Genetic Characterization of Bardet-Biedl, Ellis-Van Creveld and Acromesomelic Dysplasia Type Maroteaux Syndrome in Consanguineous Families

by

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A dissertation submitted in the partial fulfillment of the requirements for the degree of MASTER OF PHILOSOPHY

by

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In the name of Allah, The Most Gracious,

The Most Merciful

So only those of His servants who have knowledge (of these realities with a vision and outlook) fear Him. Surely, Allah is Almighty, Most Forgiving. [Fatir, 35:28.]

Say: 'Can those who have knowledge and those who do not be alike?' So only the wise do receive the admonition. [al-Zumar, 39:9.]

Approval Certificate

This is certified that the dissertation titled "Genetic Characterization of Bardet-Biedl, Ellis-van Creveld and Acromesomelic Dysplasia type Maroteaux Syndromes in Consanguineous Families" Submitted by **Maryam Khalid** to the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad Pakistan, is accepted in its present form as it is satisfying the dissertation requirement for the degree of Master of Philosophy in Biotechnology.

Supervisor — **Prof. Dr. Waseem Ahmed**

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Chairman — **Dr. Muhammad Naeem**

Dated: _____

I always find you by my side
From the day I open my eyes
Even here thick or thin
You were the only holding me tight
In the sunny day
You the only shady way
Whoever I am, whatever I be
On the stage where I am standing now
No doubt, it's all due to you
You give your rest plus sweat
For my success
Just saying thanks is not enough
Sacrifices whole life for you haven't worth
For what you did for me
I haven't words
To express my love
You my world, you are my heart
You my soul, you my freedom
I use to pray my Lord
To Bless my parents a lot
Here and here after

Written By: Maryam Khalid

I dedicate my research work to my loving and caring parents "Ch Khalid Mehmood and Mis. Tahira Khalid", without their continuous and unconditional encouragement and support I could have done nothing.

LIST OF ABBREVIATIONS

*	Stop codon
µg	Microgram
µl	Microlitre
A	Adenine
aa	Amino acid
ACVR1	Activin A receptor type 1
AL	Alstrom syndrome
AMD	Acromesomelic dysplasia
AMDM	Acromesomalic Dysplasia Maroteux
APC	Anaphase promoting complex
APS	Ammonium per sulphate
Arg	Arginine
ARL	ADP-ribosylation factor (ARF)-like-6 (ARL6)
Asp	Abnormal spindle gene
ATPase	Adenine triphosphatase
BBS	Bardet biedl syndrome
BMP	Bone morphogenetic protein
BMP14	Bone Morphognetic Protein
BMPR1B	Bone Morphogenetic Protein Receptor Type 1B
bp	Base pair
C	Cytosine
C- Terminus	Carboxyl terminus
CDMP1	Cartilage-derived morphogenetic protein-1 (CDMP1)
cDNA	Complementary deoxyribonucleic acid

cM	Centimorgan
CNP	2',3'-Cyclic Nucleotide 3' Phosphodiesterase
CTSA	Cathepsin A
del	Deletion
DNA	Deoxy ribonucleic acid
Dup	duplicate
E	Extra cellular matrix
EBP	Emopamil binding protein (sterol isomerase)
EMP	Epithelial membrane protein
EvC	Ellis-van creveld
FGF	Fibroblast growth factor 1
fs	frameshift
g	Gram
G	Guanine
GALNS	Galactosamine (N-acetyl)-6-sulfatase
GDF5	Growth differentiation factor 5
GHR	Growth harmone regulator
GLB1	Galactosidase beta 1
GMP	Guanine mono phosphate
GSK3	Glycogen synthase kinase 3
GTP	Guanine tri phosphate
His	Histidine
IF74	Intraflagellar Transport 74
Ift	Intra Flagella Transport
IFT27	Intraflagellar Transport 27
Igf1	IGF1 insulin like growth factor 1

IHH	Indian hedhog
INCDB	International Nomiculture Of Constitutional Disease Of Bone
IRB	Institutional Review Board
Kb	Kilobase
KCl	Potassium chloride
kDa	KiloDalton
KPK	Khyber pukhtunkhawa
Leu	Leucine
M	Molarity
M	Methionine
mA	Milliampere
mg	Milligram
MgCl2	Magnesium chloride
MKKS	Mukusick- kufman syndrome
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MSC	Mesenchyme stem cell
N	Any nucleotide
NaCl	Sodium Chloride
NEU1	Neuraminidase 1
NORD	National organization of rare syndrome
Npr2	Natriuretic peptide receptor 2
OFDI	Orofaciodigital syndrome type 1
P	Short arm of chromosome
PAGE	Poly acrylamide gel electrophoresis

pc1 and 2	Polycystine 1 and 2
PCDKS	primary ciliary dyskinnesia and kartagener syndrome
PCP	Planar- cell polarity pathway
PCR	Polymerase chain reaction
PK	Proteinase K
Pro	Proline
PTHrp	Parathyroidharmone- related peptide
Q	Long arm of chromosome
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Ser	Serine
SIT-R	Slosson intelligence test
SLS	Sample Loading Solution
SMAD	Mad (SMAD) Mothers against dpp
SNPs	Single nucleotide polymorphisms
T	Thymine
Taq	Thermus aquaticus
TBE	Tris borate EDTA
Tcp	T-complex 10
TE	Tris- EDTA
TEMED	N, N, N', N'-Tetra Methyl Ethylene Diamine
TGFβ	Transforming growth factor beta
Thr	Threonine
Tm	Annealing temperture

TMC1	Transmembrane channel-like protein 1
TRIM32	Tripartite Motif Containing 32
TTC8	Tetratricopeptide repeat domain 8
Tyr	Tyrosine
US	United State
UV	Ultra violet
V	Valine
v/v	Volume/volume
VA	Visual Acuity
Val	Valine
WES	Whole exome sequencing

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Maryam khalid

Abstract

ABSTRACT

The adult human skeleton is composed of 206 bones. All skeleton units' such as muscles, ligament and tendon are formed within 5-7 weeks of embryo development. There are many genes, regulatory regions; transcriptional factors, signaling pathways, and transducer involve information and modification of human skeleton. Skeletal dysplasia or bone deformities include a large category of genetically inherited disorders involving the abnormalities in the growth and modeling of bones. 364 genes have been reported, causing 436 skeletal diseases that have been grouped into 42 categories. Abnormal cilia formation is also one of main reason of the skeleton dysplasia i.e. BBS and EvC syndrome. There are twenty one genes (involved in the cilia formation, BBSome complex or the basal bodies of cilia) which cause BBS syndrome. Similarly *EVC* genes product is acting as a barrier between ciliary and plasma membrane. They use to transfer signals through hedgehog signaling pathway from extra cellular to the nucleus.

In the present study, three families (A, B, C) of Pakistani origin having autosomal recessive bone deformities were evaluated on clinical and genetic basis. Clinical analysis of the phenotypes of affected members showed Acromesomelic Dysplasia Maroteaux Type in family A. Affected members of family B were found to have Bardet-Biedl Syndrome, while family C was clinically observed to be affected with Ellis-Van Creveld. The hunt of the mutated genes in the affected members of these families was done through homozygosity mapping based on Sequence-Tagged Site (STS) markers and Sanger sequencing. Family A affected with AMDM did not show linkage to any reported known gene or loci concluded that the novel gene might be involved. Haplotype analysis showed linkage of family B to *MKKS* gene and family C to *EVC* gene. All exons of *MKKS* and *EVC* gene were Sanger sequenced. Analysis of the sequencing results showed novel pathogenic variant (c.775delA; p.Thr259Leufs*21) in exon 1 of *MKKS* in family B. This deletion of A nucleotide causes a frame shift and a pre-mature stop codon thus spoiling the MKKS protein structure. In family C no pathogenic mutation were identified in sequenced exons of *EVC/EVC2*, suggesting presence of mutation in rest of the unsequenced exons.

Introduction

INTRODUCTION

Human skeleton

The human skeleton is internal framework of the body. Neonate human skeleton consist of round about 300 bones, later on decreased to 206 due to the fusion of some bones (Marshall, 2010). The density of bone increased to its maximum level at the age of 20. There are two main divisions of human skeleton, axial skeleton and the appendicular skeleton. The axial skeleton (forms body structure) consists of vertebral column, the rib cage, the skull and some associated bones. The axial skeleton composed of 80 bones. 22 major and 7 associated bones forms a skull, 32–34 bones forms vertebral column, 12 pair of ribs and a sternum forms a rib cage and a sacral and coccygeal (the lower two bones may vary in length). The main function of axial skeleton is to maintain up right posture. Appendicular skeleton is that portion of skeleton which attaches with axial skeleton (support the appendages), contains 126 bones. The appendicular skeleton consist of shoulder girdle, the pelvic girdle, upper and lower limbs bones. It helps in movement and protects many major organs i-e organ of digestion, excretion and reproduction. Six major functions are performed by the human skeleton i-e movement, protection, support, erythropoietin, endocrine regulation and storage of minerals. 3 different sites are involved in skeleton formation. The axial originates from the somites, the craniofacial bones originates from neural-crest whereas the appendicular skeleton arises in MSC which is located in the lateral plate (Kornak and Mundlos, 2003) Unlike many other primate species the human skeleton is not sexually dimorphic, there are very less difference exist in the morphology of human skeleton i-e dentition, pelvis, skull and long bones. In comparison of male and female skeleton, female skeleton tend to be smaller and less strong. The females use to give birth to child that is why female pelvis is different from that of males (Thieme Atlas of Anatomy, 2006) more over the human male skeleton don't have penile bone like other primates i-e chimpanzees, gorilla (Harper and Row, 1951).

Formation of Human Skeleton

The ovum contain all the genetic information relevant to the development of whole organism, many transcription and helping factors are involves in the process of cell division and cell differentiation for the proper growth and development of bones. The interaction between developing tissues is one of the primarily factor which help in gene expression, metabolism, organization and development of bones. Epigenetic events as well as mechanical factors take part in attaining the shape, pattern of developing tissues (Uhthoff *et al.*, 1978). Within 5-7 weeks of embryo development, almost all major skeleton units' i-e muscles, ligament and tendon are formed. The physiochemical regulation ossification of skeleton is also observed during this period of time. The muscles movement is clearly observed within 15 weeks of gestation. Muscles contraction and relaxation help in increasing the ossification of the bones. It is also observed that cease in muscles movement cause abnormal skeleton development (Devries *et al.*, 1982).

Skeletogeny

Skeletogeny required correlate action of multiple cell types in mammals and is organized by two defined developmental processes. Endochondral ossification; mostly of the skeleton is attained by this process i-e long bone and other one is Intramembranous ossification; part of clavicle, pubic and craniofacial skeletal elements are primarily obtained by this process (Amjad *et al.*, 2010). During the period of embryonic advancement the osteoblasts arise from local mesenchyme cells and after the birth oseoblast arises from connective tissue mesenchymal stem cell (MSC). In response to specific stimuli, these precursor cells act to osteogenic lineage and discriminate into mature osteoblasts. The vivo and in vitro culture, model studies and using certain modifications in gene expression of normal and affected bone tissues shows different stages of osteoblast which is defined by the expression of specific genes. In-vivo profiling of gene expression further explains the sub-stages of osteoblast maturation. Genetic mutation can result in alteration of these sub-stages (Karsenty, 2008).

Steps Involved in Osteoblastogenesis

In general, there are four major phases of osteoblastogenesis. Lineage commitment, proliferative, expansion, synthesis of extracellular matrix (ECM) and mineralization. These all stages are controlled by a gene which expressed continuously i-e help in advancement of osteoblast differentiation through developmental transit points. The first transit needs MSC commitment to osteogenic lineage, second transit is related with expansion of osteoprogenitor, and its mitotic duplication. The third transit is the extra cellular matrix production by osteoblast (required to exit from the cell cycle). The final and the last transit is osteoblastogenesis which is characterized by minerals deposition in the ECM (Boskey and Wright, 1999). The pluripotent mesenchymal stem cells can give rise to lineages of multiple type of tissue such as chondroblast, myoblast, adipocytes, osteoblast, odontoblast, and tendon cells.

Cellular Pathways Involved in Normal Skeleton Formation

The growth plate consists of 3 main zones; resting, proliferating and hypertrophic zone (Karsenty *et al.*, 2009). There are no proper orientations of cell division in resting zone (Kronenberg, 2003). The least differentiated cells exist in resting zone, these cells enter to the next zone (proliferating zone) in which the cell proliferation occur, the cell start dividing perpendicular to the plane of growth plate to form well oriented columnar cells. These cells start intercalating each other and convert into the pillars of discoid chondrocyte (Romereim *et al.*, 2011). Non canonical WNT signaling, PCP (Planar – cell polarity pathway) cadherin and β catenin helps in cell adhesion for column formation (Romerein *et al.*, 2014). During cell division, the daughter cell remains close with each other, with the help of adhesion-laden surface the cells spread on one another (Romerein *et al.*, 2014). After receiving the signals cell enter in to next zone (hypertrophic zone), in which they differentiate into hypertropic chondrocytes (rapid increase in cell volume).

Signaling Pathways Involved in Regulation of Chondrocyte Proliferation

The regulation of chondrocyte proliferation through growth plate is done by many signaling pathways (Baldridge *et al.*, 2014). These pathways include IHH (Indian hedhog), PTHrP (parathyroid hormone – related peptide), fibroblast growth factor, C –

type natriuretic peptide, transforming growth factor B, notch, bone morphogenetic protein (BMP) (Kanaenty *et al.*, 2010), WNT canonical and non canonical. They play important role in well orientation of cell division in different zone.

Skeletal Dysplasia

Disorganization of bones and cartilage has been assigned as skeletal dysplasia. The skeletal dysplasia belongs to anomalies in the arrangement, size, maintenance, and development of the axial and appendicular skeleton and normally results in disproportionate short stature. Up till now there are 436 well-defined skeletal dysplasia classified on the basis of radiographic, molecular criteria and clinical characterization whereas 364 genes are involved in causing different type of skeleton dysplasia (Bonafe *et al.*, 2015). These anomalies are due to the mutation in the genes of different families that encode transcription factors, extracellular matrix proteins, tumor suppressors, ligands, channel proteins, receptors, enzymes, cellular transporters, intracellular binding proteins, chaperones, RNA processing molecules, cytoplasmic proteins, cilia and a number of many other gene involved, whose function are still unknown.

Reason behind Skeletal Dysplasia

Bone and cartilage disorders are normally known as skeleton dysplasia. There are 436 skeletal dysplasia (Bonafe *et al.*, 2015) every 1/5000 child is affected by this disorder that ranges from mild to severe abnormality (Warman *et al.*, 2011). As it is discussed earlier, there are many complex signaling pathways which play major role in skeletongenesis i-e FGF, TGFβ, BMR, WNT, Notch and hedhog pathways. These signaling series help in formation of joint, cartilage and bone. Any problem (primary or secondary) or dysregulation in these pathways will lead to skeletal dysplasia (Dustin *et al.*, 2010).There are four main groups into which these disorders can be placed. These are Osteodysplasia, Chondrodysplasia, Dysostoses and Reduction.

The Osteodysplasia or skeletal dysplasias is recognized by the lower density of bones than normal bones (Osteopenia), or a condition having higher density of bones than normal bones Osteosclerosis (Jane *et al.*, 2005). The chondrodysplasia results in short stature bones are, therefore, grown linearly caused by pathogenic variants in the

organogenetic genes (Mortier, 2001) whereas those that affect specific bone or class of bones have been known as dysostoses i-e Triphalangism, syndactyly, and polydactyly (Schramm *et al.*, 2009). Sub classes of Dysostoses are explained by Zelzer and Olson (2003) on the bases of abnormalities in appendicular, axial, and cranio-facial skeleton. Secondary malformation of the bones is referred to as Reduction (Schramm *et al.*, 2009). Here, in the present dissertation, we will discuss two syndromic and one non syndromic forms of skeletal dysplasia (BBS, EVC and acomesomelic dysplasia).

