Pathway Analysis Using Information from Allele-Specific Gene Methylation in Genome-Wide Association Studies for Bipolar Disorder

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Abstract

Bipolar disorder (BPD) is a complex psychiatric trait with high heritability. Despite efforts through conducting genome-wide association (GWA) studies, the success of identifying susceptibility loci for BPD has been limited, which is partially attributed to the complex nature of its pathogenesis. Pathway-based analytic strategy is a powerful tool to explore joint effects of gene sets within specific biological pathways. Additionally, to incorporate other aspects of genomic data into pathway analysis may further enhance our understanding for the underlying mechanisms for BPD. Patterns of DNA methylation play important roles in regulating gene expression and function. A commonly observed phenomenon, allele-specific methylation (ASM) describes the associations between genetic variants and DNA methylation patterns. The present study aimed to identify biological pathways that are involved in the pathogenesis of BPD while incorporating brain specific ASM information in pathway analysis using two large-scale GWA datasets in Caucasian populations. A weighting scheme was adopted to take ASM information into consideration for each pathway. After multiple testing corrections, we identified 88 and 15 enriched pathways for their biological relevance for BPD in the Genetic Association Information Network (GAIN) and the Wellcome Trust Case Control Consortium dataset, respectively. Many of these pathways were significant only when applying the weighting scheme. Three ion channel related pathways were consistently identified in both datasets. Results in the GAIN dataset also suggest for the roles of extracellular matrix in brain for BPD. Findings from Gene Ontology (GO) analysis exhibited functional enrichment among genes of non-GO pathways in activity of gated channel, transporter, and neurotransmitter receptor. We demonstrated that integrating different data sources with pathway analysis provides an avenue to identify promising and novel biological pathways for exploring the underlying molecular mechanisms for bipolar disorder. Further basic research can be conducted to target the biological mechanisms for the identified genes and pathways.

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Introduction

Bipolar disorder (BPD) is a severe and complex psychiatric disorder, with high heritability around 0.6 to 0.7 [1,2]. Prior individual linkage studies and meta-analyses suggested a number of susceptible regions in human genome for the risk of developing BPD. However, most of these findings are inconsistent and rarely pointed to specific chromosomal locations for replication [3]. Recently, large scale genome-wide association (GWA) studies, which scanned half or a million single nucleotide polymorphisms (SNPs), were frequently employed. Although the GWA studies were anticipated to provide comprehensive genetic information for complex traits, previous GWA studies for BPD reported limited numbers of susceptible loci with small effect size. The odds ratios of significant findings in GWA studies for BPD in populations of European, American and Han Chinese were between 1.2 and 2.0 [4,5,6,7], which are in accordance with the observations from other GWA studies in complex traits [8]. In addition, the reported associated variants from GWA studies often explain a small proportion of heritability for complex traits, a so called 'missing heritability' phenomenon [9]. Missing heritability may be owing to lack of power to detect common variants with very small effect, not including rare variants for their effects in whole-genomic array, or not considering other genomic mechanisms, such as complex gene-gene interaction and epigenetic influences [10].

In most of the genetic studies, a commonly applied strategy is to analyze single markers or specific haplotypes for their associations with disease of interests. This often produces limited success in identifying putative loci for BPD, especially for variants with small to moderate effect. The genetic causes of BPD are likely involved with a large collection of genetic variants in certain biological pathways to jointly exhibit their effects for the trait. Therefore, pathway-based approach becomes a useful and complementary method in addition to single locus analyses. A pathway is
considered as a specific gene set that is defined according to certain biological function or process. Analyzing GWA dataset with pathway-based approach could provide integrating information of multiple loci with similar physiological functions to bring biological insights into the mechanisms of BPD. Previously, pathway analysis has been successfully conducted using GWA datasets for several complex traits, such as schizophrenia, major depressive disorder, and breast cancer [11,12,13] to reveal important biological mechanisms underlying the diseases.

