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Identification of Single Nucleotide Polymorphisms Regulating Peripheral Blood mRNA Expression with Genome-Wide Significance: An eQTL Study in the Japanese Population

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Abstract

Several recent studies have reported that expression quantitative trait loci (eQTLs) may affect gene expression in a cell-dependent manner. In the current study, a genome-wide eQTL analysis was performed in whole blood samples collected from 76 Japanese subjects. RNA microarray analysis was performed for 3 independent sample groups that were genotyped in a genome-wide scan. The correlations between the genotypes of 534,404 autosomal single nucleotide polymorphisms (SNPs) and the expression levels of 30,465 probes were examined for each sample group. The SNP-probe pairs with combined correlation coefficients of all 3 sample groups corresponding to $P<3.1\times10^{-12}$ (i.e., Bonferroni-corrected $P<0.05$) were considered significant. SNP-probe pairs with a high likelihood of cross-hybridization and SNP-in-probe effects were excluded to avoid false positive results. We identified 102 *cis*-acting and 5 *trans*-acting eQTL regions. The *cis*-eQTL regions were widely distributed both upstream and downstream of the gene, as well as within the gene. The eQTL SNPs identified were examined for their influence on the expression levels in lymphoblastoid cell lines by using a public database. The results showed that genetic variants affecting expression levels in whole blood may have different effects on gene expression in lymphoblastoid cell lines. Further studies are required to clarify how SNPs function in affecting the expression levels in whole blood as well as in other tissues.

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Introduction

Advances in high-throughput genotyping and gene expression platforms have enabled genome-wide analysis of gene expression quantitative trait loci (eQTLs), allowing investigation of both *cis* and *trans* effects. Previous eQTL studies have examined the association between genetic variants and gene expression levels in various biological samples, including human whole blood [1,2], lymphocytes [3], the liver [4], and, primarily, in lymphoblastoid cell lines [5,6]. Recently developed web tools such as SNPexp [7] and Genevar [8] have enabled analysis of the correlation between SNP genotypes in HapMap genotype data and genome-wide expression levels in lymphoblastoid cell lines. Development of such tools in other cell types is also anticipated, as a substantial fraction of eQTLs are cell type-specific [9,10,11,12].

Despite these advances, several challenges still remain in the field of genome-wide eQTL research. The large number of gene expression traits and genomic loci requires enormous calculations, raising issues of computer efficiency and statistical power. Another challenge is the varying genetic backgrounds in study populations, which may be one of the causes of the poor reproducibility observed across studies. Furthermore, confounding variables, such as the time of day at which sampling was performed, may also affect gene expression patterns in peripheral blood [13]. In addition, microarray probes may contain one or more SNPs in the target sequence. These probes may cause hybridization differences due to sequence polymorphisms present in the mRNA region, resulting in the occurrence of false positive results [14]. Other probes may undergo cross-hybridization, also resulting in false positive results for *trans*-eQTLs. The large number of probes and

SNPs cause difficulties in accounting for these confounding and influencing variables. A limited number of studies have overcome these methodological issues; therefore, further accumulation of data is required. Specifically, genome-wide eQTL data for Asian population is scarce [15].

Gene expression in whole blood could function as biomarkers for several disease conditions such as diabetes [16] and attention deficit hyperactivity disorder [17]. Elucidation of the genetic basis affecting such gene expression may be important in uncovering the etiological factors and pathophysiology of the diseases. Taking the aforementioned issues into consideration, we have examined the correlations between the genotypes of every SNP from a genome-wide scan and the expression levels of genes in the whole blood of Japanese individuals. To avoid the influence of batch effects, which is often ignored in eQTL studies, microarray data collected in different batches were first analyzed separately and then integrated. After strict corrections for multiple testing and exclusion of potential false-positive eQTLs, we investigated whether the SNPs found to have an effect on the expression levels in whole blood also influenced the expression levels in lymphoblastoid cell lines. Public data from the HapMap project of SNP genotypes and gene expression levels in lymphoblastoid cell lines were used for the analysis.

Materials and Methods

Genomic DNA was collected from 24 subjects (13 men and 11 women, mean age [SD] = 39.9 [7.6] years) in sample group 1, 24 subjects in sample group 2 (12 men and 12 women, 34.1 [11.5] years), and 28 subjects (14 men and 14 women, 41.4 [11.8] years) in sample group 3. The blood samples of each of the 3 sample groups were collected at different times and the microarray data of each sample group were obtained separately. Approximately half of the subjects suffered from depressive disorder (11, 12, and 16 subjects in sample groups 1, 2, and 3, respectively), but all were physically healthy and without clinically significant systemic disease (e.g., malignant disease, diabetes mellitus, hypertension, renal failure, or endocrine disorders). Subjects were recruited from the outpatient clinic of the National Center of Neurology and Psychiatry Hospital, Tokyo, Japan, through advertisements in free local information magazines or through our website announcement. All the subjects were biologically unrelated Japanese individuals who resided in the same geographical area (western Tokyo). The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. Written informed consent was obtained from every subject after the study was explained to them.

Venous blood was collected between 1100 and 1200 h in PAXgene tubes (Qiagen, Valencia) from each subject and was incubated at room temperature for 24 h for RNA stabilization. RNA was extracted from whole blood according to the manufacturer's guidelines by using the PAXgene Blood RNA System Kit (PreAnalytix GmbH, Hombrechtikon, Switzerland). The RNA was quantified by optical density readings at A260 nm by using the NanoDrop ND-1000 (Thermo Scientific, Rockford). Gene expression analysis was performed using Agilent Human Genome 4×44 K arrays (Agilent Technologies, Santa Clara). Raw signal data for each of the 3 independent sample groups were analyzed separately by the GeneSpring GX software (Agilent Technologies). Data were filtered according to the expression level for quality control to eliminate genes that were below the 20th percentile threshold. The expression value of each gene was normalized to the median expression value of all genes in each chip. A total of 30,465 probes were included in the analysis.

Genomic DNA was obtained from venous blood samples. Genotyping was performed by Riken Genesis (Yokohama, Japan) using the Illumina HumanOmni1-Quad BeadChip (Illumina, Inc., San Diego). A total of 713,495 autosomal SNPs were assessed for quality using the PLINK v1.07 software [18]. All SNPs with a call rate below 95%, a deviation from Hardy-Weinberg equilibrium at an error level of $P<0.001$, or a minor allele frequency of less than 10% were excluded. The remaining 534,404 SNPs were used for further analysis. RNA expression and DNA genotype data are available at NCBI's Gene Expression Omnibus under accession number GSE42488.

Since RNA expression arrays of the 3 sample groups were performed at different times, the correlation between the genotype and expression levels was calculated separately in each sample group to avoid the influences of batch effects. The Pearson's correlation coefficient (r) between the genotype (coded as 0, 1, or 2) and gene expression level was calculated for each of the 1.63×10^{10} SNP-expression probe pairs in the 3 sample groups. The correlation coefficients of the 3 sample groups were averaged according to the following equation [19]:

$$\bar{r}=e^{2\bar{z}}-1/e^{2\bar{z}}+1$$

where $\bar{z}=\Sigma_i\ [1/2\times\ln\{(1+r_i)/(1-r_i)\}\times n_i]/\Sigma_i\ n_i$

n_i = the number of individuals in sample group i.

r_i = the correlation coefficient between the genotype and expression level in sample group i.

To minimize the possibility of false positives, the SNP-expression probe pairs with $\bar{r}$ corresponding to a Bonferroni-corrected P value of <0.05 (i.e., uncorrected $P<0.05/[30{,}465\times534{,}404]=3.1\times10^{-12}$) were also examined using Spearman's rank correlation in a similar method as described above. The SNP-probe pairs with Bonferroni-corrected P value of the average Spearman's rank correlation <0.05 (i.e., uncorrected $P<3.1\times10^{-12}$) were considered significant.

To determine the potential for cross-hybridization of the probes, a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed against the human genome by using the online Ensembl database. Probes with greater than 50% homology with other genomic regions were excluded.

