Table 1. Demographic data of the subjects

	Discovery cohort		Replication cohort	
	MDD	CTRL	MDD	CTRL
Number of samples	20	19	12	12
Age (year)	44.2 ± 15.2	42.4 ± 12.3	45.4 ± 12.2	44.3 ± 10.8
Sex (M:F)	2:18	2:17	3:9	3:9
Age at disease onset (year)	42.9 ± 16.2	NA	44.3 ± 13.1	NA
MDD episode (Single: Recurrent)	17:3	NA	9:3	NA
HAM-D	23.6 ± 6.2	NA	19.2 ± 6.7	NA

Abbreviations: MDD, Major depressive disorder; CTRL, control; M, male; F, female; HAM-D, Hamilton Rating Scale for Depression; values expressed as the mean ± SD.

psychiatric problems and a past history of mental illness. For our replication set of samples, 12 medication-free patients with MDD (3 males and 9 females, mean age: 45.4 ± 12.2 y) were recruited from the same university hospitals. Among these 12 patients, 9 had no history of taking antipsychotics, and 2 had not taken any antipsychotics for at least six months, and one patient sometimes took Tandospirone in the previous six months. Twelve control subjects (3 males and 9 females, mean age: 44.3 ± 10.8 y) were selected from volunteers under the same conditions as the first set of control subjects. The demographic data of all samples analyzed in this study are presented in **Table 1**. All subjects who participated in this study were of unrelated Japanese origin and signed written informed consent forms that were approved by the institutional ethics committees of the University of Tokushima Graduate School and Kochi Medical School to participate in this study.

DNA methylation methods

Genomic DNA was extracted from peripheral blood. Bisulfite conversion of 500 ng genomic DNA was performed using the EZ DNA methylation kit (Zymo Research). DNA methylation level was assessed according to the manufacturer's instructions using Infinium® HumanMethylation450 BeadChips (Illumina Inc.). The technical schemes, accuracy, and high reproducibility of this array have been described in a previously published paper.[42] Quantitative measurements of DNA methylation were determined for 485,764 CpG dinucleotides, which covered 99% of the RefSeq genes and were distributed across the whole gene regions, including promoter, gene body, and 3′- untranslated regions (UTRs). They also covered 96% of CGIs from the UCSC database with additional coverage in CGI shores (0–2 kb from CGI) and CGI shelves (2–4 kb from CGI). DNA methylation data was analyzed with the methylation analysis module within the BeadStudio software (Illumina Inc.). DNA methylation status of the CpG sites was calculated as the ratio of the signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value, known as β, ranges from 0 (completely unmethylated) to 1 (fully methylated). Qualified CpG sites used in statistical analyses were defined as follows: 1) detected in 80% of subjects, 2) excluded sex chromosome, 3) excluded nonspecific probes as shown in a previous paper,[43] and 4) excluded probes with SNPs at the CpG site with minor allele frequency MAF > 0.1% in the Japanese population. The final data set included 431,489 CpG sites. For validation of array data, we used 8 samples from the current study and measured DNA methylation status by using next-generation methylome sequencing. We observed a high correlation between Infinium HumanMethylation450 BeadChips and a next-generation sequencing ($r^2 = 0.81$; **Supplementary Fig. S1**).

RNA expression methods

The control subjects and patients with MDD who were chosen for the expression experiments were identical for the discovery set of samples in our methylation experiments with the exception of one patient, whose RNA was not available. Total RNA was extracted from peripheral leukocytes of whole blood samples using the PAX gene Blood RNA tube (Qiagen, Tokyo, Japan) and the PAX gene Blood RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. In brief, the PAX gene Blood RNA kit is for purification of total RNA from 2.5 ml of human whole blood collected in the PAX gene Blood RNA tube. Purification begins with a centrifugation step to pellet nucleic acids in the tube. The pellet is washed and resuspended, followed by automated RNA purification using the QIAcube (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. RNA concentration and its integrity were analyzed with an Agilent 2100 Caliper LabChip Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Two micrograms of total RNA was used for cDNA synthesis by random (N6) primers and Quantiscript Reverse Transcriptase (Qiagen). Quantitative real-time PCR was performed with the ABI 7500 Fast system. The GAPDH gene, β-actin gene and ABL1 gene were used as candidate housekeeping genes for normalization. Measurements of each gene expression were conducted in triplicate. Primers were selected and optimized at the exon-intron boundary of each gene. The primers for *GSK3B* were as follows: forward primer: 5′- GGTCTATCTTAATCTGGTGCTGG-3′; reverse primer: 5′- TGGATATAGGCTAAACTTCGGAAC -3′. The primers for GAPDH were as follows: forward primer: 5′-CAGCCTCAAGATCATCAGCA-3′; reverse primer: 5′-TGTGGTCATGAGTCCTTCCA-3′. The primers for β-actin were as follows: forward primer: 5′-GGAATTATGTGTCTACCCTCGC-3′;

reverse primer: 5′-TCCTGTCTTTGCGTTCCTG-3′. Finally, the primers for ABL1 were as follows: forward primer: 5′-CTCAGATCTCGTCAGCCATG-3′; reverse primer: 5′-ATCAGCTACCTTCACCAAGTG-3′. The stability of the reference genes was evaluated by a statistical program, NormFinder.[44] GAPDH showed the best stability score, so they can be used as the optimal reference genes in our study. Proper amplification of PCR products was confirmed by sequencing and single band in agarose gel electrophoresis.

Bioinformatics and statistical computing

Bioinformatics analyses were performed on 64-bit Linux platforms (CentOS/Scientific Linux 6.4). Data manipulations, including data extraction, depletion and merging, were performed with Shell (GNU bash, version 4.1.2) and/or Perl (version 5.10.1) scripts. The statistical computations were performed with R (version 3.0.3) and Bioconductor (version 2.13) packages. All statistical analyses were performed after a Z score transformation. Significances of differential DNA methylation of each methylation site between patients and healthy volunteers were estimated by the Mann-Whitney U test. To correct for multiple testing, the q-value was computed for each nominal P value by controlling the false discovery rate (FDR) at 0.05 in DNA methylation analysis.[45] The performances of linear discriminant analyses (LDA), with a combination of methylation levels of multiple sites of DNA methylation as explanatory variables, were evaluated. Gene ontology (GO) and pathway analyses were performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 to detect functionally significant genes, GO terms and/or pathways.

Disclosure of potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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ORIGINAL PAPER

Aberrant DNA Methylation of Blood in Schizophrenia by Adjusting for Estimated Cellular Proportions

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Abstract DNA methylation, which is the transference of a methyl group to the 5′-carbon position of the cytosine in a CpG dinucleotide, is one of the major mechanisms of epigenetic modifications. A number of studies have demonstrated altered DNA methylation of peripheral blood cells in schizophrenia (SCZ) in previous studies. However, most of these studies have been limited to the analysis of the CpG sites in CpG islands in gene promoter regions, and cell-type proportions of peripheral leukocytes, which may be one of the potential confounding factors for DNA methylation, have not been adjusted in these studies. In this study, we performed a genome-wide DNA methylation profiling of the peripheral leukocytes from patients with SCZ and from non-psychiatric controls ($N = 105$; 63 SCZ and 42 control subjects) using a quantitative high-resolution DNA methylation microarray which covered across the whole gene region (485,764 CpG dinucleotides). In the DNA methylation data analysis, we first estimated the cell-type proportions of each sample with a published algorithm. Next, we performed a surrogate variable analysis to identify potential confounding factors in our microarray data. Finally, we conducted a multiple linear regression analysis in consideration of these factors, including estimated cell-type proportions, and identified aberrant DNA methylation in SCZ at 2,552 CpG loci at a 5 % false discovery rate correction. Our results suggest that altered DNA methylation may be involved in the pathophysiology of SCZ, and cell heterogeneity adjustments may be necessary for DNA methylation analysis.