To perform pathway analysis using GWA dataset, an important first step is to extract SNPs information for each gene region. A commonly adopted method is to select the most significant SNP within a gene region to represent the gene [14]. Often, tens to hundreds of common SNPs are found in a typical gene region. Only a few of them are functional variants, thus, SNP with the maximum statistic may lack direct biological meaning and connection with the trait of interest. Nevertheless, non-structured variants may still regulate gene functions through other mechanisms. Epigenetic changes, one of regulatory mechanisms, can modify gene activity or gene expression without altering the genomic structure, including stable DNA methylation, post-translational modifications of histone proteins, and non-coding RNA [15]. Among these epigenetic modifications, changes of DNA methylation patterns at CpG sites are considered heritable and may play important roles in regulating gene functions [16,17].

A pilot study of the Human Epigenome Project reported that more than 50% of CpG sites have greater than 50% variation within the region of major histocompatibility complex [19]. Compelling evidence also reveals a commonly observed phenomenon called allele-specific methylation (ASM) to describe different status of DNA methylation of a nearby CpG site by the two alleles presenting in a cell [19].

The profiles of DNA methylation are dynamic and tissue specific. Using human adult cerebellum samples, Zhang and colleagues (2010) conducted whole genome genomic polymorphisms and methylation quantitative association analysis to identify SNPs that regulate DNA methylation of CpG sites through cis- or trans-regulation [20]. Their findings documented that variation in genetic polymorphism affects the degree of DNA methylation in coding or non-coding region of specific genes. A considerable proportion of CpG sites were regulated by specific genetic variants distributed in the whole genome. Incorporating the ASM information into pathway-based analysis using GWA dataset may provide a new avenue to search for important biological pathways and to investigate the underlying pathogenesis of BPD.

The present study aimed to integrate brain-specific ASM information into whole genome genotyping data to identify important pathways for bipolar disorder. We used two GWA datasets of BPD in the Caucasian populations, the Wellcome Trust Case Control Consortium (WTCCC) and the Genetic Association Information Network (GAIN). The list of brain-specific ASM was obtained from the Zhang’s (2010) study. We applied comprehensive pathway-based statistical approaches with novel weighting scheme to incorporate the impacts of ASM to evaluate the enrichment of annotated pathways for BPD. The present study successfully identified significant and novel pathways for BPD. Our strategy to explore potential mechanisms for BPD through integrating information from different genomic aspects can be well applied to other complex traits.

Materials and Methods

Genome-wide association (GWA) datasets

In the current study, two GWA datasets of BPD in the Caucasian populations were used, the WTCCC [4] and the GAIN data [21]. We used these two individual GWA datasets to search for consistent pathway findings for BPD. The details of subject enrollment and genotyping of the two GWA studies were provided in their primary articles. In brief, all participants in the WTCCC were self-identified as white Europeans who lived in the United Kingdom. These included 1,968 subjects with BPD and 2,938 healthy subjects from the 1958 British Birth Cohort or United Kingdom Blood Donors. In the GAIN dataset, individuals were Americans with European ancestry, including 1,001 cases of BPD and 1,034 controls. The genotyping platform was Affymetrix GeneChip Human Mapping 500K Array and Affymetrix Genome-Wide Human SNP Array 6.0 for the WTCCC and GAIN, respectively. After quality control procedures implemented, a total of 485,263 (WTCCC) and 698,227 (GAIN) autosomal SNPs were retained in the following analyses [4,21]. All single marker association analyses with additive model were conducted using PLINK versions 1.07 [22].

Brain specific allele-specific gene methylation (ASM) list and computing gene-wise statistic values

Information of ASM in human brain tissues was obtained from Zhang and colleagues [20]. Regulating SNPs within 1 Mb region of both ends of each CpG site were considered cis-acting, and all the other regulating SNPs were trans-acting. SNPs with region-wise p-value less than 0.05 in cis-acting and SNPs with genome-wide p-value less than 0.05 in trans-acting were selected in the ASM list in the present study. In total, we had 9,414 SNP-CpG pairs in autosomal chromosomes, which included 9,042 cis-acting and 372 trans-acting pairs (In the Table S5 of the Zhang et al’s study). Figure 1 described our analysis flow-chart. To obtain gene level significance for BPD in the two GWA datasets, we first mapped SNPs to a gene region (using NCBI build 35 for the WTCCC and build 36 for the GAIN due to different genotyping platforms) if SNPs were located within 5 kb of both ends of the gene. In the WTCCC dataset, there were 193,837 SNPs mapped to 15,054 genes. Among these SNPs, 6,324 SNPs in the ASM list locate in 1,785 genes. In the GAIN dataset, there were 304,343 SNPs mapped to 16,385 genes, and 6,992 SNPs in the ASM list locate in 1,961 genes. We therefore divided genes into two sets, ASM and non-ASM set, in the subsequent pathway analysis. We defined the ASM set to include 1) genes with SNPs in the ASM list, and 2) genes with CpG site that is regulated by SNPs in the ASM list. Genes not in the ASM set were assigned into the non-ASM set. In total, we had 2,327 and 2,298 genes in the WTCCC and GAIN datasets, respectively.

