Preliminary Examination of MicroRNA Expression Profiling in Bipolar Disorder I Patients During Antipsychotic Treatment

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Although major progress has been achieved in research and development of antipsychotic medications for bipolar disorder (BPD), knowledge of the molecular mechanisms underlying this disorder and the action of atypical antipsychotics remains incomplete. The levels of microRNAs (miRNAs)—small non-coding RNA molecules that regulate gene expression, including genes involved in neuronal function and plasticity—are frequently altered in psychiatric disorders. This study aimed to examine changes in miRNA expression in bipolar mania patients after treatment with asenapine and risperidone. Using a miRNA microarray, we analyzed miRNA expression in the blood of 10 bipolar mania patients following 12 weeks of treatment with asenapine or risperidone. A total of 16 miRNAs were differentially expressed after treatment in the asenapine group, 14 of which were significantly upregulated and the other two significantly downregulated. However, all three differentially expressed miRNAs in the risperidone group were downregulated. 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 suggest that candidate miRNAs may be involved in the mechanism of action of both antipsychotics in bipolar mania. © 2016 Wiley Periodicals, Inc.

Key words: bipolar; mania; asenapine; risperidone; miRNA

INTRODUCTION

Bipolar disorder (BPD) is a severe mental disorder with profound social and economic impacts. It is characterized by recurrent episodes of mania and depression, and the lifetime prevalence is estimated to be at 0.6% [Merikangas et al., 2011]. The polygenic nature of BPD has been investigated in a large number of studies of various sample sizes and populations. These studies have resulted in a list of candidate genes associated with BPD, each with a relatively small effect in contributing to the complexity of the disorder. The search for novel targets for pharmacological interventions for BPD is made more difficult, however, because of interaction and overlap with genes associated with other psychiatric illnesses [Maier et al., 2005].

MicroRNAs (miRNAs) are small non-coding RNA molecules of approximately 21 nucleotides involved in regulation of post-transcriptional gene expression, targeting one third of human coding RNA [Lewis et al., 2005]. MiRNAs are abundantly located in the nervous system, where they have vital roles in neuronal function and plasticity that are relevant for brain function and mental health [Kosik, 2006]. This is supported by a number of studies that reported altered miRNAs level in BPD patients [Rong et al., 2011], as well as other psychiatric disorders such as schizophrenia and depression [Bocchio-Chiavetto et al., 2013; Song et al., 2014]. In addition to this, Walker et al. [2015] noticed altered miRNAs level in individuals with high familial risk for BPD. These miRNAs were previously associated with BPD and...
schizophrenia. In view of these studies, alteration in miRNA might contribute to the genetic and molecular basis of neuropsychiatric disorders.

Although alterations in miRNAs expression have been reported, the pharmacological impact of antipsychotics on the miRNAs in BPD is still poorly understood. In this study, two antipsychotics have been chosen for comparison in miRNA expression, namely asenapine and risperidone. Risperidone has been a first-line treatment in acute mania. In a comprehensive meta-analysis, Cipriani et al. [2011] proposed that risperidone was among the most effective atypical antipsychotics in this regard. However, risperidone is also associated with some unwanted side effects such as hyperprolactinemia [Eberhard et al., 2007].

Asenapine, on the other hand, is a relatively new atypical antipsychotic on the market, and is described as a tetracyclic antipsychotic [Shahid et al., 2009]. It is administered sublingually, which enhances its absorption and increases its bioavailability compared with other orally administered atypical antipsychotics that have much lower bioavailability as a result of hepatic metabolism [Reynolds, 2011]. Compared to risperidone, asenapine has a relatively favorable weight and metabolic profile [Citrome, 2011].

In this study, we examined changes in miRNA expression in bipolar mania patients following 12 weeks of treatment with asenapine or with risperidone. The aim of our study was to provide more insights into the molecular mechanisms underlying the action of the atypical antipsychotics under investigation, as well as to have a better understanding of the pathophysiology underlying bipolar mania.

