Genetic Analysis of Hereditary Primary Microcephaly in Selected Families of Pakistani Origin

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2019

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A dissertation submitted in the partial fulfillment of the

Requirements for the degree of

MASTER OF PHILOSOPHY

in

Biochemistry/Molecular Biology

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CERTIFICATE

This thesis submitted by **Mr. Waseem Ullah Khan** is accepted in its present form by the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy (M. Phil) in Biochemistry/ Molecular Biology.

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Declaration

I hereby declare that the work presented in the following thesis is my own effort and hard work except, where otherwise acknowledged and that the thesis is my own composition. No part of this thesis has been previously published or presented for any other degree or certificate.

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Waseem Ullah Khan

In the name of ALMIGHTY

ALLAH, the Entirely Gracious, the Especially

Merciful

We created man from an essence of clay: then turned him into a sperm-drop in a safe lodging: then changed the sperm drop into a clot of blood and the clot into a little lump of flesh: then turned the little lump of flesh into bones: then clothed the bones with flesh, and then brought him forth as quite a different creation (from the embryo). So blessed be Allah, the best of all creators.

(Surah Al-Mu'minun 23: 12-14)

Dedicated

To

"My Loving Parents"

Whose Guidance, Prayers, Love and

Affections

are

Source

of strength and Success for Me in

Every Step of My Life.

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List of Abbreviations

A	Adenine
Aa	Amino Acid
APS	Ammonium persulphate
ASD	Autism spectrum disorders
ASPA	Aspartoacylase
ASPM	Abnormal spindle-like microcephaly associated
bp	Base Pair
C- Terminus	Carboxyl terminus
CASC5	Cancer susceptibility candidate 5
CCDC88C	Coiled-coil domain containing 88C
CD	Canavan Disease
CDK	Cyclin dependent kinase
CDK5RAP2	CDK5 regulatory subunit-associated protein 2
CDK6	Cyclin-dependent kinase 6
CENPE	Centromere protein E, 312kDa
CENPF	Centromere protein F, 350/400kDa
CENPJ	Centromere protein J
CEP135	Centrosomal protein 135kDa
CEP152	Centrosomal protein 152kDa
CEP63	Centrosomal protein 63kDa
cM	Centimorgan
CpG	Cytosine-Phosphate- Guanine
CSF	Cerebrospinal fluid
CT	Computed tomography
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylene-diamine-tetra-acetic acid
	family member 1
g	Gram
G	Guanine
HCl	Hydrochloric acid
HERC1	HECT and RLD domain containing E3 ubiquitin protein ligase

ID	Intellectual disability
Indel	Insertion/deletion
IQ	Isoleucine glutamine
kDa	Kilo Dalton
KPTN	Kaptin (actin binding protein)
M	Molarity
Mb	Mega base
MCD	Malformations of cortical development
MCPH	Autosomal recessive primary microcephaly
MCPH1	Microcephalin 1
mg	Milligram
MgCl2	Magnesium Chloride
MIM	Mendelian Inheritance in Man
ml	Milliliter
MLC	Megalencephalic Leukoencephalopathy with Subcortical Cysts
MLC1	Megalencephalic leukoencephalopathy with subcortical cysts 1
mM	Millimolar
MPDZ	Multiple PDZ domain protein
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic Acid
NDE1	nudE neurodevelopment protein 1
NSD1	Nuclear receptor binding SET domain protein 1
OFC	Occipital-frontal circumference
p	Short arm of chromosome
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pH	Negative log of hydrogen ion concentration
PHC1	Polyhomeotic homolog 1
Plk4	Polo-like kinase 4
PPP1R15B	Protein phosphatase 1, regulatory subunit 15B
PTEN	Phosphatase and tensin homolog
q	Long arm of chromosome
rpm	Revolutions per minute

SASS-6	Spindle assembly 6 homolog
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
STIL	SCL/TAL1 interrupting locus
T	Thymine
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	Tetra Methyl Ethylene Diamine
Tm	Melting temperature
TUBGPC4	Tubulin, gamma complex associated protein 4
UV	Ultra Violet
WDR62	WD repeat domain 62
ZNF335	Zinc finger protein 335
Ml	Microliter
Mm	Micrometer

Acknowledgements

All praises for **Allah Almighty**, the Most Merciful, the Most Gracious, Who helped and blessed me all the way along till the completion of my research work. Without His grace and mercy, I am nothing. Countless Blessings upon the **Holy Prophet Hazrat Muhammad** (**P**eace **B**e **U**pon **H**im), the city of knowledge who guided his UMMAH to seek knowledge from cradle to grave and who is an ever ember of guidance for humanity.

First and foremost, I would like to express my deep sense of gratitude and obligation to my supervisor **Dr. Mariam Anees** for her guidance, ideas and cooperative and friendly nature throughout the research. I most gratefully acknowledge her constant encouragement and help in different ways to complete the research successfully.

I am indebted to **Prof. Dr. Wasim Ahmad** for his kind support to carry out this work in Human Molecular Genetics Lab, overseeing this research and for his immense guidance, assistance, advice and constructive comments throughout my research.

I am thankful to **Prof. Dr. Muhammad Rashid Khan**, Chairperson, Department of Biochemistry, Faculty of Biological Sciences, QAU for providing research facilities to accomplish this work.

I am extremely thankful to all my teachers especially **Prof. Dr. Bushra Mirza**, best teacher I've ever met! I am thankful to Dr. Muhammad Tahir Waheed, Dr. Iram Murtaza, Dr. Samina Shakeel, Dr. Aneesa Sultan, Dr. Muhammad Rizwan Alam and Dr. Hussain Mustatab Wahedi. Thank you all for sharing your knowledge.

My acknowledgements would never be complete without mentioning my lab mates. I would like to acknowledge Dr. Asmat Marwat, Pashmina Waqar Shah, Hammal Khan, Farooq Ahmad, Muhammad Zahid, Muhammad Bilal, Abdullah, Zain Ali, Amir Hayat, Feroz Khan, Amjad, Shazia, Sidra Bukhari, Rubab Raza, Romana Liaqat, Warda Nawal and Hamid Siddique.

I am most indebted to my parents, my sisters and my brother for their endless prayers, matchless love and care. Words become meaningless when I have to say thanks to my family; their prayers gave me strength and hope to accomplish this task and to pursue my goals. The occasion needs special mention of my beloved parents whose uninterrupted support in various aspects of my life and studies, kept me going with

grace and honor under the shadow of their prayers. I can never repay their unlimited love and precious prayers. You have always encouraged me when I lost heart.

My deepest feelings and love for my friends especially Q. Ijaz Ahmad, Kaleem Khan, Akmal Zubair, Hashim Khan, Inam Banochi, Obaid Ullah, Gul Saeed, Fayyaz Ahmad, Nawab khan, Muhammad Irfan, Athar Aitazaz, Rehman Nawaz, Mati Ullah, Aziz-ur-Rehman and Nasir Abbas for their support and providing relaxation in extremely tense situations. It has been a great privilege to spend two years in the Department of Biochemistry, Quaid-i-Azam, University, Islamabad and its members will always remain dear to me.

I convey my heartiest thanks to the departmental clearical staff especially Mr. Tariq, Mr. Fayyaz, and Mr. Shehzad for their cooperation during my study.

Finally, I thank all those who have helped me directly or indirectly in the successful completion of my research work. Anyone missed in this acknowledgement are also thanked.

Thanks to all of you!

Waseem Ullah Khan

Abstract

Microcephaly is defined as the neurological distortion in which affected individuals have an unusually smaller head as compared to other children of the same age and sex. There are several environmental and genetic factors leading to microcephaly. Autosomal recessive primary microcephaly is a genetically heterogeneous neurodevelopmental imperfection described by a significantly reduced head circumference existing at birth, intellectual disability and mental retardation. Up till date, 18 MCPH loci (MCPH1–MCPH18) have been discovered in various populations around the world. MCPH genes play a significant role in centriole biosynthesis, DNA replication and genome stability. Blood samples from three families (A, B, C) segregating with MCPH were obtained from different remote regions of Pakistan. Family A and C genotyping examination displayed that normal and affected individuals were heterozygous within these known genes, thus excluding the linkage. Linkage analysis of family B indicated MCPH5 locus on chromosome 19 harboring ASPM gene. Sequence analysis of specific exon in these MCPH5 linked families resulted in the identification of an already known non-sense mutation in the exon 17 of ASPM gene. In such mutation, G to A conversion occurs at 3978 position of nucleotide, creating premature stop codon. Exact nomenclature for this mutation is 3978G>A W1326*. The emphasis of current genetic study was investigation of causative gene and DNA variant at nucleotide level which will help in future genetic testing and genetic counseling to reduce the risk of genetic disorders in Pakistani population.

1. Introduction

Human being is an amazing living entity in the whole animal kingdom because of the presence of miraculously large brain and biggest cerebrum (Thornton and Woods, 2009), where the chief cognitive capabilities occur. It is the principal advanced feature that distinguishes human beings from remaining species (Luo *et al*., 2012). It has been observed within human population that brain volumes vary from 98ml-1,7695 on average in men and women (Rushton, 1992; Luo *et al*., 2012). There are various brain anomalies, for instance, microcephaly, schizophrenia, attention deficit and lissencephaly, which occur due to reduction in brain volume (Darvish *et al.*, 2010). The humans have remarkably larger brain as compared to all other mammals including primates. The evolutionary expansion mostly affected the cerebral cortex, which is liable to numerous human cognitive abilities, such as language (Gilmore and Walsh, 2013).

1.1. Microcephaly

Clinical diagnosis defines microcephaly as a smaller head size before or after birth. This examination does not essentially designate irregular development of brain, as some individuals with microcephaly are fit and healthy (Leviton *et al.*, 2002; Ashwal *et al*., 2009). Microcephaly is defined as the neurological malformation in which individuals have a remarkably smaller head as compared to other children of the same gender and age (Gilmore and Walsh, 2013). It can occur before birth or after birth and is linked with different brain abnormalities with over 300 genetic, sporadic and chromosomal syndromes. The prevalence for isolated microcephaly is determined as 1 in 6,000–10,000 births. The prognosis is based on the etiology of microcephaly and its magnitude is associated with brain abnormalities. Mental retardation may be moderate to severe resulting mostly because of microcephaly syndromes and isolated genetic microcephaly. Microcephalic individuals can have mild to severe mental retardation along with loss of hearing, seizures and squat physique (Darvish *et al*., 2010)

1.2. Causes of Microcephaly

There are numerous environmental and genetic factors linked to microcephaly during prenatal and postnatal development of life (Krauss *et al*., 2003; Gilmore and Walsh, 2013).

1.2.1. Genetic factors

Genetic factors involved in microcephaly include chromosomal irregularities, impulsive chromosomal condensation or scratched DNA as a result of improper mitotic spindle arrangement.

1.2.2. Environmental factors

Microcephaly resulting from environmental factors is attributed to infectious and noninfectious agents. There are various congenital infections associated with microcephaly which include rubella virus, herpes simplex virus (HSV), cytomegalovirus, Toxoplasma gondii, HIV, Zika virus, varicella zoster virus and arboviruses such as chikungunya (Gérardin *et al*., 2014; Von der Hagen *et al*., 2014; Devakumar *et al*., 2017)

There are more cases of microcephaly in developed countries resulting from noninfectious factors (Szabó *et al*., 2010). These include nutritional deficiencies, hypothyroidism, brain injury, alcohol drinking, drug utilization during pregnancy, metabolic disorders etc. (Faheem *et al*. 2015). Complications of metabolism are mostly responsible for secondary microcephaly and are frequently connected with other clinical signs and symptoms (Hagen *et al*., 2014). Abnormality of metabolism includes mitochondriopathies, defect in serine biosynthesis, congenital glycosylation disorders and imperfection in sterol biosynthesis (Von der Hagen *et al*., 2014)

1.3. Classification of Microcephaly

Microcephaly is classified based on its onset into primary microcephaly and secondary microcephaly (Woods, 2004).

1.3.1. Primary Microcephaly

Microcephaly that develops at birth, usually because of neurodevelopmental defect, is known as primary microcephaly (*MCPH*, for "microcephaly primary hereditary"). Primary microcephaly may be an isolated condition which shows that it can happen with or without any other minor or major birth imperfections or it may be combined with other major birth defects. During neurogenesis, primary microcephaly shows a decrease in production of neurons. Individuals with MCPH usually have intellectual disability and language delay, with varying degrees of motor delay (Gilmore and Walsh, 2013). Although microcephaly is a relatively rare condition, understanding of its etiology has shed important light on core questions in the field of neurocortical development. Environmental factors like drugs, alcohol or infections are the main entities responsible for causing primary microcephaly (Kaindl *et al.*, 2010; Opitz and Holt, 1990). As a result of zika virus epidemic in different countries, researchers have been taken keen interest in primary microcephaly since last few years (Kleber *et al*., 2015).

1.3.2. Secondary Microcephaly

Microcephaly that develops after birth, also known as secondary microcephaly, is a progressive condition associated with white matter developmental irregularities or neurodegenerative processes. Secondary microcephaly, associated with additional sign and symptoms, is frequently a result of metabolic disorders (Von *et al*., 2014). Investigation of metabolic screening should mainly emphasize on 2-ketoglutaric aciduria, maternal phenylketonuria and scarcity of phosphoglycerate dehydrogenase in order to determine secondary microcephaly (Kelley *et al*., 2002).

1.4. Autosomal Recessive Primary Microcephaly (MCPH)

MCPH is a disorder of fetal brain growth, with no apparent abnormalities in other systems and shows extreme genetic heterogeneity by virtue of extensive clinical phenotype (Cowie, 1960). There are now 18 MCPH loci involved in MCPH (OMIM, 2015), accompanied by highest incidence of *ASPM* gene mutation which is responsible for microcephaly in 25% to 50% of the cases (Bond *et al*., 2002; Darvish *et al*., 2010). ASPM is followed by WDR62 gene regarding the frequency of mutations (Nicholas *et al*., 2010; Yu *et al*., 2010). The recent definition of MCPH

from clinical point of view includes: 1) Congenital microcephaly, where OFC is 4 SD below age and gender means; 2) mentally retarded but still have no other neurological finding; 3) normal body physical phenotype, body weight, brain scan and examination of chromosome. Moreover, patients who have MCPH1 mutations, show reduction in height and OFC (Woods *et al*., 2005)

1.5. Prevalence of Primary Microcephaly

The occurrence of MCPH ranges from 1:30,000–1:250,000 in population (Zaqout *et al*., 2017). The consanguineous marriages are more common in Arab and Asian populations resulting in more cases of MCPH than in whites. (Mahmood *et al*., 2012). The region with high frequency of consanguinity, such as Morocco, have relatively higher rate of MCPH, which is around 1 per 1000 (Jaouad *et al*., 2009, Thorton and Woods., 2009).

1.6. Phenotypic Features of Primary Microcephaly

It has been evident through worldwide studies that more than 300 families suffering from microcephaly have inadequate phenotype details. It includes hyperactivity, tapered slanting forehead, devotion deficit, dialogue deferral and intellectual disability (Kraemer *et al*., 2016). In primary microcephaly, neurogenesis and brain development are affected along with short stature and intellectual damage; generally, other somatic or neurological aberrations are not present (Alcantara, 2014; Barbelanne and Tsang, 2014). However, some individuals manifest neurological anomaly for example speech problem and mild seizures, and dysmorphism for example cryptorchidism and sloping forehead. In addition, some individuals who are associated with Seckel syndrome, display more severe phenotypes for instance mental retardation and bird-headed appearance with dwarfism (Hussain *et al*., 2012).

1.7. Clinical Manifestations of MCPH

MCPH is a neurodevelopmental imperfection that is identified by reduction in OFC of head up to 3 or 4 SDs below ethnically matched age and sex related means and mental retardation without any significant neurological deficit (Passemard *et al*., 2009; Thornton and Woods, 2009). It has been explained by ultrasound of affected pregnancies that normal head measurement is found up to 20 weeks of gestation, and

a reduction in OFC is observed by 32nd week (Woods *et al*., 2005). The most common diagnostic tool for MCPH is measurement of OFC. In MCPH patients, OFC ranges from 4 to 12 SD below the age and sex means. The relative degree of microcephaly does not vary throughout life and within a family OFC usually does not vary by >2 SD between affected individuals (Roberts *et al*., 2002). The sharply sloping forehead is present in many cases but not always associated with MCPH (Cowie, 1960). Seizures and epilepsy have higher incidence in patients with *ASPM* mutations but are very rarely found in patients affected due to other MCPH genes and are no more common than in general population (Roberts *et al*., 2002; Shen *et al*., 2005; Woods *et al*., 2005; Passemard *et al*., 2009). The affected child is microcephalic at birth but development in the first year of life is often normal. After the first year of life, the development is only slightly delayed but progress in speech is more delayed. Magnetic resonance imaging (MRI) and computerized tomography (CT) scans of the brain show that MCPH patients have an apparently normal architecture of brain but with reduced size, especially cerebral cortex is severely affected.