Sequence polymorphisms in the mRNA region targeted by the microarray expression probes may cause hybridization differences due to SNP-in-probe effects. Therefore, SNP-probe pairs were excluded from the analysis if the 60-mer probe was mapped to a genomic location that contained a known SNP showing linkage disequilibrium (LD; $r^2>0.1$) with the SNP of the SNP-probe pair.

We also examined whether the eQTL SNPs affecting the expression levels in whole blood also influence expression levels in lymphoblastoid cell lines. The SNPexp [7] software was used to retrieve public data from the HapMap project (release 23) of SNP genotypes and the gene expression levels in lymphoblastoid cell lines of 45 Japanese subjects. Pearson's correlation coefficients were used to assess the influence of SNPs on expression levels in lymphoblastoid cell lines.

Results

Identification of eQTLs

The procedure used for SNP-probe pair selection (Figure 1) generated 1,554 pairs, which are listed in Table S1. These SNP-probe pairs consisted of 1,153 SNPs, defined as eQTL SNPs, and 185 probes. For 122 of these 185 probes, we could identify the corresponding gene from the HapMap database (Release 28).

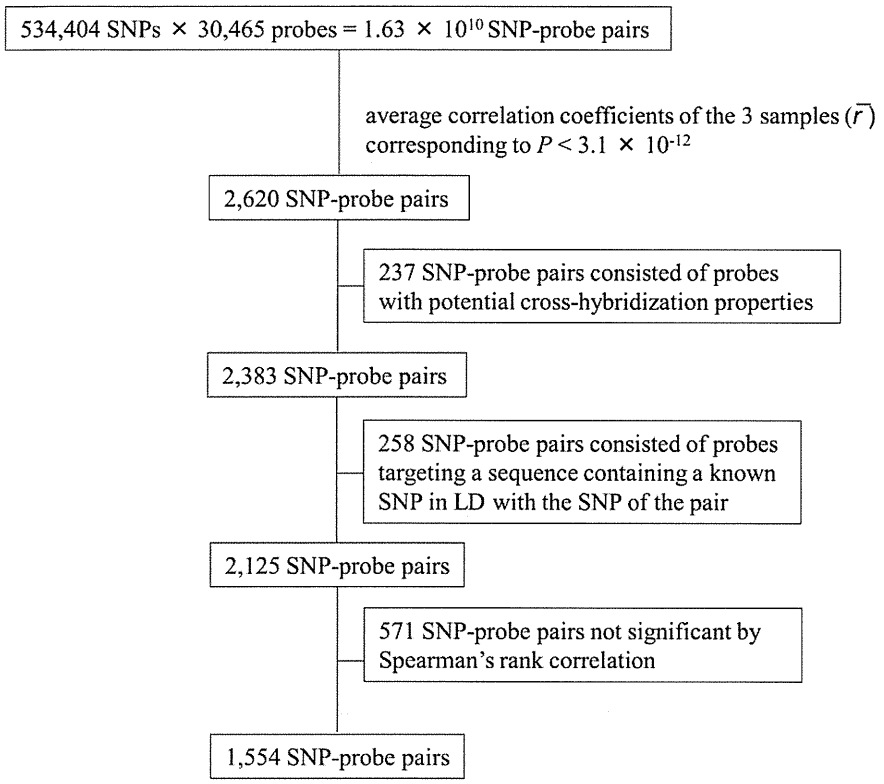

Figure 1. Procedure for selecting significant SNP-probe pairs. The procedure for selecting significant SNP-probe pairs is shown. SNP-probe pairs with a high likelihood of cross-hybridization and SNP-in-probe effects were excluded to exclude false positive results. The SNPs of the remaining 1,554 SNP-probe pairs were considered as eQTL SNPs.
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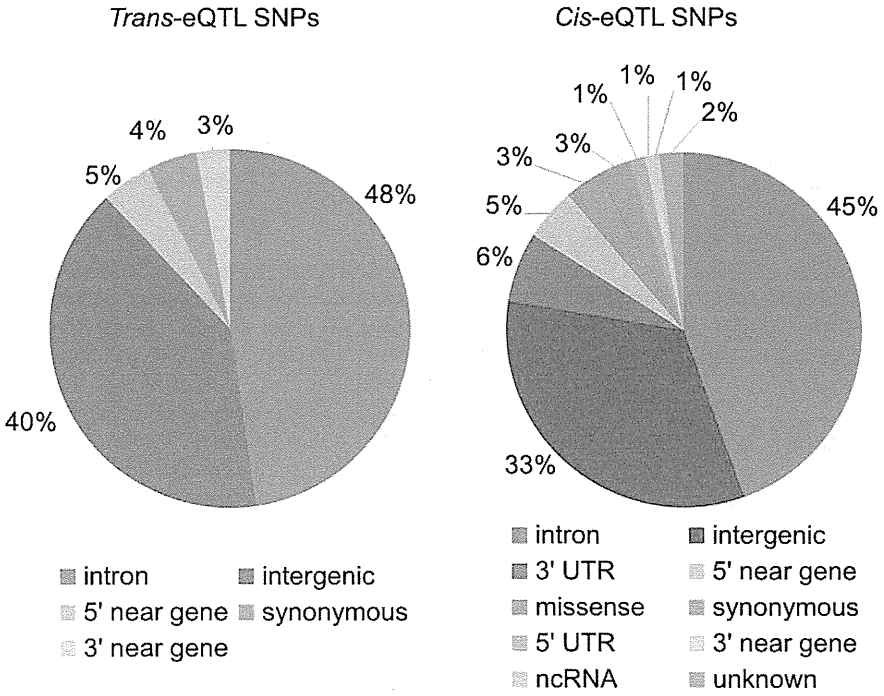

Figure 2. Functional types of the eQTL SNPs. The percentage of SNP types is shown for *cis*- and *trans*- eQTL SNPs.
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Since several of the probes targeted the same gene, the total number of genes identified was 107. As shown in Figure 2, the majority of the eQTL SNPs were located in intronic (45% and 48% for *cis*- and *trans*-eQTL SNPs, respectively) or intergenic (33% and 40% for *cis*- and *trans*-eQTL SNPs, respectively) regions.

Table S2 shows the names and properties of the 107 genes whose expression levels in whole blood were affected by SNPs. The SNPs affecting expression levels of the same gene were primarily in high LD with each other. Furthermore, investigation of combined Chinese and Japanese (CHB+JPT) panels from the 1000 Genomes Pilot 1 SNP data set and the HapMap release 22 data set showed a greater number of SNPs in high LD ($r^2>0.8$) with the eQTL SNPs identified in the current study. Since the high intermarker correlations cause difficulties in determining which SNP is responsible for the regulation of gene expression, we defined the eQTL region of a gene as the genomic range in which the SNPs in LD ($r^2>0.8$) with the eQTL SNPs of the gene are located. LD was determined by SNAP [20] using the population panel CHB+JPT from the 1000 Genomes Pilot 1 SNP data set and the HapMap release 22 data set.

Locational Relationships between the eQTL and the Gene

Regarding the locational relationships between the eQTL and the gene, 102 of the eQTLs were *cis*-acting (within 1 Mb upstream or downstream of the gene), and 5 were *trans*-acting, of which 4 were located on a different chromosome from the gene that they influenced. When the genome was divided into 3 segments (i.e., upstream, intragenic, and downstream), 69 *cis*-acting eQTL regions covered multiple segments that included the intragenic segments, 13 were confined to upstream segments, 7 were confined to downstream segments, and 13 were confined to intragenic segments.

Comparison of Results with Previously Reported Whole Blood eQTLs

We compared our results with those of the study by Fehrmann et al. [21], which performed a genome-wide eQTL analysis on 289,044 SNPs in whole blood expression data of 1,469 unrelated individuals from the United Kingdom and the Netherlands. The genotyping platform which they used (Illumina HumanHap300 platform) included only 24% of the 534,404 SNPs analyzed in the current study and 15% of the 1,153 eQTL SNPs identified in the current study. Therefore, 85% of the eQTL SNPs identified in the current study had not been identified by Fehrmann et al. [21], because they were not included in the Illumina HumanHap300 platform. On the other hand, 84% of the eQTL SNPs identified in the current study which were included in the Illumina HumanHap300 platform had also been identified as eQTL SNPs in their study. The high replication rate supports the robustness of our findings.

