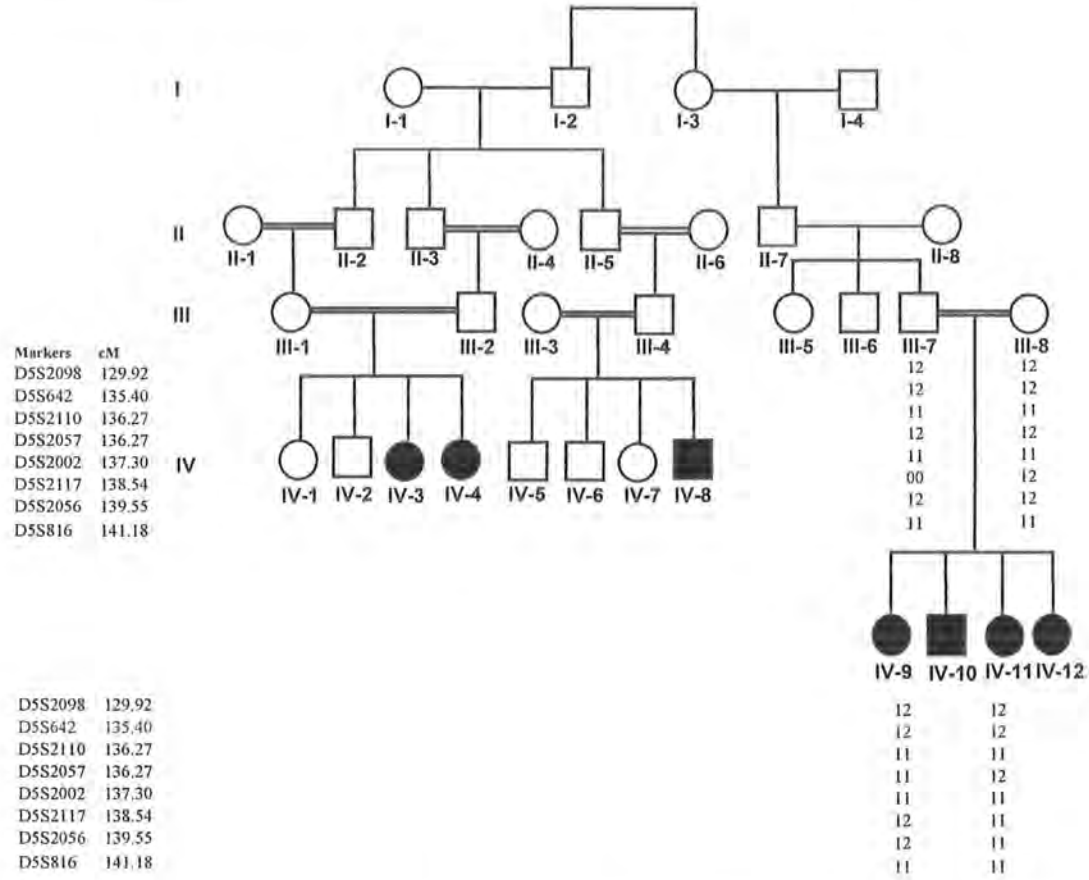


Figure 3.2: Pedigree drawing of family SD-1. Haplotype for the closely linked markers to PITX1 gene locus in Club foot family.