Keywords Epigenetics · DNA methylation · Schizophrenia · Microarray · Leukocyte · Blood · Cell heterogeneity

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Introduction

Schizophrenia (SCZ) is a complex psychiatric disorder characterized by auditory hallucinations, delusions, and cognitive dysfunctions. Its reported morbid rate is 1 % in the general population (Jablensky et al. 1992). Epigenetics is defined as the study of mitotically or meiotically heritable variations in gene function that cannot be explained by changes in DNA sequence (Petronis et al. 2000). DNA methylation, which is the transference of a methyl group to the 5-carbon position of the cytosine in a CpG dinucleotide, is one of the major mechanisms of epigenetic modifications, and DNA methylation patterns are affected by environmental factors and genetic variants (Roth and Sweatt 2011; Numata et al. 2012), both of which are involved in the pathophysiology of SCZ (Khojasteh-Fard et al. 2011; Rutter et al. 2006; Gejman et al. 2011).

A number of studies have demonstrated altered DNA methylation of blood in SCZ in previous studies (Tolosa et al. 2010; Carrard et al. 2011; Chen et al. 2012; Dempster et al. 2011; Bönsch et al. 2012; Kordi-Tamandani et al. 2012; Melas et al. 2012; Xu et al. 2012; Kordi-Tamandani et al. 2013; Nishioka et al. 2013; Kinoshita et al. 2013; Aberg et al. 2014; Cheng et al. 2014). However, most of these studies have been limited to the analysis of the CpG sites in CpG islands (CGIs) in the gene promoter regions, although accumulating evidence has shown the relationship between DNA methylation in the regions flanking CGIs or in gene bodies and gene expression (Irizarry et al. 2009; Illingworth et al. 2010; Maunakea et al. 2010; Sandoval et al. 2011; Rao et al. 2013; Zouridis et al. 2012; Lambert et al. 2013). Furthermore, the effect of cell-type proportions on the measurement of DNA methylation has not been adjusted in any previous studies of SCZ using blood, although several studies have demonstrated the influence of cellular heterogeneity on DNA methylation status (Adalsteinsson et al. 2012; Bock 2012; Houseman et al. 2012; Lam et al. 2012; Reinius et al. 2012; Liu et al. 2013).

In this study, we conducted a genome-wide profiling of DNA methylation by using the peripheral leukocytes from patients with SCZ and from non-psychiatric control subjects and identified aberrant DNA methylation in SCZ after correcting the estimated cell-type proportions of each sample.

Materials and Methods

Subjects

Sixty-three SCZ patients (50 males and 13 females; mean age: 48.6 ± 9.6 years) were recruited from the Tokushima, Osaka, and Kochi University Hospitals in Japan. The diagnosis of SCZ was decided according to DSM-IV criteria by at least two experienced psychiatrists on the basis of extensive clinical interviews and reviews of the patients' medical records. All of the patients had no psychiatric comorbidity, and all patients were treated with various antipsychotic drugs. The mean chlorpromazine equivalent dose was 935.7 ± 579.3 mg/day. Forty-two control subjects (25 males and 17 females; mean age: 46.9 ± 10.2 years) were recruited from volunteers who were hospital staff, university students, and company employees. All of the control subjects were free from psychiatric problems, a past history of mental illness, and medications. All subjects who joined this study were of unrelated Japanese origin and signed written informed consent forms that were approved by the institutional ethics committees of the University of Tokushima Graduate School, the Osaka University Graduate School of Medicine, and Kochi Medical School.

DNA Methylation Methods

Genomic DNA was prepared from peripheral blood samples. A bisulfite conversion of 500 ng of genomic DNA was performed with the EZ DNA methylation kit (Zymo Research). DNA methylation level was assessed with Infinium HumanMethylation450 Beadchips (Illumina Inc.) according to the manufacturer's instructions. The technical schemes, accuracy, and high reproducibility of this array have been described in previous papers (Bibikova et al. 2011; Dedeurwaerder et al. 2011; Sandoval et al. 2011). Quantitative measurements of DNA methylation were determined for 485,764 CpG dinucleotides that covered 99 % of the RefSeq genes and were distributed across whole gene regions, including promoters, gene bodies, and 3′-untranslated regions (UTRs). The arrays also covered 96 % of the CGIs from the UCSC database with additional coverage in CGI shores (0–2 kb from CGI) and CGI shelves (2–4 kb from CGI). Detailed information on the contents of the array is available in the Infinium HumanMethylation450 User Guide, the HumanMethylation450 manifest (www.illumina.com), and previous papers (Bibikova et al. 2011; Sandoval et al. 2011). DNA methylation data were analyzed using the methylation analysis module within the BeadStudio software (Illumina Inc.). The DNA methylation status of the CpG sites was calculated as the ratio of the signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value, known as β, ranges from 0 (completely unmethylated) to 1 (fully methylated). For intra-chip normalization of the probe intensities, we performed color balance and background corrections on every set of 12 samples from the same chip by using internal control probes. X-chromosome CpG sites in the CGIs in the *AR* gene as well as

the internal control probes were checked to validate the DNA methylation measurements, as in a previous study (Supplementary Figure S1) (Siegmund et al. 2007). For quality control, β values with detection p values ≥ 0.05 were treated as missing values. The qualified CpG sites used in the statistical analyses were defined as follows: (1) autosomal CpGs with no missing values in all subjects; (2) CpGs with no probe SNPs at minor allele frequencies ≥ 5 % in the HapMap-JPT population; (3) CpGs with no probe cross-reactivity, and (4) no SNPs at CpG sites and single-base extension sites in a previous paper (Chen et al. 2013). The final data set included 338,876 CpG sites (promoter: 149,193; gene body: 102,880; 3′-UTR: 10,088; intergenic region: 76,715; CGI: 114,649; CGI shore; 83,125; CGI shelf: 29,741; others: 111,361).

Statistical Analysis

Cell-type proportions (CD4 + T cell, CD8 + T cell, CD56 + NK cell, CD19 + B cell, CD14 + Monocyte, and Granulocyte) for each of the samples were estimated using a published algorithm (Aryee et al. 2014; Houseman et al. 2012) implemented in an R-package 'Minfi'. Surrogate variable analysis (SVA), which is a method for modeling potential confounding factors that may or may not be known, including technical factors such as batch effects, enable to increase the biological accuracy and reproducibility of analyses in microarray studies (Leek and Storey 2007; Teschendorff et al. 2011). We used SVA to identify potential confounding factors in our microarray data as surrogate variables (SVs). Then, the influences of diagnosis on DNA methylation were examined by a multiple linear regression analysis, adjusted for age, sex, 14 significant identified SVs, and 6 estimated cell-type proportions. A false discovery rate (FDR) correction was applied at the 0.05 level for multiple testing. The gene-ontology analysis was performed with the database for annotation, visualization and integrated discovery (DAVID) (da Huang et al. 2009). A significant criterion of this analysis was considered with at a FDR 5 % correction.

Results

Estimated Cell-Type Proportions of Each Sample and Each CpG Site

We estimated 6 cell-type proportions using 'Minfi', a flexible and comprehensive bioconductor package for the analysis of Infinium DNA methylation microarrays developed by Aryee et al. 2014. The estimated cellular proportions of each sample are shown in Supplementary Table S1, and the average estimated cellular proportions of the two groups (SCZ and control) are shown in Fig. 1. Of the 6 cell types, 4 (CD8 + T cell, CD4 + T cell, CD14 + Monocyte, and Granulocyte) showed significant differences between the two groups (Welch's t test $p < 0.05$).