Bardet-Biedl Syndrome (BBS)

Bardet–biedl syndrome is one of the autosomal recessive diseases that cause abnormalities of multiple tissues of the body. The ratio of existence of BBS in North America and Europe is 1/140,000 to 1/160,000 in live birth. It is most common in Bedouin population of Kawait i.e 1/13,500 newborn, whereas 1/17000 in island of Newfoundland. Bardet-biedl syndrome is one of the rare genetic diseases which includes multiple systems and broad spectrum of clinical features. Principal (primary) feature of BBS are retinitis pigmentosa (rod-cone dystrophy), renal dysfunction, truncal obesity, hypogonadism, mental retardation, postaxial polydactyly and secondary features (features not always present) includes dental anomalies, cardiovascular abnormalities, facial dysmorphism, psychomotor delay, type 2 diabetes mellitus, learning problem, language deficits, hearing loss, hepatic fibrosis, neurological problem, speech deficits (Beales *et al.*,1999). BBS diagnosed on the bases of presence of at least four primary features or presence of three primary features with two secondary features (Beales *et al.*, 1999).

In BBS the retina gets damaged continuously and therefore causes the loss of vision. This problem starts (apparent) in mid childhood, first night vision damage followed by peripheral vision (blind sport develop in the side). Many of the patients suffer loss of visual acuity (VA) and become blind in their adulthood (Ansley *et al.*, 2003). BBS patient also suffer from the renal abnormalities, learning difficulties, severe mental impairment, and truncal obesity. Continuous weight gain leads to many problem i-e type 2 diabetes, hypertension and hypercholesterolemia, hypogonadism, postaxialpolydactyly, facial dysmorphism, behavioral traits, dental anomalies, neurological and speech deficits.

The disease symptom varies both within and between families (Beales *et al.*, 1999). 69% of patients are affected by postaxial polydactyly whereas obesity begins to develop in age of 2-3 and vision problem begins in age of 8- 8.5 years (Beales *et al.*, 1999).

Genes Involved in BBS

Twenty one different genes are reported up till now which are involve in BBS syndrome (also known as BBS genes) (Elise *et al.*, 2016). These genes are involved in formation of cell structure known as cilia. Cilia are microscopic finger like projection; they are present on the surface of the cell. They help in cell movement and plays major role in sensation that are sight, hearing and smell (Mullery *et al.*, 2010). Mutation in BBS genes causes deformation in the function as well as the structure of cilia. Cilia defect disturb chemical signaling pathway and also cause abnormalities in sensory perceptions (Tobin, 2007). Most of the BBS syndrome are due to the mutation in *BBS1* gene (about ¼ of BBS cases), whereas 20% of disease are due to the mutation in *BBS10* gene. Rest of disease is due to the mutation in other genes, whereas 25% of patient carries mutation other than BBS known gene (Katsain *et al.*, 2004). It is also observed in some families for BBS syndrome that triallelic inheritance is necessary (combination of 3 mutation allele) (Beales *et al.*, 2003) Mutated BBS genes also result in isolated (non-syndromic) diseases whose phenotype is similar to BBS or ciliopathies syndromes i-e retinitis pigmentosa, cone –rod dystrophy etc (Mockel *et al.*, 2011). Inherited retinal dystrophies (non syndromic) genetic heterogeneity involves 250 associated genetic loci (RETNET, http:// www.RETNET.org, last accessed April 19, 2016)

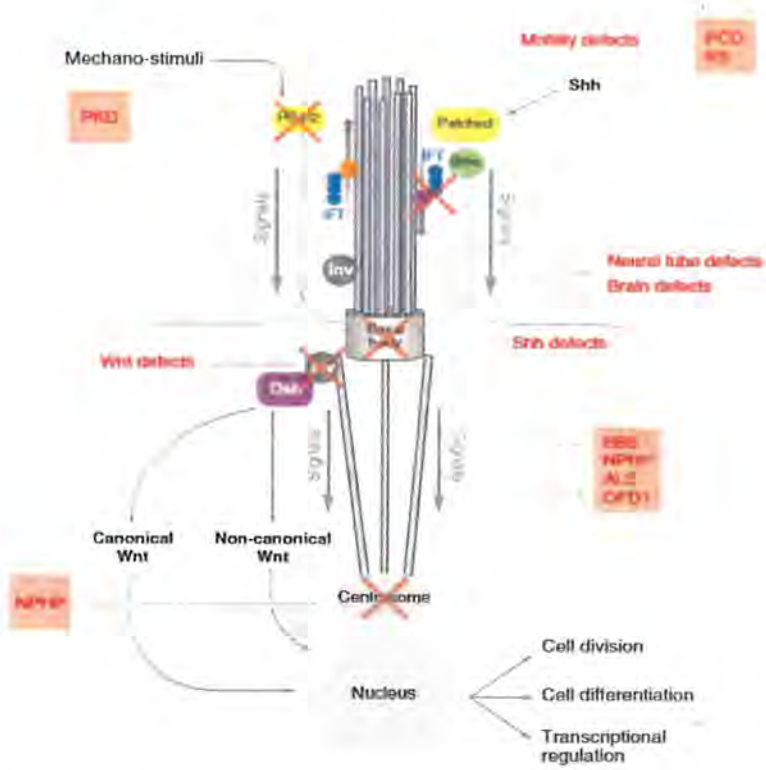


Figure 1.1: The figure shows that how different signals defect cilia formation, the microtubule extends from the nucleus to the tip of cilia. There are some important signaling pathways which cause normal formation of cilia. The internal signaling (Shh, Wnt, NTHP and some other) sense and transduce in the cell and then they reaches to the nucleus and cause the regulation of gene, cell differentiation and cell division. The phenotypic outcome of different cilia will perturbed (disturb) depending upon the signal effect i-e the defect (mutation) in the motile cilia will leads to immotile cilia cause PCDKS (primary ciliary dyskinnesia and kartagener syndrome). Similary the Shh defect cause neural tube and brain defect, Wnt defect cause NPHP and basal body defect cause BBS, NPHP, ALS (Alstrom syndrome), OFDI (orofaciodigital syndrome type 1) mutation or defect in (pc1 and 2) polycystine 1 and 2 will leads to polycystic disease (Bandano *et al.*, 2006).

BBS1

BBS1 is also known as *BBS2L2, FLJ23590. BBS1* gene involved in protein formation found in cells throughout the body, also play role in the formation of cilia (a finger like projection) (Mullery *et al.*, 2010). 76 mutations have been reported up till now in *BBS1* (Khan *et al.*, 2016), the mutation in *BBS1* is one of the main reasons of causing BBS syndrome. The most common mutation which is reported up till now is replacement of amino acid methionine with the amino acid argnine at the position 390 (M390R) (Beales *et al.*, 2003). Symptoms are obesity, vision loss, extra finger or toes (polydactyly intellectual disability and abnormalities), chromosomal location is 11q13.2 and molecular location 66,510,648-66,533,613 bp on chromosome 11. BBS1 plays main role in promoting biogenesis of ciliary membrane by increasing the level of Rab8-GTP as well as play role in the signaling pathway of leptin receptor (Khan *et al.*, 2016)

BBS2

Bardet-biedl syndrome 2 (*BBS2*) also known as *RP74*, plays important role in cilia formation, chromosomal location is16q21. *BBS2* consist of 17 exons, encodes a protein consist of 721 amino acid (Khan *et al.*, 2016). Mutation in *BBS2* results in similar phenotype of BBS. Many of BBS protein included BBS2 move to the basal bodies, pericentriolar, ciliary axonemes region of cell. *BBS2* play role in intracellular trafficking by microtubule- related transport. This gene helps in the formation of BBSOME complex with 7 other gene (National Center of Biotechnology Information 2014).

BBS3

BBS3 also known as *ARL6* gene cause BBS3 due to homozygous mutation in *BBS3* locates on chromosome 3q11. *ARL6* consist of 9 exon encodes a long cystolic protein consist of 186 amino acid also known as ADP-ribosylation factor like protein 6 (Khan *et al.*, 2016), mutation results in ciliary dysfunction. The symptoms are polydactyly, obesity, hypergenitalism, mental retardation, retina pigmentosa and some also suffering from unilateral dialated renal pelvis (Kwitek *et al.*, 1993).

BBS4

Cytogenic location of *BBS4* is 15q24.1 (Mykytyn *et al.*, 2001). *BBS4* is also involved in BBSOME plays role in the formation of protein complex required for ciliogenesis (Nachury *et al.*, 2007) immunohistochemical analysis revealed that *BBS2* and *BBS4* is also involved in cellular structure associated with motile cilia (Shah *et al.*, 2008). *BBS4* gene consist of 16 exon around 52kb (Mykytyn *et al.*, 2001) encodes a protein consist of 519 aminoacid. It resembles to the gene (acetyle- glucosamine transferase) of many species i-e plants, arehea- bacteria). Atleast 10 TPR exists in *BBS4* which helps in protein- protein interaction (Khan *et al.*, 2016).

BBS5

BBS5 also involved in formation of protein complex which is required for ciliogensis (Nachury *et al.*, 2007) cytogenetic location 2q31.1, *BBS5* gene contain 12 coding exon. BBS protein is involved in the cilia and flagella formation (Li *et al.*, 2004) and it also help in the formation of the BBSome complex. There are two domains of BBS5 pleckstrin homology domains and binds to phosphoinositides. Inhibition of phosphoinositide production results in the inhibition of ciliogenesis, which proves the link between IFT (intraflagellar transport) machinery and ciliary membrane. Up till know 20 different mutations which includes splice site, deletion, missense/nonsense, insertion and indels are reported in *BBS5* gene and cause BBS syndrome (Khan *et al.*, 2016).

BBS6

BBS6 is also known as Mukusick-kufman syndrome (*MKKS*). Cytogenetic location is 20p12.2 (Stone *et al.*, 2000). Translation of *MKKS* gene produces long chain of 570 amino acid (open reading frame). It is predicted that the amino acid of MKKS protein resembles to chaperonin family of protein, which are associated with limbs, reproductive system and cardiac development. It is closest protein which resembles to alpha subunit of thermoplasma acidophilum thermosome (Stone *et al.*, 2000). *MKKS* gene consists of 6 exon, coding region starts from exon 3 (Stone *et al.*, 2000). It is observed that *MKKS* is rapidly shuttled and highly mobile between cytosol and centrosome.

BBS7

The cytogenetic location is 4q27. *BBS7* is also the part of BBSome which plays major role in ciliogenesis consists of 19 exon (Badano *et al.*, 2003). *BBS7* is similar to *BBS2*. There are 252 amino acids between residues 147 and 398 similar to *BBS2* (Bandano *et al.*, 2003). It is also discovered that conserved area between 171 -351 residue uses to encode six-bladed beta –propeller structure. The part of this domain overlapped with *BBS1* region indicating that these genes *BBS1, BBS2, BBS7* belongs to distinct sub family of protein, mutation in these domains will leads to similar disease (Badano *et al.*, 2003).

BBS8

BBS8 (Bardet-biedl syndrome 8) is caused by the mutation (homozygous) in *TTC8* gene (Ansley *et al.*, 2003), cytogenetic location is 14q31. Symptoms are retinitis pigmantosa, obesity, postaxial polydactyly (at least 2 limbs), hypogonadism, and developmental delay. This was observed in 1 of Pakistani and 2 of Saudia Arabia lineage (Ansley *et al.*, 2003). The mutation in *BBS8* causes deformities in the basal body of ciliated cells.

BBS9

BBS9 Syndrome is caused by the mutation in gene known as *PTHBI* which is located on the chromosome 7p14 (homozygosity or compound hetrozygosity). *BBS9* consist of 23 exons use to encode a protein consist of long chain of 887 amino acid. It is also one of those genes that form BBSome and use to expressed in skeleton muscles, adult heart, lungs, liver, placenta, kidney and brain. The mutation in gene PTHBI cause similar symptoms of BBS, 3 sibs with BBS9 are reported in consanguineous Arab family (Abu-safieh *et al.*, 2012).

BBS10

A novel chaperonion subfamily protein (723 amino acid) is encoded by a gene *C120RF58*, Composed of 2 exon gives map on the 12q (Stoelzel *et al.*, 2006). Immunohistochemical revealed that BBS10 and BBS12 both located in unciliated fat cell. Knock down expression of *BBS10* and *BBS12* by interference of RNA cause reduction in amount of ciliated cell whereas increase the amount of GSK3 (UN phosphorylated active)

key regulation of adipogenesis that repress by wnt signaling (Marion *et al.*, 2009). Defected BBS10 cause increase in triglyceride and differentiation of fibroblast in to fat accumulating cell, this shows that primary dysfunction of adipogenises cause obesity in BBS.

BBS11

BBS11 is due to the mutation in *TRIM32* gene, cytogenetic location 9q33. *BBS11* have been expressed in adipose tissue; consist of two exons, encodes a protein consist of 652-amino acid. BBS11 (TRIM32) belongs to TRIM family, it have domain familiar to TRIM family consist of a RING finger, coiled-coiled motif, B-box. TRIM32 is considering one of the adjusting gears of the cytoskeleton so mutation in *BBS11* resulted in muscle dystrophy i-e BBS syndrome. (Locke *et al.*, 2009, Kudryashova *et al.*, 2009). Up till know only a missense mutation has been reported in *BBS11/TRIM32* gene leads to BBS syndrome. The homozygous mutation in which proline is replaced by serine at codon 130 in all affected (4) member of same family in TRIM32 cause BBS11 syndrome (Chiang *et al.*, 2006).

BBS12

FLJ35630 gene renamed as BBS12 shows resemblance (homology) to group II chaperonins and with the sequences of chaperonin like families composed of *BBS10* and *BBS6.* It consist of 2 exons which encodes the protein consists of 710 amino acid. Gene maps to chromosomes 4q27 (Stoetzel *et al.*, 2007) (The role of *BBS12* is already discussed in *BBS10*). 11 % of the families affected with BBS syndrome are due to mutated *BBS12* (Khan et al., 2016).

BBS13

This is due to the heterozygous mutation in *MKS1* gene. *MKS1* is located on chromosome 17q22 consist of 100-kb region. MKS1 also consist of (76-1755 bp) open reading frame, it encodes 559-amino acid having a conserved domain B9 (Kyttala *et al.*, 2006). MKS1 exist to a narrow family which includes B9 domain- proteins that also contain B9D1 and B9D2, all 3 domain of B9-containing proteins accomplice with primary cilia and basal

bodies in mammalian cells (Bialas *et al.*, 2009). The mutation in *BBS13, MKS3, CEP290* results in BBS syndrome as well as it is also known BBS-associated loci (have a potential epistatic effect on mutations causing BBS syndromes) (Leitch *et al.*, 2008). Five out of 6 families were showing the symptoms of seizures having mutation in MKS1 where as this was not the phenotype of Bardet-Biedl syndrome. Later on the studies in zebra fish showed that MKS1 is required for gastrulation movements and also that there are genetical interaction of MKS1 with known *BBS* genes (Leitch *et al.*, 2008). Up till now four mutations (deletion mutation, compound heterozygous mutations) reported belongs to gene *BBS13/MKS1* which caused BBS syndrome (Khan *et al.*, 2016).

BBS14

BBS14 is due to homozygous mutation in *CEP290* gene, chromosomal location is 12q21. The homozygous nonsense mutation in *CEP290* gene cause BBS observed in consanguineous Saudi Arab family (Leitch *et al.*, 2008), studies on zebra fish represent that there is strong genetics interaction between *CEP290* and *MKS3* allele (Leitch *et al.*, 2008). 4% of mutation are reported in *BBS14* up ill know which includes missense and deletion.

BBS15

The homozygous mutation in *WDPCP* gene causes Bardet-Biedl syndrome-15 (BBS15). The chromosomal location is 2p15 consist of 12 exons.The patient having the homozygous mutation in the *WDPCP* gene shows BBS syndrome. Whereas parents and unaffected sib do not shows any syndrome due to the heterozygous mutation (Kim *et al.*, 2010).

BBS16

The homozygous or compound heterozygous mutations in the *SDCCAG8* gene cause BBS. The chromosome location is 1q43. Senior-Loken syndrome-7 is also due to the mutation in *SDCCAG8*. There was a case of two sib (female) observed in east Indian showing same symptoms of BBS i-e renal disease that required transplantation respectively, obesity, short stature, and mild cognitive impairment. The smaller one had

nonalcoholic fatty liver disease, Visual acuity, retinitis pigmentosa, rod function to be more severely affected than cone function in both cases but Polydactyly was absent (Billingsley *et al.*, 2012). There was also another 2 cases of BBS16 in consanguineous families observed in gypsy and a French family having obesity, chronic renal failure, cone-rod dystrophy, and developmental delay. Also present were conductive hearing loss/recurrent otitis, respiratory infection, and asthma. But Polydactyly was absent in all affected individuals (Otto *et al.*, 2010).