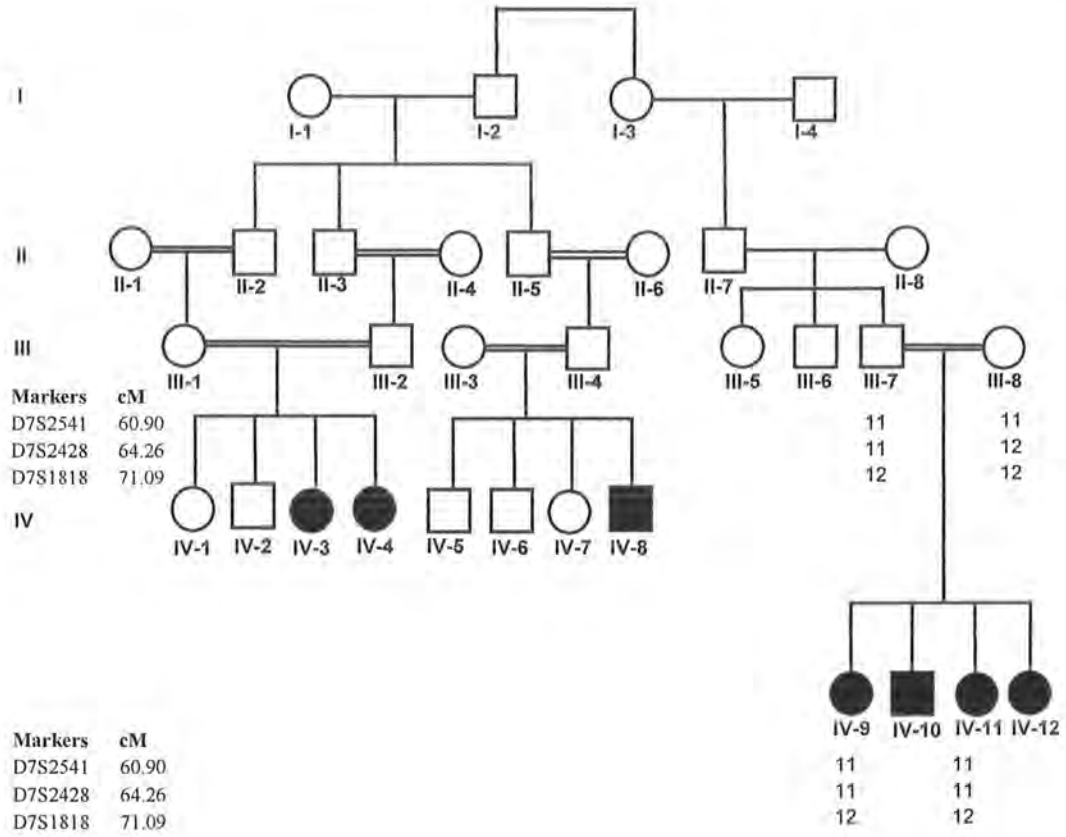


Figure 3.3: Pedigree drawing of family SD-1. Haplotype for the closely linked markers to Gli3 gene locus in Club foot family.

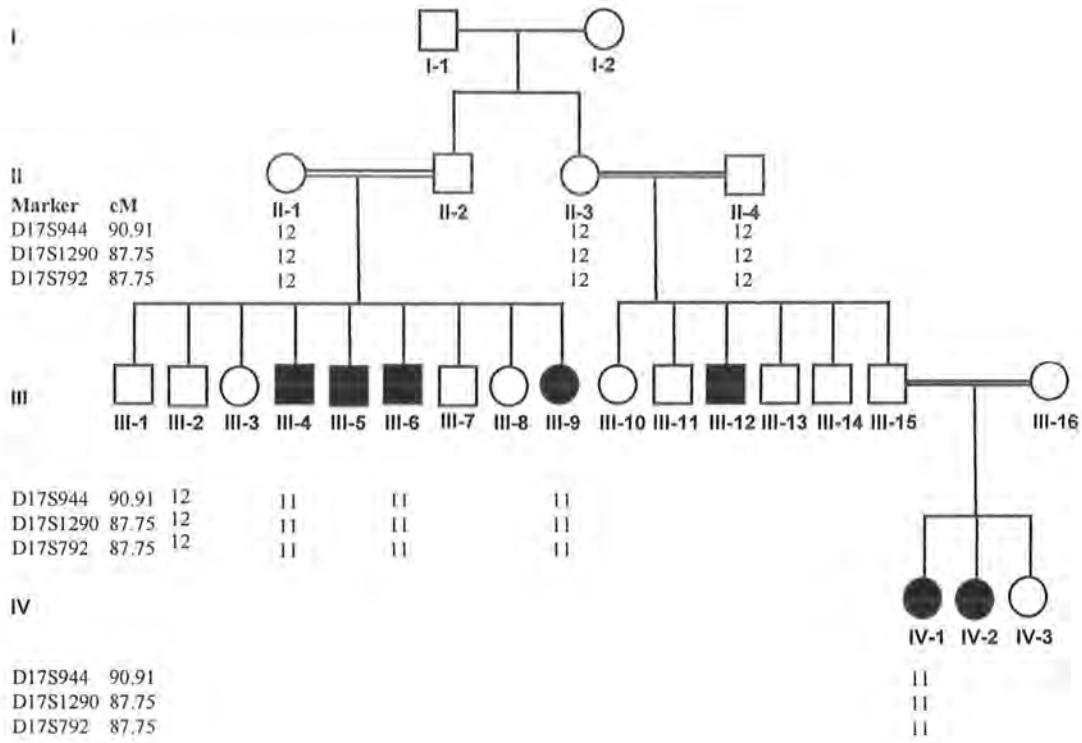


Figure 3.4: Pedigree drawing of family SD-14 showing linked markers to *GH1* gene locus in family. Shaded circles and squares represent affected female and male individuals respectively.

