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Monozygotic twins affected with major depressive disorder have greater variance in methylation than their unaffected co-twin

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Our understanding of major depressive disorder (MDD) has focused on the influence of genetic variation and environmental risk factors. Growing evidence suggests the additional role of epigenetic mechanisms influencing susceptibility for complex traits. DNA sequence within discordant monozygotic twin (MZT) pairs is virtually identical; thus, they represent a powerful design for studying the contribution of epigenetic factors to disease liability. The aim of this study was to investigate whether specific methylation profiles in white blood cells could contribute to the aetiology of MDD. Participants were drawn from the Queensland Twin Registry and comprised 12 MZT pairs discordant for MDD and 12 MZT pairs concordant for no MDD and low neuroticism. Bisulphite treatment and genome-wide interrogation of differentially methylated CpG sites using the Illumina Human Methylation 450 BeadChip were performed in WBC-derived DNA. No overall difference in mean global methylation between cases and their unaffected co-twins was found; however, the differences in females was significant \(P = 0.005\). The difference in variance across all probes between affected and unaffected twins was highly significant \(P < 2.2 \times 10^{-16}\), with 52.4% of probes having higher variance in cases (binomial \(P\)-value \(< 2.2 \times 10^{-16}\)). No significant differences in methylation were observed between discordant MZT pairs and their matched concordant MZT (permutation minimum \(P = 0.11\)) at any individual probe. Larger samples are likely to be needed to identify true associations between methylation differences at specific CpG sites.

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Introduction

Major depressive disorder (MDD) is a complex disorder with a considerable impact on the quality of patient’s lives including an increased rate of mortality. Lifetime prevalence is estimated to be 15% and it is twice more common in women than in men. Heritability of MDD is estimated to be around 40%. There have been no common genetic variants convincingly associated with MDD, and most of the variants identified for complex psychiatric disorders contribute to a small fraction of the genetic variation and explain only a small proportion of the heritability. Growing evidence suggests that variations in epigenetic profiles (that is, DNA methylation) influence complex trait variation. Epigenetic modifications do not directly interfere with the gene transcript but control tissue and temporal specificity of gene expression. DNA methylation is the most common epigenetic variation in the mammalian genome and is not entirely stable; stochastic and environmental events can also generate variations over time. Non-heritable influences on depression may mediate their biological effects through epigenetic mechanisms such as methylation of CpG dinucleotides.

Several epigenetic studies have attempted to identify differences in DNA methylation associated with complex traits. Associations between variations in DNA methylation profiles and various psychiatric disorders (for example, schizophrenia, MDD, bipolar disorder and so on) have been reported. Some limitations should be considered from these studies (for example, small samples sizes, sample heterogeneity, epigenome coverage). Tissues involved in complex psychiatric disorders are not directly accessible from living patients; epigenetic studies often rely on peripheral tissue biomarkers such as buccal, gut and white blood cells (WBC). Post-mortem tissue can also be used in epigenetic studies to compare central and peripheral tissue; however, samples are not easily accessible. Moreover, post-mortem epigenetic measures may reflect treatment rather than disease. More research is needed to determine the accuracy of peripheral tissue biomarkers.

Discordant monozygotic twin (MZT) pairs constitute a powerful design in epigenetic studies. Single-nucleotide polymorphisms and other DNA sequence variations, which are abundant in singleton-based studies, are not a confounding source of variation in MZT-based epigenetic studies. The aim of our study was to examine whether diverse epigenetic profiles, specifically DNA methylation profiles, are associated with increased risk of MDD. We analysed genome-wide methylation patterns after bisulphite conversion of CpG sites using the Illumina Human Methylation (HM) 450
BeadChip (HM 450 BeadChip) of WBC-derived DNA from 12 discordant MZT (6 male and 6 female) pairs and 24 concordant MZT pairs.

Materials and methods

Sample selection/study design. Participants were drawn from the Queensland Twin Registry. Phenotypic information was collected as part of studies undertaken at the Queensland Institute of Medical Research each approved by the Institute’s ethics committee. On the basis of the study, depressive symptoms were evaluated with either the Semi-Structured Assessment for the Genetics of Alcoholism,22 (adapted for use in Australia) or the Composite International Diagnostic interview23 questionnaires. A diagnosis of MDD was constructed from these questionnaires according to the Diagnostic and Statistical Manual of Mental Disorders-IV criteria.24 A total of 17 958 individuals from 6855 families have completed psychiatric interviews, including a total of 1812 MZT pairs, of whom 261 were found to be discordant for MDD.

