Author’s response to reviews

Title: Genome-wide DNA methylome reveals the dysfunction of intronic microRNAs in major Psychosis

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Version: 1 Date: 26 Aug 2015

Author’s response to reviews:

Respond to Reviewers

Reviewer #1

I suggest that the authors make the following changes before the manuscript is accepted for publication:
1. Abstract, page 2: Background, first sentence: At present the study of the role of DNA methylation in major psychosis is in its early stages and nothing has been confirmed in this area of research. Hence I suggest that the authors insert the words "thought to be" between "is" and "extensively"

REPLY: Thanks for your comments. Following your suggestion, we inserted the words "thought to be" between "is" and "extensively" in the revised manuscript.

On page 2

Background: DNA methylation is thought to be extensively involved in the pathogenesis of many diseases, including major psychosis.

2. Background, page 3: First sentence: At present it has not been confirmed that SZ and BD are etiologically correlated although some studies do suggest this to be the case. Hence I suggest that the authors insert the words "could be" before the words "etiologically correlated".

REPLY: Following your suggestion, we inserted the words "could be" before the words "etiologically correlated" in the revised manuscript.

On page 3

Schizophrenia (SZ) and bipolar disorder (BD), together termed “major psychosis”, could be etiologically correlated psychiatric conditions [1], and characterized by long-lasting behavioral abnormalities.


REPLY: Thanks for your comments. We corrected the sentence in the revised manuscript.

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Preliminary investigations of DNA methylation mainly focused on disease-related genes, such as RELN [2], SOX10 [3], MB-COMT [4] and GAD1 [5], which exhibited methylation alterations between major psychosis and normal controls.

4. References: The references are not uniform in style and do not follow Journal style. Please change suitably.
REPLY: Thanks for your comments. We downloaded BMC Medical Genomics reference style (Endnote style file) provided by BMC Medical Genomics. And we changed the reference style suitably in the revised manuscript.

5. The English grammar is incorrect in many of the sentences of the manuscript. Please change suitably.

REPLY: Thanks for your comments. We checked the English grammar thought the full text and carefully revised the manuscript. The manuscript was also proof-read and edited by a native English speaker.

Special thanks to you for your good comments.

Reviewer #2

This manuscript examines in schizophrenia and bipolar patients genome-wide differences in DNA methylation and gene expression. The authors find that differentially methylated regions (DMRs) are only able to account for a small fraction of the gene expression differences found. They indicate that DMRs in introns leading to alterations in miRNA and lincRNA expression are more plausible explanations for the gene expression differences. The manuscript addresses a topic of interest to the field, namely the role of non-coding RNA in major mental illnesses. The language of the article is overall satisfactory with some minor grammatical errors. I have the following specific comments.

1) What was the average RIN score for the subjects and were there differences based on diagnosis?

REPLY: Thanks for your comments. In our study, the average RIN scores for SZ, BD and control samples were 7.6, 7.3 and 7.4, respectively. And there were no significant differences of RIN scores between disease (SZ or BD) and control samples (P value =0.81 and 0.67, respectively, two-tailed Student t test). We added the corresponding information in the Method section of the revised manuscript.

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RNA-seq

Beads with oligo(dT) were used to isolate poly(A) mRNA after total RNA was collected from all samples (RNeasy Lipid Tissue Mini Kit (Qiagen # 74804) and QIAzol Lysis Reagent (Qiagen # 79306)). In the study, only high-quality RNA samples that yielded RNA integrity numbers (RIN) scores >= 6.0 by Agilent Bioanalyzer were used for subsequent analysis. The average RIN scores
for SZ, BD and control samples were 7.6, 7.3 and 7.4, respectively. And there were no significant differences of RIN scores between disease (SZ or BD) and control samples (P value = 0.81 and 0.67, respectively, two-tailed Student t test). mRNA was fragmented in fragmentation buffer as previously described [26].

2) On page 13 the authors state "more than 50% of the intronic DMRs were highly associated with strongly or weakly transcribed regions." The language of this sentence is somewhat confusing. Are the authors saying that 50% of hyper- or hypomethylated regions in schizophrenia or bipolar patients are associated with either strongly or weakly transcribed regions, but not necessarily hypermethylation with low transcription and hypomethylation with high transcription. Some clarification would be helpful.

REPLY: Thanks for your comments. As you said, more than 50% of hyper- or hypomethylated regions located in intron in schizophrenia and bipolar patients are associated with either strongly or weakly transcribed regions, but not necessarily hypermethylation with low transcription and hypomethylation with high transcription. We modified the description in the revised manuscript.