Influence on Expression Levels in Lymphoblastoid Cell Lines

Next, we examined whether the eQTL SNPs affecting the expression levels in whole blood also influence expression levels in lymphoblastoid cell lines. We selected representative SNPs in eQTL regions and examined their effects on the expression of the corresponding gene in lymphoblastoid cell lines. The SNPs that showed the strongest correlation with the expression levels in whole blood for each eQTL region were selected for examination of the possible effects on expression levels in lymphoblastoid cell lines. If there were any additional eQTL SNPs in the same region that were not in LD with the selected SNP ($r^2<0.1$), then one of the SNPs with the strongest correlation with the expression levels in whole blood was also selected. In the eQTL regions for *MICA*, *MICB*, *HLA-DRB5*, *HLA-DQB1*, and *HLA-DQA2*, 2 representative SNPs, which were not in significant LD with each other ($r^2<0.1$), were selected. For other genes, the eQTL SNPs in the same eQTL region were in LD with each other ($r^2>0.1$); therefore, 1 representative SNP was selected for each region. If the genotype data of the selected SNP were not available in the HapMap data, the SNP within the same eQTL region having the next strongest correlation with the expression levels in whole blood was selected.

Genotype and expression levels in lymphoblastoid cell lines were retrieved from public data for 45 Japanese individuals for 88 (86 *cis* and 2 *trans*) of the 112 representative SNPs. The average number of individuals with applicable data for genotype and the expression levels of lymphoblastoid cell lines in the 88 retrieved SNP-gene pairs was 43.8. The Pearson's correlation coefficients between the eQTL SNPs and the expression levels of the corresponding genes in lymphoblastoid cell lines were calculated and have been shown in Table S3. A positive correlation coefficient indicates that the SNP has a similar effect on expression levels in whole blood and lymphoblastoid cell lines. Of the 86 *cis*-eQTL SNPs, 34 showed a significantly positive correlation, whereas 13 showed a significantly negative correlation with the expression levels of lymphoblastoid cell lines (FDR-corrected, $P<0.05$). None of the *trans*-eQTL SNPs identified in the current study significantly affected expression levels in lymphoblastoid cell lines.

Functional Properties of the eQTL SNPs

We examined whether the regulatory effects of eQTL SNPs were caused by mutations in transcription factor-binding sites (TFBSs), splicing-affecting sites, or microRNA (miRNA)-binding sites. The proportion of SNPs in LD ($r^2>0.8$) with a SNP predicted to be located on such sites was compared between the 37 eQTL SNPs affecting expression levels in both whole blood and lymphoblastoid cell lines; 49 eQTL SNPs affecting only whole blood expression levels; and 5,681 non-eQTL SNPs located within 100 kB of the 107 genes that were regulated by the eQTL SNPs identified in the current study. A web-based tool (FuncPred; http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm) was used to predict the functional properties of the SNPs. As shown in Table 1, eQTL SNPs were more likely to be in LD with SNPs located on TFBSs, splicing-affecting sites, and miRNA-binding sites.

Cis-only Analysis

The small-effect eQTL SNPs are likely to have remained undetected in the present study due to the strict correction procedures for multiple testing. In order to reduce the number of unreported *cis*-eQTL SNPs, we also performed *cis*-only analysis by examining only SNPs 1 Mb upstream or downstream of the targeted gene. A total of 955,370 SNP-probe pairs were examined, and those with an average Pearson's correlation ($\bar{r}$) of the 3 sample groups corresponding to $P<5.23\times10^{-9}$ (i.e., Bonferroni-corrected $P<0.05$) were considered significant. As shown in Table S4, the *cis*-only analysis resulted in 3,883 SNP-probe pairs consisting of 3,161 SNPs and 347 probes.

The Influence of Depressive Disorder on Gene Expression Regulation

In order to investigate whether depressive disorder was a major confounding factor for gene expression regulation, we calculated the Spearman's correlation coefficients separately in depressed and non-depressed subjects. All the 1,554 SNP-probe pairs identified

Table 1. Percentage of SNPs that are in linkage disequilibrium ($r^2>0.8$) with a SNP predicted to be located on TFBS, splicing-affecting site, or miRNA binding site.

	TFBS	Splicing	miRNA binding site
eQTL SNPs affecting expression levels in both whole blood and LCLs (37 SNPs)	73.7% ‡	42.1% ‡	44.7% ‡
eQTL SNPs affecting expression levels in only whole blood (49 SNPs)	58.8% ‡	43.1%‡	29.4% †
non-eQTL SNPs (5,681 SNPs)	34.8%	17.3%	14.1%

The following abbreviations are used: TFBS, transcription factor binding site; miRNA, micro RNA; LCL, lymphoblastoid cell line.
†$P<0.01$,
‡$P<0.001$: Significantly higher compared to non-eQTL SNPs (χ^2 test).
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as eQTL in the present study achieved high correlations for both depressed and non-depressed subjects (average Spearman's correlation of the 3 sample groups $\overline{\rho}>0.4$, FDR-corrected $P<0.01$ in non-depressed subjects and $\overline{\rho}>0.5$, FDR-corrected $P<0.005$ in depressed subjects for all 1,554 SNP-probe pairs).

Discussion

To our knowledge, this is the first genome-wide eQTL study in Asian subjects that examined the association of SNPs with expression levels in whole blood. The genome-wide investigation uncovered 1,153 SNPs affecting gene expression levels in human whole blood. Although the number of eQTL regions identified in the current study was relatively small, the likelihood of false positives is low because of the strict correction procedures for multiple testing and exclusion of SNPs with potential cross-hybridization or SNP-in-probe effects.

Since SNPs in strong LD with a SNP directly responsible for regulating gene expression levels are also correlated with gene expression levels, it is difficult to determine which SNP is the causative one. We assumed that the genetic regulatory locus would be included in the eQTL region, defined as the genomic range in which the SNPs in LD ($r^2>0.8$) with the eQTL SNPs identified in our study are found. Although the numerous SNPs in LD with each other hindered the identification of the responsible SNP, the locations of the eQTL regions indicated that eQTLs are widely distributed both upstream and downstream of the gene, as well as within the gene.

The current study showed that several of the SNPs affecting the expression levels of a gene in whole blood also influenced the expression levels of the same gene in lymphoblastoid cell lines. A recent study by Powell et al. [22] has shown that the genetic control mechanisms of gene expression in whole blood and lymphoblastoid cell lines are largely independent. Despite the evidence of low genetic correlation of regulatory variation averaged across the genome, our results suggest that a subset of eQTLs commonly affect expression levels in whole blood and lymphoblastoid cell lines. Conversely, our findings suggest that some of the whole blood eQTL SNPs do not regulate expression levels in lymphoblastoid cell lines. This is in line with a previous study that reported that 69–80% of the identified regulatory variants operated in a cell type-specific manner [9]. Compared to SNPs affecting only expression levels in whole blood, higher, although not statistically significant, proportion of SNPs affecting expression levels in both whole blood and lymphoblastoid cell lines were in LD with SNPs located on TFBSs and miRNA-binding sites. The finding suggests that these functional properties affect expression levels across multiple cell types.

Intriguingly, 13 of the 88 eQTL SNPs in whole blood were observed to have opposite effects on expression levels in whole blood and lymphoblastoid cell lines. Dimas et al. [9] compared gene expression variation in fibroblasts, lymphoblastoid cell lines, and T cells and reported that the same directional effect in each cell type was observed for eQTLs shared between multiple cell types. However, 2 recently published studies reported that some eQTL SNPs have opposite allelic effects on gene expression in the liver, adipose tissue, skeletal muscle [10], or in B cells and monocytes [11]. Our findings also suggest the possibility that some SNPs may exert opposite effects on gene expression in different cell types. However, an alternative explanation may be that the eQTL SNPs identified may function to alter the splicing of the mRNA. Since the gene expression microarray platform used in the previous eQTL study examining LCL expression levels in Japanese subjects was different from ours, the different probes may have detected different splicing variants, resulting in seemingly opposite allelic effects. A comparison using the same platform would be necessary to uncover cell-specific effects on expression levels.