**METHODS**

**Subjects**

The subjects who participated in this study were recruited from the University Malaya Medical Centre (UMMC) inpatient psychiatric ward. The inclusion criteria for recruitment were patients who were currently in the manic phase of BD II, as diagnosed by psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (5th edition); who were 18–65 years old; and who were either treatment-naive or had not received medication for at least 3 months prior to admission. The exclusion criteria were as follows: a history of substance abuse and psychiatric disorders other than BPD, currently pregnant or actively seeking pregnancy, and severe mania that required electroconvulsive therapy. A patient was recruited into the study if the patient and his or her family member consented; patients were then randomly divided into two groups. One group of patients was prescribed asenapine, while the other group was given risperidone. Each patient began by taking the optimum dose for his or her condition, with the patient’s progress being monitored by the psychiatrists; the dose of antipsychotics was then escalated, depending on the patient’s mental state. Active treatment was initiated with asenapine 20 mg (10 mg twice daily) and risperidone 4 mg (2 mg twice daily). Thereafter, treatment continued with flexible dosing (asenapine 10–20 mg and risperidone 1–4 mg). Both groups were also prescribed sodium valproate as a mood stabilizer. The dose of sodium valproate was begun and maintained at 20 mg/kg body weight of the patient. This study was approved by the Medical Ethics Committee of UMMC, and all participants provided written consent to be enrolled in this study. A post hoc power analysis indicated that this sample has a power >75% to detect a minimum fold change of 2.

We assessed patients from their sociodemographic profiles and their score on the Young Mania Rating Scale (YMRS) in order to monitor improvement in their condition at baseline (week 0) and at weeks 1, 4, and 12 of treatment with asenapine or risperidone. Blood samples were collected in Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA). Patients were discharged on the basis of clinical improvement and a greater than 50% reduction in their YMRS score. The assessor was not blinded to the randomized drug.

**RNA Extraction**

Total RNA was extracted by using the Tempus Spin RNA Isolation Kit (Applied Biosystems) according to the manufacturer’s standard protocols. RNA integrity and concentration were assessed with the NanoDrop spectrophotometer (Nanodrop 2000; Thermo Scientific, Waltham, MA) and the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). To ensure that the quality of the microarray result was not affected, we accepted only those samples with an RNA integrity number equal to or exceeding eight for subsequent analysis.

**MiRNA Microarray Expression Analysis**

An Affymetrix GeneChip miRNA 4.0 Array (Affymetrix, Santa Clara, CA), containing probes of 2025 mature human miRNAs in total, was used to profile miRNA expressions. The RNA was labeled by using FlashTag biotin-HSR RNA labeling kits (Genisphere, Hatfield, PA). Sample labeling, microarray hybridization, and washing were performed according to the manufacturer’s standard protocols. Briefly, total RNAs were tailed with PolyA and then labeled with biotin. The labeled RNAs were then hybridized onto the GeneChip miRNA 4.0 microarrays at 48˚C for 16 hr. Following washing and staining with Affymetrix Fluidics Station 450 (Affymetrix), the arrays were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix) by using Command Console software (Affymetrix). The scanned images were analyzed with Affymetrix Expression Console software (version 1.3.1, Affymetrix).

**RT-qPCR Analysis**

Three selected candidate miRNAs that reached a significant difference in expression and were biologically important were validated by using a real-time PCR method in the same sample set. A customized TaqMan miRNA expression assay (Applied Biosystems, Carlsbad, CA) was used for the quantification of the expression level of selected miRNAs. All experiments were run in triplicate and RNU48 was selected to act as the endogenous control. Total RNA was transcribed into cDNA by using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The results were analyzed with StepOne Plus software (version 2.3, Applied Biosystems). Relative gene expression levels were calculated by using the ΔΔct method [Livak and Schmittgen, 2001].
Statistical Analysis and Target Prediction

MiRNA expression values were background adjusted and normalized across arrays by using Robust Multichip Average (RMA) method [Irizarry et al., 2003]. Median of the log-transformed value of each probe were calculated and then subtracted from all samples, and baseline was set to median. For each array, the miRNA probes were compared with distribution signals for anti-genomic probes that had matching GC content, followed by Mann–Whitney test of $P$-value of less than 0.05 to identify miRNAs above background. Subsequent analysis were restricted to miRNAs that exceeded background levels. The regulatory targets of differentially expressed miRNAs were identified with the ingenuity pathway analysis (IPA) miRNA Target Filter function, which queries TarBase and miRecords for experimentally validated miRNA targets and queries 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 Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) [Huang da et al., 2009] for gene ontology annotation analysis and identification of related biological pathways. The changes in the YMRS score during weeks 0, 1, 4, and 12 were examined by using one-way analysis of variance (ANOVA). The difference between YMRS scores for the two groups was compared by independent t-test.