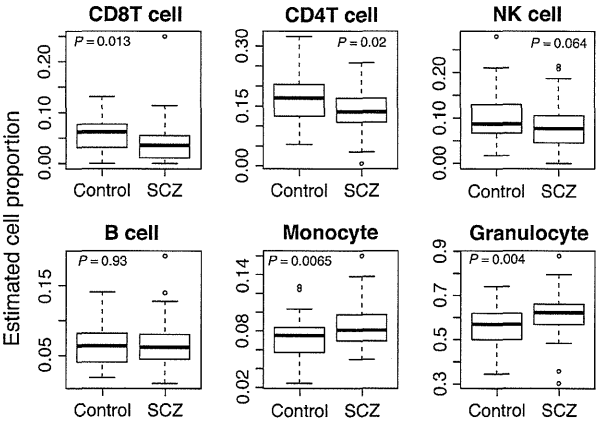

Fig. 1 Average estimated cellular proportions of SCZ and control groups. The y-axis is each of average estimated cellular proportions of CD8 + T cell, CD4 + T cell, CD56 + NK cell, CD19 + B cell, CD14 + Monocyte, and Granulocyte. Significant differences between the two groups were observed in 4 cell types (CD8 + T cell, CD4 + T cell, CD14 + Monocyte, and Granulocyte) (Welch's t test $p < 0.05$)

Diagnostic Differences of DNA Methylation Between Patients with SCZ and Controls

The DNA methylation levels of 63 SCZ patients were compared to the levels of 42 control subjects using Infinium HumanMethylation450 BeadChips. Of the 338,876 CpG sites, significant diagnostic differences in DNA methylation were observed at 16,220 CpG sites at a FDR 5 % correction in SVA. After adjusting for estimated cell-type proportions, significant diagnostic differences were observed at 2,552 CpG sites at a FDR 5 % correction (Supplementary Table S2). Of these 2,552 CpG sites, 1,161 (45.5 %) demonstrated higher DNA methylation in patients with SCZ than in the controls. Figure 2 shows volcano plots of differentially methylated CpG sites between diagnosis before and after adjustment for estimated cell-type proportions. When these 2,552 CpG sites were classified into four different categories according to their locations in the genes (promoter, gene body, 3′-UTR, and intergenic region), 1,607 sites (63.0 %) were located in the promoter regions, 525 sites (20.6 %) in gene bodies, and 30 sites (1.2 %) in 3′-UTRs. When these 2,552 CpG sites were classified into four categories according to the CpG content in the genes (CGI, CGI shore, CGI shelf, and others), 1,491 sites (58.4 %) were located in the CGIs, 432 sites (16.9 %) in CGI shores, and 209 sites (8.2 %) in CGI shelves.

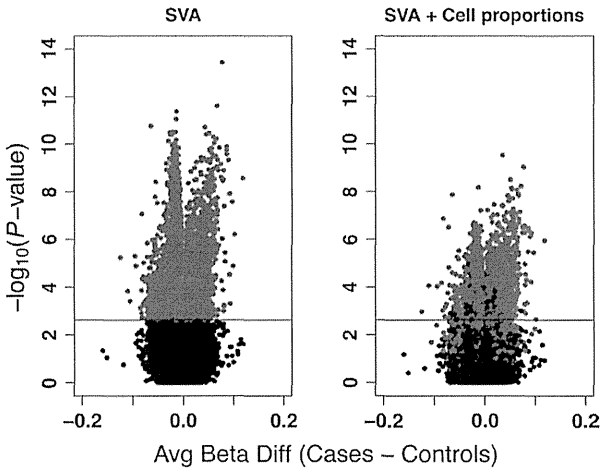

Fig. 2 Volcano plots of differentially methylated CpG sites between diagnosis before and after adjustment for estimated cell-type proportions. The *x*-axis represents average beta differences ($\Delta\beta$ value) between control subjects and patients with schizophrenia (SCZ). $\Delta\beta$ value >0 corresponds to higher DNA methylation in patients with SCZ than in the controls, whereas $\Delta\beta$ value <0 corresponds to lower DNA methylation in patients with SCZ than in the controls. The *y*-axis represents −log10 *p* values. Each dot represents an individual CpG site (a total of 338,876 CpG sites). *Red dots* represent 16,220 CpG sites that showed significant diagnostic differences between patients with SCZ and the controls at a 5 % FDR correction using SVA without adjustment for cellular mixture proportion. After adjustment for estimated cellular proportions, a total number of 16,220 differentially methylated CpG sites were decreased to 2,552 (Color figure online)

Aberrant DNA methylation in SCZ is more likely to occur at CpG sites in CGIs in gene promoter regions (45.7 % of 2,552 loci vs. 21.3 % of 338,876 loci, χ^2 test $p < 0.0001$) (Table 1).

Gene-Ontology Analysis

We used DAVID to perform a gene-ontology analysis of the genes, which showed significant diagnostic differences in DNA methylation. Table 2 lists the significant gene-ontology categories. Seven of the top-10 of most significant gene-ontology terms included the functions related to transcription such as, regulation of transcription, transcription regulator activity, and regulation of transcription from RNA polymerase II promoter.

Discussion

In this study, we conducted a genome-wide DNA methylation profiling of peripheral leukocytes from patients with SCZ and from non-psychiatric controls using Infinium HumanMethylation450 BeadChips and identified the CpG sites associated with SCZ by adjusting for estimated cell-type proportions. This is the first DNA methylation study of SCZ to correct the cellular proportions in data analysis. Our study revealed that cellular proportions were one of the major confounding factors that affected DNA methylation status, and this finding suggests that the adjustment of cellular heterogeneity may be necessary to identify accurate abnormalities associated with diseases. Recently, trials to adjust for cellular heterogeneity in epigenome-wide association studies have been started (Houseman et al. 2012; Liu et al. 2013; Guintivano et al. 2013; Aryee et al. 2014; Houseman et al. 2014; Langevin et al. 2014). Houseman et al. (2012) originally developed the statistical method to estimate relative proportions of immune cells in unfractionated white blood and applied this method to a DNA methylation array data set from blood studies of head and neck cancer, ovarian cancer, Down syndrome, and obesity. Liu et al. (2013) slightly modified this method and identified DNA methylation differences between patients with rheumatoid arthritis and controls. Guintivano et al. (2013) generated a set of bioinformatics tools designed to identify and correct for cellular heterogeneity bias in the brain. More recently, Houseman et al. (2014) developed and proposed reference-free cell mixture adjustments for the analysis of DNA methylation data when a reference dataset is unavailable.

There are three epigenome-wide association studies of SCZ using blood, and none of them have adjusted for cellular heterogeneity in their analysis. When we compared the present study with 234 CpG sites identified in our previous study using medication-free patients with SCZ

Table 1 Number of CpG sites which showed differentially methylated between medicated SCZ patients and controls

	Illumina Infinium HumanMethylation 450 Beadchip	SVA	SVA plus adjustment for estimated cellular proportions	χ^2 test *p* value (Beadchip vs. SVA plus adjustment for estimated cellular proportions)
Promoter	149,193	4,398	1,607	<0.0001
CpG islands	114,649	3,269	1,491	<0.0001
Promoter associated and CpG islands	72,320	3,270	1,166	<0.0001
Total CpG sites	338,876	6,846	2,553	

SVA surrogate variable analysis

Table 2 Gene ontology analysis of the genes which showed significant diagnostic differences in DNA methylation in this study

Category	Term	Gene count (%)	FDR corrected *p* value	Fold enrichment
GOTERM_BP_FAT	Regulation of transcription	325 (19.95)	1.50.E−06	1.35
GOTERM_MF_FAT	Transcription regulator activity	204 (11.90)	7.19.E−05	1.42
GOTERM_BP_FAT	Regulation of transcription from RNA polymerase II promoter	112 (6.53)	6.56.E−04	1.60
GOTERM_BP_FAT	Transcription	257 (14.99)	1.84.E−03	1.31
GOTERM_BP_FAT	Negative regulation of gene expression	83 (4.84)	2.55.E−03	1.70
GOTERM_BP_FAT	Positive regulation of macromolecule metabolic process	125 (7.29)	3.68.E−03	1.51
GOTERM_BP_FAT	Regulation of transcription, DNA-dependent	220 (12.83)	4.34.E−03	1.33
GOTERM_MF_FAT	Transcription factor binding	82 (4.78)	8.53.E−03	1.65
GOTERM_BP_FAT	Regulation of RNA metabolic process	222 (12.94)	1.03.E−02	1.31
GOTERM_BP_FAT	Negative regulation of transcription	75 (4.37)	1.14.E−02	1.69
GOTERM_BP_FAT	Positive regulation of transcription	87 (5.07)	2.57.E−02	1.58
GOTERM_BP_FAT	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	94 (5.48)	2.90.E−02	1.55
GOTERM_BP_FAT	Positive regulation of biosynthetic process	102 (5.95)	2.93.E−02	1.51
GOTERM_BP_FAT	Positive regulation of cellular biosynthetic process	101 (5.89)	3.11.E−02	1.52
GOTERM_BP_FAT	Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	80 (4.66)	3.13.E−02	1.61
GOTERM_BP_FAT	Positive regulation of nitrogen compound metabolic process	96 (5.60)	3.13.E−02	1.54
GOTERM_BP_FAT	Positive regulation of macromolecule biosynthetic process	97 (5.66)	3.28.E−02	1.53
GOTERM_BP_FAT	Negative regulation of cellular biosynthetic process	85 (4.96)	4.76.E−02	1.57
GOTERM_BP_FAT	Negative regulation of nitrogen compound metabolic process	80 (4.66)	4.93.E−02	1.59
GOTERM_CC_FAT	Nucleoplasm	116 (6.76)	4.41.E−02	1.45