The strength of the current study is that a relatively homogeneous Japanese population was used, which may have minimized the effects of differential genetic backgrounds. The major limitation of the current study is that the conservative corrections for multiple testing may have missed a large proportions of eQTL SNPs. Increasing power allows better detection of weaker and more distantly located *cis*-regulatory elements [23]. Greater than 82% of the significant eQTL-probe pairs identified in the current study had $P<3.1\times10^{-13}$, which far exceeded the predetermined significance level ($P<3.1\times10^{-12}$). Our findings should not be generalized to more weakly associated eQTLs since they may have different regulatory mechanisms. Another limitation is that approximately half of the samples were collected from patients with a depressive disorder. However, analyzing healthy and depressive subjects separately also resulted in achieving high correlations (FDR-corrected $P<0.01$) for all the 1,554 SNP-probe pairs identified in the current study. Therefore, it is unlikely that depressive disorder has a major impact on gene expression regulation of the identified eQTL SNPs. Further investigation on the influence of depressive symptoms on gene expression levels is underway using a larger sample size.

In summary, we have presented the results on genome-wide investigations of SNPs affecting the expression levels in whole blood. Both *cis*-acting and *trans*-acting eQTL SNPs were identified for a total of 107 genes. The eQTL regions were widely distributed upstream, downstream, and within the gene sequence. The findings of this study are valuable if gene expression levels in whole blood are used as biomarkers for disease conditions. Gene expression levels and their connection with disease-associated SNPs may lead to a better understanding of genetic predisposition to disease and may be used to predict disease susceptibility. Further studies are required to clarify how SNPs function in

affecting the expression levels in whole blood as well as in other tissues.

Supporting Information

Table S1 Significant SNP-probe pairs. The SNP-probe pair selection procedure generated 1,554 significant pairs, consisted of 1,153 SNPs, defined as eQTL SNPs, and 185 probes.
(XLSX)

Table S2 Genes whose expression levels in whole blood are affected by SNPs. The names and properties of the 107 genes whose expression levels in whole blood were affected by SNPs are shown.
(XLSX)

Table S3 The Pearson's correlation coefficients between the eQTL SNPs and the expression levels of the corresponding genes in lymphoblastoid cell lines. A positive correlation coefficient indicates that the SNP has a similar effect on expression levels in whole blood and lymphoblastoid cell lines. Of the 86 *cis*-eQTL SNPs, 34 showed a significantly positive correlation, whereas 13 showed a significantly negative correlation with the expression levels of lymphoblastoid cell lines (FDR-corrected, $P<0.05$).
(XLSX)

Table S4 The results of the *cis*-only analysis. The *cis*-only analysis resulted in 3,883 SNP-probe pairs consisting of 3,161 SNPs and 347 probes.
(XLSX)

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Author Contributions

Conceived and designed the experiments: DS HH HK. Performed the experiments: DS SN RM NY. Analyzed the data: DS HH SN RM TT KH MO. Contributed reagents/materials/analysis tools: DS HH TT KH MO NY HK. Wrote the paper: DS TH NA HK.

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Negative correlation between cerebrospinal fluid oxytocin levels and negative symptoms of male patients with schizophrenia

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ABSTRACT

Background: Accumulating evidence indicates that oxytocin plays an important role in social interactions. Previous studies also suggest altered oxytocin function in patients with schizophrenia and depression. However, few studies have examined the central oxytocin levels in these disorders.
Methods: Cerebrospinal fluid (CSF) oxytocin levels were measured by ELISA in male participants consisting of 27 patients with schizophrenia, 17 with major depressive disorder (MDD), and 21 healthy controls.
Results: CSF oxytocin levels of patients with schizophrenia or MDD did not differ significantly with healthy controls. The antidepressant dose or the Hamilton depression rating scale score did not significantly correlate with the oxytocin levels in MDD patients. CSF oxytocin levels in schizophrenic patients significantly negatively correlated with second generation antipsychotic dose ($r=-0.49$, $P=0.010$) but not with first generation antipsychotic dose ($r=-0.13$, $P=0.50$). A significant correlation was observed between oxytocin levels and negative subscale of PANSS ($r=-0.38$, $P=0.050$). This correlation remained significant even after controlling for second generation antipsychotic dose ($r=-0.47$, $P=0.016$).
Conclusions: We obtained no evidence of altered CSF oxytocin levels in patients with schizophrenia or those with MDD. However, lower oxytocin levels may be related to higher second generation antipsychotic dose and more severe negative symptoms in schizophrenia.

1. Introduction

Oxytocin is produced in the supraoptic and paraventricular nuclei of hypothalamus and is secreted into the blood stream from the posterior pituitary. Its release is induced by a variety of stressful stimuli, including noxious stimuli, conditioned fear, and exposure to novel environments (Onaka, 2004). Accumulating evidence indicates that oxytocin plays an important role in social interactions (Lim and Young, 2006; Bartz et al., 2010). Deficits in social functioning observed in psychiatric disorders including schizophrenia (Couture et al., 2006; Sparks et al., 2010) and mood disorders (Inoue et al., 2004; Montag et al., 2010; Wolkenstein et al., 2011) imply the possible involvement of oxytocin in the pathophysiology of these disorders.

Many studies have investigated the possible link between oxytocin and psychiatric disorders. Some previous studies reported altered oxytocin function in patients with schizophrenia (Linkowski et al., 1984; Beckmann et al., 1985; Mai et al., 1993). Higher plasma oxytocin levels in schizophrenic patients were associated with lower symptom severity (Rubin et al., 2010). A clinical study showed that administration of this hormone ameliorated symptoms of schizophrenia (Feifel et al., 2010). In a preclinical study, systemically administered oxytocin reversed prepulse inhibition deficits induced by amphetamine and the phencyclidine analog in rats (Feifel and Reza, 1999). Oxytocin dysfunction has been implicated in the pathophysiology of depression as well. Two studies have shown that peripheral oxytocin levels and depressive symptoms were significantly correlated in patients with major depressive disorder (MDD) (Scantamburlo et al., 2007; Cyranowski et al., 2008). Moreover, oxytocin knock-out mice have shown dysregulated stress responses to psychological stimuli (Mantella et al., 2005) and enhanced anxiety behaviors (Mantella et al., 2003).

Oxytocin secreted from the pituitary gland generally does not re-enter the brain through the blood-brain barrier (Ermisch et al., 1985). Therefore, the behavioral effects of oxytocin are likely to be due to the release from centrally projecting oxytocin neurons. Since

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oxytocin in the nervous system can be transported to blood (Durham et al., 1991), peripheral oxytocin levels may reflect brain levels to some extent. However, central and peripheral oxytocin is regulated independently, and the half-life of oxytocin is less than 5 minutes in the blood (Ryden and Sjoholm, 1969) while that in the brain is 19.1 - minutes (Durham et al., 1991). Therefore, measurement in the CSF is necessary for the direct assessment of central oxytocin levels.

To our knowledge, two studies have previously examined the cerebrospinal fluid (CSF) levels of oxytocin in patients with schizophrenia. One reported elevated oxytocin levels in schizophrenia compared with controls (Beckmann et al., 1985), while the other did not obtain such a finding (Glovinsky et al., 1994). Only one study has examined the CSF levels of oxytocin in patients with depression, in which no difference was found compared with controls (Pitts et al., 1995). No study to date has examined the association of CSF oxytocin levels with symptom severity of these disorders. Since symptom severity forms a continuous spectrum ranging from mild to severe state, an association with the severity of the disease would suggest that oxytocin levels reflect the state of the disease.