The gene-wise p-value for each gene was defined by the most significant SNP within a gene region, which was commonly adopted in conducting pathway analysis [14]. For genes in the ASM set, the smallest p-value (min-p) among all SNPs in the ASM list in a gene region was used to represent the gene-wise statistic value. Similarly, for genes in the non-ASM set, the smallest p-value (min-p) of all SNPs in a gene region was used to represent the gene-wise statistic values. To account for potential bias caused by using minimum p-values to represent gene-wise statistic for genes with various sizes, we adopted the method by Yang and colleagues [23] to calculate normalized gene scores via 10,000 permutations for all the genes we analyzed. For each gene, a gene-size adjusted gene score was calculated and used in the following pathway analyses.
Statistical methods for pathway enrichment analysis

We downloaded canonical pathway information from Molecule Signature Database (msigDB). The msigDB consists of several available online sources of pathway databases and manually curated pathways from the literature, including Kyoto Encyclopedia of Genes and Genomes (KEGG), BioCarta, Reactome, Gene Ontology (GO) terms, and gene sets compiled from published biomedical literature [24], which listed 4,726 pathways and 22,429 genes. Pathways with extreme numbers of genes (i.e., 10th percentile of pathway size distribution, less than 10 or more than 300) were removed from analysis to avoid stochastic bias or testing too general biological process. After mapping genes in GWA datasets into pathways, we tested in total 3,917 pathways for the WTCCC and 4,051 pathways for the GAIN datasets.

We applied both competitive and self-contained pathway analyses approaches [14]. Competitive method compares the statistics of genes in a given pathway with the rest of genes not in the pathway. Self-contained method compares the statistics of genes in a given pathway with the null genomic background [25]. To obtain more comprehensive information in pathway analyses, three statistical methods were performed to evaluate the enrichment of all pathways, Gene Set Enrichment Analysis (GSEA), sum-square-statistic and sum-statistic [26,27,28]. The details of calculation procedures were provided in our previous study [13]. In brief, GSEA method ordered a set of genes by the min-p, and the gene-wise statistic values ($t_i$) were defined as the chi-square statistic of the corresponding most-significant SNP. For each examined pathway, an enrichment score (ES) was calculated to evaluate association signals for all genes in the pathway. The sum-statistic or sum-square-statistic methods were to sum (the square) all gene-wise statistic values over the set of genes ($\sum_{i=1}^{S} t_i^2$ or $\sum_{i=1}^{S} t_i^2$) [28]. The three pathway-based approaches were analyzed with or without weighting procedures using the ASM information.

We performed five thousand permutations to obtain empirical p-values for each pathway. The Benjamini and Hochberg (BH) multiple comparison procedure was used to control for the false discovery rate (FDR) [29] in pathway analyses. A p-value less than 0.01 after FDR correction was considered significant in the present study. To examine the common processes or underlying biological themes among significant pathways, we also analyzed functions of genes in enriched pathways using GO terms, including domains in biological process, cellular component, and molecular function (http://www.broadinstitute.org/gsea/msigdb/annotate.jsp).