and monozygotic twins (Kinoshita et al. 2013), common DNA methylation changes were observed at 4 CpG sites in the *HCG18*, *MYO5C*, *SLC6A9*, and *ZNF461* genes. The *SLC6A9* (Solute carrier family 6 (neurotransmitter transporter, glycine), member 9) gene encodes *GLYT1* (Glycine transporter 1). Glycine is a co-agonist of the NMDA glutamate receptor and is up-taken by glial cells close to the synaptic cleft in the central nervous system (Harvey et al. 2008). A high synaptic level of glycine leads to NMDA receptor hyperactivity. Accumulating evidence suggests the therapeutic efficacy of *GLTY1* inhibitors in patients with SCZ (Tsai and Lin 2010). When we compared the present study with 360 CpG sites identified in a previous study using first-episode patients with SCZ (Nishioka et al. 2013), common DNA methylation changes were observed at 5 CpG sites in the *ABL2*, *C6orf114*, *LETM1*, *PURA*, and *SLC35E1*genes. *PURA* is a multifunctional single-stranded DNA-and RNA-binding protein and is implicated in diverse cellular functions, including transcriptional activation and repression, translation, and cell growth (Gallia et al. 2000), and altered expression of this gene in bipolar disorder has been reported (Nakatani et al. 2006). When we compared the present study with 112 genes identified in a recent study using large samples with SCZ (Aberg et al. 2014), common DNA methylation changes were observed in the *ACSL1*, *ARL3*, *BRP44L*, *FOXN3*, *MYT1L*, and *TMEM131* genes. The *MYT1L* (myelin transcription factor 1-like) gene encodes the neural transcription factor. *MYT1L* is essential to neurogenesis and differentiating neurons in the mammalian brain (Kim et al. 1997; Nielsen et al. 2004; Vierbuchen et al. 2010). Genetic variants and copy-number variants in the this gene have been associated with SCZ (Vrijenhoek et al. 2008; Lee et al. 2012; Li et al. 2012; Van Den Bossche et al. 2013).

There are several limitations to this study. First, our sample size was not large. Studies with larger samples will be needed. Second, cell-type specific studies will be needed even though we adjusted for estimated cellular proportions in the present study. Third, medication might affect our results because antipsychotic drugs are known to influence DNA methylation status (Dong et al. 2008; Melas et al. 2012; Murata et al. 2014). Fourth, the number of CpG sites that we analyzed in the present study was limited, although the 450 K microarray is one of the highest resolution tools currently available for assessing DNA methylation changes. Finally, bisulfite conversion, which we employed for this study, is not possible to differentiate methylation from the 5-hydroxymethylation of cytosine, which also plays a critical role in gene regulation (Bhutani et al. 2011).

In summary, we identified aberrant DNA methylation in SCZ by conducting a comprehensive DNA methylation

profiling of blood and adjusting for estimated cellular proportions. Our findings provided further evidence that altered DNA methylation may be involved in the pathophysiology of SCZ, and our results suggest that taking the influence of cellular heterogeneity bias into consideration in DNA methylation studies may be necessary to reveal accurate abnormalities associated with diseases.

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SHORT COMMUNICATION

Microarray analysis of global gene expression in leukocytes following lithium treatment

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Objectives To elucidate the molecular effects of lithium, we studied global gene expression changes induced by lithium in leukocytes from healthy subjects.
Methods Eight healthy male subjects participated in this study. Lithium was prescribed for weeks to reach a therapeutic serum concentration. Leukocyte counts and serum lithium concentrations were determined at baseline (before medication), after 1 and 2 weeks of medication and at 2 weeks after stopping medication. Gene expression profiling was performed at each time point using Agilent G4112F Whole Human Genome arrays (The Agilent Technologies, Santa Clara, CA, USA). Expression of some candidate genes was also assessed by real-time polymerase chain reaction (PCR).
Results Gene ontology analysis revealed that the cellular and immune responses to stimulus and stress indeed played a major role in the cellular response to lithium treatment. Pathway analysis revealed that the interleukin 6 pathway, the inhibitor of differentiation pathway, and the methane metabolism pathway were regulated by lithium. Using real-time PCR, we also confirmed that five candidate genes in these pathways were significantly changed, including suppressor of cytokine signaling 3 and myeloperoxidase.
Conclusions Our investigation suggests that the molecular action of lithium is mediated in part by its effects on the cellular and immune response to stimulus and stress followed by the interleukin 6, inhibitor of differentiation, and methane metabolism pathways. Copyright © 2014 John Wiley & Sons, Ltd.

KEY WORDS—lithium; leukocyte; gene expression; interleukin 6

INTRODUCTION

Lithium is typically prescribed as a mood stabilizer for bipolar disorder and as an augmentation agent for refractory depression; however, the exact molecular mechanisms remain unknown (reviewed in Serretti and Drago, 2010). Several studies have recently demonstrated altered messenger RNA (mRNA) expression in the peripheral leukocytes of patients with mood disorders, and their application as biological markers has been suggested (reviewed in Iga *et al.*, 2008). There are various advantages associated with leukocyte gene expression biomarkers. For example, mRNA is stable for long-term storage, and using DNA microarrays, numerous genes can be examined quickly and simultaneously with only a small amount of blood. Furthermore, the test is simple to repeat during disease progression. By examining the effects of lithium on gene expression in healthy subjects, we were able to investigate the precise action of lithium without relevant confounding factors, such as disease pathophysiology and other medications (patients are often prescribed several medications simultaneously). Thus, we used microarray analysis and real-time PCR to analyze global gene expression changes in the leukocytes of healthy subjects undergoing 2 weeks of lithium treatment. Our aim was to perform a comprehensive analysis of changes induced by lithium treatment, which may reveal new targets for the molecular mechanisms underlying lithium action.

METHODS

Lithium treatment for healthy subjects

The healthy subjects chosen for this study were eight men with no present Diagnostic and Statistical Manual of Mental Disorders-IV axis I psychiatric disorders. Additional information about lithium treatment

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parameters and subjects is detailed in our earlier paper (Kikuchi *et al.*, 2011) and in Table 1. All individuals provided signed informed consent, which was approved by the university ethics committee.

Tissue processing, RNA purification, and preparation for microarray analysis

Total RNA was extracted from peripheral leukocytes of whole blood samples using the PAXgene Blood RNA tube (Qiagen, Tokyo, Japan) and the PAXgene Blood RNA kit (Qiagen, Tokyo, Japan). RNA concentration and its integrity were analyzed with an Agilent 2100 Caliper LabChip Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity numbers (RIN) of all samples were above 7.0 (cutoff RIN), and the mean RIN was 7.8. For Agilent G4112F Whole Human Genome array preparation and analysis, we followed the protocols provided by Agilent Technologies. We used two micrograms of RNA from each of the leukocytes from eight subjects per group for microarray experiments, because it has been reported that pooled RNA samples on microarrays showed comparable quality criteria when compared with individual RNA samples (Port *et al.*, 2012). All microarray methods and results may be found at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE44987.

Quantitative real-time PCR

Transcripts from 17 different genes were quantified by real-time PCR. In these experiments, we did not use pooled RNA samples but analyzed individual RNA specimens from each sample. Two micrograms of total RNA was used for complementary DNA synthesis by random (N6) primers and Quantiscript Reverse Transcriptase (Qiagen, Tokyo, Japan) after assessing RNA quality and quantity with NanoDrop (NanoDrop Technologies, DE, USA). Quantitative real-time PCR was performed with the ABI 7500 Fast System (Applied Biosystems, Foster City, CA, USA). NORMFINDER, a statistical application, was used to evaluate the stability of reference genes (Andersen *et al.*, 2004). glyceraldehyde-3-phosphate dehydrogenase showed the best stability score and was used as reference gene in our study. Measurements of each gene expression were conducted in triplicate. Primers for each gene are summarized in Table 2.