1.8. Inheritance Pattern of MCPH

MCPH has an autosomal recessive approach of inheritance in which both copies of the gene at same locus on homologous pair of chromosomes have same mutation in each cell. Afflicted individual with MCPH receives one copy of the mutated allele from each parent, while the parents of affected child are apparently normal and do not display any symptoms or signs of the illness. The parents in this condition are carriers of causative gene (Klapfer *et al*., 1964).

1.9. Molecular Genetics of Microcephaly

Several genes have been identified as causes of MCPH, including *MCPH1, CEP 152, WDR62, CENPJ, CDK5RAP2, ASPM, CEP63*, and *STIL* (Jayaraman *et al*., 2018). Table 1.1 provides a more complete list of loci (numbered *MCPH1–MCPH18* at last count and growing) and their associated genes. These genes are generally expressed in the primary germinal zone in the cerebral cortex, called the ventricular zone (VZ), during cortical neurogenesis, which is consistent with a role in proliferation of neural progenitor cells (NPCs) (Bond and Woods, 2006). Intriguingly, the centrosome has been implicated in the pathogenesis of several MCPH syndromes (Megraw *et al*.,

2011). Since these genes encode proteins especially expressed ubiquitously in the centrosomes of most mitotic cells of the body (Mirzaa *et al*., 2014), it is obvious why mutations in them preferentially affect centrosomal proteins (Jayaraman *et al*., 2018).

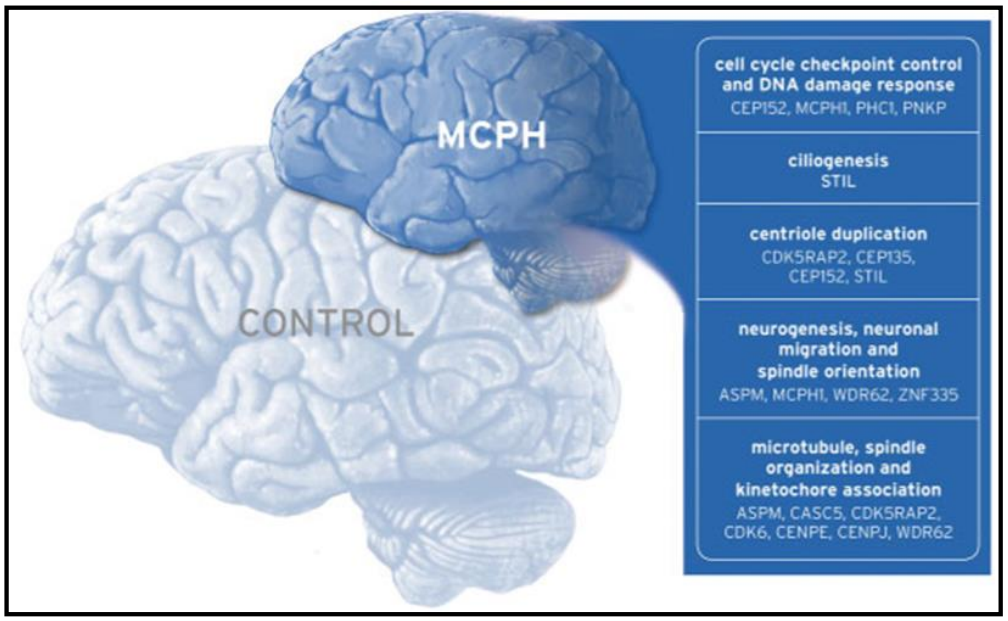


Figure 1.1: Illustration of the brain phenotype in MCPH patients and the main roles of MCPH proteins. Note the typical reduction in the brain volume and the simplification in cortical gyration of an otherwise architecturally normal brain. MCPH proteins are involved in cell cycle dynamics, ciliogenesis, the centrosome, neurogenesis, and neuronal migration. MCPH, microcephaly primary hereditary (Zaqout et al.,2017)

1.9.1. Microcephalin *(MCPH1)*

Microcephalin gene is situated on chromosome No 8p23 comprising 835 amino acids, 14 exons, molecular weight of 92,877 Da and genome size of 241,905bp (Faheem *et al*., 2015). It plays an essential role in neurogenesis, DNA damage repair mechanism, tumor suppression in various cancers, brain development, regulation of telomere integrity and size determination of cerebral cortex (Pulvers *et al*., 2015). Several research findings revealed that mutation in *MCPH1* could lead to delayed de-condensation post mitosis, instability of genome and dysregulated chromosomal condensation (Liu *et al*., 2016).

1.9.2. *WDR62 (WD Repeat-Containing Protein 62)*

WDR62 protein encoding gene is positioned on chromosome No. 19q13.12. It includes 1518 amino acids and 35 exons, along with molecular weight and genome size of 165,954 Da and 50,230 bp, respectively (Faheem *et al*., 2015; Pervaiz and Abbasi, 2016). This gene plays a significant role in proliferation, migration of neurons, duplication of centrioles, cortical development and human brain evolution. (Pervaiz *et al*., 2016; Sgourdou *et al*., 2017). Mutation in *WDR62* can lead to a broad range of disorders comprising of cortical malformation, cognitive disability and microcephaly. Several research findings suggested that mutation in *WDR62* is only responsible for 10% cases of microcephaly (Naseer *et al*., 2017).

1.9.3. *CDK5RAP2 (CDK5 Regulatory Subunit-Associated Protein 2)*

MCPH3 is a protein coding gene positioned on chromosome No 9q33.2, including1893 amino acids, 39exons, and a molecular weight of 215,038 Da (Graser *et al*., 2007). *MCPH3* is also known as *CDKRAP2, Cep215* and *C48.* This gene plays a vital role in regulating CDK5 activity in the cell organelles such as Golgi complex and centrosomes, microtubule nucleation and centriole engagement (Faheem *et al*., 2015; Sukumaran *et al*., 2017). Mutation or any damage to *CDK5RAP2* displays spindle checkpoint protein expression and chromosomal segregation problems (Faheem *et al*., 2015).

1.9.4. *CASC5 (Cancer Susceptibility Candidate 5)*

CASC5 gene is positioned on chromosome No 15q15.1, including 2342 amino acids, 27 exons and 265 kDa molecular mass. It is also known as *MCPH4, spc7, D40, PPP1R55, hKNL-1, CT29, AF15Q14* and *hSpc105*. This gene plays an important role in checkpoint functioning, kinetochore assembly and spindle assembly. Mutation or any other damage to *CASC5* protein can lead to misalignment and disrupt chromosome segregation (Petrovic *et al*., 2014; Szczepanski *et al*., 2016).

1.9.5 ASPM (Abnormal Spindle-Like Microcephaly-Associated Protein)

ASPM is a protein encoding gene that is present on chromosome No 1q31.3. It consists of 62,567 bp, 28 exons and 3477 amino acids (Saunders *et al*., 1997;

Ponting, 2006). It plays an important role in cytokinesis, pole organization and focusing and organization of spindles (Tungadi *et al*., 2017). Several research studies identified that *ASPM* knockdown leads to reduction of head size in various species for instance Zebrafish, increases thickness of white matter and reduce surface area (Kim *et al*., 2011; Passemard *et al*., 2016)

*1.9.6. CENPJ (*Centromere Protein J)

CENPJ gene is positioned on chromosome No 13q12.2. It is comprised of 40,672 bp, 17 exons, 1338 amino acids and 153 kDa molecular mass (Saunders *et al*., 1997). This gene has almost 204 orthologs. The most convincing and significant role of *CENJP* is microtubule assembly in the centrosome as it stabilizes centriole integrity (Kirkham *et al*., 2003; Hung *et al.,* 2004). It has been revealed that mutation in *CENPJ* gene enhances the rate of apoptosis, destruction of centrioles and mitotic arrest in Drosophila (Koyanagi *et al*., 2005; Basto *et al*., 2006).

1.9.7. STIL (SCL/TAL1-Interrupting Locus)

This gene is situated on chromosome No 1p33, consisting of 63,018 bp, 20 exons, 1287 amino acids and molecular mass of 150 kDa (Kaindl *et al.,* 2010). This gene plays a vital role in progression of cell cycle, apoptosis regulation, ciliogenesis and centriole duplication.

1.9.8. CEP152 (Centrosomal Protein 152)

It is situated on chromosome No 15q21.1. It contains 149,368 bp, 1710 amino acids, 38 exons, and a molecular weight of 195,626 Da (Jamieson *et al*., 2000). This gene is also represented as *Asterless*, *MCPH9, SCKL5* and *KIAA0912* (Dzhindzhev *et al*., 2010). It plays a vital role in receptivity, cellular division, cell polarity and shaping the cell (Cizmecioglu *et al*., 2010)

1.9.9. ZNF335 (Zinc Finger Protein 335*)*

This protein encoding gene is situated on chromosome No 20q13.12. It comprises of 24,258 bp, 1342 amino acids, 28 exons and molecular weight of 144,893 Da (Deloukas *et al*., 2001). It is also known as *MCPH10, NIF-1 and NIF-2* (Mahajan *et al*., 2002; Garapaty *et al*., 2008)

1.9.10. *PHC1* (Polyhomeotic-Like Protein 1)

The *PHC1* is a protein encoding gene positioned on chromosome 12p13.31, including 1004 amino acids and 105,534 Da molecular mass. This gene is also identified as *MCPH11, PH1, HPH1, RAE28, EDR1* and early developmental regulator1 (Chavali *et al.*, 2017). The most essential and vital function of this gene is in cell cycle regulation and chromosome remodeling. (Awad *et al*., 2013; Srivastava *et al*., 2017).

1.9.11. *MCPH12 (CDK6)*

MCPH12 is a protein encoding gene situated on chromosome No 7q21.2. It comprises of 231,707 bp genome size, 10 exons, 326 amino acids and molecular mass of 36,983 Da. It is also recognized as a *CDK6* (cyclin dependent kinase 6). This gene appears to be crucial in cell differentiation and cell cycle mechanism. Depletion of *CDK6* gene affects nuclei organization of centrioles and cell proliferation (Hussain *et al*., 2013)

1.9.12. MCPH13 (CENPE*)*

This gene is present on chromosome No 4q24, containing 2701 amino acids and size of 492,604 bp. Research studies indicate that this protein may play an essential role in transcription factor activity and signal transducer activity (Ahmad *et al*., 2017).

1.9.13. *MCPH14 (SASS6)*

This gene is situated on chromosome 1p21.2, containing 49,552 bp, 17 exons, 657 amino acids and a molecular mass of 74,397 Da. This gene is present inside the centrosome, cytoskeleton, centrioles, cytoplasm and microtubule organizing center. The most important role of *SASS6* is to support the formation of centriole (Gupta *et al*., 2015; Arquint and Nigg, 2016).

1.9.14. *MCPH15 (MFSD2A)*

MCPH15 gene is mapped to chromosome 1p34.2, encompassing 543 amino acids, 14 exons, a molecular weight of 60,170 Da and size of genome 14,857 bp. This gene is mainly present in plasma membrane, cytosol, cytoplasmic bodies, blood brain barrier and cerebral cortex (Reiling *et al*., 2011). *MFSD2A* gene mutation results in microcephaly leading to smaller brain volume than usual (Nguyen *et al*., 2014).

1.9.15. *MCPH16 (ANKLE2)*

ANKLE2 gene is located on chromosome 12q24.33 with a molecular mass of 10,000 Da and 938 amino acids. This gene plays a convincing role in nuclear envelope development. Recent studies revealed that Mutation in *MCPH16* can lead to abnormal nuclear envelope formation (Faheem *et al*., 2015)

1.9.16. MCPH17 (CIT)

CIT is a protein encoding gene mapped to chromosome 12q24.23, encompassing 2027 amino acids, 50 exons, molecular mass of 231,431 Da, and genome size of 191,501bp. This gene plays a meaningful role in cytokinesis and cell division (Naveed *et al*., 2018).

1.9.17. *MCPH18 (WDFY3)*

WDFY3 gene is present on chromosome 8p23, with a genome size of 296,855 bp, 3526 amino acids, molecular mass of 395,258 Da and has two isoforms. The subcellular location is nucleus and cytoplasm and it plays a role in autophagy and regulation of Wnt signaling (Naveed *et al*., 2018).

1.10. Role of Primary Microcephaly Genes in a Centriole Biogenesis Pathway

Most of the microcephaly protein coding genes confine to the centrosome and play a meaningful function in centriole duplication or biogenesis. In mammalian cell, centrosome is a very important microtubule-organizing center. Centrosome comprises of a pair of centrioles: a less immature daughter and mature mother centrioles, encircled by an amorphous pericentriolar matrix (PCM) (Bornens, 2012). It plays a significant role in various cellular processes for instance, cell shape, intracellular transport, cell cycle progression, positioning of cellular organelles, polarity, DNA damage response, adhesion and motility, mitotic spindle formation, cilia assembly, genome stability, positioning and orientation (Jayaraman *et al.*, 2018). It has been revealed through recent studies that mutations in all these genes *CPAP/CENPJ/SAS-4*, *CEP 63* and *CEP152* can lead to autosomal recessive MCPH in humans (Jayaraman *et al.*, 2018). *CENPJ/SAS-4/CPAP/* plays an elucidative part in centriole lengthening and biogenesis (Jayaraman *et al.*, 2018). Recent research conducted on mutant sas-4 mice

proposed that secondary loss of cilia and damage to centrioles is the major cause of neurogenesis defect in microcephaly. p53-mediated apoptosis was shown by *SAS-4* mutant mice (Bazzi and Anderson, 2014; Insolera *et al.,* 2014). Two proteins *CEP63* and *CEP152* physically interact with each other and colocalize in a ring-shaped design (Sir *et al.,* 2011). *CEP 63* and its mouse ortholog and *CEP 152* its fly ortholog Asterless are vital for productive centriole duplication in mouse and human cells (Sir *et al.,* 2011; Brown *et al.,* 2013).

Recent research has revealed the role of most common genes that lead to MCPH. *ASPM* and *WDR62* play a crucial role in neurocortical development and centriole biogenesis (Jayaraman *et al.,* 2016). MCPH with fairly cortical architecture and well preserved gyral design results due to mutations in *ASPM* gene (Bond *et al.,* 2002), while *WDR62* mutation can lead to microcephaly with further developmental shortcomings, including irregular development of the gyri (Nicholas *et al*., 2010). It was discovered by using cell and mouse models that *ASPM* and *WDR62* not only share a mutual, crucial role in centriole duplication but loss of both *ASPM* and *WDR62* is embryonically lethal. The centriole duplication becomes impaired due to total or partial loss of either or both *ASPM or WDR62.* The severity of microcephaly is directly proportional to the severity of the cellular imperfection and leads to a decrease in cilia and centrosomes in the early mouse brain.

Table 1.1: Genes associated with primary microcephaly that encode centrosomal proteins functioning in centriole biogenesis (Jayaraman *et al*., 2018).

Locus	Gene	Chromosomal location	Subcellular location	Pathway
MCPH1	*MCPH1*	8p23.1	Nucleus	DNA damage response and regulation of chromosome condensation
MCPH2	*WDR62*	19q13.12	Centrosome (interphase) and spindle poles (mitosis)	Centriole biogenesis
MCPH3	*CDK5RAP2*	9q33.2	Centrosome	Centriole biogenesis
MCPH4	*CASC5*[a]	15q15.1	Kinetochore	Microtubule attachment to centromere and spindle-assembly checkpoint activation in mitosis
MCPH5	*ASPM*	1q31.3	Centrosome (interphase)	Centriole biogenesis
MCPH6	*CENPJ* (also known as *CPAP* or *SAS-4*)	13q12.12–12.13	Centrosome (interphase)	Centriole biogenesis
MCPH7	*STIL*	1p33	Centrosome	Procentriole formation and centriole biogenesis
MCPH8	*CEP135*	4q12	Centrosome	Centriole assembly
MCPH9	*CEP152*	15q21.1	Centrosome	Centriole biogenesis
MCPH10	*ZNF335*	20q13.12	Nucleus	Transcriptional regulation of brain-specific genes controlling cell fate via REST/NRSF
MCPH11	*PHC1*	12p13.31	Nucleus	Negative regulation of GMNN (which itself regulates the cell cycle and inhibits DNA replication)
MCPH12	*CDK6*	7q21.2	Centrosome (mitosis)	Unknown
MCPH13	*CENPE*	4q24	Kinetochore/ centromere	Unknown
MCPH14	*SASS6*	1p21.2	Centrosome	Centriole assembly with CEP135 and CENPJ/CPAP/SAS-4
MCPH15	*MFSD2A*	1p34.2	Plasma membrane	Omega-3 fatty acid transport across blood–brain barrier
MCPH16	*ANKLE2*	12q24.33	Not well characterized	Fly model shows decreased proliferation and increased apoptosis
MCPH17	*CIT*	12q24.23	Midbody	Cytokinesis
MCPH18	*WDFY3* (also known as *ALFY*)	4q21.23	Nucleus and cytoplasm	Autophagy and regulation of Wnt signaling

Even the trans heterozygote (Wdr62+/−; Aspm+/−) has a minor cellular and brain phenotype, consistent with nonallelic non-complementation between the two genes, which frequently indicates a physical contact between the gene products. In human cells, during interphase *ASPM* and *WDR62* protein encoding gene confine to the proximal end of the mature mother centriole and form a physical complex. Finally, Jayaraman *et al.* also found that the *WDR62* mutant mouse shows a decrease in centrosomes and cilia and other associated defects during neurogenesis. Together,

these results implicate microcephaly genes like *WDR62* and *ASPM* in centriole biogenesis as well as regulation of brain size.

