BIPOLAR DISORDER: MICRORNA PROFILING AND GENETICS COMPARISON WITH SCHIZOPHRENIA IN A MALAYSIAN POPULATION

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF MEDICAL SCIENCES

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITY OF MALAYA

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Registration/Matric No: MGN 130003

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Field of Study: Pharmacogenomics

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ABSTRACT

Recent studies have shown that bipolar disorder (BPD) and schizophrenia (SZ) share some common genetic risk factors. This study aimed to examine the association between candidate single nucleotide polymorphisms (SNPs) identified from genome-wide association studies (GWAS) and risk of BPD and SZ. A total of 715 patients (244 BPD and 471 SZ) and 593 controls were genotyped using the Sequenom MassARRAY platform. We showed a positive association between LMAN2L (rs6746896) and risk of both BPD and SZ in a pooled population (P-value=0.001 and 0.009, respectively). Following stratification by ethnicity, variants of the ANK3 gene (rs1938516 and rs10994336) were found to be associated with BPD in Malays (P-value=0.001 and 0.006, respectively). Furthermore, an association exists between another variant of LMAN2L (rs2271893) and SZ in the Malay and Indian ethnic groups (P-value=0.003 and 0.002, respectively). Gene-gene interaction analysis revealed a significant interaction between the ANK3 and LMAN2L genes (empirical P=0.0107). Significant differences were shown between patients and controls for two haplotype frequencies of LMAN2L: GA (P=0.015 and P=0.010, for BPD and SZ, respectively) and GG (P=0.013 for BPD). Our study showed a significant association between LMAN2L and risk of both BPD and SZ. Although major progress has been achieved in terms of research and development, there stills exists gap in the knowledge of molecular mechanisms underlying bipolar disorder (BPD) and action pathway of atypical antipsychotics. MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression, including genes involved in neuronal function and plasticity. This study aimed to examine the changes in miRNA expression in the blood of 14 bipolar mania patients following 12 weeks of treatment with asenapine and risperidone using miRNA microarray. A total of 24 miRNAs were differentially expressed after treatment in asenapine group, 22 of which were significantly up-regulated and the other two were

significantly down-regulated. However, all three differentially expressed miRNAs in the risperidone group were down-regulated. MiRNA target gene prediction and gene ontology analysis revealed significant enrichment for pathways associated with immune system response and regulation of programmed cell death and transcription. Our results show that miRNAs were involved in the pathway mechanism of both antipsychotics.

ABSTRAK

Kajian menunjukkan bahawa gangguan bipolar dan skizofrenia berkongsi factor beriski yang sama. Kajian pada kali ini bertujuan untuk mengkaji pertalian antara Single nucleotide polymorphisms (SNP) terpilih daripada kajian GWAS yang terdahulu dengan risiko gangguan bipolar dan skizofrenia. Sejumlah 715 pesakit (244 pesakit bipolar dan 471 skizofrenia) degan 593 subjek control menjalani genotyped menggunakan platform Sequenom MassARRAY. Kami menunjukan terdapat pertalian positif antara LMAN2L (rs6746896) dengan risiko mendapat gangguan bipolar dan skizofrenia di populasi berkongsi (p = 0.001 dan 0.009). Berikut perpecahan kepada kaum, SNP dari ANK3 gen (rs1938516 dan rs10994336) telah didapati pertalian dengan bipolar di kaum Melayu (p = 0.001 dan 0.006). Selain itu, terdapat juga petalian antara rs2271893 dari LMAN2L gen dengan risiko skizofrenia di kaum Melayu dan India (p =0.003 dan 0.002). Interaksi antara gen menunjukkan terdapat pertalian antara ANK3 dengan LMAN2L gen (p=0.0107). Perbezaan yang penting juga terlihat dalam dua frekuensi haplotype antara pesakit dan control bagi LMAN2L gene, iaitu GA (p=0.015 dan 0.010 bagi Bipolar dan Skizofrenia) dan GG (p =0.013 bagi BPD). Kajian kami menunjukkan terdapat pertalian yang penting antara LMAN2L dengan risiko bipolar dan skizofrenia.Walaupun perkembangan dalam kajian terhadap antipsychotic telah bermaju, namun masih terdapat perkosongan dalam perfahaman mekanisme molekul bipolar dengan antipsychotic. MicroRNA (miRNA) adalah rna molekul yang kecil dan mampu mengawal ekpressi gen, termasuk gen yang terlibat dalam fungsi neuron yang mempunyai kepentingan bagi fungsi otak dan kesihatan mental. Kajian ini bertujuan untuk mengkaji perubahan dalam expressi miRNA di darah 14 pesakit bipolar setelah 12 minggu dirawat dengan asenapine dan risperidone menggunakan miRNA microarray. Terdapat 24 miRNA yang berbeza expres setelah rawatan di kumpulan asenapine, di mana 22 miRNA di atas express dan 2 lagi di bawah express. Manakala

hanya 3 miRNA yang terdapat di kumpulan risperidone, dan semuanya telah di bawah express. Kajian target miRNA menunjukkan terdapat 35 gen yang bertalian dengan bipolar di kumpulan asenapine, manakala terdapat 6 gen yang bertalian dengan bipolar di kumpulan risperidone. Kajian kami menunjukkan bahawa miRNA berkenanan terlibat dengan tindakan mekanisme kedua-dua antipsychotic berkenaan.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my foremost gratitude to both my supervisors, Prof Datin Dr Zahurin Mohamed and Prof Dr Nor Zuraida Zainal for their continuous guidance to allows me to finish my research project.

Next, I would like to thanks Prof Gavin Reynolds, the visiting professor in the department, who is also the project advisor for my project. He always advised me on improving my research design. I would also like to thanks Dr Shamsul Mohd Zain, for his efforts in assists me in analyzing the data.

My sincere gratitude also goes to Assoc. Prof Datin Dr Sharmilla Kanagasundram, all the medical officers and staff nurse of Psychological Medicine Department in providing any kinds of assistance in helping me to finish my research project.

I would like to acknowledge the financial assistance and technical support provided by University Malaya, Department of Pharmacology and also all the members of Pharmacogenomics Laboratory. Last but not least, I would like to thanks my parents for their continuous support in pursing my study.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
±	plus minus
µg	microgram
µL	microliter
AD	alzheimer disease
ANK3	Ankyrin-3
ANOVA	Analysis of variance
ATP	Atypical Antipsychotics
BDNF	Brain-derived neurotrophic factor
BPD	Bipolar Disorder
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit
CACNB2	calcium channel, voltage-dependent, beta 2 subunit
cDNA	Complementary Deoxyribonucleic acid
CI	Confidence Interval
ct	comparative threshold
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
g	Gravity force
GABA	gamma-aminobutyric acid
GMDR	Generalized Multifactor Dimensionality Reduction
GWAS	Genome Wide Association Studies

HWE	Hardy–Weinberg equilibrium
IPA	Ingenuity Pathway Analysis
KCTD12	Potassium channel tetramerization domain containing 12
LMAN2L	lectin, mannose-binding 2-like
LD	Linkage disequilibrium
MAF	Minor Allele Frequency
MARK1	MAP/microtubule affinity-regulating kinase 1
MeCP2	methyl CpG binding protein 2
miRNA	microRNA
ml	Millilitre
NAPG	N-Ethylmaleimide-Sensitive Factor Attachment Protein, Gamma
NPAS4	neuronal PAS domain protein 4
NRG1	Neuregulin 1
OR	Odds ratio
PDE10A	phosphodiesterase 10A
PER1	period circadian protein homolog 1
PTSD	Post-traumatic stress disorder
RGS4	Regulator of G protein signaling 4
RNA	ribonucleic acid
SD	Standard Deviation
SNP	Single nucleotide Polymorphisms
SP4	SP4 transcription factor
SP8	specificity protein 8
ST8SIA2	ST8alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2

SZ	Schizophrenia
TAE	Tris-acetate-EDTA
TBE	Tris/Borate/EDTA
TRANK1	tetratricopeptide repeat and ankyrin repeat containing 1
UMMC	University Malaya Medical Centre
UV	Ultra violet
YMRS	Young Mania Rating Scale

LIST OF APPENDICES

CHAPTER ONE: INTRODUCTION

Bipolar Disorder is a chronic affective disorder with significant social burden. Although the exact causes of BPD are unknown, genetics factors have been proposed to be one of the risk factors. This has been well established through twin and family studies. Despite the fact that development of drugs for the treatment for BPD has achieved major advancement, such as the atypical antipsychotic agents, which have long been the first-line treatment for acute mania, there stills exists variability in terms of treatment response and the existence of various adverse effects. These include correct diagnosis and medications, adequate dose given, optimum duration and good compliance. Genetic variability in different individuals has been suggested as the possible causes of the variability in occurrence of treatment response and occurrence of adverse effects.

Single nucleotide polymorphism (SNP) have been the subject of research since the past decades, as variation in DNA sequence could bring about varying degree of susceptibility to the diseases, as well as in treatment response. The fact is, BPD, like other psychiatry illness, is a complex disorder that can be influenced by multiple gene interactions, and also by environmental factors. More recently, microRNA (miRNA) has gained important insights in molecular research of BPD, most probably due to its ability to regulate post translational gene expression.

With the involvement of advance genetic research tools, the molecular mechanisms underlying BPD, as well as treatment mechanism have begun to unfold. This is rather important, as brain disorders, like BPD, may involve changes in the brain long before the first symptoms emerged. With early detection and early intervention, this can bring about better treatment outcome.

Objectives:

The aims of this study are:

1. To examine the association of SNP of the candidate genes with susceptibility to BPD and SZ in a multi-ethnic Malaysian population.

2. To study the genetic overlap between BPD and SZ in a multi-ethnic Malaysian population.

3. To investigate the gene-gene interaction of the candidate SNPs.

4. To examine the changes in the expression of miRNA in the patients of BPD mania before and after the treatment of atypical antipsychotics.

5. To examine the changes in the scores of YMRS in the patients of BPD mania before and after the treatment of atypical antipsychotics.

Research Hypothesis

1. There is an association of SNP of the candidate genes with susceptibility to BPD and SZ in multi-ethnic Malaysian population.

2. There is genetic overlap between BPD and SZ in multi-ethnic Malaysian population.

3. There is gene-gene interaction between the candidate SNPs.

4. There are changes in the expression of miRNA in the patients of BPD mania before and after the treatment of atypical antipsychotics.

5. There are changes in the scores of YMRS in the patients of BPD mania before and after the treatment of atypical antipsychotics.

CHAPTER TWO: GENETIC ASSOCIATION OF LMAN2L GENE IN BIPOLAR DISORDER AND SCHIZOPHRENIA AND ITS INTERACTION WITH ANK3 GENE POLYMORPHISM

2.1 INTRODUCTION

Bipolar Disorder (BPD) is a severe mental disease with profound social and economic impacts. BPD accounted for 7% of disability-adjusted life years worldwide, as indicated by the Global Burden of Diseases 2010 report (Whiteford et al. 2013). It is a condition where the patients have excessive mood swings between depression and mania, in addition to changes in daily activities.

The possible causes of BPD are still under study, but among those, the changes in the brain structure and genetics factors seems to be important factors. BPD tends to run in families. Person with a family history of BPD are at a higher risk of having BPD. Besides that, studies also suggests that BPD share some common genetic roots with another psychiatric illness, schizophrenia (SZ). (Berrettini 2000, Lichtenstein et al. 2009)

Every individual carries different genetic material. This contributes to genetic variations among individual and allows one to differentiate from the other. There are a few types of genetic variations in human, such as single nucleotide polymorphism (SNP), structural alterations, and chromosome alterations. Among these, SNP are the most extensively studied. SNP is a single alteration in the DNA sequence. Since DNA sequence are translated into RNA, and followed by protein synthesis, any alteration in DNA sequence will result in subsequent alteration in the protein synthesis, and hence alteration in function of the body. SNP are preferably studied compared to other variation due to its occurrence at significant frequency (>1%) in human population, and complete SNP study are attainable through high-throughput genotyping (Wang et al. 1998). Although not all SNP will cause diseases, there are some SNP, however that are associated with certain diseases. This is known via studies done through case-control

studies in which comparison is made of the SNP genotyping between cases and healthy control of particular disease.

As psychiatric illness is a complex disease that results from the interaction of environment factors and genetic factors, searching for the exact patho-physiology of BPD seems far beyond reach. The environmental factors include early parental, particularly maternal loss, which increases the risk for BPD in the offspring (Tsuchiya et al. 2005) and also exposure to childhood traumatic event (Etain et al. 2008). However, through identifying SNP that are associated with susceptibility to the disease, a few things can be achieved. First, the mechanisms and molecular pathway underlying the disease can be further studied through identification of the gene involved. Second, a particular genotyping test can be developed to help in improved diagnosis of the disease, and also personalized treatment with better treatment outcome and lesser side effects.

Considering the possible genetic overlap between BPD and SZ (Skibinska et al. 2004, Cordeiro et al. 2005, Green et al. 2005), we set out to examine the association between variants within these genes and in these two disorders. We hypothesized the root genetic cause of BPD and SZ by highlighting genetic variants that overlap in both disorders, as well as the genetic variants that are distinctly different between the two conditions. The BPD and SZ patients in our study were of three major ethnic groups in Malaysia: Malays, Chinese, and Indians; each of which are presumably from a different genetic pool. Hence, this sample provided a good opportunity to study ethnic differences in susceptibility to BPD and SZ as well as the association with gene polymorphisms.

2.1.1 Objectives

The aims of this study are:

1. To examine the association of SNP of the candidate genes with susceptibility to BPD in a multi-ethnic Malaysian population.
2. To examine the association of SNP of the candidate genes with susceptibility to SZ in a multi-ethnic Malaysian population.
3. To study the genetic overlap between BPD and SZ in a multi-ethnic Malaysian population.
4. To investigate the gene-gene interaction of the candidate SNPs.

2.1.2 Justification of Study

BPD affects 2.4 percent of the world's population, with the highest rates of prevalence in the United States and lowest in India (Merikangas et al. 2011). In Malaysia, there is still lack of research data regarding the genetic susceptibility of BPD in the Malaysian population. According to best estimates, about 1% of the Malaysian population suffers from BPD, that is, there are about 250,000 people with BPD in Malaysia. Thus, genetic study on BPD is necessary to provide more genetic information on BPD especially in the Malaysian population. Besides, this study also aims to recognize susceptible genes for BPD and also SZ. It is hoped that the result of this study can provide more clues on the genetic overlapping of both BPD and SZ, and further the molecular mechanisms underlying both diseases.

2.1.3 Research Hypothesis

The null hypothesis

1. There is no association of SNP of the candidate genes with susceptibility to BPD in multi-ethnic Malaysian population.
2. There is no association of SNP of the candidate genes with susceptibility to SZ in multi-ethnic Malaysian population.
3. There is no genetic overlapping between BPD and SZ in multi-ethnic Malaysian population.
4. There is no gene-gene interaction between the candidate SNPs.

The research hypothesis

1. There is no association of SNP of the candidate genes with susceptibility to BPD in multi-ethnic Malaysian population.

2. There is no association of SNP of the candidate genes with susceptibility to SZ in multi-ethnic Malaysian population.

3. There is no genetic overlap between BPD and SZ in multi-ethnic Malaysian population.

4. There is no gene-gene interaction between the candidate SNPs.

2.2 LITERATURE REVIEW

2.2.1 Bipolar Disorder

BPD refers to a group of affective disorders, in which patients experience two different episodes of mood changes, namely the episode of mania/hypomania and the episode of depression, with the episodes alternating between each other (Phillips and Kupfer 2013). According to Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV), there are four main subtypes of BPD:

i. BPD type I (episodes of depression and at least one episode of full-blown mania)
ii. BPD type II (several prolong episodes of depression and at least one hypomanic episode but no manic episodes)
iii. cyclothymic disorder (many periods of hypomanic and depressive symptoms, in which the depressive symptoms do not meet the criteria for depressive episodes)
iv. BPD not otherwise specified (depressive and hypomanic-like symptoms and episodes that might alternate rapidly, but do not meet the full diagnostic criteria for any of the above mentioned illnesses).

BPD I and II have high prevalence of being wrongly diagnosed, especially in their early stages (Hirschfeld et al. 2003). The main reason being the difficulty to differentiate BPD patients experiencing depressive phrase from Major Depression, especially those with no history of mania or hypomania (Hirschfeld et al. 2003). Increasing evidence have shown that patients diagnosed with Major Depression may actually have a misdiagnosed BPD (Goldberg et al. 2001).