Statistical analysis

The transcripts represented on the array together with quantitative data were analyzed using the GENESPRING GX 11.5.1 (Agilent Technologies, Palo Alto, CA, USA). Data were normalized against the 75th percentile for per-chip normalization.

For the Gene Ontology (GO) analysis, the significance of the association between the dataset and the pathway was measured using two methods. First, only changes equal to or greater than plus/minus two-fold at any time point (80 upregulated and 54 downregulated genes) were included in GO analysis. Second, Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes and the GO may be explained by chance alone. GO annotations were obtained from the Gene Ontology website (http://www.geneontology.org/).

For pathway analysis, the significance of the association between the dataset and the pathway was measured using two methods: First, only changes equal or greater than positive/negative two-fold were included for the pathway analysis. Second, Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the pathway may be explained by chance alone. Pathway annotations were obtained from WikiPathways (http://www.wikipathways.org/index.php/WikiPathways).

For real-time PCR analysis, statistical calculations were carried out using the SPSS Statistical Software Package 11.5 (SPSS, Tokyo, Japan). Changes during lithium treatment were calculated with the Wilcoxon matched-pairs signed-rank test. The criterion for significance was set at $p < 0.05$ for all tests.

Table 1. Lithium concentration and leukocyte count

Parameter	Baseline	1 week	2 weeks	Post-2 weeks
Lithium dose (mg/day)	None	1200 ± 0.0	1375 ± 225	None
Lithium conc (mEq/l)	None	0.55 ± 0.23	0.73 ± 0.27	None
Leukocyte count (cells/μl)	5513 ± 1382	6538 ± 1016*	6400 ± 1021*	5438 ± 835
Granulocytes (%)	50.4 ± 8.7	53.6 ± 10.3	47.3 ± 9.4	49.3 ± 7.3
Lymphocytes (%)	39.1 ± 9.1	34.4 ± 10.4	39.3 ± 10.1	38.6 ± 7.6
Monocytes (%)	6.5 ± 2.1	5.6 ± 2.4	7.1 ± 2.6	7.2 ± 2.7

Leukocyte counts were significantly increased at 1 and 2 weeks (Wilcoxon matched-pairs signed-rank test) compared with baseline and were decreased at post-2 weeks compared with 1 and 2 weeks. Mean percentages of the leukocyte fraction were not significantly altered.
*$p < 0.05$

Table 2. List of primers used in real-time PCR experiments

Name	Gene symbol	Forward primer	Reverse primer
Interleukin 6	IL6	CCATCTCACAGTTTCATTGGTG	GCCAATTGAAACAGCTACAAAAG
Suppressor of cytokine signaling 3	SOCS3	CAAGGACGGAGACTTCGATTC	GAAACTTGCTGTGGGTGAC
FBJ murine osteosarcoma viral oncogene homolog	FOS	TTGTGAAGACCATGACAGGAG	CCATCTTATTCCTTTCCCTTCGG
Jun proto-oncogene	JUN	AGCCCAAACTAACCTCACG	TGCTCTGTTTCAGGATCTTGG
Myeloperoxidase	MPO	TCTGAACATGCAGCGCAG	CATCAGTTTCCTCGCCAATTTC
cAMP responsive element binding protein 1	CREB1	GGCAGACAGTTCAAGTCCATG	CGCTTTTGGGAATCAGTTACAC
Tumor necrosis factor	TNF	ACTTTGGAGTGATCGGCC	GCTTGAGGGTTTGCTACAAC
Microtubule-associated protein tau	MAPT	GACAGAGTCCAGTCGAAGATTG	GCGACTTGTACACGATCTCC
Signal transducer and activator of transcription 3	STAT3	TGCAGAAAACTCTCACGGAC	TGTTGACGGGTCTGAAGTTG
Radical S-adenosyl methionine domain containing 2	RSAD2	AGAATACCTGGGCAAGTTGG	GTCACAGGAGATAGCGAGAATG
Ubiquitin specific peptidase 28	USP28	GAGTTAAGGAGCCCAGTCAAG	CAGTAGACTCAAAGCAATGGC
Zinc finger protein 507	ZNF507	GTGTCCAGGAAGGGAATAAGTC	CACATACCCACACAGAGAGC
Heat shock protein 90 kDa alpha (cytosolic), class A member 1	HSP90AA1	GTCTGTGAAGGATCTGGTCATC	CAGCAGTAGGGTCATCTTCATC
Apolipoprotein L, 6	APOL6	AGAAAGTGAGGCTGGTGTTG	CAGATCTCCGTCTTGTAGCTC
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA
Beta-actin	ACTB	GGAATTATGTGTCTACCCTCGC	TCCTGTCTTTGCGTTCCTG
C-abl oncogene 1	ABL1	CTCAGATCTCGTCAGCCATG	ATCAGCTACCTTCACCAAGTG

cAMP, cyclic adenosine monophosphate.

RESULTS

Leukocyte counts in lithium-treated healthy subjects

As reported in a previous paper (Kikuchi *et al.* 2011), the mean leukocyte count in lithium-treated healthy subjects was significantly increased, but the leukocyte fraction was not significantly altered during lithium treatment (Table 2). This indicates that the change in leukocyte gene expression was not influenced by changes in the leukocyte fraction.

Microarray gene expression

In total, 44 000 genes were present on the Agilent G4112F Whole Human Genome arrays at all four time points, that is, baseline, 1, 2, and 2 weeks after the end of lithium treatment (post-2 weeks). A threshold of two-fold at any time point was used as a minimum expression change from baseline. Lithium treatment for 2 weeks led to upregulation of 80 genes (0.18% of all detected genes) and downregulation of 54 genes (0.12% of all detected genes) (Figure 1). All upregulated or downregulated genes are shown in Supplemental Table 1. All microarray results may be found at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE44987.

Gene ontology analysis

The top five GO categories for upregulated genes include response to stimulus (GO:0050896|GO:0051869), cellular response to stimulus (GO:0051716), response to stress (GO:0006950), immune system process (GO:0002376), and cell surface receptor signaling pathway (GO:0007166) (Figure 2). The top five GO categories for downregulated genes include response to

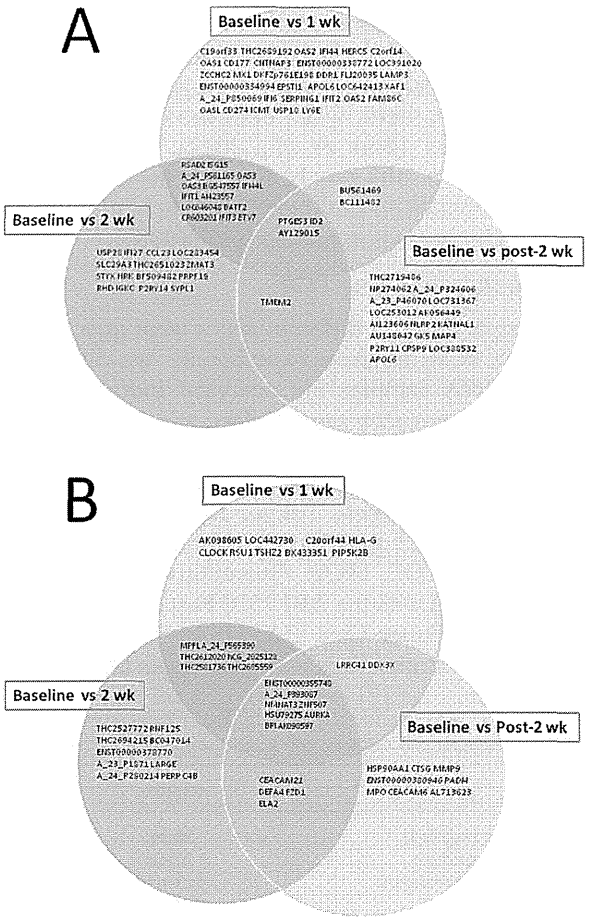

Figure 1. Venn diagram of genes regulated by lithium. Only those genes that showed at least two-fold upregulation (A) or downregulation (B) in comparison with baseline are illustrated. A total of 80 and 54 genes were upregulated and downregulated, respectively, by lithium treatment at any time point