BBS17

Bardet-Biedl syndrome-17 is due to the mutation (homozygous or compound heterozygous) in the *LZTFL1* gene the chromosome location is 3p21.The mutation in *LZTF1* gene shows the phenotype of BBS, study of two sibs belongs to consanguineous Algerian family shows with developmental delay, obesity, unilateral central polydactyly, bilateral postaxial polydactyly of feet, situs inversus, and hypogenitalism with micropenis and atrophic testes (Deffert *et al.*, 2007). There was another family reported in 2014 by Schaefer discusses about 36 year old dizygotic twin sisters affected with BBS. Both shows polydactyly were having 6 toes on each foot whereas 7 fingers on both hand having mesoaxial polydactyly. There are Fusion and hypoplasia between the third and fourth metacarpal bones. In the age of 3 month the extra digits were removed. Both were having retinitis pigmentosa, cognitive impairment, and renal dysfunction. One of the sib were affected by Anosmia whereas hyposmia in the other; there was abnormal enlargement of cilia anosmic where as many cilia having normal morphology exist in the nose of the hyposmic sib (Schaefer *et al.*, 2014).

BBS18

Bardet-Biedl syndrome-18 (BBS18) is caused as a result of homozygous mutation in the *BBIP1* gene and located on chromosome 10q25. Case study of consanguineous Italian family in which there was a patient (only affected sib out of 4) affected with Bardet-Biedl syndrome (BBS). He was diagnose on the bases of 4 major features (retinitis pigmentosa, obesity, kidney failure, cognitive disability) as well as retinal dystrophy. Severe Visual

impairment and 1 minor feature (brachydactyly) at the age of 49 whereas renal failure happens after 4 years of diagnosis (Scheidecker *et al.*, 2014).

BBS19

Bardet-Biedl syndrome-19 (BBS19) is due to the homozygous mutation in the *(BBS19)* gene. Chromosomal location is 22q12. There was a consanguineous Saudi family who were showing the phenotype of BBS brother and sister (Aldahmesh *et al.*, 2014) phenotypes were similar in both sib i-e obesity, mild intellectual disability, brother have polydactyly of all extremities whereas in sister have a polydactyly of 3 limbs ,borderline renal failure, retinitis pigmentosa, and brother was affected with hypogonadism. Molecular genetics prove that this is due to the homozygosity for a missense mutation at a highly conserved residue of *IFT27* (Aldahmesh *et al.*, 2014).

BBS20

Bardet-Biedl syndrome-20 (BBS20) is due to compound heterozygous mutation in gene known as *IFT74* gene located on chromosome 9p21. Transfer in autosamal recessive fashion from parents to offspring, one of the cases is reported with the phenotype of obesity, polydactyly, hypogonadism, intellectual disability, microcephaly, and retinitis pigmentosa. This is due to splice site mutation and the other was intragenic deletion in *IFT74* gene (Lindstrand *et al.*, 2016).

BBS21

The homozygous mutation in the *C8ORF37* gene cause Bardet-Biedl syndrome-21 (BBS21) gene position on chromosome is 8q22. *C8ORF37* gene mutation causes cone-rod dystrophy as well as isolated retinitis pigmentosa.

A case study of 17 year old girl daughter of a consanguineous parents, have phenotype similar to BBS syndrome i-e including slowly progressive rod-cone dystrophy, 3-limb postaxial polydactyly, obesity, mild learning difficulty, abnormally positioned uterus, and horseshoe kidney, included high myopia and elevated liver enzyme (Heon *et al.*, 2016). For molecular genetics Heon et al. perform the whole-genome sequencing and discover the homozygosity for a nonsense mutation in the *C8ORF37* gene (Hoen *et al.*,

2016). Another case was also reported a 6-year-old Saudi Arabian boy was diagnosis of BBS due to the phenotype i-e reduced and progressively worsening vision, and obesity. Chorioretinal atrophy consistent with myopia, speech delay, postaxial polydactyly of all 4 extremitie, dental anomalies, presence of 3 primary features of BBS (retinal dystrophy, polydactyly, and obesity) and 2 secondary features (speech delay and abnormal dentition). Next generation sequencing proves that this is due to homozygous for a missense mutation in the *C8ORF37* gene (Khan *et al.*, 2016).

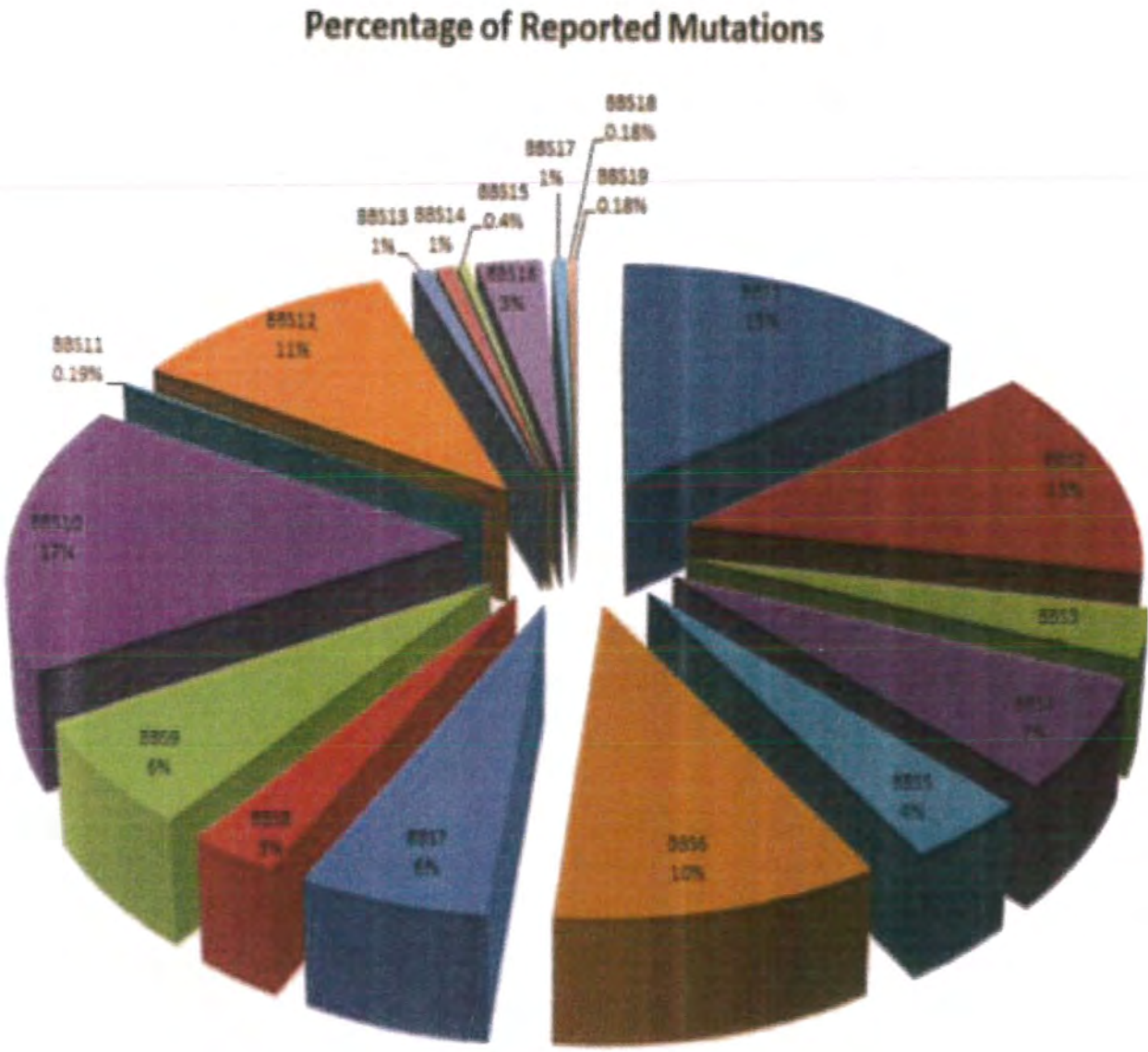


Figure 1.2: Mutation in BBS gene reported up till now (Khan *et al.*, 2016)

Ellis Van-Creveld Syndrome

Ellis–van Creveld Syndrome EVC also known as chondroectodermal or mesoectodermal dysplasia, EVC is one of rare genetic syndrome the common phenotypes are short limb dwarfism, postaxial and/or toes (polydactyly), abnormal development of fingernails and in many cases, congenital heart disorder whereas motor development and intelligence are normal. This is autosomal recessive syndrome (NORD national organization of rare syndrome).

100 (affected member) cases of this disease was first fully explained and reported by Richard W. B. Ellis (1902–1966) belongs to Edinburgh and Simon van Creveld belongs to Amsterdam in 1940, the prevalence of this disease is still unknown (Ellis and van Creveld, 1940) up till now about 50 of the cases have been reported in the literature. EVC s more common among the Amish community in Lancaster Country, Pennsylvania, US reported by McCusick he observed the largest pedigree of EVC among Amish population so far (McCusick *et al.*, 1964).

Clinical Description

People who are affected with Ellis-Van Creveld syndrome have multiple effected organs; have short arms and legs whereas head and trunk are normal. Polydactyly mostly hexadactyly are seen in all patients mostly both hands are affected. EVC patients also show the phenotype of Ectodermal abnormalities i-e abnormal growth and development of hairs, nails and teeth. EVC phenotype is variable and affects multiple organs (Mac Kusick, 2000). Prenatal abnormalities may be discovered early with in 18 week of gestation contains narrow thorax, extreme shortening of the long bones, hexadactyly (polydactyly) of hands and feet as well as cardiac abnormality(Horigome *et al.*,1997). Increased fetal nuchal translucency increased thickness during first trisemester is association with ECV (Venkat *et al.*, 2005). About 50%-60% (Blackburn *et al.*, 1991) patient affected with EVC have congenital heart malformations consist of single atrium, of the mitral and tricuspid valves, patent ductus, ventricular septal defect, atrial septal defect and hypoplastic left heart syndrome. These abnormalities help in diagnoses of EVC syndrome. The presence of congenital heart disease may support the diagnosis of

the EVC syndrome and appears to be the main determinant of longevity (Digilio, 1999). These additional findings may include polycystic kidneys, underdevelopment (hypoplasia) of the lungs, vertebral, genitourinary abnormalities, central nervous system abnormalities, cleft lip and cleft palate.

Some boys affected with EVC also shows the phenotype of undescended testicles (cryptorchidism) or an abnormal opening of the urine canal in the penis (epispadias) whereas defect in the chest wall, respiratory system and spine is also observed in EVC. The oral symptoms spectrum is wide which includes malocclusion (misalignment of teeth between the two dental arches which reaches to each other when the jaws are closed) gingival hypertrophy, labiogingival adherences, gingval hypertrophy. Abnormal teeth diagnose after birth, or exfolliate prematurely (Winter *et al.*, 1967).

Large number of affected member by EVC syndrome belongs to Pakistani family was first analyzed by kalsoom *et al.*, 2010 showed the five missense mutation in *EVC* gene. The novel case was reported in family (consanguineous family) of Pakistan, was showing the additional phenotype i-e profound deafness. Whole exome sequencing (WES) represented two homozygous mutation in genes *EVC2* (c.30dupC; p.Thr11Hisfs*45) and *TMC1* (c.1696-1G>A). WES of another family (non consanguineous family) belongs to Pakistan showed compound heterozygous novel mutation in *EVC* gene (p.Ser307Pro, c.2894+3A>G) (Umair *et al.*, 2017)

Inheritance Pattern

Ellis-Van Creveld autosomal recessive genetic disorder means the affected person inherit 2 abnormal copies of gene from both of parents. Inherit one affected copy of gene from their parent means he/she will be a carrier for the disease. There are 25% chances that the individual may be affected if both parents are affected similarly there are 25% chances that individual may be normal of both carrier parents but 50 % chances are there that offspring may be carrier of 2 carrier parents with each pregnancy. The chances are same for males and females. This disorder is more common in the offspring close relatives (consanguineous) parents.

Roie of *EVC* genes

As discussed before that Ellis Van-Creveld syndrome is one of rare skeletal disorders belongs to the ciliopathies group. These disorders are characterized by growth deficiency resulting in short stature, abnormally short ribs, extra fingers and toes (polydactyly) and variable visceral manifestations.

EVC/ EVC2

EVC is one of ciliopathies group of disease in which the genes which help in cilia formation are mutated or unable to perform function properly; these cilia are present on many cells which perform many functions. The deformities in cilia have numerous affect on signaling pathways results in multi-symptom syndrome. Ciliopathies group include polycystic kidney, primary ciliary dyskinesia, liverdisease, nephronophthisis, Bardet–Biedl syndrome, Alstrom syndrome, Meckel–Gruber syndrome and few forms of retinal degeneration (Badano *et al.*, 2006).We do not know too much about *EVC, EVC2* proper role and function in body. *EVC* genes product play main part in cell-to-cell signaling throughout the development. The product of the EVC gene helps in the regulation of the Sonic Hedgehog signaling pathway in developing embryo. The abnormal regulation of Sonic Hedgehog signaling pathway is one of the reason which result in abnormal formation and development of teeth and long bone. This signaling pathway plays major role in cell shaping (differentiation) cell growth, cell specialization of many parts of the body (Ruiz-Perez *et al.*, 2003).

Gene Involved In EvC

Up till know there are 2 gene reported in which the mutation cause Ellis-Van Creveld syndrome located on chromosome 4 called *EVC* and *EVC2*. The mutation in these genes causes abnormal synthesis of EVC and EVC2 (small) proteins. There are also such cases in which individual are affected with EVC syndrome but *EVC* and *EVC2* gene are normal indicating that there are also some unknown gene which cause EVC.

EVC and EVC2 both are the type 1 transmembrane proteins (single pass). Both genes are important co-work with each other. They are in a ring-like pattern with the ciliary transition zone, they are playing role of protein barrier between the ciliary and plasma

membranes. EVC and EVC2 use to transfer the extracellular signals to the nucleus through the hedgehog signaling pathway (Dorn *et al.*, 2012).

Gene Structure

The *EVC* gene consist of 21 exons and chromosomal position is 4p16 (Ruiz-Perez *et al.*, 2000), it seems that the *EVC* and *EVC2* genes are the nearest gene to each other, one after other in a head-to-head configuration. 2,624 bp are present between EVC and EVC2 in the human who separate the transcription site and 1,647 bp in mouse (Ruiz-Perez *et al.*, 2003). In situ hybridization and immunofluorescence studies in mouse tissues proves that EVC and EVC2 play major role in heart development i-e mesenchymal structures of the atrial septum and the atrioventricular cushions (Sund *et al*, 2009).

Acromesomelic Dysplasia

Acromesomelic dysplasia is an autosomal resessive inherited disease escalating skeletal disorder which results in a specific form of short stature (Haliloglu *et al.*, 1999) distinguish by dwarfism associated with deviation of middle and distal segments of the extremities (Demirhan *et al.*, 2005). AMD occurs both in syndromic as well as non syndromic forms. In the syndromic case, it is associated with genital, cardiac, respiratory, and neurological abnormalities (Kurt *et al.*, 2013)

Acromelia means the shortening of the bones of the hands and feet where as the meaning of mesomelia is the shortening of the bones of the lower legs forearms relative to the upper part of those limbs. So the short stature of affected individuals is the result of abnormal shortening of bones of the lower legs and unusual short arm. The very short hands, feet, fingers, and toes are initial finding .These findings are clearly appear during the first years of life (NORD national organization of rare disease, 2013).

