Association of a Chromosomal Susceptibility Locus to Bipolar Affective Disorder (BPAD): A case-control study in Kashmir

Dissertation Submitted for the Award of the Degree of Masters of Philosophy in Biochemistry

By

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CERTIFICATE

Certified that the work embodied in the dissertation entitled ***"Association of a Chromosomal Susceptibility Locus to Bipolar Affective Disorder (BPAD): A case-control study in Kashmir"*** is the bonafide work of Ms. Mutahar Andrabi and has been carried out under our guidance and supervision in the Department of Biochemistry, University of Kashmir and Department of Psychiatry, SMHS, Srinagar. The work is suitable for the award of M. Phil degree in Biochemistry.

It is further certified that no work under this heading has previously been submitted to the University of Kashmir for the award of any degree or diploma, to the best of our belief.

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DECLARATION

I, Mutahar Andrabi, declare that the work embodied in this dissertation entitled **"Association of a Chromosomal Susceptibility Locus to Bipolar Affective Disorder (BPAD): A case-control study in Kashmir"** has been carried out by me in the Department of Biochemistry, University of Kashmir and the Department of Psychiatry, SMHS, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

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ABBREVIATIONS

bp	Base pair
BPB	Bromophenol blue
BPAD	Bipolar Affective Disorder
BD	Bipolar Disorder
CSF	Cerebrospinal fluid
DA	Dopamine
^{o}C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide –triphosphate
dUMP	Deoxy thymidine monophosphate
DSM	Diagnostic and Statistical Manual of Mental Disorders
DDW	Double distilled water
EDTA	Ethylene diamine tetra acetic acid
EMF	Electro magentic field
ESTs	Expressed Sequence Tags
EtBr	Ethidium bromide
Fig.	Figure
g	Grams
5-HIAA	5-Hydroxy indole acetic acid
Hr	Hours
HGP	Human Genome Project
HVA	Homo vanillic acid
Kb	Kilo base pair
Kd	Kilo Dalton
LD	Linkage Disequilibrium
$KHCO_3$	Potassium bicarbonate

$MgCl_2$	Magnesium chloride
MHPG	3-methoxy-4-hydroxyphenylglycol
M	Molar
m	Meter
MMR	Mismatch repair
µg	Microgram
µM	Micromolar
µl	Microlitre
mg	Miligram
min	Minutes
ml	Mililitre
mM	Mili molar
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NE	Norepipherine/ norepipherenic
ng	Nanogram
NH_4Cl_2	Ammonium chloride
O.D	Optical density
O.R	Odds ratio
nsSNPs	Non-synonymous SNPs
ppm	Parts per million
PCR	Polymerase Chain Reaction
PKC	Protein kinase C
pmol	Picomole
rpm	Revolutions per minute
RFLP	Restriction Fragment Length Polymorphism
RT	Room temperature

RUP	Recurrent Major (Unipolar) Depression
SAM	Schizoaffective–Manic Disorder
SDS	Sodium dodecyl sulphate
Sec	Second
SMHS	Sri Maharaja Hari Singh Hospital
SNP	Single Nucleotide Polymorphism
SPECT	Single Photon Emission Computed Tomography
STE	Sodiumchloride-tris-ethylenediamine-tetraacetic acid
SSR	Simple Sequence Repeat
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus* DNA polymerase
TdT	Terminal deoxynucleotidyltransferase
Tm	Melting temperature
Tris	Tris (hydroxyl methyl amino methane)
UTR	Untranslated region
UV	Ultra violet
VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization
YLD	Years lost due to disability

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ABSTRACT

The etiology of bipolar disorder (BPAD) is still unknown, but family, twin, and adoption studies strongly suggest the involvement of genetic risk factors. Linkage studies have revealed a number of loci to be linked with BPAD. Of those, several investigators confirmed 18p11 as one of the susceptibility loci for BPAD. Thus, chromosome 18p11 is one of the targets of the genetic association study of BPAD. The aim of our study was to investigate whether the tandem repeat polymorphism in D18S452 microsatellite marker at locus 18p11.2 is a risk factor for the development of BPAD in Kashmiri population. The repeat polymorphism in D18S452 was evaluated in 74 patients with BPAD and 74 control (age, sex and region matched) individuals. The repeat polymorphism was evaluated by PCR analysis of DNA obtained from the blood of the subjects. We observed that the tandem repeat (300bp*) allele frequency was found to be 1.35 % in the controls and 8.108% in cases. The tandem repeat (250bp*) allele frequency was found to be in 91.89% in cases and 98.65% in controls. The 252bp/252bp genotype was found to be present in 89.18% of the cases and 98.64% of the controls, the 300bp/300bp genotype in 5.40% of cases and 1.35% of controls and the 252bp/300bp variant in 5.40% of the cases and none among the controls.

It was observed that although the proportion of patients homozygous for the tandem repeat (300bp/300bp) was higher in cases than in controls, the difference was not statistically significant when using 252bp/252bp genotype as a reference (OR= 4.4242; 95% CI, 0.4822-40.5924); P = 0.1529). However, it was observed that the frequency of the heterozygous genotype (252bp/300bp) when compared with 252bp/252bp showed statistical significance (OR=8.0603; 95% CI, 1.1112-58.4646; P = 0.0383).

Chromosome 18 harbors many candidate genes that are involved in the pathophysiology of BPAD. It may be possible that this marker is directly or indirectly involved in the regulation of neighboring genes. It is also possible that this locus may be in linkage disequilibrium with other genes. Although, this is the first study reporting the association of the marker D18S452 in heterozygous condition (252bp/300bp) with BPAD. Yet, it would be too early to associate this genotype with the predisposition to BPAD. Therefore, further studies with larger sample size should be carried to validate the result, taking into account the various disease phenotypes, endophenotypes and environmental conditions.

CHAPTER: 1

INTRODUCTION

A mental disorder or mental illness is a psychological or behavioral pattern generally associated with subjective distress or disability that occurs in an individual, and which is not a part of normal development or culture e.g., people at different times experience fluctuations in mood in response to events and situations that occur. However, this is different in the medical condition of bipolar disorder where the individual does not respond to common life situations (mild or severe) in a normal way, as there are severe swings in mood which have an impact very significant on life roles; disrupts the ability to make decisions and adversely affects social interactions.

Bipolar (BPI) disorder is a severe psychiatric illness that affects approximately 0.5 to 1% of the population (Goodwin *et al.,* 1990). The features of the illness include mania, a condition with expansive grandiose (or irritable) moods with elevated energy and pressured speech. (Goodwin *et al.,* 1990) The depressive phase includes a depressed mood, decreased energy, and anhedonia. A milder form of the illness, bipolar II (BPII) disorder, is characterized by hypomania (symptoms of mania but less severe) and depression.

Symptoms of a manic episode may include:

- Elevated mood
- Feeling invincible
- Increased energy
- Unable or unwilling to sleep
- Overfriendliness
- Disorganised and racing thoughts
- Flight of ideas
- Creative and fanciful ideas
- Grandiose beliefs
- Distractability
- Use of illicit substances
- Excessive gambling, spending or alcohol use
- Social or sexual disinhibition
- Excessive, loud and pressured speech
- Impulsive actions
- Physical agitation

Symptoms of a depressive episode may include:

- Depressed and sad
- Cranky or irritable
- Worthless or an extreme sense of guilt
- Reduced confidence and self esteem
- Diminished enjoyment in pleasurable activities
- Physically unwell
- Physically exhausted
- Negative or pessimistic thoughts
- Persecutory thoughts
- Indecisiveness
- Difficulty concentrating
- Difficulty thinking through and sorting out problems
- Thoughts of self harm, suicide or death.
- Sleep disturbances (unable to sleep or excessive sleep)
- Changes in appetite (over or under eating)
- Fatigue and physical slowing
- Difficulty tackling day to day activities and work
- Reduced contact with friends and community

The condition affects 1% of the adult population worldwide at some point during life, and is approximately equally frequent in males and females (Weissman *et al.*, 1996) and is the sixth highest cause of disability worldwide (Murray *et al.*, 1996). An estimated 20%-25% of patients commit suicide, giving BPAD a mortality rate higher than that of many types of heart disease and cancer (Goodwin *et al.*, 1990). The pathophysiology of bipolar disorder is poorly understood, but the illness has long been an attractive candidate for molecular genetic analysis, with an elevated familial incidence being noted in some of the earliest descriptions of the disorder. Family, twin and adoption studies have indicated that genetic factors contribute significantly to the disorder (Kelsoe, 1997 and MacKinnon *et al.*, 1997). Although, a single gene may occasionally have a major effect in some families, multiple genes and environmental factors are generally considered to play roles in the development of bipolar disorder (Craddock *et al.*, 1999). Family studies indicate that there is a seven-fold increase in the risk to first-degree relatives while twin studies show that there is an average four-fold increase in risk for monozygotic *vs* dizygotic twins

(Kelsoe, 1997 and MacKinnon *et al.*, 1997).The disorder is oligogenic and heterogeneous, though the mode of inheritance remains unclear, it is likely that the pattern of inheritance is consistent with the epistatic action of several genes with modest individual effect sizes (Bennett *et al.,* 2002).

Identification of genes underlying mental disorders is complicated by numerous factors typical of most common traits: unknown number of loci and pattern of inheritance, difficulties in measuring any environmental influence and perhaps most importantly for psychiatric disorders, the lack of biological diagnostic tests; there are several explanations for this discrepancy. First and foremost, observing pathological changes within the brain remains markedly more difficult than for other organs. Secondly, the limited understanding of its etiology is reflected as a list of risk factors, such as stressful life events, endocrine abnormalities (hypothyroidism and hypercortisolism), cancers (pancreatic adenocarcinoma), side effects of drugs (isotretinoin for acne and interferon-ά for hepatitis C), sheer heterogeneity of its phenotypes (Krishnan *et al.,* 2008) and endophenotypes (Lenox *et al.,* 2002) representing as a state rather than a trait marker among many others. Although psychiatric and behavioral traits represent, perhaps the greatest challenge to molecular investigation of complex genetic disorders, they also suffer arguably the greatest potential reward.

Genetic linkage studies in bipolar disorder suggests that a susceptibility locus exists on chromosome 18p11 (Lohoff *et al.,* 2009). Further, support for this region comes from many whole genome wide linkage analyses (Bennett *et al.,* 2002) as well as association studies suggesting potential locus on 18p11.2 (Shinsuke *et al.,* 2004). Chromosome 18 remains very interesting, and could be one of the first chromosomes to yield a susceptibility gene for a major psychiatric disorder.

Microsatellites (also called short tandem repeat polymorphisms) are tandem arrays of short stretches of non-coding nucleotide sequences that are usually repeated between 15 and 30 times. The obvious advantages of microsatellites are high heterozygosity, ubiquity throughout the genome, and PCR typability. Association analysis using microsatellite markers is a powerful yet cost-efficient method for mapping candidate susceptibility genes in multifactorial genetic diseases. If the frequencies of microsatellite markers differ significantly between patients and

controls, there may be susceptibility or resistance genes near them, which can be further analyzed by sequencing.

The search for the genes involved has been both encouraging and discouraging with replication studies failing to validate earlier promising results. How many mutations are needed? Are there some mutations more potent than others? How much of an influence does the environment have? Does the amount of environmental impact required vary depending on the genes affected? Is there a simple "on or off" threshold or does the severity of the illness increase as the number of mutations increase? Questions like these need to be answered in order to aid the research but can't be answered without the results of the research.

Finding susceptibility loci / genes for bipolar affective disorder becomes important because of its high prevalence and destructiveness and also absence of better ways to explore pathogenesis of the disorder. The payoff will be substantial as finding even one gene could lead to improvements in the management of BPAD at several levels. Little is known about pathophysiology of BPAD, finding a gene involved in this disorder will lead to an examination of the functions of its protein product in the neurons. This could lead to the elucidation of a cascade of neuronal events at work in the disorder. Understanding of these basic processes may guide the search for treatments of the disorder, and could illuminate the mechanisms and functions of mood regulation in general.

Hence, we are interested in understanding the potential relationship between suspected polymorphisms and their association with Bipolar Affective Disorder in Kashmir. The study is first of its kind in the valley and aims to aid in the understanding of the genetics of BPAD.

CHAPTER: 2

REVIEW OF LITERATURE

In 1860's, Gregor Mendel carried out a systemic experimental analysis of plant hybridization and inheritance patterns. The crucial feature of his work was the realization that the gene is a distinct entity. Mendel studied the effect of single genes, in terms of dominant and recessive hereditary traits. His discoveries were ignored for three decades. At the beginning of the twentieth century however, "Mendel's laws" were rediscovered by Hugo Marie de Vries, Karl Correns and Erich von Tschermak-Seysenegg.

The era of molecular biology took a new turn in 1944 with the publication of a paper by Oswald Avery and his colleagues in which they proved that DNA not protein, as many believed at that time, is the agent of heredity. Shortly after, Erwin Schrodinger developed the view that the properties of the genetic material are stable during countless generations of inheritance.

The discovery of the double helix structure of DNA (Deoxyribonucleic acid) molecule by Watson and Crick in 1953 (Watson *et al.,* 1953) had a major impact on the field of molecular genetics, followed by discovery of genetic code (Caskey *et al.*, 1968). It was in 1977 that the desire to determine the exact nucleotide order in every gene became possible when two methods were independently reported by Sanger and by Maxam and Gilbert (Maxam *et al*., 1977 and Sanger *et al*., 1977). Knowledge brought about by these discoveries has led to identification of thousands of genes (Peltonen *et al*., 2002).

Today, we know that the DNA sequence is composed of over 3 billion chemical building blocks called nucleotides. Each nucleotide consists of three structures: a nitrogenous base, a sugar and a phosphate group. There are four types of bases: adenine (A), guanine (G), cytosine (C) and thymine (T) which can be subdivided into two classes, purines (A and G) and pyrimidines (C and T).

The DNA molecule contains "recipes" with instructions needed to compose other cell components such as proteins. These recipes are called *genes* and they contain coding regions called *exons.* The exons are composed of tri-nucleotide sequences called *codons,* and each codon codes for one of the 20 amino acids required to form a protein. Interestingly, the exons only cover about 1.5% of the human genome. The rest is comprised of RNA-genes, non-protein coding sections (*introns*), non-coding repetitive sequences that are involved in the regulation of gene expression and

sequences with no known function. To date, the number of identified protein coding genes in our genome is approximately 20,000-25,000 (http://www.ebi.ac.uk/genomes).

On average, two unrelated individuals share about 99.9% of their DNA sequence (Levy *et al.,* 2007) thereby resulting in 0.1% of inter-individual variation in the genome. DNA sequence variation can occur in several forms and each possibility for a specific variant is called an allele.

The majority of DNA variations are harmless; but in some rare cases they alter the code in a gene in a way that it affects the proper production of its specific product which in turn may result in a disease. However, it is important to mention that some of the variations that occur during gamete formation (meiosis) are actually beneficial and favored by *natural selection* contributing to human evolution.

DNA sequence variants are used in all forms of genetic investigation including linkage and association studies for mapping genes predisposing to disease.