Table 2.1.1 Symptoms of Bipolar Disorder

Symptoms of mania or a manic episode include:	**Symptoms of depression or a depressive episode include:**
Mood Changes A long period of feeling "high," or an overly happy or outgoing mood. Extreme irritability **Behavioral Changes** Talking very fast, jumping from one idea to another, having racing thoughts Being easily distracted Increasing activities, such as taking on new projects Being overly restless Sleeping little or not being tired Having an unrealistic belief in one's abilities Behaving impulsively and engaging in pleasurable, high-risk behaviors	**Mood Changes** An overly long period of feeling sad or hopelessness. Loss of interest in activities once enjoyed, including sex. **Behavioral Changes** Feeling tired or "slowed down" Having problems concentrating, remembering, and making decisions Being restless or irritable Changing eating, sleeping, or other habits Thinking of death or suicide, or attempting suicide.

(National Institute of Mental Health, United States)

2.2.2 Genetics of Bipolar Disorder

Research from twins, family and adoption studies have established powerful evidence for considerable contribution of genetic factor to the risk of BPD. (Kato et al. 2005) There have been reports that the approximate lifetime risk of having BPD in the family of BPD patient are: monozygotic co-twin 40-70%; first degree relative 5-10%; and unrelated person 0.5-1.5% (Craddock and Jones 1999). Over the years, numerous studies have reported various linkage loci and candidate genes in association with BPD. These results, however, are not consistent and the rate of successful replication in different sample sets is low. (Kato 2007) Thus, there is yet to discover any well-established genetic risk factor for BPD.

Among the reported candidate genes, the following have been studied in more than two population: DRD2, DRD3, DRD4, HTT, DRD1, MAOA, DAT1, TPH1, HTR2A, and IMPA2 (Leszczyńska-Rodziewicz et al. 2005, Lai et al. 2005, Keikhaee et al. 2005). The promising candidate genes that are gaining support from various studies include Val66Met polymorphism of BDNF (Kunugi et al. 2004, Green et al. 2006, Harrisberger et al. 2015). In terms of the pathophysiology of BPD, a number of theories have been proposed. Considering that BPD is a mood disorder, sleep disturbance and circadian rhythms disrupts had been proposed to play a role in BPD (Harvey 2008). Numerous studies have yielded some candidate genes, which include TIMELESS (Mansour et al. 2006) and PERIOD3 (Nievergelt et al. 2006). Besides, a theory that have gaining much attention is the one related to mitochondrial dysfunction (Kato 2011), based on the observation that the occurrence of mood disorders were statistically higher in maternal relatives of children with mitochondrial diseases, as compared to their paternal relatives (Boles et al. 2005, Burnett et al. 2005). Studies on this

hypothesis have alluded to the involvement of some candidate genes in BPD among the Japanese population, such as NDUFV2 (Washizuka et al. 2006).

2.2.3 Schizophrenia

SZ is a mental disorder that causes huge human suffering and abundant financial and societal burden (Mueser and McGurk 2004). The diagnosis criteria of SZ as according to DSM-IV includes:

- Delusions
- Hallucinations
- Disorganized speech
- Grossly disorganized or catatonic behaviour
- Social /occupational dysfunction

There should be two or more symptoms, with each present for a significant portion of time during a one month period.

Clinical management of SZ includes primarily the use of antipsychotics. The use of first generation antipsychotics have been associated with profound extrapyramidal (EPS) side effects (Pierre 2005). This led to the development of atypical antipsychotics, with much less EPS. However, the atypical antipsychotics was later found to be associated with some metabolic side effects (Patel et al. 2009), and the high variability in treatment outcome have suggested the role of genetic factor in treatment response to antipsychotics (Reynolds et al. 2002).

2.2.4 Genetics of Schizophrenia

Studies of identical twins and meta-analysis have revealed that heritability of susceptibility to SZ can achieve as high as 80% (Cardno and Gottesman 2000). Research on genetic risk factor of SZ have been carried out extensively, but to date, there is still lack of linkage between the DNA variants of proposed candidate genes with specific protein of biological process alterations. In spite of these challenges, several positions of the chromosome have given consistent results for the linkage with SZ. Some of these studies, which incorporate the linkage analysis and association studies in the same family set have yielded several candidate genes, such as DTNBP1, NRG1, G72/G30, TRAR4 (Chumakov et al. 2002, Stefansson et al. 2002, van denOord et al. 2003, Duan et al. 2004). Furthermore, numerous studies combining associations of candidate genes with their functional roles have also yielded several promising candidate genes, which include COMT, RGS4, PPP3CC, and AKT1 (Egan et al. 2001, Mirnics et al. 2001). Results of the potential candidate genes as discussed above have yielded an important theory for the pathophysiology of SZ, which is through the glutamate neurotransmitter dysfunction (Javitt 2010).

2.2.5 Overlap in susceptibility genes between Schizophrenia and Bipolar Disorder

In considering a possible association of BPD with genetic variants, their interplay with schizophrenia (SZ) cannot be overlooked. Although the two disorders may present distinct symptoms, growing evidence has shown that they share some common genetic variations (Berrettini 2000, Maier et al. 2005, Maier et al. 2006, Lichtenstein et al. 2009). Some of the candidate gene for SZ have been replicated in BPD, and have shown some promising results. These include NRG1, DISC1, DAOA (G72) and BDNF

(Skibinska et al. 2004, Cordeiro et al. 2005, Green et al. 2005). Furthermore, antipsychotics, which are initially being used for the treatment of SZ, have been increasingly important as the first line treatment for BPD (Derry and Moore 2007). Whereas different theory for the pathophysiology of BPD and SZ have been proposed, and absence of any overlap between these two, studies on the promising common candidate genes may shed light on the molecular mechanisms underlying both diseases.

The molecular aetiology of BPD has yet to be fully understood, although one hypothesis that is gaining much support is the involvement of ion channelopathies (Ferreira et al. 2008). Genome wide association studies (GWAS) have identified susceptibility genes associated with BPD, a few of which are in a class of genes related to the structure and regulation of ion channel (Ferreira et al. 2008, Group 2011). The notion of ion channel involvement is further supported by findings of increased intracellular calcium in the lymphocytes of BPD patients (Brotman et al. 1986, Goodnick 2000) and the effectiveness of calcium channel blockers in the treatment of BPD (Hough et al. 1999). These findings add more weight to the evidence for cellular calcium imbalance and calcium ion channelopathy in the aetiology of BPD.

2.3 MATERIALS AND METHODS

2.3.1 Materials

2.3.1.1 Blood collection

Purple top vacutainer blood collection tube with EDTA, syringe and needle, tourniquet, alcohol swab, cotton wool, plasters, disposable examination gloves were used.

2.3.1.2 Buccal Swab

DNA buccal swab with cotton head and plastic handle, 1.5 ml microcentrifuge tubes, scissors, disposable examination gloves.

2.3.1.3 DNA Extraction

QIAamp DNA Blood Mini Kit, (QIAGEN, Hilden, Germany), GeneAll Exgene Tissue DNA Extraction Kit (for buccal swab), 1.5 ml microcentrifuge tubes

2.3.1.4 Agarose Gel Electrophoresis

10X TBE buffer (Biorad); 6X Loading Dye Solution (Fermentas); GelRed (Biorad); 100bp DNA ladder (Fermentas); PCR agarose powder (Biorad)

2.3.1.5 DNA measurement

Ultrapure water

2.3.1.6 Real time PCR

PCR strip tubes with cap, TaqMan Genotyping Master Mix, TaqMan SNP Genotyping Assays.

2.3.1.7 Sequenom MassArray

96-well plate and plate seal film (for quality control process). Subsequent experiments were carried out by the Centre for Genomic Sciences, Hong Kong University.

2.3.1.8 Instruments

BIO-RAD UV Transluminator; Gel Electrophoresis System with Power Pack; Vortex machine, Microcentrifuge; Microwave; Freezer; Refrigerator, Weighing Machine, Ice-maker Machine , micropipettes (20 µ L,200 µ L and 1000 µ L); Step One Plus Real-Time PCR (Applied Biosystems), Thermoblock, Thermo Scientific Nanodrop 2000c Spectrophotometers. ; Milli-Q® Water Purification Systems.

2.3.2 Methods

2.3.2.1 Subject recruitment

A case control study was conducted at the University Malaya Medical Centre (UMMC). A total of 244 unrelated BPD patients and 471 SZ patients were recruited, together with 593 healthy controls. The patients were from the psychiatry outpatient clinic of UMMC, whereas healthy controls were recruited from healthy volunteers from the University of Malaya, as well as from blood donors at UMMC. All patients were diagnosed through consensus by at least two experienced psychiatrists, according to the criteria for BPD and SZ, as set out by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Subjects were excluded from the study if they had a history of substance abuse or alcoholism, or other chronic diseases, including hypertension and diabetes. Most of the BPD patients (90%) were BPD Type I patients and all were in euthymic state during the period of sample collection. The controls were interviewed by a psychiatrist to confirm that they were free from any mental disorders and had no family history of mental illness (at least for one generation). Ethnicity of the cases and controls was confirmed by the absence of mixed marriage for at least three generations through self-report by the subjects. Subjects provided written informed consent after they were given a full explanation of the research outline. The study protocol was reviewed and approved by the Medical Ethics Committee of UMMC.

2.3.2.2 DNA Extraction

Blood samples collected were centrifuged at 3000 rpm for 10 minutes. The blood samples were then separated into plasma and buffy coat layer. The buffy coat layers were then used for DNA extraction using QIAamp DNA Blood Mini Kit, (QIAGEN, Hilden, Germany). A volume of 20 µl QIAGEN Protease (or proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube. Then, 200 µl volume of sample was added to the microcentrifuge tube. Buffer AL (200 µl) was then added to the sample. Next, the sample was mixed by pulse-vortexing for 15 s. Following this, the sample was incubated at 56°C for 10 min. The 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

Next, 200 µl ethanol (96–100%) was added to the sample, and mixed again by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. Next, the QIAamp Mini spin column was carefully opened and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was put in a clean 2 ml collection tube, and the collection tube containing the filtrate were discarded. Next, the QIAamp Mini spin column was carefully opened and 500 µl Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was carefully opened and 200 µl Buffer AE or distilled water was added. It was then incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min., Elution of DNA in Buffer AE and stored at –20°C were recommended for long-term storage of DNA. The quality of DNA was checked using Nanodrop 2000 c (Thermo Scientific)

2.3.2.3 Agarose Gel Electrophoresis

Gel electrophoresis was carried out to determine the quality of DNA and also the absence of other impurities. First, 1% of agarose was prepared by adding 1 g of powdered agarose and 6 ul GelRed into 100 ml 1X TAE buffer. Then, the agarose gel was cooked in a microwave on high power for 40-60 sec. The gel was then allowed to cool down. In the meantime, the casting tray and comb were assembled. The mixture was quickly poured onto the casting tray and left to solidify. When the gel was solidified, the gel tray was moved into the gel tank filled with TAE buffer enough to cover the top of the gel. A volume of 1ul of the genomic DNA was mixed with 1.5ul of loading dye and then loaded into the well. The loading dye was first prepared by diluting a volume of 1 ul of GelRed into 599ul of 6x loading buffer. The sample was run together with a 100bp ladder at 110 V for 30 minutes. After that, the sample was viewed under ultraviolet (UV) light using BIO-RAD UV Transluminator.

2.3.2.4 Genotyping

Genomic DNA was extracted from peripheral white blood cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). LMAN2L gene variants (rs6746896 and rs2271893), a BTF3L1/KCTD12 gene variant (rs2073831), and ANK3 gene variants (rs10994336 and rs1938526) were genotyped at the University of Hong Kong Genome Research Centre using the Sequenom MassARRAY technology platform with iPLEX Gold chemistry (Sequenom, San Diego, CA, USA) according to the manufacturer's protocols. Briefly, the MassARRAY Assay Design software package (v4.0) was used to design the specific assays with proximal SNP filtering. The quality of PCR fragment amplification and extension primer specificity was checked prior to running the reaction.

Residual nucleotides were dephosphorylated prior to the iPLEX Gold reaction. Following a single-base extension, reaction products were desalted with SpectroClean

resin (Sequenom), and 10 nL was spotted onto the SpectroCHIP using the MassARRAY Nanodispenser. A MassARRAY Analyzer Compact MALDI-TOF mass spectrometer was used to determine product mass. For proper data acquisition and analysis, MassARRAY Typer 4.0 software was used. Genotypes were called after cluster analysis by using the default setting of the Gaussian mixture model. The clusters were inspected to ensure a clear cluster separation with good signal-to-noise cut-off. A manual review was done to further clarify uncertain genotype calls. An assay with a call rate of less than 80% within the same SpectroChip was considered to have failed. A blank and five duplicates were introduced as quality controls. A SpectroChip with a call rate of more than 25% in the blank control or concordance of less than 99.5% in the duplicate checks, along with a call rate of more than 10% in the blank check, was considered to have failed and would need to be repeated. Any significant result will be validated using real time PCR.

2.3.2.5 Statistical analyses

The genotype distribution was assessed for Hardy-Weinberg equilibrium (HWE) using a chi squared test. A P-value of more than 0.05 indicates agreement with HWE. Determination of allele associations was performed by using logistic regression. In order to avoid false discoveries due to population difference, we performed the association analysis for each SNP marker separately by ethnicity. Linkage disequilibrium (LD) and haplotype analysis were performed with the Haploview 4.2 program. Pairwise LD was used to investigate the inter-marker relationship through D' values in case and control subjects. A permutation test with 5000 replications was used to obtain empirical levels of Significance. To investigate the influence of gene-gene interaction on BPD and SZ, the Generalized Multifactor Dimensionality Reduction (GMDR) method (Lou, Chen et al. 2007) was employed. All possible interactions were

tested by using 10-fold cross-validation with an exhaustive search that considers all possible variable combinations. GMDR provides a cross-validation consistency score, which is a measure of the degree of consistency with which the selected interaction is identified as the best model among all possibilities considered. The testing-balanced accuracy generated is a measure of the degree to which the interaction accurately predicts case-control status. Testing accuracy is a measure of the strength of gene-gene interaction with a power of 80% with accuracy of 0.58-0.60, given a sample of 500(Chen, Xu et al. 2011). Ethnicity was used as the covariate in the gene-gene interaction analysis. Power analysis was carried out by assuming a gene-only effect. A calculated sample size of 244 bipolar cases and 593 controls was used to provide a desired power of 80% at a P-value of 0.05 with the following assumptions: the allele frequency range was 0.20-0.80, the baseline risk for the Malaysian population was 0.16, and the minimum detectable odds ratio (OR) was 1.5. As for SZ, a sample size of 471 SZ cases and 593 controls would provide 80% power at a P-value of 0.05 with the following assumptions: the allele frequency range was 0.31-0.41, the baseline risk for the Malaysian population was 0.15, and the minimum detectable OR was 1.5.

2.3.2.6 List of SNPs Studied

No.	Gene	RS Number	Location in Chromosome
SNP1	SP8	Rs2709736	7p21.2
SNP2	ST8SIA2	Rs8040009	15q26
SNP3	CACNB2	Rs11013860	10p12
SNP4	KCTD12	Rs 2073831	13q22.3
SNP5	ANK3	Rs1938526	10q21
SNP6		Rs 10994336	
SNP7	Chromosome 3	Rs 11720452	3q21-q25
SNP8	TRANK1	Rs 9834970	3p22.2
SNP9	LMAN2L	Rs 6746896,	2q11.2
SNP10		Rs 2271893	2q11.2
SNP11	*SP4*	Rs12673091	7p15.3
SNP12		Rs3735440	
SNP13	NF1A	Rs41350144	1p31.3-p31.2
SNP14	SCN8A	Rs 303810	12q13
SNP15	NAPG	Rs 473938	18p11.22
SNP16		Rs229079	
SNP17		Rs 495484	
SNP18	ODZ4	Rs 12576775	11q14.1
SNP19	RGS4	Rs951436	1q23.3
SNP20	CACNA1C	Rs1006737	12p13.3
SNP21	PDE10A	Rs 1039002	2p16.3
SNP22	MARK1	Rs12563333	1q41
SNP23	COMT/Val158Met	Rs4680	22q11.21
SNP24	NRG1	Rs35753505	8p12
SNP25		Rs1081062	

2.4 RESULTS

2.4.1 Demographic Data

Table 2.4.1 shows the demographic data of the patients and controls. The 244 BPD patients consisted of 73 Malays, 98 Chinese, and 73 Indians, while the 471 SZ patients were made up of 132 Malays, 222 Chinese, and 117 Indians. Out of the 593 controls, 173 were Malays, 286 Chinese, and 134 Indians. There is a significant difference in the mean age between BPD patients and healthy controls (P= 0.027). Likelihood tests indicated a significant effect of ethnicity, but no significant effect of age and gender. None of the genotypes for the tested SNPs deviated from Hardy – Weinberg equilibrium for BPD patients, SZ patients, and controls.

