Linkage Analysis of Loci/Gene Involved in Hereditary Mental Retardation in Kashmiri Families

BY

MARIA FAZAL-UL-HAQ
236-FBAS/MSBT/F15

Department of Bioinformatics & Biotechnology
Faculty of Basic and Applied Sciences
International Islamic University Islamabad

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Mental retardation.
Developmental disabilities.
DNA (Deoxyribonucleic acids)

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Researcher
MARIA FAZAL-UL-HAQ
236-FBAS/MSBT/F15

Supervisor
Dr. Shaheen Shahzad
Assistant Professor
IIUI

Co-Supervisor
Dr. Asma Gul
Associate Professor
IIUI

Department of Bioinformatics & Biotechnology
Faculty of Basic and Applied Sciences
International Islamic University Islamabad

(2017)


بسم الله الرحمن الرحيم

Department of Bioinformatics and Biotechnology International Islamic University, Islamabad

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Final Approval

It is certificate that we read and evaluated the thesis, " Linkage Analysis of Loci/Gene Involved in HereditaryMental retardation in Kashmiri Families" submitted by Maria Fazal-ul-haq and it is our judgement that this project is of sufficient standard to warrant its acceptance by International Islamic University, Islamabad for M.S Degree in Biotechnology

Committee

External Examiner
Dr. Muhammad Jawad Hassan
Assistant Professor Healthcare Biotechnology
Atta-ur-Rehman School of Applied Biosciences (ASAB)
National University of Science & Technology (NUST)

Internal Examiner
Dr. Asif Mir
Associate Professor
Department Of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Supervisor
Dr. Shaheen Shahzad
Assistant Professor
Department Of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Chairperson/Co-Supervisor
Dr. Asma Gul
Associate Professor
Department Of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Dean
Professor Dr. Muhammad Arshad Zia
Faculty of Basics and Applied Sciences
International Islamic University, Islamabad

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DEDICATION

I DEDICATE THIS THESIS, BY THE CORE OF MY HEART, TO MY BELOVED PARENTS, BROTHERS, SISTERS AND MY KIND SUPERVISOR. WITHOUT THEIR SUPPORT, BUNCH OF SINCERE PRAYERS AND SACRIFICES IT WOULD NOT HAVE BEEN POSSIBLE FOR ME TO ACCOMPLISH MY WORK.

Declaration

I hereby declared that the work presented in this thesis is my own effort and hard work and it is written and composed by me. No part of this thesis has been previously published or presented for any other degree or certificate.

Date: _____ _____

Maria Fazal-ul-haq

TABLE OF CONTENTS

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List of Abbreviations

AAIDD	**American association on intellectual developmental disabilities**
AD	**Autism disorder**
ADMR	**Autosomal dominant Mental Retardation**
AMR	**Autosomal Mental Retardation**
APS	**American Psychiatric Association**
APS	**Ammonium persulphate**
ARMR	**Autosomal recessive Mental Retardation**
ARNS-MR	**Autosomal recessive non-syndromic Mental Retardation**
ASD	**Autism spectrum disorder**
bp	**basepair**
CC2D1A	**Coiled-coil and C2 domains protein 1A**
cM	**centimorgan**
CRBN	**Cereblon**
DNA	**Deoxyribonucleic acids**
dNTPs	**Deoxynucleotide Phosphates**
ECG	**Electrocardiogram**
EDTA	**Ethylenediaminetetraacetic Acid**
g	**gram**
GRIK2	**Glutamate receptor ionotropic kainite 2**
IQ	**Intellectual Quotient**
Kb	**Kilo base**
kDa	**kilo Dalton**
M	**Molar**
MAN1B1	**Mannosidase alpha class 1B member 1**

Mb	**Mega Base**
mg	**Milligram**
MgCl2	**Magnesium Chloride**
MIM	**Mendelian Inheritance in man**
min	**Minute**
mL	**Milliliter**
mM	**Millimolar**
mm	**Millimetre**
MR	**Mental Retardation**
mRNA	**Messenger Ribonucleic Acid**
NCBI	**National Center for Biotechnology and Information**
ng	**nanogram**
NSMR	**Non-syndromic Mental Retardation**
OD	**Optical Density**
OMIM	**Online Mendelian Inheritance in man**
PAGE	**Polyacrylamide Gel Electrophrosis**
PCR	**Polymerase Chain Reaction**
pH	**Potential of Hydrogen Ions**
PRSS12	**Protease serine 12**
Rpm	**Revolutions per minute**
SDS	**Sodium Dodecyl Sulphate**
SZ	**Schizophrenia**
TBE	**Tris-Borate EDTA**
TECR	**Trans-2,3-enoyl-CoA reductase**
TEMED	**Tetramethylethylenediamine**

Temp	**Temperature**
TRAPPC9	**Trafficking protein particle complex, subunit 9**
TUSC3	**Tumor suppressor candidate 3**
U	**Unit**
UCSC	**University of California Santa Cruz**
UV	**Ultra Violet**
VLCFA	**Very long chain Fatty acids**
WHO	**World Health Organization**
XLMR	**X-Linked Mental Retardation**
µg	**Microgram**
µl	**Microlitre**
°C	**Degree Celsius**
	**Normal Male**
	**Affected Male**
	**Normal Female**
	**Affected Female**

LIST OF FIGURES

LIST OF TABLES

Abstract

Mental retardation is a common disorder with an onset of cognitive impairment before the age of 18 years. It is characterized by certain limitations in intellectual functioning especially social abilities, language and cognition with IQ score of below 70. On the basis of pattern of inheritance mental retardation is divided into X-linked, autosomal dominant and autosomal recessive mental retardation. Mental retardation has variation in degrees ranging from mild to profound. Causes of 40% of mental retardation are not clear, in rest of 60% half are caused by environmental factors, poor nutrition, prenatal brain ischemia, postnatal infections, brain injury and other 50% cases are caused by genetic reasons like chromosomal abnormalities and mutations in specific genes. Several genes have been identified in which mutations produce mental retardation. These genes are involved in certain molecular processes and changes in these genes can damage the brain function.

The study presented here includes homozygosity mapping of three consanguineous families (A, B and C) with Mental retardation. The Family A and B were recruited from Muzaffarabad district and Family C was sampled from Poonch district of Azad Jammu and Kashmir. Clinical assessments indicate segregation of autosomal recessive mental retardation in all three families. The affected members were suffering from mild to severe mental retardation, with low IQ level, memory, learning, speech and balancing disabilities. They didn't show any other dystrophy, height and head circumference was normal. They didn't show any history of epileptic fits.

Homozygosity mapping was performed by using microsatellite markers of the gene PRSS12, CC2D1A, TECR, GRIK2, MAN1B1 and TRAPPC9 in family A and B while in family C we applied the markers of these genes PRSS12, CRBN, CC2D1A, TECR, TUSC3, GRIK2, MAN1B1 and TRAPPC9. The genotyping results of the families (A, B and C) did not show any homozygous region in the affected members of families (A, B and C) and indicates the exclusion of the currently tested gene/loci. These mapping results indicate that there are some other gene/loci responsible for the disorder.

INTRODUCTION

1. Introduction

Neurological disorders occur in 6% of the global population with greater prevalence of mortality and disability-adjusted life (Lopez *et al.,* 2006). It is the disorder of nervous system which causes abnormalities in brain, spinal cord or other nerves. Despite the fact that brain and spinal cord are surrounded by tough membranes which are enclosed by skull bones and vertebrae but they are vulnerable if these barriers are compromised. Some causes of neurological disorders include genetic disorders, infections, congenital disorders or brain, spinal cord and nerve injury (Figure 1.1).

Symptoms of neurological disorder are vast consisting of paralysis, poor coordination, seizures, pain, difficulty reading and writing, muscle weakness etc. Some neurological disorders are common while some are rare that may be revealed by neurological examination. Neurological disorders depict very diverse classification like alzheimers, migrane, parkinson's disease, huntington's disease, autism, microcephaly, mental retardation (MR)/intellectual disability (ID) etc. Autism spectrum disorders (ASD, nearly 1%), schizopherinia (SZ, ~ 1%) and epilepsy (~ 0.85%) are quiet frequent in population (Mitchell, 2011).

Autism disorder is major type of neurodevelopmental disorder (Geschwind, 2008). It starts from memory loss and cognitive deficit in the early stage and gradually evolves into severe dementia in later stage. The pathological reason of autism disorder includes extracellular deposit of amyloid plaques and intra-neuronal neurofibrillary tangles (NTF). Autism spectrum disorder is one of the most widespread neurodevelopmental disorders. It is characterized by impairment in social interactions, verbal/nonverbal communications and repetitive behaviour. Autism spectrum disorder is usually diagnosed before the age of three years and no effective treatment is available (Freitag, 2007; Abrahams and Geschwind, 2008). Schizophrenia is also a common neurodevelopmental disorder that is characterized by delusions, hallucinations, paranoia and extensive cognitive impairments, with estimated nearly 80% heritability (Milev *et al.,* 2005). Epilepsy is a brain disorder characterized by persistent predisposition to generate seizures and is also strongly linked with cognitive and behavioral comorbidities. Epilepsy affects approximately 50 million people worldwide, of all ages range from neonates to elderly people (Engel, 2001). Microcephaly is a neurodevelopmental disorder defined as head circumference of more than two

standard deviation is below the mean for gender and age (Kaindl *et al.,* 2010), with the occurrence of 1 in 10,000 in consanguineous populations (Thomson and Woods, 2009).

Mental retardation (MR) is a neurodevelopmental disorder; it is a common disorder with an onset of cognitive impairment before age of 18 years. Mental retardation is characterized by considerable limitations in at least two adaptive skills like communication, reading writing and self care (American Psychiatric Association, 2000). Mental retardation is subcategorized into mild, moderate, severe and profound, on the basis of IQ level (WHO, 1992). The causes of nearly 40% of Mental retardation are unknown (Molinari *et al.,* 2008). Among the known causes ~50% have environmental factors, like poor nutrition, infections during pregnancies, inadequate medical services and little gap in pregnancies. Other halves of Mental retardation cases have genetic factors which can be mutations in specific genes or it can be because of chromosomal abnormalities (Ropers, 2010). It is believed that ~25% of genetic Mental retardation patients are thought to have autosomal recessive mode of inheritance (Bartley *et al.,* 1978). By 2011 thirty loci including six known genes have been reported to be involved in autosomal recessive non syndromic mental retardation (ARNS-ID) (OMIM database). Which are PRSS12; MIM#606709 on chromosome 4q26, CRBN; `MIM#609262 on chromosome 3p26.2, CC2D1A; MIM# 610055 chromosome 19p13.12, GRIK2; MIM# 138244 on chromosome 6q16.3, TUSC3; MIM# 601385 on chromosome 8p22 and TRAPPC9; MIM# 611966 on chromosome8q24.3 (Mir *et al.,* 2009).

The prevalence of Mental retardation in Pakistan is relatively higher than other European countries and America because of consanguineous marriages. Consanguinity is a marriage in which couples having at least one common ancestor. Consanguinity is derived from Latin word "*consanguinitas*" meaning "blood relation". Consanguineous marriages are tradition and they are higly respected in most communities of Middle East, West Asia and North Africa, here marriages in family are 20-50% (Hamamy *et al.,* 2011; Tadmouri *et al.,* 2009). Pakistani, Turkish, Lebanese emigrants who now reside in Europe, Australia and North America also practice consanguineous marriages (Hamamy *et al.,* 2011). Primary reason for the consanguineous marriage is social. People of Azad Jammu and Kashmir prefer inbreeding, cousin marriages are common here (Darr and Modell, 1988). The identification of genes responsible for syndromic and non-syndromic mental retardation is an approach to improve knowledge and add information about medical and biological patterns of mental retardation.

Pakistan is among those countries in which consanguinity is very high (>60%) with 17-38% first cousin marriages (Hussain and Bittles, 1998). In Pakistan several factors play important role in consanguineous marriages like social factors, religious factors and demographic factors (Bittles, 2001). Genetics specialists and health care providers consider consanguineous marriages responsible for increase in the genetic risks to the offspring (Hamamy *et al.*, 2011). First cousins are predicted to share 12.5% (1/8) of their genes and on an average; their progeny will be homozygous at 6.25% (1/16) of gene loci (Bennett *et al.*, 2002). Stillbirths and birth defects frequency is higher in consanguineous marriages as compaired to the general population. Consanguineous marriages can lead to the autosomal recessive disorders as the expression of autosomal recessive gene mutation is inherited from both the parents (Tadmouri *et al.*, 2009).

Some mutations are identified in Mental retardation genes in Pakistani population. A homozygous splice-site mutation within CC2D2A in Pakistani family with five affected individuals is identified by Noor *et al.*, (2008). Mir *et al.*, (2009) identified TRAPPC9, a gene for autosomal-recessive Mental retardation, in a large consanguineous Pakistani family with verification in an Iranian family. Rafiq *et al.*, (2011) reported a homozygous missense mutation (c. 1189G>A) in MAN1B1 in three consanguineous Pakistani families of same village and a homozygous nonsense mutation (c. 1418G>A) in another Pakistani family. Khan *et al.*, (2011) reported a deletion mutation in TUSC3 gene associated with NS-ARID that include the deletion of almost entire TUSC3 gene (minus the promoter and exon 1) in a large consanguineous Pakistani family with six individuals. Recently, in another study Khan *et al.*, (2012) identified a homozygous substitution (c. 2035G>A) within exon 19 NSUN2 gene which resulted in the missense change p.Gly679Arg.

The aim of the present research is to identify the loci/gene responsible for the autosomal recessive non syndromic Mental retardation in Kashmiri population.

The main objectives of this study are:

- To study the linkage of known loci/gene responsible for inherited Mental retardation in Kashmiri families.
- To find out the correct genotype-phenotype correlation that will be helpful in diagnosis.

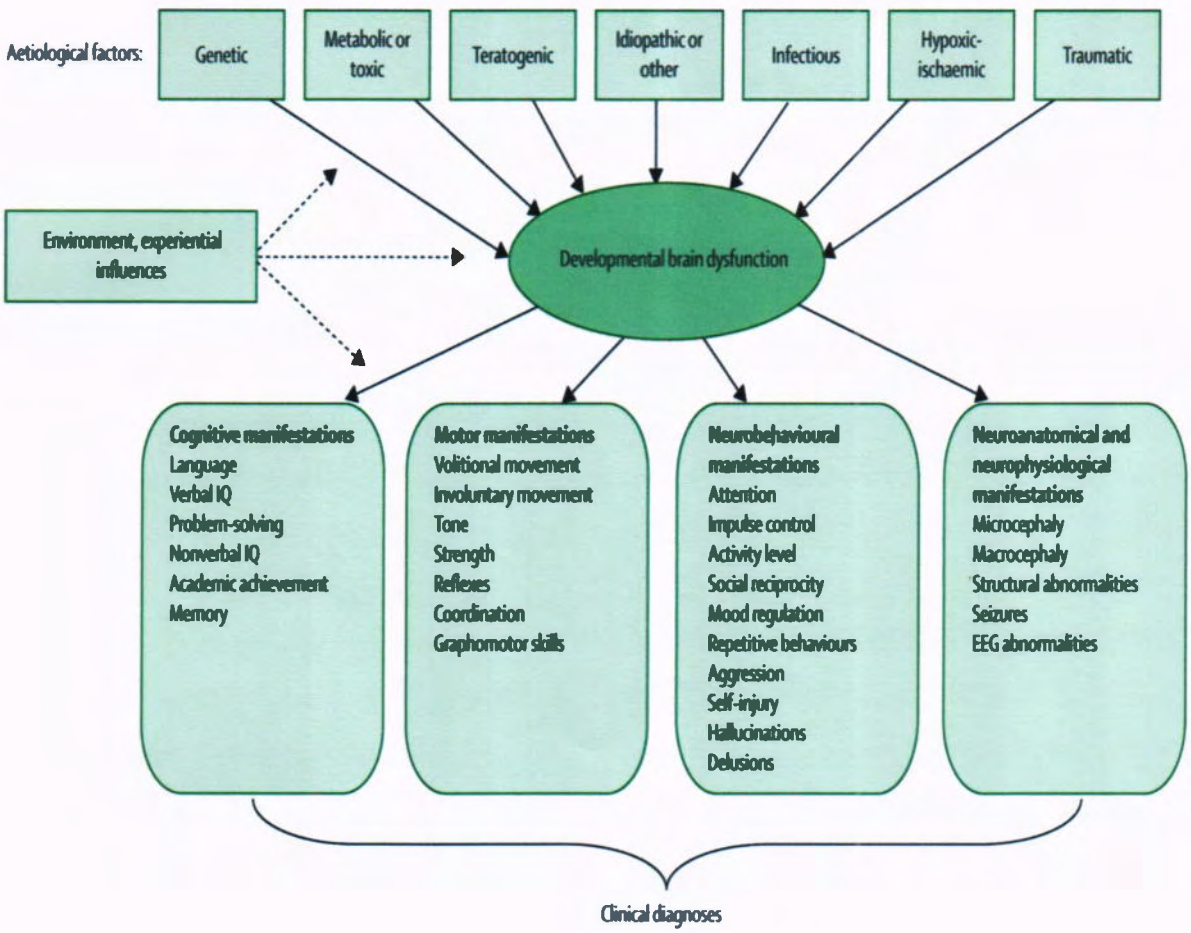


Figure 1.1 Developmental brain dysfunction etiological factors and symptoms (Adapted from Myers, 2013).