Six MZT female pairs (including five MZT female pairs previously epityped with the Illumina HM 27 BeadChip; Illumina, San Diego, CA, USA) and six MZT male pairs discordant for MDD were selected as ‘case pairs’. Where possible, we selected case probands with (the more severe) recurrent MDD; all six MZT females and two of the six males met this criterion. Further, we aimed to select twins who were recorded as non-smokers and not alcohol or drug dependent, criteria that imposed severe restrictions on MZT selection, and so selection criteria were extended to include MZT that were matched for these criteria (for example, both smokers with similar daily consumption and age of initiation). Six MZT female pairs and six MZT male pairs concordant for no MDD and low neuroticism were selected as ‘control pairs’. Neuroticism is a domain of personality easily measured by self-report. High neuroticism scores are associated with anxiety and depression; therefore, low scores are expected to represent those with a low liability for depression.25 This gives our study more power to detect variations in methylation status associated with MDD. Environmental factors such as smoking, alcohol or drug abuse are associated with altered methylation patterns.26,27 Each concordant MZT pair was selected to match as closely as possible to a discordant MZT pair on a number of key variables (Supplementary Tables 1 and 2). Matching criteria were date of blood sample collection, (as methylation differences increase with age20,25) smoking and alcohol as well as previous history of drug use. A total of 24 MZT pairs (48 individuals) were selected for our study (Table 1). All individuals were of Northern European ancestry.

Bisulphite conversions. Bisulphite conversions of previously extracted WBC-derived DNA from the 48 individuals, 6 technical replicate samples and technical control samples (described below) were performed following the protocol and using reagents of the EZ DNA methylation Kit (Zymo Research, Irvine, CA, USA). Each conversion included 10 samples and DNA recovery after conversion was quantified using Nanodrop (Thermo Scientific, Wilmington, DE, USA).

In total, eight bisulphite conversions were undertaken, each comprising three to four MZT pairs, technical replicates and control samples. Later runs repeated eight MZT pairs that had generated <80% DNA recovery in earlier runs (Supplementary Table 3).

Technical controls consisted of commercial DNA samples: Centre Etude Polymorphism Humain (CEPH) male (GM07029), CEPH female (GM06997) and FSK standard (Coriell Institute of Medical Research, Camden, NJ, USA). DNA recovery for the CEPH female sample after bisulphite conversion was ~50%: for this reason the CEPH male and the FSK standard were included in the later bisulphite conversions (Supplementary Table 3). Only FSK standard samples were included in the HM 450 BeadChips as controls because of their high percentage recovery of the bisulphite-converted DNA.

Infinium methylation assay. Bisulphite-converted DNA from 24 MZT pairs, 4 technical replicate samples and 4 FSK standard controls were hybridised to the Illumina HM 450 BeadChip, following the Illumina Infinium HD methylation protocol and using reagents and conditions supplied by Illumina. The HM 450 BeadChip-assessed methylation status was interrogated at 485 577 CpG sites across the genome. It provides coverage of 99% of RefSeq genes. Methylation scores for each CpG site are obtained as a ratio of the intensities of fluorescent signals and are represented as β-values.

\[
\beta \text{-value} = \frac{\max M}{\max M + \max U}
\]

where \(\max M\) = maximum intensity of fluorescence, M = methylated allele and U = unmethylated allele.28 The 100 in the denominator prevents division by 0 when both max M and max U are very small, but has little impact otherwise as max M + max U > 1000 in >95% of sites.29 β-values range between 0 and 1, where 0 means that no DNA molecules with a methylated CpG at the site of interest were detected in the sample, while 1 means that all DNA molecules had a methylated CpG site in the sample.

Each MZT pair was placed on the same array and in neighbouring wells to avoid batch effects contributing to

<table>
<thead>
<tr>
<th>Table 1 Summary of cases and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cases</strong></td>
</tr>
<tr>
<td>6 (5 + 1) MZT ♀ pairs discordant for MDD</td>
</tr>
<tr>
<td>6 MZT ♂ pairs discordant for MDD</td>
</tr>
<tr>
<td>Total 24 MZT discordant for MDD</td>
</tr>
</tbody>
</table>

Abbreviations: MDD, major depressive disorder; MZT, monozygotic twin.
differences between pairs. A total of five HM 450 BeadChips were placed on the same array in non-adjacent wells from the corresponding MZT whereas the other two replicate samples were placed on different arrays (Supplementary Table 4). The FSK male and female samples were also duplicated and placed on different chips. A total of 56 samples were included in the arrays. Arrays were scanned with the Illumina iScan (Software Version 3.3.28) and the intensities of the images were extracted using the GenomeStudio (2010.3) methylation module (1.8.5) software.