RESULTS

Patient Demographic Data

Initially, 14 patients were recruited, seven in each group (two females and five males). The asenapine group consisted of two Malays and five Chinese, whereas the risperidone group consisted of two Malays, three Chinese, and two Indians. However, four patients dropped out during the assessment, two patients were lost to follow-up and withdrawn from the study with each patient’s consent, and there was lack of efficacy in two patients (one in each group). Out of four patients who dropped out, two were Indians, one Malay and one Chinese. Hence, at week 12, a total of 10 patients completed the study consisted of only Malays and Chinese. There was no significant difference between the two treatment groups in terms of gender and age (mean age of asenapine group = 28.64 years, of risperidone group = 29.83 years).

YMRS Score

For the four patients who dropped out, the final observation was carried forward for the calculation of the mean YMRS score. As shown in Figure 1, at week 12, the mean change from the baseline total YMRS scores was $-28.20$ in the asenapine group and $-28.42$ in the risperidone group. Statistical analysis indicated no significant difference between the asenapine and risperidone groups. In terms of the efficacy of antipsychotics, the percentage of YMRS responders is defined as a 50% reduction from the baseline YMRS total score [McIntyre 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 time during the study period (all $P$s $>0.05$).

Analysis of MiRNA Expression Changes During Antipsychotic Treatment

Using Transcriptome Analysis Console software, we analyzed miRNA expression profiling by using a one-way ANOVA test and adjusting the $P$-value for multiple testing with the Benjamini–Hochberg test. Expression of miRNAs during week 0 was compared between treatment naïve patients and patients who defaulted in treatment; however, no significant result was observed. During the 12th week of the study, differentially expressed miRNAs were identified, 16 in the asenapine group and three in the risperidone group. Of the 16 miRNAs from the asenapine group, 14 were upregulated, with the fold change ranging from 2.18 to

![FIG. 1. Mean YMRS scores during 12 weeks of treatment.](image-url)

<table>
<thead>
<tr>
<th>Transcript ID (array design)</th>
<th>Fold change</th>
<th>ANOVA $P$-value</th>
</tr>
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<tr>
<td>hsa-miR-18a-5p</td>
<td>8.56</td>
<td>0.010761</td>
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<tr>
<td>hsa-miR-19b-3p</td>
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<td>hsa-miR-145-5p</td>
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<td>hsa-miR-20a-5p</td>
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<tr>
<td>hsa-miR-339-5p</td>
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<td>0.002185</td>
</tr>
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<td>hsa-miR-1343-5p</td>
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8.56, whereas two were downregulated with the fold change ranging from -2.36 to -2.44. On the other hand, all miRNAs from the risperidone group were downregulated, with the fold change ranging from -2.41 to -4.2. None of the identified miRNAs overlapped between the two treatment groups and were considered unique to the respective group. The findings are summarized in Tables I and II.

Validation of MiRNA
In the 19 miRNAs that were significantly different, 3 were selected for further validation by using real-time PCR. The miRNAs were chosen for the following criteria: high fold change detected, and of biological importance. In Figure 2, all three miRNAs selected for validation demonstrates fold change in the same direction as in the microarray (fold change for miR-15a = 2.53; fold change for miR-210 = 4.58; fold change for miR-146b = -2.79; however, only miR-210 demonstrates a significant difference (P = 0.032).

MiRNA Target Prediction and Pathway Analysis
To further understand the role of miRNAs and their target genes, we performed a bioinformatics analysis by using IPA software. In the asenapine-treated group, initial analysis showed that there were approximately 3,000 target genes predicted. When the confidence level was set to those that were experimentally observed, only genes that were identified in previous studies were shown from the database. This filtered the target genes to 444. Although the initial analysis of the risperidone-treated group showed that about 300 target genes were predicted, when the confidence level was set to those that were experimentally observed, this filtered the target genes to 77.

The predicted target genes were submitted to DAVID for gene ontology annotation, which includes biological processes, cellular component, and molecular function. The top 10 of the most enriched items for each category are shown in Figures 3 and 4. In the asenapine group, the enriched gene list was mainly focused on regulation of cell death and of transcription. However, in the risperidone group, the list was enriched for response of the immune system and the body defence mechanism.