2.1 DNA SEQUENCE VARIATION

One of the biggest achievements in modern genetics since DNA was first discovered over fifty years ago, has been the sequencing of the human genome, the so-called 'Human Genome Project' (HGP) (Venter *et al.,* 2001). The completion of this immense undertaking carried out by an international group of top scientists was eagerly awaited by the scientific and medical communities and indeed the world as it had been heralded as having the capacity to unlock the secrets of our genomes, and importantly not least for the funding bodies was the hope that it would be the key to rapid advances in medical genetics, the elucidation of the genes underlying many of the most common ailments of our species.

Genetic variations can determine disease susceptibility and provide the tools to understand basic biological processes. DNA fragments of variable sequence at specific positions in the genome, which may also vary between individuals, are called genetic markers. 'Polymorphism' (from Greek: *poly* "many", *morph* "form") in the context of Human Genetics refers to variation in the DNA sequence among individuals that may or may not affect its phenotype. Among polymorphisms, those have a population frequency of 1% or more are considered to be common

polymorphisms. There are several different types of genetic markers, the most common being: short or long insertion/deletion variants, transposable elements, microsatellites, variable number of tandem repeats (VNTR), restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP).

Transposable elements are also called "jumping genes" or "mobile genetic elements" since they are sequences of DNA that can move around to different positions within the genome of a cell. There are a variety of mobile genetic elements, and they can be grouped based on their mechanism of transposition. Class I mobile genetic elements, or retrotransposons, move in the genome by being transcribed to RNA and then back to DNA by reverse transcriptase. Class II mobile genetic elements move instead directly from one position to another within the genome using transposase to "cut and paste" them within the genome. Transposons are very useful to researchers as a means to alter DNA in a living organism.

***Single Nucleotide Polymorhisms (*SNPs)** are single base pair changes from one of the four bases making up human DNA to another, for example a G to an A. SNPs arise due to mutation, normally due to a misincorporation of a nucleotide during replication, or by chemical or physical mutagenesis. They are biallelic that is two alleles or forms of the SNP exist in human populations with a certain percentage in a population carrying one of the alleles and the rest carrying the other. The allele found at highest frequency is referred to as the 'major allele' and the lower frequency allele is referred to as the 'minor allele'. SNP may be found in non-genic regions of the genome in addition to being found in genes. SNPs that are located in coding regions of a gene are called coding SNPs (cSNPs). It is estimated that the average human gene contains 126 SNPs, 5 of which are found in coding regions (Crawford *et al.,* 2005). Non-synonymous coding SNPs comprise a group of SNPs that, together with SNPs in regulatory regions, are believed to have the highest impact on phenotype. Non-synonymous SNPs (nsSNPs) have an effect on protein structure and/or function by causing an amino acid substitution. SNP markers have become one of the tools of choice for many different types of genetic studies. Their popularity has much to do with their abundance and density in the human genome.

Variable Number of Tandem Repeats* (VNTR) *or minisatellites are molecular marker loci consisting of tandem repeat units of 10-50 base motif, flanked by conserved endonuclease restriction sites. VNTR units are considered to be the main

cause of length polymorphisms. Due to high mutation rate of minisatellites, the level of polymorphism is substantial, generally resulting in unique multilocus profiles. Therefore, minisatellites are particularly useful in studies involving genetic identity, parentage, clonal growth and structure (Jeffreys *et al.*, 1985).

Microsatellites or simple sequence repeats (SSRs) are molecular marker loci consisting of very short tandem repeat units of for example di-, tri-, tetra-, or pentanucleotide repeated several to hundred times along the DNA. Due to their high level of polymorphism, microsatellites are informative markers that can be used for several population genetic purposes, ranging from the individual level to closely related species.

Numerous lines of evidence have demonstrated that genomic distribution of simple sequence repeats (SSRs) is *nonrandom*, presumably because of their effects on chromatin organization, regulation of gene activity, recombination, DNA replication, cell cycle, mismatch repair (MMR) system, etc (Li *et al.*, 2002). SSRs may provide an evolutionary advantage of fast adaptation to new environments as evolutionary tuning knobs (Kashi *et al.*, 1997 and Trifonov 2003). These SSRs with putative functions may be located in gene or regulatory regions. However, the reviews published to date have not clearly discussed SSR polymorphism and evolution *in gene*, because available information about SSR locations on chromosomes has been limited. Recently, however, many reports have demonstrated that a large number of SSRs are located in transcribed regions of genomes, including protein-coding genes and expressed sequence tags (ESTs) (Morgante *et al.,* 2002), although in general, repeat numbers and total lengths of SSRs in these regions are relatively small (Kantety *et al.*, 2002 and Thiel *et al.*, 2003). In protein-coding regions of all known proteins, 14% proved to contain repeated sequences, with a three times higher abundance of repeats in eukaryotes as in prokaryotes (Marcotte *et al.*, 1999). Noteworthy, prokaryotic and eukaryotic repeat families are clustered to non homologous proteins. This may indicate that repeated sequences emerged after these two kingdoms had split (Marcotte *et al.*, 1999).

SSR occurrence in coding regions seems to be limited by non-perturbation of the reading frame. This has been proved by the following facts: (1) in a human cDNA database, more than 92% of the predicted SSR polymorphisms within coding sequences have repeat-unit sizes that are a multiple of three (Wren *et al.*, 2000); (2)

in many species, exons (unlike other genomic regions) contain rare dinucleotide and tetranucleotide SSRs, but have many more triplet and hexanucleotide SSRs than other repeats (Field *et al.*,1996; Edwards *et al.*, 1998; Metzgars *et al.*, 2000; Wren *et al.*, 2000; Young *et al.*, 2000; Cordeiro *et al.*, 2001 and Morgante *et al.*, 2002). Triplet repeats show approximately twofold greater frequency in exonic regions than in intronic and intergenic regions in all human chromosomes except the Y chromosome (Subramanian *et al.*, 2003). Such dominance of triplets over other repeats in coding regions may be explained on the basis of the suppression of non-trimeric SSRs in coding regions, possibly caused by frame shift mutations (Metzgar *et al.*, 2000). Simple sequence repeat variation within genes should be very critical for normal gene activity because encoding SSR expansion or contraction directly affects the corresponding gene products and even causes phenotypic changes. In eukaryotes, SSR effects in coding regions on phenotypes have been extensively studied only in human diseases, revealing abundant evidence on human neuronal disorders and cancers.

In transcribed regions, according to available large-scale observation in humans databases, UTRs harbor more SSRs than the coding regions (Wren *et al.*, 2000 and Morgante *et al.*, 2002). The 5′-UTRs and 3′-UTRs contain more monomer and dimer motifs than those in coding regions. For dimeric repeats in UTRs, 3′-UTRs show a bias to AC/G Wren *et al.*, 2000). The 3′-UTRs also display an obvious bias to $(A/T)_7$ (27.0%) compared to $(G/C)_7$ (0.7%) (Olivero *et al.*,2003). The 5′-UTRs contains more triplets than the 3′-UTRs (31.1% vs. 4.6%; Wren *et al.*, 2000). The 5′-UTRs also exhibit a strong bias toward specific triplet repeats in different mammalian genomes (Stallings 1994). For instance, of 136 triplet repeats identified in 5′-UTRs of human cDNA, 100 were CGG or CCG (Wren *et al.*, 2000), serving as binding sites for nuclear proteins (Richards *et al.*, 1993 and Stallings, 1994).

Substantial data indicates that SSR expansions and/or contractions in protein-coding regions can lead to a gain or loss of gene function via frameshift mutation or expanded toxic mRNA. SSR variations in 5′-UTRs could regulate gene expression by affecting transcription and translation. The SSR expansions in the 3′-UTRs cause transcription slippage and produce expanded mRNA, which can be accumulated as nuclear foci, and which can disrupt splicing and, possibly, disrupt other cellular function. Intronic SSRs can affect gene transcription, mRNA splicing, or export to

cytoplasm. Triplet SSRs located in the UTRs or intron can also induce heterochromatin-mediated–like gene silencing. All these effects caused by SSR expansions or contractions within genes can eventually lead to phenotypic changes. SSRs within genes evolve through mutational processes similar to those for SSRs located in other genomic regions including replication slippage, point mutation, and recombination. These mutational processes generate DNA changes that should be corrected by DNA mismatch repair (MMR) system. Mutation that has escaped from the MMR system correction would become new alleles at the SSR loci, and then regulate and/or change gene products, and eventually lead to phenotype changes.

2.1.1 Forces shaping Genetic Variation

Genetic variation initially arises by genomic based mechanisms such as mutation, recombination and chromosomal rearrangements. The array of extant human genetic variation that can be observed today has been shaped by forces from our evolutionary past including demographic factors and selection.

Demographic forces: Human genetic variation in the form of Allele Frequencies, Haplotype Diversity and Linkage Disequilibrium has been shaped by a number of population based forces over the course of a population's history. These forces include genetic drift, which is the tendency of allele frequencies to fluctuate randomly over time due to statistical variation (Wright 1931, 1938). Eventually, after a certain number of generations the drift will carry an allele to fixation or eliminate it.

Another demographic factor affecting genetic diversity and genetic differences between populations is gene flow. Gene flow between human populations occurs due to migration of individuals into (immigration) or out of (emigration) a population. Immigration logically increases genetic diversity and emigration results in a loss of diversity.

Natural Selection: One of the basic premises upon which modern day population genetic thought is based is the so called 'Neutral theory of molecular evolution' (Kimura, 1968). Positive selection is the tendency of beneficial traits to become more frequent in a population over time (Darwin, 1859) and is likely to have aided in our ability to adapt to new and diverse environments. Negative selection (also known as purifying selection or stabilising selection) is conversely the selective

removal of rare deleterious alleles from a population and balancing selection refers to when the advantage is in having a number of polymorphisms at relatively high frequency in a population such as in the case of heterozygote advantage (Aidoo *et al.,* 2002). Each of these types of selective forces will leave their characteristic signals on the shape of genetic diversity around the gene or allele under selection. Some of the most easily distinguished traces left by the forces of selection are those left by selective sweeps (Nurminsky, 2001). Selective sweeps occur when an allele becomes more frequent in a population as a result of positive selection. As the positively selected allele increases in frequency so too will linked nearby alleles, a phenomenon known as genetic hitchhiking. A strong selective sweep will result in a region of the genome where the positively selected haplotype (of the selected variant and linked neighbouring alleles) is at high frequency, thus leading to a reduced haplotype diversity in the region. Thus the occurrence of a selective sweep can be investigated by measuring LD or by observing if a haplotype is overrepresented in a population. As a selective sweep carries an allele on a specific haplotype to high frequency faster than the rate at which it is broken down by recombination, then high frequency haplotypes will be observed longer than expected under neutrality (Sabeti *et al.,* 2002).

2.2 Mendelian and Complex Disorders

The human body has an extra ordinarily complex biology which requires subtle control of a number of biochemical and physiological mechanisms, regulated by genes. Aspects of such can be thought of as hierarchies, with genes at the lowest level and clinical endpoints that define disease at the highest (Sing *et al.,* 1996), which principally consists of two major categories of diseases; Mendelian and complex disorders.

Mendelian disorders or Monogenic disorders.: Monogenic disorders.are those showing the most straightforward mode of genetic transmission, and therefore there has been most success in the identification of genes underlying these type of diseases. A Monogenic trait is one which is controlled by a single locus and is passed on from parents to offspring in Mendelian fashion. They are normally fully penetrant, that is presence of the allele corresponds with presence of the trait, and no environmental factors are required for expression of the trait. Sickle-cell anaemia, Tay-Sachs disease, cystic fibrosis and xeroderma pigmentosa are all examples of

Monogenic diseases. Initial successes in discovering the genes underlying diseases with a heritable component were almost entirely centred on simple monogenic disorders following a Medelian pattern of inheritance. However these disorders are relatively rare, the vast majority of the common illnesses instead have a complex mode of inheritance.

Non Mendelian Disorders or Complex Disorders: Complex Disorders are caused by one or more genes in conjunction with environmental factors, with some estimates putting the genetic component of susceptibility at around 40%-70% (Goldstein *et al.,* 2005). Complications of the mode of inheritance such as gene-gene interactions (epistasis), gene-environment interactions, and non-additive gene effects abound. In complex disease there may be multiple genes of small effect. Complicating factors may also include genetic or allelic heterogeneity; where different genes or different alleles within the same gene give rise to increased susceptibility to the disease phenotype, this may be the case in particular, in different populations. There may also be distinct environmental factors interacting with the genetic component in different populations. Penetrance, that is, lack of a one to one transmission between the genotype and the phenotype (Merikangas *et al.,* 2003) may also be a factor, as may phenocopy (phenotype observed but not due to genotype).

However it is precisely with complex diseases that the greatest challenge lies, as complex diseases such as cancer, cardiovascular disease and psychiatric disorders represent some of the most common illnesses in humans of our times.

Most Psychiatric disorders are complex traits with both genetic and environmental factors influencing an individual's susceptibility to suffering disease. The heritability estimate for some of the commonly studied psychiatric disorders ranges from 0.28 for depression to 0.90 for autism (Merikangas *et al.,* 2003).

The recent advances in molecular genetics including the Human Genome Project promised to greatly increase our understanding of psychiatric disorders and human behavior. However, these expectations have yet to be fulfilled (Owen *et al.,* 2000). This failure is due to many reasons, the difficulty of identifying genes for complex disease in general and due to characteristics specific to psychiatric disorders such as the difficulty of phenotype definition.

2.3 BIPOLAR AFFECTIVE DISORDER

Bipolar affective disorder (BPAD, BAD or BD), bipolar disorder or 'manic depression' as it is also widely known [MIM 125480] is a major psychiatric disorder of multifactorial etiology (Goodwin *et al.,* 1990). It is characterized by episodes of mania and depression (Ekholm *et al.,* 2003). The original diagnosis of "manic depressive insanity," described by Emil Kraepelin in his 1899 edition of *Clinical Psychiatry*, has evolved through the years to the current classification system of four subtypes: Bipolar I Disorder, Bipolar II Disorder, Cyclothymic Disorder and Bipolar Disorder Not Otherwise Specified (Diagnostic and Statistical Manual of Mental Disorders, 2000). This evolution and elucidation of subtypes rests on the realization that not all cases of the illness are the same. These subtypes are not based on the underlying biology of the illness but rather on the consensus opinion of mental health professionals and have changed significantly over the years. These subtypes will continue to change until there is a biological basis for the diagnoses. The primary mood disturbance in Bipolar I Disorder is either mania or a mixed episode and it is usually accompanied by episodes of depression.

The primary mood disturbance in Bipolar II disorder is depression and it is accompanied by at least one episode of a mild form of mania called hypomania. An individual with Cyclothymic Disorder cycles between periods of hypomanic symptoms and periods of depressive symptoms. The hypomanic symptoms are never severe enough to be considered a manic episode and the depressive symptoms are never severe enough to be considered a depressive episode.