Table 2.4.1 Demographic Table of BD and SZ patients with healthy controls

	BPD (N=244)	**Schizophrenia (N=471)**	**Controls (N=593)**
Age (mean± SD)	42.95 ± 11.975	40.49 ± 12.082	41.09 ± 10.255
Gender			
Male	128 (53%)	270 (57%)	375 (63%)
Female	116 (48%)	201 (43%)	218 (37%)
Ethnicity			
Malay	73 (30%)	132 (28%)	173 (29%)
Chinese	98 (40%)	222 (47%)	286 (48%)
Indian	73 (30%)	117 (25%)	134 (23%)

2.4.2 Single Nucleotide Polymorphisms: LMAN2L

LMAN2L rs6746896 shows significant differences in allelic distribution between both BPD and SZ patients and controls for the pooled subjects (P =0.009 and 0.001, respectively). The LMAN2L rs2271893 C allele frequency was significantly higher in SZ patients compared with healthy controls, a condition observed in Malays and Indians (P = 0.002 and P = 0.003, respectively).

After Bonferroni correction, LMAN2L rs6746896 remained significant for both BPD and SZ (P = 0.009 for BPD and 0.001 for SZ), ANK3 rs1938526 and rs10994336 for BPD in Malays (P= 0.001 and 0.006, respectively), and LMAN2L rs2271893 for SZ in Malays (P = 0.003) and Indians (P= 0.002).

When we examined the haplotypes for the LMAN2L gene, the GA, AG, and GG showed frequencies of 68%, 17% and 15%, respectively. A significant difference exists between BPD and SZ patients and controls for haplotype frequencies of GA (P = 0.015 and 0.010 for BPD and SZ, respectively), even after the permutation test correction with 5000 permutations. There also exists a significant difference between BPD patients and controls for haplotypes frequencies of GG (P = 0.013).

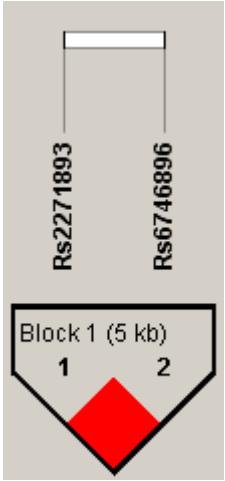

Figure 2.4 1 Haplotype view for LMAN2L gene rs2271893 and rs6746896 in BPD

Figure 2.4 2 Haplotype view for LMAN2L gene rs2271893 and rs6746896 in SZ

Table 2.4.2 Genotype Distribution of *LMAN2L* gene in BPD, SZ and Controls

	All ethnicities			**Malay**			**Chinese**			**Indian**		
	Controls	Patients BPD	SZ	Controls	Patients BPD	SZ	Controls	Patients BPD	SZ	Controls	Patients BPD	SZ
LMAN2L												
Rs6746896												
AA	270	137	259	73	32	69	111	53	105	86	51	80
AG	247	85	170	72	31	53	133	37	87	42	17	30
GG	76	22	42	28	10	9	42	8	30	6	5	6
A	787	359	688	218	95	191	355	143	297	214	119	190
G	399	129	254	128	51	71	217	53	147	54	27	42
Rs2271893												
GG	417	173	350	132	64	113	186	85	130	90	57	100
AG	153	63	109	34	5	13	83	6	81	36	8	15
AA	23	8	12	8	7	6	17	6	12	7	6	5
G	987	409	809	298	133	239	455	176	341	216	122	215
A	199	79	133	50	19	25	117	18	105	50	20	25

Table 2.4.3 Odds ratio of SNPs of LMAN2L gene in BPD and SZ in pooled population

ALL ETHNICITY				
LMAN2L	BPD		SZ	
Rs6746896	0R (CI)	P-value	OR (CI)	P-value
A	1	–	1	–
G	1.36 (1.08 – 1.71)	0.009	1.34 (1.12 – 1.61)	0.001
AA	1	–	1	–
AG	0.59 (0.35 – 1.00)	0.048	1.35 (1.04 – 1.75)	0.024
GG	0.88 (0.51 – 1.51)	0.634	1.55 (1.03 – 2.32)	0.036
Rs2271893				
G	1	–	1	–
A	1.12 (0.85 – 1.48)	0.429	1.24 (0.99 – 1.56)	0.06
GG	1	–	1	–
GA	0.69 (0.29 – 1.62)	0.388	1.22 (0.92 – 1.62)	0.164
AA	0.73 (0.30 – 1.78)	0.482	1.73 (0.87 – 3.42)	0.117

Table 2.4.4 Odds ratio of SNPs of LMAN2L gene in BPD and SZ according to ethnicity

	Malay				Chinese				Indian			
LMAN2L	BPD		SZ		BPD		SZ		BPD		SZ	
Rs6746896	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value
A	1	–	1	–	1	–	1	–	1	–	1	–
G	1.09 (0.74 – 1.60)	0.678	1.50 (1.07 – 2.10)	0.02	1.63 (1.14 – 2.34)	0.007	1.21 (0.94 – 1.55)	0.148	1.21 (0.73 – 2.00)	0.471	1.59 (0.98 – 2.58)	0.061
AA	1	–	1	–	1	–	1	–	1	–	1	–
AG	1.05 (0.58 – 1.91)	0.876	1.30 (0.80 – 2.12)	0.295	1.80 (1.09 – 2.96)	0.021	1.45 (0.99 – 2.13)	0.058	1.49 (0.75 – 2.95)	0.251	1.39 (0.78 – 2.45)	0.261
GG	1.29 (0.56 – 3.00)	0.549	2.53 (1.13 – 5.63)	0.023	2.42 (1.05 – 5.57)	0.037	1.28 (0.74 – 2.19)	0.379	0.91 (0.23 – 3.54)	0.89	5.71 (0.67 – 9.96)	0.112
Rs2271893												
G	1	–	1	–	1	–	1	–	1	–	1	–
A	0.83 (0.49 – 1.41)	0.49	2.63 (1.40 – 4.97)	0.003	1.15 (0.77 – 1.71)	0.495	0.86 (0.64 – 1.15)	0.297	1.31 (0.73 – 2.30)	0.363	2.76 (1.46 – 5.20)	0.002
GG	1	–	1	–	1	–	1	–	1	–	1	–
GA	0.77 (0.40 – 1.50)	0.444	2.28 (1.14 – 4.58)	0.02	1.00 (0.60 – 1.67)	1	0.72 (0.49 – 1.06)	0.091	1.42 (0.69 – 2.92)	0.34	2.51 (1.28 – 4.95)	0.008
AA	1.02 (0.19 – 5.49)	0.983	1.30 (0.00 – 2.13)	0.999	1.75 (0.49 – 6.20)	0.39	0.99 (0.46 – 2.16)	0.997	0.96 (0.15 – 6.06)	0.961	1.74 (0.00 – 2.15)	0.999

2.4.3 Single Nucleotide Polymorphisms: ANK3

The ANK3 rs1938526 and rs10994336 were significantly different between BPD and SZ patients and controls of Malay ethnicity (P = 0.001 and 0.012, for BPD and SZ, respectively). ANK3 rs1938526 was associated with BPD in Indians (P = 0.015).

In the Malay ethnic subgroup, the ANK3 rs1938526 C allele and rs10994336 T allele were found to be associated with a relatively reduced risk of BPD and SZ (ANK3 rs1938526: OR0.471, 95% CI 0.30 – 0.74, P = 0.001 and OR 0.60, 95% CI 0.41 – 0.89, P = 0.012, for BPD and SZ, respectively; ANK3 rs10994336: OR0.49, 95% CI 0.30 – 0.81, P = 0.006 and OR 0.62, 95% CI 0.40 – 0.95,P = 0.029, for BPD and SZ, respectively). The LD of the ANK3 gene was not significantly different between BPD and SZ patients and controls.

Table2.4.5 Genotype Distribution of *ANK3* gene in BPD, SZ and Controls

	All ethnicities			**Malay**			**Chinese**			**Indian**		
	Controls	Patients BPD	SZ	Controls	Patients BPD	SZ	Controls	Patients BPD	SZ	Controls	Patients BPD	SZ
ANK3 Rs1938526												
TT	357	141	263	111	34	69	167	52	116	79	50	77
TC	202	87	174	57	30	53	96	40	88	48	15	32
CC	34	16	34	5	9	10	23	6	16	7	8	7
T	916	369	700	279	98	191	430	144	320	206	115	186
C	270	119	242	67	48	73	142	52	120	62	31	46
Rs10994336												
CC	411	168	325	128	41	87	194	63	152	85	51	86
TC	164	70	130	39	26	38	81	28	66	44	16	26
TT	18	6	16	7	6	7	10	7	5	5	6	4
C	986	406	780	295	108	212	469	154	370	214	118	198
T	200	82	162	53	34	52	101	42	76	54	28	34

Table 2.4.6 Odds ratio of SNPs of ANK3 gene in BPD and SZ in pooled population

ANK3	ALL ETHINICITY BPD		SZ	
Rs1938526	OR (CI)	P value	OR (CI)	P value
T	1	–	1	–
C	0.92 (0.72 – 1.17)	0.483	0.86 (0.71 – 1.04)	0.123
TT	1	–	1	–
TC	1.23 (0.65 – 2.32)	0.518	0.87 (0.67 – 1.12)	0.273
CC	1.11 (0.58 – 2.13)	0.76	0.79 (0.48 – 1.30)	0.347
Rs10994336				
C	1	–	1	–
T	1.01 (0.76 – 1.33)	0.976	0.98 (0.78 – 1.23)	0.84
CC	1	–	1	–
CT	0.94 (0.67 – 1.31)	0.698	1.02 (0.78 – 1.34)	0.878
TT	1.11 (0.43 – 2.87)	0.825	0.85 (0.43 – 1.68)	0.643

Table 2.4.7 Odds ratio of SNPs of ANK3 gene in BPD and SZ according to ethnicity

	MALAY				CHINESE				INDIAN			
ANK3	BPD		SZ		BPD		SZ		BPD		SZ	
Rs1938526	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value
T	1	–	1	–	1	–	1	–	1	–	1	–
C	0.47 (0.30 – 0.74)	0.001	0.60 (0.41 – 0.89)	0.012	0.92 (0.64 – 1.32)	0.646	0.87 (0.66 – 1.15)	0.334	2.03 (1.15 – 3.58)	0.015	1.21 (0.79 – 1.83)	0.384
TT	1	–	1	–	1	–	1	–	1	–	1	–
TC	0.59 (0.33 – 1.08)	0.085	0.71 (0.44 – 1.15)	0.166	0.72 (0.44 – 1.18)	0.193	0.76 (0.52 – 1.11)	0.149	1.94 (0.99 – 3.83)	0.054	1.36 (0.77 – 2.37)	0.289
CC	0.13 (0.04 – 0.46)	0.001	0.21 (0.06 – 0.72)	0.013	1.14 (0.43 – 2.98)	0.795	1.00 (0.50 – 1.99)	0.999	5.52 (0.63 – 7.52)	0.122	1.01 (0.33 – 3.08)	0.984
Rs10994336												
C	1	–	1	–	1	–	1	–	1	–	1	–
T	0.49 (0.30 – 0.81)	0.006	0.62 (0.40 – 0.95)	0.029	1.19 (0.76 – 1.86)	0.439	1.05 (0.76 – 1.46)	0.776	1.81 (1.01 – 3.26)	0.047	1.45 (0.91 – 2.32)	0.116
CC	1	–	1	–	1	–	1	–	1	–	1	–
CT	0.49 (0.27 – 0.89)	0.02	0.71 (0.42 – 1.20)	0.2	0.95 (0.57 – 1.60)	0.852	0.96 (0.65 – 1.42)	0.829	1.78 (0.89 – 3.54)	0.102	1.68 (0.94 – 3.00)	0.082
TT	0.24 (0.05 – 1.16)	0.076	0.27 (0.07 – 1.09)	0.066	2.95 (0.37 – 3.57)	0.309	1.48 (0.49 – 4.44)	0.488	4.29 (0.47 – 5.96)	0.199	1.19 (0.30 – 4.70)	0.804

2.4.4 Single Nucleotide Polymorphism: KCTD12

KCTD12 rs2073831 was genotyped in this study. All the genotype distribution are in Hardy-Weinberg Equilibrium. No allelic associations were observed with BPD or SZ in the pooled population. However, after stratification by ethnicity, there is a significant association between the allele T with SZ in both Malays and Chinese (P = 0.024 and 0.025, respectively).

Table 2.4.8 Genotype distribution of rs2073831 of KCTD12

	All ethnicities			**Malay**			**Chinese**			**Indian**		
	Control	Patient BPD	SZ	Control	Patient BPD	SZ	Control	Patient BPD	SZ	Controls	Patient BPD	SZ
KCTD12 Rs2073831												
CC	226	95	191	72	44	41	99	83	92	55	8	58
TC	280	109	216	82	6	66	133	5	107	65	57	43
TT	87	40	64	20	27	25	52	8	24	15	6	15
C	732	299	598	226	94	148	331	171	291	175	73	15
T	454	189	344	122	60	116	237	21	155	95	69	9

Table 2.4.9 Odds ratio of rs2073831 of KCTD12 in BPD and SZ in pooled population

	ALL ETHNICITY			
KCTD12	BPD		SZ	
Rs2073831	0R (CI)	P-value	OR (CI)	P-value
C	1	–	1	–
T	0.99 (0.80 – 1.23)	0.94	1.07 (0.90 – 1.28)	0.445
CC	1	–	1	–
CT	1.05 (0.76 – 1.47)	0.752	1.05 (0.81 – 1.37)	0.695
TT	0.91 (0.58 – 1.43)	0.683	1.12 (0.77 – 1.62)	0.57

Table 2.4.10 Odds ratio of rs2073831 of KCTD12 in BPD and SZ according to ethnicity

	Malay				Chinese				Indian			
KCTD12	BPD		SZ		BPD		SZ		BPD		SZ	
Rs2073831	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value	OR (CI)	P value
C	1	–	1	–	1	–	1	–	1	–	1	–
T	0.90 (0.40 – 1.35)	0.602	0.68 (0.49 – 0.95)	0.025	0.92 (0.07 – 1.26)	0.612	1.35 (1.04 – 1.74)	0.024	1.16 (0.74 – 1.80)	0.524	1.18 (0.81 – 1.70)	0.387
CC	1	–	1	–	1	–	1	–	1	–	1	–
CT	1.13 (0.60 – 2.10)	0.86	0.69 (0.41 – 1.14)	0.147	1.08 (0.63 – 1.84)	0.783	1.15 (0.78 – 1.69)	0.492	1.13 (0.60 – 2.10)	0.711	1.69 (0.98 – 2.93)	0.061
TT	1.47 (0.50 – 4.28)	0.668	0.41 (0.20 – 0.84)	0.015	0.74 (0.39 – 1.41)	0.362	1.99 (1.13 – 3.51)	0.018	1.47 (0.50 – 4.28)	0.483	1.16 (0.50 – 2.67)	0.727

2.4.5 Single Nucleotide Polymorphism: SP4

Two SNPs of the SP4 gene (transcription factor gene) were genotyped in this study, rs12673091 and rs3735440. Both of the SNP are intron variants, located on chromosome 7. Table 2.4.11 shows the association test between cases and controls in BPD and SZ. All the genotype distributions are in Hardy-Weinberg Equilibrium. No allelic association with BPD and SZ were observed in the pooled population, or after stratification by ethnicity.

Table 2.4.11 Odds ratio of SNPs of SP4 gene in BPD and SZ in BPD and SZ

SNP	BPD		SZ	
Rs12673091	OR (CI)	P value	OR (CI)	P value
Overall	0.563 (0.432-0.734)	0.06	1.159 (0.974-1.379)	0.096
Malay	0.455 (0.274-0.754)	0.08	1.060 (0.769-1.462)	0.722
Chinese	0.633 (0.372-1.075)	0.09	1.246 (0.957-1.621)	0.103
Indian	0.723 (0.471-1.111)	0.139	1.079 (0.777-2.554)	0.595
Rs 3735440				
Overall	0.841 (0.639-1.108)	0.219	0.878 (0.695-1.109)	0.275
Malay	0.779 (0.482-1.258)	0.307	0.826 (0.536-1.272)	0.386
Chinese	1.828 (0.969-3.448)	0.062	0.957 (0.642-1.427)	0.829
Indian	0.655 (0.413-1.039)	0.072	0.886 (0.585-1.343)	0.569

2.4.6 Single Nucleotide Polymorphism: NAPG gene

Three SNPs of the NAPG gene (N-ethylmaleimide-sensitive factor attachment protein, gamma) were genotyped in this study, rs473938, rs229079 and rs495484. All of the SNP are intron variants, located on chromosome 18. Table 2.4.12 shows the association test between cases and controls in BPD and SZ. All the genotype distributions are in Hardy-Weinberg Equilibrium. No allelic association with BPD and SZ were observed in the pooled population, or after stratification by ethnicity.