Results

In the GAIN dataset, 88 pathways were significant, in which 32 were identified only by weighting the ASM information (Table S1). Similarly, we identified 15 pathways (11 of them were identified only when weighting procedure is applied for the ASM set) in the WTCCC dataset with p-value less than 0.01 after BH correction (Table S2). Among the 88 enriched pathways in the GAIN dataset, there were 32 (36.4%) from GO, nine (10.2%) from KEGG, and eight (9.1%) from Reactome. Among the 15 enriched pathways in the WTCCC dataset, there were six (40.0%) from GO and one (6.7%) from BioCarta. Three pathways consistently exhibited their biological relevance for BPD in both GWA datasets (Table 1). These 3 pathways were caton channel activity, gated channel activity, and metal ion transmembrane transporter activity. Additionally, the enriched pathways in the GAIN dataset (Table S1) were
involved in a series of biological procedures and mechanisms, such as brain development and neuron function (e.g. nervous system development, neurological system process, axon guidance, etc.), component of extracellular matrix, ECM (e.g. cell matrix adhesion, ECM receptor interaction, focal adhesion, integrin cell surface interactions, etc.), neurotransmitter (e.g. glutamate signaling pathway), and ion channel activity (e.g. potassium channel activity, voltage gated cation channel activity, calcium signaling pathway, etc.). Most of the enriched GO pathways in the WTCCC dataset were associated with serotonin receptor and channel and transporter activity, such as serotonin receptor activity, gated channel activity, cation channel activity, and metal ion transmembrane transporter activity (Table S2).

Using GO term analysis, we further examined genes in the enriched pathways of GAIN and WTCCC datasets (other than the original significant GO pathways) to search for common functions of these genes. In the GAIN datasets, there were 4,600 unique genes in 56 non-GO pathways. Table S3 shows the top 50 significant GO terms with p-value less than 0.05. Most of these significant GO terms were associated with cytoskeleton structure (e.g. actin cytoskeleton organization and biogenesis, actin filament based movement, etc.), ECM (e.g. extracellular matrix structural constituent, collagen, integrin complex, integrin complex, etc.), and cation and gated channel activity (e.g. cation channel activity, nicotinic acetylcholine gated receptor channel complex, voltage gated calcium channel activity, etc.). In the WTCCC datasets, there were 990 genes in 9 significant non-GO pathways. Table S4 exhibits the top 50 significant GO terms. Most of these significant GO terms were associated with ion channel activity (e.g., calcium, potassium, sodium, chloride channel activity, etc.), transporter activity (e.g. cation transmembrane transporter activity, inorganic cation transmembrane transporter activity, etc.), and neurotransmitter receptor activity such as serotonin receptor activity. Table 2 displays the significant GO terms that were concordantly identified for BPD in both GWA datasets. The 29 GO terms were mainly associated with ion channel activity, such as calcium channel, ligand gated channel, nicotinic acetylcholine gated channel, voltage gated channel, etc.

We further identified genes that were over-represented in enriched pathways. We selected genes that were commonly involved in more than 20% out of all enriched pathways for each GWA dataset and had at least one SNP having p-value less than 0.05 in the GAIN or the WTCCC dataset, that is, more than 18 pathways in the GAIN and 5 pathways in the WTCCC datasets. The proportion of significant SNPs in these genes ranged from 1.2% to 54.5%. In total, there were 26 concordant genes that satisfied these criteria between the two GWA datasets (Table 3). They were mainly associated with calcium and potassium channel. In the WTCCC datasets, the over-represented genes were also associated with synaptic transmission (e.g. ACCN1, CHRNA6, HTR3B, HTR3A), mediation of calcium ion release (e.g. RYR1, RYR2, RYR3, TRPC3, TRPC4), and channels of calcium, potassium, and sodium.

**Discussion**

For complex trait like bipolar disorder, the whole-genome screening provides comprehensive genetic data and pathway-based approaches offer complementary information to reveal underlying complex biological connections in the whole-genome scale. Results of pathway-based analysis not only can verify prior causal hypotheses for BPD (e.g. neurotransmitter processes and neuron activity dysfunction in brain, etc) but also to explore novel biological pathways [30,31]. In the present study, we found enriched pathways for BPD to be related to ion channel activity such as calcium, potassium, and sodium ion. These findings are consistent with some of the presumed pathological mechanisms for BPD.

