Archival Report

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DNA Modification Study of Major Depressive Disorder: Beyond Locus-by-Locus Comparisons

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ABSTRACT

BACKGROUND: Major depressive disorder (MDD) exhibits numerous clinical and molecular features that are consistent with putative epigenetic misregulation. Despite growing interest in epigenetic studies of psychiatric diseases, the methodologies guiding such studies have not been well defined.

METHODS: We performed DNA modification analysis in white blood cells from monozygotic twins discordant for MDD, in brain prefrontal cortex, and germline (sperm) samples from affected individuals and control subjects (total $N = 304$) using 8.1K CpG island microarrays and fine mapping. In addition to the traditional locus-by-locus comparisons, we explored the potential of new analytical approaches in epigenomic studies.

RESULTS: In the microarray experiment, we detected a number of nominally significant DNA modification differences in MDD and validated selected targets using bisulfite pyrosequencing. Some MDD epigenetic changes, however, overlapped across brain, blood, and sperm more often than expected by chance. We also demonstrated that stratification for disease severity and age may increase the statistical power of epimutation detection. Finally, a series of new analytical approaches, such as DNA modification networks and machine-learning algorithms using binary and quantitative depression phenotypes, provided additional insights on the epigenetic contributions to MDD.

CONCLUSIONS: Mapping epigenetic differences in MDD (and other psychiatric diseases) is a complex task. However, combining traditional and innovative analytical strategies may lead to identification of disease-specific etiopathogenic epimutations.

Keywords: DNA modification, Epigenetic outliers, Epigenetics, Heteroscedasticity, Major depressive disorder, Molecular networks

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Major depressive disorder (MDD) is a psychiatric disease characterized by an all-encompassing low mood accompanied by low self-esteem, loss of interest or pleasure in normally enjoyable activities, and a variety of other associated symptoms (1). MDD affects one in seven individuals (2) and has been projected to become the second leading cause of disability worldwide by 2020 (3).

A meta-analysis of twin studies on MDD estimated heritability at 37% (4), which is consistent with a recent large epidemiologic study (5). This significant heritability provided a basis for molecular genetic studies; however, identification of specific MDD risk genes has proven difficult. A recent genome-wide association study with >18,000 subjects in the discovery phase did not detect any genome-wide significant single nucleotide polymorphisms (SNPs). The study followed up on the top 554 SNPs ($p < .0001$) in an independent set of >57,000 subjects but failed to replicate any of the SNPs at genome-wide significance (6).

MDD exhibits numerous non-Mendelian features that can be reviewed from an epigenetic perspective (7). Such features include partial heritability, discordance of monozygotic (MZ) twins, sexual dimorphism (8,9), disease onset following major hormonal changes (e.g., postpartum depression) (10), and fluctuating course of disease (11). Epigenetics refers to the regulation of various genomic functions that are controlled by heritable but reversible chemical modifications of DNA and histones (12). Environmental factors such as stress, diet, and drugs can alter the epigenetic profile (13,14). Even in the absence of environmental exposures, stochastic epigenetic changes may influence phenotypic outcomes (15). Furthermore, there is increasing evidence that epigenetic factors, in addition to DNA sequences, account for heritability (16,17). In short, we postulate that inherited and acquired epigenetic misregulation may play an etiological role in MDD (7).

In this study, we attempted to identify MDD specific epigenetic changes using a series of experimental and analytical

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approaches, from traditional locus-by-locus comparisons to new systems biology-based strategies, such as epigenomic networks and machine-learning based classification.

METHODS AND MATERIALS

Samples

Tissue samples were collected from individuals diagnosed with MDD and from matched control subjects. Inclusion criteria involved patients between the ages of 18 and 75 diagnosed with MDD according to DSM-IV criteria. Individuals with a prior history of other mental illnesses, addiction and substance abuse, or a family history (first-degree relatives) of schizophrenia were excluded from the study. The 100 discordant MZ twin samples consisted of peripheral blood DNA from 40 pairs of MZ twins from Australia, 46 pairs from The Netherlands, and 14 pairs from the United Kingdom (for detailed description, see Supplement 1). Seventy-one prefrontal cortex samples were received from the Stanley Medical Research Institute (SMRI) and Quebec Suicide Brain Bank (QSBB). Thirty-three sperm samples from bipolar disorder patients, a disease that may be etiologically related to MDD (18,19), and control subjects were obtained from an ongoing study at the Centre for Addiction and Mental Health (Toronto, Ontario, Canada). More information on the samples can be found in Table S1 in Supplement 1.