Clinical Description

Inhibition of growth of certain long bones also occurs in acromesomelic dysplasia (i.e. bones of the forearms and lower legs) which results in having unusually short forearms, lower legs and short stature in affected individuals know as short-limbed dwarfism. Affected neonate may have normal birth weight but in many cases it is observed that in addition to broad short hands and feet infants may have facial abnormality. These findings typically become apparent during the first years of life. Abnormal development of bones and cartilage also damage bones of feet and hand (i.e. metacarpals, phalanges, metatarsals) the progressive abnormal development of bone and cartilage in the hands and feet i-e the bones within the fingers and toes (phalanges), as well as in the body of the hands (metacarpals) and feet (metatarsals), become shorter and broader during the initial years of life. During the second year of life, the rounding ends of these bones (epiphyses) often begin to appear abnormally shaped as square or a cone and often prematurely fuse. The fingers and toes appear short and stubby (brachydactyly); .the greater toe is relatively larger than other (brachydactyly) where as the hands, feet, figure and toe nail often seem unusually broad, square and short, and the feet may appear abnormally flat in many individuals. In early childhood, extra, loose (redundant) skin may develop over the fingers. Acromesomelic dyplasia features include a relatively enlarged head (macrocephaly), unusually prominent forehead (frontal bossing), and pronounced back portion of the head (occipital prominence); a slightly flattened midface; and/or an abnormally small, pug nose (NIH/National Institute of Arthritis and Musculoskeletal and Skin Diseases, 2014).

During the first years of life, distal limbs do not use to grow proportionally with the rest of the body. Due to abnormal development and premature fusion of the growth portions and the shafts of the long bones of the arm, the bones on the outer aspect and the thumb side of the forearm (ulna and radius, respectively) may be markedly shortened (hypoplastic) and abnormally curved. As well as, the

end portion of the radius which normally meets with the long bone of the upper arm humerus to form part of the elbow joint) is often completely or partially dislocated (subluxation) is known as Madelung deformity. As a result, individuals mostly not able to fully extend their arms, rotate the arms so the palms face down (pronation), or rotate their arms so the palms face upward (supination). Affected individuals often experience progressive degeneration, tedness, pain and stiffness of the elbows known as osteoarthritis (European Skeletal Dysplasia Network, 2015).

Types of Acromesomalic Dysplasia

1. Acromesomelic dysplasia, Osebold-Remondini type
2. Acromesomelic dysplasia, Maroteaux type
3. Grebe dysplasia (including Hunter-Thompson type)
4. Acromesomelic dysplasia with genital anomalies
5. Fibular hypoplasia and complex brachydactyly (Du Pan syndrome) Grebe dysplasia (including Hunter-Thompson type)

The most common are Grebe, Maroteaux, Hunter-thompson type are discussed below

Table 1.1: Types of Acromesmelic Dysplasia.

Types of Acromesomelic dysplasia		Clinical diagnoses	Radiological diagnoses	Participant Gene
GREBE Gene frequency of disease is still un known , Most common in brazil , gene frequency 1/50 and prevelance is 1/2000 live birth (D-valeria *et al* .,2003)	Average Adult height	100 cm		GDF5 & BMPR1B
	Bones Distal (bones) limbs:	Mostly polydactyly, very short hands having finger toe-like, lack of metacarpophalangeal joints, valgus disfiguration of feet, hexadactyly	fused tarsals and metatarsals, fused carpals and metacarpals, lack of exclusive metacarpal	

Types of Acromesomelic dysplasia		Clinical diagnoses	Radiological diagnoses	Participant Gene
			and metatarsal bones, no movement of toes, lack of middle and proximal phalanges	
	Proximal bones of the limbs:	Legs are highly affected than arms, Severe reduce and disfiguration femoral and tibial bones	reduce femoral neck, lack of fibular and tibial diaphyses, hypoplasia of the ulna, malformed radial head	
	Axial Skeleton:	Normal	Vertrebrae bones are normal,	
			appearance of face is normal, normal intelligence and head circumferenc e	
	Other		Normal	
HUNTER–THOMPSON	Adult Average Height	100–130 cm		GDF5
	Distal (bones) limbs:	Hands are comparatively less affected than feet. Feet are small and averted with globular toes. distal phalanges are normal	Severely short metacarpals and phalanges, phalanges are nearly square in shape, 5	

Types of Acromesomelic dysplasia		Clinical diagnoses	Radiological diagnoses	Participant Gene
			digit-Single phalangeal bone, carpal bones with abnormal shaped	
	Proximal (bones) limbs:	dislocated joints, Proximal to distal shortening	Short humerus, Hypoplastic femoral condyles, Curved radius with dislocated head and ulna, short femur, tibia and fibula along with dislocated ankle	
	Axial Skeleton:	Normal		
MAROTEUX	Average Adult Height	120 cm		NPR2
	Distal bones of the limbs:	Small and wide fingers without fusions	Small and wide phalanges, metacarpal and metatarsal bones	
	Proximal bones of the limbs:	Reduced middle and distal segments of proximal bones	Ulna is smaller than radius, bending of the radius	
	Axial Skeleton:		Squeezing of vertebral bodies with	

Types of Acromesomelic dysplasia		Clinical diagnoses	Radiological diagnoses	Participant Gene
			the dorsal margins being shorter than the ventral margins	

Acromesomelic Dysplasia, Maroteaux Type

The compound heterozygous or homozygous mutations in the *NPR2* gene cause AMDM. NPR2 produce protein known as natriuretic peptide receptor B, located on chromosome 9p13. AMDM is an autosomal recessive disorder describe by serious dwarfism (height less than 120 cm) as well as the shortage of the middle and distal portion of the limbs. These anomalies can easily diagnose after birth and becomes more prominent in the starting 2 years of life. X-rays studies represent short broad fingers, square flat feet, and shortening of the long bones (specially the forearms), the dolichocephalic skull is there with short trunk. The radius is curved whereas ulna is smaller than the radius. The narrowing and decreased height of the lumbar interpedicular distance in the vertebrae is frequently observed whereas there is no impact on Facial appearance as well as intelligence level (Faivre *et al.*, 2000). The aspect of skeletal growth in AMDM shows that the length, breadth and weight of the bone are normal whereas clinic and radiographic examination shows the minute reduction of long bones in some affected infants. Not possible to diagnose newborns by radiograph because it do not expose the anomaly of bones or abnormal growth plates. AMDM patient rapidly falls off of skeletal growth after birth, so that why we are easily able to diagnose AMDM in patients at the age of 2 have abnormal growth plates and short, misshapen bones in the limbs and spine. AMDM affected baby lengths only within 2 SD of the mean (round about equal to normal), whereas adults with AMDM have heights that are more than 5 SDs below than mean (comparatively very small height). There are also the damaged organ system with

this disorder, whereas the carrier parents either have less height as compare to normal but organ system are normal (Bartels *et al.*, 2004).

NPR2

NPR2 consist of 22 coding exons of 16.5 kb. The 5-prime flanking region do not contains TATA box consist of10 potential SP1 (binding sites) a microsatellite repeat CA/GT present in Intron 2 (Rehemudula *et al.*, 1999). Genomic analysis of DNA from somatic cell hybrids by PCR assigned the *NPR2* gene to 9p22-p11 (Lowe *et al.*, 1990), in situ hybridization further narrowed its localization to 9p21-p12. The mutation in *NPR2* results in AMDM. The *NPR2* which transcription results in homodimeric transmembrane receptor natriuretic peptide receptor B

produce guanylate cyclase that involves in the production of cytoplasmic cyclic GMP from GTP by bind with its extracellular ligand CNP (C-type natriuretic peptide) by the studing the role of CNP in transgenic and knockout mice it is observed CNP involved in the regulation of skeletal growth (Miura *et al.*, 2014).

DNA sequencing of twenty one affected families due to AMDM revealed 4 nonsense mutations, 4 frame shift mutations, 2 splice site mutations, and 11 missense mutations in the *NPR2* gene (Bartels *et al.*, 2004). It is also observed that the carrier of NPR2 mutations had also small heights below the mean (Bartels *et al.*, 2004). By the homozygosity mapping, linkage was established to *NPR2* in Pakistani family affected by AMDM. DNA sequencing revealed 2 novel missene mutation in Pakistani (a. b) families (p.Arg601Ser; p.Arg749Trp) and (c.2986+2T>G) in the third family (which was reported before) (Irfanullah *et al.*, 2015). Homozygous mutation was also reported in AMDM affected patient due to pathogenic variant in 7th exon of *NPR2* gene c.1435C>T p.R479X (Sag *et al.*, 2015). Sequence missense mutation in *NPR2* gene (p.T907M) and splice donor site mutation c.2986 + 2 T > G also causes AMDM. This pathogenic variant was first reported in five families which were affected with AMDM of Pakistan (Khan *et al.*, 2012).A deletion frameshift mutation (p.Cys586Ter), nonsense mutation (p.Arg479Ter), missense mutation (p.Val187Asp) in *NPR2* gene was reported in

different indian families who were affect by AMDM and one reported missense mutation (p.Tyr338Cys) (Srivastava *et al.*, 2016)

BMPR1B

BMPR1B belongs to the bone morphogenetic protein (BMP) receptors, BMP are a transmembrane family of serine/threonine kinases that contain 2 receptor , type I and type II receptors BMPR1A and BMPR1B are type I receptor whereas type II receptor is BMPR2 These receptors closely resemble to the activin receptors *ACVR1. BMPR1B* encodes 502-amino acid long polypeptide that includes an intracellular serine/threonine kinase and singal transmembrane domain. The chromosomal location of *BMPR1B* is 4q22.3 (Ide *et al.*, 1998). Its mutation leads to Acromesomelic dysplasia, Demirhan type, Brachydactyly, type A1, D, Brachydactyly, type A2(https://www.omim.org/)

GDF5

GDF5 stand for growth differentiation factor 5 also known as *OS5; LAP4; BDA1C; BMP14; CDMP1; LAP-4; SYM1B; SYNS2.* The chromosomal location I 20q11.22 and consist of 5 exon. *GDF5* gene translation result in ligand of the protein super family transforming growth factor-beta (TGF-beta). The Ligands binds with several TGF-beta receptors which result in activation of SMAD family (a transcription factors which help in recruitment of gene expression). The gene encoded protein cause the regulation of many developing tissue and cell type, including, joints, , teeth, cartilage, the growth of neuronal axons, brown fat and dendrites, the growth of neuronal axons and joints. Mutations in gene results in brachydactyly ,acromesomelic dysplasia, susceptibility to osteoarthritis, multiple synostoses syndrome, chondrodysplasia and proximal symphalangism (NCBI, 2017).

GLB1

GLB1 is about 62.5 kb consist of 16 exon (Santamaria *et al.*, 2007).The beta-galactosidase-1 enzyme is encoded by *GLB1.* A type of lysosomal hydrolase that the terminal beta-galactose from glycoconjugates and ganglioside substrates (Yoshida *et al.*, 1991). Beta-galactosidase also exists in a complex with (NEU1) neuraminidase and

cathepsin A (CTSA)/ protective protein, which is the part of several cell surface receptors (Hinek, 1996). The -related protein a 67 kD (S-Gal) of small protein related to short beta-galactosidase was identify as (EBP) elastin-binding protein, a major of surface nonintegrin cell receptor complex involved in expression in leukocytes, fibroblasts, chondroblasts, certain cancer cell types, and certain cancer cell types (Privitera *et al.*, 1998).

GALNS

GALNS known as glactoamsine-6-sulfate sulfatas, the chromosomal location is 16q24.3. *GALNS* gene consist of 14 exon and length is about 50Kb (Nakashima *et al.*, 1994) the promoter region of *GALNS* gene have GC instead of TATA box (Morris *et al.*, 1994). The mutation in GALNS results in Morquio A syndrome (not enough GALNS there use to recycle or break down of karatan sulfate). The waste that's collect inside the cell known as GAGS. Limiting GALNS cause the gathering of GAGS in organs, bones and other main organ of bodies' results in Marquio-A-Syndrome (https://www morquiosity. com/resources).

GHR

Growth hormone receptor (*GHR*), chromosomal location is 5p13-p12. *GHR* is one of the major genes which use to regulate signal transduction pathways, leads to anabolic and mitogenic response in growth. The binding of growth hormone and growth transmembrane receptor result s in activation of intracellular signal transduction pathways, which leads to the production of IGFI insulin like growth factor 1 and the IGFI binds to IGF1 receptor results in growth . *GHR* consist of 9 exons (87kb) encodes 246 amino acid (Godowski *et al.*, 1989). Mutation in *GHR* will result in small stature.

Three families (A, B, and C) of Pakistani origin were examined on clinical and genetic basis in this study. Family A showed the phenotypes of Acromesomelic Dysplasia Maroteaux Type (AMDM). Family B showed the phenotype of Bardet-Biedl Syndrome and family C depicted the characteristic features of Ellis-Van Creveld Syndrome (EvC). Microsatellite markers were used to analyze the linkage in the respective candidate genes. DNA sequencing was performed in family B and C after linkage was found on the

respective regions. Family A was excluded as none of the reported genes for autosomal recessive AMD. This study will provide knowledge about the molecular pathogenesis of these disorders. It will also help physicians in counselling the affected families in their future planning, rehabilitation, and treatment of their affected individuals as well as to avoid occurrence of these disorders in their future generations.

Materials And Methods

MATERIALS AND METHODS

Subject Study and Clinical Analysis

This research presents the clinical and molecular characterization of three families A, B and C belongs to remote area of Pakistan where the consanguineous marriages are very frequent. Affected member of family A showed the phenotype of Acromesomelic Dysplasia Maroteaux Type (AMDM) belongs to Khyber pukhtunkhawa (KPK). Affected members of family B depicted the characteristic features of Bardet-Beidl Syndrome belongs to District of Khairpur Sukkur Sindh whereas affected member of family C showed the phenotype of Ellis-Van Creveld syndrome belongs to village of Sindh. The common hereditary phenotype in all families was polydactyly (skeletal dysplasia). All families were completely and carefully examined i.e. age, height, weight, gender. Intelligence level, dimorphic feature, head circumference, and other related information that's appearing abnormal in the affected member was recognized and documented.

Ethical Approval

The study was initiated after obtaining approval from Institutional Review Board (IRB) of Quaid-i-Azam University, Islamabad, Pakistan. All the formalities and complete information relevant to three families was assembled before commenced of studies i-e Performa filling photograph of affected member, phenotype and pedigree construction.

Pedigree Designing

The information relevant to family i.e. affected member, marriage information, expired member (whether affected or not), number of sibling, medical history were required for the designing of pedigree. This information was collected after interaction with eldest (oldest) member of family who was able to clearly explain the genetic relation and inheritance pattern among families. Comprehensive pedigree was drawn after collecting complete information (Bennet *et al.*, 1995). The hallow circle and square were representing normal member of family. The circles in pedigree were representing females and the squares were representing the male. Crossed lines on symbols were representing expire members. Dot in symbols were representing the carrier. Filled

symbols (black) were representing affected individuals. The double lines among male and female were showing consanguineous marriage. Generation number were represented in descending order by Roman numerals and the individual order from left to right were showed by Arabic numeral.

Blood Sample Collection

Venous blood were collected from affected and normal member of family through 5-10 ml of air tight and sterilized syringes (BD 0.8 mm x 38 mm 21 G x 1 ½ TW, Franklin lakes, USA), the extracted blood were transferred to the vacutainer sets having potassium ethylene diamine tetra acetate (EDTA). Before the extraction of the DNA, the blood samples were stored at 4°C in Human Molecular Genetics lab, Department of Biochemistry, Quaid-i-Azam University Islamabad.

Extraction and Purification of Genomic DNA

Phenol- Chloroform Method were used (Manual Extraction Method) for the extraction of genomic DNA (Sambrook *et al.*, 1989). The blood samples stored at 4°C were first incubated for 1 hr at room temperature to settle all the blood components before starting genomic DNA extraction via phenol-chloroform method.

- Equal amount (750 µl) of solution A and human blood were taken in 1.5 ml of eppendorf tubes (Axygen, Union, USA).
- These eppendrof tubes were inverted several times for thoroughly mixing of solution A and human blood.
- Eppendrof were left for 40-45 minute at room temperature than eppendrof tube were centrifuged (Eppendorf, Hamburg, Germany) at 13,000 rpm (revolution per minutes) for 1 minute.
- After discarding the supernatant, the nuclear pellet was re-suspended in 400 µl of solution A, the mixture was centrifuged again at 13,000 rmp for 1 minute.
- The nuclear pellet was suspended in 400 µl of solution B, 9 µl of proteinase K (20 mg/ml stock) and 14 µl of 20% SDS after discarding the supernatant.
- The samples were placed at 37°C for overnight incubation (at least 12 hrs).
- On the next day (2nd day) equal volume of (0.5 ml) freshly prepared mixture of solution C plus D were added to the tube.

