MicroRNA Microarray Analysis Combined with Interaction Network Analysis to Investigate the Influence of Clozapine to Metabolic Syndrome

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Abstract: The Metabolic Syndrome (MetS) could significantly increase the risk of morbidity and mortality from type II diabetes and cardiovascular disease. We carried out a systematical study to investigate the potential drug targets for effective treatment of clozapine-induced MetS. In our study, the differentially-expressed miRNAs (DERs) between schizophrenia patients with MetS and without MetS after the treatment of clozapine were identified. Target genes of these miRNAs were then retrieved from two miRNA databases (miRecodes and miRTarBase) to identify the underlying mechanisms involved in the development of MetS. Interactors of the target genes were identified and a network was constructed using Osprey. Functional enrichment analysis was performed for all the genes in the network with DAVID (Database for Annotation, Visualization and Integrated Discovery). The results revealed that 3 DERs including Hsa-miR-330-3p (p = 0.00934), has-miR-18a (p = 0.001 686) and has-miR-106b (p = 0.007 531) were identified. In total, 4 target genes (E2F1, CTGF, ESR1 and ITCH) shared by both databases were picked out. What’s more, 8 significant pathways were revealed in the interaction network and the most significant pathway was positive regulation of macromolecule metabolic process (FDR = 1.80E-15). In conclusion, the 3 identified miRNAs might be the potential targets for treatment of MetS. And the results of our study provide ways to monitor the progression of MetS (e.g., serum levels of proteins), predict the outcome (e.g., polymorphisms) and even block the emergence of MetS.

Key words: Metabolic syndrome, differentially expressed miRNA, target gene, interaction network, functional annotation enrichment analysis

INTRODUCTION

The metabolic syndrome (MetS) is a clustering of metabolic, anthropometric and haemodynamic abnormalities that, when occurring together, significantly increase the risk of morbidity and mortality from type 2 diabetes and cardiovascular disease (CVD). The features which include hyperglycemia, central obesity, hyperlipidemia and hypertension may share a similar pathogenesis and in combination they pose more damage to the heart, brain, kidneys and other vital organs than simple high blood pressure or diabetes. For example, the risk of cerebrovascular disease is higher for patients with MetS compared with the patients without MetS and mortality is 5-6 times higher (Eckel et al., 2005). In fact, MetS-induced cardiovascular and cerebrovascular disease, myocardial infarction and pulmonary diseases are expected to become the three leading causes of death in the future (Mottullo et al., 2010).

One possible antecedent of MetS in psychiatric settings is the mainstream pharmacological treatment of psychotic disorders (e.g., schizophrenia) and severe behavioral disorders. Although psychotropic drugs are necessary to treat these disorders, among the side effects are an increased incidence of cardiovascular disease (Anonymous, 2004a), diabetes (De Hert et al., 2008) and weight gain, abnormal glucose and lipid metabolism (De Hert et al., 2009; McEvoy et al., 2005; Pramynothin and
Khaodhia, 2010; Walss-Bass et al., 2008). As the results, patients with schizophrenia who are subjected to long-term use of psychotropic drugs, such as clozapine and olanzapine, are at risk of and suffer from a higher incidence of MetS than general population (Anonymous, 2004b).

Many studies involving patients with clozapine-induced MetS have been conducted, but most of the studies have had a limited focus. For example, (Mulder et al., 2009) explored the association between 5-hydroxytryptamine (serotonin) receptor 2C polymorphisms and MetS in patients with schizophrenia (Mulder et al., 2009). Other investigations have explored the polymorphisms of leptin and its receptor (Boumaiza et al., 2012), pyruvate dehydrogenase kinase 4 (Moon et al., 2012), fibroblast growth factor 21 (Zhang et al., 2012), paraoxonase-1 (Kordi-Tamandani et al., 2012) and so on among this patient group. Expression levels of critical genes have also been investigated. Gormez et al. (2011) suggested that pathogenesis of MetS was associated with the expression levels of adiponectin, necrosis factor alpha and leptin (Gormez et al., 2011). However, it is likely that there are global changes in gene expression in patients with schizophrenia with clozapine-induced MetS and that these changes cannot be explained by simply one means. Therefore, Grayson and others have focused on the gene expression profile of peripheral blood (Grayson et al., 2011) and Bahr and others collected miRNA profiles of adipose tissue from rats (Bahr et al., 2011). Additionally, a range of tissues have been studied, including pancreatic islets (Dreja et al., 2010) and mononuclear cells (Camargo et al., 2010). Though considerable data have been obtained, the understanding of the progression of MetS is not yet sufficiently adequate to inform clinical interventions.

A global description of the changes in MetS including upstream regulatory factors (such as miRNAs and transcriptional factors) and downstream “effector” is beneficial. In recent years, miRNAs have been identified as key regulators of gene expression (Carnell et al., 2008) and have been implicated in many metabolic processes (Rottiers and Naar, 2012), such as lipid metabolism (Scocco and Adeli, 2012) and vascular biology (Schober et al., 2012). And these findings could be useful in helping us to understand the pathogenesis of MetS.