Bipolar Disorder Not Otherwise Specified captures all of the other variants of the disease that do not fit neatly into one of the above categories. A major depressive episode lasts at least two weeks. The major symptom is either a depressed mood or a loss of enjoyment in activities nearly every day. An individual also needs to have at least four other symptoms. Three of the possible symptoms involve either an increase or a decrease in normal functioning. They are a change in appetite, a change in sleeping patterns and a change in the speed of physical movements**.** Other possible symptoms include fatigue, feeling guilty or worthless, poor concentration and thoughts of death or suicide. A manic episode lasts at least one week and consists of an abnormally elevated mood with at least three other symptoms. Probably the most noticeable symptoms are a decreased need for sleep, being

unusually talkative and having an inflated self esteem. The other possible symptoms are racing thoughts, being easily distracted, an increase in activity and an excessive involvement in activities that are enjoyable but could result in serious consequences. A hypomanic episode lasts at least four days and, with the exception of the mood disturbance, has the same possible symptoms as a manic episode. Whereas in a manic episode the mood is abnormally elevated, in a hypomanic episode the mood is only persistently elevated. A mixed episode occurs when an individual has symptoms of both a major depressive episode and a manic episode nearly every day for at least a week.

Currently, a patient afflicted with bipolar disorder is diagnosed based on the displayed symptoms. While blood tests and physical exams can be used to rule out other illnesses with similar symptoms, there are no medical tests that can diagnose bipolar disorder. The diagnosis is based on an interview with the patient and, if possible, input from the patient's friends and family members. The focus of the interview is to review the list of possible symptoms with the diagnosis dependent on the answers. Although this subjective method of diagnosis is inherently flawed, unfortunately it is the best method available. An accurate diagnosis depends on the patient's recognition and recollection of symptoms along with the physician’s knowledge and experience. Communication issues, especially if the patient and the physician do not speak the same primary language, can lead to confusion about the symptoms. One of the biggest challenges is a direct result of the cyclic nature of the illness. If a patient with bipolar disorder has symptoms of depression and either hasn't experienced a manic or hypomanic episode, or doesn't recall having experienced one, an incorrect diagnosis of unipolar depression can be made. Treatment decisions are equally difficult with medication choices largely dependent on the physician's knowledge and experience. In many cases, the choice of medication is based on what works for other people. A small number of very fortunate patients will respond to the first medication tried and start to experience relief from symptoms within two months. For many of the patients, this trial and error method of medication selection will last much longer since most of the medications take up to two months to be effective. For some patients, the choice of medication can make the illness worse.

Some individuals experience a manic episode as a result of taking certain antidepressants. While this information might be widely known among researchers and psychiatrists, it is possible that primary care physicians are unaware of the danger. This risk to patients will continue to increase as more and more of them seek treatment from a primary care physician instead of a psychiatrist. A primary care physician is also unlikely to be able to provide the same level of follow-up care as a psychiatrist.

Family **(**Gershon *et al.,* 1982), twin (Bertelsen *et al.,* 1977) and adoption studies (Mendlewicz *et al.,* 1977) have established that there is a genetic contribution to bipolar disorder, but no mechanism of transmission has been established. It is widely believed that multiple genes contribute to the increased familial risk (Berrettini *et al.,* 2000, Risch *et al.,* 1996). Although the limits of phenotypic expression are not known, family studies suggest that BPI, BPII, recurrent major (unipolar) depression (RUP), and schizoaffective–manic disorder (SAM) are part of a bipolar spectrum (Gershon *et al.,* 1982). Since major depression is so widely prevalent, many RUP cases, even in families ascertained through BPI probands, may be genetically unrelated to bipolar disorder (Blacker *et al.,* 1993). Therefore, genetic linkage analyses have generally used at least two definitions of the affected phenotype, including and *excluding RUP* (Detera-Wadleigh, 1997).

Genome-wide linkage analyses of family samples with bipolar disorder provide convincing evidence that single locus forms of the disorder, if they exist, are uncommon (Friddle *et al.,* 2000 and Nurnberger *et al.,* 1997).

However, several susceptibility loci throughout the genome have been implicated in independent samples. These include 18p11, 18q21–23, 21q22, 4p16, 4q35, 12q24, 13q31–32, 22q11–13, and Xq24–28 (Berrettini *et al.,* 2000 and Craddock *et al.,* 1999). Recently, a genome-wide screen identified 8q24 and 10q25–26 as regions of suggestive linkage to bipolar disorder (Cichon *et al.,* 2001.)

Schizophrenia, another devastating mental illness, is also believed to have genetic and environmental causes. Currently classified as two separate disorders, schizophrenia and bipolar disorder have a common set of symptoms such as hallucinations, a change in sleeping patterns and diminished concentration. (Diagnostic and Statistical Manual of Mental Disorders, 2000) In fact, diagnosis of

one subtype of schizophrenia, schizoaffective disorder, requires that the individual have an episode of mania or depression. Linkage analysis studies of schizophrenia have identified chromosomal regions of susceptibility that have also been identified as regions of susceptibility for bipolar disorder. Furthermore, many of the linkage analysis studies of bipolar disorder include individuals who have been diagnosed as having schizophrenia. The common sets of symptoms, combined with the overlapping regions of susceptibility, have led some researchers to believe that the illnesses are part of one broader spectrum rather than two distinct disorders.

2.3.1 Epidemiology of Bipolar Disorder

Bipolar disorder is a chronic psychiatric illness characterized by recurrent episodes of mania, hypomania, mixed states, and depression. Various studies have estimated the prevalence of bipolar disorder to be between 1% and 2%. The estimated disability-adjusted life years of bipolar disorder outrank all cancers and primary neurologic disorders, such as epilepsy and Alzheimer's disease, primarily because of its early onset and chronicity across the lifespan (World Health Report, 2002). A reanalysis of data from the National Epidemiological Catchment Area survey in the United States, however, suggested that 0.8 percent experience a manic episode at least once (the diagnostic threshold for bipolar I) and 0.5 a hypomanic episode (the diagnostic threshold for bipolar II or cyclothymia). Including sub-threshold diagnostic criteria, such as one or two symptoms over a short time-period, an additional 5.1 percent of the population, adding up to a total of 6.4 percent, were classed as having a bipolar spectrum disorder (Judd *et al.,* 2003*).* A more recent analysis of data from a second US National Comorbidity Survey found that 1% met lifetime prevalence criteria for bipolar 1, 1.1% for bipolar II, and 2.4% for sub threshold symptoms (Merikangas *et al.,* 2007). Many estimates from community surveys may not include milder forms of bipolar disorder (e.g., bipolar II disorder, bipolar disorder not otherwise specified), which could result in underestimation of the true prevalence of the spectrum of the disorder (Dunner, 2003)

Men and women have similar rates of bipolar illness (Weissman *et al.,* 1996 and Hendrick *et al.,* 2000). Some evidence suggests that women are more likely to be hospitalized during manic episodes, and that rapid cycling occurs more often in women than in men (Leibenluft *et al.,* 1996 and Hendrick *et al.,* 2000). Women also

appear more likely to have predominantly depressive features, rather than manic features over the course of the illness (Lish *et al.,* 1994).

Bipolar disorder can occur for the first time at any age. However, the peak period of onset is between the ages of 15 and 19 **(**Bebbington *et al.,* 1995). A National Depressive and Manic-Depressive Association (NDMDA) survey found that 59% of patients with bipolar disorder experienced their first symptoms during childhood or adolescence (Lish *et al.,* 1994). However, bipolar disorder can also manifest for the first time in later years. One study showed that new onset of mania occurred in 9.3% of a group of patients older than 60 years of age with affective disorders (Yassa *et al.,* 1988). Another report noted that 6.1% of adults aged 60 and older with bipolar disorder appear to have illness of relatively recent onset **(**Sajatovic *et al.,* 2005).

Recurrence rates for bipolar disorder are high even with ongoing therapy. One study found a 73% relapse rate at 5 years, and two thirds of the patients had multiple relapses **(**Gitlin *et al.,*1995). Other estimates place the relapse rate at about 90%, with nearly half of the relapses occurring within 2 years.

Bipolar disorder is the sixth leading cause of medical disability worldwide among people 15 to 44 years of age. Bipolar disorder is associated with a greater degree of disability than a number of prominent chronic medical conditions, including osteoarthritis, human immunodeficiency virus infection, diabetes, and asthma **(**Murray *et al.,* 1997).

Bipolar disorder is associated with high rates of unemployment, job-related difficulties, and interpersonal stress. Two large surveys revealed unemployment rates of about 60%, even among college-educated patients **(**Hirschfeld *et al.,* 2000 and Kupfer *et al.,* 2002). In one of the surveys, 88% of respondents reported occupational difficulties **(**Hirschfeld *et al.,* 2000). The other survey also showed that 65% of respondents had difficulty maintaining long-term relationships, and 64% had difficult relationships with their children **(**Kupfer *et al.,* 2002).

Suicide

Patients with bipolar disorder have a higher risk of suicide than patients with any other psychiatric or medical illness (Woods *et al.,* 2000). According to one study, the odds ratio for suicide attempts in bipolar disorder was 6.2, which was higher

than any other axis I psychiatric disorder, including depression **(**Chen *et al.,* 1996). Another study showed that patients with bipolar disorder had a higher lifetime history of suicide attempt than patients with any other psychiatric disorder (Kessler *et al.,* 1999). Rates of suicidal ideation and attempts range between 35% and 50% **(**Woods *et al.,* 2000; Suppes *et al.,* 2001 and Kupfer *et al.,* 2002). The Stanley Center Bipolar Disorder Registry reported that 50% of patients enrolled had attempted suicide, and 35% of the attempts resulted in hospitalization Kupfer *et al.,* 2002). The rate of completed suicide is approximately 20% (Goldberg *et al.,* 2004).

2.3.2 Comorbidity and Bipolar Disorder

Patients with bipolar disorder have high rates of medical and psychiatric comorbidity. The ECA study found that among patients with bipolar disorder, 46% had alcohol abuse or dependence, 41% had drug abuse or dependence, 21% had panic disorder, and 21% had obsessive-compulsive disorder **(**Regier *et al.,* 1990; Chen *et al.,* 1995 and Dilsaver *et al.,* 1995). Persons with bipolar I disorder were more than 3 times as likely to have alcohol abuse or dependence and 7 times more likely to have drug abuse or dependence compared with the general population **(**Regier *et al.,* 1990). Patients with bipolar disorder were 26 times more likely to have panic disorder and 8 times more likely to have obsessive-compulsive disorder than were people in the general population without a mood disorder (Chen *et al.,* 1995 and Dilsaver *et al.,* 1995).

In another study, 65% of a group of patients with bipolar disorder also met Diagnostic and Statistical Manual of Mental Disorders (DSM, Fourth Edition) criteria for at least 1 comorbid lifetime axis I psychiatric disorder. The most common comorbidities were anxiety disorders and substance use. The authors found evidence that axis I comorbidity was associated with an earlier age of onset of bipolar disorder and worsening course of bipolar illness **(**McElroy *et al.,* 2001).

Other studies have confirmed frequent comorbidity of bipolar disorder with anxiety, substance use, and conduct disorders. Various reports have documented co-occurrence of bipolar disorder with a variety of other psychiatric disorders, including anorexia nervosa, bulimia nervosa, binge-eating disorder, attention-deficit/hyperactivity disorders, sexual disorders and addictions, impulse-control disorders, autism spectrum disorders, and Tourette's syndrome (McElroy, 2004).

The clinical implications of comorbidity are profound. One report noted that in bipolar patients the presence of substance use disorder doubles the risk of suicide (Comtois *et al.,* 2004) and another report found that quality of life among individuals with bipolar disorder and substance abuse is significantly affected by the severity of substance dependence (Singh *et al.,* 2005)

General medical disorders also frequently occur with bipolar disorder. These include migraine, thyroid disease, and type 2 diabetes. Community studies have demonstrated associations between bipolar disorder and migraine, Tourette's syndrome, multiple sclerosis, and obesity. Bipolar disorder has been associated with increased mortality from cardiovascular disease and some forms of cancer (McElroy, 2004). It has been suggested that the risk of developing dementia is increased when there are a greater number of episodes in bipolar disorder (Kessing *et al.,* 2004).

2.3.3 Neurobiology of Bipolar Disorder

Although genetic factors play a major, unquestionable role in the etiology of bipolar disorder (BD), the biochemical abnormalities underlying the predisposition to and the pathophysiology of BD remain to be fully elucidated. A true understanding of the pathophysiology of BD must address its neurobiology at different physiological levels, i.e. molecular, cellular, systems, and behavioral. Abnormalities in gene expression undoubtedly underlie the neurobiology of the disorder at the molecular level. Assessments of cerebrospinal fluid chemistry, neuroendocrine responses to pharmacological challenge, and neuroreceptor and transporter binding have demonstrated a number of abnormalities in the amine neurotransmitter systems in this disorder. However, recent studies have also implicated critical signal transduction pathways as being integral to the pathophysiology and treatment of BD, in addition to a growing body of data suggesting that impairments of neuroplasticity and cellular resilience may also underlie the pathophysiology of the disorder.

2.3.3.1 Classical Monoaminergic Neurotransmitter and Neuroendocrine Systems:

Noradrenergic system

Despite methodological difficulties in assessing central nervous system (CNS) noradrenergic or norepipherenic (NE) functions in humans, extensive investigation supports the presence of NE systems abnormalities in BD (Goodwin *et al.,* 1990; Schatzberg *et al.,* 1995 and Manji *et al.,* 1997). Postmortem studies have shown an increased NE turnover in the cortical and thalamic areas of BD subjects (Young *et al.,* 1994 and Vawter *et al.,* 2000 1), whereas in vivo studies have found plasma levels of NE and its major metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), to be lower in bipolar than unipolar depressed patients, and higher in bipolar patients when manic than when depressed (Goodwin *et al.,* 1990 and Manji *et al.,* 1997). The same occurs with urinary MHPG levels, which are lower in bipolar depressed patients, while longitudinal studies show that MHPG excretion is higher in the manic compared to depressed state (Goodwin *et al.,* 1990; Bowden, 1997; Schatzberg *et al.,* 1995 and Manji *et al.,* 1997). Finally, in a consistent mode, cerebrospinal fluid (CSF) NE and MHPG are also reported to be higher in mania than in depression.

Other paradigms studying NE receptor function tend to suggest the possibility of an altered sensitivity of α_2- and β_2- adrenergic receptors in mood disorders (Schatzberg *et al.,* 1995 and Manji *et al.,* 1997). Genetics studies have also been carried out, showing that polymorphic variation of enzymes involved in amine metabolism (i.e. tyrosine hydroxylase, catechol-O-methyltransferase) could confer different susceptibility to develop bipolar symptomatology (Kirov *et al.,* 1998; Rotondo *et al.,* 2002 and Serretti *et al.,* 1998). However, although promising, these findings need to be replicated and subgroups of bipolar patients to whom these alterations may apply need to be identified.

Serotonergic system

There is a consistent body of data from CSF studies, neuroendocrine challenge studies, serotonin receptor and reuptake site binding studies, pharmacologic studies, and most recently, brain imaging studies supporting a role for alterations of serotonergic neurotransmission in major depressive episodes (Goodwin *et al.,* 1990; Maes *et al.,* 1995 and Garlow *et al.,* 1999). Overall, investigators have reported reduced levels of 5-hydroxyindoleacetic acid (5-HIAA) in a subgroup of patients,

especially those with impulsivity, aggression and suicide attempts. In BD subjects, studies of CSF 5-HIAA in manic patients have generally produced variable and inconsistent results (Goodwin *et al.,* 1990 and Shiah *et al.,* 2000). Thus, baseline CSF 5-HIAA levels in manic patients, compared to nondepressed controls, have been reported as decreased in four studies, unchanged in nine studies, and increased in three studies; by contrast, most studies find no difference in the levels of CSF 5-HIAA between manic and depressed patients. Of the four studies that examined CSF 5-HIAA accumulation following administration of probenecid in manics, depressives and controls, two reported that both manic and depressed patients have diminished CSF 5- HIAA formation compared to controls, and one reported that manic patients have significantly lower CSF 5-HIAA accumulation than depressives and controls (Goodwin *et al.,* 1990).