Quality control. Background was subtracted from our data using GenomeStudio (2010.3). Illumina’s specification for the HM 450 BeadChip is 98% detection of CpG with \( P < 0.05 \). All of our samples included in the five BeadChips exceeded specification, with >99% of CpG loci detected; therefore, no samples were excluded from the study. Three hundred seventy-three probes for which 75% of the samples had a detection \( P \)-value > 0.05 were removed. A total of 485,204 probes remained.

The \( P \)-values were transformed using a logit transformation in the IMA\(^{29}\) package in the R environment for statistical computing (version 2.13.2); http://www.R-project.org). Transforming via a logit transformation increases the validity of statistical tests of differential methylation.\(^{29}\) Supplementary Figure 1 shows a histogram of the transformed \( P \)-values across all samples. The peak correction method of Deunderwaerder et al.\(^{31}\) was applied to correct for differences in results between the two chemistries utilised on the HM 450 chip. QC plots produced by the IMA package are shown in Supplementary Figures 2–4. Unsupervised clustering of all samples showed that male and female samples clustered together. Furthermore, replicate samples and twin samples clustered together providing an indication of the accuracy of the methylation detection procedure.

To improve the accuracy of our results, we excluded potential sources of technical and biological biases before analysing the methylation data. There is currently no standard procedure for filtering the samples and probes before methylation analysis.\(^{32}\) Our experimental design, including replicate samples enabled stringent quality control (QC) steps. These QC steps are described in detail in the Supplementary Methods. The number of sites annotated by probe type that were removed by the QC steps are shown in Table 2. After removing probes with large amounts of missing data and high levels of discordance between replicate pairs, a total of 462,002 (95.1%) probes remained for analysis.

Statistical analysis. A two-sample \( t \)-test was used to test for differences in overall mean methylation between monozygotic cases and their unaffected co-twin. In order to test for differences in variance between cases and their unaffected co-twin separately, and the distributions were compared using the Wilcoxon signed-rank test. All statistical tests were implemented in R.

Testing for differences between MZTs owing to MDD at each individual probe. In order to test for differences in methylation between monozygotic co-twins owing to MDD, a linear model was fitted. For each probe, the absolute difference between each twin pair was taken and regressed on matched twin set and case–control status.

\[
|y_{jk1} - y_{jk2}| = \text{Set}_i + \text{MDD}_i + \varepsilon_{jk}
\]

where, \( y_{jk}: \) the residual transformed \( P \)-value of a probe after adjusting for batch and chip effects of the \( l \)th (\( l = 1, 2 \)) twin of the \( j \)th twin pair (\( j = 1, 2 \)) from the \( i \)th set (\( i = 1, ..., 12 \)). \( MDD \) is an indicator such that \( MDD_i = 1 \) for the discordant pairs and \( MDD_i = 0 \) for the concordant pair \( \varepsilon_{jk} \) is the random error term. By taking the absolute difference in the residuals of the transformed \( P \)-value between MZT, the test can reflect both probes that are associated with more or less methylation or probes that are more variably methylated probes associated with MDD. Under the MZT design we assume that any confounding variables are independent of the differences between MZT.

To establish the significance of the top results, a set of 100 permutations were performed. In each permutation, the concordant pair and discordant label were randomly assigned within each twin pair set, the analysis was performed for each probe, and the \( P \)-value for case–control status was extracted. The results of each permutation were extracted and ranked. The mean of the \( P \)-values with the same rank across all 100 permutations was calculated and the distribution of the means was taken as the null distribution. An empirical \( P \)-value was calculated by counting the proportion of permutations that had at least one probe more significant than the top probe identified in the real data set.

Pathway analysis. All genes for which at least one probe had a \( P \)-value < 0.001 for association with MDD status were uploaded to the commercial pathway analysis software Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA) and the freely available software DAVID,\(^{33}\) to test for enrichment of associations in functional pathways and gene ontology terms.

Results

Global differences in mean and variance. There was no significant difference in overall methylation between discordant pairs (\( P = 0.56 \)). As there were highly significant differences in overall methylation of autosomal probes between males and females (\( P < 2.2 \times 10^{-16} \), Supplementary Material) as previously reported by others,\(^{34,35}\) we tested for differences in overall methylation levels by sex within
The difference in mean methylation was not significant ($P = 0.05$), but was significant in females ($P = 0.005$), with cases being less methylated when compared with controls (whereas the trend of differences in males was the opposite).