Microarray Experiment

The unmodified DNA fraction was enriched using modified cytosine (^{mod}C)-sensitive restriction enzymes, which collectively interrogate 5-methylcytosine and 5-hydroxymethylcytosine (20) (it is assumed that 5-carboxylcytosine and 5-formylcytosine are rare and unlikely to significantly contribute to the estimates of the modified/unmodified cytosines). Three aliquots of 250 ng of genomic DNA were digested individually with HpaII, HinP1I, and HpyCH4IV and pooled together after digestion was completed. For the twin samples, 500 ng of genomic DNA was digested using only HpaII. All other steps were identical to those described in our published protocol (21). The microarray experiment was conducted using a common reference pool design. The enriched polymerase chain reaction products were labeled with Cy3 for the reference and Cy5 for the sample hybridized onto 8.1K human CpG island microarrays (22,23). A detailed description of the bioinformatic methods can be found in Supplement 1.

Bisulfite modification and pyrosequencing-based fine mapping of ^{mod}C was performed using a standard protocol (24). The primers for the bisulfite polymerase chain reaction were designed using either the MethPrimer (25) or the Pyrosequencing Assay Design Software v1.0.6 (Qiagen, Valencia, California) (Table S2 in Supplement 1). For pyrosequencing, Gold Q96 Reagents and Pyromark Q24 were used (Qiagen).

Ethics Statement

Centre for Addiction and Mental Health Research Ethics Board granted approval to protocol # 024/2005-01 entitled "Molecular epigenetic studies of major depression." All experiments were performed in accordance with relevant guidelines and regulations.

RESULTS

Locus-Specific Analysis of DNA Modification in the Brain, White Blood Cells, and the Germline

In the human brain samples from the SMRI, a locus-by-locus comparison between MDD or MDD with psychosis (MDD + Psy) and control subjects using analysis of variance revealed 40 differentially modified loci (nominal $p = 4 \times 10^{-5} - .01$; Table S4 in Supplement 1); 22 loci showed differential modification between MDD and control subjects, and 18 loci showed differential modification between MDD + Psy and control subjects (Tukey's honestly significant difference, $p < .05$). Eight loci were differentially modified for both MDD and MDD + Psy compared with control subjects. One gene, *FOXD3,* was previously implicated in MDD (26). The analysis of the brain samples from the QSBB revealed 35 loci with differential modification (nominal $p = 5 \times 10^{-4} - .01$). In white blood cells (WBCs) from MZ twins discordant for MDD, we identified 44 loci with nominal $p = 9 \times 10^{-5} - .01$. Lastly, in the sperm samples from individuals affected with bipolar disorder and control subjects, we found 34 loci (nominal $p = 6 \times 10^{-4} - .01$), one of which had already been implicated in bipolar disorder (*SMAD3*) (27).

We did not find significant overlaps between any of the samples tested above. However, we found a statistically significant number of overlapping loci between our study and a previously published epigenome-wide study using the same SMRI brain samples but different enrichment technique and platform (at nominal $p < .05$ for both studies) (28). We performed permutation analysis and found that our microarray probes that were either directly on or nearest neighbors (median distance = 12 kb) to the gene of interest were overrepresented than by chance ($n = 14$; permuted $p = .04$; Table S5 in Supplement 1). Even when the parameters were made more stringent to only include microarray probes that were either directly on or within a short distance away (<10 kb or <5 kb) from the gene of interest, we still found a significant number of overlaps between the two studies ($n = 12$ for both; $p = .03$ and $p = .02$, respectively).

None of the detected loci survived correction for multiple testing, although 13 loci with nominal $p < .05$ overlapped with either the SMRI or the QSBB brain samples and the WBC samples of the MDD twins. Among the 13 loci, probes for *LRRC41* and *LIN28A* contained regulatory sequences, nuclear factor-κB transcription factor binding site, and a predicted insulator CTCF binding site, with ^{mod}C sensitive sites (Figure 1) (29). These two loci, plus three different types of repetitive elements (*LINE-1*, *NBL-2*, and *D4Z4*) as proxies for global modification changes (30), were finely mapped using bisulfite pyrosequencing. A total of 29 CpG sites (11, 4, 3, 6, and five CpG sites for *LRRC41*, *LIN28A*, *LINE-1*, *NBL-2*, and *D4Z4*, respectively) were interrogated from the two unique DNA loci and three repetitive DNA elements.

Bisulfite pyrosequencing revealed that ^{mod}C density at *LIN28A* was different in the SMRI MDD + Psy samples compared with control subjects (Mann-Whitney test, $p = .01$). While the pooled MDD samples (MDD and MDD + Psy) also showed significant differences ($p = .01$), MDD alone versus control subjects did not reach significance ($p = .08$), and the same was detected in the QSBB samples ($p = .77$). We also