In the present study, the oxytocin levels in the CSF of patients with schizophrenia and those with depression were measured and compared to that of healthy controls. Furthermore, we investigated the correlation between CSF oxytocin levels and symptom severity of these disorders. From the findings of previous studies examining peripheral oxytocin levels (Scantamburlo et al., 2007; Rubin et al., 2010), we hypothesized that CSF oxytocin levels would be lower in patient groups compared to healthy controls and that symptom severity would be negatively correlated with the oxytocin levels.

2. Materials and methods

2.1. Subjects

Participants were 27 patients with schizophrenia (mean age (standard deviation): 42.6 (8.5) years), 17 patients with major depressive disorder (MDD) (age: 39.5 (8.0) years), and 21 healthy controls (age: 38.3 (15.3) years). Demographic and clinical characteristics of the subjects are summarized in Table 1. All subjects were males to avoid gender effects and were biologically unrelated Japanese recruited from the outpatient clinic of the National Center of Neurology and Psychiatry Hospital, Tokyo, Japan or through advertisements in free local information magazines and by our website announcement. None of the healthy controls were on psychotropic medication, while 70.6% of the patients with MDD were treated with antidepressant medication at the time of the study. Most of the schizophrenic patients were prescribed antipsychotic medication, and all of those prescribed antipsychotics were on the medication for more than 3 years. Consensus diagnosis by at least 2 psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria (American Psychiatric Association, 1994), on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers with no current or past history of psychiatric treatment and were screened using the Japanese version of the Mini International Neuropsychiatric Interview (Sheehan et al., 1998; Otsubo et al., 2005) by a research psychiatrist to eliminate the possibility of any axis I psychiatric disorders. Participants were excluded if they had prior medical histories of central nervous system diseases or severe head injury or if they met the criteria for substance abuse or dependence or mental retardation. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After describing the study, written informed consent was obtained from every subject.

2.2. Clinical measures

Schizophrenic symptoms and depressive symptoms were assessed immediately after the lumbar puncture by an experienced research psychiatrist using the Japanese version of the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987; Yamada et al., 1991) and the Japanese version of the GRID Hamilton Depression Rating Scale, 17-item version (HAMD-17) (Hamilton, 1967), which have both been demonstrated to show good inter-rater reliability (Igarashi et al., 1998; Tabuse et al., 2007). Medication status at the time of lumbar puncture was recorded. Daily doses of antipsychotics in patients with schizophrenia and antidepressants in patients with MDD were

Table 1
Demographic and clinical characteristics.

	Controls ($N=21$)	Schizophrenia ($N=27$)	Depression ($N=17$)	Analysis
Age (years)	38.3 (15.3)	42.6 (8.5)	39.5 (8.0)	ANOVA: $F=0.97$, n.s.
BMI	23.9 (4.1)	26.0 (6.2)	23.9 (4.5)	ANOVA: $F=1.06$, n.s.
Duration of illness (years)		16.3 (9.8)	7.7 (7.3)	t-test: $t=2.8$, $P<0.01$
Treatment duration (years)		15.5 (9.1)	5.8 (6.9)	t-test: $t=3.4$, $P<0.01$
Medication status				
on antipsychotic medication				
first generation (%)	0	59.3	11.8	
second generation (%)	0	66.7	23.5	
first and/or second generation (%)	0	96.3	35.3	
on antidepressant medication (%)	0	25.9	70.6	
on benzodiazepine medication (%)	0	81.5	76.5	
on mood stabilizer medication (%)	0	14.8	5.9	
CP equivalent dose				
first generation (mg/day)		361.8 (445.0)		
second generation (mg/day)		402.4 (498.3)		
total (mg/day)		764.2 (591.6)		
IMI equivalent dose (mg/day)			167.2 (141.5)	
PANSS				
Positive symptoms score		12.5 (3.8)		
Negative symptom score		16.0 (5.8)		
General symptom score		6.8 (1.3)		
Total score		55.6 (12.6)		
HAMD-17 score			13.4 (9.6)	

Values are shown as mean (standard deviation).
BMI: body mass index; CP: chlorpromazine; IMI: imipramine.
PANSS: Positive and Negative Syndrome Scale; HAMD-17: 17 item Hamilton Rating Scale for Depression.
ANOVA: analysis of variance; n.s.: not significant.

converted to chlorpromazine and imipramine equivalent doses, respectively, using published guidelines (Inagaki et al., 1999).

2.3. Lumbar puncture and oxytocin assay

Lumbar puncture was performed with the subject in the left decubitus position. CSF was withdrawn from the L3–L4 or L4–L5 interspace. After the removal of 2 ml of CSF, a further 6 ml of CSF was collected and immediately transferred on ice to be centrifuged at 4 °C and aliquoted for storage at −80 °C until assay. CSF oxytocin levels were analyzed using a commercial ELISA kit (Enzo Life Sciences, INC., NY). Using the results from two separate runs of standard concentrations, the inter-assay coefficient of variation (CV) was less than 10%.

2.4. Statistical analysis

Statistical differences between groups were calculated using Student's *t*-test, Welch's *t*-test, or one-way analysis of variance (ANOVA). Correlations were assessed using Pearson's correlation coefficient. Since the CSF oxytocin levels were not normally distributed, log transformation was applied prior to statistical analyses to achieve normal distribution. Because previous studies suggest that some antipsychotic and antidepressant medications increase oxytocin secretion (Uvnas-Moberg et al., 1992, 1999), chlorpromazine and imipramine equivalent doses were examined as possible confounders. Statistical analyses were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS Japan, Tokyo, Japan). All statistical tests were two-tailed, and $P<0.05$ indicated statistical significance.

3. Results

Fig. 1 shows the CSF oxytocin levels in each diagnostic group. A one-way ANOVA using the transformed oxytocin levels as the dependent variable indicated no significant difference between diagnostic groups ($F=1.08$, $P=0.35$). The transformed oxytocin levels showed no significant correlation with age or body weight. Figs. 2 and 3 show the relation of CSF oxytocin levels with symptom severity and psychotropic dose, respectively. The antidepressant dose or the HAMD-17 score did not significantly correlate with the transformed oxytocin levels in patients with MDD (antidepressant dose: $r=-0.15$, $P=0.57$; HAMD-17: $r=-0.19$, $P=0.46$). The transformed oxytocin levels were significantly negatively correlated with negative subscale of PANSS ($r=-0.38$, $P=0.050$). Correlations between transformed oxytocin levels and other subscales of PANSS were not statistically significant. The transformed oxytocin levels in schizophrenic patients were significantly negatively correlated with chlorpromazine equivalents of total antipsychotic dose ($r=-0.51$, $P=0.0064$) and second generation antipsychotic (SGA) dose ($r=-0.49$, $P=0.010$) but not with chlorpromazine equivalents of first generation antipsychotic (FGA) dose ($r=-0.13$, $P=0.50$). Those prescribed SGA had significantly lower CSF oxytocin levels compared to those not prescribed SGA (Welch's *t* test: $t=2.6$, $df=10.4$, $P=0.024$). Comparison between patients prescribed and not prescribed FGA did not yield significant difference (Student's t test: $t=1.1$, $df=25$, $P=0.27$). Although none of the subscales of PANSS were correlated with FGA, SGA, or total chlorpromazine equivalent dose in the present study (all $P>0.1$), a previous study (Sim et al., 2009) reported an association between antipsychotic dose and the severity of positive as well as negative symptoms of schizophrenia. Therefore, we considered antipsychotic dose as a confounding factor for the association between oxytocin levels and symptom severity. Thus, we also examined the correlation between the oxytocin levels and PANSS scores controlling for prescribed antipsychotic dose. Partial correlation between transformed oxytocin levels and negative subscale of PANSS, removing the linear effects of total antipsychotic dose, was statistically significant ($r=-0.39$, $P=0.047$). Removing the linear effects of SGA dose instead of total antipsychotic dose also resulted in significant correlation of transformed CSF oxytocin levels with negative subscale ($r=-0.47$, $P=0.016$) as well as with total PANSS score ($r=-0.47$, $P=0.016$). SGA dose-controlled partial correlations between transformed oxytocin levels and other subscales of PANSS were not statistically significant (positive subscale: $r=-0.24$, $P=0.23$; general subscale: $r=-0.33$, $P=0.099$).