Table 2.4.12 Odds ratio of SNPs of NAPG gene in BPD and SZ

SNP	BPD		SZ	
	OR (CI)	P value	OR (CI)	P value
Rs473938				
Overall	1.148 (0.835-1.410)	0.187	0.924 (0.784-1.089)	0.347
Malay	1.394 (0.966-2.012)	0.076	0.991 (0.730-1.346)	0.954
Chinese	0.893 (0.631-1.264)	0.522	0.9 (0.693-1.169)	0.431
Indian	1.008 (0.663-1.532)	0.971	0.741 (0.530-1.035)	0.078
Rs229079				
Overall	1.067 (0.840-1.356)	0.595	1.119 (0.921-1.360)	0.257
Malay	1.037 (0.667-1.612)	0.872	0.887 (0.626-1.286)	0.554
Chinese	1.053 (0.729-1.522)	0.783	1.209 (0.905-1.617)	0.199
Indian	1.087 (0.689-1.717)	0.719	1.283 (0.855-1.924)	0.228
Rs495484				
Overall	0.993 (0.803-1.229)	0.951	0.974 (0.820-1.157)	0.765
Malay	1.039 (0.694-1.556)	0.853	0.806 (0.583-1.116)	0.194
Chinese	0.821 (0.593-1.137)	0.236	1.037 (0.800-1.345)	0.782
Indian	1.155 (0.763-1.747)	0.495	0.934 (0.665-1.311)	0.693

2.4.7 Single Nucleotide Polymorphism: NRG1 gene

Two SNPs of the NRG1 gene (neuregulin 1) were genotyped in this study, rs35753503 and rs1081062. Both of the SNP are intron variants, located on chromosome 8. Table 2.4.13 shows the association test between cases and controls in BPD and SZ. All the genotype distributions are in Hardy-Weinberg Equilibrium. No allelic association with BPD and SZ were observed in the pooled population, or after stratification by ethnicity.

Table 2.4.13 Odds ratio of SNPs of NRG1 gene in BPD and SZ

SNP	BPD		SZ	
Rs35753503	OR (CI)	P value	OR (CI)	P value
Overall	1.136 (0.924-1.397)	0.226	0.878 (0.695-1.109)	0.275
Malay	1.305 (0.902-1.888)	0.158	0.826 (0.536-1.272)	0.386
Chinese	1.016 (0.735-1.404)	0.925	0.927 (0.642-1.427)	0.829
Indian	0.998 (0.660-1.511)	0.994	0.886 (0.585-1.343)	0.569
Rs1081062				
Overall	1.031 (0.750-1.418)	0.851	0.986 (0.800-1.214)	0.893
Malay	0.911 (0.516-1.611)	0.749	1.162 (0.727-1.857)	0.530
Chinese	1.061 (0.648-1.738)	0.814	1.021 (0.706-1.478)	0.911
Indian	1.732 (0.608-2.107)	0.696	0.866 (0.610-1.229)	0.421

2.4.8 Single Nucleotide Polymorphism: Other candidate genes

For the SNP of CACNA1C, PDE10A, MARK1, COMT/Val158Me, NRG1, there is no allelic association with BPD and SZ observed in the pooled population, or after stratification by ethnicity. The genotype distribution of the SNP of SP8, ST8SIA2, CACNB2, TRANK1, and NF1A are deviated from Hardy Weinberg equilibrium. For the SNP of SCN8A, ODZ4 and RGS4, the association test do not proceed as more than 30% of the samples failed to be genotyped.

2.4.9 Overall Results

Overall, rs1938526 and rs10994336 of the ANK3 gene and rs6746896 of the LMAN2L gene were found to be significantly associated with BPD and SZ. It is also important to note that the homozygous mutant genotype of the LMAN2L rs6746896 confers greater risk of both disorders, while the ANK3 rs1938526 confers lower risk, indicating stronger effects of risk alleles in their homozygous form.

2.4.10 Gene-gene Interaction between LMAN2L and ANK3 genes

Results from this study suggest that variants of the LMAN2L and ANK3 genes confer increased risk to BPD and SZ in our study population. To add strength to these results, we further investigated interaction between the two risk genes on the occurrence of BPD and SZ. As indicated in Table 2.4.14, we derived one best model with perfect cross-validation consistency: a two-locus model (ANK3 rs10994336, LMAN2L rs6746896) that would fit best for SZ (empirical P= 0.0107). Interaction is however not observed in BPD.

Table 2.4.14 Best fitted gene-gene interaction model

Locus Number	Model	Cross-validation consistency	Testing Accuracy (%)	P value
2	LMAN2L (Rs6746896), ANK3 (Rs10994336),	8/10	56.80	0.0107

*P values based on 1000 permutations. Analysis of GMDR with adjustment of ethnicity

2.5 DISCUSSIONS

In this study, we investigated the association of gene variants involved in ion channel transport and ER transport with BPD and SZ.

2.5.1 Single Nucleotide Polymorphisms: LMAN2L

We showed a significant association between LMAN2L rs6746896 and both BPD and SZ in the pooled population. However, following ethnic stratification, this association was modified, with a strong and significant association that was apparent in the Chinese for BPD and in the Malays for SZ. Our pooled result on BPD is consistent with two previous GWAS: one by (Chen et al. 2013) on the European and Asian ancestry samples and another by the Psychiatric GWAS Consortium BPD Working Group (Group 2011). However, to date, no study has investigated the association between LMAN2L rs6746896 and SZ; ours is the first to report a significant positive association. LMAN2L rs2271893 was also found to be associated with SZ but not with BPD in Malays and Indians.

These findings are partially consistent with those of Andreassen et al. (2013) in that the authors reported an overlap of rs2271893 with BPD and SZ, whereas we found an association with SZ but not BPD. Our finding is inconsistent, however, with the significant association with BPD reported by (Chen et al. 2013). Nevertheless, our results suggest that the A allele of rs2271893 confers an over 2.5-fold increased risk of SZ in ethnic Malays and Indians. The GG haplotype frequency of the LMAN2L gene was found to be significantly different between patients and controls in both BPD and SZ. Our study provides an early report of such a haplotype finding and needs to be confirmed by other studies.

The role of the lectin, mannose-binding 2-like (LMAN2L) gene, also known as the VIPL (VIP36-Like) gene, as an ER export receptor has been suggested in a study by (Neve et al.

2003). Studies have demonstrated that LMAN2L may act as a regulator for the ERGIC-53 (LMAN 1) gene. Mutation of the latter gene has been associated with a genetic bleeding disorder with a combined deficiency of coagulation factors FV and FVIII (F5F8D) (Nicholas et al. 1999). However, the functional role of LMAN2L in the pathophysiology of BPD has yet to be discovered. Nonetheless, two large-scale GWAS have shown that this gene is associated with BPD (Group 2011, Chen et al. 2013).

2.5.2 Single Nucleotide Polymorphisms: ANK3

The variants ANK3 rs1938516 and rs10994336 were not significantly associated with the occurrence of BPD and SZ in the pooled subjects. However, after ethnic stratification, significant differences were observed between BPD patients and controls and between SZ patients and controls among ethnic Malays. The rs1938526 variant was also significantly associated with BPD in Indians. The association between ANK3 gene variants and BPD has gained support from various studies (Ferreira et al. 2008, Schulze et al. 2009, Paez-Gonzalez et al. 2011, Tesli et al. 2011, Dedman et al. 2012) while other studies have failed to report any significant association (Gella et al. 2011, Lett et al. 2011, Takata et al. 2011, Gonzalez et al. 2013, Kondo et al. 2013), suggesting that ethnicity contributes to the risk of these disorders. Among supporting studies were those by (Takata et al. 2011), who showed an association in the East Asian population, including Han Chinese, Japanese, and Koreans, and by (Gella et al. 2011) and (Kondo et al. 2013), who reported an association of rs1938526 and rs10994336 with SZ. These studies are in contrast, however, with the work of Tesli et al. (2011), who reported no association. We failed to show any association between rs1938526 and rs10994336 with SZ and BPD in our Chinese subgroup.

Ankyrin-G protein (ANK3) is an adaptor protein expressed in the axonal initial segment and the nodes of Ranvier in the central and peripheral nervous systems (Bennett and Baines 2001). It has been shown to regulate the assembly of voltage-gated sodium channels. (Zhou et al. 1999) reported that Purkinje cells with knockout ANK3 failed to initiate action potential to support rapid and repetitive firing. Furthermore, a study with mouse brain samples treated with lithium, one of the drugs commonly used to treat BPD, found that ANK3 and subunits of the calcium channel were down-regulated (Baum et al. 2008).

2.5.3 Single Nucleotide Polymorphisms: KTCD12

Variant BTF3L1/KTCD12 rs2073831 was found to be associated with SZ but not with BPD in the Malays and Chinese. This result is in contrast with the significant positive finding in the GWAS of the Han Chinese BPD (Yuan et al. 2012). There are, however, no reports of association of rs2073831 and risk of SZ.

An ion channel gene, potassium channel tetramerization domain containing 12 (KCTD12), is involved in potassium ion transport. KCNC2, another potassium channel, was previously identified in the Wellcome Trust Case Control Consortium Study as being associated with BPD (Baum, Akula et al. 2008). The role of potassium channel mutations has also been found to be particularly prominent in human channelopathies (Ryan and Ptácek 2010). In addition, an association between altered gene function related to potassium ion transport and BPD was reported in a study by (Goldstein et al. 2009). Sodium- and potassium-activated adenosine triphosphatases (Na+, K+ – ATPase), major plasma membrane transporters for sodium and potassium, were found to be associated with BPD (Goldstein et

al. 2009). These studies have clearly suggested a role for potassium ion transport in the etiology of BPD.

2.5.4 Single Nucleotide Polymorphisms: Other candidate genes

The present study also investigated a number of SNP in various candidate genes, which includes CACNA1C, PDE10A, MARK1, COMT/Val158Me, NRG1, SP8, ST8SIA2, CACNB2, TRANK1, NF1A, SCN8A, ODZ4 and RGS4. None of the SNP of these candidate genes was significantly associated with susceptibility to BPD and SZ. These genes and their related SNP were chosen to be candidate genes in this study because before this current study were conducted, a literature search was carried out and a number of research have shown that these candidate genes were significantly associated with susceptibility to BPD and SZ in other populations. Therefore these SNP were chosen as a prescreening test for susceptibility to BPD and SZ in Malaysia population. The non-significant result may be due to different genetic make-up between the Malaysian population and other populations being studied.

2.5.5 Gene-gene interaction

The result from gene-gene interaction analysis suggests that the ANK3 and LMAN2L genes interact with each other in the pathophysiology of BPD and SZ. Surprisingly, a literature search failed to provide any reports that link these two genes. However, gene ontology enrichment analysis (Huang et al. 2009) identified protein localization as a common shared biological pathway.

2.5.6 Justification of Study

There were more Chinese participants in both of our BPD and SZ samples than other ethnicities, which could be due to the catchment area having a higher proportion of Chinese patients in UMMC as compared with other races. However, sampling was done without bias or preference for any ethnic group. A contributing factor in the failure to replicate a positive association with either BPD or SZ, as reported in other studies, in our Chinese ethnic group may be the differences in genetic variability between Southeast Asian Chinese, Chinese from Shanghai, and Beijing Han Chinese (Chen et al. 2009). Allele frequency differences between each population may also be a factor. The smaller sample size in our study could also explain the failure to replicate findings. Nonetheless, overall, our result is consistent with previous studies in the Asian population.

2.5.7 Genetic Overlapping of Schizophrenia and BPD

Genetic association studies have provided clues on possible risk genes associated with psychiatric disorders including BPD and SZ (Berrettini 2000). These genetic variations are responsible for the pathophysiology of the disorders mainly by altering the protein functions, thereby regulating brain activity (Meyer-Lindenberg et al. 2006, Bigos et al.2010). Expression studies have shown that the activities of the BPD/SZ genes were altered following treatments (Pandey et al. 2008, Rueckert et al. 2013). These observations have strongly hinted that the genetic factor may play a significant role in etiology of these two disorders.

2.5.8 Strength and limitation of the Study

A limitation of our study is the small sample size compared with earlier GWAS samples, which could have contributed to the small effect size of the associations. Taking into consideration that BPD and SZ develop as a result of the interplay of genetic and environmental factors, the lack of environmental background information of the subjects such as exposure to nicotine, advanced paternal age and stressful or traumatic events could be a further limitation of the study (de Leon and Diaz 2005, Goldberg and Garno 2005, Torrey et al. 2009). One of the objectives of the study is to examine the gene-gene interaction of the candidate SNPs. In order to achieve this objective, we employed the GMDR method. The limitation was that only one to several SNPs per candidate genes were chosen. These numbers of SNPs were not sufficient to capture most genetic information of the candidate genes. Future studies should elaborate on the findings by including more functional SNPs in the studied candidate genes.

On the other hand, its strength lies in the genotyping of polymorphisms across the BPD and SZ population simultaneously, which enabled us to study common genetic variants involved in the pathophysiology of both BPD and SZ. The results of our study favor the hypothesis that both BPD and SZ share some common genetic risk factors. Moreover, comparison of ethnicities in relation to BPD and SZ allowed us to investigate how ethnicity modifies the association between gene variants and BPD and SZ.

2.6 CONCLUSION

To our knowledge, this study is the first to report an association between LMAN2L rs6746896 and SZ. Further evidence is needed to confirm this interaction in a larger population. We found a positive association between LMAN2L (rs6746896) with both BPD and SZ. We also showed that variants of the ANK3 gene (rs1938516 and rs10994336) are associated with BPD in Malays and that LMAN2L rs2271893 is associated with SZ in Malays and Indians. The results of our study favour the hypothesis that both BPD and SZ share some common genetic risk factors. In addition to our result, functional study of the role of candidate gene in animal model and in-vitro study could provide more insights of the molecular mechanisms of BPD. As BPD and SZ are complex diseases with interaction of huge amount of genetic factor, searching for the causative risk factor may be difficult. Nevertheless, with the recent advancement of high throughput technology, and introduction of other aspect of genetic variation in human diseases, such as microRNA, understanding of pathophysiology of BPD can be achievable.

CHAPTER THREE: DIFFERENTIAL MICRORNA EXPRESSION IN BIPOLAR DISORDER I MANIC PATIENT FOLLOWING TREATMENT WITH ASENAPINE AND RISPERIDONE

3.1 INTRODUCTION

Mood stabilizers and atypical antipsychotic agent have long been the first-line treatment for acute mania with or without psychotic features. The atypical antipsychotic have several advantages in that they do not induce depressive episodes compared to mono-therapy with mood stabilizers, while several studies have revealed that some atypical antipsychotic shows improvement in depressive symptoms in manic patients (Szegedi et al. 2011). Over the years, numerous reviews and studies have shown that these atypical antipsychotics were significantly more effective than placebo in the treatment of acute mania, although these studies do not usually seek to align the efficacy of these individual atypical antipsychotics (Scherk et al. 2007, Correll et al. 2010, Yildiz et al. 2011).

Although major progress have been achieved in terms of research and development, there stills exists gap in the knowledge of molecular mechanisms underlying bipolar mania and action pathways of atypical antipsychotic are still waiting to be filled. Asenapine is a relatively new atypical antipsychotic in the market, and its molecular structure was described as a tetracyclic antipsychotic (Shahid et al. 2009). Being administered as a sublingual preparation, which enhances its direct absorption, this increases asenapine bioavailability compared to other atypical antipsychotic that are administered orally and thus possess much lower bioavailability due to hepatic metabolism (Reynolds 2011). This makes asenapine rather unique than its fellow atypical antipsychotic.

MicroRNAs (miRNA) are small non-coding RNA molecules that regulate gene expression, including genes involved in neuronal function and plasticity that have relevance for brain function and mental health (Chan and Kocerha 2012). In several studies, miRNA

have been shown to be altered in BPD patient and other psychiatric disorder (Kim et al. 2010, Moreau et al. 2011). There are vast evidence of miRNAs' regulatory roles in a number of central nervous system processes, including neurogenesis and synaptic plasticity (Kolshus et al. 2014).

Being a complex disorder, the polygenic characteristics of BPD have invited a large number of studies carried out in various sample sizes and population subjects, and results in a list of candidate genes, each acts in small effects contributing to the complexity of the disorder. The search for novel intervention targets for pharmacology interventions is further made difficult by its interaction and overlapping with genetics of other psychiatric illness (Maier et al. 2005). Given that miRNA levels are frequently altered in psychiatric disorders and also findings that they have been able to be influenced by psychotropic medications (Dinan 2010), the ability of a single miRNA to target whole network of proteins may make it fruitful for psychiatric disorders studies where it is the sum of small effects of many genes that is thought to underlie habitability.

In contrast to postmortem studies and other invasive techniques, uses of peripheral blood as a research subject in psychiatric disorders provides advantages of easy access and of being relatively non-invasive. In addition to that, stability of miRNA in human blood is assured (Turchinovich et al. 2011). While peripheral blood provides an easy assessable and non-invasive way for biochemical investigation of BPD, there have been concerns as to whether the peripheral changes reflects the exact alterations in the brain function. In respond to this, interaction between the central nervous system (CNS) and immune system, particularly lymphocytes have been proposed. In view of this, there have been correlation and similarity in pattern between the receptor expression, altered metabolism and cellular

function of the cells in CNS with lymphocytes (Gladkevich et al. 2004). Moreover, miRNA that are specific to brain disorders are detectable in peripheral blood (Lai et al. 2011, Leidinger et al. 2013).