- The tubes were then subjected to centrifugation at 13,000 rpm for 10 minutes after being mixed thoroughly, the upper layer was separated by the help of 250 µl pipette and was collected in to new sterile microeppendrof tube.
- The tubes were centrifuged for 10 minutes at 13,000 rpm after addition of 500 µl of solution.
- Two visible layers were seen after centrifugation, the upper layer was collected into new microeppendrof tube.
- The DNA was precipitated by addition of 500 µl of iso-propanol (that was kept at -20°C) and of 55 µl of sodium acetate (3 M, pH 6).
- The tubes were inverted 5-8 times for ensure of DNA precipitation then tubes were again centrifuged for 10 minute at 13000 rpm.
- Supernatant was discarded carefully.
- 200 µl of chilled 70% ethanol (BDH, Poole, England) was added in the microeppendrof tube having DNA pellet, the tube were centrifuged for 7 minute at 13,000 rpm.
- The detachment of the pellet was avoided while discarding the ethanol.
- After discarding, the remaining ethanol was evaporated by putting the tube in a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany) for 5-10 minutes at 45°C.
- 120-140 µl of dissolving buffer (Tris-EDTA, Sigma-Aldrich MO, USA) was added to the dried DNA and stored at 4 °C The tubes were kept in incubater for 24 hr at 37°C for wholly dissolved of DNA pallet in buffer.
- On 3rd day the extracted genomic DNA was examined on 1 % agarose gel electrophoresis and visualized by the help of Ultra Violet Transilluminator (Biometra, Gottingen, Germany) and then stored at 4°C.

Composition of solution used in genomic DNA extraction

Table 2.1: Chemical composition of solution used in genomic DNA extraction

Sr-no	Solution use for DNA extraction	Chemical composition
1	Solution A	$MgCl_2$ 5mM Tris (p H 7.5) 10 mM 1% (v/v) Triton x =100 Sucrose 0.32 M
2	Solution B	NaCl 400 mM Tris (p H 7.5) 10 mM EDTA (p H 8.8) 2mM
3	Solution C	Saturated phenol
4	Solution D	24 volumes of chroloform :1 Volume of isoamylalcohol i-e 24:1
6	DNA Dissolving Buffer	10 mM Tris (p H 8.0) 0.1 mM EDTA
6	10% SDS	Tris (p H 8.0) 10 Mm EDTA 0.1Mm
7	Bromophenol blue	0.25 g bromophenol blue 40g Sucrose

Agarose Gel Electrophoresis

The DNA qualitatively was analyzing by 1% agarose gel. Gel was composed by putting 0.5 g (weighed with the help of electric balance) of agarose in 45 ml of DH_2O distilled water and 5 ml of 10 X TBE (0.89 M Tris-Borate, 0.032 M EDTA) in same beaker. Agarose was dispersed by heating the beaker in microwave until the transparent solution was obtained (1.00 minutes). Gently shake the beaker after adding 7 µl of ethidium bromide (stain the DNA bands) so that dye entirely mixed in the solution after that the solution was carefully poured into the gel tank. The gel was solidified within 20-25 minutes at room temperature. 5 µl of extracted DNA was mixed with 5 µl of loading dye (0.25% bromophenol blue with 40% sucrose) and then loaded into the gel wells. 1X TBE buffer was poured in the gel tank; the gel electrophoresis was run at 120 volts for 30-40 minutes than DNA bands was visualized under U.V Transilluminator (Biometra, Gottingen, Germany). The picture was captured with the help of Digital Camera EDAS 290 (Kodak, New York, USA) for thoroughly examination of the DNA bands.

Composition of solution use in gel loading

Table 2.2: Chemical composition of solution use to visualize the DNA on gel

Sr-no	**Solutions required for the visualization of DNA**	**Composition of solution**
1	10 x TBE Buffer	0.025 M Boric Acid EDTA 0.5 M(p H 8.3) 0.89 M Tris
2	Gel loading Dye	0.25 Bromophenol Blue 40g sucrose

Genotyping and Linkage Analysis

Highly polymorphic microsatellite markers were used for linkage analysis or genetic mapping. Linkage analysis of three families with hereditary Ellis-van Creveld syndrome, Bardet Biedl syndrome and Acromesomelic Dysplasia type Martoux was performed to find out the pathogenic variant in known gene/loci. The markers genetic map distance was obtained by the help of UCSC Genome browser and Rutgers's Combined Linkage-Physical Map (Matise *et al.*, 2007). Markers were obtained via Invitrogen Genelink (USA).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used for highly accurate amplification of specific DNA sequences. STS markers were used for the genotyping of DNA sequences. 0.2 ml volume of PCR tube (Axygen, California, USA) was used in which the total volume of reaction was set 25 µl. The reaction mixtures were composed of different chemical reaction plus 2 µl diluted DNA. 2.5 µl PCR buffer 10X (200 mM $(NH_4)_2SO4$, 750 mM of Tris-HCl pH 8.8, 0.1% Tween 20), a DNA sample dilution of 2 µl, 2 µl of $MgCl_2$ (25 mM), 0.4 µl each forward and reverse microsatellite markers (20 ng/µl), 0.5 unit Taq Polymerase (Perkin-Elmercetus, Ferments, Burlington, Canada) in amount of 0.4 µl and 0.5 µl of (10 mM) dNTPs (MBI Fermentas, UK) in 17.5µl PCR water. The reaction mixture was subjected to centrifugation at 4,000 rpm for 30 second in way to get complete mixing reaction mixture. T3 thermocyclers (Biometra, Gottingen, Germany) was used to accomplish the reaction with the following terms.

The standard thermo cyclic conditions included:

Initial step was denaturation of template DNA. PCR cycle begin with denaturation of DNA double stand at 96°C for 7 minutes, DNA product was obtain after 40 cycles of amplification, Which is further divided in 3 sub-cycles.

1 - Denaturation of DNA double stranded into DNA single stranded at 96°C for one minute,

2- "Annealing" or hybridization, attachment of microsatellite markers with their complementary sequences of DNA at a temperature of 57-60°C for one minute.

3-For cyclic extensions (The Taq DNA polymerase enzyme use to amplify the DNA sequence present between two primers) at 72°C for one minute.

Final extension or polymerization was accomplished at 72°C for 10 minutes to fulfill the left over amplifications.

Polyacrylamide Gel Electrophoresis (vertical gel)

The DNA product was observed on polyacrylamide gel 8% after the amplification. the gel was prepared by N, N' Methylene-bisacrylamide (BDH, Poole, England), 5 ml 10 X TBE (Tris 0.89 M, Borate 0.89 M, EDTA 0.02 M), 0.5 ml of 10% APS (Ammonium persulphate) (Sigma-Aldrich St Louis, MO, USA), 13.5 ml of 30% acrylamide solution comprising 29:1 ratio of acrylamide (MERCK, Darmstadt, Germany) and 20-25 ml TEMED (N, N, N', N'-Tetra methylethylene diamine) (Sigma-Aldrich, St Louis, MO, USA). All the chemicals solutions were putted together into the flask; the volume of the gel solution was raised up to 50 ml by adding distilled water. Two glass plates (spacers of 1.5 mm thickness were present between glass plates) and absolute wells were produce by pushing the combs between the two plates. After setting of glass plate the gel was poured in to the plates and then allowed the gel to solidify at room temperature for 30-40 minutes. Equal quantity of loading dye(40% sucrose with 0.25% of bromophenol) and amplified PCR product (4-5µl) were mixed and were loaded into the wells of gel, Vertical gel apparatus was used (Whatman, Biometra, Gottingen, Germany) for electrophoresis (2-3 hours at 140 volts). 1X TBE buffer (used as running media) was poured in to gel tank. After the complete running of sample on gel, the gel was removed from gel plate and was stained with ethidium bromide solution (10 mg/ml), for the visualization of DNA; the Ultra Violet transilluminator (Biometra, Gottingen, Germany) was used. The picture was captured by using digital camera EDAS 290 (Kodak, New York, USA) and haplotypes were assigned by visual inspection of the gel.

Composition of chemicals used in PAGE GEL

Table 2.3: Chemical composition of solution used in PAGE gel

Sr-no	Solution	Stock concentration and composition	Amount for

			1 gel
1	30% Acrylamide	N,N' Methylene- bisacrylamide (BDH, Poole, England) 29:1 ratio of acrylamide (MERCK, Darmstadt, Germany)	13.5ml
2	TEMED	N, N, N', N'-Tetra methylethylene diamine) (sigma- Aldrich, USA)	25 µl
3	10XTBE	Tris 0.89M, EDTA 0.02M, Borate 0.89	5ml
4	10%APS	Ammonium persulphate(5g/45 ml distill water)(sigma- Aldrich St Louis, MO, USA)	400µl
5	Distilled water		Up to 50ml of $D.H_2O$

Mutation Screening and DNA Sequencing

Family B and C showed linkage to *BBS6* located on chromosome 20p12.2, *EVC and EVC2* gene located on chromosome at 4p16.2, thus samples were subjected to Sanger sequencing. To find out disease causing mutation (pathogenic variant) we were required to sequence the exon-intron boundaries and coding exons of the gene. Specific primers were constructed by using intronic region. The sequences of the primers along with the temperatures at which they anneal to their complimentary sequences are given in the table 2.8, 2.9 and 2.10. Online primer 3 software (Rozen and Skaletsky, 2000) was used for designing of primer.

DNA Sequencing

The Standard protocol of DNA sequencing was pursued to figure out any pathogenic mutation.

First Sequencing PCR

All exons of *MKKS, EVC* and *EVC2* gene of affected individuals were amplified by using 50 µl reaction mixture, the chemical mixture was prepared by adding 5 µl of PCR buffer, 2.0-2.5 µl genomic DNA dilution, 0.4-0.6 µl of Taq DNA polymerase (one unit) (MBI Fermentas,

Burlington Canada, UK), 2.5 µl each of forward and reverse primer, 3-4 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs (MBI Fermentas, UK) and then raised the reaction volume up to 50 µl by using PCR water. These conditions (as mentioned earlier) were utilized for genotyping PCR. The PCR samples were visualized after amplification on 2% agarose gel (2g of agrose in 90 ml of $D.H_2O$ +10ml of10XTBE), equal quantity (4-6 µl) of the PCR products and Bromophenol blue were mixed together and were electrophorized on an agarose gel (2%) at 120 volts to check exon non-specificity and their band intensity. The chemical composition is mention in table 2.4.

First Purification of Amplified PCR Products

- When the Amplified PCR product showed single and clear band on agarose gel than it was purified by the help of Gene Jet PCR Purification Kit (Fermentas, USA).
- 120 µl of binding solution was mixed and vortexed with amplified PCR product for 1-

  2 minutes or the tubes were centrifuged for 30 seconds at 4000 rpm. The whole reaction was transferred to a purification column having silica based membrane and centrifuged for 1 minute at 13,000 rpm.
- Unreacted primers and waste off dNTPs were removed by treating with 600µl washing solution (H2) that contained H2 alcohol (Tris-HCl, NaCl and EDTA). This happened in micro centrifuge collecting tubes and than they were centrifuged for 1 minute at 13,000 rpm.

- Flow through was discarded from collected tube, the washing step was repeated again by adding 500 µl of washing buffer to remove the remained undesired product (1 minute centrifugation at 13,000 rpm).
- The flow-through was again discarded from collected tube, empty spin was done for 2 minute for 13,000 rpm and the membranes (having purified DNA) were shifted to 1.5ml micro centrifuge tubes.
- The purified DNA was eluted out by using 20-30 µl of elution buffer (10 mM Tris-HCl, 0.1 mM EDTA with pH 8.0) to the silica based column. The elution buffer must be preheated at 65°C before use.
- The samples were incubated for 5 min at room temperature, after that centrifuged for 2 minute at 13,000 rpm, purified product were collected and examined on 2% agarose gel.

Composition and concentration of chemicals used in PCR reaction

Table 2.4: Chemical composition concentration and quantity of solution used in PCR reaction

Sr-no	Required chemicals	Concentration of stock solution	The quantity used in PCR
1	PCR Buffer	750 mM Tris- HCL (pH 8.8) 0.1% % (Tween 20) 10 X (200 mM Ammonium Sulphate)	**2.5 µl**
2	MgCl2	25 mM	**2.0 µl**
3	DNTPs	10 mM each dNTPs	**0.4 µl**
4	Microsatellite Marker (R & F)	20 ng/µl	**Each 0.3 µl**
5	Taq	Polymerase 0.5 U/µl	**0.3 µl**

	Polymerase		
6	Genomic DNA	Dilution with PCR water (20%)	**2 µl**

Second Sequencing PCR

The PCR of the purified product was carried out with forward or reverse primer by adding 1 µl of Sequencing buffer, 2.5 µl of DTCS quick start kit, 1.5-2 µl of one out of both primers (F/R), 2-3 µl of template DNA and 3-4 µl of PCR water to make the total volume of 10 µl. These conditions were utilized for sequencing PCR.

- Initial denaturation for 5 minute at 96°C pursued by 29 cycles of denaturation for 20 seconds at 95°C of template DNA stand
- primers annealing for 20 sec at 57°C
- primers extension for 4 minutes at 60°C
- final extension for 10 minutes at 65°C

Second Purification of Sequencing PCR Products

- The PCR product purification was done by following ethanol precipitation protocol (POP6 Protocol).
- 70 µl 100% chilled ethanol, 5 µl stop solution (2 µl Sodium Acetate, 2 µl Sodium EDTA and 1 µl Glycogen) and10 µl PCR product was added to 1.5 ml eppendorf tube.
- The eppendorf tube was vortexed for proper mixing and then tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C in refrigerated centrifuge.
- Supernatant was discarded and pellet was washed by adding (150-160) µl 70% chilled ethanol, tubes were again centrifuged at 13,000 rpm for 7-8 minutes.
- The supernatant was discarded again, 130-140 µl of 70% chilled ethanol was added and the samples were centrifuged again at 13,000 rpm for 5-6 minutes.
- After that, supernatant was again discarded and the pellet was dried by using vacuum concentrator for 5-8 minutes at 45 °C to remove the residual ethanol.

- At the end, 15-16 µl of SLS (stored at -20 °C) was added to dry the pellet and vortexed gently.
- Then the sequencing of the purified product was carried out using DTCS Quick Start sequencing kit (Beckman Coulter, Fullerton, CA, USA) on CEQ8800 DNA sequencer (Beckman Coulter, CA, USA).

Mutation Screening

The result Chromatograms of both affected and normal individuals which were obtained from CEQ8800 DNA sequence (Beckman Coulter, USA) were compared with relevant control gene sequences from Ensemble Genome Browser database (http://www.ensembl.Org/index. html) to figure out any nucleotide change (mutation) present inaffected individual. BioEdit sequence alignment editor version 6.0.7 was used to figure out any mutation in the abnormal individuals and sequencing alignment results was performed by "Clustal W Multiple Alignment" tool.