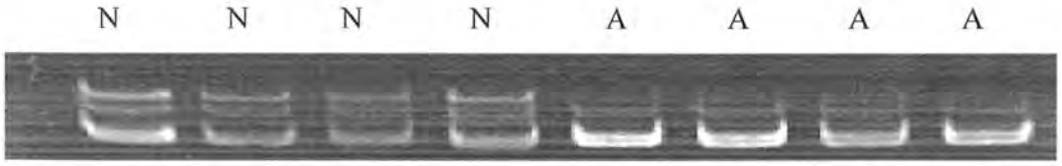


Figure 3.5: Linked marker D17S944 to *GH1* locus in family SD-14. N= Normal, and A=affected individuals.

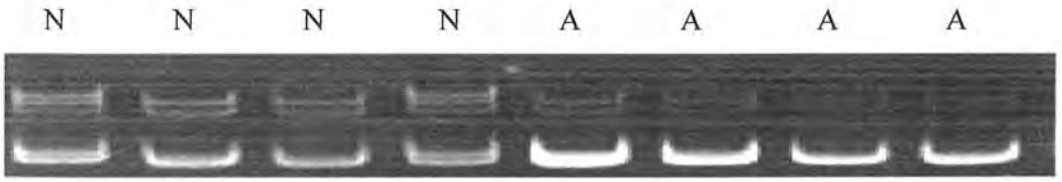


Figure 3.6: Linked marker D17S1290 to *GH1* locus in family SD-14. N= Normal, and A= affected individuals.

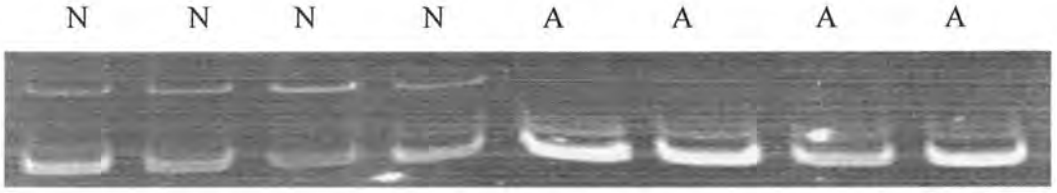


Figure 3.7: Linked marker D17S792 to *GH1* locus in family SD-14. N= Normal, and A= affected individuals.