**Table 1.** Concordant enriched pathways among GWA datasets of the GAIN and the WTCCC by different pathway-based methods.

| Pathway name | Total genes in pathway | No. of GAIN genes on list | No. of WTCCC genes on list | % of ASM genes on list | Gene Set Enrichment Analysis (GSEA) | SUMST | SUMSQ | GSEA | SUMST | SUMSQ |
|--------------|------------------------|--------------------------|----------------------------|-----------------------|------------------------------------|-------|-------|-------|-------|-------|
| GO: Calcium channel activity | 131 | 13 | 13 | 9.7% | 0.0001 | 0.3080 | 0.3080 | 0.0001 | 0.3080 | 0.3080 |
| GO: Gated channel activity | 109 | 109 | 19.3% | 0.0661 | 0.0000 | 0.0559 | 0.0559 | 0.0000 | 0.0559 | 0.0559 |
| GO: Metal ion transmembrane transporter activity | 129 | 129 | 0.039 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| GO: Nicotinic acetylcholine receptor channel complex | 136 | 136 | 136 | 0.039 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |

ASM: Gene set of allele-specific methylation; GSEA: Gene Set Enrichment Analysis; SUMST: sum-statistic; SUMSQ: sum-square-statistic; GSEA: Gene Set Enrichment Analysis; SUMST: sum-statistic; SUMSQ: sum-square-statistic; GSEA: Gene Set Enrichment Analysis; SUMST: sum-statistic; SUMSQ: sum-square-statistic.
Additionally, our results in the GAIN datasets suggest for the roles of extracellular matrix in brain to be involved in the development of bipolar disorder.

Among our reported significant pathways in the two datasets, some of them were identified through GO database, which include nervous system development, ion channel and transporter activity, extracellular matrix, etc in the GAIN dataset and gated channel activity in the WTCCC dataset. These enriched GO pathways for BPD are in line with findings from some of previous studies [32,33,34]. The other pathways we identified are mainly based on gene sets that were compiled from published biomedical literature (discussed later). We noted that few studies applying pathway based approaches for BPD utilized pathway sources only from KEGG or GO, which include only 25% to 40% genes in the whole human genome. Thus, a large proportion of genes with potential impacts for the trait of interest might be excluded from pathway analyses. This is clearly the restriction that results of pathway findings depend on the completeness and correctness of Table 2. Concordant gene sets in the two GWA datasets of the GAIN and the WTCCC using Gene Ontology analysis.

| Gene get name                              | NO. of gene in gene Set | GAIN (4,600 genes) | WTCCC (945 genes) |
|--------------------------------------------|-------------------------|--------------------|-------------------|
| Calcium channel activity                   | 33                      | 90.9               | 87.9              |
| Cation transmembrane transporter activity   | 211                     | 78.2               | 62.1              |
| Cation transport                           | 146                     | 65.8               | 53.4              |
| Delayed rectifier potassium channel activity | 12                    | 100.0              | 91.7              |
| Excitatory extracellular ligand gated ion channel activity | 21          | 85.7               | 81.0              |
| Extracellular ligand gated ion channel activity | 21          | 85.7               | 81.0              |
| Gated channel activity                     | 121                     | 86.0               | 86.8              |
| Inward rectifier potassium channel activity | 12                    | 100.0              | 91.7              |
| Ion channel activity                       | 147                     | 83.7               | 82.3              |
| Ion transmembrane transporter activity      | 275                     | 70.2               | 53.8              |
| Ion transport                              | 184                     | 64.7               | 47.8              |
| Ligand gated channel activity              | 39                      | 79.5               | 79.5              |
| Metal ion transmembrane transporter activity| 145                    | 86.9               | 86.9              |
| Monovalent inorganic cation transport       | 93                      | 69.9               | 61.3              |
| Nicotinic acetylcholine activated cation selective channel activity | 11              | 100.0              | 81.8              |
| Nicotinic acetylcholine gated receptor channel complex | 11          | 100.0              | 81.8              |
| Potassium channel activity                 | 50                      | 96.0               | 92.0              |
| Potassium ion transport                     | 58                      | 84.5               | 77.6              |
| Sodium channel activity                    | 17                      | 82.4               | 76.5              |
| Substrate specific channel activity         | 154                     | 80.5               | 78.6              |
| Substrate specific transmembrane transporter activity | 341 | 67.5               | 43.7              |
| Substrate specific transporter activity     | 388                     | 63.4               | 39.2              |
| Transmembrane transporter activity          | 371                     | 66.6               | 40.7              |
| Voltage gated calcium channel activity      | 18                      | 94.4               | 88.9              |
| Voltage gated calcium channel complex       | 15                      | 93.3               | 86.7              |
| Voltage gated cation channel activity       | 66                      | 93.9               | 90.9              |
| Voltage gated channel activity             | 73                      | 90.4               | 90.4              |
| Voltage gated potassium channel activity    | 36                      | 100.0              | 94.4              |
| Voltage gated potassium channel complex     | 40                      | 90.0               | 82.5              |

**GAIN:** The analysis of biological gene sets by Gene Ontology was among 4,600 genes from 56 enriched pathways; **WTCCC:** The analysis of biological gene sets by Gene Ontology was among 945 genes from 9 enriched pathways.