Table: 2.5: List of microsatellite markers used for genetic analysis of AMDM loci

Sr-no	Candidate Gene loci	Chromosome Location	Markers	Distance (cM)
1	*NPR_2*	9q13.3	D9S169	51.71
			D9S1678	54.78
			D9S319	55.51
			D9S1118	57.1
			D9S1817	59
			D9S200	61.8
			D9S229	62.66
			D9S55	62.66
			D9S862	66.55
2	*BMPR1B*	4q22.3	D4S1534	98.67
			D4S2284	100.28
			D4S423	105.65
			D4S433	107.3
			D4S2909	107.35
			D4S1560	108.65
			D4S2986	109.89
			D4S2634	110.05
			D4S1572	113.05
			D4S1570	114.16

3	*GDF5*	20q11.22	D20S432	47.48
			D20S54	50.36
			D20S477	51.14
			D20S865	55.42
			D20S34	56.41
			D20S478	58.9
			D20S881	58.9
			D20S107	60
			D20S108	61.97
4	*GLANS*	16q24.3	D16S486	126.17
			D16S3077	128.98
			D16S3063	129.12
			D16S3123	129.12
			D16S3026	132.03
5	*GLB1*	3p22.3	D3S2432	57.43
			D3S3623	62.36
			D3S1260	63.37
			D3S3527	64.17
			D3S3521	64.17

			D5S577	64.49
			D5S418	64.56
			D5S856	65.07
6	*GHR*	5p13-p12	D5S430	65.19
			D5S822	66.83
			D5S660	66.83

Table 2.6: Microsatellite markers list used for genetic analysis of (family B) BBS loci

SR-no	Candidate gene/loci	Chromosomal Location	Markers	Distance (cM)
1	*BBS3* *ARL6*	3q11.2	D3S3633 D3S2392 D3S2386 D3S1536 D3S2419 D3S3655 D3S1753	108.97 109.62 110.69 110.75 112.38 113.56 113.56
2	*BBS5*	2q31.1	D2S2302 D2S2370 D2S1281 D2S294 D2S1274	156.96 169.3 175.37 177.18 182.19
3	*BBS6* *MKKS*	20p12.2	D20S602 D20S851 D20S188 D20S66 D20S1143	23.25 28.32 32.07 37.86 15MB

			D4S2945	119.57
			D4S1651	120.94
			D4S2937	122.7
			D4S2297	123.65
4	*BBS7*	4q27	D4S1522	125.35
			D4S2392	125.36
			D4S191	125.89
			D4S3024	126.56
			D4S427	127.68
			D4S1612	128.25
			D4S2394	133.28
			D14S616	81.65
			D14S128	82.69
			D14S612	83.66
			D14S1066	87.16
5	*BBS8*	14q31.3	D14S1044	88.33
			D14S553	95.43
			D14S749	95.57
			D7S2525	41.05

6	*BBS9*	7p14.3	D7S2449	41.30
			D7S2564	42.92
			D7S2440	42.92
			D7S1808	42.92
			D7S2515	44.58
			D7S2496	47.04
			D7S817	50.85
7	*BBS10*	12q21.2	D12S1722	86.22
			D12S2206	88.05
			D12S1684	90.98
			D12S326	92.06
			D12S293	95.15

Table 2.7: Microsatellite markers list used for genetic analysis of (family C) EVC loci

SR-no	Candidate gene/loci	Chromosomal location	Markers	Distance (cM)
1	*EVC and EVC2*	4p16.2	D4S1614	2.86
			D4S127	3.6
			D4S3034	3.6
			D4S179	3.99
			D4S2957	5.72
			D4S3023	6.54
			D4S2925	7.17
			D4S2375	7.26
			D4S2285	7.97
			D4S3007	13.21
			D4S394	14.94

Table 2.8: primer used for the amplification of *MKKS* gene exons

Exon	Forward Primer 5′ 3′	Reverse Primer 5′ 3′	Tm
1A	GCCACAATGCTGCATATTCA	GGTTGTCGAATCCCAGTGG	57
1B	CAGCTCTGCTCAGTCACC	CGTCAAAGAGTTATAGATTCCAC	57
1C	GGTTGTCGAATCCCAGTGG	GACCCAGCATGTCAGTATCA	56-57
2	ATGGTTGTTTGCTCCACT	TCCCTAGGTAGTATAGAAAGCA	57
3	CACTATGATGCTTTGAGAGC	ACCTGAATTCTCAGCAACT	57
4	CCAACCTAGTTAAGGGTTTGC	ACTAGTCTGTTGGCAATTGAGA	56-57

Table 2.9: Primers used for the amplification of *EVC* gene exons

Exon	Forward primer 5′ 3′	Reverse primer 5′ 3′	Tm
1	AGTTTTGAGCGGTGATCCAG	AACGGAGTGGGATGTAACAG	57
2	CAAGCTTGAGAAGCACAGAG	GAGGCTTGTCTACATGACAC	57
3	CCTTTCTGAGGCTGCTATTAC	CAGGACCAAACATCACACAG	57
4	CTAGCGTGAATCACTGGTAG	CCTCCTCTGACAATGATGTG	57
5	CTGTGGCTTGTACTGATGAG	ACTGATGTCTGCCTCTCAAG	56-57
6	TCGGAGTTCCTTTCCTTCAG	CAGTGACCGTCTTTGAGTAG	57
7	GGTCAAACCTCATGTACAAC	TAGCATCAGAAACTGGATCC	57
8	GAACCAGACAATGGAACCAG	GATGGATGGAAGGGTCAATG	56
9	AGATCTCGCTCATGAAGGAG	GTACAGAGAAGGGAAGAGAG	56

10	ACTACTAGTGGGACCTACAG	GCAGCACAACTCTTATCACC	56
11	TGCCTCAGTTTCCTTGTGTG	AACTGCCTCGGTTTCTGATG	55
12	CTTGAGAGTTCCTGTCTGTG	CAACAGTCATCTCCGTAGAG	55
13	ATCCCTTCCTTACTCCACAG	CACTGTACAGATGAGGACAC	57
14	AATAGACAAGCAGATGGCGG	ATGTGTGCAGTGTAAGCTGC	57
15	CACAGACACCTAACAGACAC	AAGTCATGGAGAAGTCAGGC	56-57
16	TGGTGAGTAGGTGGAAGATC	CACTGGATGTGTGCTTATGC	56-57
17	GGTGGAAGGATACACTCTTG	TCACTCATTTGTGCCAGGTG	56-57
18	CTCTCTAAACTCCCGTGTTG	TGTCAGCTCCATAGATGAGG	56-57
19	CTCTCTGGGATGTATGTTGG	AGTCAGTTAGGAGACACAGC	57
20	AGTTGAGTGGCTGCAATAGC	AAACAGGAAGGGTAACAGCC	57
21	CACTTGGATGACCTCCTACC	TTCTCTGTCCCTCTCAGAAG	57

Table 2.10: Primers used for the amplification of *EVC2* gene exons

Exon	**Forward primer 5'-3'**	**Reverse primer 5'-3'**	**Tm**
1	ATGTCCTCTCCATGTGAGTC	ACTGCCCTAGTTCTGTAAGG	57
2	TCCATGTACCCTGTAGTCTG	AGTCAAACCTCCTCTCCATC	57
3	TGGTGTAAGCTCATGTCCAC	AGACTTGTGTAGGTAAGCCC	56-57
4	TTCCTGGATGCCTAATGTGC	CTGACCCAGAACACATTCAC	56-57
5	TACAAAGGGCTTCCTTGCTG	CTTATCTGCTAGGTGGCATC	56-57
6	TTCACAAGCTCTGACCTGTG	CCAGTCTCAACGCATAACTG	55
7	GACTCAAATGCAAGCGAGT	GTTCTGTGATTGTGCCTAGG	55
8	TTACTGTGGTCACAGTGGTG	GACTTCCTTAGAGCAGGAAG	56
9	GGATGAAGCAGAAGTGAGC	AATGAGTGGGTGGTTGGATG	57
10	GCCTGTACACATTGCATTGC	AGGACTGAACTCTGAGAGAG	57
11	CATCTGGGTCTAGAATGCAC	CCAGTCTGTGGTATTCTGTC	57
12	GGAGATGAATGTTCAGGCA	TAGATGGCACATCATGGTGC	56
13	TGGGCAACAGAGCAAGATT	CGCTGGTAATCTCATCTGTC	55
14	TGCATCTCTGGCTTCTTCAC	GAATAGCTGGACACCAATGC	55
15	AACTGAGCTCCTTCCAGAAG	TGTGTCAGCTATCAGAGCAG	57
16	ATGGAATGAAGGATGGCTG	GTTGAGAGAGTGCCAAGTAC	57
17	ATCGTGGTGCACTCACATTG	AGCTCGTGTTGGAGCAATG	57
18	ATGGCTGAGATGTGTGCTTC	TAGAGCTTCGCACACATCTG	56

19	TCACTCCAGGACAGAATGTC	AACACATGCTCCCACACTTG	56
20	TCTTGAACTCCTGACCTCAG	TGTCTGTTTGCAGCAACCTC	55
21a	ACAGCAATCTGGTGACCAA	TCTGTGCCTTCTGCATGTGC	57
21b	GGAGCATCTGAAGAGAGAA	TTGAACTGGTGACGGTAGAC	57
22	TGTTTATGGAGTGCTCCTGC	ATTACGCAAACAGAGGCTCC	57

Results

RESULTS

Family Description

Family A

Family A (Fig 3.1) belongs to District Lakki Marwat, Khyber pukhtunkhawa (KPK) province of Pakistan. The pedigree was constructed after complete investigations with family elders to avoid any error (providing information and history relevant to inherited disease). IV-2 member (male) of family A was affected and rests of them were normal. The parents and grand parent of affected individual were also analyzed very carefully. The parents were normal but one of their children (IV-2) showed 120cm height, short stature associated with deviation of middle and distal segments. Teeth abnormality, swollen knew/joint, pigeon shape chest and facial abnormality was also observed. The greater toe was relatively larger than other whereas the hands, feet, toe nail often seem unusually broad, square and short. The feet appeared abnormally flat, enlarged head, frontal bossing, occipital prominence, a slightly flattened midface, and an abnormally small, pug nose (Figure 3.2) which suggest that AMDM (Acromesomalic Dysplasia Maroteux type syndrome) in this family has autosomal recessive mode of inheritance. The blood samples were taken from three member of the family (III-1, III-3, IV-2). III-1 and IV-2 were male, while III-3 was female where as two (III-1, III-3) were normal and one (IV-2) was affected. The genomic DNA was extracted from blood by standard Phenol-Chloroform method.

Family B

Family B (Fig 3.3) belongs to Ghot Khki, Tehsil Sobodehro, and District of Khairpur Sukkur Sindh (remote area of Pakistan). Consanguineous marriages are very common in this area of Pakistan. The family contains sixteen members. The pedigree was constructed after discussion with elders and is representing four generation of family consisting of ten males and six females. Four member of family B were affected, one of the four affected

member (III-1) was expired and one of four affected member shows mild mental retardation (IV-I). Discussion and clinical finding revealed that members of family B are affected by BBS syndrome. The parents were normal but two of their children (IV-4, 1V-5) had phenotype such as post axial polydactyly (bilateral) in hands and feet, dental anomalies, speech deficits, weaken eye sight, obesity, neurological, mental retardation, facial dysmorphism, behavioral traits, and nystagmus. Analysis of pedigree suggests that BBS in this family has autosomal recessive mode of inheritance (Figure 3.4, 3.5). Blood samples were taken from four members (III-5, IV-3, IV-4, and IV-5) of family. Two (III-5, IV-3) were normal while two (IV-4, IV-5) were affected member.

Family C

The family C (Fig 3.6-3.7) belongs to village Sormer Khan Kalar of Sindh province of Pakistan. The pedigree was constructed after gathering complete information from the elders of family. The pedigree is representing four generation including four females and six males. The clinical findings revealed that affected member of family C was affected by EvC syndrome, the phenotype showed by affected individual post axial polydactyly (bilateral) in hands, syndactyly in feet, dental anomalies, short stature, nail dysplasia (Fig 3.7). The parents were normal but one of their children (III-1) showed phenotype of EvC which suggest that EvC in this family has autosomal recessive mode of inheritance. (Fig 3.6). The affected member of family C was label as III-1, and rest of the members of family were normal. The blood was taken from three member of family. Two (II-2, III-3) normal while one (III-1) was affected.

Genetic Mapping of Candidate Genes involved in Hereditary Skeleton Dysplasia

In this study, all families (A, B, C) were analyzed for linkage to the known gene or loci for skeleton dysplasia by the help of mapping technique (genotyping microsatellite markers) within the candidate interval.

Microsatellite markers for genotyping were selected on the bases of similar region to candidate genes. Genotyping was carried out using 5 to 10 microsatellite markers. Analysis of the microsatellite markers was performed using standard polymerase chain reaction and electrophoresis on 8% non-denaturing polyacrylamide gel as discussed in materials and methods. Amplified PCR products were visualized by staining the gel with ethidium bromide and genotypes were assigned by visual inspection (Materials and Methods; chapter 2). These candidate genes *NPR2, BMPR1B, GDF5, GALNS, GLB1, GHR, BBS2, BBS3, BBS4, BBS5 BBS6, BBS8, BBS7, BBS9, BBS10, BBS11, BBS12, BBS13,* and *EVC/EVC2* were tested for linkage analysis of family A, B, C. The microsatellite markers list used for the genetic analysis and chromosomal location are mention in Table 2.5-2.7. The banding pattern of normal and affected individual was visualized very carefully, the family was considered to be linked if all the normal individual of a family showed heterozygous banding pattern whereas the affected individual of family showed homozygous banding pattern of same markers, if all are showing same banding patterns means no linkage and candidate gene is normal in both affected and normal individual of family.

Mapping Genes Involved in Acromesomalic Dysplasia Type Maroteaux

Family A

Haplotypes (genotyping) results of family A did not show any linkage to the known genes including *NPR2* at chromosome 9p13.3, *BMPR1B* at chromosome 4q22, *GDF5* at chromosome 20q11.22, *GALNS* at chromosome 16q24.3, *GLB1* at chromosome 3p22.3, *GHR* at chromosome 5p13-p12 (Table 2.5). This indicates involvement of a novel gene in causing hereditary AMDM in this family the haplotype results are shown in (Figure 3.8-3.13)

Mapping Genes Involved in Bardet-Biedl Syndrome

Family B

In family B four DNA samples, including two normal (III-5, IV-3) and two affected (IV-4, IV-5) were tested for linkage by typing microsatellite markers for respective candidate genes (**Table 2.6).** The family B was tested with the reported gene causing BBS i.e. *BBS3* at chromosome 3q11.2, *BBS5* at chromosome at 2q31.1, *BBS6* at chromosome 20p12.2, *BBS7* at chromosome 4q27, BBS8 at chromosome 14q31.3, *BBS9* at chromosome7p14.3, *BBS10* at chromosome 12q21.2. Haplotypes analysis of family B showed linkage to the *BBS6 (MKKS)* locus on chromosome 20q12.2. Five markers D20S602, D20S851, D20S188, D20S66, D2OS1143 results represented heterozygous pattern in normal individual while homozygous pattern in affected individual of family B. Haplotypes of family B is shown in (Fig 3.14).

Mapping Genes Involved in Ellis-Van Creveld

Family C

Family C genotyping shown to be linked on *EVC* gene (*EVC and EVC 2*) both genes are very close to each other *EVC/EVC 2* both are present at chromosome 4p16.2. The family C was mapped by using this marker D4S1614, D4S127, D4S3034, D4S179, D4S2957, D4S3023, D4S2925, D4S2375, D4S2285, D4S3007, and D4S394 (Table 2.7). The markers D4S179, D4S2957 haplotype results represented heterozygous pattern in normal individual while homozygous pattern in affected individual of family C (Fig 3.17).

Sequencing Analysis of *MKKS*

Family B was linked to *MKKS* gene at chromosome 20p12.2 and subjected to Sanger sequencing. All four coding exons of *MKKS* were sequenced. Sequence analysis of *MKKS* gene revealed a novel deletion mutation (c.775delA; p.Thr259Leufs*21) in exon1. The deletion of A caused

frameshift due to which Threonine at position 259 was replaced by leucine T259Lfs*21 and further changes occur in downstream region results in abnormal formation of MKKS protein (Fig 3.16).

Sequence Analysis of *EVC/EVC2*

After establishing linkage in family C to *EVC/EVC2* gene was then followed by Sanger sequencing 18 exons of (2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21) out of 21 coding exons of *EVC* and 16 (1, 2, 3, 4, 5, 6, 7, 10, 13, 14, 15, 16, 17, 18, 20, 21) out of 22 exons of *EVC2* to hunt down a pathogenic mutation. No disease causing DNA sequence variant was found in these exons suggesting the presence of mutation in rest of the unsequenced exons.