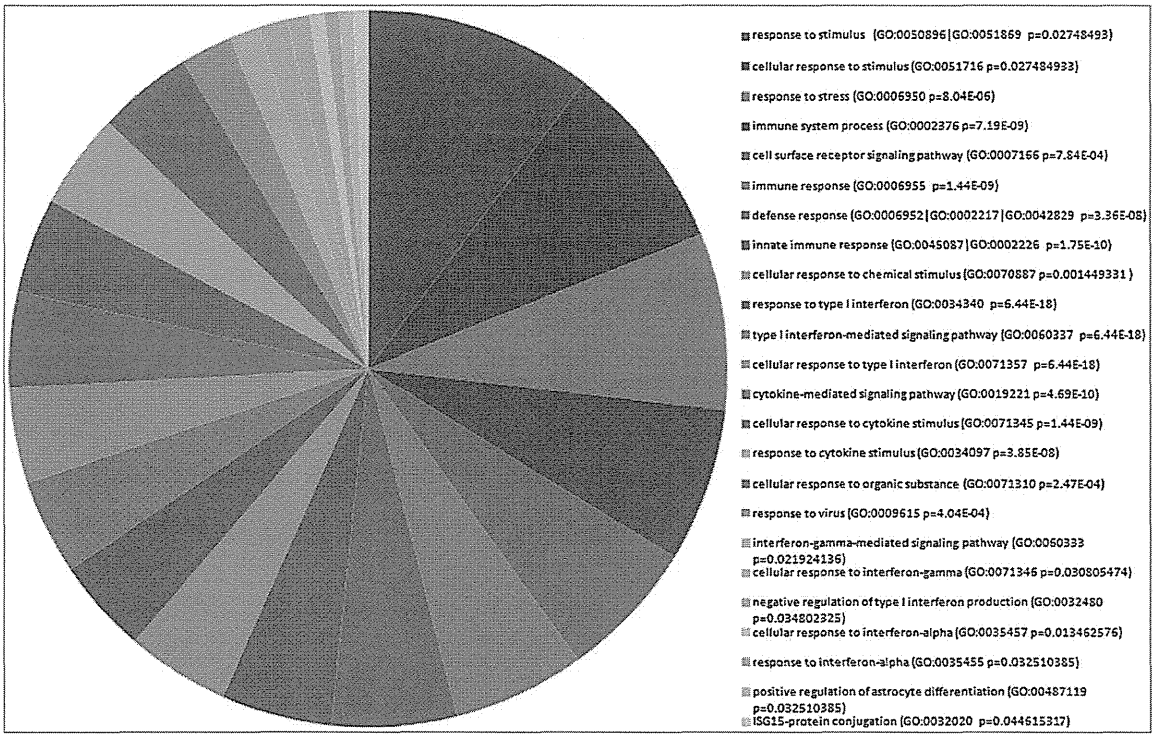

Figure 2. Gene Ontology (GO) analysis of 80 genes upregulated by lithium. Pie chart shows the percentage of upregulated genes, which were categorized on the basis of their involvement in biological processes. *p*-values were adjusted for multiple testing

bacterium (GO:0009617|GO:0009618|GO:0009680), modification of morphology or physiology of other organisms involved in symbiotic interactions (GO:0035821), cell killing (GO:0001906), response to fungus (GO:0009620|GO:0009621), and killing of cells of other organisms (GO:0031640|GO:0001908) (Figure 3).

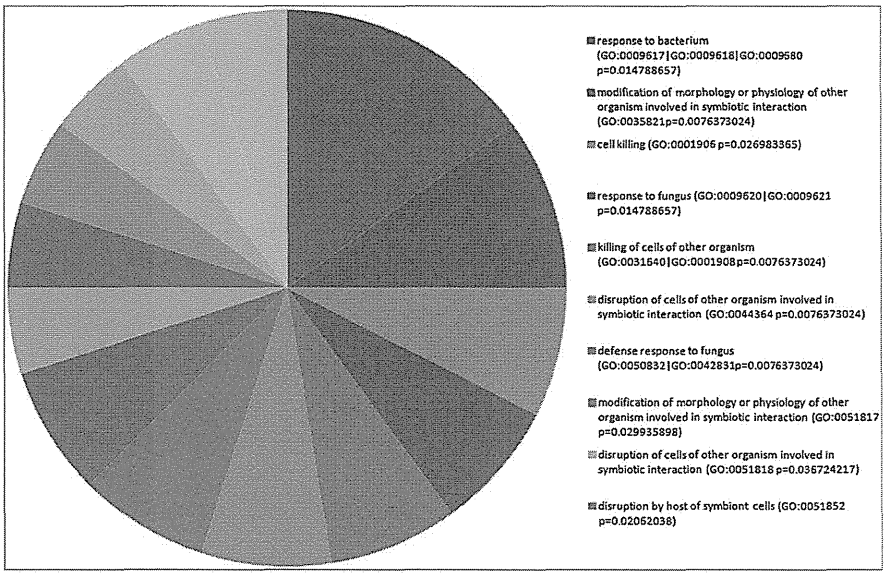

Figure 3. Gene Ontology (GO) analysis of 54 genes downregulated by lithium. Pie chart shows the percentage of downregulated genes, which were categorized on the basis of their involvement in biological processes. *p*-values were adjusted for multiple testing

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Pathway analysis

Of the genes that showed changes greater than positive/negative two-fold from baseline versus 1, 2, and post-2 weeks, the three pathways that emerged were the interleukin 6 (IL6) pathway, the inhibitor of differentiation pathway, and the methane metabolism pathway (Figure 4). All pathways were significantly altered by lithium treatment after adjusting for multiple testing ($p < 0.05$).