Studies have also reported decreased radioligand binding to the serotonin transporter (which takes up serotonin from the synaptic cleft) both in platelets and in the midbrain of depressed patients (Maes *et al.,* 1995 and Garlow *et al.,* 1999).

Neurotransmitter depletion models, specifically in this case tryptophan depletion to lower serotonin levels, permit a more direct strategy to clarify the involvement of serotonergic systems in mood disorders. Tryptophan depletion (achieved by the ingestion of preparations containing high levels of other amino acids, but devoid of tryptophan) results in reversal of the response to certain antidepressant medications and recurrence of depression; however, depletion in healthy subjects without evidence of mental illness and in nonmedicated patients with depression does not consistently cause or intensify depression (Delgado *et al.,* 1999). These studies again substantiate the underlying complexity of neurobiologic systems not only in depression but by analogy in BD. With respect to BD, recent studies have investigated the effect of tryptophan depletion in lithium-treated euthymic patients and have generally found no recurrence of symptoms (Hughes *et al.,* 2000). Thus, although lithium has often been postulated to exert many of its beneficial effects via an enhancement of serotonergic function, the tryptophan depletion studies suggest that other mechanisms may be more important.

Dopaminergic system

Several lines of evidence point to a role of dopamine (DA) system in mood disorders. A relevant preclinical model derives from the crucial role of mesoaccumbens DA in the neural circuitry of reward and/or incentive motivational behavior (Wightman *et al.,* 2002). Loss of motivation is one of the central features of depression and indeed anhedonia is one of the defining characteristics of melancholia. Thus, a deficiency of DA systems stands out as a prime candidate for involvement in the pathophysiology of depression (Fibiger, 1991and Willner *et al.*, 1991). The strongest direct finding from clinical studies implicating DA in depression is reduced homovanillic acid (HVA, the major DA metabolite) in the CSF; indeed, this is one of the most consistent biochemical findings in depression (Goodwin *et al.*, 1990 and Manji *et al.*, 1997). There is also evidence for a decreased rate of CSF HVA accumulation in subgroups of depressed patients, including those with marked psychomotor retardation versus agitation (Willner *et al.,* 1991). Furthermore, depression occurs in up to 40% of patients with idiopathic Parkinson's disease and may precede motor symptoms.

Pretreatment plasma HVA has been shown to predict neuroleptic treatment response in manic psychosis, where those with higher HVA responded better (Mazure *et al.*, 1998). Lithium, being the first line treatment in BPI, has been shown to decrease dopamine formation (Friedman and Gershon, 1973). Agents that increase dopamine availability, on the other hand have been shown to trigger hypomania-mania, exemplified by amphetamine (Gerner *et al.,* 1976 and Jacobs *et al.*, 1986). A dopamine deficiency in depression has been supported by consistent findings of a reduction of the CSF HVA in depressed subjects.

In a SPECT (single photon emission computed tomography) study where euthymic bipolar patients and healthy controls were subjected to an amphetamine challenge, intriguingly, they did not observe any mood-lowering effects of but observed a 'rebound hypomania' in a significant percentage of the patients (Anand *et.al.,* 2000). The authors concluded that they diid not find evidence for increased striatal dopamine release but suggested that the data was consistent with enhanced postsynaptic dopamine responsivity in patients with bipolar disorder.

Although these data point at an important role of dopamine in bipolar disorder, there is not considered to be a primary abnormality in bipolar disorder in euthymia, but a defect in the system of dampening and fine-tuning (Goodwin *et al.,* 2007).

Cholinergic system

Much of the evidence supporting the involvement of the cholinergic system in mood disorders comes from neurochemical, behavioral and physiologic studies in response to pharmacologic manipulations. These studies, carried out in the early 1970s, showed that the relative inferiority of noradrenergic compared to cholinergic tone was associated with depression, while the reverse was associated with mania (Janowsky *et al.,* 1995). Additional support is found from a study on the central cholinesterase inhibitor physostigmine (administered intravenously), in which transient modulation of symptoms in manic cases and induction of depression in euthymic bipolar patients stabilized with lithium were observed.

A decrease in the cholinergic tone during mania has also been described when increased requirements of the cholinergic agonist pilocarpine were needed to elicit pupillary constriction: consistently, this responsiveness increased after lithium or valproic acid treatment (Sokolski *et al.,* 1999 and Sokolski *et al.,* 2000), adding evidence on the effects of lithium perhaps potentiating brain cholinergic systems (Lenox *et al.,* 1998 and Jope, 1999). However, the therapeutic responses observed with antidepressant and antimanic pharmacological agents are not reliably matched with effects on the cholinergic system.

2.3.3.2 Signaling Networks:

More recently, research into the pathophysiology and treatment of mood disorders has moved from a focus on neurotransmitters and cell surface receptors to intracellular signaling cascades.

Complex signaling networks may be especially important in the CNS, where they 'weigh' and integrate diverse neuronal signals and then transmit these integrated signals to effectors, thereby forming the basis of a complex information processing network (Bourne *et al.,* 1993; Bhalla *et al.,* 1999 and Weng *et al.,* 2000). The high degree of complexity generated by these signaling networks may be one mechanism by which neurons acquire the flexibility for generating the wide range of responses

observed in the nervous system. These pathways are thus undoubtedly involved in regulating such diverse vegetative functions as mood, appetite and wakefulness and are therefore likely to be involved in the pathophysiology of BD.

The Gs/cAMP generating signaling pathway

Several independent laboratories have reported abnormalities in G protein subunits in BD. Postmortem brain studies have reported increased levels of the stimulatory G protein ($G\alpha_s$) accompanied by increases in post-receptor stimulated adenylyl cyclase (AC) activity in BD (Young *et al.,* 1993). Several studies have also found elevated $G\alpha_s$ protein levels and mRNA levels in peripheral circulating cells in BD, although the dependency on clinical state remains unclear (Lenox *et al.,* 1998 and Wang *et al.,* 1999). It should be emphasized, however, that there is at present no evidence to suggest that the alterations in the levels of $G\alpha_s$ are due to a mutation in the $G\alpha_s$ gene itself (Manji *et al.* 2003). There are numerous transcriptional and post-transcriptional mechanisms which regulate the levels of G protein subunits, and the elevated levels of $G\alpha_s$ could potentially represent the indirect sequelae of alterations in any one of these other biochemical pathways (Manji *et al.* 2003).

There is growing consensus that the ability of a 'simple' monovalent cation like lithium to treat multiple aspects of an illness as complex as BD arises from its major effects on intracellular signaling pathways, rather than on any single neurotransmitter system per se (Manji *et al.* 2003). Although speculative, it might be postulated that these G protein effects - which would theoretically attenuate excessive signaling through multiple pathways - likely contribute to lithium's long-term prophylactic efficacy in protecting susceptible individuals from spontaneous-, stress-, and drug (e.g. antidepressant, stimulant)- induced cyclic affective episodes.

The protein kinase C signaling pathway

Protein kinase C (PKC) exists as a family of closely related subspecies, has a heterogenous distribution in brain (with particularly high levels in presynaptic nerve terminals), and, together with other kinases, appears to play a crucial role in the regulation of synaptic plasticity and various forms of learning and memory (Manji *et al.* 2003).

To date, there have only been a limited number of studies directly examining PKC in BD (Hahn *et al.,* 1999). Although undoubtedly an over-simplification, particulate (membrane) PKC is sometimes viewed as the more active form of PKC, and thus an examination of the subcellular partioning of this enzyme can be used as an index of the degree of activation. Friedman et al. (Friedman *et al.,* 1993) investigated PKC activity and PKC translocation in response to serotonin in platelets obtained from BD subjects before and during lithium treatment. They reported that the ratios of platelet membrane-bound to cytosolic PKC activities were elevated in the manic subjects. In addition, serotonin-elicited platelet PKC translocation was found to be enhanced in those subjects. With respect to brain tissue, Wang and Friedman (Manji *et al.* 2003) measured PKC isozyme levels, activity and translocation in post-mortem brain tissue from BD patients; they reported increased PKC activity and translocation in BD brains compared to controls, effects which were accompanied by elevated levels of selected PKC isozymes in cortices of BD subjects.

Interestingly, lithium and VPA appear to bring about their effects on the PKC signaling pathway by distinct mechanisms (Manji *et al.* 2003).These biochemical observations are consistent with the clinical observations that some patients show preferential response to one or other of the agents, and that one often observes additive therapeutic effects in patients when the two agents are co-administered.

In view of the pivotal role of the PKC signaling pathway in the regulation of neuronal excitability, neurotransmitter release, and long-term synaptic events (Manji *et al.* 2003), it was postulated that the attenuation of PKC activity may play a role in the antimanic effects of lithium and VPA. In a pilot study it was found that tamoxifen (a non-steroidal antie- strogen known to be a PKC inhibitor at higher concentrations (Couldwell *et al.,* 1993) may, indeed, possess antimanic efficacy (Manji *et al.* 2003). Clearly, these results have to be considered preliminary, due to the small sample size thus far. In view of the preliminary data suggesting the involvement of the PKC signaling system in the pathophysiology of BD, these results suggest that PKC inhibitors may be very useful agents in the treatment of mania. Larger double-blind placebo-controlled studies of tamoxifen and of novel selective PKC inhibitors in the treatment of mania are warranted.

Abnormalities of calcium signaling

Calcium ions play a critical role in regulating the synthesis and release of neurotransmitters, neuronal excitability, and long-term neuroplastic events, and it is thus not surprising that a number of studies have investigated intracellular Ca^{2+} in peripheral cells in BD (Manji *et al.* 2003). These studies have consistently revealed elevations in both resting and stimulated intracellular Ca^{2+} levels in platelets, lymphocytes and neutrophils of patients with BD. The regulation of free intracellular Ca^{2+} is a complex, multi-faceted process, and the abnormalities observed in BD could arise from abnormalities at a variety of levels (Manji *et al.* 2003). Ongoing studies should serve to delineate the specific regulatory sites at which the impairment occurs in BD.

2.3.3.2 Abnormalities in circadian system:

It has been hypothesized for quite some time that bipolar disorder is associated with abnormalities in the circadian system (Roybal *et al.,* 2007). Virtually all individuals with bipolar disorder have major alterations in circadian functions including sleep, activity, hormonal secretions, and appetite. Appearance of mania can cycle with a regular, even seasonal, pattern, further suggesting a circadian component to its pathology. In addition, normalization of both sleep/wake cycles and social zeitgebers (periodic environmental condition or event that acts to set or reset an innate biological rhythm of an organism) often is essential for mood stabilization, while disruptions in these rhythms can trigger manic episodes. Some successful treatments for mood disorders rely on altering the circadian cycle. Depression symptoms are also diurnal, being more prevalent during the winter months (termed seasonal affective disorder) and in parts of the world that receive little sunlight for extended periods of time. Lithium, a mood stabilizing drug lengthens the circadian period in several organisms, including *Drosophila,* rodents, and humans, and this effect may be important for its therapeutic efficacy. Furthermore, another mood stabilizer, valproate, alters the expression of several circadian genes in the amygdala and chronic treatment with the antidepressant fluoxetine increases expression of *Clock* (Circadian Locomotor Output Cycles Kaput) and *Bmal1* (Brain And Muscle Aryl Hydrocarbon Receptor Nuclear Translocator ARNT-like) genes in the hippocampus. Together, these findings support the view that circadian genes in multiple regions of the brain are important in the development and treatment of

mood disorders. Individual genetic characteristics of the molecular mechanisms of the biological clock are also determinants of core features of mood disorders, including age at onset, recurrence, symptoms of insomnia and its treatment and response to sleep deprivation. Such parallel findings point to an intimate relationship between the neurotransmitter systems targeted by drugs and the circadian rhythms targeted by chronotherapeutics (Wirz-Justice *et al.*, 2006).

2.3.3.3 Impairments of Neuroplasticity and cellular resilience:

Structural imaging studies have demonstrated reduced gray matter volumes in areas of the orbital and medial PFC, ventral striatum and hippocampus, and enlargement of third ventricle in patients with mood disorders relative to healthy controls (Drevets *et al.*, 2001). Complementary post mortem neuropathological studies have shown abnormal reductions in cortex volume, glial cell counts, and/or neuronal densities/sizes in the subgenual PFC, orbital cortex and dorsal anterolateral PFC in unipolar and bipolar patients. However, many of these preliminary reports, although extremely interesting, require further replication.

The marked reduction in glial cells in these regions is particularly intriguing in view of the growing appreciation that glia plays critical roles in regulating synaptic glutamate concentrations and CNS energy homeostasis, and in releasing trophic factors that participate in the development and maintenance of synaptic networks formed by neuronal and glial processes (Coyle *et al.*, 2000).

However, it is not presently known whether this evidence of neuronal deficits constitutes developmental abnormalities that may confer vulnerability to abnormal mood episodes, compensatory changes to other pathogenic processes, or other factors that are difficult to control in patient populations.

Underlying mechanism for cell loss

Activation of the HPA axis appears to play a critical role in mediating hippocampal atrophy. In addition to directly causing neuronal atrophy, stress and glucocorticoids also appear to reduce cellular resilience, thereby making certain neurons more vulnerable to other insults, such as ischemia, hypoglycemia, and excitatory aminoacid toxicity.

The reduction in the resilience of hippocampal neurons may also reflect the propensity for various stressors to decrease the expression of brain derived neurotrophic factor (BDNF) in this region (Duman, 2002). BDNF and other neurotrophic factors are necessary for the survival and function of neurons, implying that a sustained reduction of these factors could affect neuronal viability. Increasing evidence suggests that neurotrophic factors inhibit cell death cascades by (in large part) activating the mitogen activated protein (MAP) kinase signaling cascade, and upregulating major cell survival proteins such as bcl-2 (Manji *et al.,* 2003). Bcl-2 is now recognized as a major neuroprotective protein, since bcl-2 overexpression protects neurons against diverse insults, including ischemia, the neurotoxic agent methyl-phenyl-tetrahydropyridine (MPTP), β-amyloid, free radicals, excessive glutamate, and growth factor deprivation (Manji *et al.,* 2000). Accumulating data suggests that bcl-2 is not only neuroprotective, but also exerts neurotrophic effects and promotes neurite sprouting, neurite outgrowth and axonal regeneration (Manji *et al.,* 2000). Overall, it is clear that the neurotrophic factors/MAP kinase/bcl-2 signaling cascade plays a critical role in cell survival in the CNS, and that there is a fine balance maintained between the levels and activities of cell survival and cell death factors. Modest changes in this signaling cascade or in the levels of the bcl-2 family of proteins (potentially due to genetic, illness or insult-related factors) may therefore profoundly affect cellular viability.