DISCUSSION
The development of atypical antipsychotics has provided patients with drugs with fewer side effects, and the use of these antipsychotics has consistently emerged as the first-line treatment for bipolar mania. Nonetheless, significant numbers of patients remain impaired. In general, current atypical antipsychotics concentrate their effects on several specific pathways that include dopamine receptors, 5-HT receptors, and the GABA system. The molecular

<table>
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<th>Transcript ID (array design)</th>
<th>Fold change</th>
<th>ANOVA P-value</th>
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<td>hsa-miR-664b-5p</td>
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<td>hsa-miR-146b-5p</td>
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mechanisms of these atypical antipsychotics remain elusive, however, making it difficult to personalize treatment for better therapeutic effects. Given the heterogeneity of psychiatric disorders, the emergence of research on miRNAs has been important. An increasing number of findings have shown that miRNAs are involved in the control of cellular processes, including neurogenesis, synaptic plasticity, apoptosis, and cell fate decisions [Magill et al., 2010; Bredy et al., 2011].

Several studies have shown that miRNAs can act as diverse target sites for certain psychotropic drugs. For example, miR-504 has been shown to increase dopamine D1 receptor expression [Huang and Li, 2009]. Perkins et al. [2007] compared the differential expression of miRNAs in 179 rats treated with haloperidol to that in untreated controls and found that miR-199a, miR-128a, and miR-128b were elevated in haloperidol-treated rats. Despite the findings that miRNA expression can be altered by antipsychotics and other psychotropic drugs, hardly any evidence is available to show that these miRNA changes are associated with symptom improvement in bipolar mania.

In the present study, we sought to examine miRNAs that are associated with or involved in the mechanism of action pathway for asenapine and risperidone. In terms of efficacy of the antipsychotics in treating acute mania symptoms, the YMRS scores did not show any significant differences between the two treatment groups. Although the antipsychotics were little differentiated in terms of efficacy and symptom relief, other aspects related to treatment, such as antipsychotic-induced weight gain, could be more promising in distinguishing and classifying these antipsychotics [Reynolds, 2011]. However, this is beyond the scope of our study.

In miRNA expression profiling, we identified 16 miRNAs that were significantly differentially expressed after asenapine treatment, whereas only three miRNAs were identified as such in the risperidone group. Several miRNAs were identified as being linked with neurological disorders, including miR-19b, miR145, and miR-339, which were previously reported to be dysregulated in patients with autism spectrum disorder [Mundalil Vasu et al., 2014] and with Alzheimer disease [Botta-Orfila et al., 2014; Li et al., 2014; Long et al., 2014].

Two miRNAs, miR-15a and miR-146b, are also reported to be involved in an interaction with brain-derived neurotrophic factor (BDNF) [Lohoff et al., 2005; Gao et al., 2015]. BDNF is potent in neuronal maturation and is well-known for its role in the pathological process underlying BPD and other related neuropsychiatric disorders [Post, 2007]. MiR-15a has been shown to regulate the expression level of BDNF, where exogenous BDNF is able to rescue neuronal maturation deficits resulting from overexpression of miR-15a [Zhou et al., 2006]. Furthermore, in another study of rats with an induced BDNF val 66 met polymorphism, miR-146b was downregulated in their hippocampus [Hsu et al., 2015].

MiR-30b is associated with schizophrenia, a psychiatric disorder that has been shown to share common genetic roots with BPD [Berrettini, 2000]. In a study of the relationship between estrogen signaling pathways and schizophrenia, miR-30b expression was significantly reduced in the cerebral cortex of female but not male

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**Fig. 3.** Gene ontology annotation of predicted target gene in asenapine group. The gene ontology annotation generated by DAVID in three categories, biological process, cellular component, and molecular function. Top 10 enriched item in each category is displayed.
subjects with schizophrenia [Mellios et al., 2012]. Overexpression of miR-210 was 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].