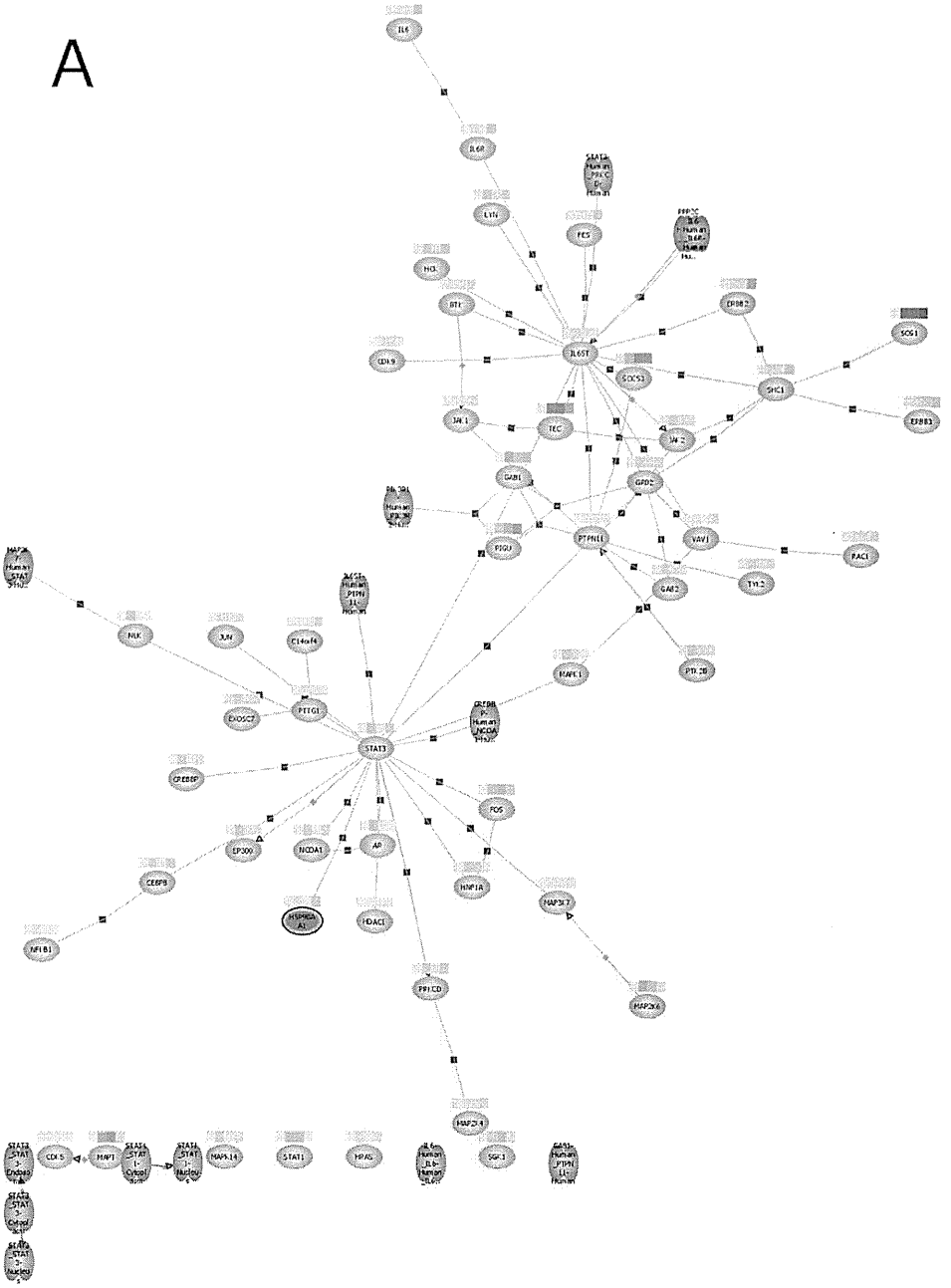

Figure 4. (A) Diagram of the interleukin 6 (IL6) pathway, which had the most significant association with expression level changes induced by lithium treatment. (B) Diagram of the ID pathway. (C) Diagram of the methane metabolism pathway. All pathways were significantly changed by lithium treatment after adjusting for multiple testing ($p < 0.05$). Expression levels at each time point are indicated by column color over each gene symbol. Red columns were upregulated, whereas green columns were downregulated. Yellow columns were detected by microarray analysis, but their expression was unaffected

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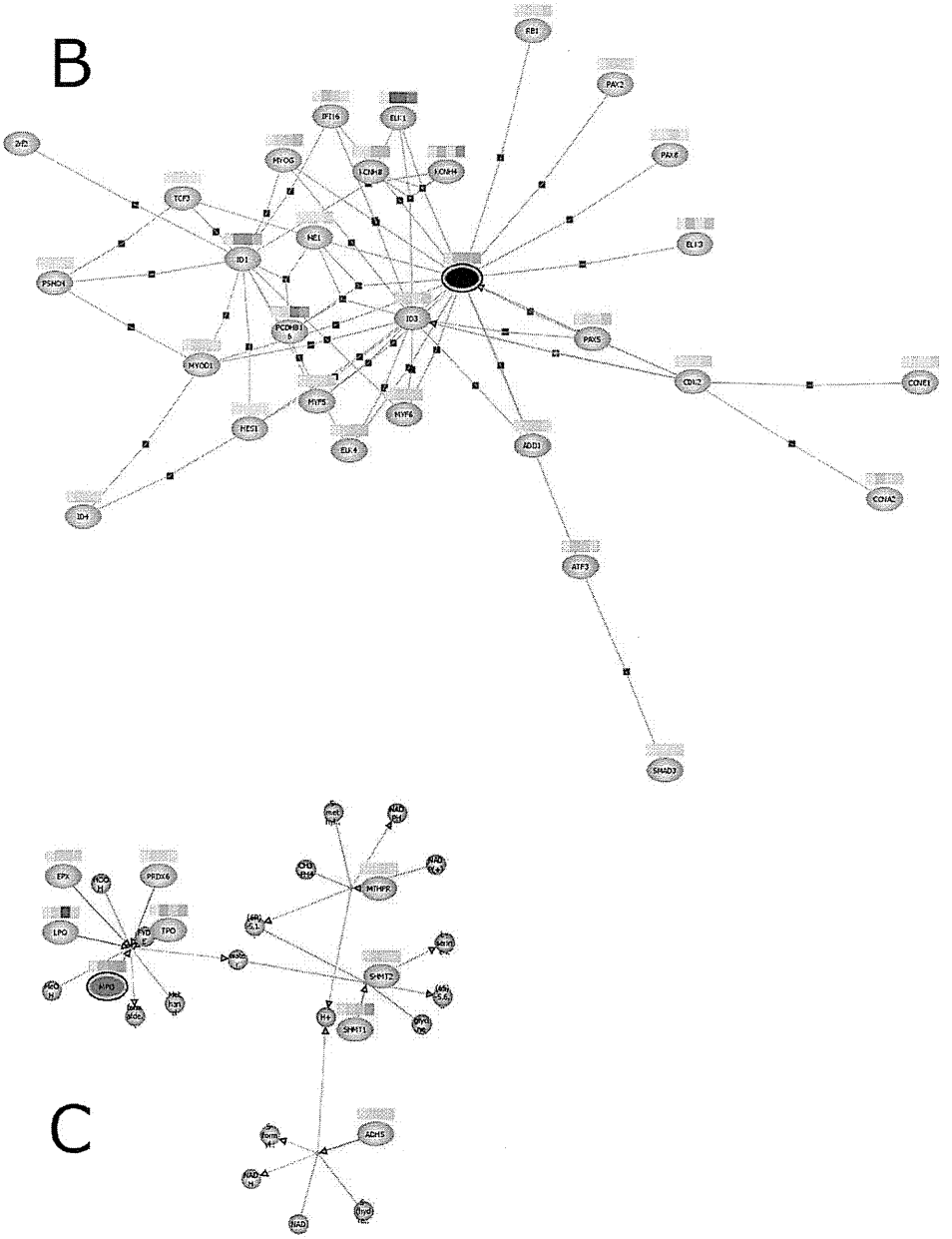

Figure 4. Continued.

Validation of the microarray data by real-time PCR

We confirmed the expression of five genes (RSAD2, USP28, ZNF507, HSP90AA1, and APOL6) in the top five upregulated and downregulated genes by real-time PCR. For statistical comparisons between PCR and Agilent data, linear regression with $r^2 = 0.39$ and $p < 0.014$ was performed (Figure 5). Nine candidate genes from the three pathways reportedly associated with the effects of lithium were also selected, and significant changes in the six genes were reconfirmed by real-time PCR. The six genes included one gene that was upregulated in the IL6 pathway (FBJ murine osteosarcoma viral oncogene homolog [FOS]) and four genes that were downregulated in the IL6 pathway (IL6, suppressor of cytokine signaling 3

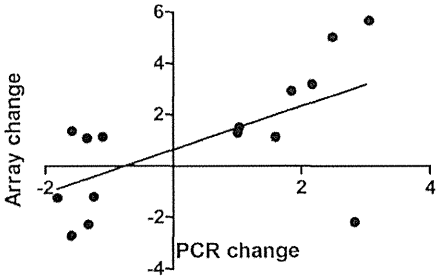

Figure 5. Five gene expression changes (RSAD2, USP28, ZNF507, HSP90AA1, and APOL6) at each time point in the top five upregulated and downregulated genes in the Agilent data could be quantified by PCR. For statistical comparison between PCR and Agilent data, linear regression with $r^2 = 0.39$ and $p < 0.014$ was calculated

[SOCS3], and jun proto-oncogene [JUN]) and in the methane metabolism pathway (myeloperoxidase [MPO]) (Table 3).

DISCUSSION

On GO analysis, categories for upregulated genes included response to stimulus, response to stress, immune system process, and response to cytokines (Figure 2), while downregulated genes included response to bacterium or fungus, cell killing, and neutrophil-mediated cytotoxicity (Figure 3). Taken together, the results indicate that the genes involved in the response to stimulus, stress, and cytokines were increased, while the genes involved in the response to infection and cell killing were decreased by lithium treatment. Our data suggest a promising role for lithium in the regulation of inflammation and cell death (Dai *et al.*, 2012, Green and Nolan, 2012, Matsebatlela *et al.*, 2012).