REVIEW OF LITERATURE

2. Mental retardation

Intelligence is the general mental ability that involves reasoning, planning, solving problems thinking conceptually, comprehending complex ideas, learning efficiency and learning from experience (AAIDD, 2010). Mental retardation (MR) also known as Intellectual disability (ID) is a common disorder with an onset of cognitive impairment before the age of 18 years (Riazuddin *et al.*, 2016). It is characterized by certain limitations in intellectual functioning especially social abilities, language and cognition with an IQ score of below 70. It can occur with or without any physical or other mental disorders. Mental retardation comprises of following general categories: mild, moderate, severe and profound (Daily *et al.*, 2000). Mental retardation can occur as a syndrome or as a broader disorder but is most commonly found as an isolated disorder. MR is particularly common in countries where parental consanguinity is common, including the involvement of autosomal recessive genetic abnormalities (Ropers *et al.*, 2011).

2.1. Etiology

The cause of almost 40% of Mental retardation (MR) is unclear (Molinari *et al.*, 2008), in more or less 50% of known cases main causes are poor nutrition, prenatal or perinatal brain ischemia, postnatal infections and other half of cases have genetic reasons like mutations in specific genes and chromosomal abnormalities (Ropers, 2010). Environmental factors consist of exposure of fetus to alcohol, congenital infections such as cytomegalovirus, herpes, syphilis and rubella and prolonged maternal fever in the first trimester (Stromme and Hagberg, 2007). During birth unusual stress may damage the brain, especially premature birth and low birth weight may predict mental retardation. During childhood, factors such as environmental toxins, disease (e.g measles), a blow on head, severe and prolonged malnutrition, and environmental toxins may cause unending damage to nervous system (Winnepenninckx *et al.*, 2003).

There are 23 pairs of chromosomes in human cells, out of which 22 pairs are autosomal and 1 pair is of sex chromosomes. The genetic factors include chromosomal number deviation, monogenic and polygenic factors (Ropers, 2008). Structural rearrangements occur they can also cause changes in whole chromosome number so this breakage and repair can give rise to a different arrangement of chromosome. It can result in neurological disorders like autism, down

syndrome etc. Genetic causes are present in more than 50% of severely disabled persons (Chelly *et al.*, 2006).

2.2. Classification of Mental retardation

Mental retardation can be classified in different ways on the basis of severity, pattern of inheritance and presence or absence of clinical symptoms.

Mental retardation is subcategorized as mild (50-55 to 70), moderate (35-40 to 50-55), severe (20-25 to 35-40) and profound (below 20-25) (American Psychiatric Association, 2000). The majority of people with Mental retardation are classified as having mild intellectual disabilities. People with mild Mental retardation are slower in all areas of conceptual development and social and daily living skills. These people are able to learn practical life skills which allow them to live with minimal levels of support. People with moderate Mental retardation can take care of themselves, they can travel to familiar places nearby and learn basic skills related to safety and health, their self-care requires moderate support. Severe Mental retardation marked as major delays, often these individuals have the ability to understand speech or else have limited communication skills (Sattler, 2002). These individuals need to live in supervision. Persons with profound mental retardation often have congenital syndromes (Sattler, 2002).

Mental retardation is subdivided into two major categories syndromic forms, which are accompanied by malformations, dysmorphic features or neurological abnormalities, whereas non-syndromic forms have no comorbid features (Ropers, 2006).

On the basis of inheritance pattern Mental retardation is classified into X-linked mental retardation (XLMR) and autosomal mental retardation (AMR), which is subdivided into autosomal recessive mental retardation (ARMR) and autosomal dominant mental retardation (ADMR) (Winnepenninckx *et al.*, 2003).

2.3. X-Linked Mental retardation

Mental retardation is more frequent in males than in females, possibly because hemizygous males cannot compensate for deleterious mutations in genes on the X chromosome (Chiurazzi *et al.*, 2004). X-linked forms of Mental retardation are easily recognizable because of their mode of

inheritance. (Ropers and Hamel, 2005). Major genes involved in X-linked Mental retardation are mentioned in Table 2.1. XLMR genes are present throughout the X chromosome and it is caused by duplications, deletions or inversions of large gene regions. However, the common cause of XLMR is monogenic mutations of X chromosome (Kleine *et al.*, 2010). The XLMR conditions are divided into two categories: syndromic form when MR is associated with clinical, metabolic, or radiologic features and non-syndromic form when MR is isolated (Chiurazzi *et al.*, 2004).

Fragile X syndrome is the most common type of X-linked MR syndromes with an incidence of 1 in 1000 to 1400 males, the phenotype may not be recognized until the adolescence years. It is caused by an expanded trinucleotide area in the Fragile X gene on the X chromosome (Torello, 2005).

2.4. Autosomal Dominant Mental retardation

The identification of genes responsible for autosomal dominant mental retardation is difficult due to lack of large families as the affected individuals rarely reproduce. Non syndromic mental retardation (NS-MR) is likely to be uncommon (Lenski *et al.*, 2004). However, autosomal dominant syndromic MR genes are more easily identified through patients with a unique chromosome translocation or carrying a small casual copy number variation, e.g NSD1, EHMT1, CBP AND MEF2C (Le Meur *et al.*, 2010). Only one gene (PPP2R2C) causing familial autosomal dominant non-syndromic MR has been reported to date (Backx *et al.*, 2010). Very little is known about the genetic abnormalities that are caused by the autosomal dominant non-syndromic mental retardation (Ropers, 2007). Major genes involved in autosomal dominant Mental retardation are mentioned in Table 2.2. Genes which are associated with autosomal dominant MR are primarily identified by mapping of chromosomal breakpoints in intellectual disable patients with balanced translocations, inversions or duplications (Basel-Vanagaite *et al.*, 2007).

2.5. Autosomal Recessive Mental retardation

Recessively inherited diseases are more extensively present in populations where cousin marriages are frequent, such as Pakistan. Some major genes involved in autosomal recessive

Table 2.1 Major genes involved in X-Linked Mental retardation

Gene	Location	References
FMR1	Xq27.3	(Devys *et al.*, 1993)
RPS6KA3	Xp22.12	(Merienne *et al.*, 1999)
MECP2	Xq28	(Amir *et al.*, 1999)
IL1RAPL1	Xp21.3-p21.2	(Carrie *et al.*, 1999)
TM4SF2	Xp11.4	(Zemni *et al.*, 2000)
FACL4	Xq23	(Longo *et al.*, 2003)
ARHGEF6	Xq26	(Kutsche *et al.*, 2000)
AGTR2	Xq23	(Vervoort *et al.*, 2002)
ACSL4	Xq23	(Meloni *et al.*, 2002)
SLC6A8	Xq28	(Salomaons *et al.*, 2003)
ZNF41	Xp11.3	(Shoichet *et al.*, 2003)
JARID1C	Xp11.22	(Jensen *et al.*, 2005)
FTSJ1	Xp11.23	(Freude *et al.*, 2004)
SYN1	Xp11.3-p12	(Garcia *et al.*, 2004)
SLC16A2	Xq13.2	(Friesema *et al.*, 2004)
PQBP1	Xp11.23	(Kalscheuer 2003)
ZNF674	Xp11.3	(Lugtenberg *et al.*, 2006)
AP1S2	Xp22.2	(Tarpey *et al.*, 2006)
GRIA3	Xq25	(Wu *et al.*, 2007)
PAK3	Xq23	(Rejeb *et al.*, 2008)
MAGT1	Xq21.1	(Molinari *et al.*, 2008)
ZNF711	Xq21.1	(Tarpey *et al.*, 2009)
SYP	Xp11.23	(Tarpey *et al.*, 2009)
RAB39B	Xq28	(Giannandrea *et al.*, 2010)
IQSEC2	Xp11.22	(Shoubridge *et al.*, 2010)
HCFC	Xq28	(Huang *et al.*, 2012)

Table 2.2 Major genes involved in Autosomal Dominant Mental retardation

Gene	Location	References
GATAD2B	1q21.3	De Light *et al.*, 2012
MBD5	2q23.1	Wagenstaller *et al.*, 2007
MEF2C	5q14.3	Zweier *et al.*, 2010
SYNGAP1	6p21.32	Hamdan *et al.*, 2011
KIRREL3	11q24.2	Bhalla *et al.*, 2008
GRIN2B	12p13.1	Endele *et al.*, 2010
CDH15	16q24.3	Bhalla *et al.*, 2008
CACNG2	22q12.3	Hamadan *et al.*, 2011

Mental retardation are mentioned in Table 2.3. In large consanguineous families chances of rare autosomal recessive disorders are very high because the offspring of these consanguineous couples have more homozygous DNA than the children of an out bred marriage, so these consanguineous families are a powerful resource for genetic linkage studies (Jaber *et al.*, 1998).

2.6. Non syndromic Autosomal Recessive Mental retardation

Non syndromic autosomal recessive mental retardation is very diverse disease and contributes much more than the X-linked mental retardation (Mhamdi *et al.*, 2011). Until recent, mutant fifty three genes have been clearly associated to non syndromic autosomal recessive mental retardation (OMIM database).

1. **PRSS12 (MIM # 606709)**

Protease serine 12 (PRSS12) is involved in NS-ARMR, located on chromosome 4q26 and contains 13 exons (Molinari *et al.*, 2002). It is an extracellular multidomain serine protease associated with neural development and plasticity. It encodes neuronal serine protease neurotrypsin associated with neural development (Molinari *et al.*, 2002). It is secretion of neuronal cells in several brain regions. Neurotrypsin have physiological and pathological roles in

Table 2.3 Major genes involved in Autosomal Recessive Mental retardation

Gene	Location	References
ST3GAL3	1p34.1	(Hu *et al.*, 2011)
HNMT	2q22.1	(Heidari *et al.*, 2015)
CRBN	3p26.2	(Higgins *et al.*, 2004)
MRT31	4q12-q13.1	(Kuss *et al.*, 2011)
PRSS12	4q26	(Molinari *et al.*, 2002)
MRT29	4q27-q28.2	(Jamra *et al.*, 2011)
NSUN2	5p15.31	(Brzezicha *et al.*, 2006)
MRT30	6q12-q15	(Jamra *et al.*, 2011)
GRIK2	6q16.3	(Motazacker *et al.*, 2007)
MED23	6q23.2	(Hashimoto *et al.*, 2011)
TUSC3	8p22	(Khan *et al.*, 2011)
IMPA1	8q21.13	(Figueiredo *et al.*, 2016)
TAF2	8q24.12	(Najamabadi *et al.*, 2011)
TRAPPC9	8q24.3	(Mir *et al.*, 2009)
MAN1B1	9q34.3	(Rafiq *et al.*, 2011)
ANK3	10q21.2	(Iqbal *et al.*, 2013)
MRT23	11p13-q14.1	(Jamra *et al.*, 2011)
CRADD	12q22	(Di Donato *et al.*, 2016)
MRT9	14q11.2-q12	(Najamabadi *et al.*, 2007)
EDC3	15q24.1	(Ahmed *et al.*, 2015)
MRT10	16p12.2-q12.1	(Najamabadi *et al.*, 2007)
MRT35	17q21.31-q22	(Al-Owain *et al.*, 2011)
MRT19	18p11.3	(Jamra *et al.*, 2011)
TECR	19p13.12	(Caliskan *et al.*, 2011)
CC2D1A	19p13.12	(Basel-Vanagaite *et al.*, 2006)

central nervous system. In hippocampus neurotrypsin secretes excessively during first postnatal week, than its secretion gradually decreases but still continues into adult life (Iijima *et al.*, 1999). In perinatal period abundant expression of neurotrypsin mRNA is observed in other regions, olfactory system, cranial nerve nuclei, spinal cord and peripheral nervous system, it shows neurotrypsin have many important roles in development of nervous system (Mitsui *et al.*, 2009). An Algerian consanguineous family having 3 affected females and 1 male was studied by Molinari *et al.*, (2002) they were moderate to severely mentally retarded. A 4-bp deletion was reported in exon 7 of the gene resulting in premature termination codon.

2. CRBN (MIM # 609262)

Cereblon (CRBN) is another gene involved in NS-ARMR, located on chromosome 3p26.2 and it contains 11 exons. It was discovered in five nuclear families and is considered as the new genetic cause of mild mental retardation (Higgins *et al.*, 2004). This type of mental retardation shows non-syndromic autosomal recessive inheritance pattern (Higgins *et al.*, 2000). DNA sequencing and Single strand polymorphism analysis revealed that this form of mental retardation is caused by a single nucleotide mutation (C→T mutation) in cereblon (CRBN) gene (Higgins *et al.*, 2004). This mutation initiates premature stop codon which results in terminated protein at arginine 419, the resulting protein CRBN R419X, lacks last 24 amino acids as compared to the full length protein which contains 442 amino acids. CRBN was identified as thalidomide-binding protein, it forms E3 ubiquitin ligase complex with damaged DNA-binding protein-1 (DDB1 and CUL4A) which are very important for outgrowth of limbs and expression of fibroblast growth factor receptor FGF8 in chicks and zebrafish. Thalidomide starts teratoginic effects by binding to CRBN and inhibits E3 ubiquitin ligase activity (Ito *et al.*, 2010).

3. CC2D1A (MIM #610055)

Coiled-coil and C2 domains protein 1A (CC2D1A) is involved in NS-ARMR and located on chromosome 19p13.12 and contains 31 exons. It encodes coiled-coil and C2 domains 1A protein also called Freud-1 which is neuronal calcium-regulated repressor of serotonim (5HTIA) receptor. Basel-Vanagaite *et al.*, (2006) identified CC2D1A while searching for non syndromic autosomal recessive mental retardation genes along chromosome 19.

In nine consanguineous families with severe mental retardation a mutation in CC2D1A gene was identified as a result of this mutation a large genomic deletion of 3,589 nucleotides occurred and created a truncated protein and presented a loss of function effect (Basel-Vanagaite *et al.*, 2006).

4. GRIK2 (MIM # 138244)

Glutamate receptor ionotropic kainite 2 (GRIK2) is another gene of NS-ARMR, located on chromosome 6q16.3 and contains 16 exons. GRIK2 encodes GLUK6 which is subunit of kainite receptors (KARs), involved in that is highly expressed in brain (Motazacker *et al.*, 2007). Along with alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors, KARs belongs to ionotropic receptors which are targets of glutamate released in excretory synapses (Hollmann and Helnemann, 1994). Loss or mutation of NMDA or AMPA receptors results in derangement of neuronal function.

Glutamate receptors mediate most excitatory neurotransmission in the brain. Paschen *et al.*, (1994) demonstrated RNA editing; they observed changes in sequence of mRNA after posttranscription in human GLUR2 and GLUR6. In GLUR2 CAG coding for glutamine was changed to CGG coding for arginine, while in case of GLUR6 10% change occurred in corpus callosum and 90% change occurred in gray matter.

In a large Iranian family a nonsense mutation in GRIK2 gene was first reported. In this family mutation was reported by removal of exon 7 and 8 which results in deletion of 84 amino acids between amino acid 317 and 402 (Motazacker *et al.*, 2007). In another consanguineous family, 2 sibs born with autosomal recessive mental retardation, a homozygous mutation was identified by Cordoba *et al.* (2015). This mutation resulted in arg198-to-ter substitution.

5. TUSC3 (MIM # 601385)

Tumor suppressor candidate 3 (TUSC3) is located on chromosome 8p22 and contains 11 exons and is involved in NS-ARMR (Garshasbi *et al.*, 2008). It consists of 1348 amino acids. MacGrogan *et al.*, (1996) isolated TUSC3 gene and called it N33, within it a homozygous deletion was associated with metastatic prostate cancer (Bova *et al.*, 1996). In the brain, the function of TUSC3 is major that it interacts with alpha isoform of catalytic subunit of protein phosphatase 1 (PPPC1A; MIM 176875), which modulate synaptic and structural plasticity (Rual *et al.*, 2005).

Molinari *et al.*, (2008) identified a homozygous mutation in TUSC3 gene in French sibs. In the second family which is a large consanguineous Iranian family with non syndromic mental retardation, a homozygous 120- to 150- kb deletion is found (Garshasbi *et al.*, 2008). In another Iranian family 3 sibs with autosomal recessive severe mental retardation, genome wide linkage analysis followed by candidate gene sequencing showed a novel nonsense mutation in the second exon of the TUSC3 gene (Garshasbi *et al.*, 2011).