The majority of clinical studies of miRNAs in psychiatric disorders are focused on schizophrenia. Being a new player in the field of atypical antipsychotic, there is a relatively lack of understanding of asenapine in terms of its molecular mechanism. This study aims to examine the changes in microRNA expression in bipolar mania patient following 12 weeks of treatment with asenapine and in comparison with risperidone. Risperidone have been among the first-line treatment in acute mania. In a recent up to dated meta-analysis, the author proposed that risperidone were among the most effective atypical antipsychotic in the treatment of acute mania (Cipriani et al. 2011). It is hoped that the results of this study were to provide more insights into the molecular mechanism underlying the action of the two atypical antipsychotics, as well as the biological pathophysiology underlying BPD mania.

3.1.1 Objectives

The aims of this study are:

1. To examine the changes in the expression of miRNA in the patients of BPD mania before and after the treatment of asenapine and risperidone.

2. To examine the changes in the scores of YMRS in the patients of BPD mania before and after the treatment of asenapine and risperidone.

3. To compare the changes in the expression of miRNA in the patients of BPD mania before and after the treatment between asenapine and risperidone.

4. To compare the changes in the scores of YMRS in the patients of BPD mania before and after the treatment between asenapine and risperidone.

3.1.2 Justification of Study

BPD, like any other psychiatric illness, is a complex diseases influenced by genetic factors that interact with environmental factors. There is no single gene that is responsible for the illness. Each genetic factor act in a very small effects, and the sum and interaction of hundreds of these genes play a role in the pathogenesis of the diseases. The ability of a single miRNA to target whole network of proteins make it an important subject of research for psychiatric disorders where it is the sum of many genes of small effects that are thought to underlie the pathophysiology of the disorder.

3.1.3 Research Hypothesis

The null hypothesis

1. There are no changes in the expression of miRNA in the patients of BPD mania before and after the treatment of asenapine and risperidone.

2. There are no changes in the scores of YMRS in the patients of BPD mania before and after the treatment of asenapine and risperidone.

3. There are no differences in the expression of miRNA in the patients of BPD mania before and after the treatment between asenapine and risperidone.

The research hypothesis

1. There are changes in the expression of miRNA in the patients of BPD mania before and after the treatment of asenapine and risperidone.

2. There are changes in the scores of YMRS in the patients of BPD mania before and after the treatment of asenapine and risperidone.

3. There are differences in the expression of miRNA in the patients of BPD mania before and after the treatment between asenapine and risperidone.

3.2 LITERATURE REVIEW

3.2.1 Antipsychotics

Antipsychotic drugs are widely used as pharmacotherapy prescribed to schizophrenia and related disorders. However, their use in the treatment of mania in BPD have increased over the past decade. This have now led to antipsychotic drugs being the first line treatment for bipolar mania. The neuronal mechanisms of mania remains ambiguous, but antipsychotic mechanisms observed in schizophrenia may provide clues on this matter. As occupancy of dopamine D2 receptor in the striatum correlates best with relief of positive symptoms of schizophrenia (Agid et al. 2007), it is likely that the similar mechanisms underlying the anti-manic effects, given the efficacy of antipsychotic in alleviation of manic symptoms in BPD. In common to other antipsychotic, asenapine have high affinity for dopamine D2 receptor. In fact, asenapine have affinity for D2 receptor that is higher than other antipsychotic except aripiprazole. (Shahid et al. 2009) Other than its affinity for D2 receptor, asenapine have been shown to have broad activities at 5-HT2 receptor. Its antagonism at 5-HT2B, 5-HT2C, 5-HT5A, 5-HT6 and 5-HT7 receptors is apparent at affinities at or greater than that for the D2 receptor; with activity at the 5-HT2C site that is particularly high (Shahid et al. 2009).

One of the side effects of atypical antipsychotic that is of most concern is weight gain. Affinity of antipsychotic at 5-HT2C has been associated with weight gain in patients (Reynolds et al. 2002). However, it is remarkable that even though asenapine possesses relatively high affinities at 5-HT2C and H1 receptors, similar to that been observed for clozapine and olanzapine, which is known for their weight gain induction, yet it avoids

inducing the profound weight gain associated with treatment using asenapine (McIntyre et al. 2009).

Another undesirable side effects associated with antipsychotic is hyperprolactinaemia. As discussed above, most antipsychotic were designed to be dopamine D2 antagonism which correlates with symptoms relief. But at the same time, this dopamine D2 antagonism by antipsychotic have introduced an unpleasant consequence, which is disinhibition of the release of prolactin via their action at receptors in the pituitary gland (Kapur et al. 2002). In view of this, the antipsychotic used in the treatment of BPD that were frequently reported to be associated with hyperprolactinaemia is risperidone (Kearns et al. 2000). This can result in galactorrhoea and gynaecomastia, and may contribute to sexual dysfunction (Haddad and Wieck 2004).

The mechanism that underlies prolactin secretion is the synergy interaction between dopamine D2 antagonism and serotonin receptors, particularly 5-HT2A and 5-HT2C receptors. It has been reported that these receptors have opposing effects to those of D2 receptors (Bagdy 1996). In addition to this, 5-HT2C agonists, are specifically effective in blocking prolactin release and this action is lacking in risperidone (Scheepers et al. 2001).In this aspect, even though asenapine has a high affinity for D2 receptor, it also possesses a strong affinity for 5-HT2C receptor, and in this way, the unwanted side effect is somewhat prevented (Shahid et al. 2009).

3.2.2 MicroRNA Production

MiRNA are non-protein coding endogenous RNA molecules, consisting of approximately 21 to 23 bases. MiRNA production starts with the transcription of miRNA genes by RNA polymerase Pol II promoters, and followed by splicing and polyadenylation. The product of the transcription is known as primary miRNA (pri-miRNA). Next, the pri-miRNA is cleaved in the nucleus by an enzyme complex that contains Drosha and Pasha. Following this, the cleavage product is known as precursor miRNA (pre miRNA). Pre miRNA is transported out to the cytoplasm from the nucleus by exportin 5 and further cleaved by an enzyme complex which consists of Dicer and Loquacious. After that, the miRNA duplex is then unwound, where it binds to a target mRNA, and the passenger strand is destroyed. (Kosik 2006)

Due to their abilities in regulating post-transcriptional gene expression, miRNAs have promptly gained important insights in molecular research since its discovery. Until now, there are over 2000 mature human miRNA sequences that have been reported (Friedman et al. 2009), and the numbers are increasing. A miRNA have the ability to target and regulate hundreds of mRNA, and reciprocally, a single mRNA can be regulated by multiple miRNAs. As reported, nearly 50 % of mammalian mRNA are potential targets for miRNAs (Friedman et al. 2009).

Generally, miRNAs act to suppress the expression of their target gene. However, the corresponding relationship between miRNA levels and their target genes are being extensively studied (Pasquinelli 2012). MiRNA are expressed not only during development stage, but also in adulthood. Their expression pattern is rather unique, in that some of the

miRNA are being expressed in specific organs (Sempere et al. 2004), or even specific region within an organs such as the brain (Olsen et al. 2009). Functional studies reveal that miRNAs play vital roles in the control of cellular processes which include neurogenesis, apoptosis, cell fate decision and synaptic plasticity (Kloosterman and Plasterk 2006, Bredy et al. 2011). Likewise, Dicer, one of the key enzymes in the production of miRNA, have been shown to be necessary for maintenance of neuron survival in the mature brain and also precise development of embryonic brain in zebra fish model (O'Connor et al. 2012).

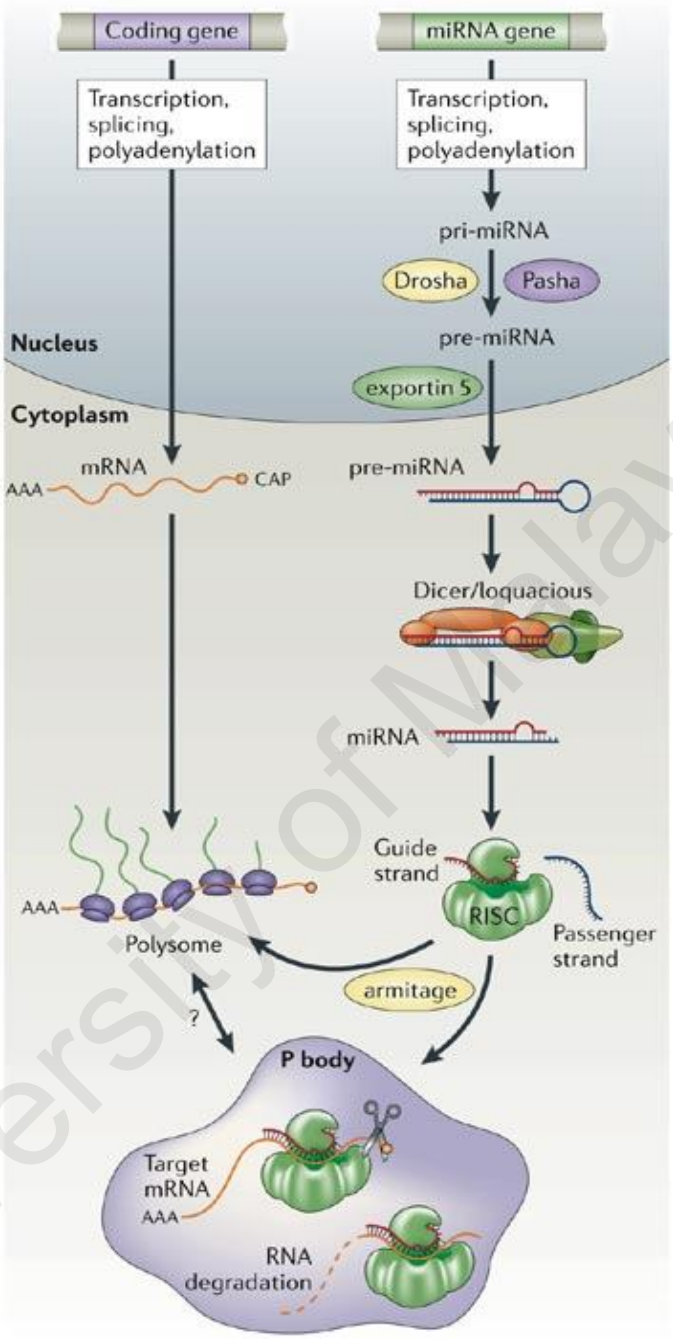

Figure 3.2 1 Production of miRNA

Adapted from Kosik KS. The neuronal microRNA system. Nat Rev Neurosci. 2006 Dec; 7(12):911–20

The ability of synapses to response to activity in their neuronal circuits by making adaptive changes is known as synaptic plasticity. These adaptive changes include modification of the strength or efficacy of synaptic transmission, modification of dendritic size and density and receptor expression. Synaptic plasticity also plays an important roles in the early development of neural circuity (Martin et al. 2000). Given that these modification are crucial parts of the mechanisms underlying higher brain function such as memory and learning, dysregulation of synaptic plasticity are postulated to be involved in the pathogenesis of neuropsychiatric disorders.

Certain miRNA, such as miR-132 and miR-134 have been identified to be associated with altered dendritic spine density and volume (Schratt et al. 2006, Edbauer et al. 2010). The target genes of these miRNA are deeply associated with synaptic function and regulation. These includes genes that encodes brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB), and LIM domain kinase-1 (LimK-1). BDNF are widely expressed throughout the brain where it promotes neuronal survival and maturation, synaptic plasticity and synaptic function; CREB is a transcription factor involved in synaptic plasticity and memory formation; while LimK-1 is an enzyme that regulates dendrite size. Furthermore, BDNF can reverse the suppression action of miRNA towards LimK-1 (Schratt et al. 2006).

In addition, miRNA also acts as a vital regulators of postnatal neurogenesis - the growth and development of neuron. One relevant example of this is miR-124, which has been shown to command adult neurogenesis in mice (Åkerblom et al. 2012). In relation to psychiatry, a study has shown that antidepressant therapies stimulate neurogenesis, while stress and depression inhibit neurogenesis (Baudry et al. 2011). Given their role in

neurogenesis, the study of miRNA is of relevance in further understanding the molecular mechanisms of psychiatric disorders.

The interaction of miRNA and antipsychotic treatment has also gained the attention from researchers, as shown by various studies that examine the miRNA changes in postmortem studies of individuals receiving antipsychotic treatment. In a studies using animal model, haloperidol was found to upregulate the level of a set of miRNAs (Perkins et al. 2007), and these results have supported the results of another similar study using cortex of SZ patient (Beveridge et al. 2010). Among those miRNA that have shown alteration in expression, miR-219 is of particular interest. Animal studies have shown that a reduction in miR-219 mediates schizophrenia-like effects similar to those demonstrated by NMDA (N-Methyl-D-aspartate) antagonists such as hyperlocomotion and stereotypy (Kocerha et al. 2009). On the other hand, this condition can be reversed via pretreatment of the animal with antipsychotics. Furthermore, miR-219 has been reported to be upregulated in the dorsolateral prefrontal cortex (DLPFC) of schizophrenic patients of the previously mentioned study.

Besides antipsychotics, studies also revealed the roles of miRNA in mood stabilizer treatment in BPD patients. Preclinical studies have shown the effects of mood stabilizers (lithium and valproate) on miRNA levels in rats, with up regulation of several miRNAs and down regulation of miR-144 in response to mood stabilizer treatment (Zhou et al. 2009). Another such studies with lithium using lymphoblastoid cell lines have revealed that three miRNAs were upregulated (miR-221, miR-152 and miR-15a) while miR-494 was shown to be down regulated (Chen et al. 2009).

3.3 MATERIALS AND METHOD

3.3.1 Materials

3.3.1.1 Blood collection

Tempus RNA tube (Applied Biosystems), syringe and needle, tourniquet, alcohol swab, cotton wool, plasters, disposable examination gloves.

3.3.1.2 RNA Extraction

Tempus RNA Extraction Kit (Applied Biosystems), 1.5 ml microcentrifuge tube

3.3.1.3 RNA measurement

Nanodrop (Thermo Scientific), Bioanlyzer

3.3.1.4 Real time PCR

PCR strip tubes with cap, Custom-made Taqman miRNA expression assay (Applied Biosystems), TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems).

3.3.1.5 MicroRNA Microarray

GeneChip® miRNA 4.0 Array, Flashtag™ Bundle, Enzyme Linked Oligosorbent Assay

3.3.2 Instruments

BIO-RAD UV Transluminator; Gel Electrophoresis System with Power Pack; Vortex machine, Microcentrifuge; Microwave; Freezer; Refrigerator, Weighing Machine, Ice-maker Machine , micropipettes (20 µ L,200 µ L and 1000 µ L); Step One Plus Real-Time PCR (Applied Biosystems), Thermoblock, Thermo Scientific Nanodrop 2000c Spectrophotometers. ; Milli-Q® Water Purification Systems.

3.3.3 Subjects

3.3.3.1 Recruitment

The subjects for this study were recruited from University Malaya Medical Centre inpatient psychiatric ward. The inclusion criteria for recruitment includes patients who have been diagnosed with BPD I, currently in manic phrase, according to DSM V by psychiatrists, aged 18 to 65, and is either treatment-naive or have defaulted medication for the past three months prior to admission. The exclusion criteria were as follows: a history of substance abuse and psychiatric disorders other than BPD, currently in pregnancy or actively seeking pregnancy and severe mania who needed ECT. The patients were recruited into the study if the patient and relatives have consented and were then divided into two groups, by randomization. One groups of patients were prescribed asenapine, while the other group were given risperidone. Both groups have also been prescribed sodium valproate as mood stabilizer. Each patients was commenced on the dose optimum for the patient's condition. The patient's progress was monitored by the psychiatrists. The dose of antipsychotics was escalated depending on the patient's mental state. The dose for sodium valproate was started and maintained on 20 mg/kg body weight of the patient. This study is approved by Medical Ethics Committee of UMMC, and all the subjects provided written consent to be enrolled into this study.

3.3.3.2 Measurement

The patients were assessed on their sociodemographic profiles and Young Mania Rating Scale (YMRS) to monitor the improvement of the patient's condition at baseline (WO), week 1, week 4 and after 12 weeks of treatment with asenapine or risperidone. Blood were taken and put into Tempus RNA blood tube (Applied Biosystems) and the miRNA expression were analyzed and compared using GeneChip miRNA 4.0 array (Affymetrix). The details of the demography of subjects were listed in Table 3.4.1. Patients were

discharged based on clinical improvement and YMRS scored showed more than 50% reduction. The rater was not blinded to the randomized drug.