Table 3. Mean expression levels of nine candidate genes in the three pathways

	Baseline	1 week	2 weeks	Post-2 weeks
SOCS3	1.00	0.98	0.66*	0.75
IL6	1.00	1.07	1.06	0.78*
MPO	1.00	0.77*	0.93	0.96
CREB1	1.00	1.12	1.01	0.81*
FOS	1.00	1.64	1.79*	0.87
JUN	1.00	0.72	0.65*	0.73
TNF	1.00	1.14	1.01	1.04
MAPT	1.00	1.26	1.06	1.18
STAT3	1.00	0.91	0.92	0.93

SOCS3, Suppressor of cytokine signaling 3; IL6, interleukin 6; MPO, myeloperoxidase; CREB1, cyclic adenosine monophosphate responsive element binding protein 1; FOS, FBJ murine osteosarcoma viral oncogene homolog; JUN, jun proto-oncogene; TNF, tumor necrosis factor; MAPT, microtubule-associated protein tau; STAT3, signal transducer and activator of transcription 3.
*$p < 0.05$ Wilcoxon matched-paired signed-rank test (vs. baseline).

On pathway analysis, the IL6 pathway was associated with the action of lithium. There is evidence indicating an association between lithium, IL6, and bipolar disorder. For example, IL6 gene expression in the monocytes of bipolar patients was significantly increased (Padmos *et al.*, 2008). Serum IL6 protein levels were significantly increased in selective serotonin re-uptake inhibitors-refractory depression (O'Brien *et al.*, 2007, Yoshimura *et al.*, 2009), which is often treated with lithium augmentation. The tumor-necrosis-factor-mediated IL6 induction was further enhanced by lithium (Vandevoorde 1992). Following lithium treatment, SOCS3 in the IL6 pathway showed marked downregulation. SOCS3 is expressed by immune cells and cells in the central nervous system (CNS) (microglia and astrocytes) that have the potential to regulate numerous CNS disease states (reviewed in Baker *et al.*, 2009). Our results suggest that lithium has neuroprotective effects by decreasing both IL6 and SOCS3 expressions in immune cells. Very recently, we found that drug-naive major depressive disorder (MDD) patients showed significantly lower SOCS3 mRNA levels when compared with healthy subjects, while SOCS3 mRNA levels were significantly increased to healthy control levels after successful treatment with antidepressants (unpublished data). Because SOCS3 expression is stimulated by cytokine signaling and SOCS3 acts as a negative feedback regulator to prevent overstimulation of cytokine-responsive cells, the decrease in SOCS3 mRNA expression in the leukocytes of MDD patients may be associated with the unsuccessful prevention of over-activated inflammation. Successful treatment with antidepressants may enhance the function of SOCS3 and recover the abnormal immune response in MDD. Thus, SOCS3 mRNA levels in leukocytes may be available as a biomarker for the diagnosis of mood disorders and responsiveness to lithium and antidepressant. FOS and JUN gene expressions in the IL6 pathway were also significantly altered. FOS and JUN protein homodimer or heterodimer assemble in the activator protein 1 (AP-1) transcription factor. Interestingly, AP-1 proteins are also known to be involved in the molecular action of lithium (Jope and Bijur, 2002).

In the CNS, inhibitor of DNA binding (ID) proteins are positive regulators of neural cell proliferation, are required for neural cell cycle progression, and also play a role in the timing of oligodendroglial differentiation (Tzeng 2003). Lithium is known to increase adult hippocampal neurogenesis (Boku *et al.*, 2010). Although we did not reconfirm significant changes in ID gene expression by real-time PCR, ID proteins may play important roles in lithium-induced cell growth and differentiation.

Significant changes were observed in the methane metabolism pathway, which include candidate genes for mood disorders. It was reported that MPO is a useful biomarker of immune activation in MDD and by promoting inflammation, may be involved in the pathogenesis of MDD (Vaccarino *et al.*, 2008). Our results suggest that lithium exerts clinical effects by decreasing MPO activity and immune activation. Thyroid peroxidase is an important enzyme in thyroid hormone synthesis, and it is reported that a high prevalence of autoimmunity for this enzyme was confirmed in patients with bipolar disorder (Padmos *et al.*, 2004). Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme for homocysteine metabolism. Homocysteine is known to be associated with geriatric depression (Almeida *et al.*, 2008), and MTHFR C677T polymorphism, which impairs homocysteine metabolism, is over-represented among depressive patients (Coppen and Bolander-Gouaille, 2005).

Although this is the first study to examine the global gene expression effects of lithium on peripheral leukocytes, Sun *et al.* (2004) found that chronic lithium treatment at a therapeutically relevant concentration decreased the expression of seven genes (alpha 1B-adrenoceptor, acetylcholine receptor protein alpha chain precursor, cyclic adenosine monophosphate-dependent 3′,5′-cyclic phosphordiesterase4D, substance-P receptor, somatostatin receptor type 2, nuclear factor kappa-B DNA binding subunit, and ras-related protein) in lymphoblasts from lithium responders (Sun *et al.*, 2004). Unfortunately, none of these seven genes are listed in our results. These discrepancies may be due to differences in the tissue sampled and/or the pathophysiology of bipolar disorder. Further research is needed to identify the lithium response markers using leukocytes from patients.

The limitations of this study include the low rate of validation. Although most of the validated genes showed similar changes as in the microarray results, we were only able to validate significant changes in five of the 17 selected genes. In addition, four of 15 data points in the top five upregulated and downregulated genes showed changes in the opposite direction from microarray to PCR. These differences may be due to differences in sample preparation, sensitivity of the two techniques, and use of different statistical analysis methods. Setting the threshold for minimum change as two-fold on microarray analysis may ignore important genes with expression changes of less than two-fold.

CONCLUSION

We show that lithium treatment significantly affects leukocyte gene expression. GO analysis revealed the promising effects of lithium in the regulation of inflammation and cell death. Pathway analysis suggests that the molecular action of lithium is mediated by the IL6 pathway, the ID pathway, and the methane metabolism pathway. Some genes in these pathways, such as SOCS3 and MPO, were also validated by real-time PCR.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site.

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ORIGINAL RESEARCH

COMT Val158Met, but not *BDNF* Val66Met, is associated with white matter abnormalities of the temporal lobe in patients with first-episode, treatment-naïve major depressive disorder: a diffusion tensor imaging study

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Abstract: We investigated the association between the Val158Met polymorphism of the *catechol-O-methyltransferase* (*COMT*) gene, the Val66Met polymorphism of the *brain-derived neurotrophic factor* (*BDNF*) gene, and white matter changes in patients with major depressive disorder (MDD) and healthy subjects using diffusion tensor imaging (DTI). We studied 30 patients with MDD (17 males and 13 females, with mean age ± standard deviation [SD] =44±12 years) and 30 sex- and age-matched healthy controls (17 males and 13 females, aged 44±13 years). Using DTI analysis with a tract-based spatial statistics (TBSS) approach, we investigated the differences in fractional anisotropy, radial diffusivity, and axial diffusivity distribution among the three groups (patients with the *COMT* gene Val158Met, those with the *BDNF* gene Val66Met, and the healthy subjects). In a voxel-wise-based group comparison, we found significant decreases in fractional anisotropy and axial diffusivity within the temporal lobe white matter in the Met-carriers with MDD compared with the controls ($P<0.05$). No correlations in fractional anisotropy, axial diffusivity, or radial diffusivity were observed between the MDD patients and the controls, either among those with the *BDNF* Val/Val genotype or among the *BDNF* Met-carriers. These results suggest an association between the *COMT* gene Val158Met and the white matter abnormalities found in the temporal lobe of patients with MDD.

Keywords: catechol-O-methyltransferase, brain-derived neurotrophic factor, 3-methoxy-4-hydroxyphenylglycol, homovanillic acid

Introduction

Catecholamines play an important role in the pathogenesis of major depressive disorder (MDD).[1] Catechol-O-methyltransferase (COMT) is a methylation enzyme that plays a role in the degradation of noradrenaline and dopamine, by catalyzing the transfer of a methyl group from S-adenosylmethionine. Biochemical research has established that the enzyme activities in patients with MDD differ from those of nondepressed subjects.[2] The *COMT* gene is located at 22q 11.21. In a multicenter European study, an association was found between the *COMT* gene Val158Met (G324A) functional polymorphism and MDD.[3] The Val allele has been reported to result in three- to fourfold higher activity than the Met allele.[4] One report suggests that there is an association between higher activity of the *COMT* gene Val158Val-type and a poor antidepressant

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