The current study aimed to systemically investigate the changes and position potential drug targets for effective treatment of clozapine-induced MetS. We analyzed miRNA chip data to identify differentially-expressed miRNAs (DERs). Target genes of the DERs were retrieved and functional enrichment analysis as well as interaction network analysis were conducted to identify any alterations in gene expression level.

MATERIALS AND METHODS

Subjects: Our sample consisted of 24 unrelated patients (12 males and 12 females) who had been diagnosed with schizophrenia according to DSM-IV criteria (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition) by psychiatrists at the Shanghai Mental Health Center in China. All patients were Han Chinese and provided their informed consent to participate in the study, which had been approved by the local research ethics committee.

All twenty-four patients had been taking clozapine for more than one year.

Criteria for MetS: The status of MetS for each patient was established using the IDF (International Diabetes Federation) criteria (Alberti et al., 2005). Those with central obesity assessed by waist circumference (Chinese <90 cm for males and ≤80 cm for females) and have any two or more of the following factors: Elevated concentration of triglycerides (>150 mg dL⁻¹), reduced concentration of high density lipoprotein cholesterol (<40 mg dL⁻¹ in men and <50 mg dL⁻¹ in women), raised fasting glucose concentration (>100 mg dL⁻¹) and raised systolic arterial blood pressure (≥140 mmHg) and/or diastolic arterial blood pressure (≥85 mmHg) were identified as having MetS.

The mean age of patients with MetS was 46.4±5.0 years and the mean age of patients without MetS was 44.4±7.1 years.

MI RNA microarray: We collected peripheral blood samples. Every four samples were pooled and analyzed with one Affymetrix micro RNA 2.0 chip. A total of 6 chip data were obtained with 3 normal metabolism and 3 abnormal metabolism.

Package Affy (Fujita et al., 2006; Troyanskaya et al., 2001) of R was used to convert original CEL format into expression profile format and then normalize the data with median method. Package milttest (Dudoit et al., 2003) was chosen for differential analysis with the t-test method. A p value of less than 0.01 was set as the significance cut-off criterion.

Retrieval of target genes: Target genes of the DERs were retrieved from two miRNA databases: MirRecords and miRTarBase, respectively. Both databases gather
experimentally verified target genes. The miRecords database contains 548 miRNAs and corresponding target genes from 9 species (Xiao et al., 2009), while the miRTarBase database includes 773 miRNAs and 2632 target genes from 14 species (Hsu et al., 2011). Since each miRNA has a range of target genes based upon different algorithms, those shared by both databases were regarded as of high confidence and were retained for the analyses.

**Interaction network analysis**: Osprey (Breitkreutz et al., 2003a) was adopted to retrieve interactors of the target genes and construct the interaction network. This network visualisation system was developed for use in studies about protein-protein interaction networks and protein complexes. Osprey currently contains more than 50,000 interactions and is connected with the BIND (Biomolecular Interaction Network Database) (Willis and Hogue, 2006) and GRID (Global Resource Information Database) (Breitkreutz et al., 2003b).

DAVID (Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2009), a clustering tool based on the hypergeometric distribution, was chosen as the analytic tool in our study. An FDR (false discovery rate) value of less than 0.05 was set as the cutoff criterion.

**RESULTS AND DISCUSSION**

**Identification of DERs**: A good performance of normalization was achieved (Fig. 1). Differential analysis was performed using t-test method from package multtest between 3 chips with abnormal metabolism and 3 with normal metabolism. A total of 3 miRNAs were identified: Hsa-of miR-330-3p (p = 0.00934), has-of miR-18a (p = 0.001686) and has-of-miR-106b (p = 0.007531).

**Target genes for DERs**: Target genes of the three DERs were retrieved through searching within miRecords and miRTarBase, individually. Then four genes shared by both databases were picked out for further analysis: E2F1, CTGF, ESR1 and ITCH (Table 1).

**Interaction network for target genes**: Interactors of the four target genes were acquired and the network consisting of 53 nodes was constructed with Osprey (Fig. 2). Database for Annotation, Visualization and Integrated Discovery was used to perform functional enrichment analysis for all the genes in the network and 8 significant pathways were uncovered (Table 2). The most significant pathway was positive regulation of macromolecule metabolic process (FDR = 1.70E-15), while others were also associated with regulation of metabolic process, such as RNA and DNA.