3.3.4 RNA extraction

The blood sample in the Tempus tube was poured into a clean 50-mL tube. A volume of 3ml of 1xPBS were pipetted into the tube to bring the total volume to 12ml. The tube was then vortexed for 30 seconds to ensure proper mixing of the contents. Next, the mixture was centrifuged at 4^0C at 3000 x g for 30 minutes. The supernatant was carefully poured off and the tube were left inverted on absorbent paper for 1 to 2 minutes. Then, the remaining drops of liquid off the rim of the tube were blotted with clean absorbent paper. A volume of 400µl of RNA purification suspension solution was pipetted into the tube, and the tube was briefly vortexed to suspend the RNA pellet. Suspended RNA was then ready for the next step. First, the RNA purification filter was inserted into a waste collection tube. The filtration membrane was pre-wet with RNA Purification Wash Solution 1. Next, the suspended RNA was pipetted into the purification filter and centrifuged at 16,000 x g for 30 seconds. Then, the purification filter was removed and re-inserted into the waste tube after the liquid waste collected in the waste tube was discarded. The steps was repeated again with RNA Purification Wash Solution 2. After the liquid waste collected in the waste tube was discarded and the purification filter re-inserted into the waste tube, the tube was centrifuged at 16,000 x g for 30 seconds. Following that, the purification filter was transferred into a new collection tube to collect the eluate. Then, the nucleic acid purification elution solution was pipetted into the purification filter and the cap was closed and incubated at 70^0C for 2 minutes followed by centrifuged at 16,000 x g for 30 seconds. The collected RNA eluate was pipetted back into the purification filter and was then centrifuged at maximum speed for 2 minutes. The purification filter was discarded, and approximately 90µl of the RNA

eluate was transferred to a new, labeled collection tube. The RNA was then stored at -80 ^{0}C for long-term storage.

3.3.5 Quantitative microRNA expression analysis

RNA integrity and concentration were assessed using NanoDrop spectrophotometer (Thermo Scientific) and Bioanalyzer (Agilent). To ensure that the quality of the result of microarray is not affected, only samples with RNA integrity number equal or exceeds 8 are accepted for subsequent analysis. The RNA was labelled using the FlashTag Biotin RNA Labeling Kit. Prior to hybridize to GeneChip miRNA 4.0 microarrays (Affymetrix Inc.) containing probes for a total of 2025 human miRNAs, the Biotin-labeled RNA was checked for its quality using the Enzyme Linked Oligosorbent Assay (ELOSA). A positive ELOSA result confirms that the biotin-labeling process of RNA was properly carried out. The sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA were tailed with Poly A and then labeled with Biotin. Afterwards, the labeled RNAs were hybridized onto the GeneChip miRNA 4.0 microarrays at 48°C for 16 hours. Having washed and stained, the arrays were scanned by the Affymetrix Scanner 3000 (Affymetrix). The scanned images were analyzed using Expression Console software (version1.3.1, Affymetrix).

Selected candidate miRNA with significant difference were validated using real time PCR method. Custom-made TaqMan miRNA expression assay (Applied Biosystems) were used for the quantification of expression level of selected miRNA candidate. All experiments were ran in triplicate and RNU 48 were selected to act as the endogenous control. The total RNA were transcripted into cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Briefly, total RNA were diluted to suitable concentration as the

volume of RNA needed for the following reaction is 5µl per reaction. Next, 5µl of RNA were mixed together with 3µl of primer and 7µl of master mix which consists of 100 mM deoxynucleotide triphosphates (dNTP) (with deoxythymidine triphosphate, 0.15 µl/reaction), Multiscribe™ Reverse Transcriptase 50 U/µl (1 µl/reaction), 10 x Reverse Transcriptase Buffer (1.5 µl/reaction), RNase Inhibitor 20 U/µl (0.19 µl/reaction) and nuclease-free water (4.16 µl/reactions). This brings up to total volume of the mixture to 15µl. The mixture were then incubated for 30 min at 16 °C and followed by 30 min of incubation at 42 °C. The reaction was terminated at 85 °C for 5 min before being held at 4 °C until required for qPCR.

After that, a PCR master mix was prepared for each miRNA gene assay. Each 20 µl reaction contained 1 µl 20X TaqMan miRNA assay mix, 10 µl TaqMan 2X Fast Universal PCR Master Mix with No Amperase UNG, 7.67 µl of nuclease-free water, and 1.33 µl of the reverse transcription product. Each reaction was performed in triplicates. For negative control of the reverse transcription template, nuclease-free water was used. PCR reactions were placed in the StepOne Plus machine, and the reaction was performed at 95 °C for 20 s, followed by 40 cycles of denaturing at 95 °C for 1 s, and then annealing and extension at 60 °C for 20 s. The results were analyzed with StepOne Plus software (version 2.3). Relative gene expression levels were calculated using the comparative threshold (ct) method, commonly known as the delta-delta ct method.

Delta Ct = Ct value of a miRNA in a sample - Ct value of the endogenous control in the Same sample

Delta-delta ct = Mean value of delta Ct of treated group - Mean value of delta Ct of Untreated group (control group).

Fold change = $2^{\text{delta-delta ct}}$

Note: The Ct or cycle threshold value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal that is significantly above the background fluorescence.

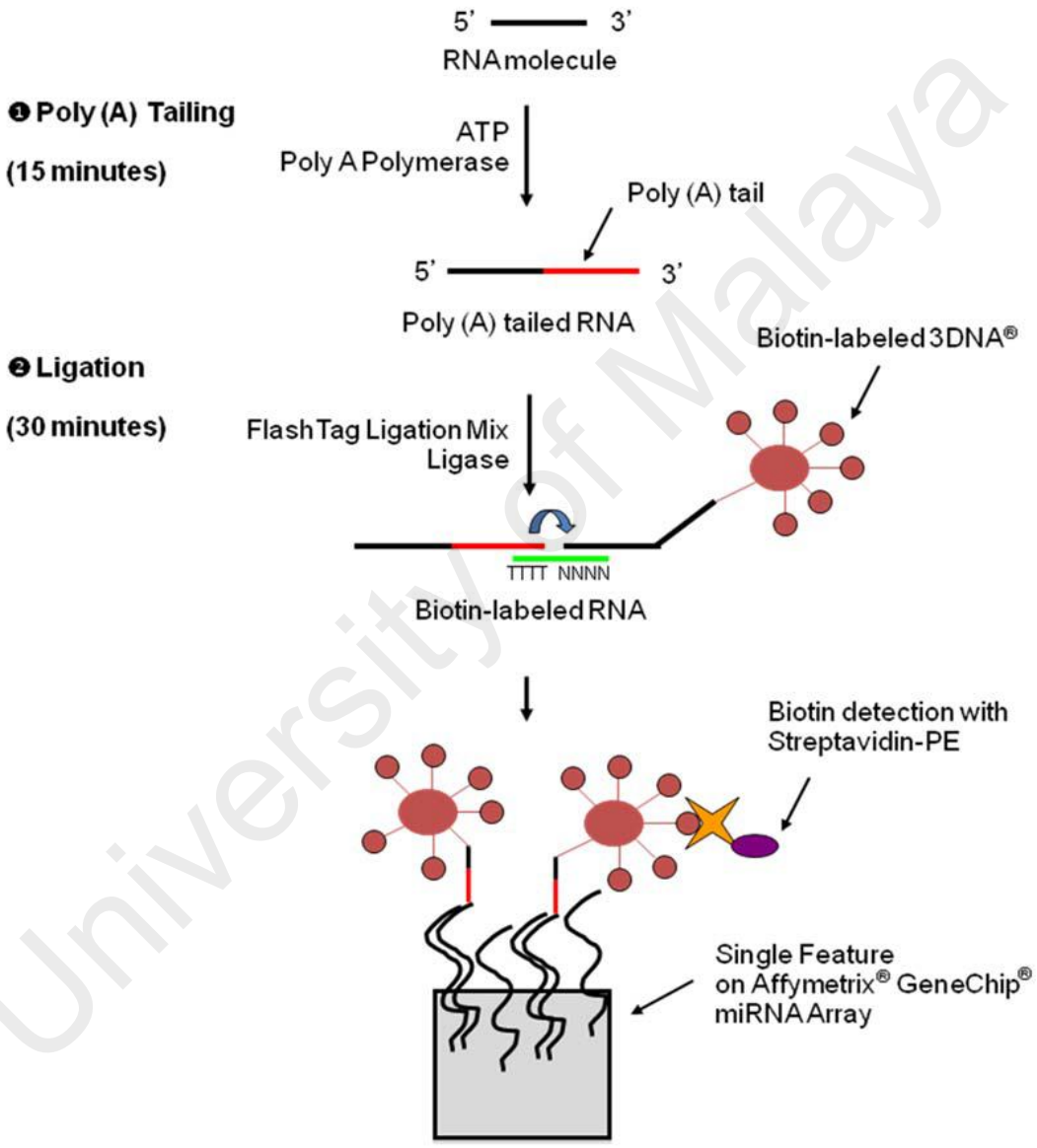

Figure 3.3.1 Overview procedure of Flashtag Biotin RNA Labelling

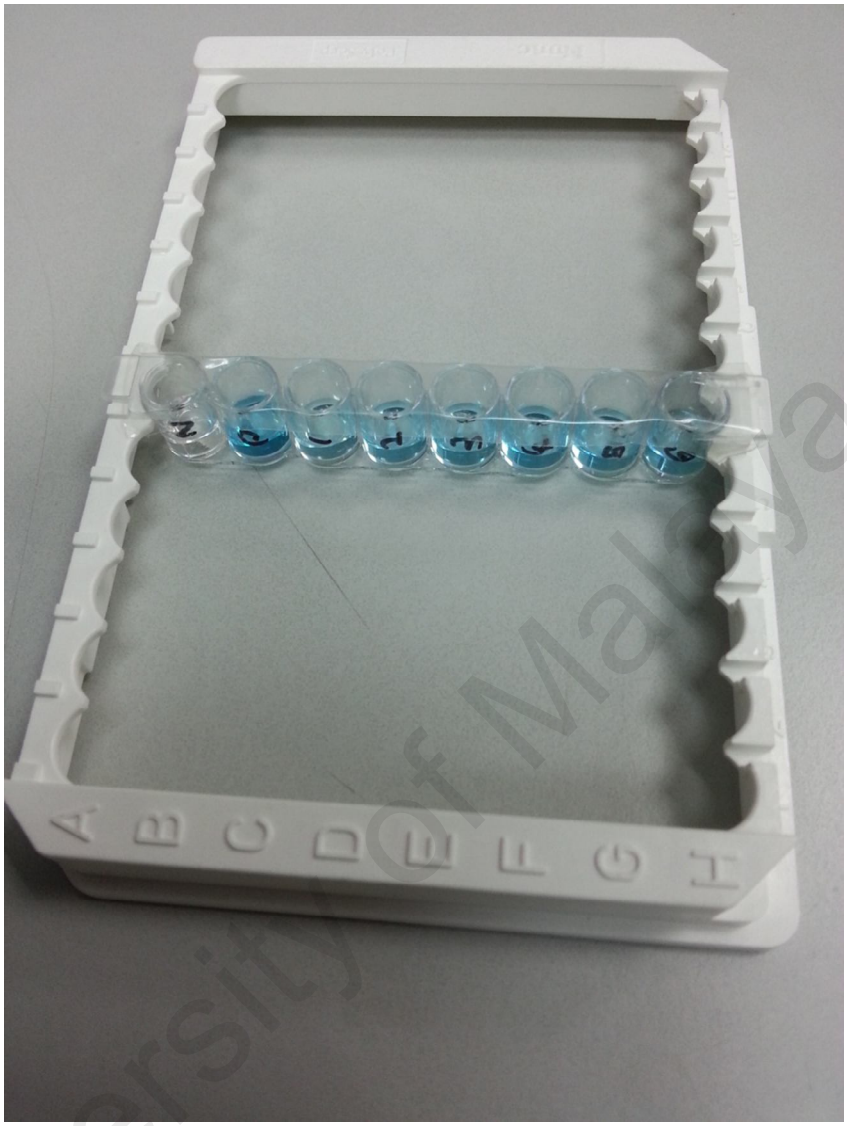

Figure 3.3.2 Enzyme Linked Oligosorbent Assay with negative and positive control.

(This serves as a quality control to ensure that the microRNA have been labeled prior to microarray scanning in order to be detected.)

3.3.6 Statistical analysis and target prediction

The statistical analysis relative to miRNA expression were performed using the Affymetrix Transcriptome Analysis Console (TAC) Software. The regulatory targets of differentially expressed miRNAs were identified using Ingenuity Pathway Analysis's (IPA) miRNA Target Filter function, which queries TarBase and miRecords for experimentally validated miRNA targets and TargetScan for predicted miRNA targets. In addition, the Ingenuity Knowledge Base contains information on miRNA targets from the peer-reviewed literature. The list of experimentally observed and predicted miRNA targets was then submitted to DAVID (Database for Annotation, Visualization and Integrated Discovery) to identify gene ontology annotation analysis and biological pathway.

The changes in YMRS score during week 0, week 4 and week 12 was examined using one-way ANOVA. The difference between YMRS score between the two groups were compared using t test.

3.4 RESULTS

3.4.1 Demographic data of the patient

There are no significant differences between the two treatment groups in terms of gender and age (asenapine group = 28.64, risperidone group = 29.83). Initially, there were a total of fourteen patients recruited, seven on each group. However, during the assessment, four patients dropped out, two were due to loss of follow up and withdrawal from the study with the patients' consent, while another two patients was due to lack of efficacy (one in each group). Hence, at week twelfth, there were a total of ten patients who completed the study.

Table 3.4.1 Demographic data of patient

	Asenapine	Risperidone
Age	28.64	29.83
Gender		
Male	2	2
Female	5	5
Ethnicity		
Malay	2	2
Chinese	5	3
Indian	-	2

3.4.2 YMRS Score

For the four patients who dropped out, last observation carried forward method (LOCF) were used for the calculation of mean YMRS scores. At week twelfth, the mean change from the baseline in total YMRS scores was -28.20 with the asenapine group and -28.42 with the risperidone group. Statistical analysis indicates no significant difference between asenapine and risperidone. In terms of measurement of the efficacy of antipsychotics, the percentage of YMRS responders is calculated. The percentage of YMRS responders is defined as 50% reduction from baseline in YMRS total score at the endpoint of study (Mclntyre et al 2009). In the present study, the percentage of asenapine and risperidone patients meeting the criteria for YMRS response was not significantly different at any assessment during the study period (all $p > 0.05$). The rates of response at twelfth week were 90% with asenapine and risperidone, respectively.

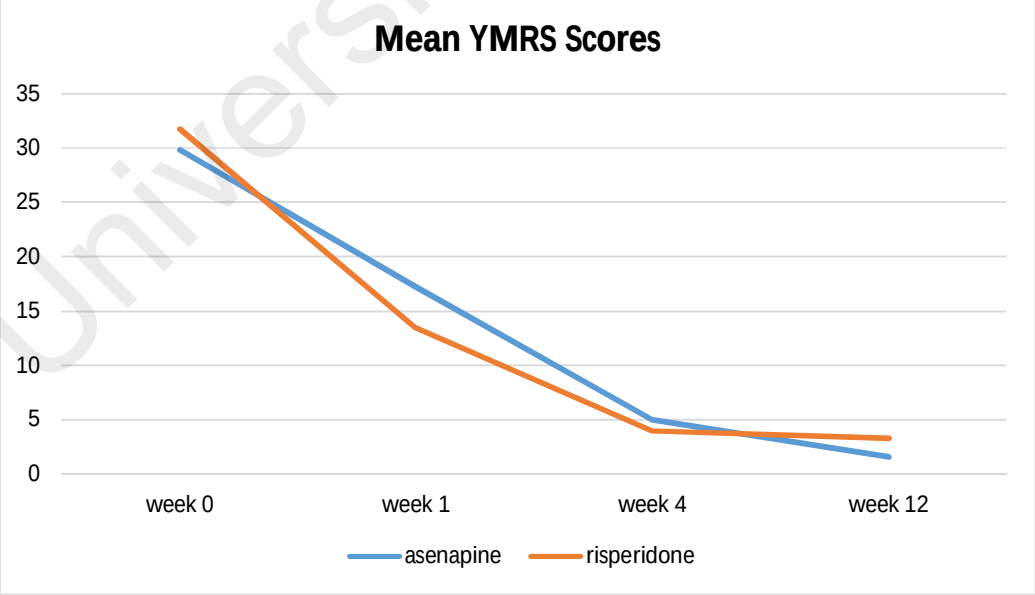

Figure 3.4.1 Mean YMRS scores during twelve weeks of treatment

3.4.3 Analysis of miRNA expression changes following treatment with asenapine and risperidone

By using the Transcriptome Analysis Console (TAC) Software, the miRNA expression profiling were analyzed using one-way ANOVA test and the p-value was adjusted for multiple testing with the Benjamini Hochberg test. At the fourth week of the study, there were no statistically significant difference in expression of miRNA compared to baseline, for both groups. However, during the twelfth week of the study, differentially expressed miRNA that were statistically significant were identified. There were 24 such miRNA in asenapine group while only three in risperidone group.