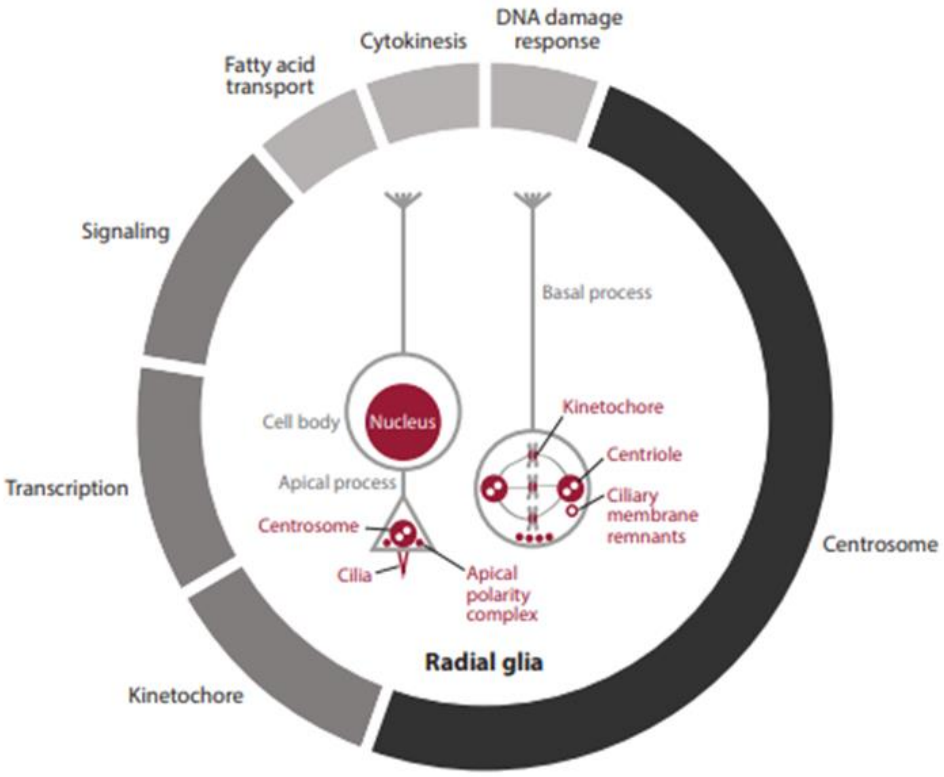


Figure 1.2: Primary microcephaly contributing genes govern the cell destiny of radial glial cells. Eighteen MCPH genes have been discovered so far and out of those, nine encode for centrosomal proteins. The other MCPH genes are implicated in various characteristics of radial glial cells. The cell organelles involved in MCPH are presented in red color (Jayaraman *et al*., 2018).

The work conducted not only discovered an innovative cellular function for *ASPM* and *WDR62* but also located both genes in a pathway in which other microcephaly causing genes are also present, whose protein products are all needed chronologically in centrosome biogenesis. In fact, *CEP63* is also part of larger protein complex comprising of *WDR62* and *ASPM*. In human cells, knockdown of *CEP63* stops the *ASPM-WDR62*– communication, telling that *CEP63* is needed to facilitate this interaction (Jayaraman *et al.*, 2016; Jayaraman *et al.*, 2018). Immunocytochemistry in

WDR62 depleted human cells and *Wdr62* mutant mouse embryonic fibroblasts presented that *WDR62* is vital for suitable centrosomal localization of *CEP63* to the centrosome, in order to direct to centrosome engaging *WDR62* between *CEP63* and *CEP152*. Similarly, RNA interference (RNAi) experimentations proved that before *ASPM* both *CEP63* AND *WDR62* localize to the centrosome, which in turn aids to localize *CPAP/ CENPJ/ SAS*-4 to the centrosome, therefore, *ASPM* is placed between *CENPJ/ CPAP/ SAS-4* and *CEP63* in order to direct to the centrosome (Jayaraman *et al*., 2016). The significant role of all these associated microcephaly gene encoding proteins is to finally carry *CPAP/CENPJ/SAS-4* to the centrosome. These and other findings (Kodani *et al*., 2015) together support a model in which MCPH-associated proteins recruit each other sequentially to the centrosome, thereby enabling centriole duplication to occur (Gonczy., 2012). Those substances which modulate the MCPH associated centrosomal proteins, for instance Plk, are also implicated in microcephaly (Martin *et al*., 2014). Plk family proteins are essential to centrosome biogenesis. In flies, frog, yeast, and humans, lack of Plk leads to monopolar spindles formation (Jayaraman *et al*., 2018). *WDR62* and *ASPM* also interact with some Plk protein members. Drosophila Plk interacts with and phosphorylates Asp, the fly homolog of *ASPM* (do Carmo *et al.,* 2001). Like WDR*62*, Plk3 in mammals at metaphase localizes to the spindle poles (Ruan *et al*., 2004), and *CEP 170* centrosomal protein interacts with the Plk (Guarguaglini *et al*., 2005). Through proteomic study it was discovered that *WDR62* is a binding partner for *CEP 170* (Hutchins *et al*., 2010). During mitosis, overexpression of endogenous *CEP170* colocalizes *WDR62* in a pericentrosomal ring like pattern (Yu *et al*., 2010). Recent research recommends that in human cells PLK1 is responsible for phosphorylation of *WDR62* (Miyamoto *et al*., 2017). *WDR62* fly homolog is crucial for maintenance of Plk positioning and action at the apical centrosome (Ramdas *et al*., 2016). *PLK4* mutation in humans could lead to a syndrome of dwarfism, retinopathy and microcephaly (Martin *et al*., 2014).

1.11. Microcephaly genes implicated in other broad pathways

There are other pathways in which MCPH genes fall in addition to centriole biogenesis. These genes are involved in DNA repair, DNA replication, maintenance of genome stability and cell cycle progression. It has been discovered through research that the first microcephaly locus was linked to the protein encoding gene

microcephalin (*MCPH1*), which concerned the DNA damage response pathway (Jayaraman *et al*., 2018). PNKP is a protein encoding gene needed for DNA repair and mutation in this gene could lead to microcephaly with seizures (Shen *et al*., 2010). This broad category also includes *ATR*, *NBS1* and *PHC1* (Table 1.2).

A cluster of syndromes collectively referred to as microcephalic primordial dwarfism (MPD) goes together with postnatal growth interruption and intrauterine growth control. The syndromes that fall under the category of microcephalic primordial dwarfism comprise Meier–Gorlin syndrome, Seckel syndrome and microcephalic osteodysplastic primordial dwarfism (MOPD) types 1–3, based on the cellular pathways and clinical phenotype involved (Klingseisen *et al*., 2011). The important function of pericentrosomal protein encoded by pericentrin (PCNT) is to nucleate spindle microtubules, and mutation in PCNT could lead to MOPD type 2 (Doxsey *et al*., 1994). The causative agents of Meier–Gorlin syndrome include *CDT1, CDC45, ORC1, GMNN ORC4 and ORC6* (Jayaraman *et al*., 2018). But, mutations in other genes such as *CPAP/SAS-4/CENPJ, CEP63, CEP152* and *CDK5RAP2* have also been connected to MCPH phenotype and classic Seckel syndrome (Bond *et al*., 2005; Kalay *et al*., 2011; Yigit *et al.*, 2016). In fact, *CPAP/CENPJ/SAS-4CEP152* are considered as both Seckel syndromes and MCPH genes (Verloes *et al*., 1993). Two recent research works identified that mutation in DONSON gene leads to MPD and involved in DNA damage response (Evrony *et al*., 2017; Reynolds *et al*., 2017). RNA sequencing was used in one study in a single huge First Nations community with a newborn fatal disorder of strong microcephaly, skeletal dysplasia, craniofacial dysmorphism, intrauterine growth limitation and craniofacial dysmorphisms to recognize an intronic variant triggering unusual splicing of DONSON (Evrony *et al*., 2017).

Table 1.2: Genes linked to microcephaly that encode proteins involved in DNA repair (Jayaraman *et al*., 2018).

Disease	Gene	DNA repair function	Neurological phenotype	Other phenotype
Nijmegen breakage syndrome	*NBS1*	DSB repair	Microcephaly	Immunodeficiency and cancer
Seckel syndrome	*ATR*	Damage sensor	Microcephaly	Severe growth retardation and cancer
Cernunnos deficiency	*XLF*	DSB repair (NHEJ)	Microcephaly	Immunodeficiency
Ligase IV deficiency	*LIG4*	DSB repair (NHEJ)	Microcephaly	Immunodeficiency and cancer
XRCC4 deficiency	*XRCC4*	DSB repair (NHEJ)	Neuronal death	Immunodeficiency and cancer
Ku70 and Ku80 deficiency	*Ku70/80*	DSB repair (NHEJ)	Neuronal death	Immunodeficiency and cancer
DNA-dependent kinase deficiency	*DNA-PK*	DSB repair (NHEJ)	Neuronal death	Immunodeficiency
XRCC2 deficiency	*XRCC2*	DSB repair (homologous recombination)	Neuronal death	Embryonic lethal
Microcephaly vera 1	*MCPH1*	DNA repair or cell cycle control	Microcephaly	Unknown
Microcephaly with seizures	*PNKP*	SSB (BER) and DSB repair	Microcephaly and seizures	No cancer or immune defects

Table 1.3: Phenotype of Seckel syndrome induced by mutations in genes encoding proteins implicated in DNA damage response and centriole biogenesis (Jayaraman *et al*., 2018).

Phenotype	Chromosomal location	Gene/locus	Pathway
SCKL1	3q23	*ATR*	DNA damage response
SCKL2	18q11.2	*RBBP8*	DNA damage response
SCKL3	Not identified	Not identified	Not identified
SCKL4	13q12.12–12.13	*CENPJ*	Centriole biogenesis
SCKL5	15q21.1	*CEP152*	Centriole biogenesis
SCKL6	3q22.2	*CEP63*	Centriole biogenesis
SCKL7	14q22.1	*NIN*	Centriole subdistal appendage protein required to anchor microtubules
SCKL8	10q21.3	*DNA2*	DNA damage response
SCKL9	3p21.31	*TRAIP*	DNA damage response
SCKL10	8q24.13	*NSMCE2*	DNA damage response

DONSON gene is also coexpressed with small interfering RNA (siRNA) and vital DNA replisome constituents. Downregulation of cyclin E2 and D2, and p21 upregulation are a result of DONSON gene knockdown. Another research work used whole-exome sequencing from multiples families in Europe, Asia, Middle east and Africa to discover biallelic DONSON mutation in 29 patients with microcephalic dwarfism (O'Driscoll *et al*., 2003). DONSON gene is considered as a constituent of the DNA replication machinery to sustain stability of genome by stabilizing replication forks and stimulating checkpoints of cell cycle. ATR gene itself is involved in the DNA damage response and Seckel syndrome and also facilitates DONSON to perform its function (O'Driscoll *et al*., 2003; Reynolds *et al*., 2017).

It has been identified recently that a few nascent microcephaly genes that play a role in cytokinesis are involved in a completely novel cellular pathway in the pathogenesis of microcephaly. Citron kinase encoded by *CIT*, which plays a significant role in cytokinesis, is the first example (Harding *et al*., 2016; Li *et al*., 2016). Fascinatingly, citron kinase colocalizes with *ASPM* during cytokinesis at the midbody (Paramasivam *et al*., 2007). Moreover, *ASPM* is the binding companion of *CIT* as confirmed by mass spectrometry (Jayaraman *et al.*, 2016). *KIF14* gene encodes a protein that plays an essential role in cytokinesis and it was discovered that *KIF14* mutations lead to microcephaly and short stature (Moawia *et al.*, 2017). These findings proposed a new role for microcephaly gene encoding proteins in cytokinesis.

It was discovered recently that a new pathway kinetochore/centromere is involved in MPD syndromes and microcephaly. In humans, mutations in *CASC5* are associated with MCPH (Genin *et al*., 2012). Likewise, *CENPE* gene encodes a protein that limits to the kinetochore/centromere, and MPD results due to mutations in *CENPE* (Mirzaa *et al.*, 2014).

For the pathogenesis of microcephaly, the two microcephaly genes *ALFY/WDFY3* emphasize the role of Wnt signaling in the autophagy and developing brain. *ALFY* gene encodes a scaffold protein which helps in autophagy. It causes MCPH due to dominantly inherited mutation. *ALFY* also negatively regulates the canonical Wnt pathway by autophagy-mediated clearance of aggregates of DVL3, a downstream target of Wnt (Kadir *et al*., 2016). NPC proliferation is partly influenced by *ASPM* through the Wnt signaling pathway. It was discovered through genome wide siRNA

screen that *ASPM* acts as a positive regulator of the Wnt pathway (Major *et al*., 2008). In utero electroporation of short hair pin RNAs (shRNAs) to *ASPM* in mice caused defects in neurogenesis and reduced Wnt mediated transcriptional activity in the developing neocortex that were successfully rescued by coexpression of stabilized β-catenin (Buchman *et al*., 2011). Transgenic mice in which β-catenin (a downstream signaling target of Wnt) was constitutively expressed in NPCs developed large brains with increased surface area and folding of the lateral ventricles analogous to gyri, consistent with an expansion of the progenitor pool (Chenn *et al*., 2002). These findings suggest that the Wnt pathway may mediate proliferation defects caused by *ASPM* loss of function, *ALFY* gain of function, and possibly mutations in other microcephaly genes yet to be identified.

There is another category of microcephaly gene encoding proteins implicated in intracellular or transmembrane transport. For instance, the first one is *COH1* that plays an important role in vesicle-aiding intracellular transport, and mutation in *COH1* could lead to retinal dystrophy, autosomal recessive disorders, dysmorphic facies, Cohen syndrome and intermittent neutropenia (Mochida *et al*., 2004; Kolehmainen *et al*., 2013). The second one is *ARFGEF2* that plays an essential role in the transport of proteins especially β-catenin from Golgi complex to plasma membrane, and mutation in *ARFGEF2* could lead to periventricular heterotopia and a microcephaly autosomal recessive syndrome (Sheen *et al*., 2004). Furthermore, *TRAPPC9* protein function is intracellular transport in postmitotic neuron. Mutation in *TRAPPC9* protein could result in variable postnatal microcephaly and autosomal recessive intellectual disability (Mochida., 2009). Next protein which plays a significant role in protein intracellular trafficking is *CHMP1A.* Mutations in this protein lead to microcephaly and small cerebrum (ponto cerebellar hypoplasia) (Mochid *et al*., 2012).

Finally, a new category of microcephaly genes has come to light in the last few years that encode proteins involved in amino acid or protein synthesis. The first of these was *QARS*, which encodes glutaminyl-tRNA synthetase and causes progressive microcephaly with atrophy of cerebellum, cerebral cortex and causes severe seizures when mutated (Zhang *et al*., 2016). Furthermore, *AARS* encodes alanyl-tRNA and loss of its function leads to intractable seizures, progressive microcephaly, spasticity and

hypomyelination (Nakayama *et al*., 2017). Finally, *PYCR2* gene encodes pyrroline-5-carboxylate reductase 2 and mutation in this gene gives rise to hypomyelination and postnatal microcephaly as a result of increased apoptosis (Nakayama *et al*., 2015). These postnatal or progressive microcephaly syndromes are best classified as secondary microcephaly and likely reflect neuronal atrophy rather than decreased proliferation or cell fate changes (unlike many MCPH syndromes), they illustrate the importance of protein and amino acid synthesis pathways in ensuring adequate neuronal survival in the developing central nervous system.