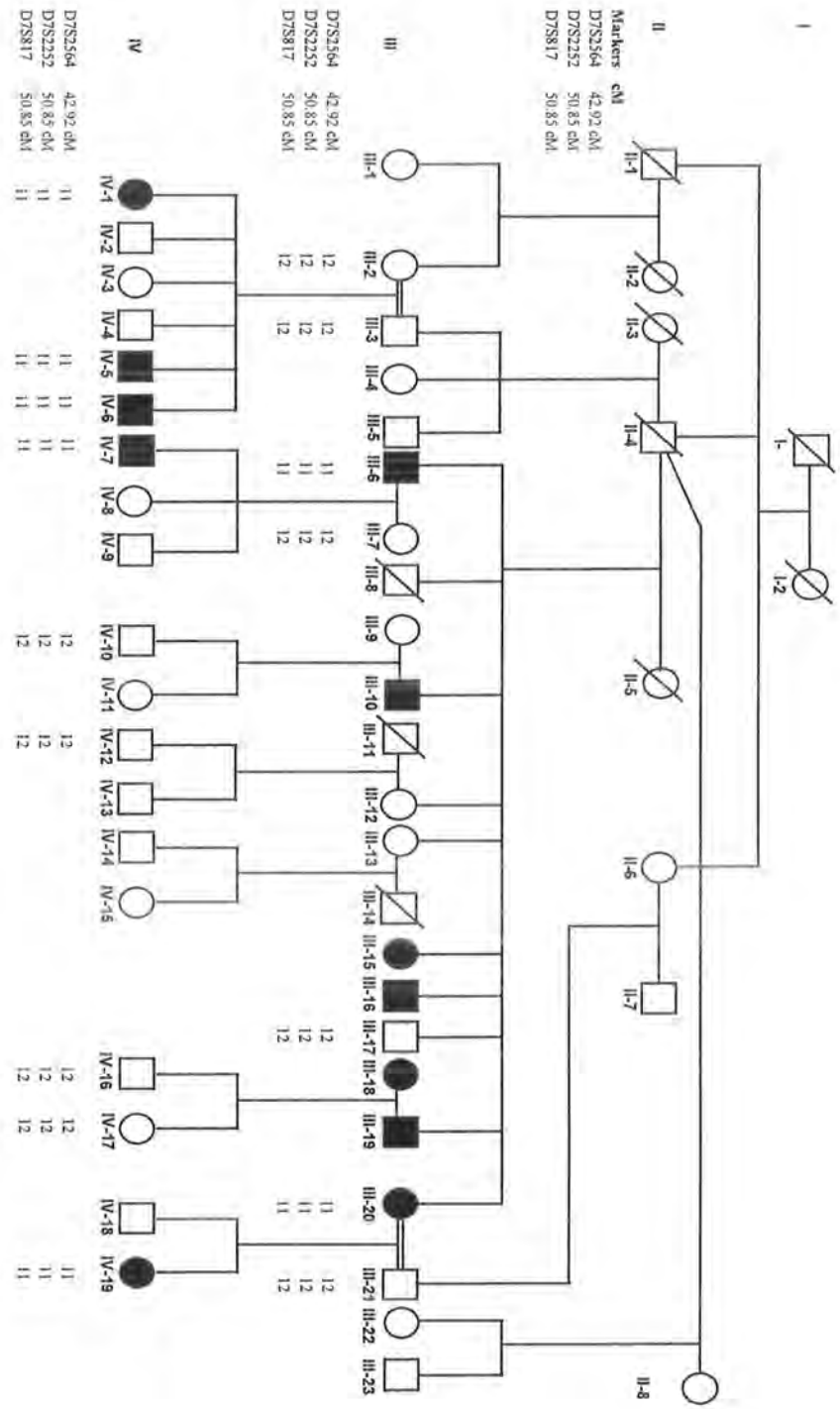


Figure 3.8: Pedigree drawing of family SD-26 showing linked markers to *GHRHR* locus. Shaded circle and square represent affected females and male individual respectively.

Chapter No. 4

Discussion

Populations that have a high frequency of inbreeding tend to show an increased prevalence of recessive disorders. The study of inbred populations has been highly successful in the fine mapping of recessive traits. The effect of consanguinity reduces allelic and non-allelic heterogeneity and potentially increases linkage disequilibrium and hence the power to detect associations between genetic markers and disease (Sheffield *et al.*, 1998). Mapping homozygous regions in affected individuals in consanguineous families is a powerful method of localizing autosomal recessive genes. This technique known as homozygosity or autozygosity mapping assumes that affected offspring co-inherit two copies of a disease related chromosomal segment from a common ancestor. Such positional cloning of recessive genes in families with rare genetic variants can provide valuable insight into the localization and identity of genetic susceptibility factors involved in the common types of disease with similar clinical features (Lander and Botstein, 1987).

Genetic disorders of the skeleton or skeletal dysplasias are a clinically diverse and genetically heterogeneous group of disorders affecting skeletal development (Horton and Hecht, 1993; Lachman, 1996). In general, they are characterized by abnormal shape, growth or integrity of bones. Tremendous advances in the knowledge of developmental pathways governing skeletal development as well as the progress in the human genome sequencing effort have resulted in the discovery of the basic genetic defects underlying several skeletal dysplasias over the past 5–10 years (Erlebacher *et al.*, 1995; Dreyer *et al.*, 1998; Warman, 2000).

The diagnosis of a skeletal dysplasia can be difficult because of its considerable heterogeneity. A thorough clinical examination and radiographic evaluation is required of the patient skeleton. Further narrowing the diagnosis, morphologic studies of the growth plate, cartilage and bone can be helpful. Biochemical and/or molecular (DNA) analysis can be performed when the defective gene (product) is known to confirm the diagnosis. An accurate diagnosis is essential both for genetic counseling and for proper management of the affected individual. The first step in the diagnostic assessment of a patient suspected for having a skeletal disorder is its complete medical history, pedigree analysis and physical examination. Knowledge of birth length and evaluation of the growth curves are very important. In case of short stature

evaluation of body proportions measurement of height, span, upper and lower segment are necessary.