In the analysis of gene ontology and biological function in the asenapine group, biological processes were mainly enriched by the regulation of programmed cell death and transcription. This finding is in concordance with studies performed in genetic model organisms, where it has been proposed that programmed cell death is an important aspect of neuronal development, supported by findings that the regulation of cell death is observed during the early development of the neural tube [Deshmukh and Johnson, 1997; Buss and Oppenheim, 2004; Kristiansen and Ham, 2014]. Bai et al. [2006] examined the effect of haloperidol and other antipsychotics on the regulation of the expression of neurotrophin factors, which are capable of mediating programmed cell death. They reported that the expression of the neurotrophin factor gene was influenced by antipsychotics. A similar result was also shown in another relevant study [Noh et al., 2000]. In addition, increased expression of pro-apoptotic genes (such as BAD or BAX) and reduced expression of anti-apoptotic genes have been observed in the prefrontal cortex of BPD patients [Benes et al., 2006; Kim et al., 2010]. These findings suggest that apoptosis in the neural circuit plays an important role in the pathophysiology of BPD [Uribe and Wix, 2012].

In the risperidone group, gene ontology analysis showed that the biological processes were enriched for immune response and body defence. This is in-line with various studies which examine the role of inflammation in BPD, including a review published recently [Rosenblat and McIntyre, 2016]. 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]. Moreover, accumulating evidence has shown an association between a dysregulated immune system and 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 cytokine levels, important components of the immune response [Miller et al., 2011]. In addition, several studies have shown that antipsychotics are capable of altering these levels [Himmerich et al., 2011; Al-Amin et al., 2013].

By cross-matching the differentially expressed miRNA predicted target genes with a database for BPD [Chang et al., 2013], we managed to find 35 predicted target genes in the asenapine group that were previously reported to be associated with BPD, as well as six predicted target genes in the risperidone group.

Our study on miRNA profiling was conducted by using peripheral blood. Since psychiatric disorders involve brain mechanisms, there exists a concern as to whether miRNA changes in the blood reflect the mechanisms occurring in the brain. This concern may have been addressed by several findings from studies of miRNAs across brain and blood samples, in which the expression pattern of these samples was examined. Liu et al. [2010] examined miRNA expression profiling in brain and blood samples from rats with ischemic stroke, brain hemorrhage, and kainate-induced seizures. They reported that the expression patterns of several miRNAs in the blood samples correlated with those of the brain samples. A related study also reported similar miRNA changes in the serum of subjects who had brain injury [Jickling et al., 2014]. In addition, peripheral blood miRNAs have been shown to be potential bio-

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**FIG. 4.** Gene ontology annotation of predicted target gene in risperidone group. The gene ontology annotation generated by DAVID in three categories, biological process, cellular component, and molecular function. Top 10 enriched item in each category is displayed.
markers in various human diseases and pathological conditions [Gupta et al., 2010; Leidinger et al., 2013]. Furthermore, the miRNA changes observed may be due to pathway interactions between the central nervous system and the immunological system, which leads to changes in the lymphocytes of peripheral blood [Gladkevich et al., 2004]. These observations, however, warrant validation from in vitro and animal model studies. In our study, there were also more Chinese participants in the samples than other ethnicities, which could be because the catchment area is more populated by Chinese patients. Our final analysis included only the Malay and Chinese patients since those patients of the Indian ethnic group had dropped out from the study. We, therefore, opted to carry out a pooled analysis (combining Malays and Chinese), the assumption being made that these two ethnic groups are relatively genetically similar as shown by Hatin et al. [2011] which found that the sub-ethnic groups of the Peninsular Malays are clustered together with the Malaysian Chinese in the population structure but not the Malaysian Indians.

Limitations of the Study

In this study, we have shown that miRNA may be involved in the mechanism of action of atypical antipsychotics and that these miRNAs regulate several target genes, including those involved in neuronal functions and the signaling pathway. However, the sample size was small, and so further validation with larger sample sizes and in other psychiatric disorders such as schizophrenia and major depression are needed to evaluate the specificity of the result. Moreover, the expression of the identified target genes needs to be examined to verify the interaction of miRNA and mRNA. Furthermore, prolonged treatment time might have given a clearer indication of differentially expressed miRNAs.

CONCLUSION

Result from the present study suggests that certain miRNAs may be involved in the mechanism of action of antipsychotics in the treatment of bipolar mania. In addition, some predicted target genes were involved in or had previously been reported to be associated with BPD and other related brain disorders. However, these findings warrant further study in larger patient sample and examine the expression of predicted target genes.

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