2.3.4 STUDIES TO IDENTIFY CHROMOSOMAL SUSCEPTIBILITY LOCI:

Current models on the etiology of psychiatric disorders support the idea of a biologic cause as well as interactions of biologic systems with the environment. The elucidation of the genetic etiology is of paramount importance to understand the cause of psychiatric disorders

Linkage analysis studies, the best method for understanding the biological causes of bipolar disorder, are used to isolate chromosomal regions of susceptibility and the genes those regions contain. The primary result of a linkage analysis study is a set of LOD scores. LOD scores, short for “logarithm of odds”, are a ratio of the likelihood that two sections of a chromosome are inherited together (Haines *et al.,* 1998) If two sections of a chromosome appear together more often in people with a particular disease compared to people without that disease then it’s possible those chromosomal locations contain susceptibility genes.

Genetic linkage in bipolar disorder was studied as early as the 1960s. Multiple molecular genetic linkage studies of bipolar disorder have yielded evidence for susceptibility loci across whole genome. While initial genetic linkage studies of bipolar disorder were fraught with inconsistencies and failures to replicate, more recently the accumulation of multiple studies on large pedigree cohorts has led to reproducible identification of several susceptibility loci. These include regions on chromosomes 4, 4q35, 12q, 13q14, 18, 21q and 22q[11, 17-26]. (Craddock *et al.,* 1994; Berretini *et al.,* 1994; Straub *et al.,* 1994 ; Stine *et al.,* 1995; Blackwood *et al.,* 1996; Adams *et al.,* 1998 ; Craddock *et al.,* 1999; Friddle *et al.,*2000; Kelsoe *et al.,* 2001 and Badenhop *et al.,* 2002) A comprehensive linkage analysis of chromosome 21q22 supports evidence for a putative bipolar disorder locus[29]. Many more genome scans and follow-up studies have identified bipolar disorder susceptibility loci on chromosome 1q42 (Macgregor *et al.,* 2004), 3p (Etatin *et al.,* 2006), 13 and 19 (Badenhop *et al.,* 2002).

A study on the patients from Faroe Islands yields support for a common risk locus on the short arm of chromosome 4 for bipolar affective disorder and schizophrenia (Als *et al.,* 2004). Another study identifies a susceptibility loci on 4q32 for bipolar disorder in isolated Finnish population (Ekholm *et al.,* 2003). A locus for bipolar affective disorder on chromosome 4p is also reported (Blackwood *et al.,* 1996). A haplotype analysis and a novel allele-sharing method has further strengthened the evidence of linkage of chromosome 4p locus to bipolar affective disorder (Hellard *et al.,* 2007)

Chromosome 22 has been implicated in schizophrenia and bipolar disorder in a number of linkage, association and cytogenetic studies. Initial genome wide scans for schizophrenia by different groups suggested possible linkage for markers on chromosome 22q although neither of the groups reported statistically significant results. CAG repeat polymorphisms on chromosome 22 are associated with schizophrenia and bipolar disorder (Saleem *et al.,* 2001). Another genome survey of bipolar disorder using 443 microsatellite markers in a set of 20 families from the general North American population has further strengthened the possibility of a susceptibility locus on chromosome 22q (Kelsoe *et al.,* 2001).

A genome wide screen designed to identify chromosomal regions that harbour susceptibility genes for BPAD found susceptibility loci at chromosomes 2, 3, 4, 6, 7,

9, 10, 12, 17, 18 and X (Bennett *et al.*, 2002). Another genome-wide scan for genes involved in bipolar affective disorder in 70 European families ascertained through a bipolar type I early onset proband supports evidence for linkage at 3p14 (Etatin *et al.*, 2006). A genome screen of 13 bipolar affective disorder pedigrees provides evidence for susceptibility loci on chromosome 3, 9, 13 and 19 (Segurado *et al.*, 2003).

The rank-based genome scan meta-analysis (GSMA) method was applied to 18 BPD genome scan data sets in an effort to identify regions with significant support for linkage in the combined data. The most significant *P* values were observed on chromosomes 9p22.3-21.1, 10q, 11.21-22.1 and 14q24.1-32.12 (McMahon *et al.*, 1995).

In contrast with some retrospective analyses, several clinical studies suggest a parent-of-origin effect in bipolar pedigrees, suggesting a basis for familial heterogeneity. Whether this is a genetic effect, and if so, by what mechanism, reflects unanswered but important questions with implications for the mode of inheritance. Possible molecular genetic explanations could involve X-linkage, mitochondrial inheritance, or other forms of non-Mendelian inheritance such as genomic imprinting (McInnis *et al.*, 2003).

Several aspects of the inheritance of bipolar disorder have puzzled researchers for decades. Although familial, the disease displays an atypical pattern of inheritance. Imprinting has been put forward as a putative explanation. Imprinting refers to an epigenetic phenomenon of differences between maternally and paternally transmitted illness. It appears to be related to a differential expression of maternally and paternally transmitted disease alleles resulting from methylation during meiosis. In one linkage study of unilineal families, a parent-of-origin effect was found with a high prevalence of the illness among maternal relatives (McMahon *et al.*, 1995). In another study (Cichon *et al.*, 1990), paternally imprinted loci were identified on chromosomal regions 2p24-p21 and 2q31-32 and maternally on 14q32 and 16q21-q23.

While a number of further reports have failed to reproduce these results, others have demonstrated positive linkage findings for markers spread throughout chromosome 18 using various methods of analyses.

A few loci have been repeatedly implicated in bipolar disorder by independent studies. One of the most intensively studied regions amongst these include several loci on chromosome 18p. In one study, chromosome 18p has been shown to be a highly susceptible locus for the affective mood disorder (Ginns *et al.*, 1998) Further, support for this region comes from evidence suggesting potential overlap with schizophrenia; Schwab *et al.*, reported a possible schizophrenia locus on 18p11.2, and demonstrated that evidence for linkage in these schizophrenia families increased when individuals with affective diagnoses were also included in the analysis (Schwab *et al.*, 1998).

There have been a paucity of studies on psychiatric difficulties experienced by persons with chromosome 18 abnormalities. A susceptibility gene on chromosome 18 and a parent-of-origin effect have been suggested for BPAD (Stine *et al.,* 1995). In studies of persons with chromosome 18p- deletions, there have been reports of paranoid psychosis, depression, and subclinical obsessive compulsive disorder (Babovic-Vuksanovic *et al.,* 2004). In studies of 18qsyndromes, there have also been individual reports of violent/aggressive behaviors (Mahr *et al.*, 1996). There is one report of a behavioral/psychiatric problem in a case of chromosome 18p tetrasomy; an individual who had a history of aggressive, self injurious, and destructive behavior (Swingle *et al.,* 2006). There is also a report of a family with apericentric inversion of chromosome 18, in which three individuals (two with dup(18p)/del(18q) and one with dup(18q)/del(18p), all displayed depression and anxiety (Vermeulen *et al.,* 2005).

Many other numerous studies have identified potential loci (Bassett, 1992; McInnes *et al.,* 1996; Escamilla *et al.*, 2001; Mukherjee *et al.,* 2006] and candidate genes (TGIF (Chavarr *et al.,* 2007, NDUFV2 (Wasizuka *et al.,* 2009), VAPA (Lohoff *et al.,* 2008), NAPG (Weller *et al.,* 2006 and Xingwang *et al.,* 2009), IMPA2 (Yoshikawa *et al.,* 2001; Sjøholt *et al.,* 2004), GNAL (Tsiouris *et al.,* 1996) related to bipolar disorder as being on chromosome 18p. Further, support for chromosome 18 at this locus comes from the fine scale mapping of a locus on chromosome 18p 11.3 in the Costa Rican Population (McInnes *et al.,* 2001). A significant evidence of linkage to markers is reported at the pericentromeric region of chromosome 18 (Berrettini *et al.,* 1994). Other studies have also reported likage of bipolar disorder to chromosome 18 (**McMahon *et al.,* 1997)** NAPG gene, located on 18p11, has

been suggested as being associated with bipolar disorder in European population. A genome wide screen designed to identify chromosomal regions that harbour susceptibility genes for BPAD has also found chromosome 18p as one of the many susceptibility loci (Bennett *et al.,* 2002) .The affected-sibpair analyses indicated excess allele sharing for markers on 18p (Stine *et al.,* 1995).Under the narrowest diagnostic model, the single most significant multipoint linkage was on chromosome 18 at marker D18S452 (MLS=1.54) (Bennett *et al.,* 2002).

Though the incidence of BPAD in Kashmir is significant, yet there has been no genetic study from Kashmir till date and the existence of any genetic predisposition in this population remains totally untouched and unexplored. The molecular and epidemiological studies remain open for research and seem to be promising in addressing the various queries of BPAD etiology in this area. Identifying susceptibility genes will pinpoint biochemical pathways involved in pathogenesis, facilitate development of more effective, better targeted treatments and offer opportunities for improving the validity of diagnosis and classification. Therefore the current work was undertaken to determine the existence of any association between the chromosomal susceptibility locus (D18S452) on chromosome 18 and genetic predisposition to BPAD.

CHAPTER: 3

MATERIALS & METHODS

3.1 Material

3.1.1 Study population

Patients who were diagnosed with the disorder by clinicians using their proforma (Diagnostics and Statistical Manual IV) at Psychiatric Diseases Hospital, Srinagar were enrolled as cases. The permission was sought from the concerned authorities for taking the blood sample from the cases and the controls. Only after getting the written consent from them or their relatives, the subjects were included in the study. Controls were recruited from the General Medicine wards of SMHS Hospital, Srinagar following the referral pattern of sex and age matched patients. None of the controls had a personal or family history of any psychiatric disorder.

3.1.1.1 Subjects

74 cases and 74 controls were included in the study.

Criteria adopted for selecting the cases and controls

The criteria for including or excluding a subject in the study were formulated prior to the commencement of the study.

Cases

All the diagnosed patients were included irrespective of any grade (whether or not on medication), age or gender.

Exclusion criteria

Under the following conditions the patients were not recruited in the study;

- Patients suffering from thyroid disorders.
- Patients not belonging to Kashmir valley.
- Who did not agree to participate

Inclusion Criteria

- Cases diagnosed through interview.
- Patients who were willing to take part in the study.
- Patients belonging to Kashmir valley.

Controls

Controls were matched to the cases by age and gender.

Inclusion criteria

- Residents of the Kashmir valley.
- Matched male and female ratio with the cases.

Exclusion criteria

- Patients who were ever in their life time treated for and/or diagnosed with schizophrenia or schizoaffective disorder.
- Patients who had family history of bipolar disorder, schizophrenia or alcohol dependence disorders.
- Patients who did not agree to participate.

3.1.2 COLLECTION OF BLOOD SAMPLES

Individuals who gave consent to participate in this case-control study were enrolled for the study. Blood sample was collected from both the cases and the controls. Around three milliliter (3 ml) peripheral blood was collected from each subject in sterilized plastic vials containing EDTA (0.5M; pH-8.0) and stored at -20^{o}C for further analysis.

3.2 Methods

3.1.3 DNA extraction from whole blood

The phenol-chloroform method for isolation of genomic DNA was employed with slight modification to suit the lab conditions. Briefly, 3ml of the whole blood was used. The cells were lysed with freshly prepared lysis solution (155mM NH_4Cl_2, 10mM $KHCO_3$ and 0.1mM EDTA), shaken for 2-3 min and then kept at -20^oC for 30 min. Lysed cell suspension was centrifuged at 6000 rpm for 10 min at 4^oC. The pellet recovered was suspended in 5ml of SE solution and 10µl of 100ng/µl of Proteinase-K and 1 ml of 10% SDS. The sample was incubated at 37^oC for overnight. Next morning 3 ml of the phenol (saturated with 200 mMTris pH 8.0, containing 0.1% of hydroxyquinoline) was added. It was gently shaken for 10 min and then centrifuged at 6000 rpm for 10 min and aqueous layer was removed to a fresh tube. To aqueous layer, 1.5 ml phenol and 1.5 ml chloroform-isoamylalcohol was added and shaken gently for 10 min and then centrifuged at 6000 rpm for 10 min at 4^oC. Aqueous layer was again collected and to it was added 3 ml of chloroform-isoamylalcohal and again shaken gently for 10 min. the tubes were centrifuged again at 6000 rpm for 10 min at 4^oC. The aqueous layer was transferred to a fresh tube and to it was added 0.3 ml of chilled sodium acetate (pH 5.2) and 3 ml of chilled isopropanol. The DNA got precipitated immediately and was washed with 70% ethanol. The DNA was retrieved and dried and then dissolved in 500µl of 10mM Tris (pH 8).

3.1.4 Determination of concentration, purity and quality of genomic DNA

3.1.4.1 Qualitative Analysis

The quality of the genomic DNA was examined by gel electrophoresis using 0.7% agarose gel. 2µl of each DNA sample was mixed with 1µl of 6X DNA loading dye (6X loading dye consists of 4.16 mg bromophenol blue,4.16 mg xylene cyanol and 0.66g sucrose in1ml water) and was loaded in the gel. Electric current was applied at 20 volt until DNA enters in to the gel and was raised to 50 volt for rest of the run. Run was stopped when the dye had travelled nearly 2/3rd of the gel. Gel was visualized by a Gel doc system (Alphaimager TM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system.

3.1.4.2 Quantitative Analysis

The quantity of the DNA was estimated by making appropriate dilutions to determine the optical density (OD) at 260nm and 280 nm by double beam spectrophotometer (Spectron 2206) and the concentration was determined using equation:

$$\text{DNA } (\mu g/ml) = A_{260} \times 50 \times \text{dilution factor}$$

The ratio of A_{260}/A_{280} was calculated and the DNA samples for which the ratio was 1.7-1.9 was considered for the future use. The DNA was stored at 4^oC for a short time but the vials were kept at -20^oC for longer duration storage.

3.1.5 Agarose Gel Electrophoresis

- The edges of clean, dry glass plate were sealed with tape to form a mold, and were set on a horizontal section of the bench.
- Sufficient electrophoresis buffer (1XTAE) was prepared to cast the gel and to fill the electrophoresis tank.
- 0.8g of agarose was dissolved in 100 ml of 1X TAE buffer and heated to boil in oven until a clear transparent solution was formed.
- After removing from the oven the solution was allowed to cool to 50-60^oC and then few (2.5µl) of ethidium bromide (92mg%) was added as visualizing agent.
- The warm agarose was poured into the mold. An appropriate comb was positioned for forming the sample slots in the gel when the agarose was added to the mold.
- The gel was allowed to set completely (30-45 min), then a small amount of TAE buffer was poured on top of the gel and comb was carefully removed. TAE buffer was poured, after removing tape also, and gel was mounted in the tank.
- Sample was mixed with 2-3 µl of 6X gel loading buffer.
- Sample was loaded slowly and carefully into the slot of submerged gel using disposable loading tips.
- Lid of gel tank was closed and electric leads connected so that the DNA will migrate toward positive anode (red lead).Voltage of 1-5 V/cm was applied (distance measured from cathode to anode). The gel was allowed to run until

the bromophenol blue and xylene cyanol migrate an appropriate distance through the gel.