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Table 3. Over-representing genes in enriched pathways in the two GWAS datasets of the GAIN and the WTCCC.

| Gene     | Set      | GAIN |   |   | WTCCC |   |   |
|----------|----------|------|---|---|-------|---|---|
|          |          | No. of SNP in gene | % of significant SNPs | Smallest p-value | No. of SNP in gene | % of significant SNPs | Smallest p-value |
| ACCN1    | ASM      | 348  | 11.5 | 1.35E-03 | 127  | 9.4 | 3.33E-05 |
| CACNA1A  | ASM      | 48   | 2.1  | 4.06E-02 | 37   | 5.4 | 3.60E-02 |
| CACNA1B  | ASM      | 20   | 30.0 | 1.11E-02 | 68   | 14.7| 4.45E-03 |
| CACNA1C  | ASM      | 205  | 3.4  | 3.82E-03 | 149  | 26.8| 5.49E-05 |
| CACNA1D  | ASM      | 123  | 3.3  | 2.52E-02 | 68   | 14.7| 4.45E-03 |
| CACNA1E  | ASM      | 45   | 6.7  | 3.92E-03 | 149  | 26.8| 5.49E-05 |
| CACNA2D1 | non-ASM  | 77   | 2.6  | 2.47E-02 | 77   | 2.6 | 2.47E-02 |
| CACNB2   | ASM      | 185  | 11.9 | 5.07E-04 | 127  | 9.4 | 3.33E-05 |
| CACNB3   | ASM      | 1    | 100.0| 3.63E-02 | 1    | 100.0| 3.63E-02 |
| CACNB4   | ASM      | 68   | 1.5  | 4.59E-02 | 37   | 5.4 | 3.60E-02 |
| CENPN    | non-ASM  | 4    | 25.0 | 5.15E-22 | 4    | 25.0 | 5.15E-22 |
| CHRNA6   | non-ASM  | 6    | 33.3 | 2.84E-02 | 6    | 33.3 | 2.84E-02 |
| HTR3B    | non-ASM  | 14   | 7.1  | 6.88E-19 | 14   | 7.1 | 6.88E-19 |
| KCNA2    | ASM      | 5    | 20.0 | 4.85E-02 | 5    | 20.0 | 4.85E-02 |
| KCNA4    | non-ASM  | 5    | 20.0 | 3.64E-02 | 5    | 20.0 | 3.64E-02 |
| KCNB2    | ASM      | 142  | 4.9  | 2.62E-03 | 107  | 3.7 | 2.34E-04 |
| KCNC1    | ASM      | 11   | 54.5 | 1.59E-02 | 9    | 22.2| 2.99E-02 |
| KCNC4    | ASM      | 10   | 10.0 | 1.60E-02 | 10   | 10.0 | 1.60E-02 |
| KCND3    | ASM      | 114  | 14.9 | 7.76E-04 | 59   | 5.1 | 1.31E-02 |
| KCNE1    | ASM      | 20   | 25.0 | 2.04E-02 | 20   | 25.0 | 2.04E-02 |
| KCNG2    | non-ASM  | 8    | 25.0 | 6.66E-04 | 8    | 25.0 | 6.66E-04 |
| KCNH1    | ASM      | 149  | 8.1  | 4.03E-03 | 79   | 3.8 | 1.19E-02 |
| KCNH2    | non-ASM  | 2    | 50.0 | 2.74E-02 | 2    | 50.0 | 2.74E-02 |
| KCNJ1    | non-ASM  | 11   | 9.1  | 3.95E-02 | 11   | 9.1 | 3.95E-02 |
| KCNJ12   |          | 5    | 20.0 | 3.02E-02 | 5    | 20.0 | 3.02E-02 |
| KCNJ15   |          | 14   | 7.1  | 3.11E-02 | 14   | 7.1 | 3.11E-02 |
| KCNJ3    | ASM      | 49   | 12.2 | 2.02E-03 | 49   | 12.2 | 2.02E-03 |
| KCNJ5    | non-ASM  | 18   | 5.6  | 7.22E-03 | 18   | 5.6 | 7.22E-03 |
| KCNJ6    | ASM      | 157  | 8.3  | 5.15E-03 | 97   | 2.1 | 2.12E-03 |
| KCNK1    | ASM      | 36   | 8.3  | 2.01E-02 | 21   | 38.1| 3.04E-02 |
| KCNK3    | ASM      | 2    | 50.0 | 3.16E-05 | 2    | 50.0 | 3.16E-05 |
| KCNMB2   | ASM      | 94   | 23.4 | 1.46E-03 | 63   | 11.1| 1.03E-02 |
| KCN2     |          | 30   | 10.0 | 3.71E-03 | 30   | 10.0| 3.71E-03 |
| KCN3     | ASM      | 83   | 15.7 | 4.28E-03 | 36   | 2.8 | 3.55E-02 |
| KCN1     | non-ASM  | 102  | 14.7 | 6.35E-04 | 64   | 4.7 | 2.53E-02 |
| KCN3     | non-ASM  | 162  | 1.2  | 1.65E-02 | 97   | 4.1 | 4.10E-03 |
| KCN5     |          | 94   | 8.5  | 2.07E-03 | 94   | 8.5 | 2.07E-03 |
| KCN1     | non-ASM  | 5    | 40.0 | 6.14E-04 | 4    | 50.0| 5.58E-05 |
| KCN5     | ASM      | 21   | 14.3 | 3.18E-02 | 24   | 20.8| 1.72E-02 |
| P2RX4    |          | 3    | 33.3 | 2.93E-02 | 3    | 33.3| 2.93E-02 |
| PKD2     | ASM      | 27   | 22.2 | 7.65E-03 | 10   | 20.0| 1.59E-02 |
| RYR1     |          | 21   | 9.5  | 4.76E-03 | 21   | 9.5 | 4.76E-03 |
| RYR2     |          | 114  | 14.0 | 1.30E-03 | 114  | 14.0| 1.30E-03 |
| RYR3     |          | 185  | 5.4  | 2.14E-03 | 185  | 5.4 | 2.14E-03 |
| SCN11A   |          | 20   | 30.0 | 1.13E-02 | 20   | 30.0| 1.13E-02 |
| SCN2A    |          | 14   | 14.3 | 4.18E-02 | 14   | 14.3| 4.18E-02 |
| SCN2B    |          | 11   | 18.2 | 7.79E-03 | 11   | 18.2| 7.79E-03 |
Pathway Analysis with Methylation Information