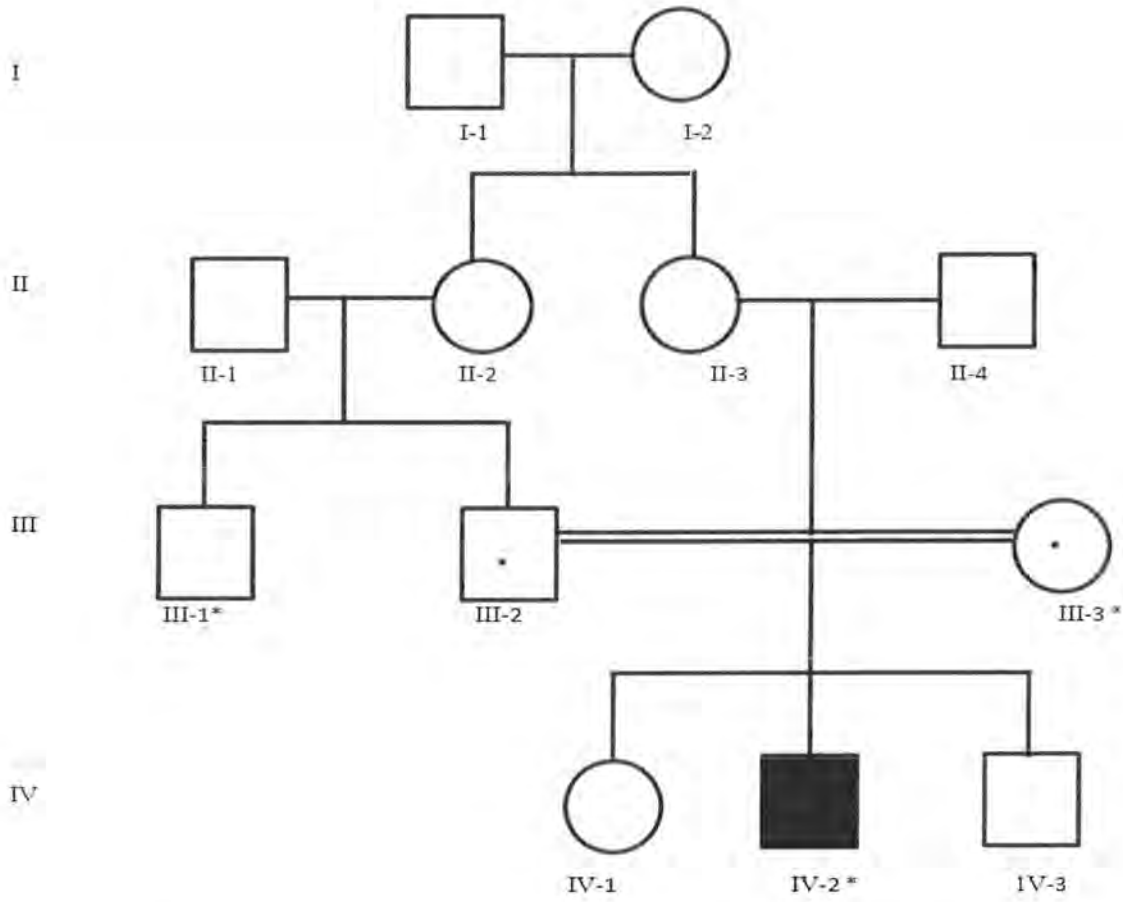
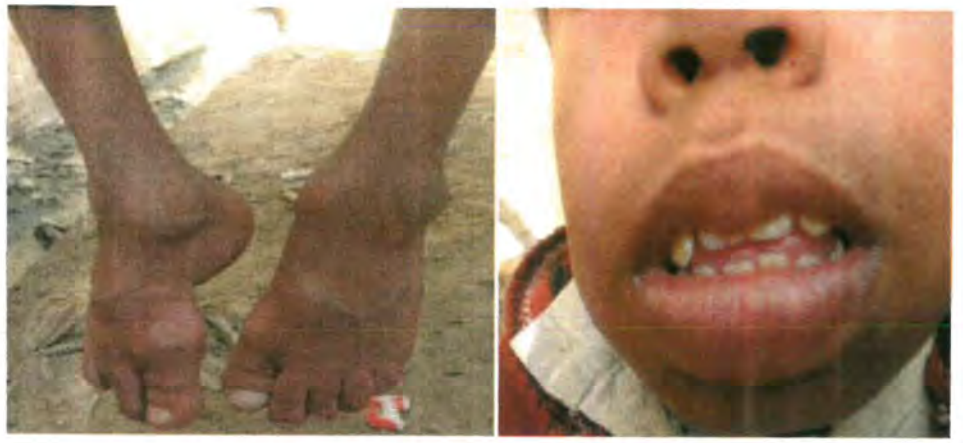
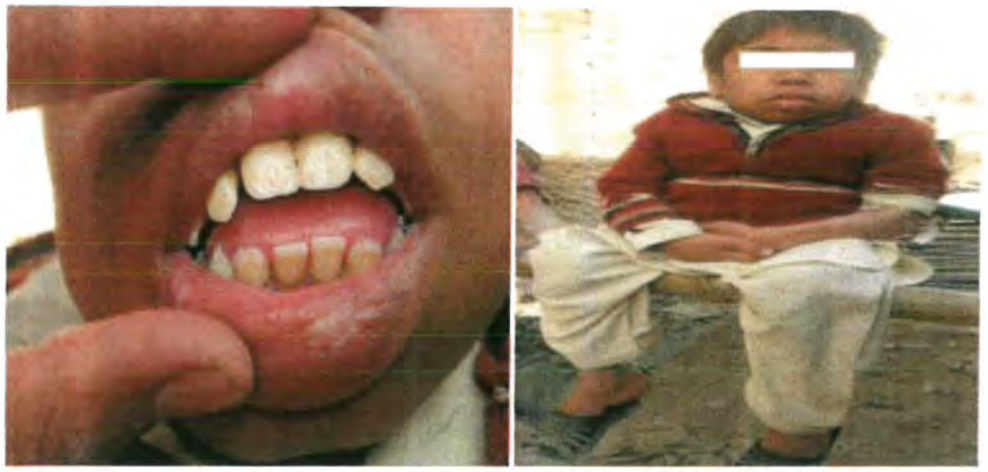


Figure 3.1: Pedigree of family A representing autosomal recessive Acromesomalic Dysplasia Type Maroteaux. Squares and circles represent males and females respectively. Double line is representing consanguineous marriage. A filled symbol represents an affected individual and unfilled symbol represents the normal Individuals. The roman numerals indicate the generation of family within a pedigree while Arabic numeral indicates individual positions within a generation. The individual numbers labeled with asterisks indicate the samples available for this study.



a b



c d

Figure 3.2: Affected individual (III-2) in family A with autosomal recessive Acromesomalic dysplasia type Maroteaux. Clinical finding revealed that the affected individual (IV-A) knew/joint swollen, broad short feet, the greater toe is relatively larger than other, the feet appeared abnormally flat (a), slightly flattened midface, an abnormally small, pug nose (b) Teeth abnormality (c), having short stature 120cm associated with deviation of middle and distal segments, pigeon shape chest, the hands, feet, toe nail often seem unusually broad, square, short, enlarged head frontal bossing, occipital prominence and facial abnormality (d).

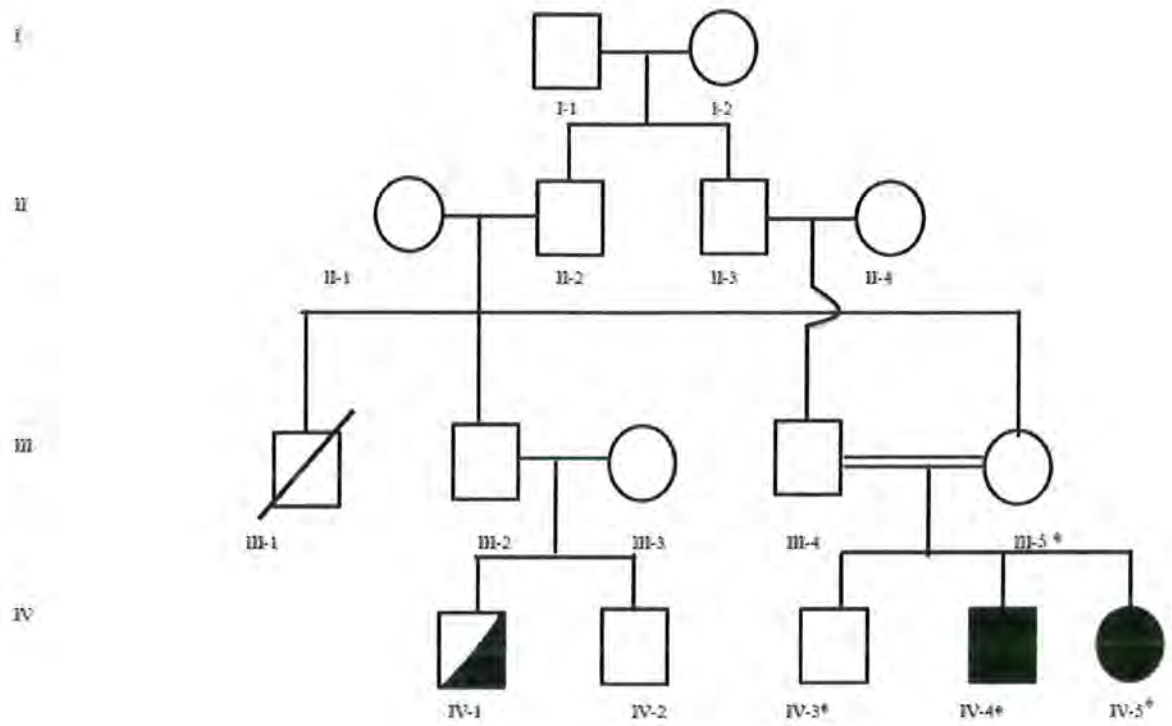
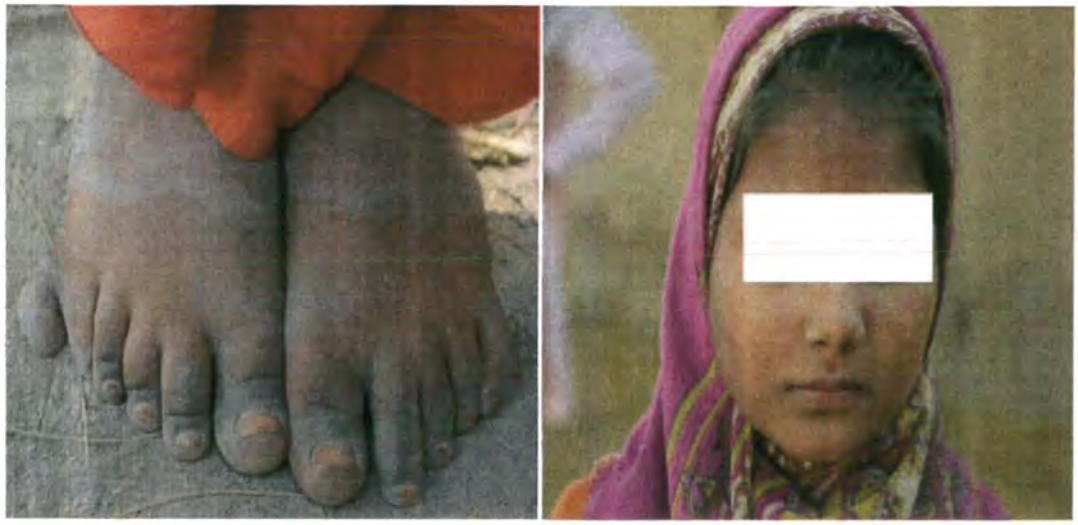


Figure 3.3: Pedigree of family B showed autosomal recessive Bardet-biedl syndrome. Females and Males are represented by circle and square; double line showed consanguineous marriage where as cross lines on circle or square showed expired individual, half filled square showed slightly mantel retardation. The roman numerals are representing the generation of family whereas the Arabic numerals are representing the number of individual with in family. The individual numbers labeled with asterisks indicate the samples available for this study.



a **b**

Figure 3.4: Family B member (IV-5) showed polydactyly in feet (a), obesity, normal height, nystagmus, rapid eye movement (b).

c **d** **e**

Figure 3.5: **The family member IV-4 showed** post axial polydactyly bilateral in hands and feet (c), (d). Dental anomalies, speech deficits, weaken eye sight, obesity, neurological, mental retardation, facial dysmorphism, and unable to move without assistance (e).

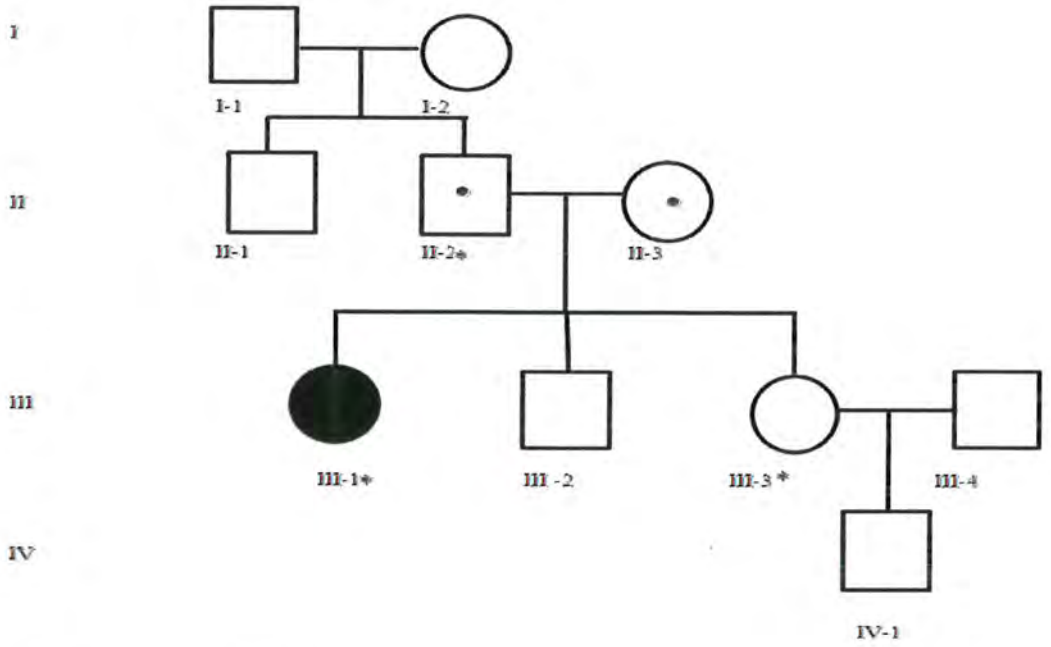


Figure 3.6: Pedigree of family C showed autosomal recessive EvC. Females and Males are represented by circle and square; the Roman numeral showed the generation number whereas Arabic numeral showed the individual position in the pedigree

a **b** **c** **d**

Figure 3.7: Affected member showed phenotype of Post axial Polydactyly (bilateral) (a), syandactyly (b), dental anomalies (c), short stature; short arms and legs whereas head and trunk are normal (d).