Pattern of separation was visualized by a Gel doc system (AlphaimagerTM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system. Samples containing high molecular weight DNA with no fragmentation/ shearing and without any apparent contamination or streaking were selected for further analysis

3.1.6 Genotyping of marker D18S452

Once it was confirmed that the genomic DNA is present and concentration and purity is also desirable, the desired fragment of DNA i.e., the microsatellite marker D18S452, was amplified by Polymerase Chain Reaction (PCR). The standard protocol for PCR was used, however the technique was standardized and optimized for available environmental conditions. After standardizing all the parameters of technique like varying annealing temperature from 55^{o}C to 65^{o}C, dNTP, primer and template concentration, the marker was amplified. PCR was performed in total volume of 25µl. The PCR reactions composed of 50-150ng genomic DNA, 0.2mM dNTPs, 0.4pmoles/µl of each primer and 1.5U of Taq polymerase in 1X PCR buffer as shown in Table-3.1.

Table 3.1: Volume and final concentration of different reagents used in PCR

Reagent	Volume required	Final concentration
Taq Buffer B (10X)	2.5 µl	1X
Taq Buffer with MgCl2 (25mM)	2.5 µl	2.5mM
dNTP mix (10mM)	0.5µl	0.2mM
Forward primer (20pmoles/µl)	0.25 µl	0.2pmoles/µl
Reverse primer (20pmoles/µl)	0.25 µl	0.2pmoles/µl
Taq DNA polymerase (1U/ µl)	0.3µl	0.06U/ µl
Genomic DNA	2.5µl	50-150ng
Milli Q *w*ater	16.2µl	
Total volume	**25.0 µl**	

Reaction mixture in PCR tubes was gently mixed and placed in a twenty-five well automated thermal cycler (Eppendorf). The different temperatures were set as given in the Table-3.2. During this procedure precautions were taken to keep the Taq polymerase active by not only preparing the reaction under 4^{o}C temperature but also adding the various reaction components as quickly as possible. The PCR conditions were selected after extensively standardizing all the PCR parameters. The primer pair used for amplification was;

Forward primer: 5′-TGG GGC ATA CAT AGT GCA AA-3′

Reverse primer: 5′-AGG CCT TTT GCT AGT TGG GT-3′

Lyophilized primer stocks were diluted first to 100pmol/µl concentration and then to 20pmol/µl using milliQ water. PCR buffer contained Tris-Cl, KCl and $(NH_4)_2SO_4$; pH 8.7. Amplification and specificity of amplicon obtained in the PCR reaction was analyzed by agarose gel electrophoresis on 3.0% gel. After the electrophoresis, the gel was visualized on UV-illuminator and photographed on a Gel Doc (Pharmacia).

Table 3.2: PCR cycling parameters

Steps	Temperature ^{o}C	Time	
Initial denaturation	95	4 minutes	
Denaturation	95	45 seconds	34Cycles
Annealing	59.2	30 seconds	
Extension	72	45 seconds	
Final extension	72	5 minutes	

The concentration of amplified PCR products was approximated by comparison with the corresponding bands in a 50 bp DNA marker. However, the total amount of DNA marker in one lane was 1µg.

3.1.7 Statistical Analysis

Results were statistically analyzed and data was expressed as mean ± SD. Allele and genotype frequencies were compared between groups using the χ^2-test. The association between D18S452 and the risk of BPAD was estimated by calculating odds ratio (OR) and their 95% confidence intervals (95% CI). A P value of <0.05 was used as a criterion for statistical significance. For the analysis statistical software GraphPad Prism version 5.0 was used.

CHAPTER: 4

RESULTS

4.1 General characteristics of study population

In the present study 74 blood samples from BPAD patients and 74 blood samples from healthy controls were analyzed. General characteristics of the BPAD patients and controls are given in table 4.1.

Table 4.1: General characteristics of the study population

Characteristics	Cases	Controls	P value*
Mean Age (years)	34.50	46.76	-
±SD	11.84	20.97	
Gender			
Males n(%)	43(58.1)	46(62.2)	0.6145
Females n(%)	31(41.9)	28(37.8)	
Dwelling			
Urban n(%)	16 (21.6)	58(78.37)	0.2589
Rural n(%)	16(21.6)	22(29.7)	
Economic Status			
Lower n(%)	51(68.9)	47(63.5)	0.4869
Middle n(%)	23(31.1)	27(36.5)	
Total	**74**	**74**	

n= No. of individuals

Values represent ±SD

*P using χ^2 Test (P<0.05, Data statistically significant)

4.2 Evaluation of DNA concentration and purity

The genomic DNA was extracted from the whole blood samples from the BPAD patients and controls of Kashmir. The integrity, concentration and purification of the genomic DNA was checked not only by UV- spectrophotometry but also by agarose gel electrophoresis, by analysing 3-4 µl of genomic DNA on the 0.8 % agarose gel (as shown in Fig 4.1), which reflected the intactness of genomic DNA, because the genomic DNA was restricted to the wells with no smear in the gel suggesting that DNA was not degraded. The results showed that DNA concentration of the samples ranged from 300ng/µl to 1200 ng/µl which was sufficient enough to carry out the other phases of the study. The purity of DNA was determined by A_{260} / A_{280} ratio and it was found that the ratio in all DNA preparations was in the range of 1.60-1.94. Agarose gel electrophoresis also demonstrated that DNA samples had high molecular weight without degradation as shown in Fig 4.1.

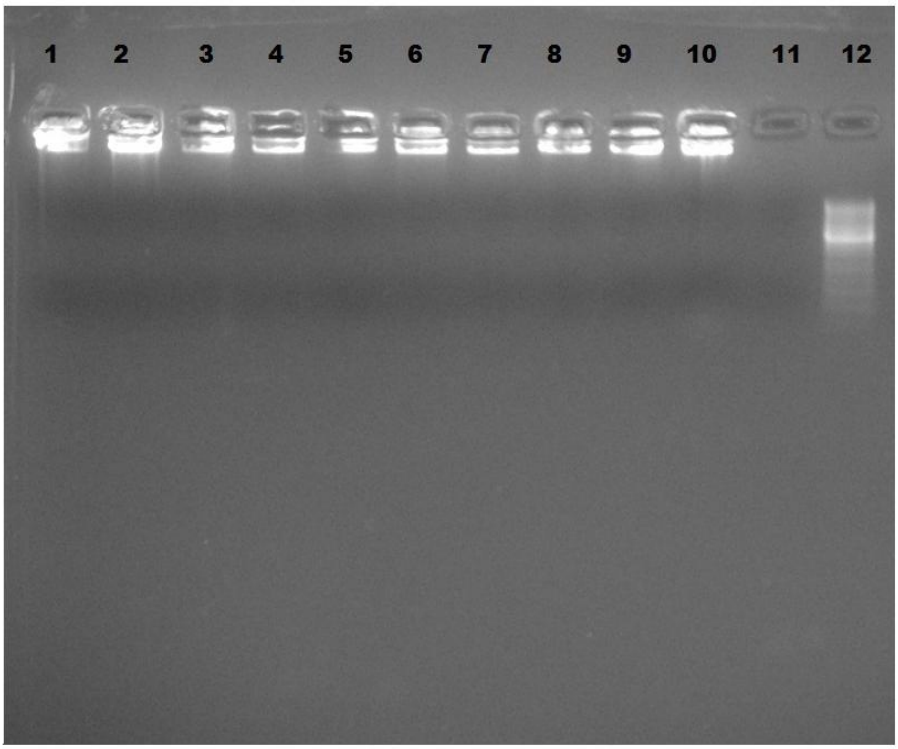

Fig 4.1: Representative gel picture showing the integrity of genomic DNA on 0.8% Agarose. Lane 1-10 represents intact genomic DNA extracted from study subjects. Lane 11 represents negative control and Lane 12 represents DNA marker (100bp).

4.3 Analysis of D18S452 252bp→300bp genotypes

Presence of the tandem repeat sequence in the microsatellite marker D18S452 gene was detected using a protocol already described in the methodology section. Amplified PCR products were visualized on a 3% agarose gel using ethidium bromide. The PCR results for both the cases and controls are shown in the fig 4.2-4.4. It was observed that the homozygotes for the repeat (252bp/252bp) produced a single band corresponding to 252 bp. Heterozygotes (252bp/300bp) produced two fragments corresponding to 300bp and 252bp respectively, and homozygotes for the repeat (300bp/300bp) produced a single 300bp fragment.

4.4.1 Genotypic and Allelic frequency of D18S452 polymorphism

In the present study 74 BPAD cases and 74 matched controls belonging to the Kashmir division were analyzed for repeat polymorphism.

We found six types of alleles corresponding to base pair size: 200 bp; between 200-252bp; 252bp; between 252-300bp; 300bp; between 300 and 350bp.

Only two samples one from case (1.35%) and one from control group (1.35%) had allelotype corresponding to 200 bp. One sample from case (1.35%) had allelotype corresponding to between 200bp to 250 bp. Again, only one sample from cases (1.35%) had allelotype corresponding to between 252bp and 300bp and one sample (1.35%) had allelotype corresponding to between 300 to 350bp. Sixty three patients (87.16%) and 72 controls (97.297%) had allelotype corresponding to 252bp, Seven patients (7.43%) had allelotype corresponding to 300bp, while as only one sample (1.37%) from controls had allelotype corresponding to 300bp.. The allelic frequencies are given in table 4.2

Since, the alleles corresponding to bp size 200, between 200-252bp, between 252-300bp, between 300 and 350bp were very rare, we considered them as minor alleles and grouped them under two main classes of major alleles; 252bp* (containing alleles of size upto252bp) and 300bp* (containing alleles corresponding to more than 252bp).

The tandem repeat (300bp*) allele frequency was found to be 1.35 % in the controls and 8.108% in cases. The tandem repeat (250bp*) allele frequency was found to be 91.89% in cases and 98.65% in controls. The 252bp/252bp genotype was found to be present in 89.18% of the cases and 98.64% of the controls, the 252bp/300bp variant in 5.40% of the

cases and none among the controls and the 300bp/300bp genotype in 5.40% of cases and 1.35% of controls (Table 4.3 and 4.4).

It was observed that although the proportion of individuals who were homozygous for the tandem repeat(300bp/300bp) was higher in cases than in controls, the difference was not statistically significant when using 252bp/252bp genotype as a reference (OR= 4.4242; 95% CI, 0.4822-40.5924); P = 0.1529). However, it was observed that the frequency of the heterozygous genotype (252bp/300bp) when compared with 252bp/252bp showed statistical significance (OR=8.0603; 95% CI, 1.1112-58.4646; P = 0.0383).

The above results were also expressed geometrically in the form of histograms (Fig. 4.5-4.6)

In Fig. 4.5, X-axis represents types of study subjects present in the population and the y-axis represents number of allelotypes. The dark pink bar represents 252* bp allele and the light pink bar represents the 300* bp allele. In cases, the dark pink bar corresponds to 136 no. of allelotypes and in controls it corresponds to 146 no. of 252* bp allele. Thus, 252* bp allele had higher frequency in both cases and controls than the 300* bp allele and the 300* bp allele had higher frequency in cases than controls.

Similarly, in Fig., 4.6, X-axis represents types of genotypes present in the population and the y-axis represents number of genotypes. The dark pink bar represents the cases and the light pink represents the controls. In cases, 252bp/252bp genotype corresponds to 66 no. of genotypes and in controls, it corresponds to 73. The 300bp/300bp genotype corresponds to 4 and 1 numbers of genotypes in cases and controls respectively. Similarly, the 252bp/300bp genotype corresponds to 4 and 0 numbers of genotypes in cases and controls respectively. Thus, 252bp/252bp genotype was higher in cases as well as controls, followed by 300bp/300bp genotype; 252bp/300bp genotype was present only in cases and none among the controls.

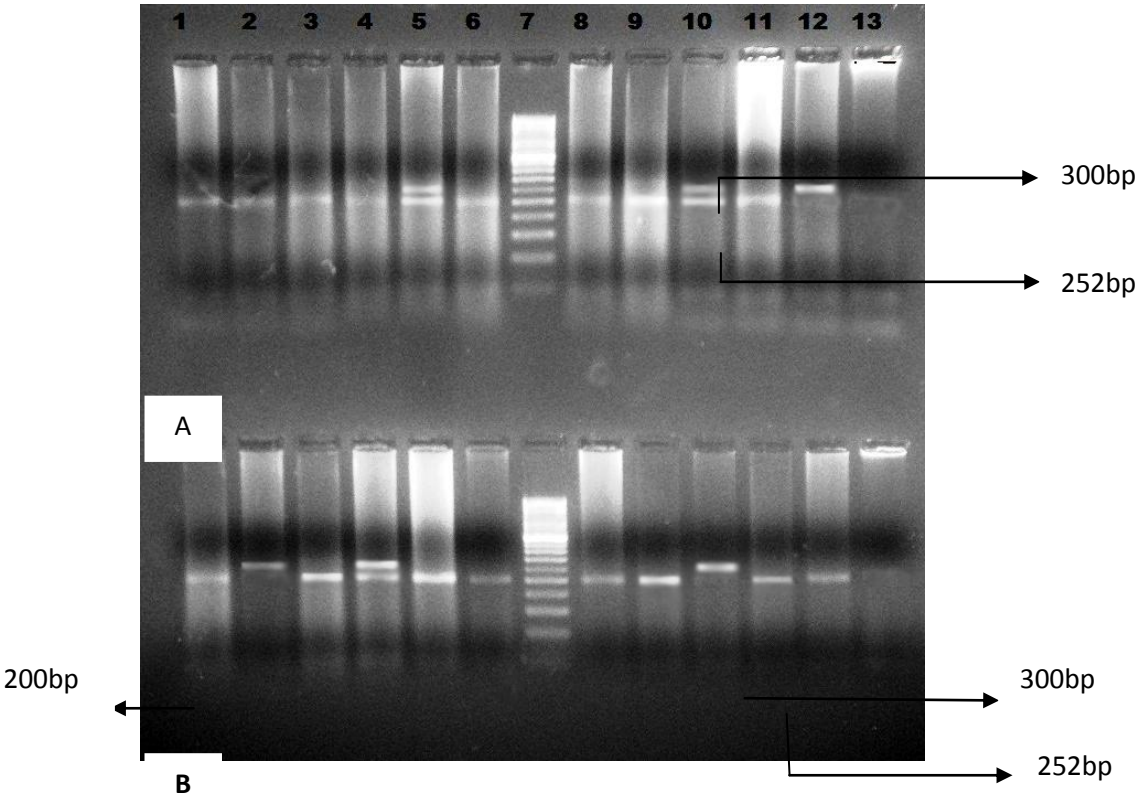

Fig 4.2: Representative gel picture showing 3% Agarose gel electrophoresis of some BPAD cases.

Gel A: lane1,2,3,4,6,8,9,11 and 12 represents homozygous condition (252bp);lane5 and 10 represents heterozygous condition(252bp/300bp);lane7 represents DNA marker (50bp); lane 13 represents negative control.

Gel B: lane1represents homozygous condition(200b)p; lane 2represents homozygous condition(252-300bp);lane3,5,6,8,9,11,12 represents homozygous condition(252bp);lane4 represents heterozygous condition(252bp/300bp); lane7 represents DNA marker (50bp); lane 13 represents negative control.