6. TRAPPC9 (MIM # 611966)

Trafficking protein particle complex, subunit 9 (TRAPPC9) is the involved in NS-ARMR located on chromosome 8q24.3 and contains 23 exons. TRAPPC9 encodes NIK and IKK-beta and plays very important function in the neuronal NF-kappa-B signiling pathway (Hu *et al.*, 2005). Mochida *et al.*, (2009) noted that in human there are 2 transcript variants of TRAPPC9 in human. Variant 1 is longer and it codes for 1246 amino acid protein, while variant 2 have an alternate 5-prime exon initiating translation at downstream start codon encodes 1148 amino acid protein. TRAPPC9 was found in neurons of hippocampus, cerebral cortex and deep gray mater. In human's embryonic brain of 11.5 week, there is high expression in developing cortical plate as compared to ventricular zone and indicates higher expression in post mitotic neurons than in progenitor cells (Mochida *et al.*, 2009).

Mochida *et al.*, (2009) found homozygous truncating mutation in Israeli Arab family with non syndromic mental retardation, by genomewide linkage analysis followed by gene sequencing. In Pakistani family with TRAPPC9, Mir *et al.*, (2009) identified the R475X mutation in affected members. In a Tunisian family with three affected brothers, Philippe *et al.*, (2009) identified a homozygous mutation in exon 9 of TRAPPC9 gene. This mutation result in arg570-to-ter substitution. Marangi *et al.*, (2013) identified a homozygous truncating mutation in TRAPPC9 gene in two sisters, born of unrelated parents in southern Italy. Parents were unaffected and heterozygous for mutation while two affected sisters were homozygous for mutation; A-to-C transversion in intron 17 of gene was observed which results in missing of exon 18 and caused premature termination.

7. TECR (MIM # 610057)

Trans-2,3-enoyl-CoA reductase (TECR) also refered to as GPSN2 (synaptic glycoprotein 2) is located on chromosome 19p13 and contains 13 exons (Caliskan *et al.,* 2011). Later on Hartz (2015) mapped the TECR gene to chromosome 19q13.12, because of alignment of TECR sequence with genomic sequence. TECR is a synaptic glycoprotein it is involved in the synthesis of very long chain fatty acids (VLCFA) in a reduction step of microsomal fatty acyl elongation process. The orthologue of TECR is highly expressed in nervous system of mouse (Moon and Horton, 2003).

Ober *et al.,* (2001) reported a Hutterite family having 5 sibs affected with NS-MR, later Caliskan *et al.,* (2011) detected homozygosity for C-to-T conversion in exon 8 of TECR gene which resulted in a pro182-to-leu (P182L) substitution in trans-2,3-enoyl-CoA reductase, other 8 unaffected members were heterozygous for mutation. From 1496 Schmiedeleut Hutterites, 103 were found heterozygous and 5 homozygous for P182L mutation in TECR gene (Chong *et al.,* 2012).

8. MAN1B1 (MIM # 604346)

Mannosidase alpha class 1B member 1 (MAN1B1) is located on chromosome 9q43.3 and encodes endoplasmic reticulum mannosyl-oligosaccharide 1, 2-α- mannosidase (ERMan1) protein. ERMan1 belongs to group of enzymes which are involved in maturation of N-linked glycans, in secondary pathways. By this process timing and removal of misfolded glycoproteins through the endoplasmic reticulum associated degradation pathway is controlled (Rafiq *et al.,* 2011).

In Pakistani consanguineous family having 3 affected members, a homozygous 1189G-A transition mutation in MAN1B1 gene is identified, this mutation is resulted in a glu397-to-lys substitution in the site of enzyme where it interacts with glycan substrate. Results showed that mutant protein was expressed 5% less than the wild type levels (Rafiq *et al.,* 2011). In another Pakistani consanguineous family having 3 sibs, a homozygous 1418G-A transition in exon 9 of the MAN1B1 gene was identified. This transition resulted in try473-to-ter substitution, which resulted in nonsense-mediated mRNA decay. In Iranian consanguineous family having 3 affected sibs a homozygous 1000 C-T transition resulting in an arg334-to-cys substitution in base of active site of enzyme from where it interact with glycan substrates (Rafiq *et al.,* 2011).

2.7. Signaling Pathways involved in Mental retardation

The mental retardation genes are involved in broad range of functions, it shows that disturbance of any of a wide array of molecular process can damage brain function and can cause mental retardation. Some of the categories are: enzymes, mediators of signal transduction, binding proteins, transcription regulation and transporters. To date no mental retardation genes were linked with immunity proteins but IKBKG codes a subunit of a signal transducer which regulates NF-κB in the immune and inflammatory response pathway (Wallach *et al.,* 2002). Translation regulator category was also not used but EIF2AK3 encodes a kinase that indirectly regulates translation by phosphorylating eukaryotic translation initiation factor-2 (Ma *et al.,* 2002). Some other important proteins products which are encoded by these genes are; intracellular signaling, chromatin remodeling, actin cytoskeleton and neurogenesis.

1. The Rho and Rab-GTPase pathway

Rho and Rab are subfamily of Ras, while Ras is super family of kinases. They are involved in regulation of actin cytoskeleton and vesicle exocytosis (Nokelainen and Flint, 2002). Structural abnormalities in dendritic and spine are associated with MR and Rho GTPases emerged as important regulators of dendritic branching and spine morphogenesis. Therefore mutations in genes of Rho signaling pathway have been found to trigger various forms of MR (Frints *et al.,* 2002). In this protein family Rho, Rac and Cdc42 are main actors in signal transduction

Mutations in genes which affect working of Rho signaling pathway have been found in non-syndromic mental retardation. Oligopherinin-1(OPHN1) (Billuart *et al.,* 1998) and ARHGEF6 gene directly affect the activation of Rho cycle (Kutsche *et al.,* 2000). OPHN1 codes a Rho-GAP protein which stimulates GTPase activity of Rho, Rac and Cdc42.ARHGEF6 gene codes a small cytoplasmic protein which is homologous to GEF for Rho-GTPases, which activate them by exchanging Rho bounded GDP for GTP. Other genes found mutated in MR families are PAK3 and GDI. PAK3 (MRX30) (Allen *et al.,* 1998) is supposed to be the downstream effector of Rho-GTPases Rac and Cdc42. GDI gene codes for one more regulator of GTP/GDP exchange cycle (D'Adamo *et al.,* 1998).

There is indication that mutation in these genes can interrupt normal development of axonal connections (Van and D'Souza *et al.,* 1997). Inhibition of Rho, Rac and Cdc42 also reduce dendrite formation (Threadgill *et al.,* 1997).

2. MAPK/ERK Signaling pathway

Another pathway which is involved in cognitive impairment is Mitogen activated protein kinases (MAPK)/ Extracellular signal-regulated kinases (ERK). This pathway is found to be involved in MR condition, Coffin-Lowry syndrome which was mapped by Linkage analysis to chromosome Xp22.2 (Hanauer *et al.,* 1988), mutations were found in RSK2 gene (ribosomal S6 kinase) (Triver *et al.,* 1996). MAPK/ERK pathway plays an important role in differentiation, inflammation, growth, apoptosis, so it is vital during neuronal maturation and plasticity. This signaling pathway is important for memory and learning and mis-expression of genes in this pathway are associated with MR (Cohen and Greenberg, 2008).

3. Epigenetic regulation

Epigenetic regulation is also an important mechanism which regulates transcription. The genes associated with NS-MR etiology and play important role in epigenetic regulation are MECP2 and ATRX. Mutations in MECP2 gene causes Rett syndrome (Amir *et al.,* 1999), MECP2 binds with methylated CpG-nucleotides right through chromosome (Bird and Wolffe, 1999), which results in chromatin compaction and turns off transcription of gene (Nan *et al.,* 1998). MECP2 also interacts with ATRX gene, it has been proved by disturbed ATRX localization in neurons in MECP2 null mice (Nan *et al.,* 2007). Some problems caused by mutations in these genes are; Rett syndrome, LUBS X-linked MR syndrome, non-syndromic MR and autism, ATRX gene mutations may cause alpha-thalassemia/MR syndrome, alpha thalassemia myelodysplasia syndrome, X-linked MR hypotonic facies syndrome and non syndromic MR (Kaufmen *et al.,* 2010).

4. Chromatin remodeling and Transcriptional regulation

The involvements of genes which affect other genes in MR are highly expected. Transcriptional regulators influence biochemical pathways like development, there are four syndromes which are known to be caused by mutation in transcriptional regulators. JARIDIC is X-linked mental

retardation gene with twenty mutations (Tahiliani *et al.*, 2007). It encodes histone demethylase which specifically demethylates di- and tri- methylated histone 3 lysine 4 residues (Christensen *et al.*, 2007; Cloos *et al.*, 2008). Trimethylation of this residue is necessary for chromatin structure and transcriptional regulation (Tahiliani *et al.*, 2007). Transcription of several serotonin and NF-κB factor is repressed by CC2D1A gene (Basel-Vanagaite *et al.*, 2006) and NF-κB pathway is activated by TRAPPC9 (Hu *et al.*, 2005). Defect in NF-κB signaling may cause isolated cognitive deficit, thus expanding the area of the pathophysiology of MR (Philippe *et al.*, 2009).

MATERIALS & METHODS

3. Materials and Methods

3.1. Identification and diagnosis of Families

In the present research study three families (A,B,C) with hereditary Mental retardation were ascertained from Muzaffarabad and Abbaspur cities of Azad Jammu and Kashmir. The affected families were visited at their resident places to collect blood samples and to obtain some relevant information about family history, onset of disease, number of affected family member severity of disease and other abnormalities associated with disease. Pedigrees of all three families were constructed for extensive genetic analysis. Informed consent was obtained from elder members of three families participating in this research for the study including presentation of photographs. The research study was approved by ethical review committee International Islamic University, Islamabad Pakistan.

3.2. Pedigree Construction

An extensive pedigree was constructed based on the standard method elucidated by (Bennet *et al.*, 1995) after collecting data about the thorough medical history of the families. On the basis of material obtained by questioning the elder members of the families pedigree were constructed. The purpose of constructing pedigrees was to understand the mode of inheritance of the disease and genetic relationships among the family members. To symbolize males and females, squares and circles were used, respectively. Unfilled circles and squares represent the normal individuals whereas filled circles and squares were used to represent the affected ones. To show the deceased females and males in the pedigree crossed circles and squares were used, respectively. Roman numerals (I, II, III) in descending order shows the generation numbers while individuals within a generation were represented by Arabic numerals (1, 2, 3) in left to right order. To indicate the consanguineous marriages between the couples double line in the pedigree was used.

3.3. Blood Sampling

Blood samples as of all accessible normal and affected members were taken from peripheral venous route through 10 ml sanitized syringes (BD 0.8 mm x 38 mm 21 G x 1 ½ TW, Franklin lakes,USA). Blood samples of individuals were transferred to the vacutainer sets having

potassium EDTA (BD Vacutainer® K3 EDTA, Franklin Lakes, NJ, USA) and stored at 4°C and then processed for DNA extraction.

3.4. Genomic DNA Extraction and Purification

Genomic DNA extraction was carried out by following two protocols:

- Standard phenol-chloroform method (Sambrook *et al.*, 1989)
- Commercially available kit method (GeneElute™ Sigma-Aldrich., St. Louis, Mo. USA).

3.4.1. Standard Phenol-Chloroform Procedure

Before starting genomic DNA extraction via phenol-chloroform method, blood samples stored at 4°C were incubated for one hour at room temperature. This was performed to settle all the blood components.

- 750µl of human blood and equal volume of solution A were taken in Eppendorf tubes (1.5 ml), then the Eppendorf tubes were inverted 4-6 times for proper mixing of human blood and solution A (Table 3.1) and then the mixture was placed for half an hour at room temperature. The Eppendorf tubes were then centrifuged at 13,000 rpm (revolution per minute) for minute and nuclear pellet was obtained while the supernatant was discarded.
- Then the nuclear pellet was resuspended by adding 400µl of solution A and again centrifuged for 1 minute at 13,000 rpm. Again the supernatant was discarded the nuclear pellet was resuspended in 400 µl of solution B, 9 µl of proteinase K (10 mg/ml stock) and 14 µl of 20% SDS. Samples were then placed at 37°C for overnight incubation or incubated at 65°C for 3 hours.
- On the day second of extraction the pellet was fully dissolved, 500 µl fresh mixture prepared from equal volume of both solutions C+D (Table 3.1) was added to each of Eppendorf tubes and mixed properly. The samples were centrifuged at 13,000 rpm for 10 minutes. As a result of centrifugation two layers were formed.
- Upper aqueous layer was collected that contained DNA with the help of 1000 µl wide pipette set at 200 µl to reduce the risk of DNA damage in a new labeled eppendorf tube,

500 µl solution D (Table 3.1) was added to each tube and centrifuged for 10 minutes at 13,000 rpm.

- The separation of two layers was carried out carefully again and upper layer was transferred to new eppendorf tubes and 500 µl of isopropanol kept at -20°C and an amount of 55 µl of sodium acetate (3 M, pH 6) was used for the precipitation of DNA.
- The tubes were gently inverted several times to certify DNA precipitation and again certified at 13,000rpm for 10 minutes. DNA pellet was obtained carefully by discarding the supernatant. 200 µl of chilled 70% ethanol was added to the tubes having the DNA pellet and centrifuged at 13,000 rpm for 7 minutes.
- Ethanol was carefully discarded to avoid detachment of the pellet, while the remaining ethanol was evaporated by keeping the tubes for 5 minutes at 45° C in a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany). An appropriate amount of Tris-EDTA (TE) buffer was added to completely dissolve the DNA pellet when the residual ethanol was evaporated completely. The tubes were kept for incubation for 24 hours so that DNA gets completely suspended in the buffer.

3.4.2. DNA Extraction Using Commercially Available Kit Method

For the extraction of genomic DNA from the blood samples, commercially available kit, Sigma's GenEluteTM Blood Genomic DNA Kit (Sigma Aldrich, USA) was used.

In this method 200 µl of whole blood, 200 µl buffer B3 (NucleoSpin®, MACHERY-NAGEL, Duren, Germany) and lysis solution C was added in 2 ml collection tube. An amount of 10 µl proteinase K was added and thorough vortex for 15 seconds was done. At 55°C the sample was incubated for 10 minutes.

In the pre-assembled GenElute Miniprep Binding Column 500 µl of Column Preparation Solution was added and for 1 minute centrifugation was carried out at 13,000 rpm. Flow-through liquid was discarded and 200 µl of 100% chilled ethanol (labscan analytical Sciences, Bangkok, Thailand) was added to the lysate and vortexing for 5-10 seconds was done for proper mixing.

In the whole solution was shifted to the treated column and centrifugation was carried out for 1 minute at 10,000 rpm. Flow-through liquid was discarded from the collection tubes and the column was shifted to a new 2 ml collection tube. An amount of 500 µl of prewash solution

(NucleoSpin, MACHEREY-NAGEL, and Duren, Germany) was added to the column and then the tubes were centrifuged for 1 minute at 10,000 rpm. The column was transferred to a new 2 ml collection tube after discarding flow-through liquid. The column was centrifuged at 10,000 rpm for 3 minutes after adding 500 µl wash solution. To elute the residual ethanol, empty spin for 1 minute was done at 12,000 rpm. 200 µl elution solutions was added in the centre of the column after transferring it into a new 2 ml collection tube after discarding flow through liquid, the tube containing column was left at room temperature for 5 minutes. In order to elute DNA the column was centrifuged for 1 minute at 10,000 rpm and the collection tube containing pure genomic DNA was stored at 4°C.

3.5. Agarose Gel Electrophoresis for Extracted DNA Quantification

Quantitative analysis of extracted DNA was done by using 1% agarose gel which resolves the DNA. Gel was prepared by adding 0.5 g of agarose to the mixture of 45 ml of distilled water and 5 ml of 10 X TBE (0.89 M Tris-Borate, 0.032 M EDTA) (Table 3.2) in the flask. 0.5 g of agarose was weighed with the help of electric balance. Agarose was dissolved by heating the flask in microwave for 2 minutes. As the transparent solution was obtained, 7 µl of ethidium bromide was added to the flask. Ethidium bromide was added to stain the DNA bands. By gentle shaking the dye was thoroughly mixed in the solution and then the solution was carefully poured into the gel tank.

The gel mixture was left for 20-25 minutes at room temperature for solidification. Equal volume (5 µl) of extracted DNA was mixed with loading dye (0.25% bromophenol blue with 40% sucrose) and the mixture of DNA and dye was loaded into the gel wells. In the gel tank 1 X TBE buffer was added and gel electrophoresis was run fo 30-40 minutes at 120 volts, horizontally. DNA bands were visualized under U.V Trans-illuminator (Biometra, Gotinngen, Germany). To properly analyse the DNA bands photographs were taken by using Digital Camera EDAS 290 (Kodak, New York, USA).