1.12. Therapy

Primary Microcephaly is a lifetime disorder. It may range from severe to mild that's why no standard cure is available so far. MCPH treatment mainly emphasize on symptoms for instance hyperactivity, vision problems and seizures rather than its etiology. Therapeutic strategies such as speech therapy, physical therapy and occupational therapy should be considered for patients who have a trouble in language and speech or motor skills. Medication for instance methylphenidate, an antiepileptic drug, can be used to control hyperactivity and epilepsy. Genetic counseling could benefit people to know the peril of microcephaly in the succeeding pregnancies (Zaqout *et al*., 2017).

1.13. Aim and Objectives

The major aim of this study was to determine the genetic basis underlying microcephaly cases in 3 Pakistani consanguineous families. The specific objectives were:

1. To recruit 3 families with individuals(s) suffering from MCPH.
2. To gather family history and construct pedigree.
3. To perform clinical examination to confirm the disease.
4. To draw blood from patients and controls and extract DNA.
5. To perform linkage analysis to unveil which gene is responsible for MCPH.
6. To perform sequencing of identified gene to determine pathogenic variant(s).

2.Materials and Methods

2.1 Study Subjects

Three families (A, B, C) belonging to Punjab and Khyber Pakhtunkhwa were selected for the study. Information about their family history was taken from the elders at home. Prior to the study onset, acceptance for the research work was acquired from IRB (Institutional Review Board) of Quaid-i-Azam University. Blood was collected from the affected individuals and other family members after obtaining their informed consent. For minors or mentally impaired individuals, consent was taken from the parents/guardians.

2.2 Pedigree Designing

Pedigrees sketch was designed after consultation with the family elders. Squares and circles were drawn in the pedigrees to represent males and females, respectively. The filled symbols represent affected individual, while unfilled represent unaffected. The generation numbers and individual numbers have been shown by Roman numbers and Arabic numbers, respectively. A circle or square crossed by a line indicate the deceased individuals. Consanguineous union of the parents has been shown by a double line.

2.3. Collection of Blood Sample

For blood (5-7ml) extraction, sterilized syringes of 10ml were used. Blood specimens of affected and normal members of the family were obtained in EDTA tubes. The tubes were kept at 4°C.

2.4. Genomic DNA Extraction

Standard phenol-chloroform method was used for extraction of DNA (Sambrook *et al*., 1989). The following steps were practiced during the extraction of genomic DNA:

- In the first step, 700 µL blood was mixed with an equal volume of solution A in an eppendorf tube of 1.5 ml by gently inverting 4-6 times. This tube was incubated at room temperature for 45 minutes after mixing.
- The eppendorf tube containing blood after incubation was rotated for 60 second at 13,000 rpm in a centrifuge.

- In the third step, supernatant was discarded, and pellet was resuspended in 500 µL of solution A.
- The pellet was again spun at 13,000 rpm for 60 seconds.
- After discarding supernatant, solution B 500 µL, proteinase k 10-15 µL and 20-25 µL of 20% SDS were mixed in the eppendorf tube containing pellet. After that, the sample was incubated in incubator overnight at 37ºC.
- On the following day, 500 µL of new mixture of solution C and D (250 µL Sol-C and 250 µL Sol-D) was added to the overnight kept tube containing sample, inverted few times and centrifuged at 13,000 rpm for 10 minutes.
- Three evident layers were formed after centrifugation. With the help of pipette, DNA containing upper layer was picked up and shifted to a new eppendorf tube.
- Then, 500 µL of solution D was added to the eppendorf tubes (containing DNA) and centrifuged at 13,000 rpm for 10 minutes. Once again, via pipette, the upper layer was picked up and transferred to new tube.
- Next, 55 µL sodium acetate and 500 µL chilled isopropanol were added to the tube containing DNA. The tubes were inverted several times in order to precipitate the genomic DNA.
- The eppendorf tube was spun at 13,000 rpm for 10 minutes in centrifuge in order to precipitate the DNA in the form of pellet.
- The upper layer was discarded, and the pellet was cleaned with 200 µl of 70% chilled ethanol.
- The ethanol was cautiously removed, and DNA was dried in vacuum concentrator for about 7-10 minutes at 45ºC.
- At last the DNA was placed in 150 µl Tris- EDTA buffer and incubated in incubator at 37ºC overnight.
- Purified DNA was kept at 4ºC after totally dissolving the pellet.

Table 2.1: Solutions' composition

Solution	Composition
Solution A	Sucrose (0.32 M), MgCl2 (5 mM), Tris (pH 7.5) 1% (v/v), Triton X- 100
Solution B	EDTA (pH 8.8) NaCl 400 mM Tris (pH 7.5) 10 mM
Solution C	Phenol
Solution D	24 parts of Chloroform: one part of Isoamyl Alcohol
20% SDS	10 g in 50 ml water
10X TBE	Tris 0.89 M, Boric acid 0.025 M, 0.1 M EDTA (pH 8.3)
Bromophenol Blue	Sucrose 40 g, Bromophenol Blue 0.25 g

2.5. Agarose Gel Electrophoresis

DNA was run on 1% agarose gel in order to investigate its quantity and quality. For preparation of agarose gel, 45 ml distilled water, 5ml 10X TBE buffer and 0.5g agarose were added in a beaker. After mixing, beaker was placed for 2-3 minutes in a microwave oven to completely dissolve the agarose to form clear solution. Then 5µl ethidium bromide was added and mixed in it. At last, for solidification of gel, the solution in the beaker was poured into the gel tank and kept at 25^{o}C for 30-40 minutes. Combs were placed for the formation of wells. When gel solidified completely, combs were removed carefully, and the gel was placed in gel running tank containing running buffer (1X TBE). 3 µl of loading dye bromophenol was added to an equal volume of purified DNA and loaded into each well of the gel. After that, next step was to connect the tank with the power supply of 100 volts and was run for 20-30 minutes. For visualization of DNA band, gel was placed in the gel dock system in order to take image.

2.6. Homozygosity Mapping

In this research work, genetic mapping of 3 MCPH families A, B, C was carried out for the known genes, using microsatellite markers. The physical distance of these known loci was identified through UCSC genome browser and the genetic distance of the microsatellite markers flanking these loci was found on the basis of the physical distance. To confirm the homozygosity and heterozygosity of candidate genes, 10 microsatellite markers harboring the respective genes were amplified in all available normal and affected members of each family. In case of linkage with a gene, the gene was subjected to Sanger sequencing for screening of pathogenic sequence variants.

2.7. Polymerase Chain Reaction (PCR)

The DNA dilutions were used for amplification of STS markers through polymerase chain reaction. For preparation of PCR reaction mixture, 200 µl PCR tubes were used (Axygen, California, USA).

Table 2.2: Composition of PCR mixture

Chemicals	Amounts
DNA	1 µL
PCR buffer	2.5 µL
$MgCl_2$	2 µL
dNTPs	0.5 µL
Taq Polymerase	0.3 µL
Primers (F and R)	0.3 µL
PCR water	18.1 Ml

The reaction mixture was vortexed and then put in a Thermocycler. The standard PCR conditions were followed, which were:

Initial denaturation at 96°C for 10 minutes

Denaturation of DNA at 96°C for 1 minute
Primer annealing at 54-58°C for 1 minute
Extension at 72°C for 1 minute] **39 Cycles**

Final amplification at 72°C for 10 minutes

2.8. Polyacrylamide Gel Electrophoresis (Vertical Gel)

8 percent polyacrylamide gel was used to examine the PCR products.

Table 2.3: Composition of polyacrylamide gel

Chemicals	**Amounts**
30% Acrylamide	13.5 microliter
10X TBE	5 microliters
APS 10 %	350 microliters
TEMED	25 microliters
Distilled water	Final volume raised to 50 microlitre with distilled water

Eight percent polyacrylamide gel (1 plate) was prepared. Firstly, gel assembly was done by combining the 2-glass plates and fixing spacers between them in order stop the leakage of gel. After that, next step was to prepare the gel by mixing 5 microlitre of 30% acrylamide, 5 microlitre of 10X TBE and distilled water to raise up the volume to 50 microlitre in a graduated cylinder. Then 400 microlitre of 10% APS and 25 microlitre TEMED were added to it. Finally, the prepared gel solution was poured into the assembled cast and a comb was fixed to create wells for DNA loading. For solidification of gel, it was placed for 30-40 minutes at 25°C. The final step was to combine 6 ml bromophenol blue, a loading dye, with the amplified PCR product, mix it and then load in the wells of the vertical gel. Gel electrophoresis was performed for 2-3 hours at 120 V in vertical gel tank. To make the DNA visible under UV light ethidium bromide was used. Gel pictures were taken by using digital camera attached with Gel Dock.

2.9. Sequencing of *ASPM*

Using online tools primer 3 (version 4), primers 17 and 18 for the exons of ASPM gene were designed. After establishing linkage to *MCPH5*, the candidate gene *ASPM* was sequenced in all the family members (normal & affected) of family B.

2.9.1. *Pre-Sequencing PCR*

For the amplification of all the exons of *ASPM*, the standard PCR protocol was followed. After PCR, the amplified PCR products were run on a two percent agarose gel to check the specificity of the products. The purification kit was used for purification of specific PCR amplified products.

2.9.2. *Purification of Pre-sequencing PCR Products*

After the confirmation of specific exonic amplification, the highly specific PCR products were purified via Gene Jet PCR purification kit. The purification protocol is as follow:

- 125 µL of binding buffer was add and mixed in the amplified PCR product.
- The mixture of PCR product and binding buffer was shifted to the column of purification and spun for 60 seconds at 13,000 rpm in a centrifuge. The fluid which passed through the column was removed after the centrifugation procedure.
- For the removal of unused primers, dNTPs, polymerase and buffer, washing buffer of 500 µL was added followed by centrifugation at 13,000 rpm for 60 seconds and again the fluid which passed through the column was removed. This step was repeated two times.
- Later, an empty spin was given at 13,000 rpm for 2-3 minutes to eliminate the washing buffer remnant.
- Column of purification was then shifted to a new eppendorf tube and 30 µL of elution buffer was added in the middle of the column and was kept for 10 minutes at 25 ^{o}C for correct elution of DNA.
- After incubation, again a spin at 13,000 rpm for 2 minutes was given to purification column and the purified final product was obtained in the eppendorf tube.
- 2 percent gel was used for identification of purified products.

2.9.3. *Sequencing PCR*

Sequencing PCR was performed using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit V 3.1 (PE Applied Biosystems). The reaction mixture contained 1-2 µl (25 ng) of DNA, 1.5-2 µl of primers (reverse/ forward), one microlitre of sequencing buffer, 6µl of distilled water and 1 µl of ready reaction mixture (RR).

For sequencing PCR, the following conditions were used:

First step: Initial denaturation of DNA at 96ºC for 3 minutes.

Second step: For amplification, 30 PCR cycles were needed, each consisting of 3 sub cycles:

- Denaturation of genomic DNA at 96ºC for half minute
- Annealing of primer (forward or reverse) at 55ºC for 30 seconds
- Extension of the DNA template at 72ºC for 4 minutes

Third step: Final extension at 72ºC for 10 minutes.

2.9.4. *Sequencing PCR Products Purification*

Ethanol precipitation method was used for the purification of sequencing PCR products. The protocol of the ethanol precipitation method is as following:

- For preparation of stop solution, 2 µL of Na-acetate ,2 µL of Na-EDTA, and one microlitre of 20 mg/ml glycogen were mixed for each 10 milliliters of sequencing PCR product.
- The product was transferred to 1.5 µL tube. Then 5 µL of stop solution and 60 mL of chilled ethanol 100% were added in the microcentrifuge tube having the sequencing product.
- For appropriate mixing, the microcentrifuge tube was vertex and incubated at 10 minutes at room temperature. The tube was then spun at 12,000 rpm for 20 minutes in a centrifuge. The supernatant was discarded.
- Freshly prepared 150 µL solution of 70% ethanol was added to the tube and again centrifugation was done at 13,000 rpm for 10 minutes. The supernatant was removed once again.

- For the removal of the remaining ethanol, the tubes were dried at 45ºC for 2-3 minutes in concentrator.
- To re-suspend the pellet, twenty microlitre of formamide was added and then the mixture was shifted to 0.5 microlitre tubes of septa for sequencing in automated DNA Sequencer.

2.9.5. Analysis of Sequencing Data

Using Bio Edit alignment editor version 7.1.3.0, the sequencing data was analyzed by comparing the sequencing data of affected individual with normal version of the corresponding gene to check nucleotide variation. The reference sequence of the gene was downloaded from Ensembl Genome Browser database (http://www.ensembl.org/index.html). The variants found were than compared with the cDNA sequence of the corresponding gene to check whether the variant was pathogenic or benign.

Table 2.4: List of microsatellite markers used for linkage analysis

S. No	Gene	Cytogenetic position	Microsatellite Marker	Genetic Location (cM)
1	MCPH1 *MCPH1*	8p23.1	D8S439 D8S518 D8S1140 D8S277 D8S561 D8S1706	9.96 c M 12.26 c M 15.08 c M 17.64 c M 18.13 c M 19.19 c M
2	MCPH2 *WDR62*	19q	D19S1170 D19S416 D19S893 D19S220 D19S422 D19S190	54.58 c M 56.28 cM 59.73 c M 62.62 c M 63.12 c M 64.03 c M
3	MCPH3 *CDK5RAP2*	9q33.2	D9S1776 D9S907 D9S170 D9S1802 D9S762 D9S2155	123.79 c M 124.24 c M 125.51 c M 126.13 c M 127.65 c M 132.42 c M
4	MCPH4 *CASC5*	15q15.1	D15S994 D15S514 D15S222 D15S659 D15S1039 D15S1028	41.73 cM 42.12 cM 42.81 cM 43.74 cM 45.72 cM 46.89 cM
5	MCPH5 *ASPM*	1q31.3	D1S3468 D1S2625 D1S408 GATA135F02 D1S1660 D1S2660	201.3 cM 201.7 cM 203.86 cM 204.64 cM 205.81 cM 206.75 cM

6	MCPH6 *CENPJ*	13q12.12	D13S1285 D13S742 D13S1243 D13S252 D13S787 D13S1254	5.41 cM 8.75 cM 11.71 cM 13.94 cM 16.05 cM 17.27 cM
7	MCPH7 *STIL*	1p33	D1S2706 D1S2130 D1S211 D1S451 D1S2874 D1S386	75.2 cM 76.12 cM 78.1 cM 79.35 cM 80.58 cM 82.61 cM
8	MCPH8 *CEP135*	4q12	D4S3025 D4S2381 D4S1594 D4S428 D4S1592 D4S3248	65.11 c M 66.43 c M 69.43 c M 70.47 c M 74.06 c M 76.56 c M
10	MCPH10 *ZNF335*	20q13.12	D20S150 D20S861 D20S911 D20S886 D20S891 D20S197	64.05 c M 65.29 c M 66.53 c M 69.36 c M 70.74 c M 71.66 c M

Table 2.5: Primers for amplifying selected *ASPM* exons

Exon No.	**Primer Sequence**		**Tm**
17	Forward	5’ TGTAGGGGTGTTTTATTTCC 3’	57 ºC
	Reverse	5’ CTTCATCACATTTTGCCTTC 3’	
18.1	Forward	5’ GAATTGGCTACAGGTATATC 3’	50 ºC
	Reverse	5’ GGTTAGTATGGACTCTTTTC 3’	
18.2	Forward	5’ AAGAGCTTTTAGAGAATGGC 3’	52 ºC
	Reverse	5’ TCATCTTAACAGTTGACTGC 3’	
18.3	Forward	5’ GCATATAGAGGGATGCAAGC 3’	58 ºC
	Reverse	5’ TGGCCATTCTAAAAGCAGAC 3’	
18.4	Forward	5’ TGGTACAGGGCGTACAAGAC 3’	60 ºC
	Reverse	5’ GTGTTGCTGCAGTCTGCATC 3’	
18.5	Forward	5’ ATTCAACACATGCACAGGGC 3’	57 ºC
	Reverse	5’ GTCTGAGATAATGCTGCCTC 3’	
18.6	Forward	5’ TTCCTTCAGGTACAAAATGCAG 3’	60 ºC
	Reverse	5’TAGCCCACTGAAGCTTTTGG 3’	

3.RESULTS

3.1 Family Description

3.1.1. Family A

Family A belongs to district Multan, Punjab, Pakistan. The information about pedigree and family history was taken from senior family figure after obtaining their willingness. Due to their cultural values and ethnicity, consanguineous marriages were prime option for the family. Autosomal recessive mode of inheritance and microcephaly at birth were found in the affected patients. After clinical examination, it was found that the disease was not triggered by a any environmental factor; nor was it related with any identified syndrome. The symptoms of affected individuals were reduced skull size, mental retardation, weak eye sight, inability to walk and stand upright, muscular dystrophy and saliva release from mouth. The pedigree sketch (Figure 3.1) showed four generations, consisting of thirteen members out of them, three were affected candidates including two boys and one girl. Blood specimens were obtained from three members of the family comprising of one normal individual (III-1), one affected patient (IV-3) and carrier mother.