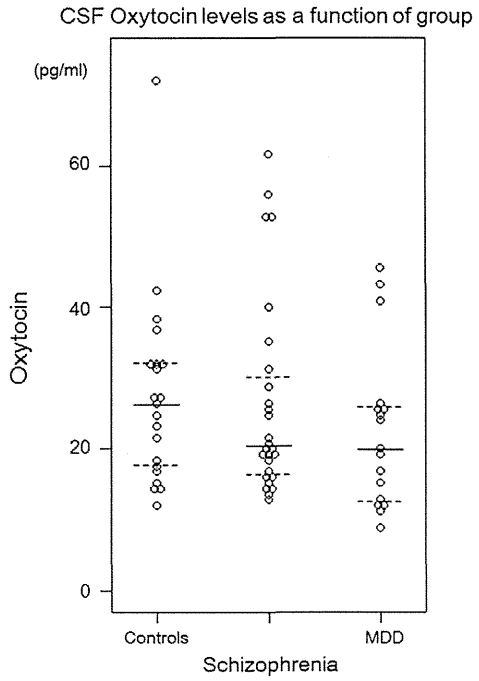

Fig. 1. Cerebrospinal fluid oxytocin levels as a function of group. The cerebrospinal fluid oxytocin levels in healthy controls and patients with schizophrenia and major depressive disorder are shown. Solid bars indicate median values and the dotted lines indicate interquartile range. No significant difference was observed between the diagnostic groups.

4. Discussion

Consistent with some previous studies (Glovinsky et al., 1994; Pitts et al., 1995), CSF oxytocin levels did not significantly differ between healthy controls and patients with schizophrenia and MDD. However, the present results showed that higher levels of CSF oxytocin may be associated with less severe symptoms of schizophrenia.

The observed negative correlation between antipsychotic dose and CSF oxytocin levels points to the possibility that antipsychotic medication lowers oxytocin levels. A recent study suggests that an inhibitory feedback loop may exist between prolactin-secreting lactrophs and oxytocinergic paraventricular neurons (Sirzen-Zelenskaya et al., 2011). Therefore, the disinhibition of prolactin secretion due to the D_2 receptor blockade by antipsychotics may have resulted in the suppression of oxytocin secretion. This, however, does not explain the stronger correlation of SGA dose compared to FGA dose. Kiss et al (2010) showed that SGAs have a more potent influence than haloperidol on the activity of oxytocin magnocellular neurons. This also seems contradictory to the present finding that SGA is negatively correlated with oxytocin levels. An alternative explanation for this negative correlation is that patients with low oxytocin levels may respond poorly to antipsychotic medication, and thus, higher dose was prescribed to such patients. Nevertheless, despite the relatively strong correlation with the antipsychotic dose, the cross-sectional design of the present study hinders any causal inferences. One previous study (Glovinsky et al., 1994) demonstrated that CSF oxytocin levels were unchanged by antipsychotic medication. Thus, further investigation is necessary to elucidate the effects of antipsychotic medication on oxytocin levels.

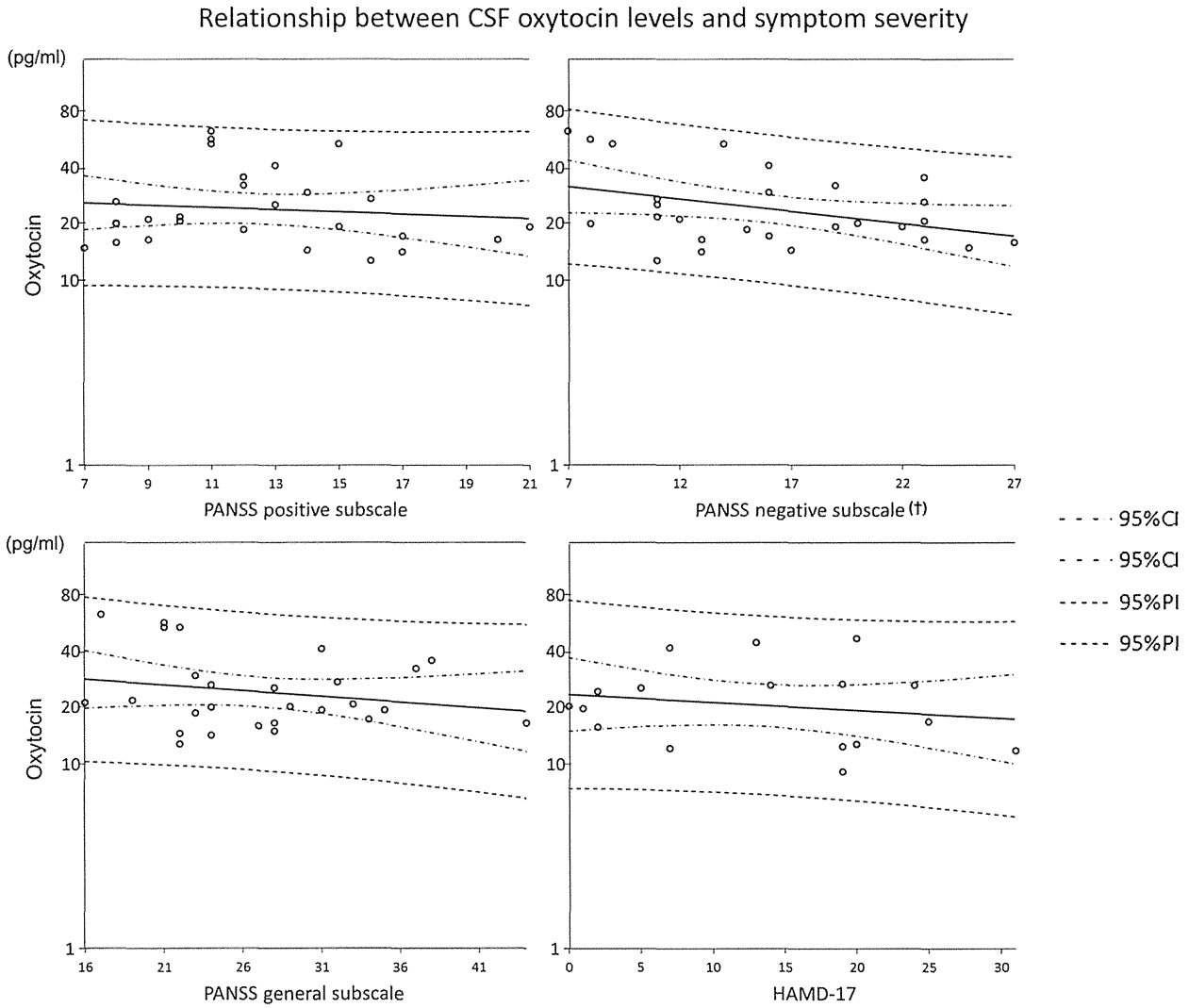

Fig. 2. Relationship between cerebrospinal fluid oxytocin levels and symptom severity. The association between cerebrospinal oxytocin levels and symptom severity is shown. Oxytocin levels are shown in logarithmic scale. Solid lines indicate fitted regression lines, unevenly dashed lines indicate 95% confidence intervals, and evenly dashed lines indicate 95% prediction intervals. (†): Correlation at significance level of $P<0.05$. PANSS: Positive and Negative Syndrome Scale, HAMD-17: Hamilton Depression Rating Scale, 17-item version, 95%CI: 95% confidence interval, 95%PI: 95% prediction interval.