On page 13

Although a part of intronic DMRs are marked by polycomb repressed, heterochromatic and repetitive states, more than 50% of the intronic hyper- and hypomethylated regions in SZ and BD are associated with either strongly or weakly transcribed regions (Figure 4A and 4B). Such association between intronic DMRs and transcriptional activity supported an important role of intronic DNA methylation in altering chromatin structure and elongation efficiency [40]. Note that it is not necessarily true that hypermethylation and hypomethylation are correlated with regression and activation of transcription, respectively.

3) One of the take home messages of this manuscript is that DNA methylation and gene expression are not necessarily always inversely correlated. However, a major focus of this manuscript is based on differences in methylation of non-coding RNA genes. The authors conclude that the differential methylation of non-coding RNA regions may explain the gene expression changes more than differences in gene promoter methylation. What is confusing is why if DNA methylation does not predictable control transcription of protein coding genes would it control transcription of non coding RNA transcripts? Does DNA methylation more reliably suppress non-coding RNA expression than protein coding expression?

The main concern is what appears to be a contradiction within the manuscript, namely if DNA methylation does not always suppress protein coding gene expression then why would changes in methylation within introns lead to alterations in noncoding RNA expression.
REPLY: Thanks for your comments. In our study, we investigated the distribution of DMRs across different genomic elements and observed that DMRs fell into 288 gene promoters for SZ and 557 gene promoters for BD. Specially, 14 (4.9%) and 73 (13.1%) genes showed expression changes in SZ and BD, respectively (Table S2 for SZ and Table S3 for BD). It suggested that DNA methylation changes in promoter regions had modest effects on gene expression. On the other hand, there are 3232 and 5107 genes associated with intronic-related DMRs for SZ and BD, respectively, of which 236 (7.3%) and 499 (9.8%) genes showed expression changes. Notably, we found that some targets of intronic DMR-related miRNAs showed expression changes, suggesting a part of intronic DMRs could influence miRNAs transcription, which in turn changed the expression of downstream target genes. Our findings indicated that differential methylation of miRNAs (especially for intronic miRNAs) represented another possible regulatory mechanism of controlling gene expression beside aberrant promoter methylation. We are sorry for our unclear descriptions about the effects of the differential methylations of non-coding RNA regions and protein coding gene promoters on gene expression. We modified the description about the findings in the Results of the revised manuscript.

Additionally, following your suggestion, we evaluated the effects of promoter methylation on protein coding genes (PCGs) and miRNAs. We downloaded an additional set of RNA-seq, smRNA-seq and MeDIP-seq data of H1 cell line from the NIH Roadmap Epigenomics Program. For RNA-seq data, we used Tophat (version 2.0.1) (C Trapnell et al. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics, 2009), to map reads to the human reference genome version hg19. Then HTSeq (S Anders et al. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics, 2015) was used to quantify the read counts mapped to PCGs. We calculated FPKM (Fragments per kilobase of transcript per million fragments mapped) values as the expression levels of PCGs. For smRNA-seq data, we converted the BED file to FASTA file and then used miRanalyzer (M Hackenberg et al. miRanalyzer: an update on the detection and analysis of microRNAs in high-throughput sequencing experiments. Nucleic Acids Research, 2011) to calculate the RPM (reads per million reads mapped) as the expression values of miRNA with default parameters. For MeDIP-seq data, we obtained the miRNA promoters from miRStart (CH Chien et al. Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. Nucleic Acids Research, 2011). Then we used MEDIPS (version 1.12.0) (L Chavez et al. Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage. Genome research, 2010) to calculate the absolute methylation scores (ams) for promoters of miRNAs and PCGs. As a result, both miRNAs and PCGs showed weak negative correlations between promoter methylation and their expression (Pearson’s correlation coefficient = -0.08, P value < 2.2e-16 for PCGs and Pearson’s correlation coefficient = -
0.08, P value = 0.1 for miRNAs, Figure S3), suggesting the similar effects of DNA methylation on expression of PCGs and miRNAs. These findings indicated DNA methylation can not only directly influence gene transcription through promoter methylation alteration, but also indirectly influence gene transcription by changing miRNAs methylation. We added a paragraph in the Discussion section and provided Table S2, Table S3 and Figure S3 in the revised manuscript.

On page 12

In addition, we extracted DMR-related genes and grouped them into different element-associated sets. By comparing these genes with differentially expressed genes, we found that there are 288 genes for SZ and 557 genes for BD with aberrant promoter methylation, of which 14 (4.9%) and 73 (13.1%) showed expression changes (Figure 2C and Table S2 for SZ; Figure 2D and Table S3 for BD). It may lead us to consider that DNA methylation had a limited role in directly regulating gene expression. Interestingly, there were 3232 genes for SZ and 5107 genes for BD with intronic DMRs, of which 236 (7.3%) and 499 (9.8%) showed expression changes (Table S2 for SZ; Table S3 for BD). These findings suggested that beside the impact of promoter DNA methylation on gene expression, DNA methylation alterations in introns might represent another mechanism by which DNA methylation influences expression.