3.1.2. Family B

Family B belonged to district Peshawar, Khyber Pakhtunkhwa Pakistan. A detail discussion with the family's elders helped in designing the pedigree of the family. Clinical examination showed that the disease was congenital and isolated. The pedigree elaborated that the pattern of inheritance was autosomal recessive. The pedigree had four generations having seventeen individuals along with 2 afflicted patients in the 4th generation. Blood specimens were collected from three (III-5, III-6, IV-4) normal members and three (IV-1, IV-2, IV-3) affected members. Reduced head size, mental retardation and inability to talk properly were significant features identified in the affected individuals of the family. Ages and head sizes of affected individuals were 23 years (MCP 167-1); 16 inches, 21 years (MCP 167-2); 15 inches and 19 years (MCP 167-3); 15 inches, respectively.

3.1.3. Family C

Family C belonged to district Dikhan, Khyber Pakhtunkhwa, Pakistan. The pedigree outline was designed after a detailed discussion with family's elders. The pedigree revealed that the disease was autosomal recessive. The clinical analysis confirmed that the microcephaly was primary and isolated. The pedigree had four generations with fifteen members (2 affected in the fourth generation). Blood samples were obtained from two normal (III-1, III-2) and two afflicted (IV-1, IV-2) individuals. Mental retardation, inability stand upright, reduced head circumference and speech impairment were the main characteristics recognized in the affected individuals. Ages of affected individuals were 8 years and 25 years, while the head circumference was 14 and 15 inches, respectively.

3.2. Homozygosity mapping of disease-causing genes for Hereditary Primary Microcephaly

Homozygosity linkage analysis guidelines recommend that firstly known loci of hereditary primary microcephaly should be checked for either linkage or exclusion mapping before exon or whole genome sequencing. Family A and C of primary microcephaly was processed for trial linkage to 10 most prominent known MCPH loci in Pakistani population. Family B of microcephaly was tested for linkage analysis to ASPM gene. Linkage analysis was done by mapping candidate genes or loci via highly polymorphic microsatellite markers segregating along with specific genes or loci. For linkage analysis, amplification of microsatellite markers was performed via typical PCR reaction and gel electrophoresis of PCR products in non-denaturing polyacrylamide gel 8% as earlier described in Materials and Methods. After electrophoresis, the gel was stained in ethidium bromide for visualizing amplified PCR products and genotyping was allotted by visual checkup. Homozygous pattern of bands was seen for diseased individuals and heterozygous pattern of bands was observed for normal members of families during linkage analysis. Affected members' homozygous banding pattern, taken through Gel Dock, was studied as linked, while heterozygous pattern of affected individuals was marked as not linked.

In family A, three DNA specimens of two normal (III-2 and IV-5) and one affected (III-2) individuals, were considered for genotyping by means of STS markers. Examination of the results revealed that the family was not linked to any of the known MCPH genes, indicating the contribution of an unknown gene so far not identified as liable for congenital primary microcephaly in this family.

Six DNA samples of family B comprising of three affected patients (IV-1, IV-2, IV-3) along with normal members (III-5, III-6, IV-4) were subjected to genotyping through highly polymorphic microsatellite markers. Family B showed linkage to ASPM gene. Homozygosity in afflicted member and heterozygosity in normal candidate was exposed through linkage analysis.

Four DNA samples of family C comprising of two affected individuals (III-1, III-2) along with normal members (IV-1, IV-2) were subjected to genotyping. Examination of results revealed that family was not linked to any of the known MCPH gene, indicating the contribution of an unknown gene so far not identified as liable to congenital primary microcephaly in this family.

3.3. Sequencing of *ASPM* gene

Family B exhibited linkage to *ASPM* gene. Only two exons 17 and 18 of ASPM gene were selected for sequencing which are frequently present in most of the times in Pakistani population. Sequencing results of these selected exons did not show any pathogenic variants in affected individual (IV-3) of family B, suggesting that functional variant may be present in other exons which are not sequenced or regulatory sequence of this gene.

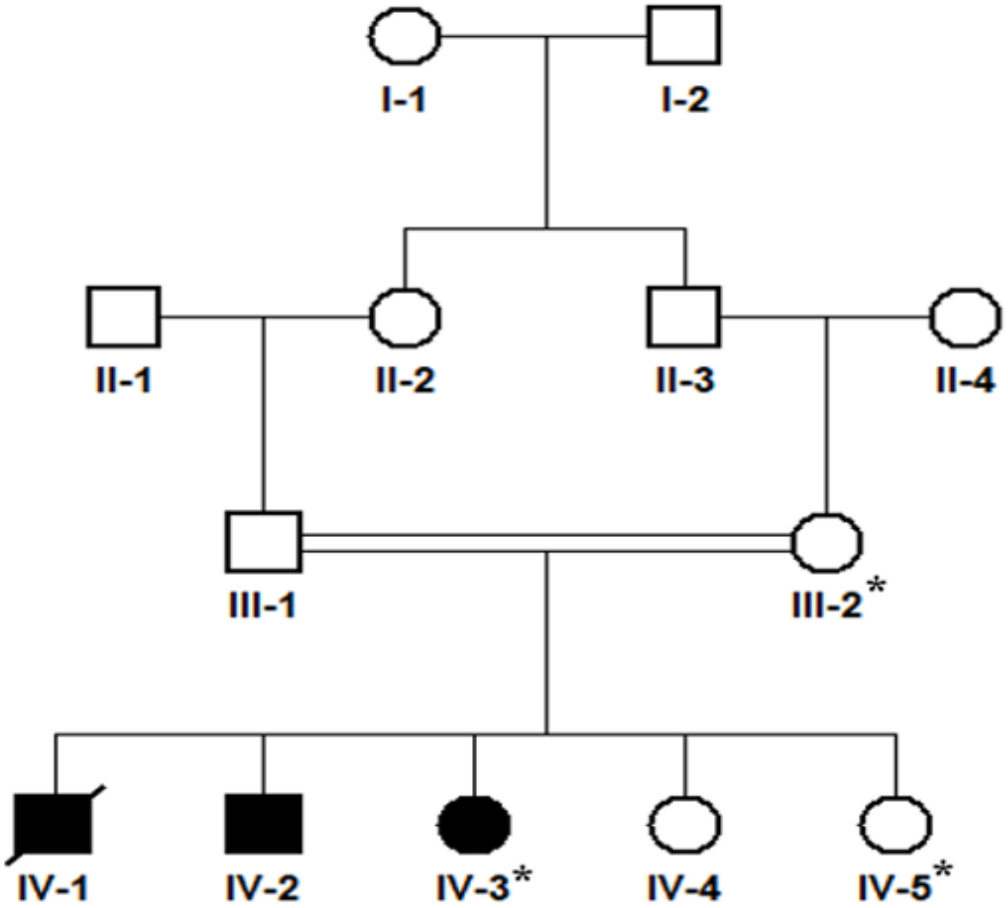


Figure 3.1: Pedigree sketch of family A having primary microcephaly. Squares and circles are used for males and females, respectively. Affected member are denoted by filled symbols and normal individuals by empty symbols. Deceased individuals are represented by crossed circles and squares, respectively. Arabic numbers are used to show the individuals within a generation whereas the Roman numbers represent the generations.

Figure 3.2: (a) Frontal view of affected individual IV-3 (b) Lateral view of affected member IV-3**.** The affected individual showed sloppy head, was mentally retarded and had weak eye sight.

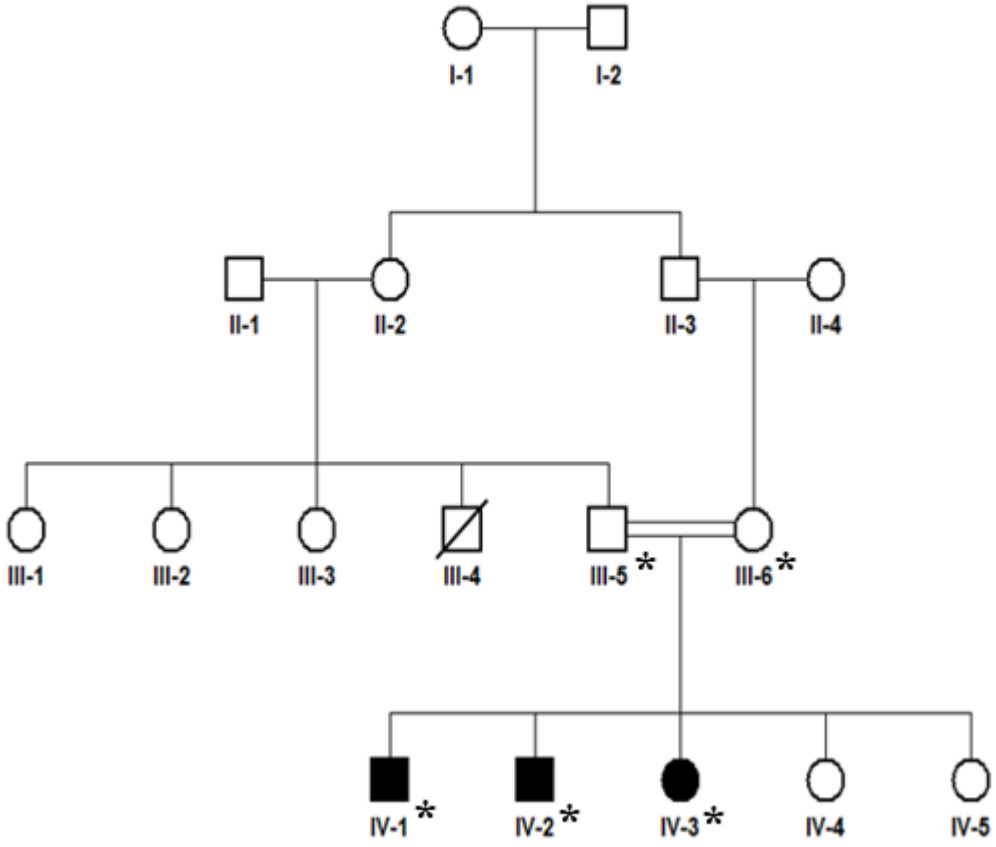


Figure 3.3: Pedigree drawing of Family B with Primary Microcephaly. Males and females are indicated by squares and circles, respectively. Filled symbols portray affected and empty symbols describe normal individuals. Deceased individuals are represented by crossed circles and squares. Arabic numbers are used to show the individuals within a generation whereas the Roman numbers show the generations.

Figure 3.4: (a) Frontal view of affected individual IV-1 (B) Frontal view of affected individual IV-2 **(**c) Frontal view of affected individual IV-3.

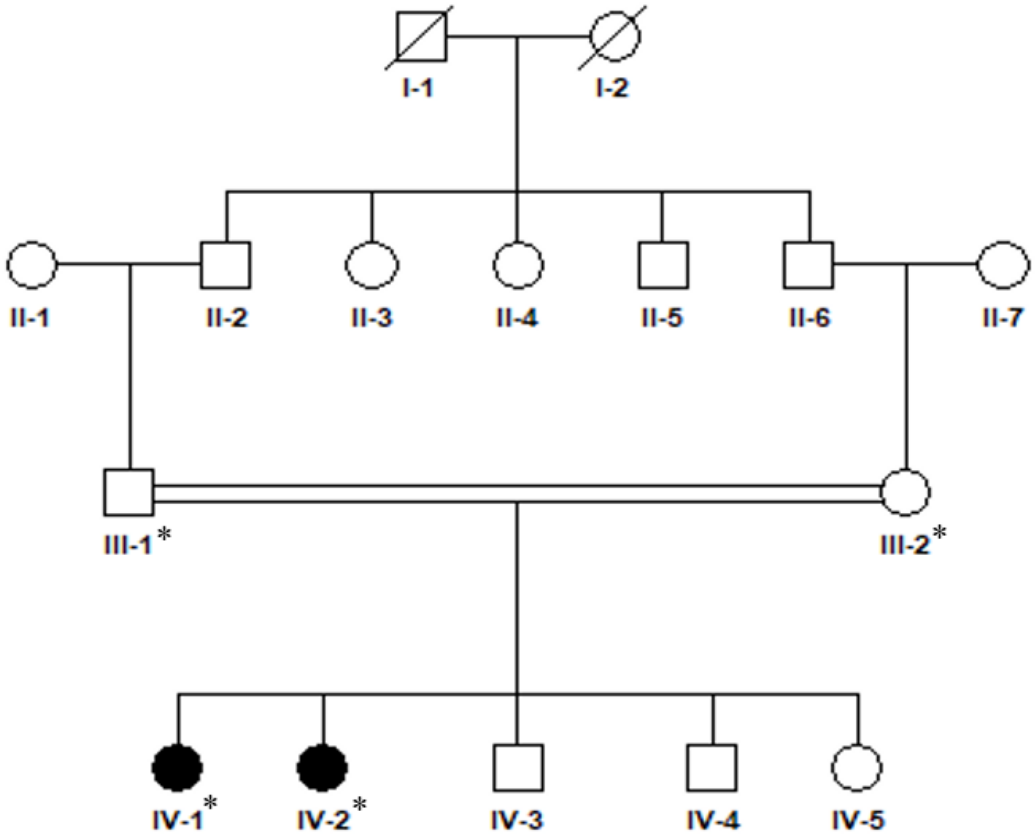


Figure 3.5: Pedigree sketch of Family C with Primary Microcephaly. Males and females are shown by squares and circles, respectively. Normal and affected individuals are indicated by empty symbols and filled symbols, respectively. Deceased individuals are represented by crossed circles and squares. Arabic numbers are used to show the individuals within a generation whereas the Roman numbers show the generations.

Figure 3.6: (a) Frontal view of affected individual IV-1 (b) Frontal view of affected individual IV-I**.**

3.4. Family A→*MCPH1* (8p23.1)

Family A was analyzed for specific alleles at *MCPH1* locus, amplified with below listed markers and results are illustrated in Figure 3.7.

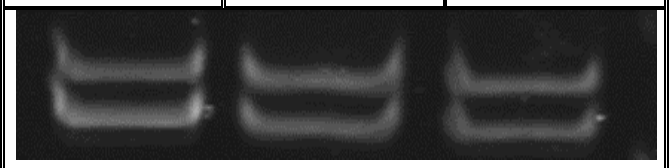
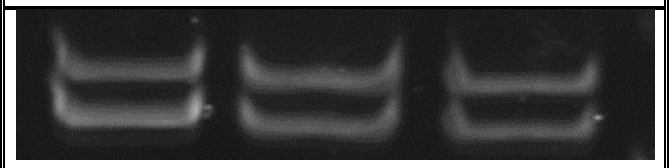
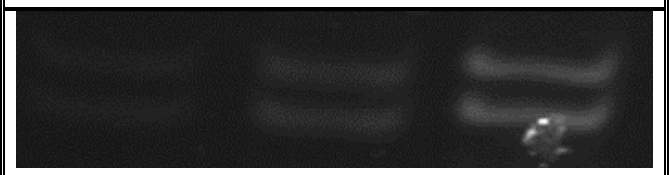
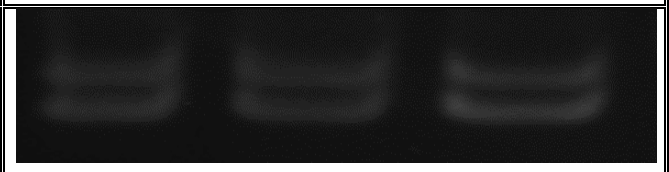
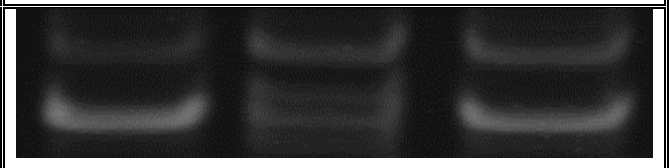
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D8S439	9.96			
D8S518	12.26			
D8S1140	15.08			
D8S277	17.64			
D8S561	18.13			

Figure 3.7: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH1* (Microcephalin) locus at chromosome 8p23.1. Roman with Arabic numerals indicate the family members of the pedigree.

3.5. Family A→ *MCPH 2* (19q13.12)

Family A was analyzed for specific alleles at *MCPH2* locus, amplified with below listed markers and results are illustrated in Figure 3.8.