The present results showed that the negative symptoms of schizophrenia were negatively correlated with CSF oxytocin levels. The correlation coefficient between CSF oxytocin levels and total PANSS score was also significant, controlling for SGA dose. Rubin et al. (2010) reported that higher peripheral oxytocin levels were associated with more prosocial behaviors in female patients with schizophrenia. Furthermore, previous studies have demonstrated improvement of social behaviors with administration of intranasal oxytocin (Macdonald and Macdonald, 2010; Pedersen et al., 2011). Since strong relationships between negative symptoms and social difficulties have been demonstrated in schizophrenia (Weinberg et al., 2009), the present finding associating higher CSF oxytocin levels with lower negative subscale is in accord with what has previously been described for peripheral oxytocin. Whether the peripheral oxytocin levels reflect the CSF oxytocin levels, or whether a different mechanisms of action in the brain and the peripheral result in a similar effect, remains to be explored.

Previous studies examining CSF oxytocin levels in patients with schizophrenia (Beckmann et al., 1985; Glovinsky et al., 1994) and depression (Pitts et al., 1995) showed mean oxytocin levels of less than 10 pg/ml, which is lower than that in the present study (>20 pg/ml). Such outcome may have resulted from some of the methodological differences between previous studies and the present one. Previous three studies measured oxytocin levels using radioimmunoassay (RIA), while the present study used a commercially available ELISA kit. A recent study that used the same ELISA kit to measure CSF oxytocin levels (Heim et al., 2009) also demonstrated higher levels of oxytocin (mean oxytocin levels of 17 pg/ml in women without a history of emotional abuse) compared to the previous studies using RIA. Thus, the different measurement techniques may have influenced the values.

A number of other methodological differences exist between the present study and previous ones examining CSF oxytocin levels (Beckmann et al., 1985; Glovinsky et al., 1994; Pitts et al., 1995). One of the major differences was that the present study did not require fasting prior to lumbar puncture, while Beckmann et al (Beckmann et al., 1985) collected CSF in patients with schizophrenia after 12 hours fasting. Although a previous study (Challinor et al., 1994) reported that peripheral oxytocin levels were not affected by 20 hours of fasting, the influence of fasting on CSF levels is unknown. Furthermore, Beckmann et al used Research Diagnostic Criteria to select a patient group consisting entirely of paranoid schizophrenia. Such difference in composition of participants may have affected the outcome of the study by Beckmann et al (1985), which showed significantly higher CSF oxytocin levels in schizophrenic patients compared to healthy controls. The findings by Glovinsky et al (1994) and Pitts et al (1995) were consistent with the present study in that no significant difference in CSF oxytocin levels was found between patients and controls. However,

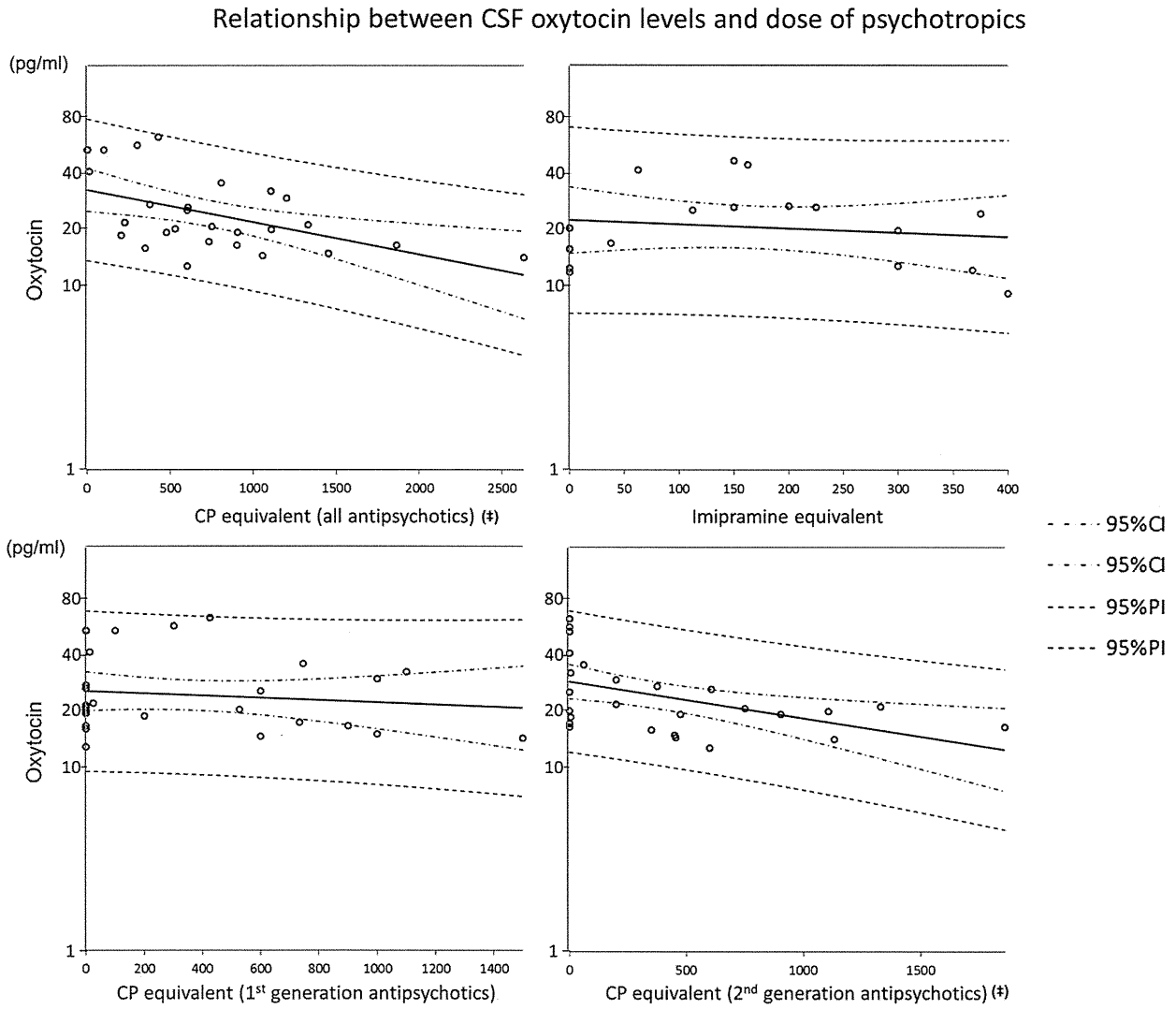

Fig. 3. Relationship between cerebrospinal fluid oxytocin levels and dose of psychotropics. The association between cerebrospinal oxytocin levels and dose of psychotropics is shown. Oxytocin levels are shown in logarithmic scale. Solid lines indicate fitted regression lines, unevenly dashed lines indicate 95% confidence intervals, and evenly dashed lines indicate 95% prediction intervals. (‡): Correlation at significance level of $P<0.01$. CP equivalent: chlorpromazine equivalent, 95%CI: 95% confidence interval, 95%PI: 95% prediction interval.

participants in these studies also differed from that of the present study in that both genders were included. Furthermore, MDD patients in the study by Pitts et al (1995) all scored 18 or above on the HAMD-17, while the MDD patients in the present study included those in a remitted state. These differences in composition of study samples should be carefully considered when comparing findings across studies.

Some limitations must be considered when interpreting the results of this study. First, the effects of medication could not be fully controlled due to the variability in types and doses. Future studies should examine oxytocin levels in untreated patients to elucidate the role of oxytocin in the pathophysiology of schizophrenia and depression. Treatment duration may also affect oxytocin levels. However, since all of the schizophrenic patients that were prescribed antipsychotics were on chronic treatment with the medication, treatment duration is unlikely to have confounded the main findings of the present study. Secondly, as mentioned above, the cross-sectional design did not allow for any definitive conclusions regarding the causal relationship between the CSF oxytocin levels, psychotropic medication, and symptom severity. Thirdly, only male participants were included in the present study. Previous studies suggest that effects of peripheral and intranasal oxytocin may differ between men and women (Domes et al., 2010; Rubin et al., 2010, 2011). Therefore, the present findings cannot be generalized to women. Finally, the risk of type II error was high due to the small sample size. The sample size in the present study was comparable to those of the previous studies that examined CSF oxytocin levels in patients with schizophrenia and depression (Beckmann et al., 1985; Glovinsky et al., 1994; Pitts et al., 1995). However, the power to detect a moderate difference (effect size of 0.50) in CSF oxytocin levels between patients and controls was relatively low (schizophrenia: 39%; MDD: 32%; calculated by G*Power 3.1.3 (Faul et al., 2007)). A larger sample may be necessary to detect small to moderate change in CSF oxytocin levels in psychiatric disorders.