3.6. Dilution of DNA

Quality of Genomic DNA was determined by taking optical density (OD) at 260nm wavelength. PCR water was used to dilute the stock DNA to 40-50 ng/µl by using GeneRay UV-Photometer (Biometra®, Germany). Generally, dilutions were made in 10 µl stock and 90µl PCR water.

3.7. Genotyping and Linkage Analysis

Linkage analysis or genetic mapping or genotyping of the families with hereditary Mental retardation was performed by using highly polymorphic microsatellite markers for the well-known gene loci (Table 3.5). The genetic map distance of markers was obtained from UCSC Genome browser and Rutgers's Combined Linkage-Physical Map (Matise *et al.*, 2007).

3.8. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was carried out to amplify the specific DNA sequences. DNA sequences were subjected to genotyping by using specific polymorphic microsatellite markers (Table 3.5). 25 µl was set as the total volume of mixture using 2 µl DNA. 0.2 ml volume PCR tubes (9Axygen, California, USA) were used. The mixture was prepared by adding different chemicals of different amounts and compositions for this reaction (Table 3.3). 2.5 µl PCR buffer 10X (200mM (NH4)2SO4, 750 mM of Tris-HCl pH 8.8, 0.1% Tween 20), a DNA sample dilution of 2 µl, 2 µl of MgCl2 (25 mM), forward and reverse microsatellite markers (20 ng/µl) in amount of 0.3 µl each, 0.4 unit Taq Polymerase (Perkin-Elmercetus, Ferments, Burlington, Canada) in amount of 0.3 µl and 0.5 µl of (10 mM) dNTPs (MBI Fermentas, UK) in 17.6 µl of PCR water. The reaction mixture was subjected to centrifugation at 4,000 rpm in order to get thorough mixing of reaction mixture. Reactions were performed by means of T3 thermocyclers (Biometra, Gottingen, Germany) with the following conditions.

The standard conditions for thermocycler are:

- To denature the double stranded DNA the cycle of template DNA denaturation at 96°C for 7 minutes.
- Amplification cycles were kept 40 each of which included 3 sub-cycles. Each cycle included denaturation of double stranded DNA into single strand.

Table 3.1 Composition of solutions used in Genomic DNA Extraction

Solution	Composition
Solution A	Sucrose 0.32 M MgCl2 5 mM Tris (pH 7.5) 10 mM 1 % (v/v) Triton X-100
Solution B	EDTA (pH 8.8) 2 Mm NaCl 400 mM Tris (pH 7.5) 10 Mm
Solution C	Saturated phenol 400µl
Solution D	Chloroform 24 volumes Isoamylalcohol 1 volume i.e. 24:1
10% SDS	10 g in 50 ml water
DNA dissolving Buffer	Tris (pH 8.0) 10 mM EDTA 0.1 mM
Bromophenol blue	Sucrose 40 g Bromophenol Blue 0.25 g

Table 3.2 Composition of solutions used in Gel Electrophoresis

Solution	Composition
Gel Loading Dye	Sucrose 40 g Bromophenol Blue 0.25 g
10X TBE Buffer	Tris 0.89 M Boric Acid 0.025 M 0.5 M (pH 8.3) EDTA

- DNA at 96°C for one minute, followed by cyclic "anneling" or hybridization of microsatellite markers to their complementary DNA sequences for 1 minute at a temperature of 55-60°C, 1 minute for cyclic elongation of specific DNA sequences from each primer at 72°C, also called cyclic extention.
- Final extension or polymerization was performed at 72°C for 10 minutes by Taq DNA polymerase to complete amplification of any leftover specific DNA sequences present in the reaction mixture.

With the help of PCR, specific DNA sequences were amplified a number of times.

3.9. Polyacrylamide Gel Electrophoresis (PAGE)

The analysis of amplified PCR product was carried out on 8% polyacrylamide gel performing electrophoresis. The gel composition is as follows.

The gel mixture (Table 3.4) was thoroughly mixed by inverting the measuring cylinder for two to three times. Two washed glass plates apprehended 1.5 mm apart by clips and spacers were set and the gel mixture was poured gently between the plates and the combs were inserted to make wells in the gel for proper polymerization and solidification the plates were left for 30-40 minutes at room temperature. The amount of 6-7 µl loading dye (40% sucrose with 0.25% of bromophenol) was gently mixed with amplified PCR products and then were loaded in wells and left for 2-3 hours at 140 volts for electrophoresis in Vertical gel apparatus (Whatman, Biometra, Gottingen, Germany). The running media used was 1 X TBE buffer. The gel was taken out from the plates, stained with ethidium bromide (0.5 µg/ml), visualized by UV Trans-illuminator (Biometra, Gottingen, Germany) after the gels were run enough at the edge along with the loading dye and finally the picture of gels were captured by using digital camera EDAS 290(Kodak,NewYork,USA).

Table 3.3 Chemicals used in Polymerase Chain Reaction

Chemical Used	Stock Concentration	Amount used in PCR
DNA dilution	2µl	2 µl
PCR water	18.7 µl	18.7 µl
PCR Buffer	10 X (200 mM Ammonium Sulphate, 750 mM Tris-HCL (pH 8.8, 0.1 %Tween 20)	2.5 µl
$MgCl_2$	25 mM	2 µl
$dNTP_S$	10 mM each dNTPs	0.5 µl
Microsatellite Markers(R & F)	20 ng/µl each	0.3 µl each
Taq Polymerase	0.5 U/µl	0.3 µl

Table 3.4 Polyacrylamide Gel Composition

Chemicals	Stock Concentration & Composition	Amount for one Gel
30% Acrylamide	29:1 ratio of acrylamide (MERCK, Darmstadt,Germany).N,N'Methylene-bisacrylamide (BDH,Poole, England)	13.5 ml
10X TBE	Tris 0.89 M, Borate 0.89 M and EDTA 0.02M	5ml
10%APS	Ammonium per sulphate (5 g/45 ml distilled water) (Sigma-Aldrich St Louis, MO,USA)	400 µl
TEMED	N, N, N', N'-Tetra methyl ethylene diamine) (Sigma-Aldrich, USA)	25 µl
Distilled Water		Raise up to 50

Table 3.5 List of microsatellite markers used to investigate the linkage to known genes/loci.

S.L NO.	Candidate Gene	Locus	Chromosomal Location	Markers	Distance cM
1.	PRSS12	MRT1	4q26	D4S407	120.94cM
				D4S2937	122.7cM
				D4S2297	123.65cM
				D4S1522	125.35 cM
				D4S191	125.89 cM
				D4S1612	128.25cM
				D4S194	129.25cM
				D4S1615	131.92cM
2	CRBN	MRT2	3p26.2	D3S1297	4.52 cM
				D3S3525	5.19cM
				D3S3630	5.97cM
				D3S4538	9.75cM
				D3S3050	10.42cM
				D3S1620	10.68cM
3	CC2D1A	MRT3	19p13.12	D19S1165	32.65cM
				D19S914	32.9cM
				D19S564	34.46cM
				D19S840	34.46cM
				D19S385	36.98cM
				D19S252	38.36cM
4	TECR	MRT3	19p13.12	D19S1165	32.65cM
				D19S914	32.9cM
				D19S564	34.46cM
				D19S840	34.46cM
				D19S385	36.98cM
				D19S252	38.36cM

5	TUSC3	MRT17	8p22	D8S26 D8S1827 D8S549 D8S1731 D8S254 D8S261	29.8 cM 30.15cM 30.41cM 30.41cM 31.68cM 34.73cM
6	MAN1B1	MRT15	9q34.4	D9S1818 D9S1826 D9S158 D9S905 D9S1838 D9S2168	152.82 cM 160.5 cM 163.08 cM 165.24cM 165.25cM 167.16 cM
7	TRAPPC9	MRT13	8q24.3	D8S1753 D8S1837 D8S1521 D8S1717	154.71cM 158.44cM 160.14cM 165.78cM
8	GRIK2	MRT6	6q16.3	D6S1716 D6S1717 D6S1565 D6S475 D6S962 D6S1709 D6S283 D6S1692	107.63cM 108.48cM 108.76cM 109.17cM 109.74cM 110.69cM 110.69cM 111.26cM

RESULTS

4. Results

4.1. Description of the Families studied

This research was conducted on three Kashmiri families, living in Azad Jammu and Kashmir. The affected members of the families had clinical presentation of Mental retardation.

4.1.1. Family A

The family A is Hindko speaking family and belongs to Muzaffarabad city of Azad Jammu and Kashmir, Pakistan. It consisted of four generation pedigree; pedigree was constructed by interviewing the senior members of the family. After drawing pedigree we came to know that three members in the family are affected (III-12, IV-2, IV-3), the female affected member (III-12) passed away few years ago and was severely mental retarded. The pedigree analysis showed that the sampled affected children were born to consanguineous parents and inherits the disease from the unaffected parents which showed it is an autosomal recessive mode of inheritance (Figure 4.1). The blood samples were taken from all the five members of family consisting of unaffected parents (III-7, III-8), two affected (IV-2, IV-3) and one unaffected children (IV-6). Parents of the affected individuals had normal IQ and didn't show any clinical symptoms of MR. Clinical analysis of affected individuals of family A showed that they are having moderate MR with poor speach, low IQ and they also had balancing problems. The affected individuals have no phenotypic features of microcephaly, vision or hearing impairment (Figure 4.2). Delays in speech and waling, they learn simple communication.

4.1.2. Family B

The family B is Kashmiri speaking family and belongs to village area of district Muzaffarabad, Azad Jammu and Kashmir, Pakistan. It consists of four generation pedigree, with two affected individuals (IV-5, IV-9). The blood samples were taken from the five members of the family consisting of two affected (IV-5, IV-9) and three unaffected (III-5, III-6, IV-3) individuals. The pedigree analysis showed that affected individuals are born to consanguineous parents and parents are unaffected which is evidence that it is autosomal recessive mode of inheritance (Figure 4.3). The parents (III-5, III-6) of affected individuals were physically and mentally normal. The affected individuals have speaking delays, uncontrolled unbalanced walking; they

show response with little or no communication skills. They didn't show any phenotypic features of microcephaly, facial abnormality, vision and hearing impairments (Figure 4.4).

4.1.3. Family C

The family C is from Abbaspur, Poonch district of Azad Jammu and Kashmir, Pakistan. Three generation pedigree was constructed after collecting data from the elder members of the family (Figure 4.5). Pedigree investigation strongly put forward the autosomal recessive mode of inheritance. The pedigree consists of 30 members, including 3 affected members; 19 females and 8 male members. The affected members were investigated very carefully. Clinical investigation shows that affected members are severely mentally retarded; they have speaking delays they cannot communicate and show very little response, they cannot take care of themselves and female member show aggressive behaviour. They didn't show any other symptoms like microcephaly or hearing or visual impairments (Figure 4.6). There disease was not caused by any environmental factor and not linked to any known syndrome hence shows non-syndromic disorder. Blood samples were taken from the four members of the family, consisting of two affected (III-1, III-3) and two unaffected individuals (II-1 and III-4).

4.2. Genetic Mapping of known Loci for Mental retardation

The known loci involved in autosomal recessive MR were checked for linkage in these three families. Microsatellite markers mentioned in Table 3.5 were used for genotyping through PCR to check for linkage in each of candidate gene. The amplified PCR products were than run on 8% polyacrylamide gel electrophoresis PAGE. Later on the gel was stained with ethidium bromide and visualized by gel documentation.

4.2.1. Family A

Five DNA samples containing two affected (IV-2, IV-3) and three unaffected individuals (III-7, III-8, IV-6) from family A were genotyped for these microsatellite markers PRSS12 (4q26), GRIK2 (6q16.3), TRAPPC9 (8q24.3), MAN1B1 (9q34.3) CC2D1A (19p13.12) and TECR (19p13.12). From the analysis of results obtained from microsatellite markers, no homozygous region was detected with any microsatellite marker (Figure 4.7-4.11). Hence on the basis of the genotyping results, linkage to six known loci was excluded in family A.

4.2.2. Family B

Five DNA samples containing two affected (IV-5, IV-9) and three unaffected individuals (III-5, III-6, IV-3) from family B were genotyped for these microsatellite markers PRSS12 (4q26), GRIK2 (6q16.3), TRAPPC9 (8q24.3), MAN1B1 (9q34.3) CC2D1A (19p13.12) and TECR (19p13.12). From the analysis of results obtained from microsatellite markers, no homozygous region was detected with any microsatellite marker (Figure 4.12-16). Hence on the basis of the genotyping results, linkage to six known loci was excluded in family B.

4.2.3. Family C

Four DNA samples containing two affected (III-1, III-3) and two unaffected individuals (II-1, II-2) from family C were genotyped for microsatellite markers specific for these microsatellite markers CRBN (3p26.2), PRSS12 (4q26), GRIK2 (6q16.3), TUSC3 (8p22), TRAPPC9 (8q24.3) MAN1B1 (9q34.3) CC2D1A (19p13.12) and TECR (19p13.12). From the analysis of results obtained from microsatellite markers, no homozygous region was detected with any microsatellite marker (Figure 4.17-4.23). Hence on the basis of the genotyping results, linkage to eight known loci was excluded in family C.

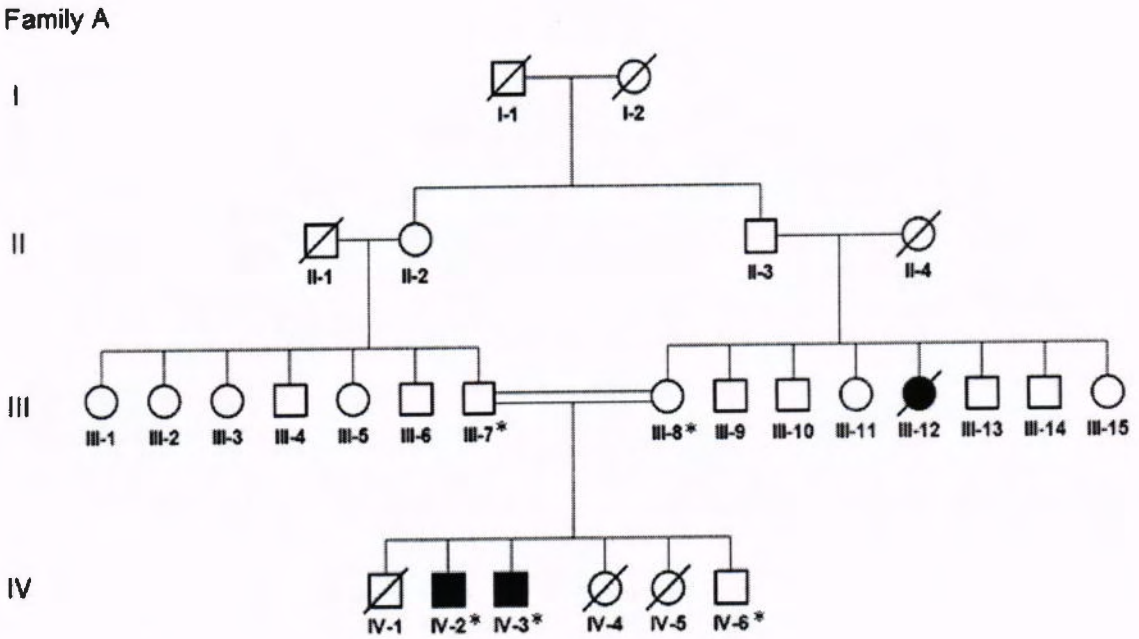


Figure 4.1 Pedigree of family A with Mental retardation, showing autosomal recessive mode of inheritance. Squares and circles represent males and females, respectively. A filled symbol represents an affected individual. Double lines indicate consanguineous marriage. The Roman numerals indicate the generation number of individuals within a pedigree while Arabic numerals indicate their positions within a generation. The samples collected from member that are mentioned with (*) in pedigree.