| Gene   | Set   | GAIN | WTCCC |
|--------|-------|------|-------|
|        |       | No. of SNP in gene | % of significant SNPs | Smallest p-value | No. of SNP in gene | % of significant SNPs | Smallest p-value |
| SCNS5A |       | 19   | 5.3   | 2.33E–02 |
| SCN9A  |       | 22   | 9.1   | 2.08E–03 |
| SERP5B |       | 21   | 4.8   | 2.98E–02 |
| TRPC3  |       | 12   | 8.3   | 3.08E–02 |
| TRPC4  |       | 57   | 10.5  | 8.55E–03 |

ASM: Gene set of allele-specific methylation; Non-ASM: Gene set of other than ASM in pathway analysis.

We also identified several important genes that over-represented in enriched pathways. Many of these genes are associated with different biological processes and functions, including synaptic transmission (e.g. ACCN1, CHRNA6, HTR3B, HTR5A, etc) and cation channels activity (e.g. ACCN1, CACNA1C, KCNA3, etc). For genes encode for synapse components, prior association studies have indicated the involvement of certain genetic variants in a variety of psychiatric disorders [33,41,42,43,44,45]. Genetic variants in genes related to serotonin transmission (e.g. HTR1B, HTR3A, HTR5A, etc) were associated with mood disorder [46]. Polymorphisms in CHRNA6 (nicotinic alpha subunit 6 of neuronal cholinergic receptor), have also been reported to be associated with bipolar disorder [47].