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Given that promoter methylation could account for a part of expression changes of protein coding genes (PCGs), it remains to be determined whether these finding is applicable to miRNAs. To address this, we downloaded an additional set of RNA-seq, smRNA-seq and MeDIP-seq data of H1 cell line from the NIH Roadmap Epigenomics Program [55] and calculated the expression levels of miRNAs and PCGs as well as their promoter methylation levels. As a result, both miRNAs and PCGs showed negative correlations between promoter methylation and their expression (Pearson’s correlation coefficient = -0.08, P value < 2.2e-16 for PCGs and Pearson’s correlation coefficient = -0.08, P value = 0.1 for miRNAs; Figure S3). These results suggested that there were no significant differences in the effects of DNA methylation on expression of PCGs and miRNAs.

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Figure S3. Scatter diagrams showing the correlations between promoter methylation levels (x axis) and log-transformed expression (y axis) of protein coding genes (left) and miRNAs (right).
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Table S2. The number (percentage) of genes with hypermethylated or hypomethylated regions in different elements showing up- or down-regulated expression in SZ.

Table S3. The number (percentage) of genes with hypermethylated or hypomethylated regions in different elements showing up- or down-regulated expression in BD.

I think a table comparing hyper vs hypomethylated gene promoters and RNA expression (up or down) would be helpful.

REPLY: Thanks for your comment. Following your suggestion, we added two tables for comparing methylation alterations and gene expression changes in SZ and BD (Table S4 for SZ and Table S5 for BD). For SZ, we found that 4 of the 168 genes whose promoters were hypermethylated showed decreased expression and 6 of 120 genes whose promoters were hypomethylated showed increased expression. For BD, there are 11 genes with promoter hypermethylation and decreased expression, and 4 genes with promoter hypomethylation and increased expression. For example, gene PLP1 showed increased promoter methylation levels and down-regulated expression in SZ patients compared with control subjects (Figure S2). Notably, decreased expression of PLP1 has been frequently observed in BA9 of patients with both SZ and BD (BP Sokolo et al. Oligodendroglial abnormalities in schizophrenia, mood disorders and substance abuse. Comorbidity, shared traits, or molecular phenocopies? Int J Neuropsychopharmacol, 2007). Our results demonstrated that promoter methylation alteration could account for a part of gene expression changes, which probably underlies the pathogenesis of SZ and BD. We added Table S4, Table S5 and Figure S2 into Additional files and corresponding descriptions in the revised manuscript.

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Consistent with previous studies, DNA methylation alternations at gene promoters could regulate a part of gene expression (Table S4 for SZ and S5 for BD). For SZ, we found that 4 of the 168 genes with promoter hypermethylation showed decreased expression and 6 of 120 genes with promoter hypomethylation showed increased expression (Table S4). For BD, 11 of the 146 genes with promoter hypermethylation showed decreased expression and 4 of 411 genes with promoter hypomethylation showed increased expression (Table S5). As an example, gene PLP1 gained increased promoter methylation levels and showed down-regulated expression in SZ patients compared with control subjects (Figure S2). Decreased expression of PLP1 has been frequently observed in BA9 of patients with both SZ and BD [52]. Interestingly, we found that many DMRs were located in introns, probably affecting promoters, transcriptional elongation and enhancer-mediated looping, in line with a recent report that intragenic methylation could play a major role in the regulation of tissue- and cell-specific alternative promoters [53].

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Figure S2. The UCSC Browser showing raw RNA-seq and MeDIP-seq reads distributions around gene PLP1 in SZ patients and controls. Yellow shadow represents the DNA methylation levels in the promoter region of PLP1.

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Table S4. The number of genes with hypermethylated or hypomethylated promoters and gene expression (up or down) for SZ.

Table S5. The number of genes with hypermethylated or hypomethylated promoters and gene expression (up or down) for BD.

5) I find figures 2-5 rather confusing.

REPLY: We are very sorry for our unclear description of figures 2-5. We modified the description of figures 2-5 in the Result section of the revised manuscript. We also provided more details about these figures in their figure legends in the revised manuscript. In addition, in order to facilitate understanding, we added labels at appropriate locations in figures 2-5.

On page 12

In addition, we extracted DMR-related genes and grouped them into different element-associated sets. By comparing these genes with differentially expressed genes, we found that there are 288 genes for SZ and 557 genes for BD with aberrant promoter methylation, of which 14 (4.9%) and 73 (13.1%) showed expression changes (Figure 2C and Table S2 for SZ; Figure 2D and Table S3 for BD).