Family A

Sr- no	Marker name	Map unit cM	Results		
			1N	2 N	3A
1	D20S432	47.48			
2	D20S54	50.36			
3	D20S477	51.14			
4	D20S865	55.42			
5	D20S34	56.41			
6	D20S478	58.9			
7	D20S881	58.9			
8	D20S107	60			
9	D20S108	61.97			

Figure 3.8: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *GDF5* candidate gene, cytogenetic location of gene is 20q11.22 were taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

Sr-no	Marker name	Map unit Cm	Results		
			1 N	2 N	3A
1	D4S1534	98.67			
2	D4S2284	100.28			
3	D4S423	105.65			
4	D4S433	107.3			
5	D4S2909	107.35			
6	D4S1560	108.56			
7	D4S2986	109.89			
8	D4S2634	110.05			
9	D4S1572	113.05			
10	D4S1570	114.16			

Figure 3.9: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *BMPR1B* candidate gene, cytogenetic location of gene is 4q22.3 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

sr -no	Marker name	Map unit cM	Results		
			1 N	2 N	3A
1	D9S169	51.71			
2	D9S1678	54.78			
3	D9S319	55.51			
4	D9S1118	57.01			
5	D9S1817	59			
6	D9S200	61.8			
7	D9S229	62.66			
8	D9S55	62.66			
9	D91862	66.55			

Figure 3.10: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *NPR2* candidate gene, cytogenetic location of gene is 9p13.3 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member

Sr-no	Markers name	Map unit cM	Results		
			1N	2N	3A
1	D3S2432	57.43			
2	D3S3623	62.36			
3	D3S1260	63.37			
4	D3S3527	64.17			
5	D3S3521	64.17			

Figure 3.11: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *GLB1* candidate gene, cytogenetic location of gene is 3p22.3 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

Sr –no	Marker name	Map unit cM	Results		
			1 N	2 N	3 A
1	D16S486	126.17			
2	D16S3077	128.98			
3	D16S3063	129.12			
4	D16S3123	129.12			
5	D16S3026	132.03			

Figure 3.12: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *GALNS* candidate gene, cytogenetic location of gene is 16q24.3 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

Sr-no	Markers	Map unit cM	Results		
			1N	2 N	3N
1	D5S577	64.49			
2	D5S418	64.56			
3	D5S856	65.07			
4	D5S430	65.19			
5	D5S660	66.83			

Figure 3.13: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *GHR* candidate gene, cytogenetic location of gene is 5p12-13 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

Family B

Sr-no	Marker name	Map unit cM	Results
			1 N | 2N | 3 A | 4A
1	D20S602	23.25	
2	D20S851	28.32	
3	D20S66	37.86	
4	D20S1143	15 MB	

Figure 3.14: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *MKKS* candidate gene, cytogenetic location of gene is 20p12.2 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

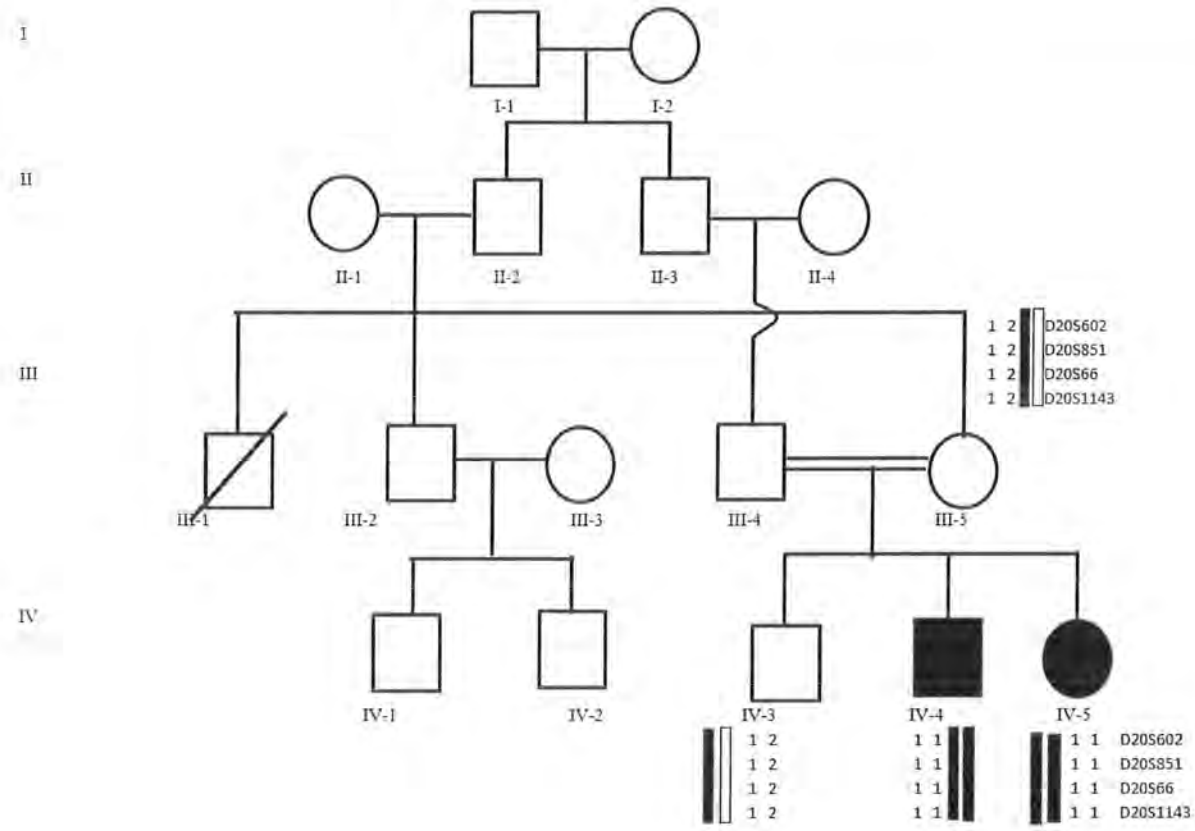


Figure 3.15: Haplotypes of Family B segregating autosomal recessive Bardet Biedl Sydrome for genotyped individuals, haplotypes of the closely linked microsatellite markers on chromosome 20p12.2 are shown beneath each symbol. Alleles causing the risk haplotype are shown with 1 1 while alleles not co-segregating are represented with 1 2.

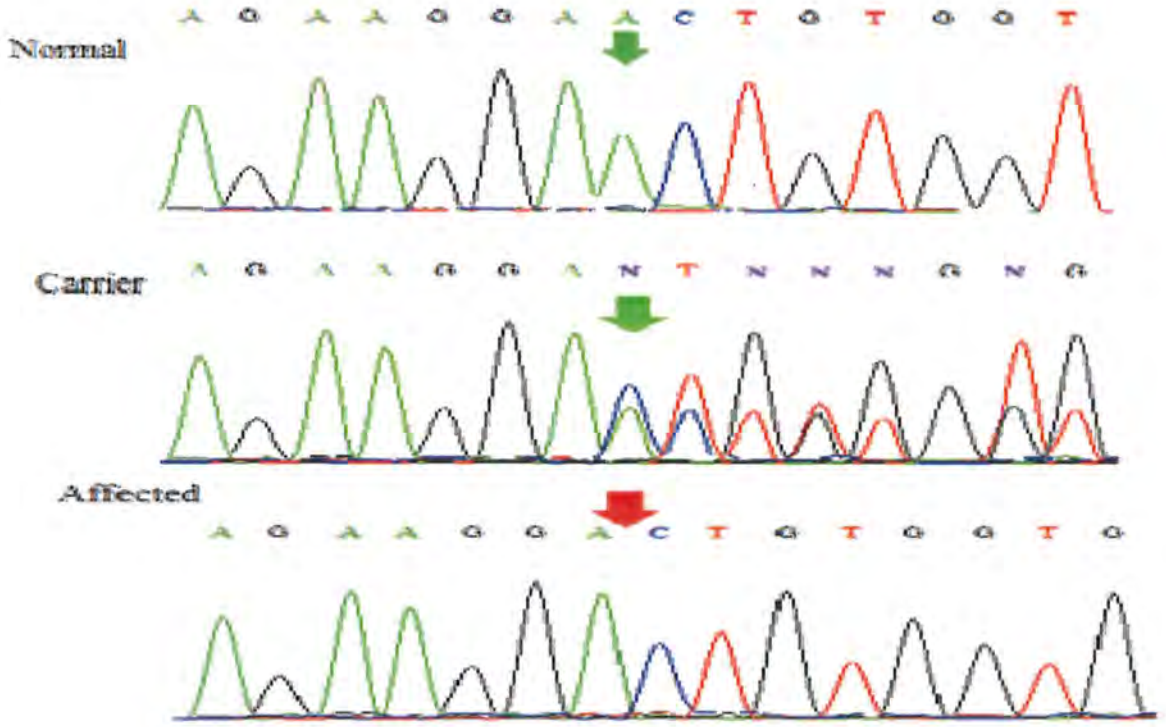


Figure 3.16: Sequence analysis of *MKKS* gene in individuals of family B. DNA sequence analysis of exon 2 of MKKS gene in (a) Control individual (b) Affected individual and (c) Heterozygous carrier. Arrow head represents the nucleotide changed in affected individual, the mutation in affected member and showing comparison with normal and carrier individual of the family.

Family C

Sr-no	Markers names	Map unit cM	Results		
			1N	2A	3N
1	D4S1614	2.86			
2	D4S127	3.6			
3	D4S3034	3.6			
4	D4S179	3.99			
5	D4S2957	5.72			
6	D4S3023	6.54			
7	D4S2925	7.17			
8	D4S2375	7.26			
9	D4S2285	7.97			
10	D4S3007	13.21			
11	D4S394	14.94			

Figure 3.17: The image of polyacrylamide gel (Electropherogram) showed allel pattern with particular markers of *EVC, EVC2* candidate gene, cytogenetic location of gene is 4p16.2 was taken after staining the gel with ethidium bromide. Homozygous banding patterns shows that the individual is affected whereas heterozygous pattern of bands shows that individual is normal, the symbol N is indicating that individual is normal while A is indicated the affected individual. The Arabic numerals are representing number of family member.

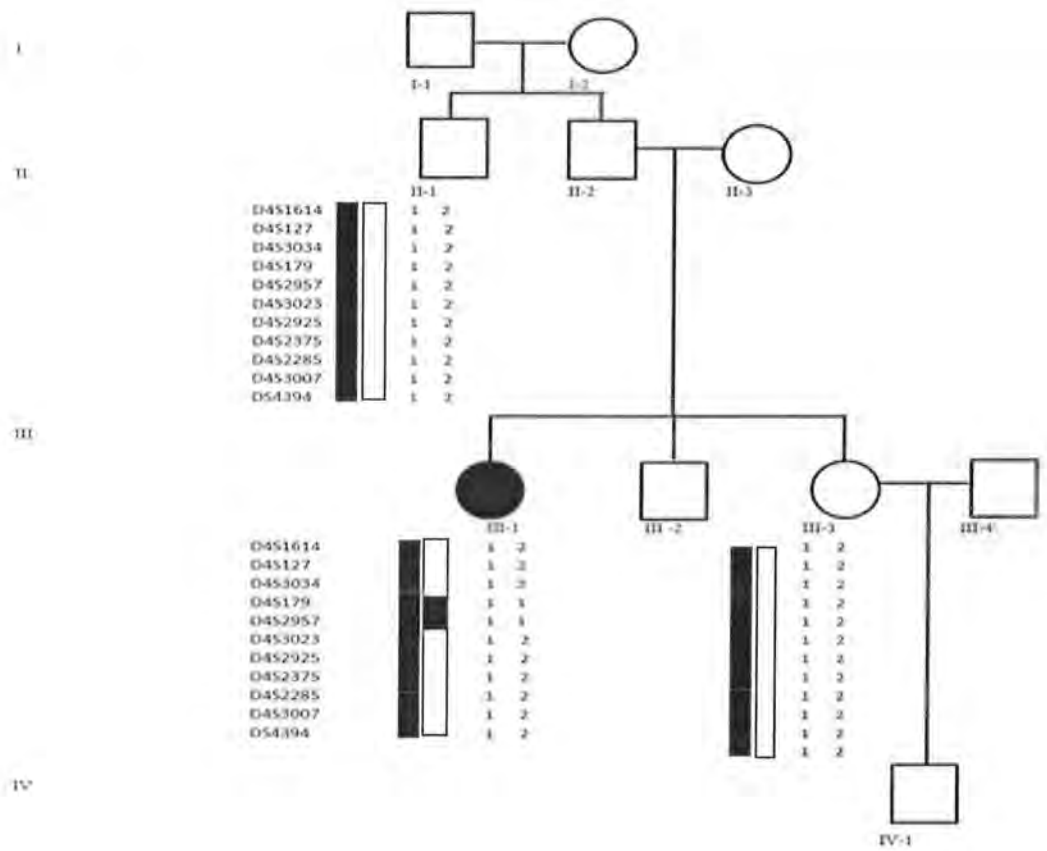


Figure 3.18: Haplotypes of Family C segregating autosomal recessive Ellis- Van Creveld Sydrome for genotyped individuals, haplotypes of the closely linked microsatellite markers on chromosome 4p16.2 are shown beneath each symbol. Alleles causing the risk haplotype are shown with 1 1 while alleles not co-segregating are represented with 1 2.

Discussion

DISCUSSION

Human skeleton is one of the most complex structure of the body and well modified than other primates. The skeleton reaches its full density at the age of 20. Number of genes (signaling pathway, extra cellular matrix protein, signaling mediators, transcription factor) are involved in the development and formation of skeleton. Any mutation that occurs in the gene or pathways leads to severe skeletal dysplasia or other skeleton disease. The human skeleton plays many major roles in movement, support, erythropoietin, protection, mineral storage and regulation of endocrine glands.

Homozygosity mapping is the best technique to figure out the recessive trait of human gene. The method involves the detection of the disease locus by simply comparing homozygous pattern of affected allele with heterozygous pattern of allele present in normal member of family. A affected child of a first-cousin marriage shows to carries the total similar information about linkage as a nuclear family with three affected children (Lander *et al.*, 1987).

Current research was focused on three consanguineous families (A, B, C) inherited different types of skeletal disorders. Family A belongs to District Lakki Marwat, Khyber pukhtunkhawa, B to Ghot Khki and C to Sormer Khan Kalar of Sindh. Due to some major social and culture issue the consanguineous marriages are preferred in these areas of Pakistan. Family A members were affected with Acromesomali Dysplasia Type Maroteaux syndrome, family B with Bardet-Biedl syndrome and family C with Ellis-Van Creveld.

To figure out the causative agent in these three families, blood samples of normal and affected individuals were collected and genomic DNA was extracted by standard extraction protocol. Homozygosity mapping was performed using highly polymorphic microsatellite markers flanking the earlier reported loci for the above mentioned skeletal diseases. After discovery of linkage, the linked gene was further subjected to Sanger sequencing to figure out pathogenic variant that causes the disease.

In family A six genes *(NPR2, BMPR1B, GDF5, GLANS, GLB1, GHR)* which are involved in causing Acromesomalic Dysplasia, were tested for homozygosity mapping using 5-10 microsatellites markers. None of these markers showed linkage to inherited AMDM in the family. This concluded the involvement of a novel gene responsible for causing AMDM in this family.

Affected individuals of family B showed phenotypes of Bardet-Biedl syndrome. Blood was collected from two affected and two normal members of family B, and DNA was subjected for further analysis. Genotyping was performed using highly polymorphic microsatellite markers to figure out gene/loci causing hereditary BBS. The linkage in the family was detected on *BBS6* at chromosomal region 20p12.2 through various markers D20S602, D20S851, D20S188, D20S66, and D20S1143. Sanger sequence analysis of *MKKS* gene at 20p12.2 revealed a novel deletion of adenine at the position 775 (c.775-delA) causing a frameshift. As a result of frameshift (c.775-delA) threonine at position 259 is replaced by leucine (p.Thr259Leufs*21) resulted in abnormal protein product of *MKKS*. To date 53 mutations have been reported in *MKKS* gene (Khan *et al.*, 2016). Mutations which are reported in *MKKS* are splicing site, nonsense, missense, indels, deletion-insertion, mutations and frame shift produces non-functional protein (Khan *et al.*, 2016)

The mutation identified in family B resides in the intermediate domain of MKKS protein and the frameshift variant (c.775-delA) will most likely result in loss of function of MKKS protein through nonsense mediated mRNA decay (NMD). As *MKKS (BBS6)* along with *BBS10* and *BBS12* helps in forming a BBS chaperone complex at the base of primary cilia, thus loss of function mutation in *MKKS* would result in non function BBS complex and result in BBS phenotype

Affected members of family C showed the phenotype of EvC. Bilateral Post axial polydactyly in hands, syndactyly in feet, abnormal development of nails, dental anomalies, short stature phenotype were observed in affected member of family C. In family C Linkage was established to *EVC/EVC2* gene on chromosome 4p16 in family C. Linkage analysis was followed by Sanger sequencing of eighteen exons (2-8, 10-21) of

EVC and sixteen exons (1-7, 10, 13-18, 20, 21) of *EVC2* gene to figure out a disease causing mutation in the candidate genes. No pathogenic variant was found in sequenced exons suggested that it might be present in the rest of unsequenced exons of *EVC/EVC2*.

Family A was excluded from known loci involved in AMDM which concludes that novel gene might be involved in causing AMDM. The family A should be further processed for whole genome scan using STS or SNP markers, and whole exome sequencing (if necessary) to figure out novel genes responsible for phenotype in family A. While in family C further Sanger sequence analysis of remaining exons of gene *EVC/EVC2* is required to figure out the causative mutation.

The objectives of the present research were to figure out the pathogenic variant causing disease. It will help to improve diagnosis, treatment, management, develop a community-generated knowledge as well as help in understanding skeletal dysplasia and related disorders. This study will support an international network of patients, clinicians and researchers seeking a better understanding of skeletal dysplasia and provides further insight in molecular pathogenesis and target for treatment. The current research was focused on inherited autosomal recessive disorders like skeletal dysplasia. Cousin marriage is considering one of the major risk factor for genetic disorders promoting recessive defected genes in off springs. Especially in Pakistan, various measures are required i.e. Prenatal diagnosis, genetic counseling and identification of carriers, to minimize the rate of affected infants.

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MPhil Thesis

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