In addition, many studies have reported that variations in gene CACNA1C (alpha 1C subunit of the L-type voltage-gated calcium channel) had strong association signals with bipolar illness [5]. Potassium channels are found in most cell types and control a wide variety of cell functions, such as regulation of action potential and resting membrane potential in neurons. Prior study reported that long repeats of KCNA3 reduce potassium channel function and modify cognitive performance in schizophrenic patients [48]. ACCN1 (neuronal amiloride-sensitive cation channel 1), a cation channel with high affinity for sodium, is also permeable for lithium and potassium ions. A recent genome-wide scan found that genetic variants in ACCN1 were associated with response to lithium treatment in bipolar patients [49].

In conclusion, our study integrated methylation information with genome-wide genotyping data to bring biological insights into the etiology of BPD. Results of GO term analysis in the GAIN dataset (Table S3) demonstrated the importance of collagen and extracellular matrix. Components of the extracellular matrix surround cell and mediate many important cellular processes such as cell differentiation, tissue rearrangement, and carcinogenesis. Neuron migration and colony from different brain areas which enrich the neuronal network with functional unit are highly associated with the extracellular matrix [38]. Disruption of this process in brain may be a potential cause of bipolar disorder. Interestingly, pathways that are related to cell movement (such as the extracellular matrix, focal adhesion, and regulation of actin cytoskeleton) were found to be associated with antipsychotic induced tremors in patient with mania episode [39]. In a GWA study of bipolar disorder, collagen type 11α2 (COL11A2), a component of extracellular matrix, also was shown to be associated with bipolar disorder comorbidity with alcohol dependence [40]. This evidence altogether points to a new possibility to further investigate the roles of brain extracellular matrix in bipolar illness.
the underlying pathogenesis of bipolar illness. We identified significant pathways that are in line with evidence from prior causal hypotheses for bipolar disorder, and also reported novel biological pathways, such as the involvement of brain extracellular matrix in bipolar disorder. The strategy we applied provides another avenue to comprehensive our knowledge for the complex network reside in the biological basis of bipolar disorder. Our findings could facilitate follow-up basic research to validate the functional and biological mechanisms for identified genes and pathways.

There are some limitations in the current study. First, the smallest p-value (as commonly adopted in other studies [14]) was used to define the gene-level statistic, thus, information of other markers in a gene region is excluded. Using a combined method to include all markers’ information in a defined gene may provide slightly different results in pathway analysis, such as the Inverse Gamma method [50], random effects model, or Bayesian statistical methods [51]. In addition, the accuracy of pathway analysis results depends on the completeness and correctness of annotated pathway database. Although we have used the more comprehensive databases, there is still likely that some pathways were not included in our analysis. Second, we incorporated methylation information in brain tissues into pathway analysis, while other genomic information such as gene expression or other epigenetic regulation was not used. Integration of genomic information from different platforms may provide additional benefit to identify enriched pathways for bipolar disorder. Third, we used two major GWA datasets of BPD in Caucasian populations to obtain concordant findings. Although these are large-scale GWA datasets, the association results from meta- or mega-analysis can be used in the near future to further increase the power to uncover the underlying biological mechanisms for BPD.

Supporting Information

Table S1 88 Significant pathways in the GAIN dataset by pathway-based methods after correction for multiple comparisons. #: The significant p-value after correction by the BH multiple comparison procedure; GSEA: Gene Set Enrichment Analysis; SUMSQ: sum-square-statistic; SUMST: sum-statistic. (DOCX)

Table S2 15 Significant pathways in the WTCCC dataset by pathway-based methods after correction for multiple comparisons. #: The significant p-value after correction by the BH multiple comparison procedure; GSEA: Gene Set Enrichment Analysis; SUMSQ: sum-square-statistic; SUMST: sum-statistic. (DOCX)

Table S3 The top 50 significant GO terms with p-value less than 0.05 in the GAIN dataset. (DOCX)

Table S4 The top 50 significant GO terms with p-value less than 0.05 in the WTCCC dataset. (DOCX)

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Author Contributions

Acquisition of data: LCC CFK. Revised manuscript critically for important intellectual content: PHK LCC CFK WLS. Final approval of the version to be published: PHK. Conceived and designed the experiments: PHK LCC CFK WLS. Analyzed the data: LCC CFK WLS. Wrote the paper: LCC CFK.

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