In Table 3.4.2, out of 24 miRNA, 22 miRNA were upregulated with the fold change ranging from 2.05 to 8.56, while two miRNA were down regulated with the fold change ranging from -2.36 to -2.44. On the other hand, in Table 3.4.3, all the three miRNA were down regulated with the fold change ranging from -2.41 to 4.2. Of these, none of the miRNA that was identified overlaps between asenapine and risperidone treatment group. Each miRNA identified were unique to respective group.

Table 3.4.2 Differential expressed miRNA in asenapine group

Transcript ID(Array Design)	Fold Change	ANOVA p-value
hsa-miR-18a-5p	8.56	0.010761
hsa-miR-19b-3p	8.07	0.013057
hsa-miR-145-5p	7.82	0.029543
hsa-miR-27a-3p	6.54	0.000161
hsa-miR-148b-3p	5.53	0.005188
hsa-miR-210-3p	5.3	0.005157
hsa-miR-17-3p	5.19	0.018034
hsa-miR-30b-5p	5.11	0.015608
hsa-miR-378g	5.11	0.047909
hsa-miR-106b-5p	3.93	0.00445
hsa-miR-505-3p	3.71	0.031197
hsa-miR-339-5p	3.55	0.002185
hsa-miR-194-5p	3.16	0.021328
hsa-miR-6068	3.05	0.047453
hsa-miR-20b-5p	2.95	0.043146
hsa-miR-106a-5p	2.58	0.006898
hsa-miR-20a-5p	2.54	0.002247
hsa-miR-17-5p	2.43	0.011219
hsa-miR-15a-5p	2.18	0.002422
hsa-miR-1231	2.11	0.005526
hsa-miR-4516	2.08	0.044268
SNORD121B	2.05	0.012617
hsa-miR-92b-5p	-2.36	0.04547
hsa-miR-1343-5p	-2.44	0.019721

Table 3.4.3 Differential expressed miRNA in risperidone group

Transcript ID(Array Design)	Fold Change	ANOVA p-value
hsa-miR-664b-5p	-2.41	0.035348
hsa-miR-6778-5p	-3.83	0.047124
hsa-miR-146b-5p	-4.2	0.005919

3.4.4 MicroRNA target prediction and pathway analysis

In order to further understand the role of miRNA and their possible gene target, we have performed a bioinformatics analysis using IPA software. In asenapine treatment group, initial analysis show that there are about 3000 target genes predicted. When the confidence level were set to experimentally observed, only those gene that are mentioned in previous studies in the database will be shown. This have filtered the target genes to 444. From this 444 target genes, we then cross-referenced with a database which includes all the susceptibility genes associated with BPD mentioned in previous publications, and this resulted in 35 genes that was reported to be associated with BPD. Out of 35 genes associated with BPD, 13 are regulated by one miRNA, which is miR-15a. Summary of the findings were listed in Table 3.4.9

While initial analysis of risperidone treatment group show that there are about 300 target genes predicted, when the confidence level were set to experimentally observed, those gene that are mentioned in previous studies in the database will be shown. This have filtered down the target genes to 77. From these 77 target genes, we further cross-referenced with a database which includes all the susceptibility genes associated with BPD mentioned in previous publications, and this resulted in 6 genes that was reported to be associated with BPD. All these six target genes are regulated by one miRNA, which is miR-146b.

The target gene were submitted to DAVID for gene ontology annotation at three levels, which is biological processes, cellular component and molecular function. The top ten of the most enriched items for each category are shown in Figure 3.4.2 and Figure 3.4.3

In the asenapine group, the enriched gene list was mainly focused on regulation of cell death and regulation of transcription. However in the risperidone group, the list was enriched on response of immune system and body defense mechanism.

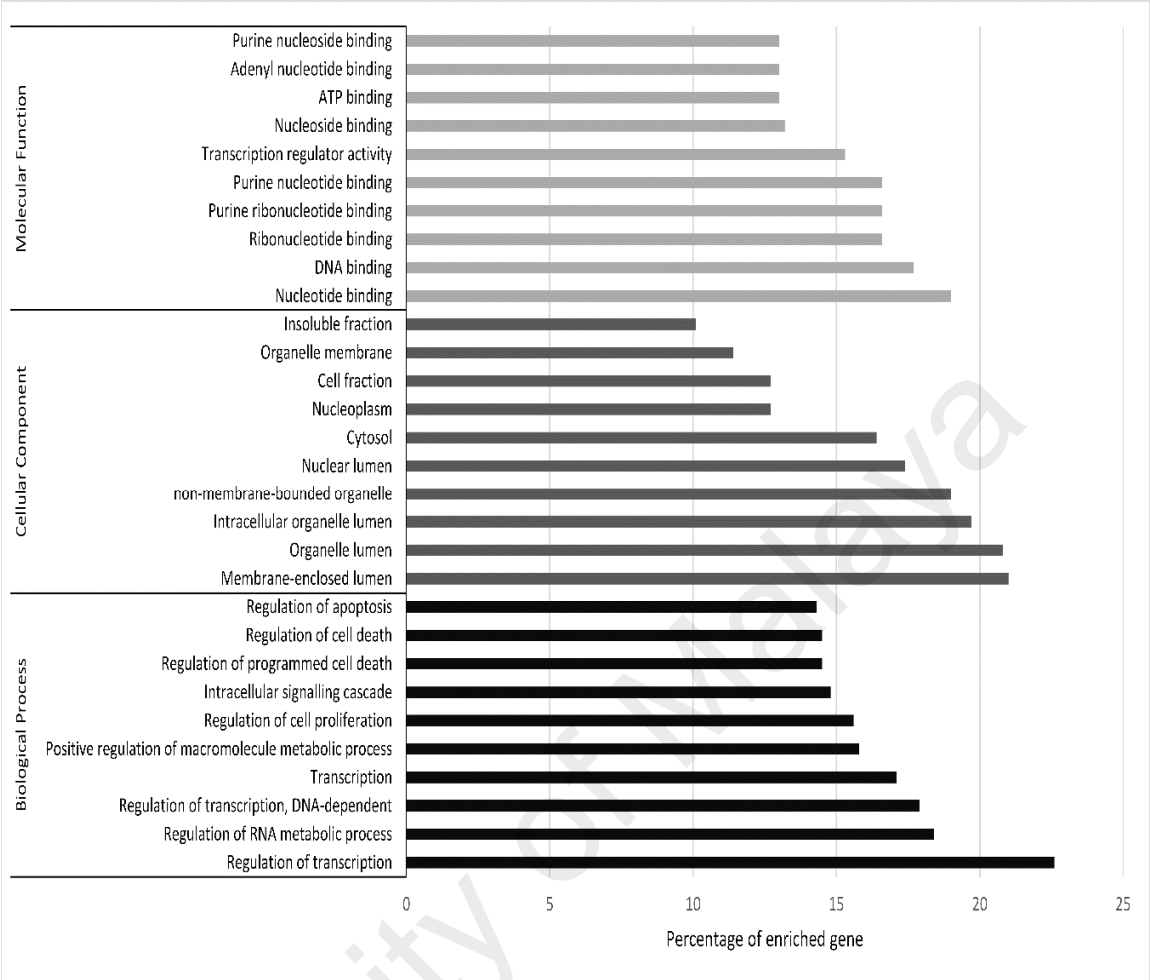

Figure 3.4.2 Top ten enriched items in three category for gene ontology in the asenapine group

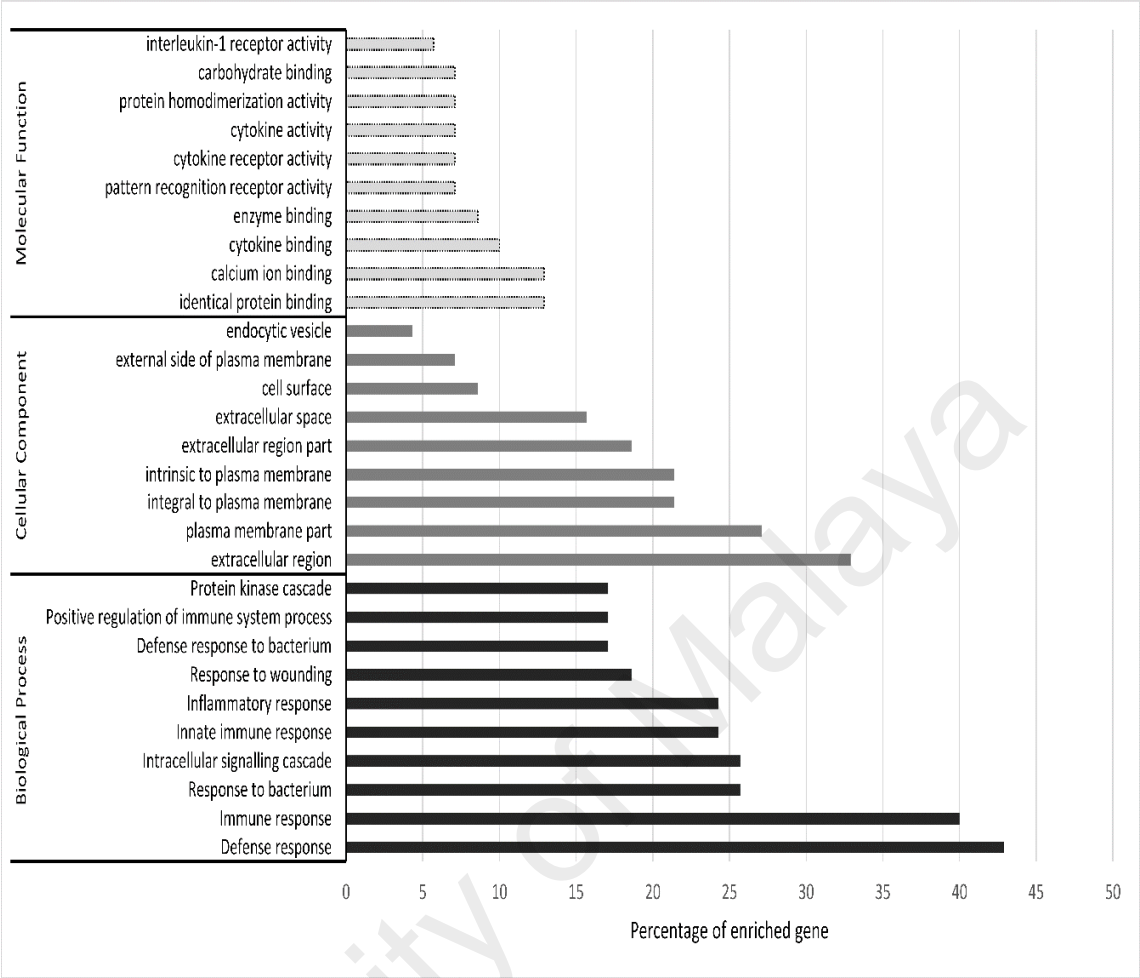

Figure 3.4.3 Top ten enriched item in three category for gene ontology in risperidone group

Table 3.4.4 Target gene prediction for selected miRNA in the asenapine group

Hsa-miR-145-5p	MYC , MMP1 ,CCNA2 , DFFA , MDM2 ,CDK4 ,EIF4E , MAPK7 , DDR1, MUC1 ,F11R ,AHNAK ,UNG , PADI1 , CES1 ,SPTB, PPP3CA , RASA1 , KLF5 , IRS1 , TPM3 , IGF1R , FSCN1 ,CLINT1, NDUFA4, USP46, EIF4EBP2, RTKN , KLF4
Hsa-miR-15a-5p	MAP2K1 ,MAP2K4 , PDCD6IP , CDC25A , TNFSF9 , ITGA2 .FGF2 , FGF7 ,CCND1 , FGFR1 ,CLDN12 , LAMC1 , HSPA1A/HSPA1B, DNAJB4 , SLC12A2 , PPP2R5C , BCL2 ,VEGFA ,CCNE1 ,CHEK1 ,CDK6 ,CCND3 ,RTN4 ,WNT3A ,EIF4E, BDNF ,E2F3 ,LAMTOR3 ,WEE1, KCNN4 ,SPTLC1, SLC7A1 ,ZYX ,NOTCH2 ,IGF2R ,NAPG ,HMGA1 ,RECK ,TXN2 ,UCP2KIF23 ,CDC14B ,UBE4A , KPNA3 ,PAFAH1B2 , ARHGDIA , ANLN , PSAT1 , PNP , TPM3 , ATF6 ,VTI1B , ECHDC1
hsa-miR-19b-3p	HIPK3 , PTEN , CCND1 , BMPR2 , ERBB4 , BCL2L11 , ESR1 , THBS1 , CTGF , MYLIP
hsa-miR-210-3p	ISCU, FGFRL1 ,EFNA3 ,E2F3 ,ACVR1B , SDHD
hsa-miR-27a-3p	GCA , ST14, ZBTB10 , FOXO1, CYP1B1 , RUNX1 , SMAD5 , PPARG , FBXW7 , MMP13, SMAD4 , RXRA , ADORA2B, BBC3 , PHB , THRB
hsa-miR-30b-5p	PPP3CA , NT5E , GNAI2 , MAP4K4 , BCL6 , RUNX2 , ATP2A2 ,AP2A1 , MAT2A , ACVR1 , PGM1 , UBE2I , SLC38A1, NEUROD1 , NAPG , RAD23B , PTPRK , PAFAH1B2, PRPF40A
hsa-miR-339-5p	HOXA 1, HOXA 11, BCL 6, GRM3

Table 3.4.5 Target gene prediction for miRNA in risperidone group

Hsa-miR-146b-5p	BRCA1 ,C8A ,CAM ,CCL8 ,CCNA2 ,CCR3, CD1D ,CD40 ,CDKN3,CFH ,CHUK ,COL13A1 ,CRP , CXCL8 ,CXCR4,DMBT1 ,FADD ,IFNA1/IFNA13 ,IFNB1 , IL10 ,IL12RB2 ,IL1F10 ,IL1R1 ,IL1RAP ,IL1, RAPL2 ,IL1RL2 ,IL36A ,IL36B, IL36 , IL36RN ,IL37 , IRAK1 , IRAK2

Table 3.4.6 Six target gene associated with bipolar disorder in risperidone group

NFIX	nuclear factor I/X (CCAAT-binding transcription factor)
TIMELESS	timeless circadian clock
POLE2	polymerase (DNA directed), epsilon 2, accessory subunit
IL10	interleukin 10
MMP16	matrix metallopeptidase 16 (membrane-inserted)
TLR4	toll-like receptor 4

Table 3.4.7 35 target genes that were associated with bipolar disorder in asenapine group

BDNF	brain-derived neurotrophic factor
GRM3	glutamate receptor, metabotropic 3
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma
NPTX1	neuronal pentraxin I
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1
ERBB4	erb-b2 receptor tyrosine kinase 4
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
BCL2	B-cell CLL/lymphoma 2
CREB1	cAMP responsive element binding protein 1
NDUFA4	NDUFA4, mitochondrial complex associated
JAK1	Janus kinase 1
FBXO28	F-box protein 28
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)
NT5DC1	5'-nucleotidase domain containing 1
MBNL1	muscleblind-like splicing regulator 1
NR4A2	nuclear receptor subfamily 4, group A, member 2
NFIA	nuclear factor I/A
CCKBR	cholecystokinin B receptor
ADORA2B	adenosine A2b receptor
HARS	histidyl-tRNA synthetase
MMP3	matrix metallopeptidase 3
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1
SPTLC1	serine palmitoyltransferase, long chain base subunit 1
USP46	ubiquitin specific peptidase 46
FGFRL1	fibroblast growth factor receptor-like 1
ESR1	estrogen receptor 1
EGFR	epidermal growth factor receptor
GNAI2	guanine nucleotide binding protein (G protein),
CRHBP	corticotropin releasing hormone binding protein
RTN4	reticulon 4
HSF2	heat shock transcription factor 2
WIPF1	WAS/WASL interacting protein family, member 1
CEP72	centrosomal protein 72kDa
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma
BCL2L11	BCL2-like 11 (apoptosis facilitator)

Table 3.4.8 Summary of the target gene for miRNA in asenapine group

MicroRNA	**Target Gene**
miR-15a	BDNF, NAPG, CACNA2D1, BCL2, NTSDC1, NF1A, HARS, HSP90B1, SPTLC1, FGFRL1, EGFR, CRHBP, RTN4, W1PF1,
miR-106b	BCL2, CREB1, JAK1, CR1M1, MMP3, ESR1, BCL2L11,
miR-30b	NAPG, ATP2A2, MBNL1, GNA12,CEP72,
miR-145	NDUFA4, FBXO28, USP46,
miR18a, miR-19b, miR-148b, miR-27a, miR-339, miR-210	GRM3, NPTX1, ERBB4, NR4A2, CCKBR, ADORA2B, HSF2, PPP2R5C

3.5 DISCUSSION

The development of atypical antipsychotics has provided patients with drugs which are better in terms of much less side effects, and the uses of atypical antipsychotics have consistently emerged as the first line treatment for bipolar mania. Despite this, there are still significant numbers of patients who are impaired. In general, the current pharmacology of the atypical antipsychotics were concentrated on several specific pathways, which include dopamine receptors system, 5-HT receptors and GABA system. The molecular mechanisms of the pharmacology of these atypical antipsychotics remain elusive, making it difficult to personalize the treatment for better therapeutic effects. Given the heterogeneity of psychiatric disorders, in which there is no single gene that exerts a huge impact on the disorders, and the pathophysiology of psychiatric disorders mainly involves dynamic interactions of several complex pathways, such as synaptic plasticity and neurogenesis, the emergence of research on miRNA have claimed its importance in this particular issues. MiRNA, which is a non-coding RNA, have the potential to regulate the expression of several hundred target genes, opens the possibility to be manipulated as the novel target for antipsychotic actions. Increasing amount of findings have shown that miRNA are involved in the control of cellular processes including neurogenesis, synaptic plasticity, apoptosis and cell fate decision (Magill et al. 2010, Bredy et al. 2011).