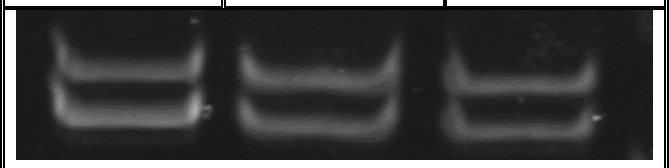
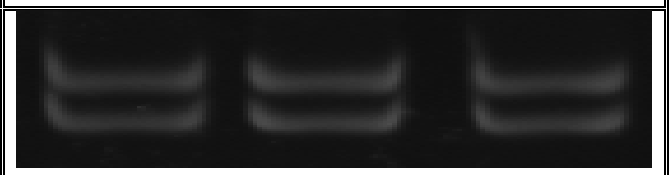
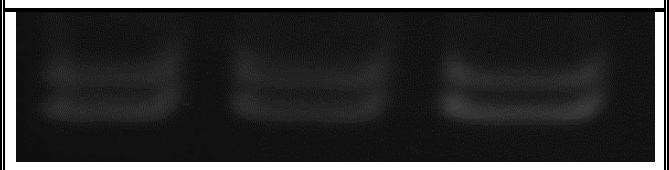
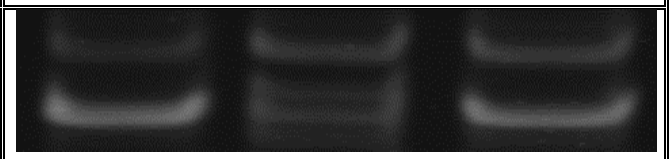
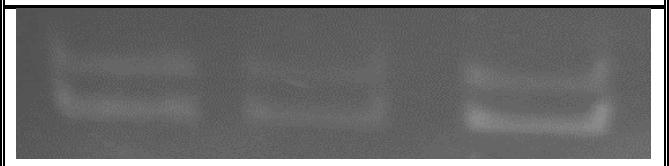
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D19S1170	54.58			
D19S416	56.28			
D19S220	62.62			
D19S422	63.12			
D19S190	64.03			

Figure 3.8: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within MCPH2 (*WDR62*) locus at chromosome 19q13.12. Roman with Arabic numerals indicates the family members of the pedigree.

3.6. Family A→ *MCPH3* (9q33.2)

Family A was analyzed for specific alleles at *MCPH3* locus, amplified with below listed markers and results are illustrated in Figure 3.9.

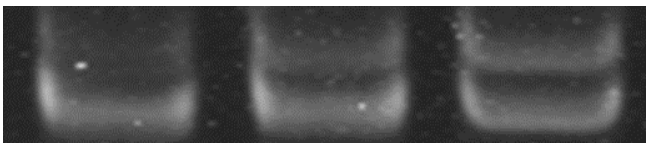
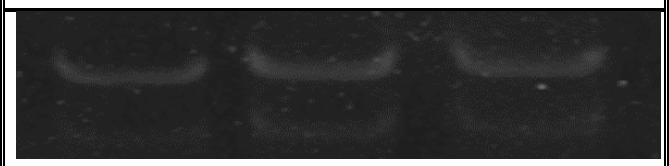
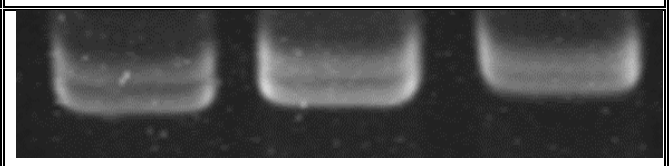
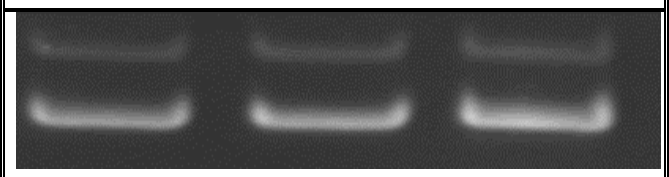
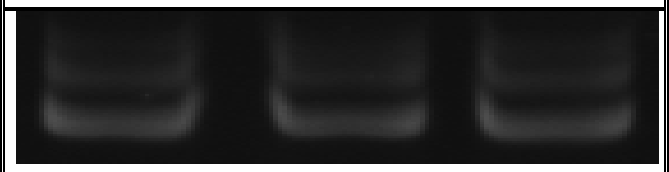
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D9S1824	122.39			
D9S170	125.51			
D9S289	121.32			
D9S907	124.24			
D9S1776	123.79			

Figure 3.9: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH3* (*CDK5RAP2*) locus at chromosome 9q33.2. Roman with Arabic numerals indicate the family members of the pedigree.

3.7. Family A→ *MCPH4* (15q15.1)

Family A was analyzed for specific alleles at *MCPH4* locus, amplified with below listed markers and results are illustrated in Figure 3.10.

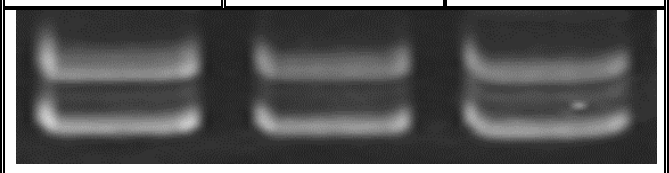
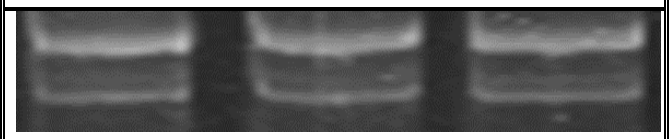
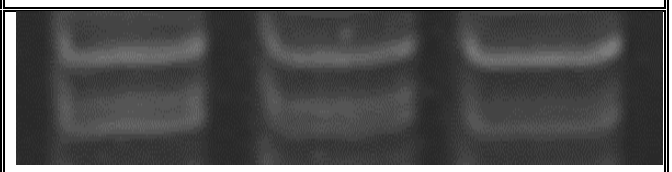
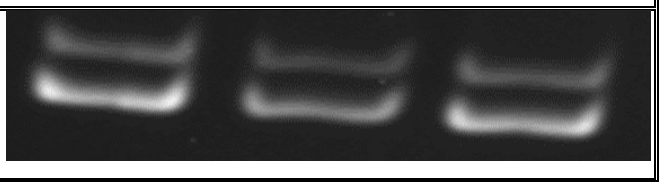
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D15S784	42.12			
D15S641	41.37			
DI5S222	42.81			
D15S1039	45.72			
D15S1044	38.97			

Figure 3.10: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH4* (*CASC5*) locus at chromosome 15q15.1. Roman with Arabic numerals indicate the family members of the pedigree.

3.8. Family A→ *MCPH5*(1q31.3)

Family A was analyzed for specific alleles at *MCPH5* locus, amplified with below listed markers and results are illustrated in Figure 3.11.

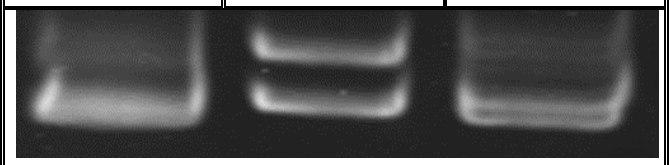
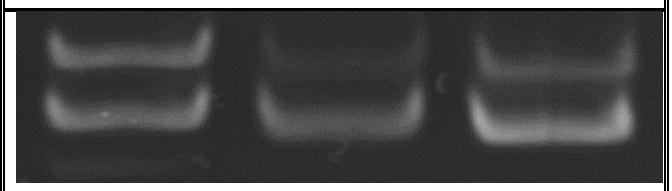
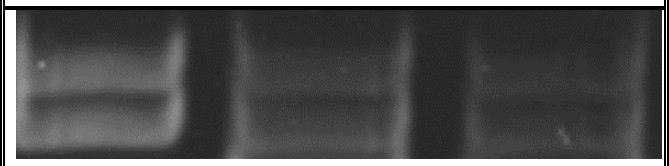
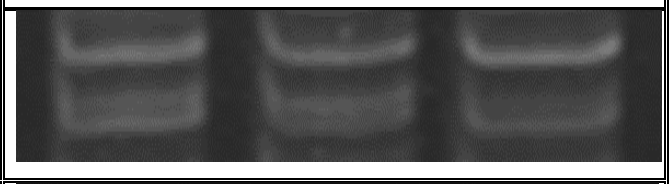
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D1S1183	204.64			
D1S3468	201.3			
DIS2622	203.86			
DIS2816	206.75			
DIS533	204.78			

Figure 3.11: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH5(ASPM)* locus at chromosome 1q31.3. Roman with Arabic numerals indicate the family members of the pedigree.

3.9. Family A→ *MCPH6* (13q12.12)

Family A was analyzed for specific alleles at *MCPH6* locus, amplified with below listed markers and results are illustrated in Figure 3.12

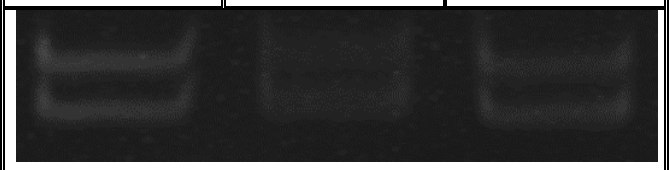
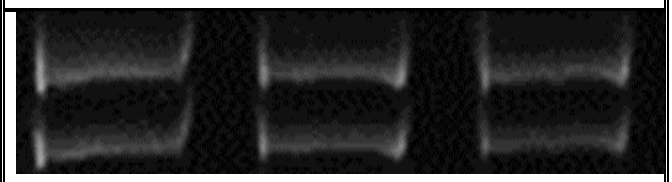
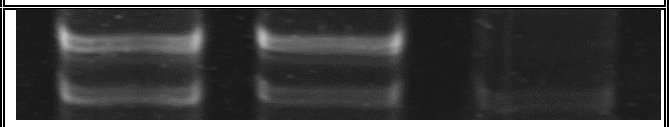
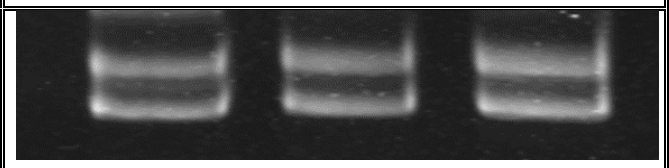
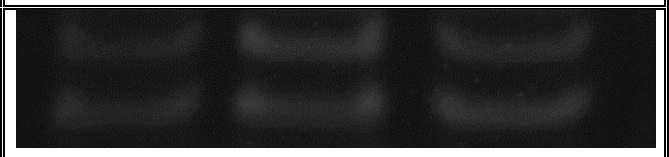
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D13S1285	5.41			
D13S742	8.75			
D13S1243	11.71			
D13S252	13.94			
D13S787	16.05			

Figure 3.12: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH6* (*CENPJ*) locus at chromosome 13q12.12. Roman with Arabic numerals indicate the family members of the pedigree.

3.10. Family A→*MCPH7*(1p33)

Family A was analyzed for specific alleles at *MCPH7* locus, amplified with listed markers and results are illustrated in Figure 3.13.

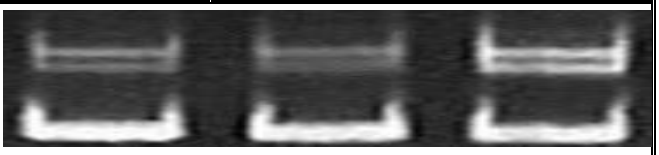
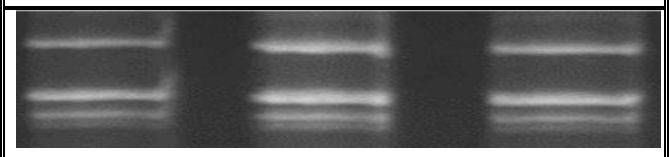
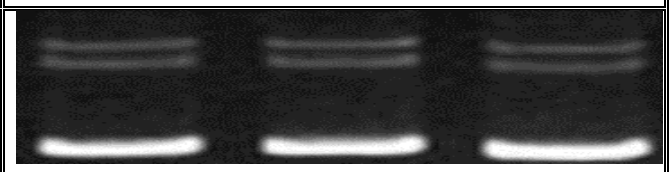
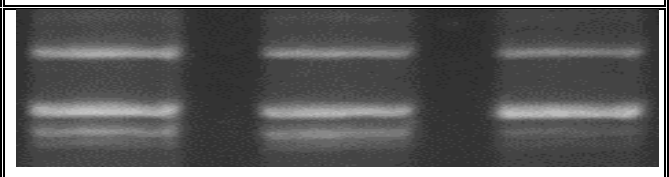
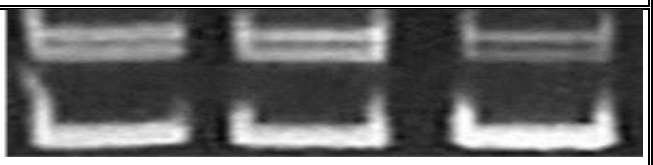
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
DIS2130	76.12			
D1S211	78.1			
D1S2797	79.35			
DIS2874	80.58			
DIS386	82.61			

Figure 3.13: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH7* (*STIL*) locus at chromosome 1p33. Roman with Arabic numerals indicate the family members of the pedigree.

3.11. Family A→*MCPH8* (4q12)

Family A was analyzed for specific alleles at *MCPH8* locus, amplified with listed markers and results are illustrated in Figure 3.14.

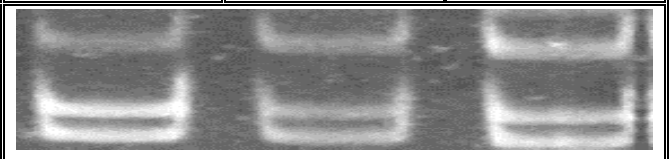
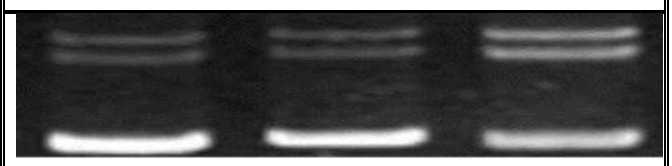
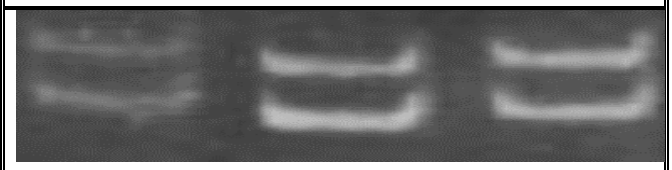
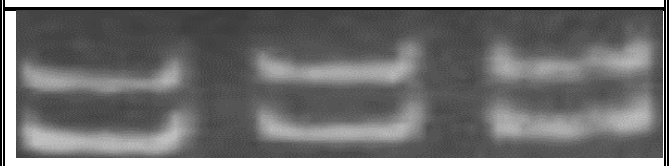
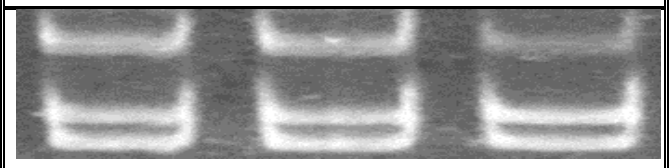
marker	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D4S428	72.47			
D4S2379	72.39			
D4S3000	73.42			
D4S3019	74.06			
D4S1596	76.56			

Figure 3.14: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within MCPH8 (*CEP135*) locus at chromosome 4q12. Roman with Arabic numerals indicate the family members of the pedigree.

3.12. Family A→ *MCPH9* (15q21.1)

Family A was analyzed for specific alleles at *MCPH9* locus, amplified with below listed markers and results are illustrated in Figure 3.15.

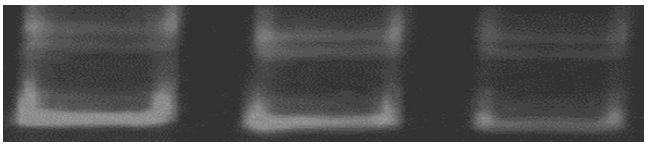
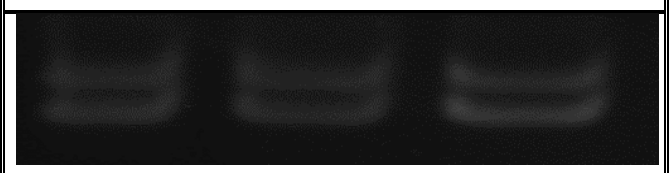
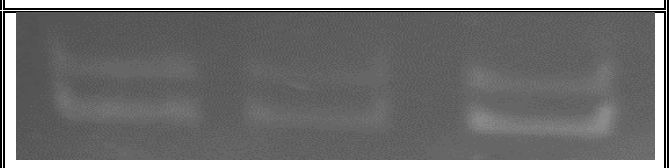
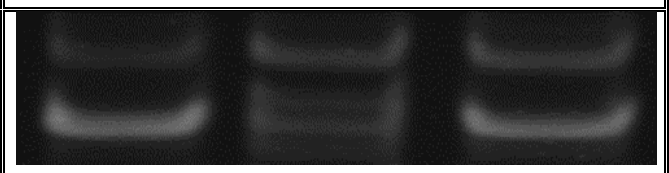
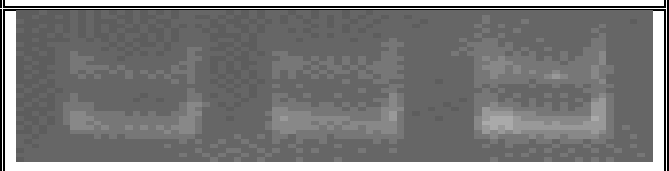
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D15S1028	46.89			
D15S978	47.92			
D15S982	48.52			
D15S170	50.28			
D15S962	51.59			

Figure 3.15: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH9* (*CEP152*) locus at chromosome 15q21.1. Roman with Arabic numerals indicate the family members of the pedigree.