![Fig. 1: Box plot for normalized gene expression data. A, B and C represent the three samples with normal metabolism while D, E and F are the three samples with abnormal metabolism. Black lines in the boxes indicate the medians. The medians are almost on the same line, suggesting a good performance of the normalization.](image)

<table>
<thead>
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<th>ID</th>
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<th>References (PMID)</th>
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<td>Hsa-miR-106b</td>
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<tr>
<td>GO:00100694</td>
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<tr>
<td>GO:0051254</td>
<td>positive regulation of RNA metabolic process</td>
<td>5.75E-07</td>
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<tr>
<td>GO:0006239</td>
<td>regulation of RNA metabolic process</td>
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<td>GO:0006259</td>
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<td>GO:0051174</td>
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<td>GO:0051247</td>
<td>positive regulation of protein metabolic process</td>
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It could be speculated that psychotrophic drugs primarily caused abnormal expression of hsa-miR-330-3p, has-miR-18a and has-miR-106b which affected target genes and their interactors that were implicated in regulation of biomolecule metabolic process.
We applied miRNA microarray technology to determine the global expression changes for patients with MetS compared with those without MetS. A total of 3 differentially expressed miRNAs were identified: Hsa-miR-330-3p, has-miR-18a and has-miR-106b.

E2F transcription factor 1 (E2F1) is the target of Hsa-miR-330-3p (Emmrich and Putzer, 2010; Lee et al., 2009). E2F1 is a member of the E2F family of transcription factors which play a critical role in the control of cell cycle and action of tumor suppressor proteins. E2F1 also takes a part in metabolic processes (Fajas and Ammicotte, 2011). E2F1 regulates the expression level of pyruvate dehydrogenase kinase 4 and thus influences the metabolism in human cardiac cells (Palomer et al., 2011). Blanchet et al. (2009) indicate that the CDK4-pRB-E2F1 regulatory pathway is involved in general glucose homeostasis and metabolism (Blanchet et al., 2009).

Perri et al. (2012) investigate the expression of miRNAs in obese patients and report that miR-18a is up-regulated in patients with obesity while miR-106b is up-regulated in non-obese individuals (Perri et al., 2012). The target of miR-18a contains connective tissue growth factor (CTGF) (Ohgawara et al., 2009) and estrogen receptor 1 (ESR1) (Loven et al., 2010). CTGF is a mitogen that is secreted by vascular endothelial cells. The encoded protein plays a role in chondrocyte proliferation and differentiation, cell adhesion in many cell types and is related to platelet-derived growth factors. Colak and others report that serum levels of CTGF may be of clinical utility for distinguishing nonalcoholic fatty liver disease patients with and without advanced fibrosis (Colak et al., 2012). The positive role of CTGF in the development of cardiac hypertrophy and fibrosis has also been demonstrated (Yoon et al., 2010). ESR1 is an estrogen receptor and also a ligand-activated transcription factor. The relationship between its polymorphisms and lipid metabolism has been extensively studied (Molvarec et al., 2007; Sertie et al., 2009).

The miR-106b is associated with cholesterol metabolism. Kim et al. (2012) indicate that miR-106b impairs cellular cholesterol efflux and increases amyloid β level by repressing ATP-binding cassette transporter A1 expression (Kim et al., 2012). Its target includes E2F1 (Trompeter et al., 2011) and itchy E3 ubiquitin protein ligase (ITCH) (Rivetti di Val Cervo et al., 2009). ITCH is a member of the Nedd4 family of HECT domain E3 ubiquitin ligases and plays a role in multiple cellular processes.

To further confirm the regulation role of these miRNAs in MetS, the interaction network was established for the four target genes using Osprey. Functional annotation enrichment analysis was then performed with DAVID for all the genes in the network and 8 significant annotations were revealed (Table 2). In functional enrichment analysis, a group of genes sharing similar or relevant functions are considered as a whole which greatly reduces the dimensions of the data analysis and facilitates the determination of changes in biological processes. Therefore, it is widely adopted in analysis of chip data (Huang et al., 2008).
All the biological pathways were associated with metabolic process. The most significantly over-represented one was positive regulation of macromolecule metabolic process, while metabolisms of phosphate and nitrogen compound were also significant. The linkages between some interactors and MetS have been reported. It has been found that v-akt murine thymoma viral oncogene homolog 1 (AKT1) is associated with glucose homeostasis and MetS (Devaney et al., 2011; Harmon et al., 2010). The polymorphism of insulin-like growth factor 1 receptor (IGF1R) is also linked to MetS (Kilpelainen et al., 2008). Tumor protein p53 (TP53) is also a regulator in lipid metabolism pathways (Goldstein et al., 2012). Further, IGF1 (Hu et al., 2010) and cyclin-dependent kinase 4 (CDK4) (Blanchet et al., 2011) are also involved in the regulation of metabolic process. Taken together, the interaction network of the target genes is involved in the regulation of various metabolic processes which validates the roles for the 3 miRNAs.

CONCLUSION

In summary, we integrated miRNA microarray analysis and interaction network analysis to identify miRNAs implicated in the regulation of metabolic process. It is likely that clozapine influences the expression pattern of gene through the regulation of miRNAs and thus results in abnormalities of metabolism which increases the risk of metabolic syndrome. Three miRNAs could be potential targets for treatment of MetS. In addition, many relevant genes were also closely related to MetS which provides ways to monitor the progression of MetS (e.g., serum levels of proteins), predict the outcome (e.g., polymorphisms) and even block the emergence of MetS.

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