Now a days it is possible to diagnose a skeletal disorder by performing molecular (DNA) analysis of the causal gene. This analysis is easy and straightforward for some disorder because of the presence of a common and recurrent mutation. While in some cases it is very time-consuming and laborious, because of the large size of the gene and the diversity of possible mutations or because of several genes involved in the condition.

The limitation of homozygosity mapping for linkage study of small families is that it detects a large disease interval that contains hundreds of genes. However, fine mapping and previous linkage reports in other families can reduce the size of the disease interval in the identification of the candidate gene in the region (Knight *et al.*, 2008; Hassan *et al.*, 2006). We performed homozygosity mapping for three families, affected with skeletal deformities.

In the family SD-1, affected with talipes equinovarus, we checked three Loci, *WNT7A, PITX1* and *Gli3* located on chromosome 3p25, 5q31 and 7p13 respectively but non of these showed linkage to the disease. It indicates that a novel gene locus may be involved in the transmission of the disease in the family. Idiopathic talipes equinovarus is a complex disorder that is caused by the interaction of several genes and environmental factors (Wang *et al.*, 1988; De Andrade *et al.*, 1998). Evidence supporting a genetic etiology underlying idiopathic talipes equinovarus comes from (1) aggregation of idiopathic talipes equinovarus in families; (2) twin studies and (3) segregation analyses, which suggest idiopathic talipes equinovarus is most likely caused by a single gene with major effects as well as other genes with minor effects and environmental factors (Yang *et al.*, 1987; Ester *et al.*, 2007). The control genes for bone and joint development are thought to lie in the Hox A and Hox D complexes. Although efforts are now underway to investigate the role of potential candidate genes in ICTEV (Barker *et al.*, 2003).

WNT7A encodes a secreted protein that stimulates dorsal patterning in the developing limb ectoderm (Tickle, 1996). Dietz *et al.* (2005) suggested that *WNT7A* is a highly plausible candidate gene for CTEV. We used thirteen markers upstream and down stream of the gene but found no significant linkage to the *WNT7A* gene. Similarly Liu

et al. (2008) studied a large series of families with club foot using three STS marker (D3S2403, D3S2385 and D3S1252) and found no significant linkage and suggested that the *WNT7A* gene is unlikely to be a major contributor to the aetiology of familial CTEV. To date, a small number of genes have been implicated in a small proportion of CTEV families. These includes the diastrophic dysplasia sulphate transporter (*DTDST*) gene (Superti-Furg, 1999), *CASP10* gene (Heck *et al.*, 2005), methylene tetrahydrofolate reductase (*MTHFR)* gene (Sharp *et al.*, 2006) and recently N-acetylation genes, *NAT1* and *NAT2* were reportedly associated with CTEV (Hecht *et al.*, 2007). Ester *et al.* (2007) genotyped, seven apoptotic genes *Casp3, Casp8, Casp9, Casp10, Bid, Bcl-2 and Apaf1,* and tested for linkage with and without association with idiopathic talipes equinovarus. No significant linkage without association was identified.

Recently Gurnett *et al.* (2008) studied a five generation family with asymmetric right sided predominant idiopathic clubfoot segregating as an autosomal-dominant condition with incomplete penetrance. They found a mutation in the *PITX1* gene that was present in all affected and three carrier individual and suggest that the gene *PITX1* located on chromosome 5q31, play a very important role in the development of hind limb. We used eight microsatellite markers for the linkage analysis of *PITX1* in family SD-1, but no linkage was observed, and we exclude the *PITX1* gene loci from further processing.

Zhang *et al.* (2006) identified *Gli3,* as a candidate gene for ICTEV. The *Gli3* promoter contains two putative Hoxd13 binding sites that directly interact with the promoter of *Gli3*. Thus, Hoxd13 can directly regulate the expression of *Gli3* during limb formation. In ICTEV patients, *HOXD13* has lower expression compared to healthy subjects (Wang *et al.*, 2005). Thus, the decrease in *HOXD13* expression may be led to a change in the expression of *Gli3*, which manifests as ICTEV. (Cao *et al.*, 2009). In our extended family SD-1, we genotyped the *Gli3* gene with three closely linked markers to the *Gli3* loci but no significant linkage was observed. From our study about the club foot it is clear that a novel candidate gene may involve in the disease transmission. So further insight into the study of club foot with different disease loci involve in the patterning and development of bones and joints are needed.