Several studies have shown that miRNA can act as diverse pharmacology target sites for certain psychotropic drugs. For example, miRNA-504 have been shown to increase dopamine D1 receptor expression, while the expression can be reversed by miR-504 inhibition (Huang and Li 2009). Perkin at el (2007) examined the differential expression of miRNA in 179 rats treated with haloperidol with untreated control, and have found that

miR-199a, miR-128a and miR-128b were elevated in haloperidol treated rats (Perkins et al. 2007). Albeit the findings that miRNA expression can be altered by antipsychotics and other psychotropic drugs, there is hardly any evidence that these miRNA changes were associated with symptoms improvement in bipolar mania.

This present study were intended to examine the miRNA that are associated with or involved in the pathway mechanism of action for both atypical antipsychotics, namely, asenapine and risperidone. In terms of efficacy of the atypical antipsychotics in treating the acute mania symptoms, the YMRS scores do not show any significant differences between the two treatment groups. Both treatment group achieved 90% responder at the twelfth weeks of study. While the atypical antipsychotics do not differentiate much in terms of efficacy and symptoms relief, other aspects relating to the treatment such as antipsychotics-induced weight gain would be more prominent in differentiating and classifying these atypical antipsychotics. (Reynolds 2011). But this is not within the scope of our study.

In the miRNA expression profiling, we have identified 22 miRNA that was significantly differentially expressed after treatment with asenapine, while only 3 such miRNA that was identified in the treatment group of risperidone. In order to further understand the role of miRNA identified in the molecular mechanisms of action of both atypical antipsychotics, a thorough literature search have been performed. In treatment group of asenapine, out of 22 miRNAs, seven miRNAs were found to be related to neuronal disorders.

MiR-19b

miR-19b was found to be associated with three neurological disorders, where it was upregulated in the serum of Autism Spectrum Disorder patient (Mundalil Vasu et al. 2014) and the serum and amygdala of animal model of PTSD (post-traumatic stress disorder) (Balakathiresan et al. 2014) ; while down-regulated in idiopathic Parkinson's Disease (Botta-Orfila et al. 2014). In the study of PTSD, the author showed that miR-19b was enriched in glial cells and was able to inhibit aberrant glial expression of neuronal proteins. The author suggested that miR-19b was able to cause neurodegenerative disease by inducing dopaminergic neuronal apoptosis. Besides, miR-19b was also shown to be down-regulated in animal model of aging, and their down-regulation was associated with increased transcription of several target genes identified to be associated with aging in a previous study (Hackl et al. 2010).

MiR-145

In a study to examine the neuronal apoptosis in Alzheimer diseases, MiR-145 was found to be upregulated in Alzheimer disease (Li et al. 2014). The author hypothesized that the role of miR-145 in Alzheimer disease is dependent on a tumor suppressor gene, p53, based on the observation that previous studies have shown that increased p53 reactivity has been associated with apoptosis in Alzheimer disease, and its inhibition has been shown to protect neurons from amyloid-induced cell death. It has therefore been postulated that up-regulation of miR-145 is associated with increased neuron apoptosis through its interaction with p53. Besides, miR-145 was found to be down-regulated in the brain of rats exposed to prenatal stress (Zucchi et al. 2013). Apart from this, miR-145 was identified to be expressed in the primary neurons of dorsal root ganglion and it was down-regulated following nerve

injury (Zhang et al. 2011). The author hypothesized that miR-145 may act as a repressor of neurite growth during the regeneration of primary neurons after injury, as it was shown that miR-145 can inhibit neurite growth in normal neuron in an in- vitro study. The down regulation of miR-145 may thus facilitate peripheral nerve regeneration in an injured neuron.

MiR-15a

MiR-15a was associated with neuronal maturation through its link with brain-derived neurotrophic factor (BDNF) (Gao et al. 2015). BDNF is a potent neurotrophic factor for neuronal maturation and is well known for its role in the pathology of BPD and other related neuropsychiatry disorders (Post 2007). Studies have shown that BDNF expression is regulated by another gene, MeCP2 (Zhou et al. 2006). MeCP2 gene regulates the expression of BDNF through its interaction with miR-15a. Overexpression of miR-15a was able to inhibit dendritic morphogenesis in immature neurons while reduction of miR-15a has the converse effect. MiR-15a was also able to regulate the expression level of BDNF, where exogenous BDNF was able to rescue the neuronal maturation deficits resulting from overexpression of miR-15a. Furthermore, inhibition of miR-15a could reverse the effect of neuronal maturation deficits in MeCP2-deficient adult-born new neurons. All of these have supported the hypothesis that miR-15a plays a role in neuronal development through its interaction with BDNF and MeCP2.

MiR-30b-5p

MiR-30b was associated with schizophrenia, a psychiatric disorder that have been shown to share common genetic roots with BPD. In a study of the relationship between estrogen signaling pathways and schizophrenia, miR-30b expression was significantly reduced in the

cerebral cortex of females but not male subjects with schizophrenia (Mellios et al. 2012). Estrogen signaling pathways was known to affect cortical function and metabolism and are thought to play a role in the pathophysiology of schizophrenia, where it exert neuro-protective effects in female subjects at risk (Cyr et al. 2002). It is then postulated that miR-30b in schizophrenia could be influenced by gender and potentially regulated by estrogen signaling. Reduced miR-30b expression causes the neuro-protective effects in females to be lost, thereby increasing the female subjects at risk of schizophrenia. Of note, miR-30b is predicted with high probability to target at least 20 genes implicated in schizophrenia, including metabotropic glutamate receptors GRM3 and GRM567.

MiR-210-3p

Overexpression of miR-210 was found to be able to induce angiogenesis and neurogenesis in the normal adult mouse brain, and both processes are crucial for brain tissue repair and remodeling after brain injury (Zeng et al. 2014). Other than the validated gene targets ISCU1/2 and COX10, which have important roles in mitochondrial respiration and function, miR-210 is also predicted to target several neuro-protective proteins, such as BDNF, SYNGAP1 and IGF-1R (Li et al. 2014). Of note, mitochondrial dysfunction were proposed to be one of the pathology of BPD by several studies (Stork and Renshaw 2005, Clay et al. 2011).

MiR-339-5p

MiR-339-5p was associated with the pathology of Alzheimer diseases, where its levels were found to be significantly reduced in brain specimens isolated from Alzheimer Disease (AD) patients as compared with age-matched controls (Long et al. 2014). The author showed that miR-339 plays a significant role in the regulation of an enzyme, β-site APP-cleaving

enzyme 1 (BACE1). Since BACE 1 was involved in the rate-limiting step of production of amyloid-β (Aβ) peptide, and excess accumulation of the peptide is recognized as one of the main contributor to the occurrence of AD, thus miR-339 is directly involved in the development of AD. Delivery of the miR-339 mimic significantly inhibited expression of BACE1 protein in human glioblastoma cells and human primary brain cultures. In contrast, delivery of target protectors designed against the miR-339-5p BACE1 3'-UTR target sites in primary human brain cultures significantly elevated BACE1 expression.

MiR-146b

MiR-146b was linked to BPD through its interaction with BDNF val 66 met polymorphism, a factor which is associated with BPD and shown by numerous studies (Lohoff et al. 2005). In a study using rats with induced BDNF val 66 met polymorphism, miR-146b was down-regulated, which subsequently increased Per1 and Npas4 mRNA levels in the hippocampus of mice (Hsu et al. 2015). Per1 is a transcriptional repressor controlling circadian rhythm (Takahashi et al. 2008). Per1 expression is also altered in postmortem brains of schizophrenia patients (Aston et al. 2004), while its homolog PER3 is considered a candidate gene for risk of BPD and schizophrenia (Nievergelt et al. 2006). Npas4 is homologous to Npas2, which is another core component of circadian regulation (DeBruyne et al. 2007). Circadian rhythm disruption were hypothesized as one of the factor in the pathology of BPD (Harvey 2008).

In the gene ontology and biological function analysis for the asenapine group, the biological process were mainly enriched on regulation of programmed cell death and transcription. This is in concordance with studies carried out in genetic model organisms, where it have been proposed that programmed cell death is one of the important aspects

during the neuronal development, supported by findings that regulation of cell death are observed during the early development of neural tube (Deshmukh and Johnson 1997, Buss and Oppenheim 2004, Kristiansen and Ham 2014). Bai and colleagues (Bai et al. 2006) examined the effect of haloperidol and other antipsychotics on the regulation of expression of neurotrophin factors, which are capable of mediating programmed cell death. They reported that the expression of the neurotrophin factors gene was influenced by antipsychotics. A similar result were also shown by another relevant study (Noh, Kang et al. 2000).

In the risperidone group, the gene ontology analysis showed that biological processes were enriched on immune response and body defense. Although BPD is a brain disorder, and it would seem that there is no direct link between brain disorder and immune systems response, this have been answered by several studies regarding this matter. In a meta-analysis covering 30 years of study, it was shown that psychological stress is associated with vulnerability of the immune system (Segerstrom and Miller 2004). Besides, accumulating evidence have shown the association between dysregulated immune system with the etiology of psychiatric disorders (Gibney and Drexhage 2013, Jones and Thomsen 2013). For instance, a proportion of schizophrenia patients were observed to have disrupted cytokines level (Miller et al. 2011). In addition, several studies have shown that antipsychotics were capable of altering the levels of cytokines, an important component of immune response (Himmerich et al. 2011, Al-Amin et al. 2013).

By cross-referencing the predicted target genes of the miRNA that were differentially expressed with a genetic database for BPD (Chang et al. 2013), we managed to find 35 genes that were previously reported to be associated with BPD in the asenapine

group, while there are 6 such genes in the risperidone group. Out of the 35 target genes in the asenapine group, 14 of such genes is being regulated by miR-15a. In accordance with a previous study who reported the role of miR-15a in neuronal maturation (Gao et al. 2015), we therefore hypothesized the possibility of miR-15a as a biomarker in treatment response in BPD.

Our study of the micro profiling were conducted in peripheral blood. Since psychiatric disorders involves the mechanisms in the brain, there exists concern as to whether miRNA changes in the blood would reflect the real mechanisms taking places in the brain. This concern could have been answered by several findings that studied the miRNA across brain and blood samples, and examine the expression pattern in these samples. Liu and colleagues (Liu et al. 2010) examined the miRNA expression profiling in ischemic strokes, brain hemorrhage and kainate-induced seizures rats using brain and blood samples. The author reported that expression pattern of several miRNA in the blood samples correlates with that of the brain samples. A related studies have also reported similar miRNA changes in the serum of subjects exposed to brain injury (Jickling et al. 2014). Besides, peripheral blood miRNA have been shown to be potential biomarkers in various human diseases and pathology (Gupta et al. 2010, Leidinger et al. 2013). Furthermore, the miRNA changes observed may possibly be due to pathway interaction between the central nervous system and immunological system, which leads to changes that can be observed in the lymphocytes of peripheral blood (Gladkevich et al. 2004). These observation, however still warrants validation from in-vitro and animal model studies.

There were more Chinese participants in the samples than other ethnicities, which could be due to the catchment area (Klang Valley) having a higher proportion of

Chinese patients attending clinics and wards at the University Malaya Medical Centre as compared with the other races.

Limitation of the study

In this study, we have shown that miRNA plays a role in the mechanism of action of atypical antipsychotics, and these miRNA regulates several target genes, including those in the neuronal functions and in the signaling pathway. However, due to the small sample size of our study, further validation in larger sample sizes and in other psychiatric disorders such as schizophrenia and major depression are needed to evaluate the specificity of the result. Secondly, the identified target genes would be needed to examine for its expression to verify the interaction of miRNA and mRNA.

In summary, our study have shown that 22 miRNAs were upregulated with the fold change ranging from 2.05 to 8.56 , while 2 miRNAs were downregulated with the fold change ranging from -2.36 to -2.44 . On the other hand, there were only 3 statistically significant miRNAs that were identified for the treatment group with risperidone. All the 3 miRNAs were downregulated with the fold change ranging from -2.41 to 4.2. Out of the miRNAs that were identified, 7 miRNAs were found to be related to neuronal disorders which includes miR-19b, miR-145, miR-15a, miR-30b, miR210, miR339 and miR-146b.

3.6 CONCLUSION

In this present study, we have shown that certain miRNAs may involve in the mechanisms of action of antipsychotics. The miRNA that were being identified were further analyzed for target prediction. Furthermore, some of the target genes have been shown to be involved in or being reported previously to be associated with BPD and other related neurological disorder. Our results support the hypothesis that miRNA have the potential to act as novel target sites for antipsychotics drugs, due to its ability to target several hundred genes, and also due to the heterogeneity of BPD. Of note, the target gene expression would be needed to replicate in larger sample size to determine the specificity of the result. Although the research on miRNA in psychiatric, and in particular, BPD, is still in its infancy, it has been shown to have promising results and huge potential as the new research area in developing personalized medicine in psychiatry.

Future studies would require the expression of predicted target genes being studied in larger sample size to further validate the functional role of specific miRNA. Besides, replication in other related psychiatric disorders such as schizophrenia and major depression would be needed to determine the specificity of the functional role of the miRNA, since overlapping of genetic roots have been hypothesized in these disorders.

CHAPTER FOUR: GENERAL CONCLUSION

Bipolar Disorder is a complex disease which is a result of the interaction of both genetic and environmental factors. The study was designed to examine the role of genetic component in the pathophysiology of BPD. It was divided into two parts; first, to examine the association of SNP of the candidate genes with susceptibility to BPD, and second, to examine the changes in the expression of miRNA in the patients of BPD mania before and after the treatment of atypical antipsychotics.

In the first study, we have managed to show the association of several gene with susceptibility to BPD, and these includes LMAN2L and ANK3. Besides, our result also support the hypothesis that BPD is in fact sharing some common genetic root cause with another psychiatric illness, SZ. The LMAN2L gene deserved much attention on its association with BPD and SZ in the pooled population. Further functional study would be required to unfold its roles in the pathophysiology of both disorders.

In the second study, the changes in the expression of miRNA observed in the BPD patients following treatment have shown that certain miRNA are involved in the mechanisms of action of the antipsychotics. Furthermore, target gene prediction also revealed that parts of the genes regulated by miRNA were associated with BPD.

The two parts of the study have enabled us to look into more details at the underlying molecular mechanisms of BPD. Interestingly, the candidate genes in part one of the study (ANK3,CACNA1C) were found to be potential target genes for miRNA in part two of the study (miRNA-27a). This has further strengthened the findings that those genes were

involved in the molecular pathway of BPD. Functional study using animal model in future studies would be able to provide a clearer picture of the functional role of these candidate genes.

Although genetic risk alleles have been identified for BPD, these have not been translated into clinical applications. This suggests that there are complex pathways underlying this disease, and larger sample size are required to replicate the findings due to genetic variability among individuals. In addition, the risk alleles were required to be associated specific treatment response. On the other hand, the introduction of miRNA, which is capable of controlling the expression of various genes, have shed a new angle into molecular research of BPD. However, the knowledge of miRNA were still in its infancy, and there is still a large amount of work to be done to uncover its function.

The discovery of risk allele and predicted target gene associated with BPD have been generated from a large numbers of studies. It is therefore the pathway that links all the proposed risk genes that needs to be identified, rather than single gene product. Besides, integration of genetic data generated with other biological information, such as neuroimaging would be needed to produce a clearer picture of the pathway underlying the disease. Any models developed from these data, be it cell model or animal model, would be required to be validated experimentally to provide a powerful tool for the understanding of BPD.

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