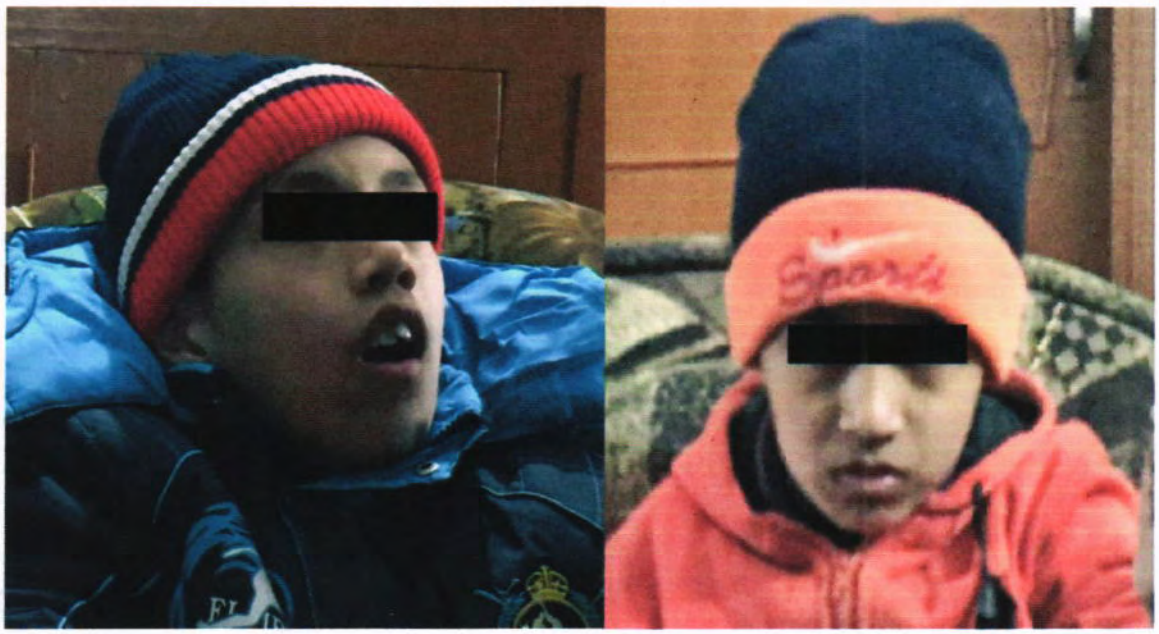


Figure 4.2 Affected individuals (IV-2 and IV-3) of family A don't show any abnormal facial features or microcephaly, which indicates it is non-syndromic Mental retardation.

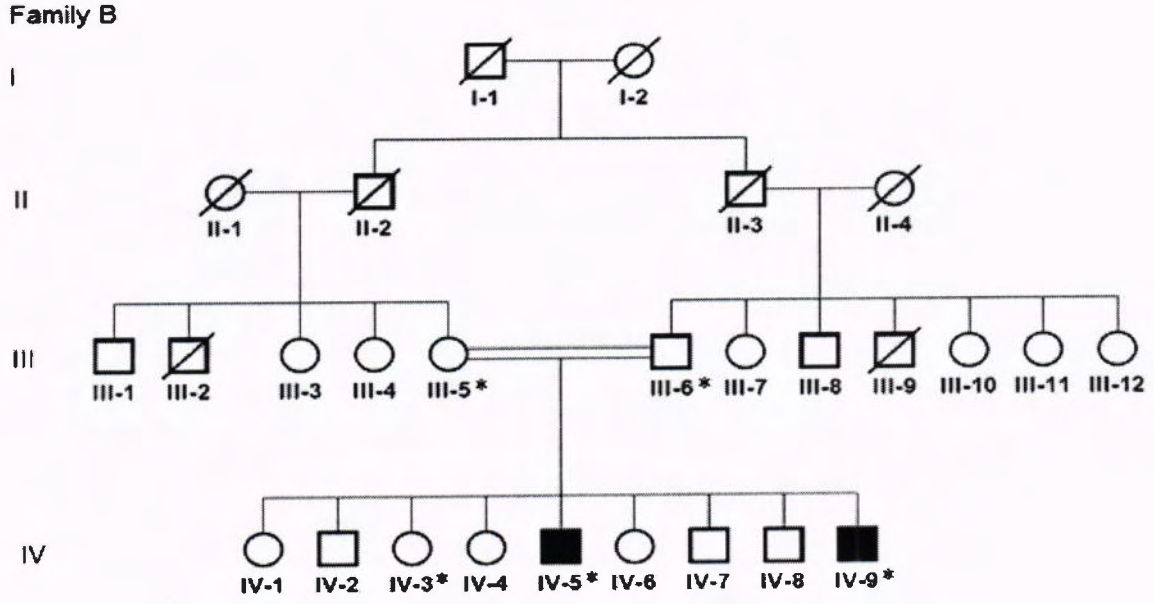


Figure 4.3 Pedigree of family B with Mental retardation, showing autosomal recessive mode of inheritance. Squares and circles represent males and females, respectively. A filled symbol represents an affected individual. Double lines indicate consanguineous marriage. The Roman numerals indicate the generation number of individuals within a pedigree while Arabic numerals indicate their positions within a generation. The samples collected from member that are mentioned with (*) in pedigree.

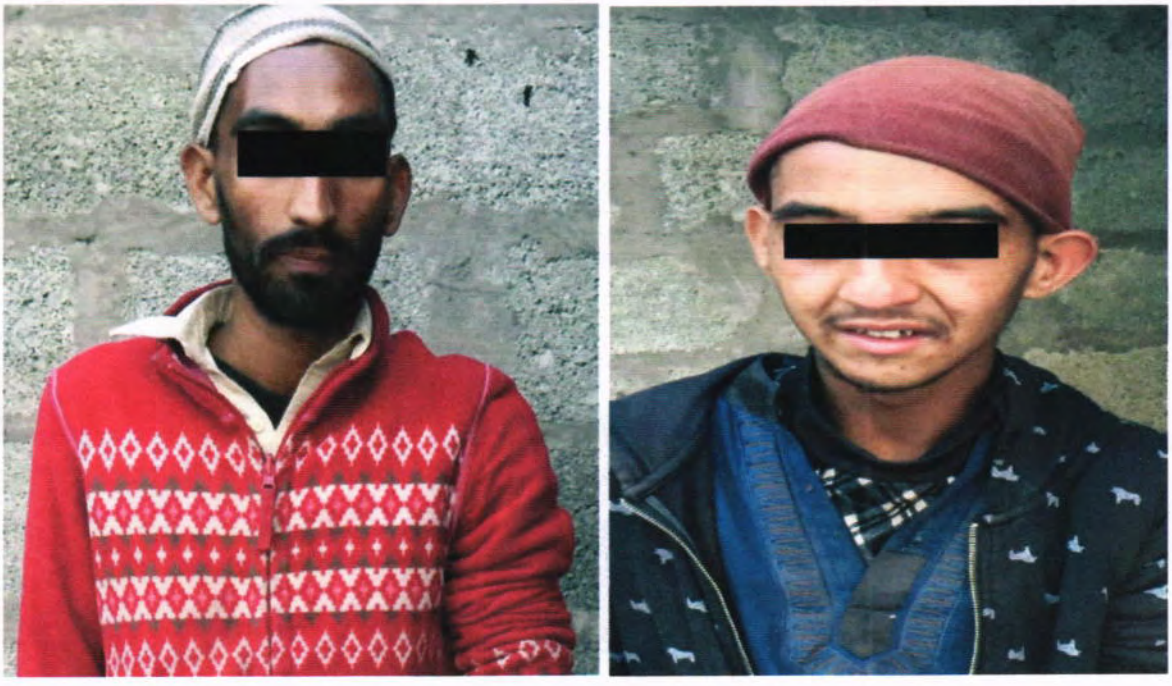


Figure 4.4 Affected individuals (IV-5 and IV-9) of family B don't show any abnormal facial features or microcephaly, which indicates it is non-syndromic Mental retardation.

Family C

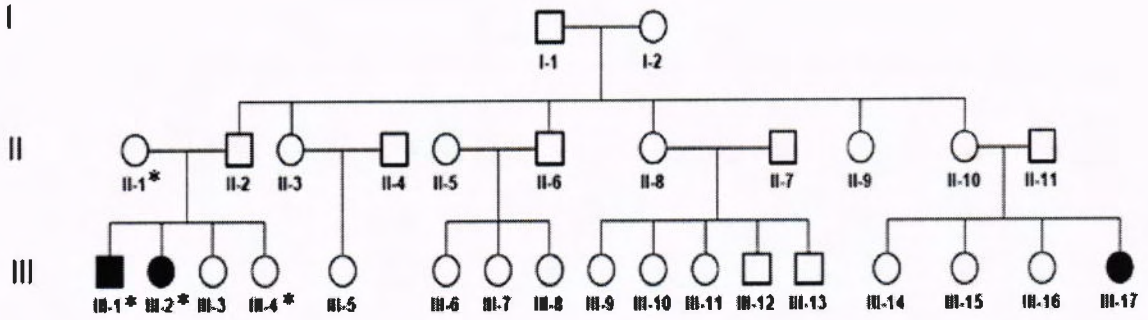


Figure 4.5 Pedigree of family C with Mental retardation, showing autosomal recessive mode of inheritance. Squares and circles represent males and females, respectively. A filled symbol represents an affected individual. The Roman numerals indicate the generation number of individuals within a pedigree with Arabic numerals indicate their positions within a generation. The samples collected from member that are mentioned with (*) in pedigree.

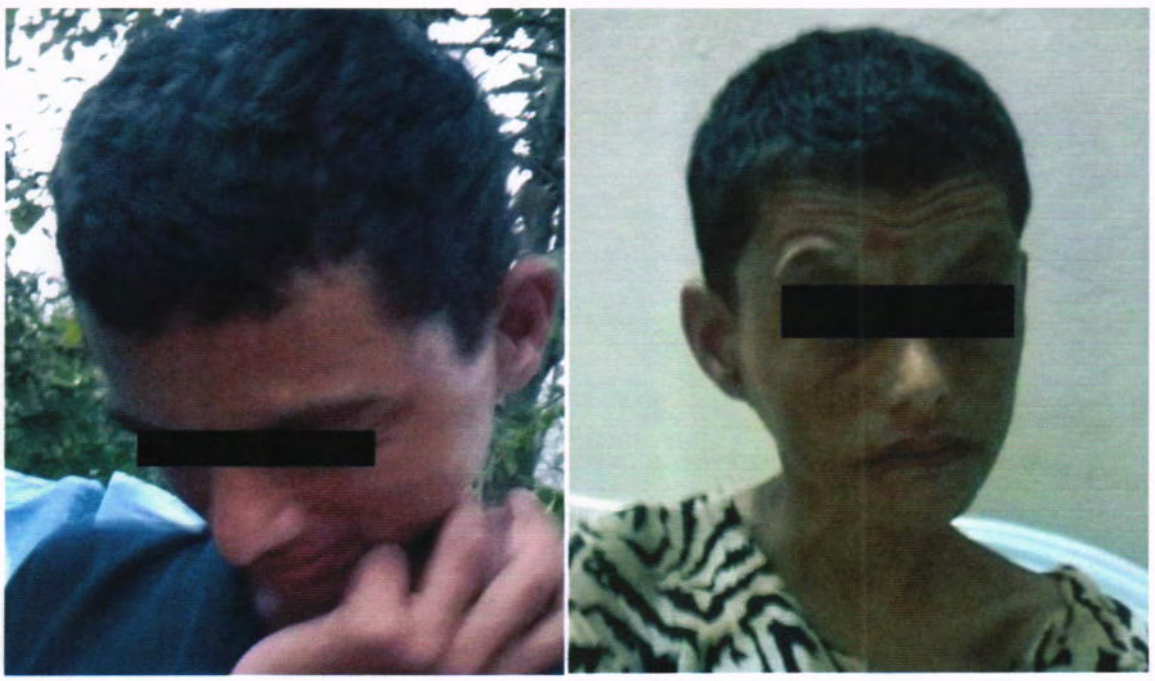


Figure 4.6 Affected individuals (III-1 and III-2) of family C don't show any abnormal facial features or microcephaly, which indicates it is non-syndromic Mental retardation.

Table 4.1 Clinical symptoms of the affected Individuals

Clinical Features	Family A		Family B		Family C	
	IV-2	IV-3	IV-5	IV-9	III-1	III-2
Sex	Male	Male	Male	Male	Male	Female
Age	11 years	9 years	27 years	15 years	16years	13 years
Speech disabilities	+	+	+	+	+	+
Facial abnormalities	-	-	-	-	-	-
Epileptic fits	-	-	-	-	-	-
Mental level	Moderate	Moderate	Mild	Severe	Moderae	Severe
Schooling	special school	special school	-	-	-	-
Growth	Normal	Normal	Normal	Normal	Normal	Normal
Self care	-	-	+	-	-	-
Can recognize	+	+	+	+	+	+
Ophthalmological	-	-	-	-	-	-
Balancing problem	+	+	+	+	+	+
Watery mouth	-	-	-	-	-	-

Absent (-)

Present (+)

Family A

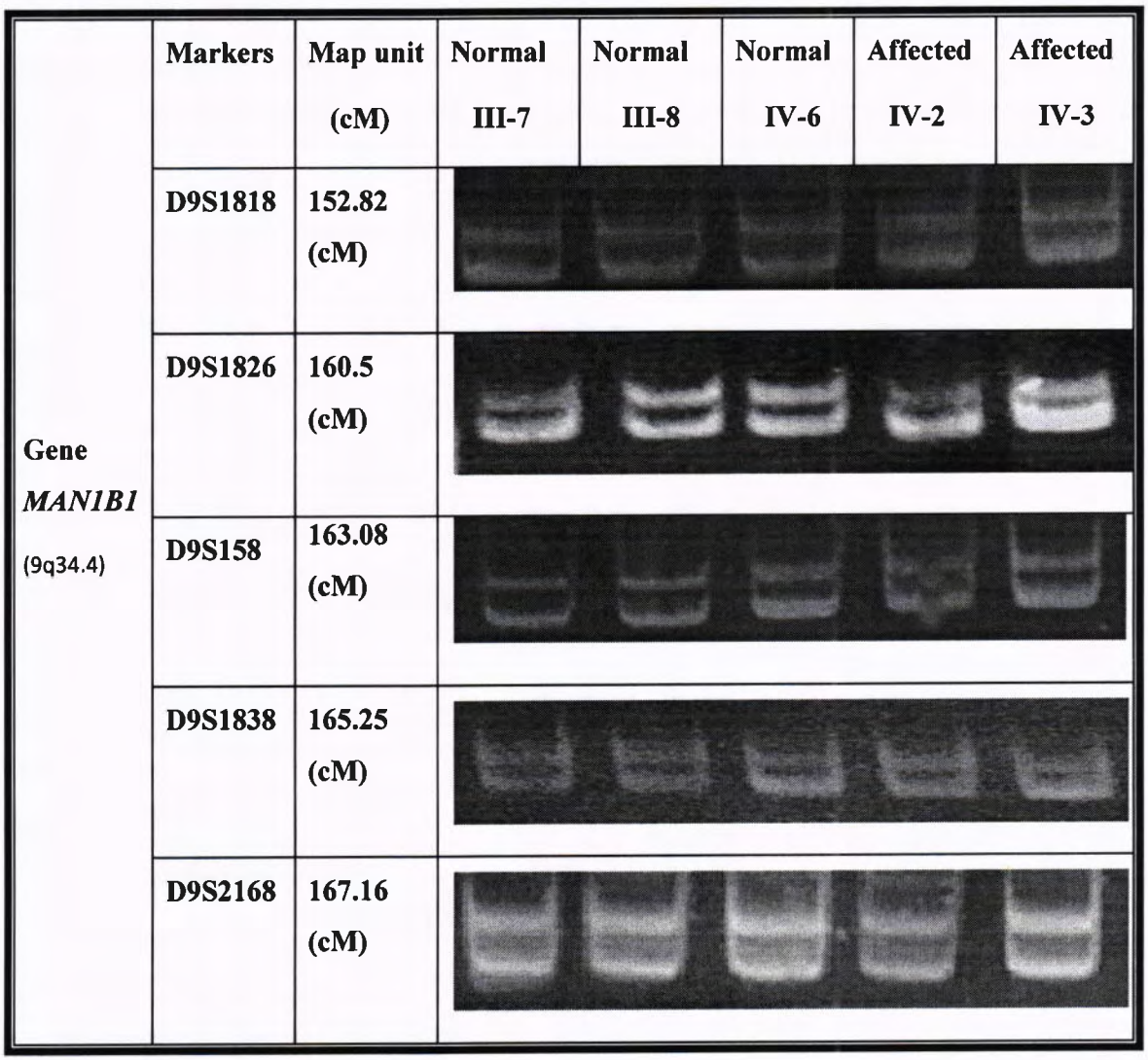


Figure 4.7 Gel images showing the band pattern of chromosome 9q34.4 markers in five member family A, these markers are used to test the *MAN1B1*gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family A