Human skeletal growth and final height attainment are results of a multifactorial regulation involving systemic and local hormones, growth and nutritional factors, lifestyle and genetic factors. Heritability estimates (Chatterjee *et al.*, 1999) and genome-wide linkage analysis (Hirschhorn *et al.*, 2001) have shown that genetic factors play a major role in determining stature (Esteban *et al.*, 2007).

GH1 gene is the main candidate gene for congenital isolated growth hormone deficiency (IGHD) in children. Short stature associated with bioinactive GH was first suggested and described by Kowarski *et al.* (1978). GH plays a major role in postnatal growth. The growth promoting effects of GH are achieved through GH's diverse and pleiotropic effects on cellular metabolism and differentiation and are mediated through the activation of a cell surface receptor (GHR) (Besson *et al.*, 2005).

The phenotypes of our family SD-14 was related to the phenotypes produce by *GH1* gene. So, we checked *GH1* gene located on chromosome 17q24.2 in family SD-14 for linkage. We found that *GH1* gene is linked to the disease in the family. All the affected individuals were homozygous and both parents and the unaffected offsprings were heterozygous for markers D17S944 (90.91cM), D17S1290 (87.75cM) and D17S792 (87.75cM) as shown in Fig: 3.5, 3.6 and 3.7.

Monogenetic recessive inheritance of isolated growth hormone deficiency (IGHD) was shown to be caused by complete deletions of the *GH1* (IGHD IA) and, more recently, by nonsense mutations of the *GHRH receptor* gene (Binder *et al.*, 2001). According to human gene mutation database (HGMD) 2010, total of 67 (86) mutation are reported in the *GH1* gene, including deletions, frame shifts, splice site, and nonsense mutations, which lead to decreased GH expression or action have been described (Rimoin and Phillips, 1997; Wagner, 1998). In family SD-14, mutational analysis was performed by direct sequencing of *GH1* gene but we could not find any pathogenic mutation.

Hereditary growth hormone deficiency GHD is a heterologous disorder with different mode of inheritance. Familial IGHD due to *GH1* gene defects is associated with four hereditary forms. Two forms are inherited as autosomal recessive traits (IGHD IA, IGHD IB), one form is inherited as an autosomal dominant trait (IGHD II), and one form is inherited as an X-linked disorder (IGHD III) (Cogan *et al.*, 1993; Phillips and Cogan, 1994; Kamijo *et al.*, 1999; Fofanova *et al.*, 2006). Both of our studied

families SD-14 and SD-26, was segregating as autosomal recessive, as shown in figures 3.4 and 3.8.

The growth hormone (GH)/insulin-like growth factor-1 (IGF1) axis is the key regulator of somatic growth in humans. Because of the importance of the GH/IGF1 axis on growth, mutations in genes of the GH and IGF1 pathways usually result in growth retardation and short stature (Walenkamp and Wit, 2006). A comprehensive survey of the literature identified ten candidate genes (*GH1, GHR, GHRH, GHRHR, IGF1, IGF1R, IGFALS, IGFBP3, JAK2, STAT5B*) for analysis of common genetic variation in the GH/IGF1 axis, and its association to adult height variation. Maheshwari *et al.* (1998) studied a dwarf family from Sindh Pakistan and found linkage of the GHRHR locus to the dwarf phenotype in the family. In the present study we performed linkage analysis for *GHRHR* gene locus in family SD-26. The microsatellite markers positively linked the disease to *GHRHR* gene loci in Family SD-26. Analysis of homozygosity by descent was consistent with linkage to that locus. So it is suggested that *GHRHR* gene will be sequenced for the purpose to find any pathogenic variant involve in the disease.

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ELECTRONIC DATABASE INFORMATION

The URLs for data presented herein are as follows:

Entrez Map Viewer

http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez hum_srch?chr=hum_chr.inf&query

Genes and Disease, http://www.ncbi.nlm.nih.gov/disease/

Human Gene Mutation Database (HGMD), Cardiff,

http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html

Mendelian Cytogenetics Network (MCN), http://mcndb.imbg.ku.dk

Mulitple sequence Alignment by CLUSTAL W, http://align.genome.jp

National Human Genome Research Institute (NHGRI), http://www.nhgri.nih.gov/

Online Mendelian Inheritance of Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

UCSC (University of California Scintes Cruze) Genome Browser,

http://genome.ucsc.edu/cgi-bin/hgGateway