Table 3

<table>
<thead>
<tr>
<th>Decile</th>
<th>Wilcoxon signed-rank test</th>
<th>Proportion of probes with increased variance in cases</th>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>$&lt; 2.2 \times 10^{-16}$</td>
<td>0.539</td>
</tr>
<tr>
<td>4</td>
<td>$&lt; 2.2 \times 10^{-16}$</td>
<td>0.530</td>
</tr>
<tr>
<td>5</td>
<td>$&lt; 2.2 \times 10^{-16}$</td>
<td>0.531</td>
</tr>
<tr>
<td>6</td>
<td>$&lt; 2.2 \times 10^{-16}$</td>
<td>0.522</td>
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<td>7</td>
<td>$&lt; 2.2 \times 10^{-16}$</td>
<td>0.519</td>
</tr>
<tr>
<td>8</td>
<td>6.50E – 04</td>
<td>0.508</td>
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<tr>
<td>9</td>
<td>8.79E – 01</td>
<td>0.501</td>
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<td>10</td>
<td>1.25E – 04</td>
<td>0.504</td>
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Table 4

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<th>LIMNID</th>
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<th>MAPINFO</th>
<th>UCSC_REFGENE_NAME</th>
<th>UCSC_REFGENE_ACCESSION</th>
<th>RELATION_TO_UCSC_CPG_ISLAND</th>
<th>P-value</th>
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<tr>
<td>cg10665379</td>
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<td>44565633</td>
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<td>S_Shore</td>
<td>2.98E – 05</td>
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<td>cg11843516</td>
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<td>Body</td>
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<td>11369615</td>
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<td>N_Shore</td>
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<td>44614496</td>
<td>MIP9</td>
<td>Body</td>
<td>N_Shore</td>
<td>5.98E – 05</td>
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<td>cg09858767</td>
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<td>14743737</td>
<td>NA</td>
<td>NA</td>
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<td>8.21E – 05</td>
</tr>
<tr>
<td>cg22618878</td>
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<td>27156656</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>8.28E – 05</td>
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<td>cg07048516</td>
<td>22</td>
<td>24105087</td>
<td>C22orf15</td>
<td>TSS200</td>
<td>NA</td>
<td>9.08E – 05</td>
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<td>27008764</td>
<td>CENPA</td>
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<td>9.48E – 05</td>
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</tbody>
</table>
significant hits for schizophrenia in this gene were found, and evidence of association with bipolar disorder was found in a recent meta-analysis (rs4332037, $P = 6.3 \times 10^{-6}$).

A total of 211 genes were uploaded to Ingenuity and DAVID pathway analysis packages. Neither pathway analysis package identified any canonical pathways or biological functions to be significantly enriched after setting a false discovery rate of 5%.

**Discussion**

Previous studies involving MZT pairs discordant for psychiatric disorders have been limited to a small number of twin pairs (for example, two MZT pairs for schizophrenia), or have assessed only a fraction of the genome-wide CpG sites. This study represents the largest twin study to date of genome-wide associated methylation differences in MZT pairs discordant for MDD. Furthermore, this is one of the first methylation studies in psychiatry that utilizes the HM 450 Beadchip, which provides coverage of a much larger number of CpG sites throughout the genome.

Our analyses included sets of discordant MZT pairs and its matched concordant pairs. Although our sample size was not sufficient to identify methylation differences that could be attributed to MDD, it revealed that cases show a highly significantly increased variation in methylation throughout the genome ($P < 2.2 \times 10^{-10}$) when compared with their control co-twins. This finding suggests that the methyolome in patients with MDD may be undergoing more changes as a result of environmental influences than that in controls as a result of a loss of epigenetic stability. Increased variability in cases is seen across many cancers, but has not been previously investigated in studies of psychiatric disorders.

One potential reason for this increased variance in cases may be due to various forms of treatment including the use of medications such as antidepressants by affected twins that are not used by the unaffected twins. However, the result remained significant when comparing those reporting antidepressant use with their co-twins and also in those reporting no antidepressant use. No information was collected on the length of time that the drugs were used for or the dosage that was prescribed. Antidepressant drugs are known to induce methylation analysis in this study imposes limitations.

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traits. However, most of the variants identified have very small effect sizes. It is likely that DNA methylation differences involved in complex psychiatric disorders will also have small effects, and that sample sizes commensurate with those utilised in genome-wide association studies may be required to detect methylation differences at individual CpG islands.

Conflict of interest

The authors declare no conflict of interest.


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