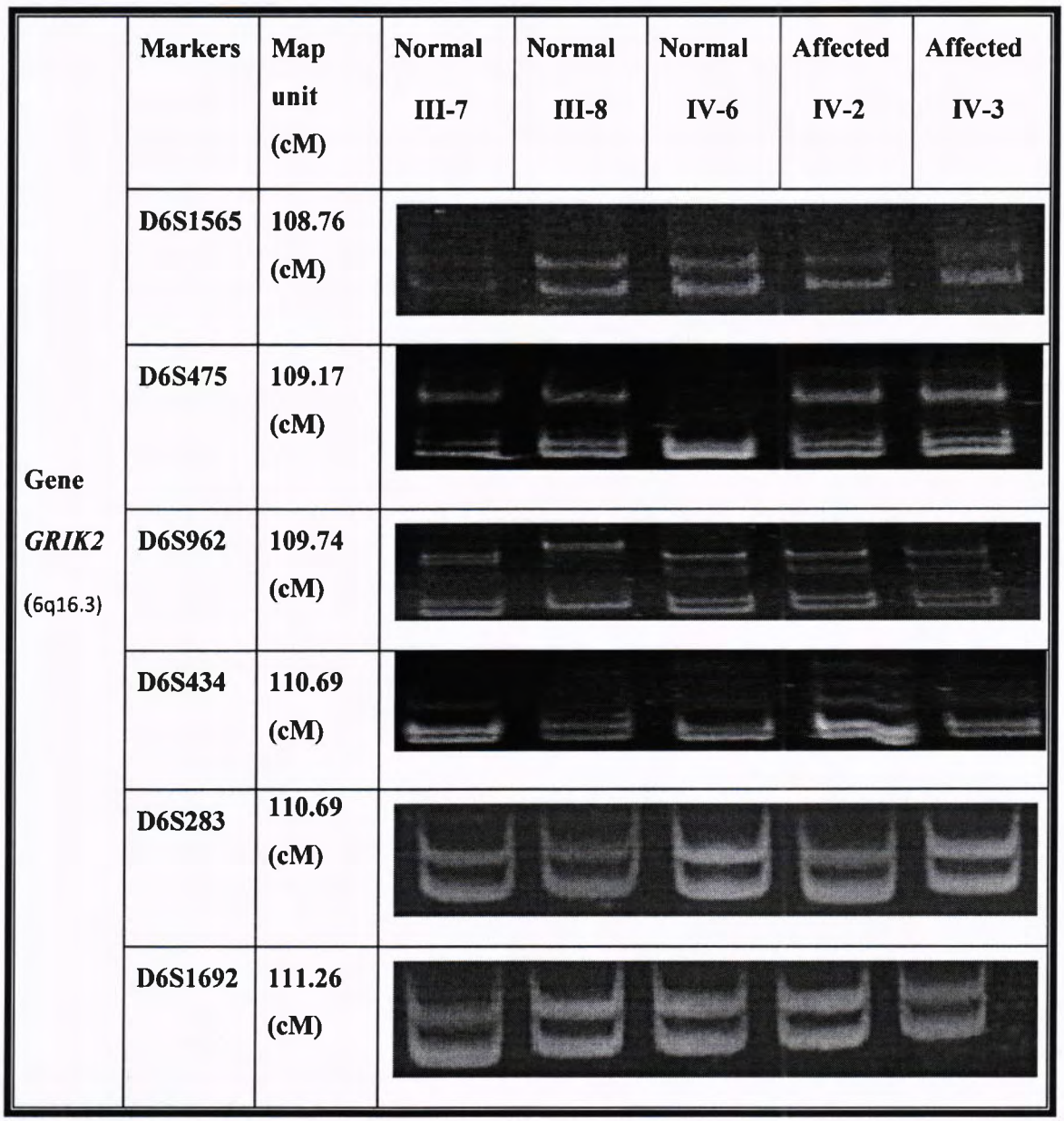


Figure 4.8 Gel images showing the band pattern of chromosome 6q16.3 markers in five member family A, these markers are used to test the *GRIK2* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family A

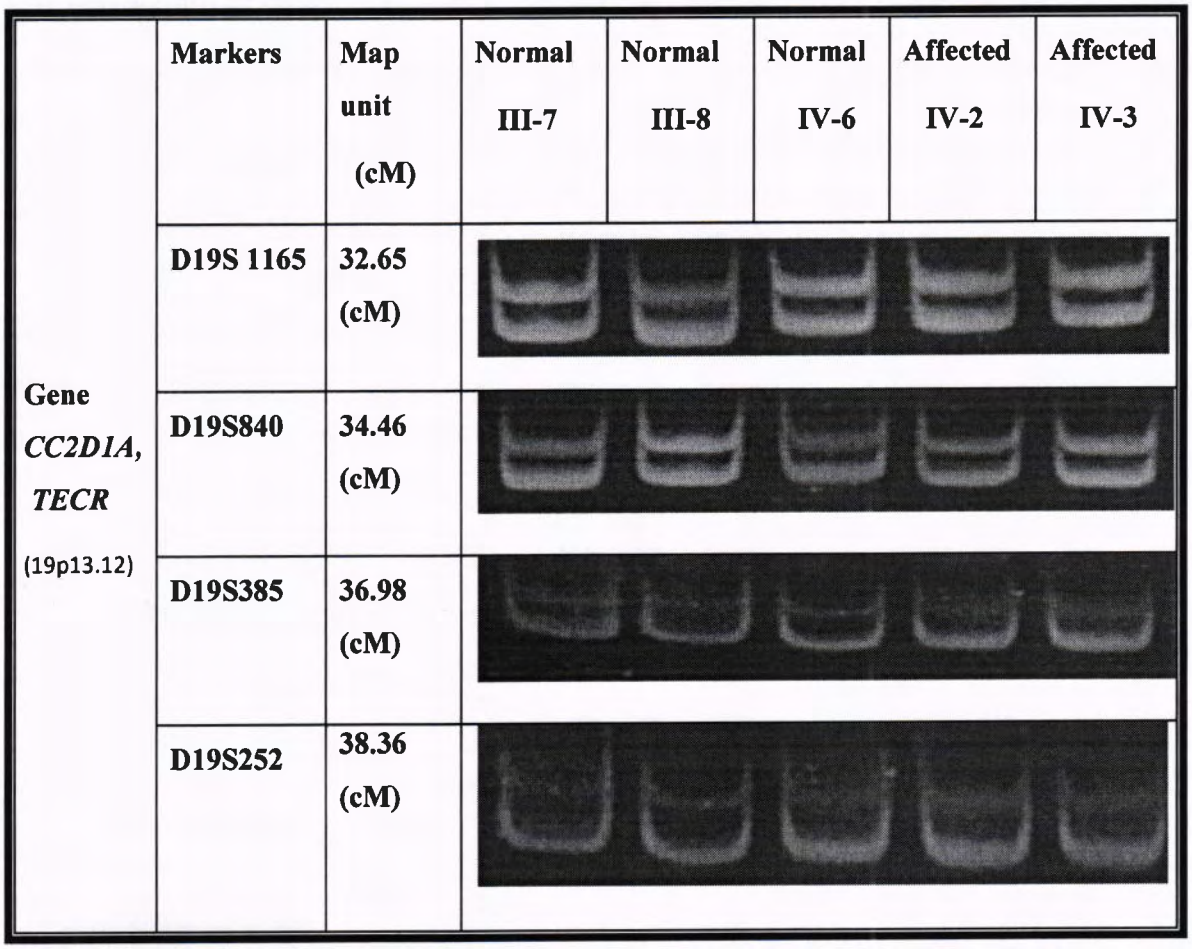


Figure 4.9 Gel images showing the band pattern of chromosome 19p13.12 markers in five member family A, these markers are used to test the *CC2D1A* and *TECR* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family A

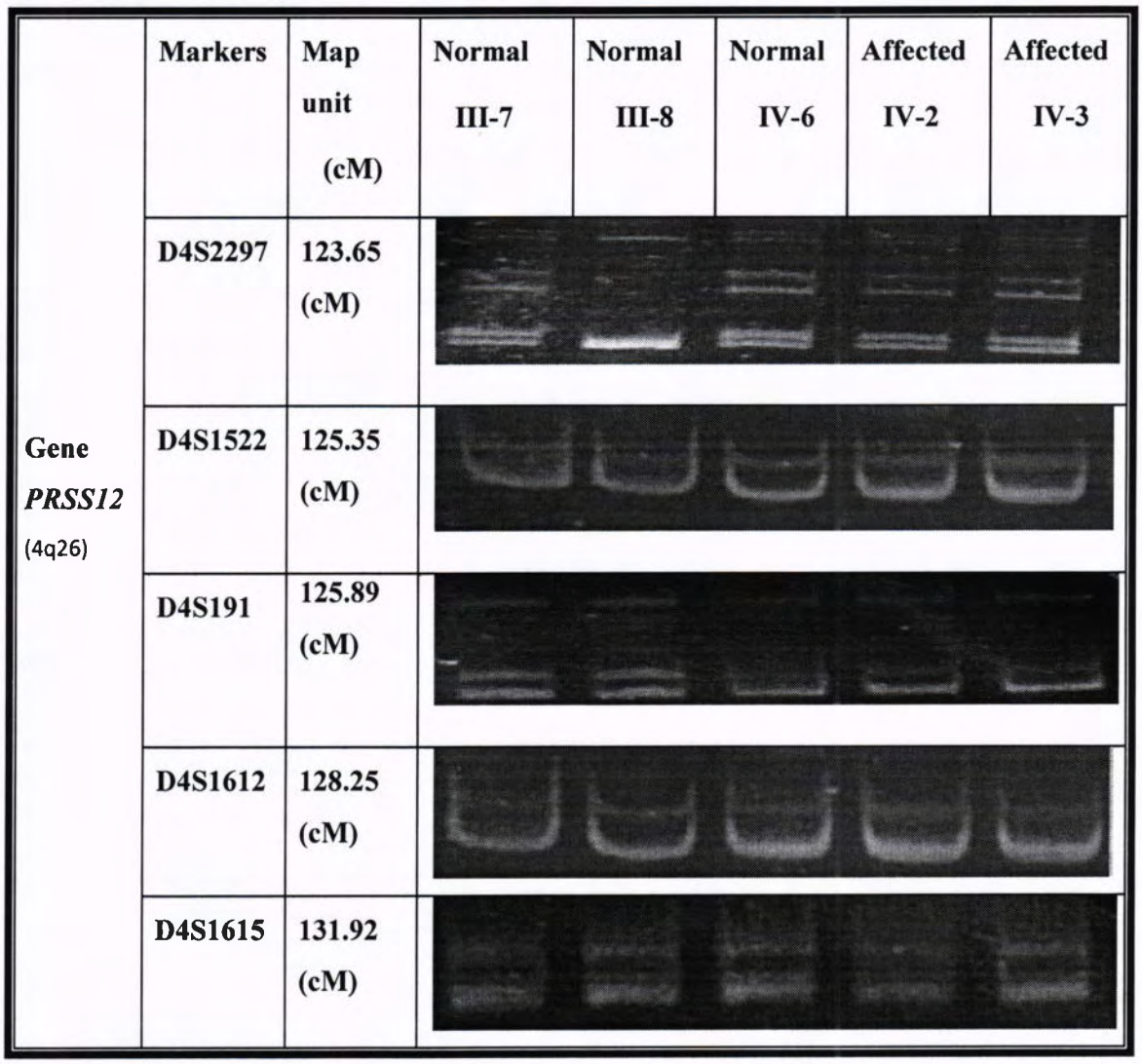


Figure 4.10 Gel images showing the band pattern of chromosome 4q26 markers in five member family A, these markers are used to test the *PRSS12* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family A

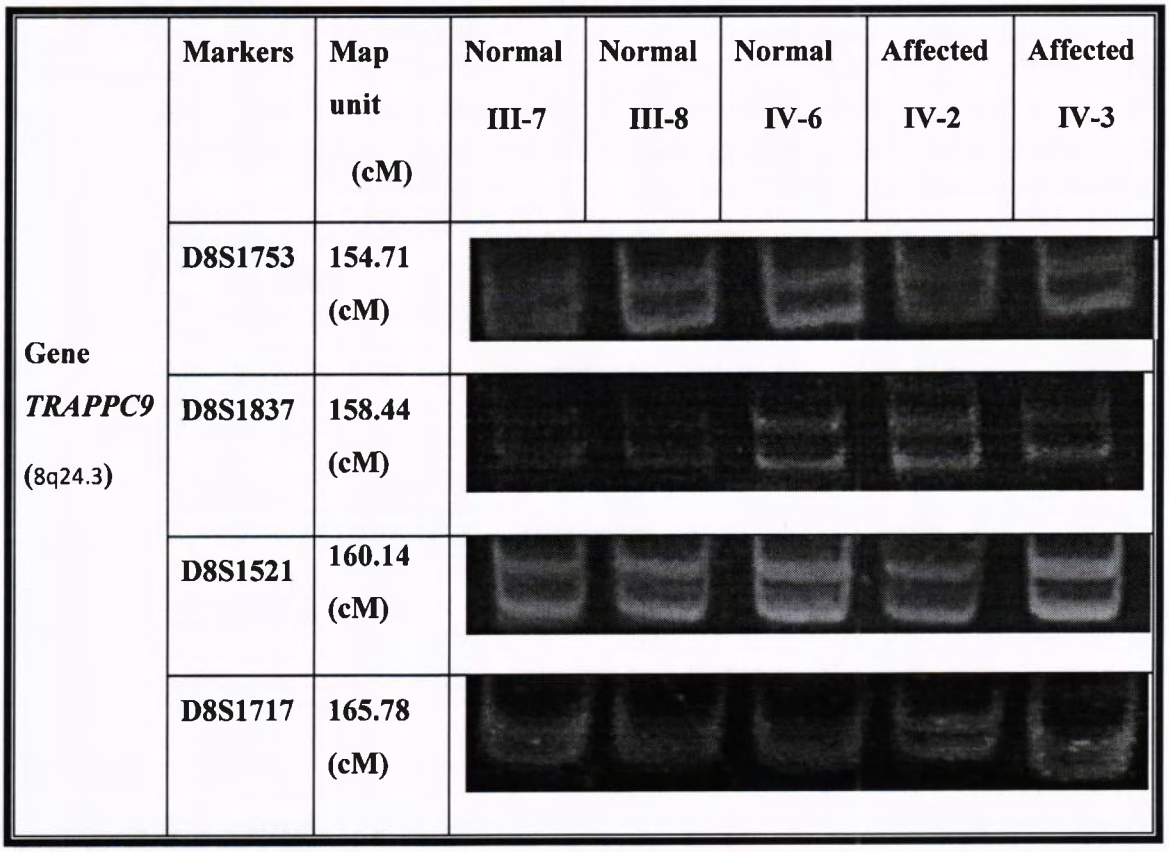


Figure 4.11 Gel images showing the band pattern of chromosome 8q24.3 markers in five member family A, these markers are used to test the *TRAPPC9* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family B

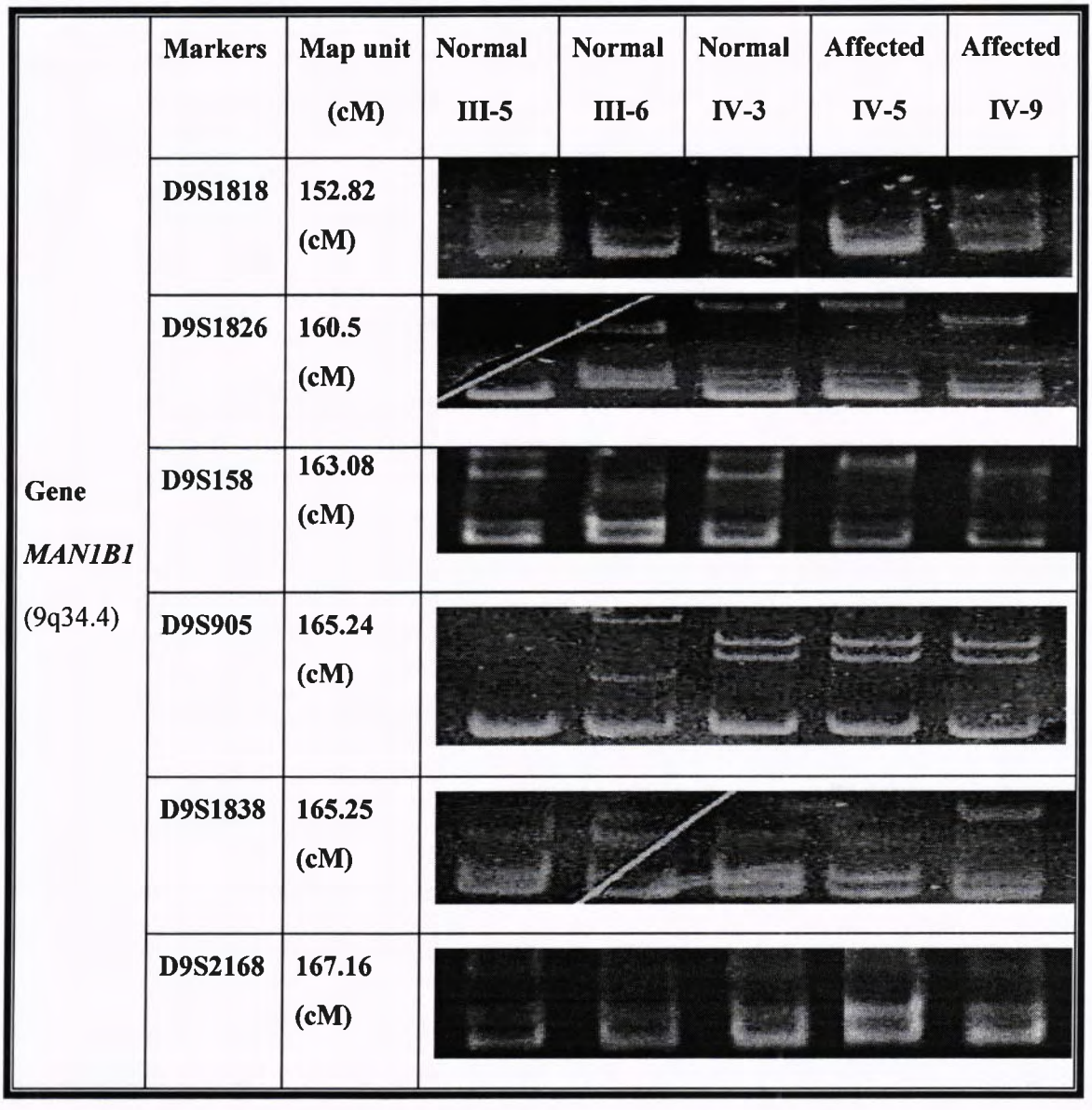


Figure 4.12 Gel images showing the band pattern of chromosome 9q34.4 markers in five member family B, these markers are used to test the *MAN1B1* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree

Family B

	Markers	Map unit (cM)	Normal III-5	Normal III-6	Normal IV-3	Affected IV-5	Affected IV-9
Gene *GRIK2* (6q16.3)	D6S1716	107.63 (cM)					
	D6S1717	108.48 (cM)					
	D6S434	110.69 (cM)					
	D6S1709	110.69 (cM)					
	D6S283	110.69 (cM)					
	D6S1692	111.26 (cM)					

Figure 4.13 Gel images showing the band pattern of chromosome 6q16.3 markers in five member family B, these markers are used to test the *GRIK2* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family B

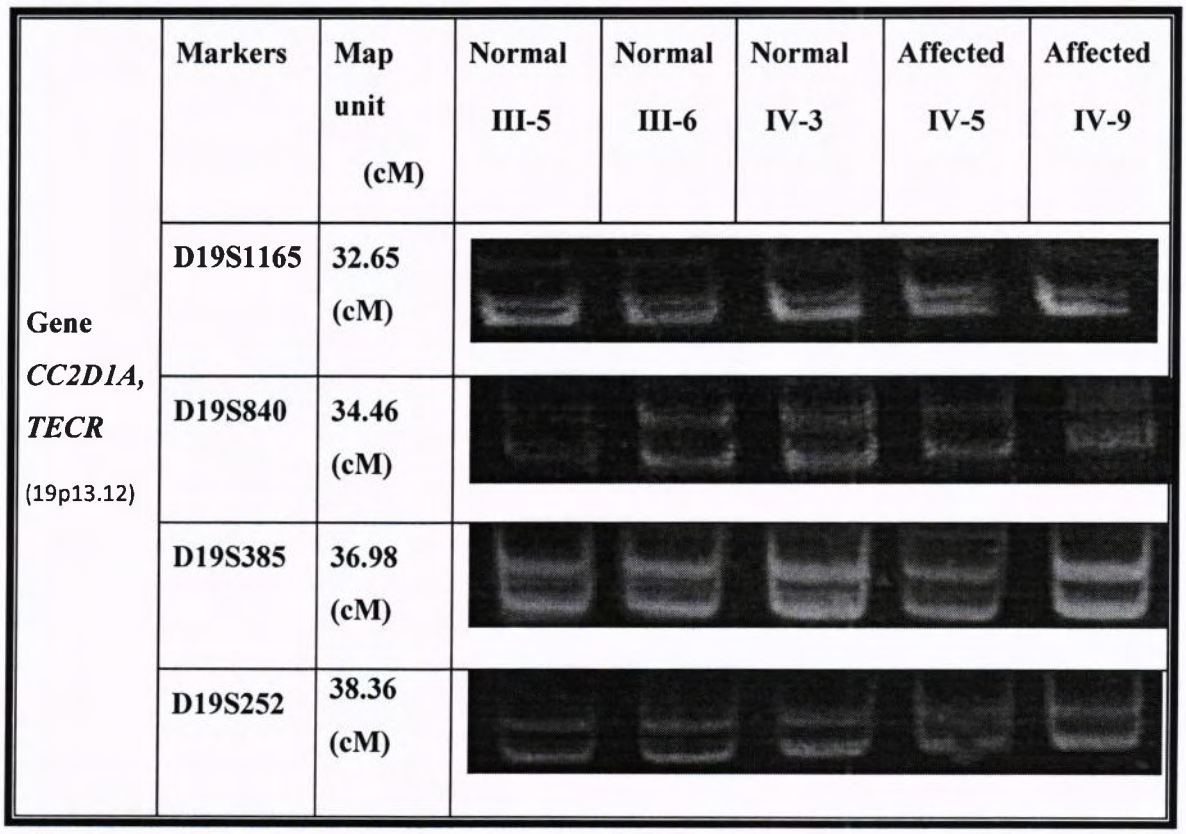


Figure 4.14 Gel images showing the band pattern of chromosome 19p13.12 markers in five member family B, these markers are used to test the *CC2D1A* and *TECR* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family B

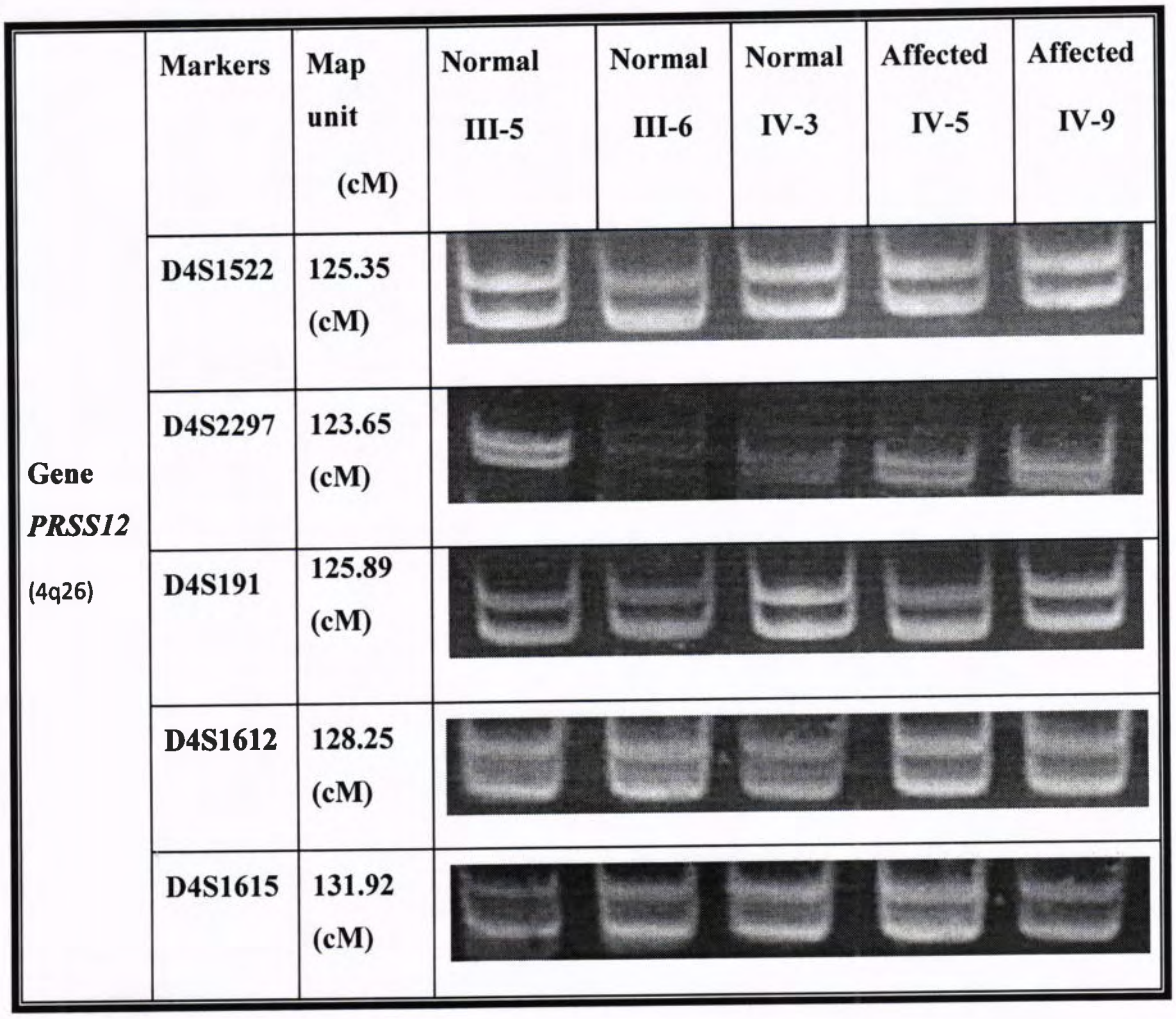


Figure 4.15 Gel images showing the band pattern of chromosome 4q26 markers in five member family B, these markers are used to test the *PRSS12* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family B

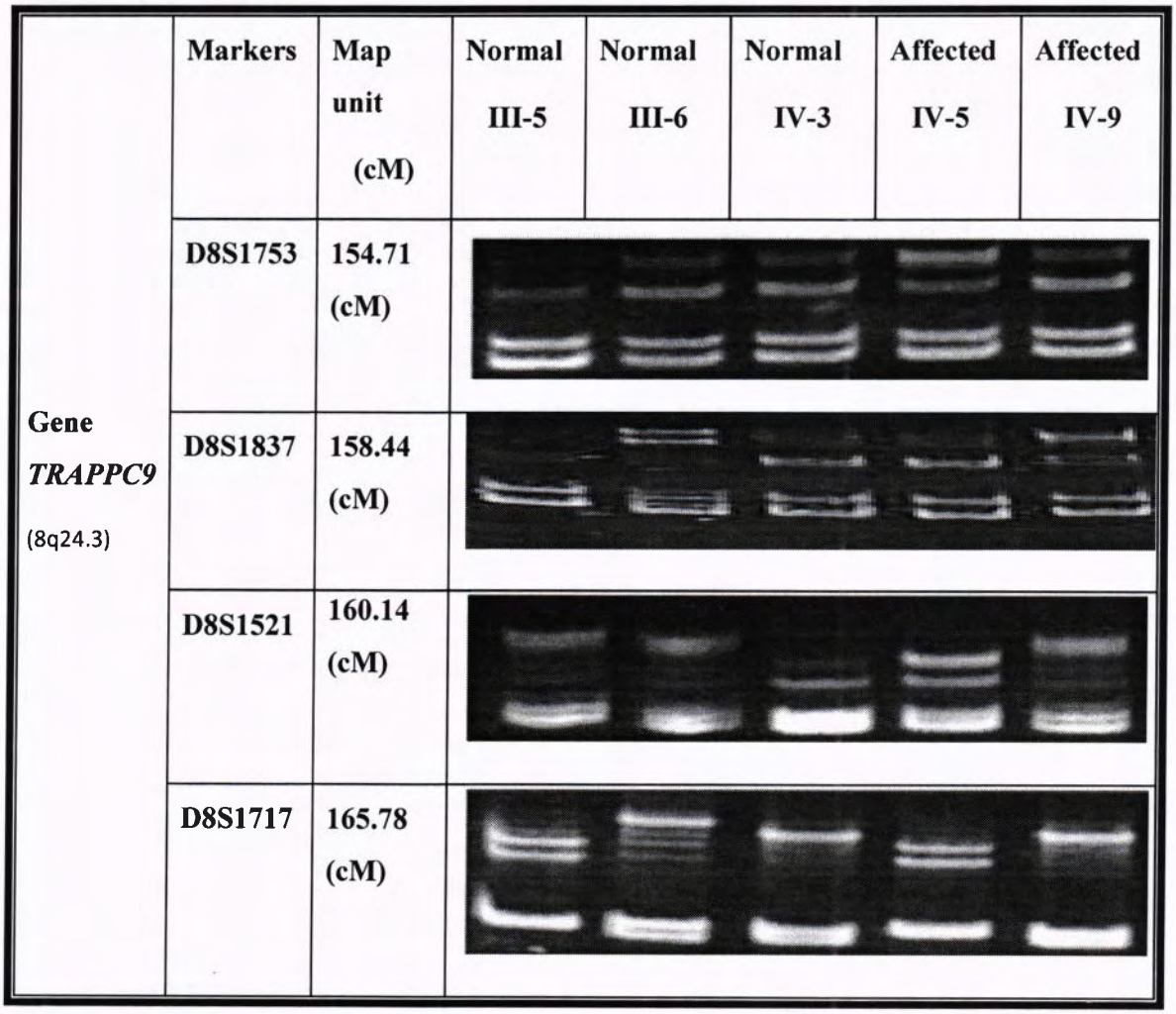


Figure 4.16 Gel images showing the band pattern of chromosome 8q24.3 markers in five member family B, these markers are used to test the *TRAPPC9* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

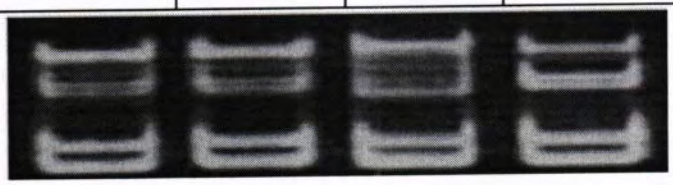
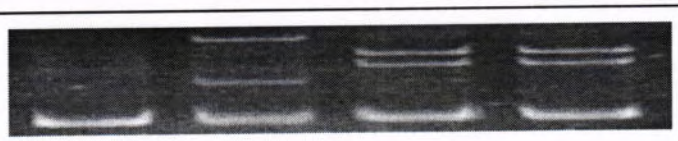
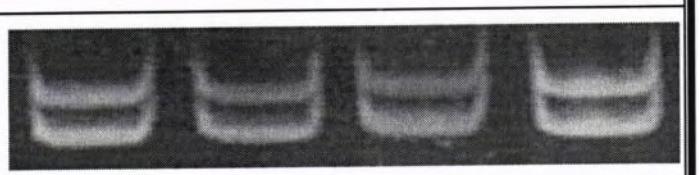
	Markers	Map unit (cM)	Normal II-1	Affected III-1	Affected III-2	Normal III-4
Gene *MAN1B1* (9q34.4)	**D9S1826**	**160.5 (cM)**				
	D9S158	**163.08 (cM)**				
	D9S905	**165.24 (cM)**				
	D9S1838	**165.25 (cM)**				
	D9S2168	**167.16 (cM)**				

Figure 4.17 Gel images showing the band pattern of chromosome 9q34.4 markers in four member family C, these markers are used to test the *MAN1B1* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

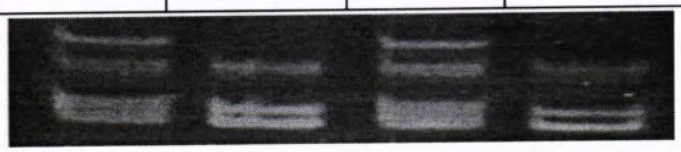
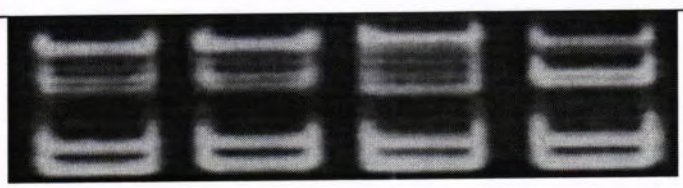
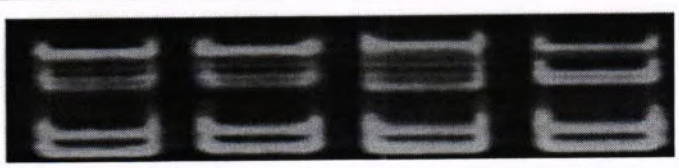
	Markers	Map unit (cM)	Normal II-1	Affected III-1	Affected III-2	Normal III-4
Gene *GRIK2* (6q16.3)	D6S1716	107.63 (cM)				
	D6S1717	108.48 (cM)				
	D6S1565	108.76 (cM)				
	D6S434	110.69 (cM)				
	D6S1709	110.69 (cM)				

Figure 4.18 Gel images showing the band pattern of chromosome 6q16.3 markers in four member family C, these markers are used to test the *GRIK2* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