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Next, we sought to examine how different genomic elements are distributed around DMRs. We analyzed the distribution patterns of different genomic elements (such as CGI, promoter and intron) around hypermethylated or hypomethylated DMRs in SZ and BD. Figure 3 shows diverse distribution patterns. Some DMRs directly overlap with promoters and some are located on their flanks. However, many DMRs (2434 hypermethylated and 1268 hypomethylated DMRs in SZ;
1951 hypermethylated and 3931 hypomethylated DMRs in BD) completely fell into introns, although a few were mapped to promoters (Figure 3).

On page 13

Although a part of intronic DMRs are marked by polycomb repressed, heterochromatic and repetitive states, more than 50% of the intronic hyper- and hypomethylated regions in SZ and BD are associated with either strongly or weakly transcribed regions (Figure 4A and 4B). Such association between intronic DMRs and transcriptional activity supported an important role of intronic DNA methylation in altering chromatin structure and elongation efficiency [40]. Note that it is not necessarily true that hypermethylation and hypomethylation are correlated with regression and activation of transcription, respectively.

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In order to investigate whether aberrant DNA methylation-associated intronic miRNAs account for gene differential expression, we obtained intronic miRNAs whose promoters are covered by DMRs and retrieved their targets from TargetScan. The results showed that targets of intronic DMR-related miRNAs captured numerous differentially expressed genes in SZ and BD (Figure 5A and 5B).

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We found that only the intronic DMR-related genes can accurately cover the majority of these functions in both SZ and BD (Figure 5C-[III-V] and 5D-[III-V]), while DMRs falling into other elements (including promoter, 5’UTR, exon and 3’UTR) almost cannot capture any functions. Unexpectedly, when combining DNA methylation alterations with gene expression changes, only down-regulated genes with hypermethylated intronic DMRs can capture limited functions enriched by differentially expressed genes in both SZ and BD (Figure 5C-[VI] and 5D-[VI]).

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Figure 2. Distribution of hyper-DMRs or hypo-DMRs in different genomic elements. (A, B) Left panel: Each bar represents the fraction of hyper-DMRs (or hypo-DMRs) map to each genomic element in SZ (A) and BD (B). Right panel: The x axis indicates O/E ratios between the observed and expected number of DMRs overlapping with a given type of genomic elements. The average number of DMRs in random sets overlapping with a given type of genomic elements was regarded as the expected value. Asterisks indicating the significance levels (* represents P < 0.05; ** represents P < 0.01). (C, D) Comparisons between differentially expressed genes and genes with aberrant DNA methylation in different genomic elements in SZ (C) and BD (D). Distribution of up- and down-regulated genes (left), and genes with hyper-DMRs (middle) or hypo-DMRs (right) located in their different genomic elements. For each type of genes, red indicates present.
Figure 3. The distribution patterns of genomic elements around DMRs in SZ (A) and BD (B). Upper and lower panels represent hypermethylated and hypomethylated DMRs, respectively. Each hyper- or hypo-DMR was divided into 10 equal-sized intervals. The regions 5 kb upstream and downstream of the DMR were divided into 200-bp intervals. Each heat map presents a distribution pattern of different genomic elements around hyper-DMRs or hypo-DMRs. Intervals overlapping with different genomic elements were indicated by different colors.

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Figure 4. Relationship between intronic DMRs and functional elements. (A, B) Heatmaps showing different types of functional elements defined by multiple epigenetic marks at or around hypermethylated and hypomethylated intronic DMRs in SZ (A) and BD (B). (C, D) Enrichment of hypermethylated and hypomethylated intronic DMRs in transcription factor binding. Each bar represents the O/E ratio between the observed and expected number of intronic DMRs overlapping with the TF binding sites in SZ (C) and BD (D). Asterisks indicating the significance levels (* represents P < 0.05; ** represents P < 0.01).

Figure 5. DMRs-mediated dysfunction of intronic miRNAs contributing to expression changes. (A, B) Distribution of targets of hypermethylated or hypomethylated intronic DMR-related miRNAs (i.e. hyper-miRNA or hypo-miRNA) and differentially expressed genes in SZ (A) and BD (B). (C, D) Heat maps of GO terms enriched by different gene sets: [I] differentially expressed genes, up- and down-regulated genes; [II] DMR-related genes; [III-V] genes with DMRs, hyper-DMRs and hypo-DMRs at different genomic elements (including promoter, 5’UTR, exon, intron and 3’UTR); [VI] hypermethylated or hypomethylated intronic DMR-related genes with expression changes; [VII] up- or down-regulated targets of hypermethylated or hypomethylated intronic DMR-related miRNAs, in SZ (C) and BD (D).

Special thanks to you for your good comments.