3.13. Family A→ *MCPH10* (20q13.12)

Family A was analyzed for specific alleles at *MCPH10* locus, amplified with below listed markers and results are illustrated in Figure 3.16.

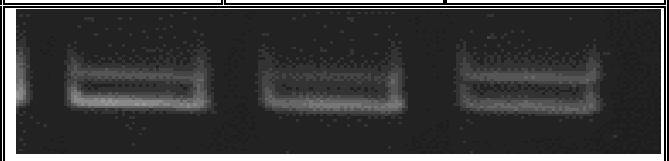
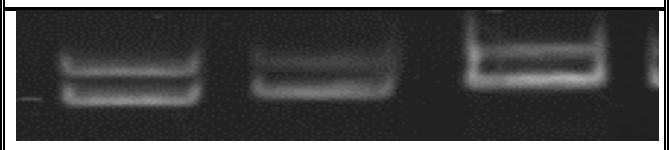
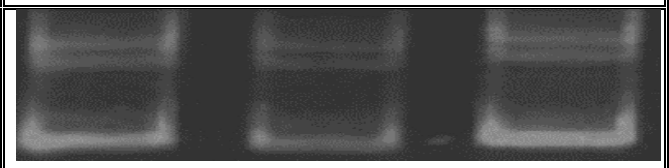
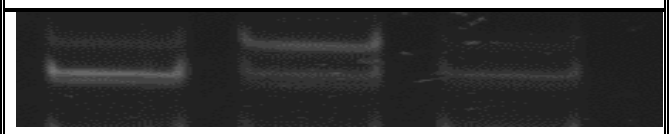
Markers	Map unit cM	IV-3 Affected	IV-5 Normal	III-2 Normal
D20S911	66.53			
D20S856	67.76			
D20S862	68.41			
D20S886	69.36			
D20S445	74.57			

Figure 3.16: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH10* (*ZNF335*) locus at chromosome 20q13.12. Roman with Arabic numerals indicate the family members of the pedigree.

3.14. Markers in family B showing linkage at ASPM Gene (MCPH5 Locus)

Family B→ *MCPH5*(1q31.3)

Family B was analyzed for specific alleles at *MCPH1* locus, amplified with below listed markers and results are illustrated in Figure 3.17.

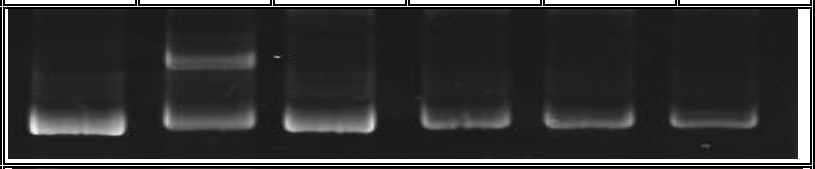
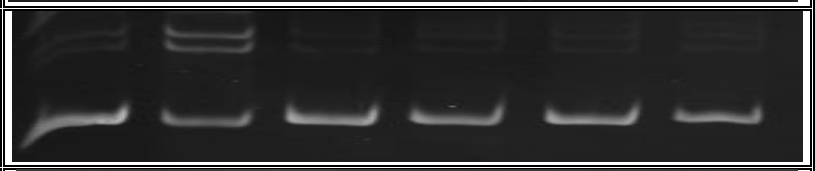
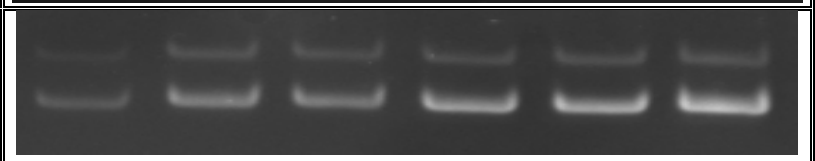
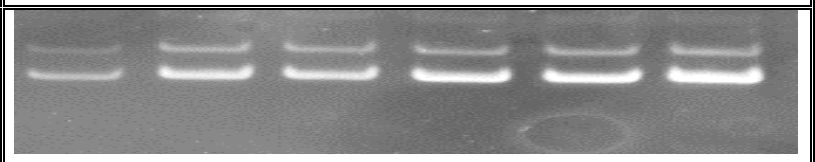
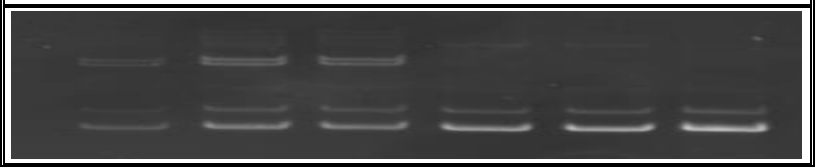
Markers	Map unit cM	III-5 Normal	III-6 Normal	IV-4 Normal	IV-1 Affected	IV-2 Affected	IV-3 Affected
DIS2625	201.1						
D1S1183	204.64						
D1S3468	201.3						
D1S2622	203.86						
D1S533	204.78						

Figure 3.17: Electropherogram of 8 percent non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH5* (*ASPM*) locus at chromosome 1q13.12. Roman with Arabic numerals indicate the family members of the pedigree.

3.15. Family C→ *MCPH1* (8p23.1)

Family C was analyzed for specific alleles at *MCPH1* locus, amplified with below listed markers and results are illustrated in Figure 3.18.

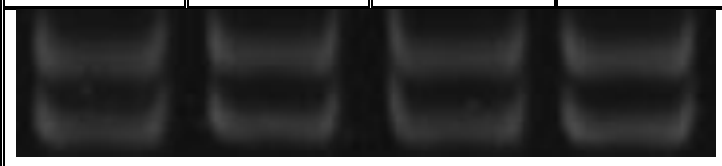
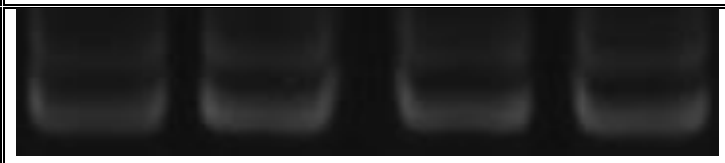
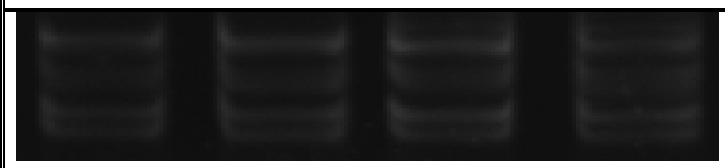
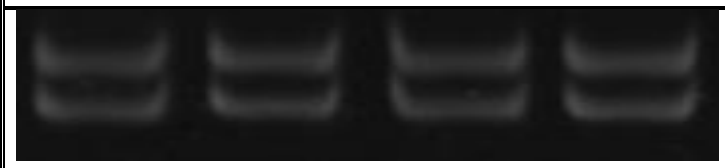
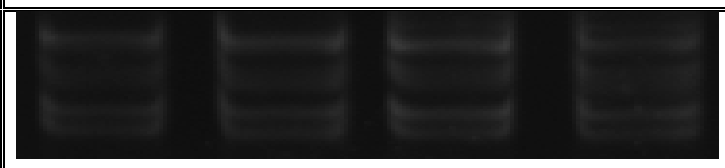
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D20S911	66.53				
D20S856	67.76				
D20S862	68.41				
D20S886	69.36				
D20S445	74.57				

Figure 3.18: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH1* (*microcephalin*) locus at chromosome 8p23.1. Roman with Arabic numerals indicate the family members of the pedigree.

3.16. Family C→ *MCPH2* (19q13.12)

Family C was analyzed for specific alleles at *MCPH2* locus, amplified with below listed markers and results are illustrated in Figure 3.19.

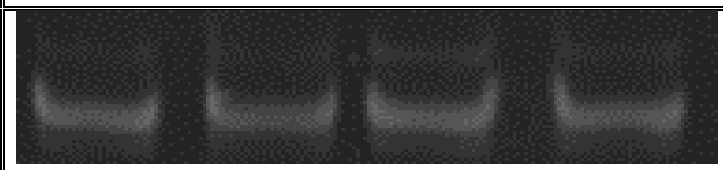
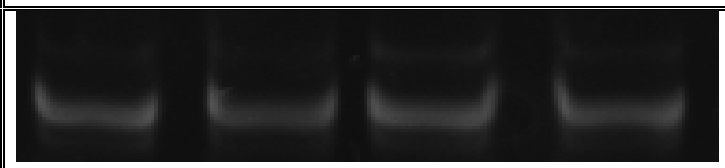
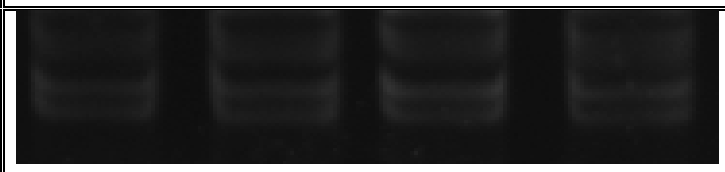
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D19S1170	54.58				
D19S416	56.28				
D19S220	62.62				
D19S422	63.12				
D19S190	64.03				

Figure 3.19: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH2* (*WDR62*) locus at chromosome 19q13.12. Roman with Arabic numerals indicate the family members of the pedigree.

3.17. Family C→ *MCPH3* (9q33.2)

Family C was analyzed for specific alleles at *MCPH3* locus, amplified with below listed markers and results are illustrated in Figure 3.20.

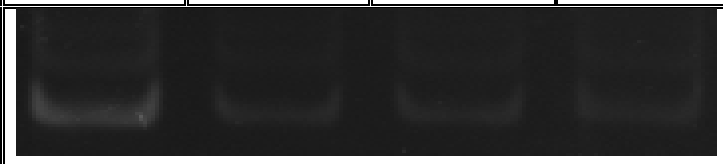
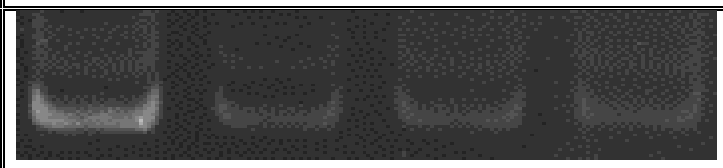
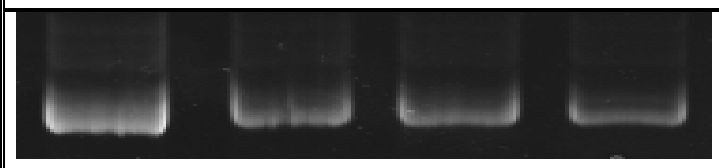
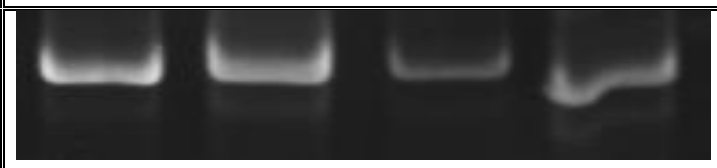
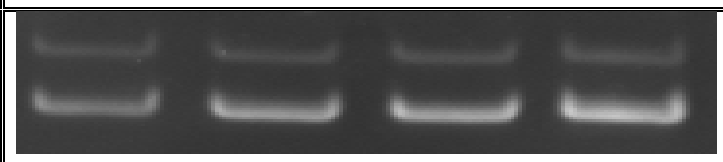
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D9S1824	122.39				
D9S170	125.51				
D9S289	121.32				
D9S907	124.24				
D9S1776	123.79				

Figure 3.20: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH3* (*CDKPAP2*) locus at chromosome 9q33.2. Roman with Arabic numerals indicate the family members of the pedigree.

3.18. Family C→ *MCPH4* (15q15.1)

Family C was analyzed for specific alleles at *MCPH4* locus, amplified with below listed markers and results are illustrated in Figure 3.21.

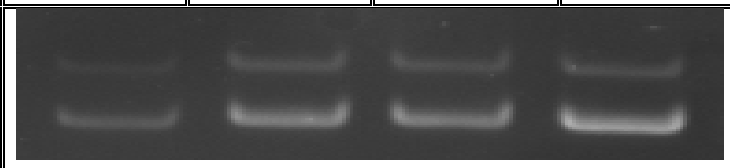
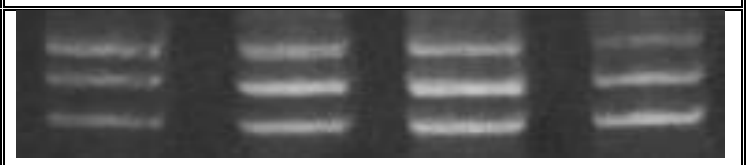
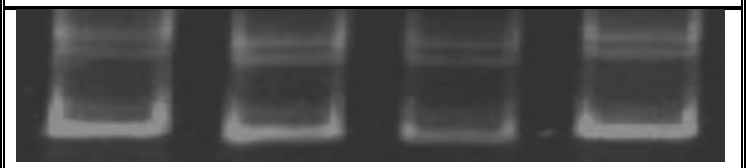
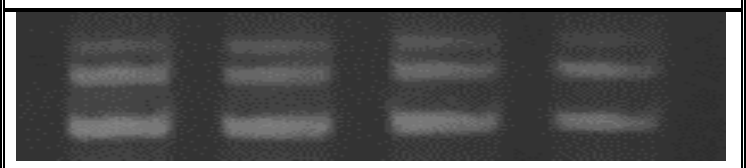
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D15S784	42.12				
D15S641	41.37				
D15S222	42.81				
D15S1039	45.72				
D15S1044	38.97				

Figure 3.21: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH4* (*CASC5*) locus at chromosome 15q15.1. Roman with Arabic numerals indicate the family members of the pedigree.

3.19. Family C→ *MCPH5* (1q31.3)

Family C was analyzed for specific alleles at *MCPH5* locus, amplified with below listed markers and results are illustrated in Figure 3.22.

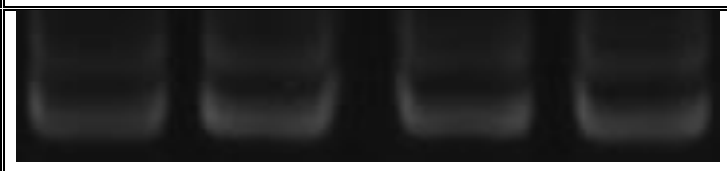
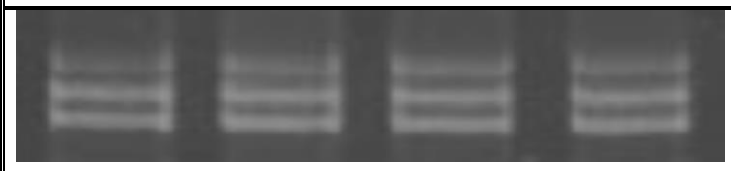
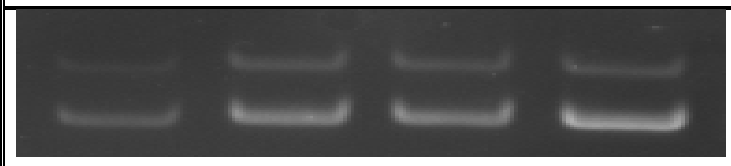
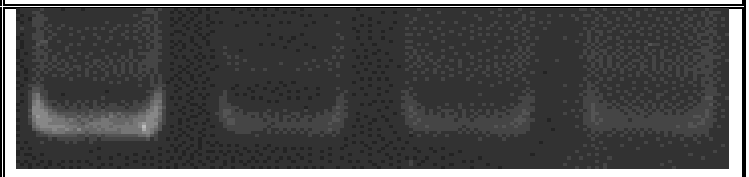
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D1S1183	204.64				
D1S3468	201.3				
D1S2622	203.86				
D1S2816	206.75				
D1S533	204.78				

Figure 3.22: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH5* (*ASPM*) locus at chromosome 1q31.3. Roman with Arabic numerals indicate the family members of the pedigree.