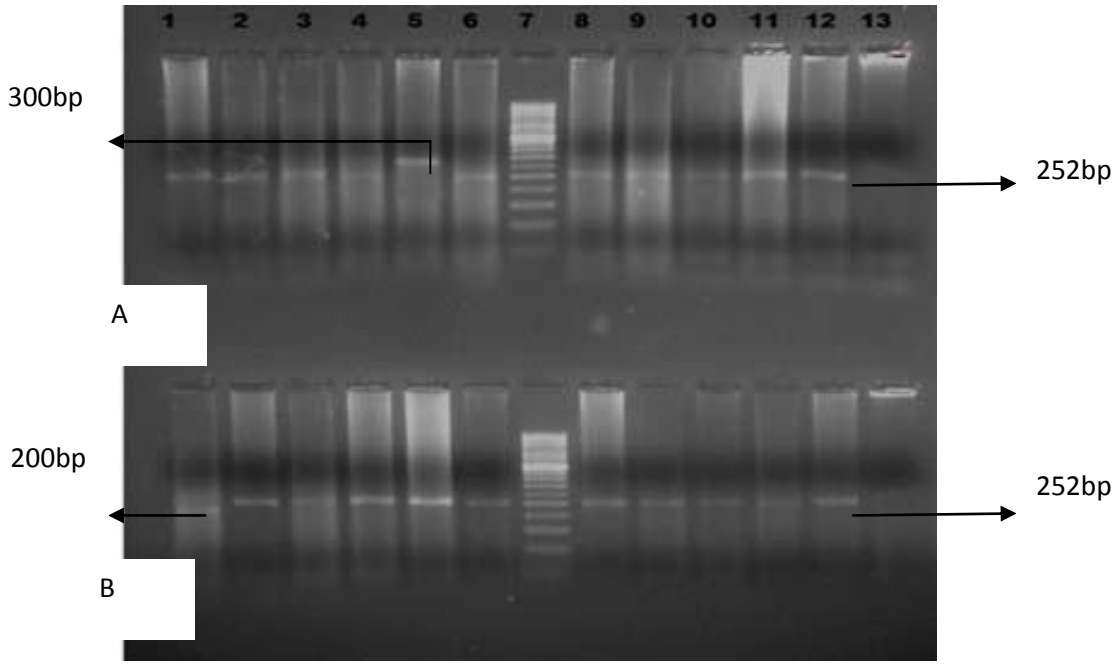

Fig 4.3: Representative gel picture showing 3 % Agarose gel electrophoresis of some controls.

Gel A: Lane1,2, 3, 4, 6,8,9,10,11and 12 represent homozygous condition (252bp); lane 5 represents homozygous condition (300bp); lane 7 represents DNA marker (50bp); lane 13 represents negative control.

Gel B: Lane1 represents homozygous condition (200bp); lane3,4,5,6,8,9,10,11,12 represents homozygous condition(252bp);lane 7 represents DNA marker (50bp); lane 13 represents negative control.

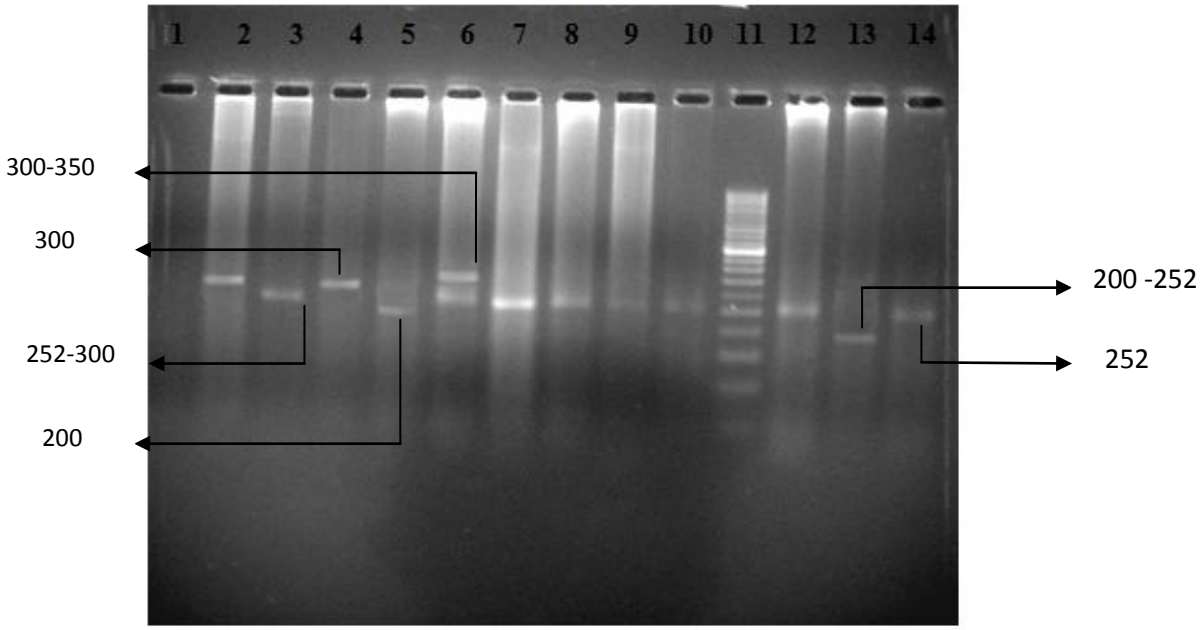

Fig 4.4: Representative gel picture showing 3% Agarose gel electrophoresis of some samples. Lane1 represents negative control; lane11 represents 50 bp ladder; lane 2 and 4 represents homozygous condition (300bp); lane 3 represents homozygous condition (252-300bp) lane 5 represents homozygous condition (200bp); lane 6 represents heterozygous condition(252bp/300-350bp); lane7,8,9,10,12, and 14 represents homozygous condition (252bp); lane13 represents homozygous condition(200-252bp).
Lanes 3,4,6,8 and 13 represents cases Lanes 2,5,7,10 and 12 and 14represents controls

Table. 4.2 Frequency of D18S452 alleles (major and minor alleles) in the study population.

ALLELOTYPE	Cases n^s (%)	Controls n^s (%)
200	2(1.35)	2 (1.35)
200-252	2 (1.35)	0 (0.0)
252	129 (85.16)	144(97.297)
252-300	2 (1.35)	0(0.0)
300	11(7.43)	2 (1.35)
300-350	2(1.35)	0 (0.0)
Total	**74**	**74**

n^s= No. of Alleles

Table 4.3: Frequency of D18S452 alleles (after grouping) in the study population.

ALLELOTYPE	Cases n^s (%)	Controls n^s(%)
252*	136(91.89)	146(98.65)
300*	12(8.108)	2(1.35)

n^S= No. of Alleles

Table 4.4: Genotypic frequencies of D18S452 polymorphism in the study population.

POLYMORPHISM	Cases n (%)	Controls n (%)	OR (95% CI)	Pvalue*
252/252	66(89.18)	73 (98.64)	1	-
300/300	4 (5.40)	1 (1.35)	4.4242 (0.4822-40.5924)	0.1529
300/252	4 (5.40)	0	8.0603 (1.1112-58.4646)	0.0383*
Total	**74**	**74**		

*P using χ^2 test

P<0.05 (Data statistically significant)

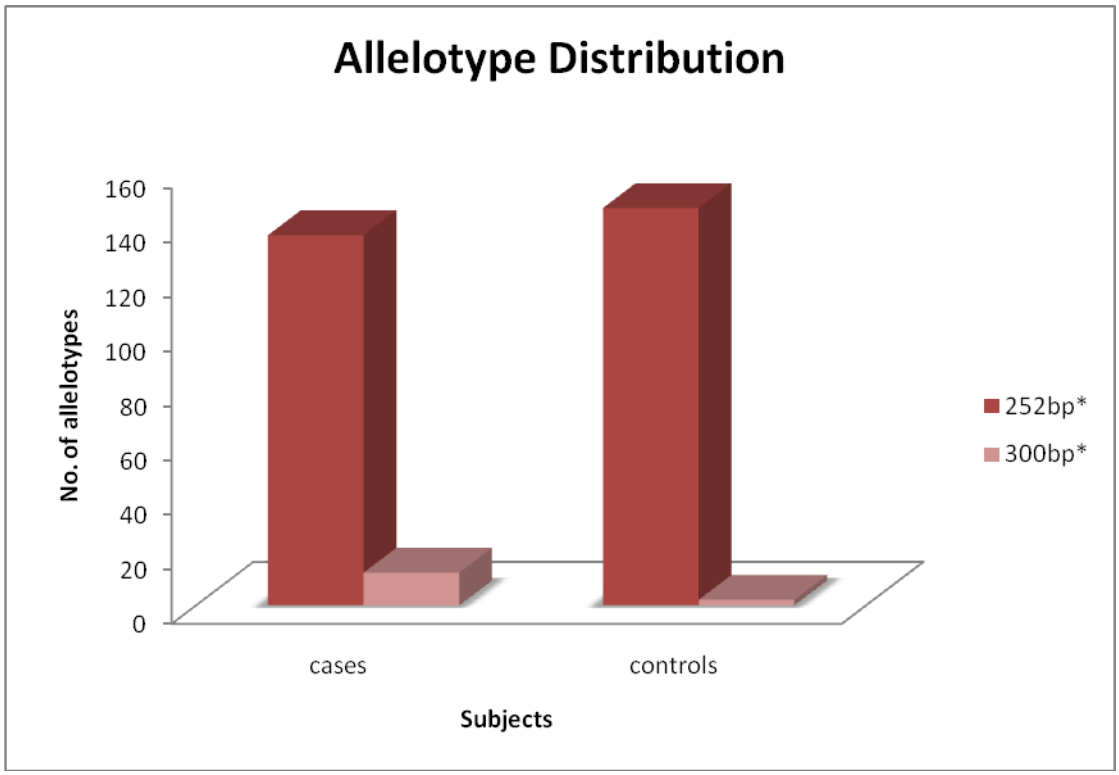

Figure 4.5: Histogram showing the allele distribution in BPAD patients and controls.

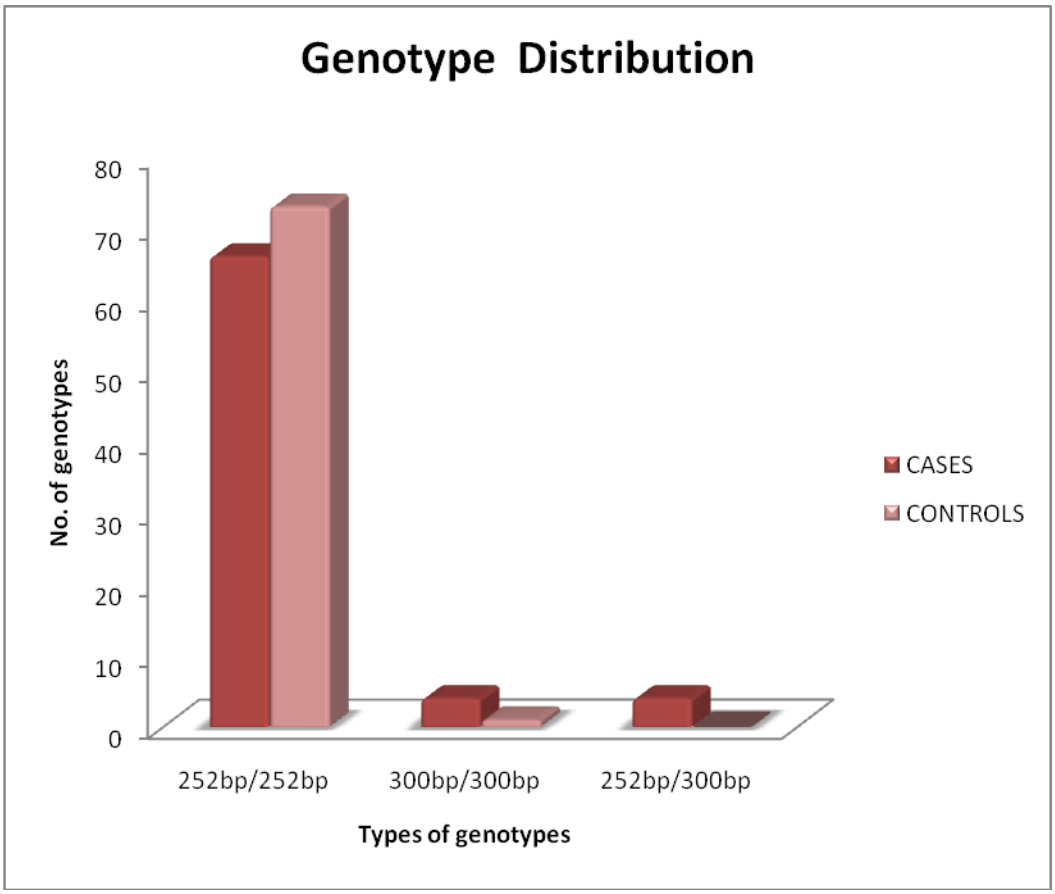

Fig 4.6: Histogram showing the genotype distribution in BPAD patients and controls.

4.4 CLINICO-PATHOLOGICAL CHARACTERISTICS OF BPAD CASES

74 blood samples from BPAD patients were analyzed and correlation made between D18S452 polymorphism and clinico-pathological characteristics as is given in the table 4.2.

Table 4.5: Correlation between D18S452 polymorphism and clinico-pathological characteristics.

VARIABLES	D18S452 POLYMORPHISM			X^2 ; P-value*
	252bp/252bp n(%)	252bp/300bp n(%)	300 bp /300 bp n(%)	
Gender				
Males n(%)	37(43.24)	4(5.40)	2(2.70)	3.105413; 0.211674
Females n(%)	29(39.2)	0(0.0)	2(2.70)	
Dwelling				
Rural n(%)	50(67.57)	4(5.40)	4(5.40)	2.474399;0.290196
Urban n(%)	16(21.62)	0(0.0)	0(0.0)	
Economic Status				
Lower n(%)	43(58.11)	4(5.40)	4(5.40)	4.045157;0.132314
Middle n(%)	23(31.08)	0(0.0)	0(0.0)	
Upper n(%)	0(0.0)	0(0.0)	0(0.0)	
BPAD Sub Type				

BPAD I **BPAD II**	62(83.78) 4(5.40)	4(5.40) 0(0.0)	4(5.40) 0(0.0)	0.512;0.773
Family History **Yes** **No**	32(43.24) 34(45.94)	2(2.70) 2(2.70)	3(4.05) 1(1.35)	1.06; 0.588
Age at onset **< or =18yrs** **>or=18yrs**	12(16.21) 55(74.32)	1 1(1.35) 3(4.05)	2(2.70) 2(2.70)	2.495; 0.287

*P using χ^2 test

P<0.05 (Data statistically significant)

CHAPTER: 5

DISCUSSION

Human genetics is now at a critical juncture. The molecular methods used successfully to identify the genes underlying rare mendelian syndromes are failing to find the numerous genes causing more common, familial, non-mendelian diseases." (Risch, 2000). Application of the gene mapping strategies which drew such success in the field of Mendelian disease to the field of complex disease is plagued by a marked lack of success (Cardon *et al.,* 2001) with just a few confirmed cases of a gene associated with disease (Hirschhorn *et al.,* 2002).