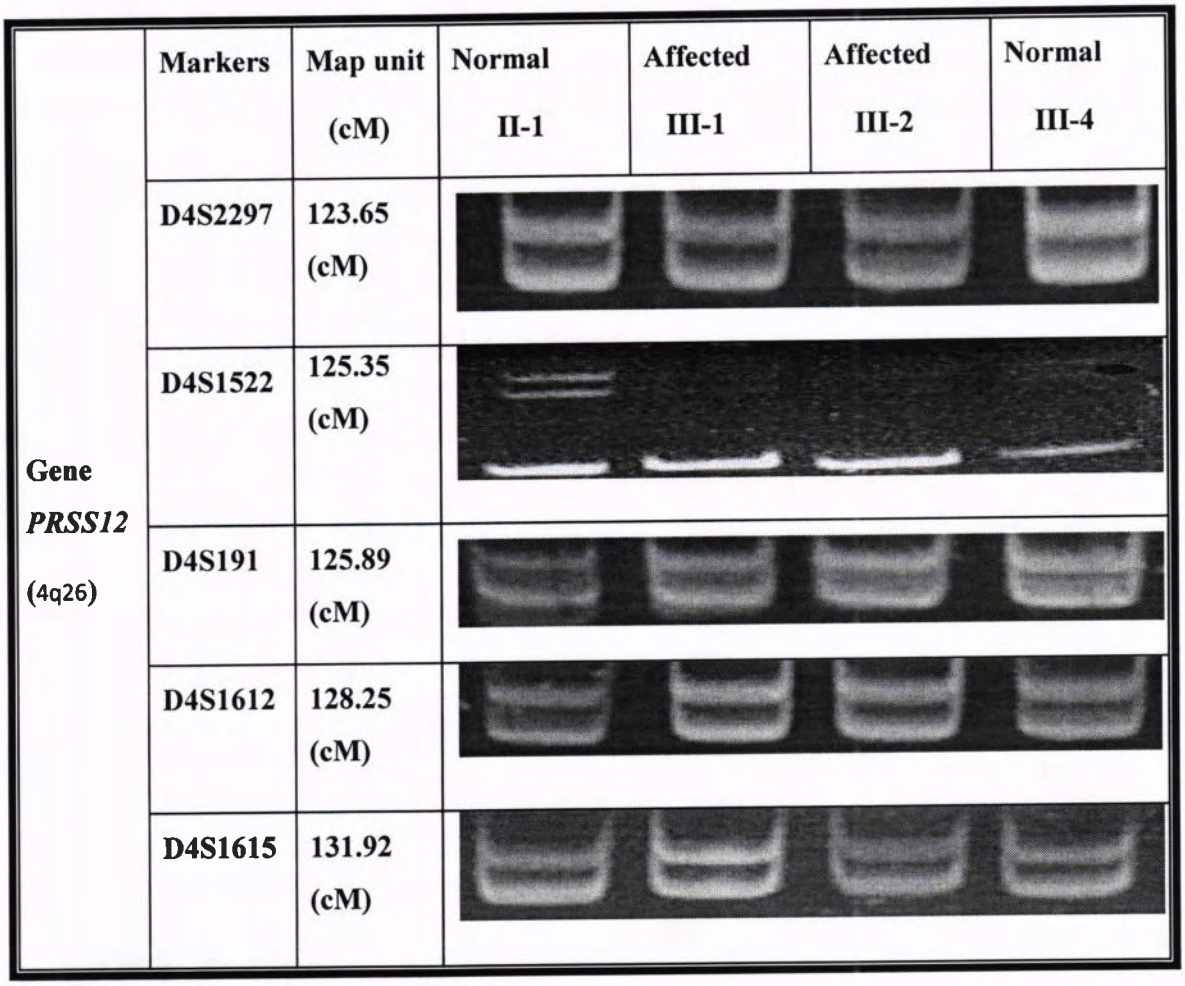


Figure 4.19 Gel images showing the band pattern of chromosome 4q26 markers in four member family C, these markers are used to test the *PRSS12* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

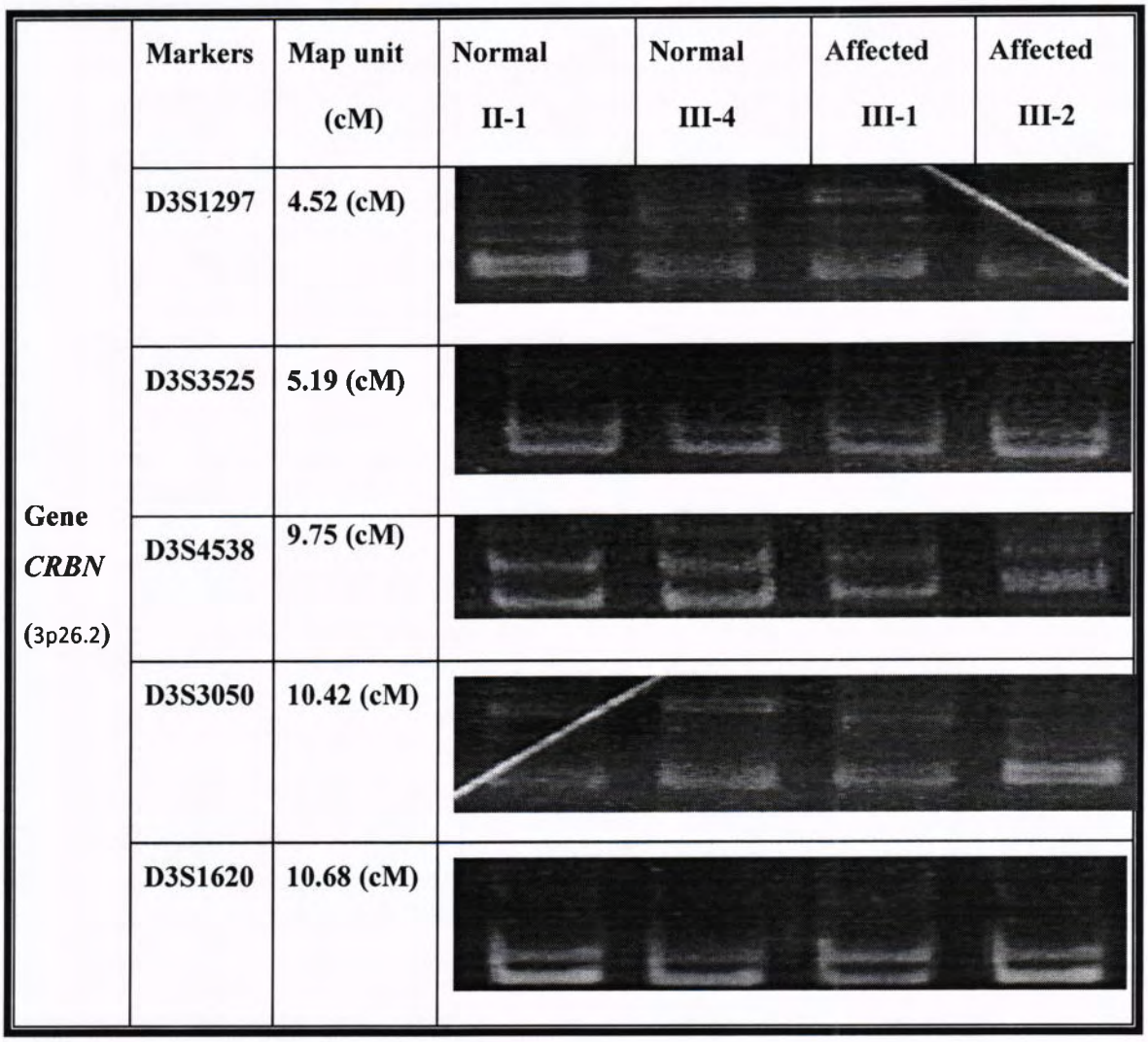


Figure 4.20 Gel images showing the band pattern of chromosome 3p26.2 markers in four member family C, these markers are used to test the *CRBN* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

	Markers	Map unit (cM)	Normal II-1	Normal III-4	Affected III-1	Affected III-2
Gene *TUSC3* (8p22)	D8S26	29.8 (cM)				
	D8S1827	30.15 (cM)				
	D8S1731	30.41 (cM)				
	D8S549	30.41 (cM)				
	D8S254	31.68 (cM)				

Figure 4.21 Gel images showing the band pattern of chromosome 8p22 markers in four member family C, these markers are used to test the *TUSC3* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

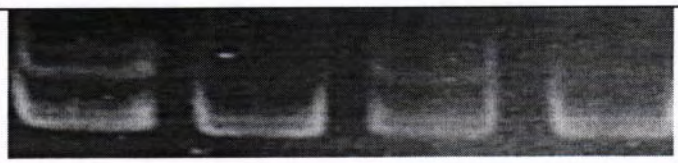
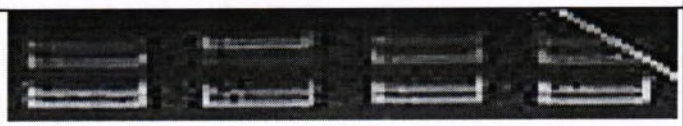
	Markers	Map unit (cM)	Normal II-1	Normal III-4	Affected III-1	Affected III-2
Gene *TRAPPC9* (8q24.3)	D8S1753	154.71 (cM)				
	D8S1837	158.44 (cM)				
	D8S1521	160.14 (cM)				
	D8S1741	164.27 (cM)				
	D8S1717	165.78 (cM)				

Figure 4.22 Gel images showing the band pattern of chromosome 8q24.3 markers in four member family C, these markers are used to test the *TRAPPC9* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

Family C

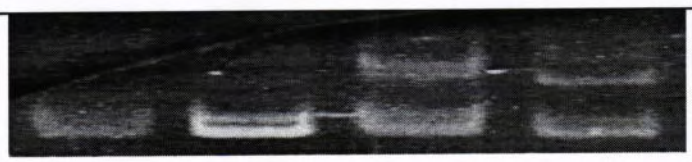
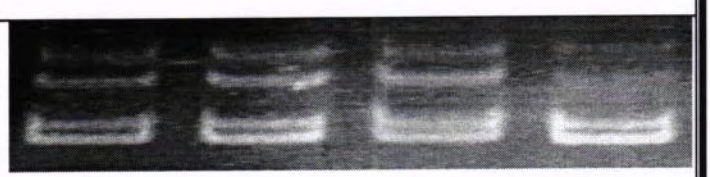
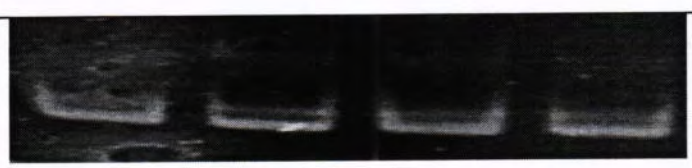
	Markers	Map unit (cM)	Normal II-1	Normal III-4	Affected III-1	Affected III-2
Gene *CC2D1A, TECR* (19p13.12)	D19S1165	32.65 (cM)				
	D19S840	34.46 (cM)				
	D19S385	36.98 (cM)				
	D19S252	38.36 (cM)				

Figure 4.23 Gel images showing the band pattern of chromosome 19p13.12 markers in four member family C, these markers are used to test the *CC2D1A* and *TECR* gene. Roman numerals indicate the number of generation while the number of the member is shown by Arabic numerals in the pedigree.

DISCUSSION

DISCUSSION

Mental retardation (MR) also known as intellectual disability (ID) is a common disorder with an onset of cognitive impairment before the age of 18 years (Riazuddin *et al.*, 2016). It is characterized by certain limitations in intellectual functioning especially social abilities, language and cognition with an IQ score of below 70. It can occur with or without any physical or other mental disorders. Mental retardation comprises of following general categories: mild, moderate, severe and profound (Daily *et al.*, 2000). MR can occur as a syndrome or as a broader disorder but is most commonly found as an isolated disorder. MR is a multifactorial disorder which is a caused by genetic as well as environmental factors (Ropers, 2008) and upto 60% of cases are without identifiable causes (Rauch et al., 2006). In almost 50% known cases main cause of MR are poor nutrition, prenatal or perinatal brain ischemia and postnatal ischemia. Other 50% cases have genetic reasons that include chromosomal abnormalities, monogenic and polygenic factors (Ropers, 2008). Most severe forms of mental retardation are caused by defects in specific genes or chromosomal abnormalities (Ropers, 2010). Mutations in a single gene may affect its function and may cause mental retardation or a variety of phenotypes linked with MR, depending on the function of the mutated gene. Polygenic mental retardation that is caused by more than a single gene is a common cause of mental retardation, especially in mildly affected patients (Winnepenninckx et al., 2003).

Mental retardation is particularly common in countries where parental consanguinity is common, including the involvement of autosomal recessive genetic abnormalities (Ropers *et al.*, 2011). Pakistan is among those countries in which consanguinity is very high (>60%) with 17-38% first cousin marriages (Hussain and Bittles, 1998). People of Azad Jammu and Kashmir prefer inbreeding, cousin marriages are common here (Darr and Modell, 1988). Several factors play important role in consanguineous marriages like social, religious and demographic factors (Bittles, 2001). In large consanguineous families chances of rare autosomal disorders are very high because the offspring of these consanguineous couples have more homozygous DNA than the children of an out bred marriage, so these consanguineous families are a powerful resource for genetic linkage studies (Jaber *et al.*, 1998).

Non-syndromic autosomal recessive mental retardation is very diverse disease and contributes much more than the X-linked mental retardation (Mhamdi *et al.*, 2011). Until Recent, fifty three

genes are clearly associated with non-syndromic autosomal recessive mental retardation. In the present research eight non-syndromic autosomal recessive genes were checked for linkage in the sampled families, PRSS12, CRBN, CC2D1A, TECR, TUSC3, GRIK2, MAN1B1 and TRAPPC9.

In the current research study three families A, B and C, suffering from mild to severe mental retardation was sampled from the Muzaffarabad and Poonch districts of Azad Jammu and Kashmir. The affected individuals were socially and academically disabled, they showed balancing and speech disabilitie. Clinical symptoms showed that the affected members were suffering from mild to severe mental retardation, with low IQ level, memory, learning, speech and balancing disabilities. They didn't show any facial abnormality, microcephaly or history of epileptic fits. They didn't show any nail or other dystrophy. Height, weight and head circumference were normal in all affected members. It showed that its non-syndromic Mental retardation.

The families A and B were excluded of six known gene/loci which are PRSS12, CC2D1A, TECR, GRIK2, MAN1B1 and TRAPPC9. While family C is excluded of the 8 known gene/loci which are PRSS12, CRBN, CC2D1A, TECR, TUSC3, GRIK2, MAN1B1 and TRAPPC9. For each gene 5-8 microsatellite markers were used and among them 4-6 markers were cleared on the basis of their results these genes were excluded. This reveals the possibility that there may be other gene/loci responsible for disease, so all the three families had considered for genome wide scan. Over the past few years use of molecular genetics technique led to improved knowledge of human heritable mental retardation disorder and disease causing mutations have been identified in several genes some mutations are mentioned here. Protease serine 12 (PRSS12) is involved in NS-ARMR, located on chromosome 4q26 and contains 13 exons (Molinari *et al.*, 2002). In an Algerian consanguineous family having 3 affected females and 1 male a 4-bp deletion was reported in exon 7 of the gene resulting in premature termination codon resulting in moderate to severe mental retardation (Molinari *et al.*, 2002). Coiled-coil and C2 domains protein 1A (CC2D1A) is involved in NS-ARMR and located on chromosome 19p13.12 and contains 31 exons. In nine consanguineous families with severe mental retardation a mutation in CC2D1A gene was identified as a result of this mutation a large genomic deletion of 3,589 nucleotides occurred and created a truncated protein and presented a loss of function effect

(Basel-Vanagaite *et al.,* 2006). Glutamate receptor ionotropic kainite 2 (GRIK2) is another gene of NS-ARMR, located on chromosome 6q16.3 and contains 16 exons. In a large Iranian family a nonsense mutation in GRIK2 gene was first reported. In this family mutation was reported by removal of exon 7 and 8 which results in deletion of 84 amino acids between amino acid 317 and 402 (Motazacker *et al.,* 2007). Tumor suppressor candidate 3 (TUSC3) is located on chromosome 8p22 and contains 11 exons and is involved in NS-ARMR (Garshasbi *et al.*, 2008). A 170.673 Kb deletion was reported in six member Pakistani family (Khan *et al.,* 2011). Trafficking protein particle complex, subunit 9 (TRAPPC9) is the involved in NS-ARMR located on chromosome 8q24.3 and contains 23 exons. In Pakistani family with TRAPPC9, Mir *et al,.* (2009) identified the R475X mutation in affected members. Mannosidase alpha class 1B member 1 (MAN1B1) is located on chromosome 9q43.3. In Pakistani consanguineous family having 3 affected members, a homozygous 1189G-A transition mutation in MAN1B1 gene is identified, results showed that mutant protein was expressed 5% less than the wildtype levels (Rafiq *et al.,* 2011).

The detection of genes that cause non-syndromic Mental retardation is necessary for understanding of human intelligence and cognition. The genes that cause intellectual impairment are expected to be associated with the process of learning and memory. In the excluded families, next generation sequencing (NGS) technology may be useful for identifying genes in short time with less labor than conventional sequencing. Therefore, this technology can be used to find out mutated gene. This study will be beneficial for potential genetic counseling of families to reduce/wipe out the disorder from the population of Azad Jammu and Kashmir.

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