3.20. Family C→ *MCPH6* (13q12.12)

Family C was analyzed for specific alleles at *MCPH6* locus, amplified with below listed markers and results are illustrated in Figure 3.23.

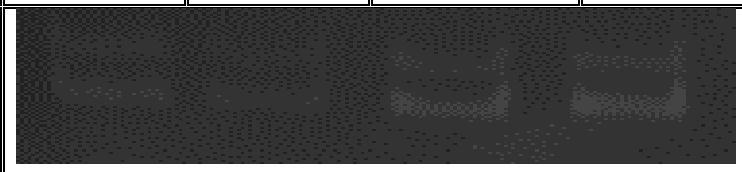
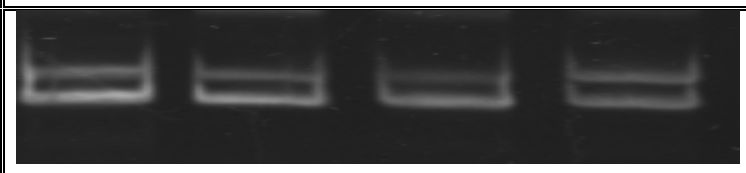
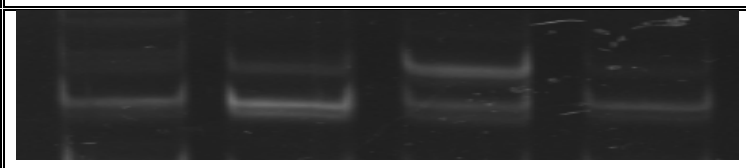
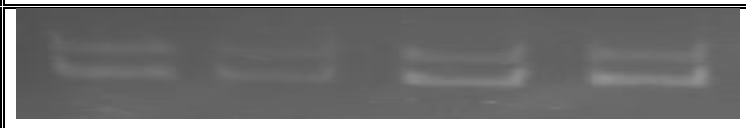
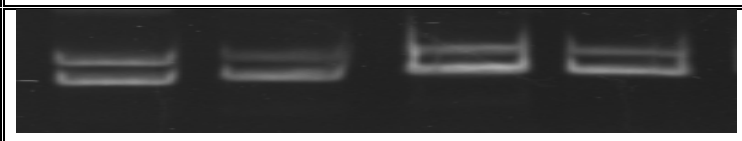
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D13S1285	5.41				
DI3S742	8.75				
D13S1243	11.71				
DI3S1254	12.23				
DI3S252	11.65				

Figure 3.23: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH6* (*CENPJ*) locus at chromosome 13q12.12. Roman with Arabic numerals indicate the family members of the pedigree.

3.21. Family C→ *MCPH7* (1p33)

Family C was analyzed for specific alleles at *MCPH7* locus, amplified with below listed markers and results are illustrated in Figure 3.24.

Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
DIS2130	76.12				
DIS211	78.1				
DIS2797	79.35				
DIS2874	80.58				
DIS386	82.61				

Figure 3.24: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH7* (*STIL*) locus at chromosome 1p33. Roman with Arabic numerals indicate the family members of the pedigree.

3.22. Family C→ *MCPH8* (4q12)

Family C was analyzed for specific alleles at *MCPH8* locus, amplified with below listed markers and results are illustrated in Figure 3.25.

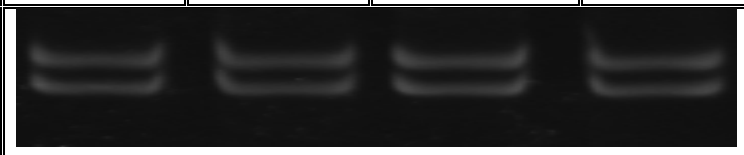
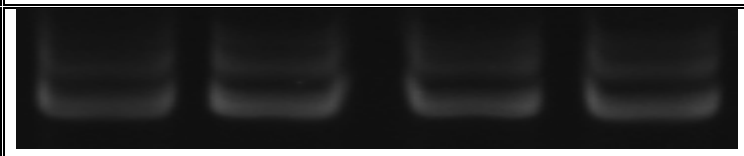
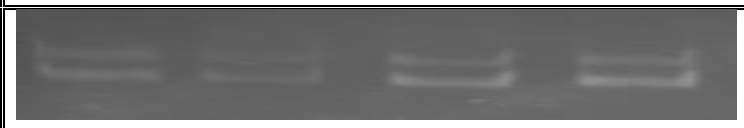
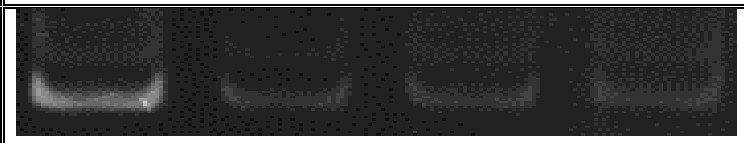
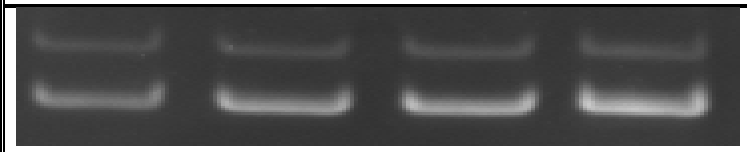
Markers	Map unit cM	III-1 Normal	III-2 Normal	IV-1 Affected	IV-2 Affected
D4S428	70.47				
D4S2379	72.39				
D4S3000	73.21				
D4S3019	74.06				
D4S1590	76.56				

Figure 3.25: Electropherogram of 8% non-denaturing polyacrylamide gel obtained by staining with ethidium bromide. Banding pattern illustrates the genotype of alleles amplified with respective markers within *MCPH8* (*CEP138*) locus at chromosome 4q12. Roman with Arabic numerals indicate the family members of the pedigree.

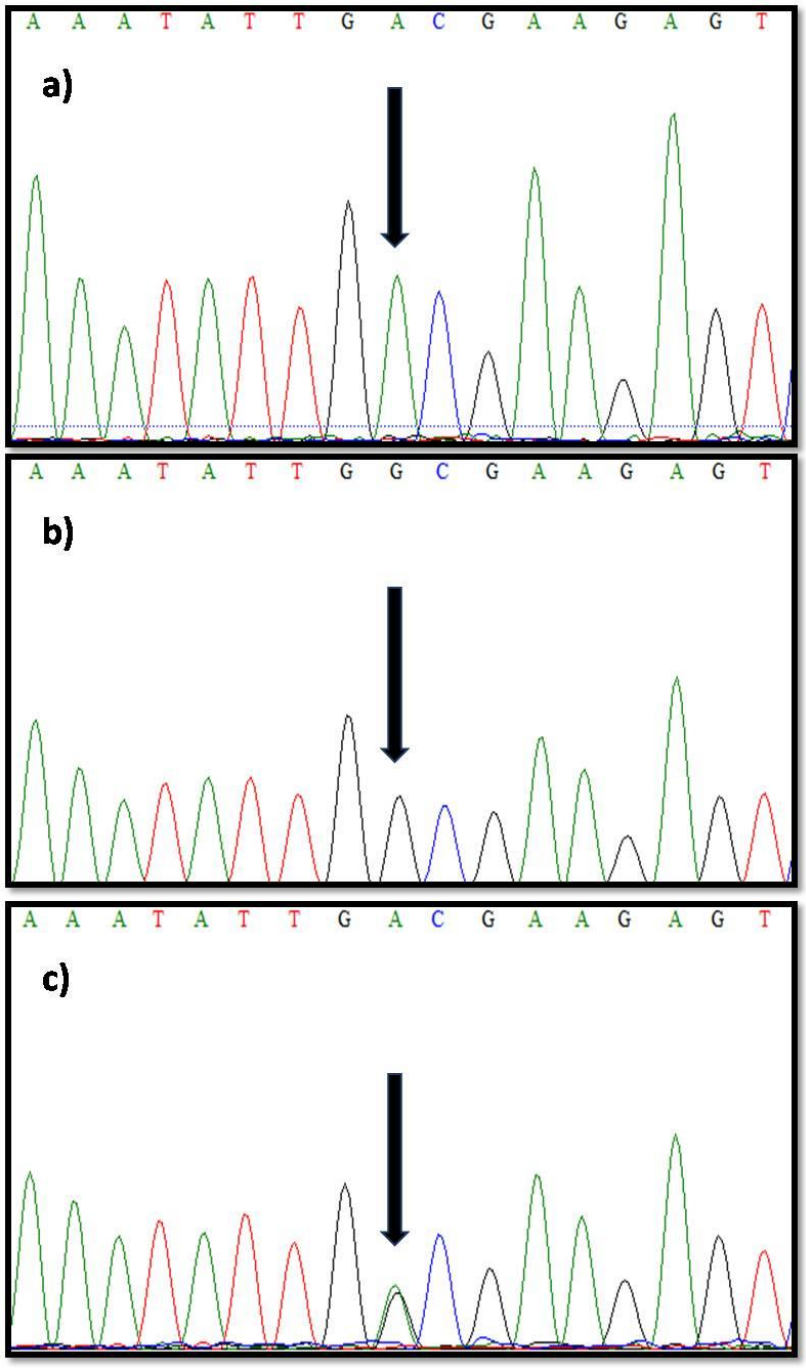


Figure 3.26: Sequencing chromatogram showing G to A substitution in exon 17 of ASPM gene in Family B. Black arrow indicates the position of nucleotide change in the sequence at 3978G>A W1326*. a) affected, b) normal, c) carrier.

4. DISCUSSION

Humans display diverse variances from other creatures in several biological areas including skin, skeleton and nervous system, especially brain. Human brain size is 3-fold larger than their closest primates (Ponting and Jackson *et al*., 2005). The development of the brain lasts in the prenatal and postnatal periods and the skull is designed to occupy the morphological variations happening throughout developmental period (Aicardi, 1998). Abnormal brain development is responsible for significantly abridged brain size and cerebral cortex but may or may not affect the gyral pattern (Mochida and Walsh, 2010). In the microcephalic condition, the gyral pattern is no longer stable and there is an obvious reduction in the cerebral cortex of the brain (Barkovich, 1997; Desire *et al*., 2008). There are various pathogenic conditions that arise consequently, comprising of microcephaly, attention deficit, lissencephaly and schizophrenia due to reduction in the brain size (Valera *et al*., 2007). During evolution cerebrum is the only part of brain that grow larger than remaining parts of brain (Finlay and Darlington, 1995; Northcutt and Kaas, 1995). During the cerebral cortex development, the symmetric and asymmetric neural progenitor cell division governs the growth, cell number and the direction of cortical expansion; and perturbation in the symmetrical and asymmetrical division can lead towards the development of brain anomalies like macrocephaly and microcephaly (Bond *et al*., 2002; Lee *et al*., 2012; Riviere *et al*., 2012).

Autosomal recessive primary microcephaly (MCPH) is described by decrease in brain size where the brain size is 4 SD lower than the mean for age and gender (Bond *et al*., 2005). Primary microcephaly has proven to be rare and static. The principal fact for markedly high rate of incidence of MCPH is consanguineous union (Finlay *et al*., 1995; Cox *et al*., 2006). Secondary microcephaly may develop after birth and could be progressive or static. Metabolic diseases, single gene disorders and chromosomal anomalies are responsible for secondary microcephaly (Woods and Parker, 2013). Autosomal recessive form of microcephaly is more heterogeneous. The affected individuals will have both alleles of the candidate genes in mutated form. Parents of the patients will be carrier with one affected allele of the causative gene (Kleopfer *et al*., 1964; Bundey, 1997).

Microcephaly has been proven to be heterogenic disorder. There are 18 MCPH loci (*MCPH1–MCPH18*) identified in various inhabitants around the world to date. It contains the following genes: *MCPHI (Microcephalin), MCPH2 (WDR62), MCPH3 (CDK5RAP2), MCPH4 (CASC5), MCPH5 (ASPM), MCPH6 (CENPJ), MCPH7 (STIL), MCPH8 (CEP135), MCPH9 (CEP152), MCPH10 (ZNF335), MCPH11 (PHC1), MCPH12 (CDK6), MCPH13 (CENPE), MCPH14 (SASS6), MCPH15 (MFSD2A), MCPH16 (ANKLE2), MCPH17 (CIT*) and *MCPH18 (WDFY3).* The discovery of the above-mentioned genes helped us in elucidating our perception about the genetic disorders at molecular level. More than half of MCPH protein-encoding genes play a noteworthy role in biogenesis/duplication of centrioles and confine to the centrosome. In addition to centriole biogenesis, there role have been also identified in cell cycle progression, genome stability, DNA repair and DNA replication.

Microcephaly may be caused by genetic or environmental factors. The genetic factors contain single chromosomal abnormalities, gene mutations and the environmental factors include maternal metabolic disorders, infections interrupting brain development after birth, malnutrition and folate deficiency etc. (Mochida and Walsh, 2001; Abuelo *et al*., 2007; Ashwal *et al*., 2009; Gilmore and Walsh, 2013)

Mutant form of *ASPM* gene was first identified in Drosophila melanogaster (do Carmo and Glover, 1999). *ASPM* defect is present in more than half of the cases of congenital primary microcephaly in Pakistani population (Roberts *et al.,* 2002; Gul *et al.,* 2006; Mahmood *et al.,* 2011). The various types of mutations comprise of duplication, substitution, variation and deletion reported in intronic region of the gene (Saadi *et al.,* 2009). Up till date, a total of 161 pathogenic *ASPM* mutations have been published, including 14 splicing mutation, 69 nonsense mutations, one missense mutation, 75 frameshift mutations and two gross deletions. Among these, 32 non-sense mutation, 28 frameshift mutations and 3 splicing mutations in *ASPM* giving rise to microcephaly were identified in Pakistani MCPH families. Additionally, the effects of mutations on phenotypic severity have not been reported (Bond *et al.,* 2003; Nicholas *et al.,* 2009; Darvish *et al.,* 2010).

The examination of inheritance pattern and phenotype-genotype association of genes segregating from parent to offspring is the central theme of our research work. I did my research work on three families A, B, C of MCPH. These families were obtained from various remote areas of Pakistan. Through clinical investigation it was found

that primary microcephaly was present at birth with mild to moderate level of mental retardation, but additional features like reduced occifrontal head circumference and weak eye sight was seen in a few affected patients. There was absence of environmental role in ailment and the inheritance pattern was found to be autosomal recessive.

The current research work was done in two phases. In the first phase, three families' (A, B, C) linkage analysis was performed in order to detect the linkage of various previously discovered *MCPH* loci by using polymorphic microsatellite markers. The families A and C with primary microcephaly were mapped to 10 most prominent known *MCPH* genes of syndromic primary microcephaly in Pakistani population. Genotyping examination displayed that normal and affected individuals were heterozygous within these known genes, thus excluding the linkage of this family within these known genes. Exclusion of linkage suggests that an unknown gene is responsible for *MCPH* in this family which is yet to be discovered.

Family B linked to the MCPH5 locus through homozygosity mapping by means of microsatellite markers suggests that the contributing *ASPM* gene at *MCPH5* 1q31.3 locus may be liable for the observable characteristics of disease. Family B linkage within *MCPH5* 1q31.3 locus identified that any type of pathogenic variant may be existing within *ASPM* gene. Affected patients were further processed for sequencing *ASPM* gene to identify pathogenic variant, which is the cause of primary microcephaly in that individual. Confirmation of linkage analysis was done in the initial step; DNA sequence analysis was performed in the second step. Out of 28 total ASPM gene exons, only two exons (17 and 18) with high frequency of mutations reported in Pakistani population were selected for sequencing. DNA sequence analysis displayed presence of already reported non-sense mutation in the exon 17 of the *ASPM* gene. In such mutation G to A conversion occurs at 3978 position of nucleotide, creating premature stop codon (Trp1326*). Truncated ASPM protein is produced due to non-sense mutation. The above non-sense mutation 3978G>A Trp1326* in the *ASPM* gene is the most commonly occurring pathogenic variant sequence in Pashtun ethnic group of Pakistani origin (Kousar *et al*., 2009).

Homozygous type of mutation in *ASPM* gene is most regularly responsible for primary microcephaly in Pakistani population (Bond *et al.*, 2002; Saadi *et al*., 2009).

ASPM gene plays an essential role in the positioning of mitotic spindle (Fish *et al.*, 2006; Higgins *et al.*, 2010), and thus effects the brain size (Cox *et al*., 2006).

In conclusion, we sampled 3 Pakistani consanguineous families suffering from MCPH and analyzed the genetic basis of the disease. 2 families (A and C) did not show linkage to any of the known loci of the reported genes. Family B however, showed linkage to ASPM gene at chromosome 17 which was confirmed by sequencing and was likely responsible for the diseased phenotype.

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