Since most psychiatric disorders show a complex mode of transmission, therefore the problems plaguing complex disease genetics are also common to the psychiatric genetics field with a general air of disappointment that despite the recent technological advances there has been a distinct lack of success in pinpointing genes underlying the common psychiatric and behavioral disorders (Owen et al. 2000; Merikangas and Risch 2003). Identifying genes involved in the pathophysiology of BPAD therefore remains a challenge, while as finding even a single gene would change the whole scenario in the treatment and management of the disorder. This is likely to have important public health implications as the mood disorder is identified as second only to ischaemic heart disease as a cause of global health burden in 2020 (Murray *et al.,* 1997). In all regions, neuropsychiatric conditions are the most important causes of disability, accounting for around one third of YLD (years lost due to disability) among adults aged 15 years and above (The global burden of disease, 2004 update).

However all hope may not be lost, as after initial disappointment the attitude seems to be 'regroup and come back stronger' and so new strategies for approaching complex disease gene mapping are emerging and this together with the falling cost of genotyping and the new influx of polymorphism data from the Hap Map Project have brought genome-wide association studies of complex diseases to within our reach (Barrett *et al.,* 2006), and it is exactly this approach which seems to hold the most promise for shedding light on the genes for complex disease.

As in other neuropsychiatric disorders, in BPAD also studies carried out so far and their results have not been replicated and the studies are fraught with inconsistencies and suffer from at least some methodological flaws. Most of our current knowledge on genetics of BPAD is hypothesis based and some important aspects remain yet to be determined. For example, the pathophysiology of BPAD

remains largely unknown. Hence a functional candidate gene cannot be determined with certainty, unless each of the perhaps 30,000 human genes are to be considered as candidate genes. We therefore, aimed to associate the susceptible chromosomal locus to BPAD by carrying a case-control study as has been done successfully at other loci in BPAD (Buttenchøn *et al.,* 2010) and other complex disorders like Alzheimer disorder (Gohlke *et al.,* 2006).

Linkage and association studies of bipolar affective disorder (BPAD) point out chromosome 18 as a region of interest and the locus 18p11 as a very potential susceptibility locus Bennett *et al.,* 2002. To investigate this region further, we conducted an association study of a marker D18S452 (locus 18p11.2) in a Kashmiri sample of 74 patients with BPAD and 74 control (age, sex and region matched) individuals. The repeat polymorphism was evaluated by PCR analysis of DNA obtained from the blood of the subjects after checking their integrity (Fig. 4.1) and purity (which would yield amplicon of 252 bp in normal population). Microsatellite marker D18S452 contains 26 dinucleotide repeats (CA) (http://genome.ucsc.edu/cgi-bin/hgTracks?org=human). If the frequencies of microsatellite markers differ significantly between patients and controls, then we can associate the polymorphism of the marker with the predisposition to BPAD.

On genotyping, several alleles for D18S452 marker were observed (Fig. 4.2 to 4.4). There were six types of alleles corresponding to base pair size: 200 bp; between 200-252bp; 252bp; between 252-300bp; 300bp; between 300 and 350bp. The allelotype 252 bp and 300bp had higher frequencies than the other alleles. We considered them as major alleles. Only two samples one from case (1.35%) and one from control group (1.35%) had allelotype corresponding to 200 bp. One sample from case (1.35%) had allelotype corresponding to between 200bp to 250 bp. Again, only one sample from cases (1.35%) had allelotype corresponding to between 252bp and 300bp and one sample (1.35%) had allelotype corresponding to between 300 to 350bp. Sixty three patients (87.16%) and 72 controls (97.297%) had allelotype corresponding to 252bp, Seven patients (7.43%) had allelotype corresponding to 300bp, while as only one sample (1.37%) from controls had allelotype corresponding to 300bp (Fig. 4.2 to 4.4, Table 4.2).

Because the alleles corresponding to bp size 200, 200-252bp, 252-300bp and 300-350bp were very rare in our study subjects, we considered them as minor alleles and

grouped them under two sets of major alleles 252bp* (containing alleles of size upto 252bp) and 300bp* (containing alleles corresponding to more than 252bp).

After grouping, we observed that the tandem repeat (300bp*) allele frequency was found to be 1.35 % in the controls and 8.108% in cases. The tandem repeat (250bp*) allele frequency was found to be 91.89% in cases and 98.65% in controls. The 252bp/252bp genotype was found to be present in 89.18% of the cases and 98.64% of the controls, the 252bp/300bp variant in 5.40% of the cases and none among the controls and the 300bp/300bp genotype in 5.40% of cases and 1.35% of controls.

It was observed that although the proportion of individuals who were homozygous for the tandem repeat (300bp/300bp) was higher in cases than in controls, the difference was not statistically significant when using 252bp/252bp genotype as a reference (OR= 4.4242; 95% CI, 0.4822-40.5924); P = 0.1529). However, it was observed that the frequency of the heterozygous genotype (252bp/300bp) when compared with 252bp/252bp showed statistical significance (OR=8.0603; 95% CI, 1.1112-58.4646; P = 0.0383) (Table 4.4).

The above results were also expressed geometrically in the form of histograms (Fig. 4.5-4.6). The 252*bp allele had higher frequency in both cases and controls than the 300*bp allele and the 300* bp allele had higher frequency in cases than controls.

For genotypes, 252bp/252bp genotype was higher in cases as well as controls, followed by 300bp/300bp genotype; 252bp/300bp genotype was present only in cases and none among the controls.

Only four samples from the cases group were found to have the heterozygous (252bp/300bp) genotype, which was not present in any of the sample from control group. This is the first study reporting the association of the marker D18S452 in heterozygous condition (252bp/300bp) with BPAD. Yet, it would be too early to associate this genotype with the predisposition to BPAD. Therefore, further study with larger sample size should be carried out to validate the result.

We also report for the first time, alleles for the microsatellite marker D18S452. Most of the alleles were found to be very rare in our population. This may be because of small sample size in our study. Therefore, further investigation with larger sample size is required.

We also tried to correlate many clinico- phenotypic conditions like, BPAD subtype, age at on set and other general characteristics with BPAD but we could not find any significant association (Table 4.5). This may be due to many reasons; small sample size, lack of the endophenotypic marker analysis, disease phenotype, co-morbid conditions, and environmental factors and therefore requires further investigation with larger sample size and keeping in consideration the various physiological, clinical and environmental parameters (Lenox *et al.*, 2002).

Overall, our study suggests that there is a significant association between tandem repeat polymorphism in heterozygous condition (252bp/300bp) and the development of BPAD in Kashmiri population. Chromosomal locus 18p11.2 harbors many candidate genes like NAPG (Weller *et al.,* 2006), GNAL (Tsiouris *et al.,* 1996) NDUFV2 (Wasizuka *et al.,* 2009) and VAPA (Lohoff *et al.,* 2008). It may be possible that this marker is directly or indirectly involved in the regulation of neighboring genes. It is also possible that this locus may be in linkage disequilibrium with other genes. However, it is too early for such hypothesis unless further studies are carried out with larger sample size.

The result of our study further adds to the data from several other studies which have also shown an association between chromosomal 18 locus and the risk of BPAD.

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APPENDIX

PROFORMA (Cases)

1. **Name: _____Code No. : _____**
2. **Age/Sex: _____**
3. **Occupation: _____**
4. **R/O: _____**
5. **C.No: _____**
6. **Economic Status:**

a) Lower b) Middle c) Upper

7. **Ethnicity/Region:**

a) Kashmiri b) Gogri c) Pahari

d) Ladakhi e) Others_____

8. **Marital Status:**

a) Single b) Married c) Widow/Widower

d) Divorced/Separated

9. **Family Status:**

a) Nuclear b) Joint c) Others

10. **Education:**

a) Nil b) less than 5^{th} standard c) 5^{th} to 8^{th} standard

d) high school e) 10 plus 2 f) Graduation

g) PG h) Others

11. **Family History:**

a) Yes b) No c) Can't say

12. **Age at onset_____**

13. Drug History:

Past (with dosage):

Present (with dosage):

14. Adverse effects with any drug:

15. Responder/ Non Responder

16. % respondance

PROFORMA (Controls)

1. **Name: _____Code No. : _____**
2. **Age/Sex:_____**
3. **Occupation:_____**
4. **R/O: _____**
5. **C.No: _____**
6. **Economic Status:**

a) Lower b) Middle c) Upper

7. **Ethnicity/Region:**

a) Kashmiri b) Gogri c) Pahari

d) Ladakhi e) Others_____

8. **Marital Status:**

a) Single b) Married c) Widow/Widower

d) Divorced/Separated

9. **Family Status:**

a) Nuclear b) Joint c) Others

10. **Education:**

a) Nil b) less than 5^{th} standard c) 5^{th} to 8^{th} standard

d) high school e) 10 plus 2 f) Graduation

g) PG h) Others

11. **Have you ever received treatment for or been diagonosed with any of the following conditions:**

a) Schizophrenia

b) Bipolar Disorder

c) Hearing voices that others cannot hear or believing things that others say were not true

No

12. Do you have a family history of bipolar disorder, schizophrenia or alcoholism:

Yes/No

13. Any other disease\disorder_____

Chemicals and other miscellaneous items

The details of chemicals and other miscellaneous items used in the study

Chemical /Enzyme	**Source**
Absolute ethanol	Bengal Chemicals& Pharmaceuticals Ltd
Ammonium Chloride	Qualgens
Bromophenol Blue	Qualgens
Chloroform	Thomas Bakers
Ethedium Bromide	SRL
Ethylene Diamine Tetra Acetate	Loba Chemie
Glacial Acetic Acid	SRL
Hydrochloric Acid	Qualgens
Isoamyl Alcohol	Thomas Bakers
Isopropanol	Thomas Bakers
Low Melting Agarose	SRL
8-Hydroxyquinoline	CDH
Phenol	SRL
Potassium Bicarbonate	Qualgens
Sodium Dodecyl Sulfate	Qualgens
Sodium Acetate	SRL
Sodium Chloride	Merck
Sodium Hydroxide	Himedia
Sucrose	SRL

Te Buffer	SRL
Tris Base	SRL
Tris Hcl	Himedia

Enzymes

Taq Polymerase	Banglore Genie
Proteinase K	SRL

Miscellaneous Material

pBR322 DNA- Msp1 Digest	New England Biolabs
50 Bp DNA Ladder	Banglore Genie

REAGENTS

Reagents for DNA Extraction

Chloroform Isoamyl alcohol (CIA)

Chloroform	24.0ml
Isoamylalcohol	1.0ml

Chloroform: isoamylalcohol, in the ratio 24:1 was prepared by mixing 24 ml of Chloroform and 1 ml of isoamyl alcohol. The solution was stored at 4˚C in dark bottle.

DNA storage buffer:

0.5 M EDTA	0.01 ml
1 M Tris	0.5 ml

Final volume was made 50 ml with sterile distilled water.

Lysis buffer

1 M Tris	2.0ml

0.5M EDTA	400µl
10% SDS	30.0ml

Final volume of the solution was made 100ml with sterile distilled water.

Lysis solution:

1 M Ammonium Chloride	15.5ml
1M Potassium bicarbonate	1.0ml
0.5 M EDTA	200µl

Final volume was made 100ml with sterile distilled water.

Proteinase K

Proteinase K	10mg

Proteinase k was dissolved in 1 ml of demonized water and stored at -20˚C in aliquots of 1 ml each.

SE Solution:

5 M Sodium chloride	3ml
0.5 M EDTA	8ml

Final volume was made 200ml with sterile distilled water. pH was adjusted to 8.

SDS (10%):

SDS	10g

SDS was dissolved in 100 ml sterile distilled water.

Sodium acetate (3M):

Sodium acetate	40.83g

Sodium acetate was dissolved in 100ml sterile distilled water; pH of the solution was adjusted to 5.0 using acetic acid.

Saturated Phenol:

0.2% β- mercaptoethanol

8-hydroxy quinoline

0.1 M Tris chloride buffer	800ml
0.5 M Tris chloride buffer	1000ml
Phenol	1000ml

The mixture obtained by adding equal volume of 0.5M Tris- Cl buffer and melted phenol was stirred for 15 min on magnetic stirrer. Two phases were allowed to separate. Upper aqueous phase was removed and equal volume of 0.1 M Tris-chloride buffer was added. The saturation with 0.1 M Tris-Cl buffer was repeated till phenol> 7.8 pH was obtained. A pinch of β-mercaptoethanol and 8-hydroxy quinoline was added. Phenol was stored at 4˚C in a dark bottle.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS:

Agarose (3%)	SRL
Agarose	3g
Buffer	100ml
EtBr	5.0µl

Agarose was dissolved in a buffer and heated till a clear solution is formed. EtBr was then added to the solution.

Bromophenol Blue:

Bromophenol Blue	0.4g
Sucrose	20.og

Bromophenol blue was dissolved in 100ml of distilled water.

From the above stock solution 31.25ml was taken and sucrose was added. Final volume was made 50ml with distilled water.

Ethidium Bromide:

Ethidium bromide 10mg

Ehidium Bromide was dissolved in 1ml of distilled water. The solution was stored in a dark bottle at 4˚C.

50X TAE (pH 8.0):

Tris base 242g

0.5M 100ml

Glacial acetic acid 57.1ml

Final volume was made 1000ml with distilled water.

1X TAE (pH 8.0):

50 X TAE 20ml

Final volume was made 1000ml with distilled water.

Reagents for PCR:

Stock

Deoxyribosenucleotidetriphosphate(dNTP) 10mM each dATP, dGTP, dCTP and dTTP.

Taq polymerase (5U/µl)

10X Taq buffer

50mM $MgCl_2$.

Primers: 100mM in sterile deionised water

pBR322 DNA- Msp1 digest (1000µg/ml)

REAGENTS FOR PURIFICATION OF PCR PRODUCTS FOR SEQUENCING

Sodium Iodide Solution

Sodium Iodide and Sodium Bisulphate

Glass Milk

Nitric acid treated glass beads (Silicon dioxide)

Wash Buffer

Tris HCl pH 7.4 and Absolute Ethanol in 1:1 ratio

Glass /Plastic ware

The details of Glass ware and Plastic ware used during the course of study

Glass/ Plastic item

Centrifuge tubes

Micro-pipettes

Glass pipettes

Syringes

EDTA-Vials

Microfuge tubes

Micro-tips

Tip-boxes

Storage racks

Measuring cylinders

Beakers

Reagent bottles

Test tube stands

Equipments

The details of equipments used during the course of study

Equipment	Source
Deep freezer (-20˚C)	Nirmal Instruments
Centrifuge	Avanti™
Spectrophotometer	ELICO
Vortex mixer	Remi
Hot plate	SHIV
Magnetic stirrer	SHIV
Thermal cycler	Eppendrof
Electrophoresis apparatus	BangloreGeni
Power supply	Genei
UV-Tran illuminator	Hoeser
Incubator	Techno
Autoclave	Techno
Refrigerator	Godrej
Ph meter	HANNA
Gel documentation unit	Alpha Innotech Corporation
Stabilizer	Philips