In conclusion, we obtained no evidence of altered CSF oxytocin levels in patients with schizophrenia or those with MDD. However, lower CSF oxytocin levels may be related to higher SGA dose and more severe negative symptoms in schizophrenia, which is in line with the possibility that central oxytocin may ameliorate the severity of some symptoms of schizophrenia by improving social functioning.

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no role in the study design; the collection, analysis and interpretation of data, in the writing of the report; and in the decision to submit the paper for publication.

Contributors

Daimei Sasayama and Kotaro Hattori designed the study. Daimei Sasayama, Kotaro Hattori, and Toshiya Teraishi performed the lumbar punctures. Daimei Sasayama, Kotaro Hattori, Toshiya Teraishi, Hiroaki Hori, Miho Ota, Sumiko Yoshida, Kunimasa Arima, and Hiroshi Kunugi screened and diagnosed the study participants. Daimei Sasayama wrote the draft of the manuscript. Hiroshi Kunugi supervised the writing of the paper. Teruhiko Higuchi and Naoji Amano gave critical comments on the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

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Proteomic Analysis of Lymphoblastoid Cells Derived from Monozygotic Twins Discordant for Bipolar Disorder: A Preliminary Study

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Abstract

Bipolar disorder is a severe mental illness characterized by recurrent manic and depressive episodes. In bipolar disorder, family and twin studies suggest contributions from genetic and environmental factors; however, the detailed molecular pathogenesis is yet unknown. Thus, identification of biomarkers may contribute to the clinical diagnosis of bipolar disorder. Monozygotic twins discordant for bipolar disorder are relatively rare but have been reported. Here we performed a comparative proteomic analysis of whole cell lysate derived from lymphoblastoid cells of monozygotic twins discordant for bipolar disorder by using two-dimensional differential in-gel electrophoresis (2D-DIGE). We found approximately 200 protein spots to be significantly differentially expressed between the patient and the co-twin (*t* test, $p<0.05$). Some of the proteins were subsequently identified by liquid chromatography tandem mass spectrometry and included proteins involved in cell death and glycolysis. To examine whether these proteins could serve as biomarkers of bipolar disorder, we performed Western blot analysis using case–control samples. Expression of phosphoglycerate mutase 1 (PGAM1), which is involved in glycolysis, was significantly up-regulated in patients with bipolar disorder (*t* test, $p<0.05$). Although PGAM1 cannot be regarded as a qualified biomarker of bipolar disorder from this preliminary finding, it could be one of the candidates for further study to identify biomarkers of bipolar disorder.

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Introduction

Bipolar disorder is a severe mental illness characterized by recurrent manic and depressive episodes. It affects approximately 1% of the general population. In bipolar disorder, contributions of genetic and environmental factors have been indicated by family and twin studies [1]. Patients with this disease have a high relapse rate, and lifelong treatment with mood stabilizers such as lithium is often needed [2]. Past studies of bipolar disorder have focused on monoamines and intracellular signal transduction pathways as related to the effects of psychotropic drugs [3,4], but recent studies focus more on neuroplasticity or resilience based on reports of neuroprotective effects of mood stabilizers [5]. Recent genome-wide association studies identified new candidate genes, such as *CACNA1C* [6,7], but the effects are relatively small.

Bipolar disorder is often misdiagnosed as depression or schizophrenia, and delayed diagnosis and treatment worsen the course of illness [8]. Thus, early diagnosis is important to prevent deterioration; however, no biomarkers for bipolar disorder are available yet. Identification of biomarkers would be indispensable for early diagnosis.

Much research on biomarkers in psychiatric diseases has been published [9,10]. Though several candidate biomarkers were identified from postmortem brains, these studies are undermined by many confounding factors, including cause of death, postmortem interval, and brain pH [11]. Postmortem interval affects degradation of mRNAs and protein levels [12,13,14,15] and significantly influences phosphorylation of signaling proteins [16]. In particular, the influence of medication cannot be ignored because a number of proteins are affected by mood stabilizers, antipsychotics, or antidepressant medication [17,18,19]. Additionally, large interindividual variations hamper the identification of biomarkers.

Biomarker research in other tissues such as serum, plasma, cerebrospinal fluids, saliva, and urine have also been performed [20,21] but has not led to a diagnostic test.

The concordance rate of bipolar disorder between monozygotic twins is approximately 70% [22,23]. By comparing monozygotic twins discordant for bipolar disorder, biochemical differences associated with bipolar disorder might be detected without interference from interindividual genetic variation [24].

Although several genome, transcriptome, and epigenome analyses in monozygotic twins discordant for bipolar disorder and other diseases have been reported [24–29], proteomic analysis has not been applied to identifying the difference between

monozygotic twins discordant for bipolar disorder, possibly due to technical difficulties. Because transcript levels do not completely correlate with protein expression levels [30,31] and aberrant post-translational modifications can cause disease, proteomic analysis is needed to supplement transcriptome and epigenome analyses.

In the present study, we performed proteomic analysis of lymphoblastoid cells derived from monozygotic twins discordant for bipolar disorder.

Materials and Methods

Subjects

For 2D-DIGE, we used lymphoblastoid cells derived from a pair of 42-year-old male monozygotic twins discordant for bipolar disorder. We initially examined their monozygosity by genotyping microsatellite repeat markers [25] and later confirmed it by single nucleotide polymorphism array [29].

For a case–control study of differentially expressed proteins using Western blot analyses, we used lymphoblastoid cells derived from eight unrelated patients with bipolar I disorder (BPI) (four men and four women, 35.6±9.0 years old [mean ± SD], Japanese) and eight unrelated control subjects (six men and two women, 36.9±10.0 years old [mean ± SD], Japanese). Table 1 gives detailed information for each subject used in Western blot analysis. Patients and controls with a history of alcohol or illicit drug abuse were excluded from the study. The patients were treated with various medications. Diagnoses were made by the consensus of two senior psychiatrists using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. Controls were selected from among students, nurses, office workers, and doctors in participating institutes, their friends, and other volunteers. A senior psychiatrist interviewed control subjects and found that they did not have major mental disorders. Written informed consent was obtained from all subjects. The ethics committees of RIKEN approved the study.

Cell culture and extraction of proteins

Lymphocytes were separated from the peripheral blood and transformed by Epstein-Barr virus using previously described standard techniques [32]. These cells were cultured and kept frozen until experiments. Lymphoblastoid cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Sigma-Aldrich), 50 U/mL penicillin, 50 μg/mL streptomycin (GIBCO, Invitrogen/Life Technologies Corporation, Grand Island, NY), and 60 μg/mL tylosin solution (Sigma-Aldrich). Cells were cultured at approximately 1×10^8 cells. Total proteins were extracted from the lymphoblastoid cells using the Q-proteome mammalian protein preparation kit (Qiagen, QIAGEN, Hilden, Germany). After the supernatant was precipitated with acetone, the pellet was dissolved using the lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM Tris-HCl [pH 8.5]) (GE Healthcare Bio-Sciences, San Francisco, CA, USA).

Two-dimensional difference gel electrophoresis (2D-DIGE) and imaging analyses

Protein concentration was determined by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA) and the Pierce 660 nm protein assay (Pierce, Rockford, IL, USA). Proteins were set to a final concentration of 5 mg/mL with the lysis buffer and labeled separately with 400 pmol of CyDye (Cy3 or Cy5) (GE Healthcare Bio-Sciences), vortexed, and incubated on ice in the dark for 30 min. A mixed sample composed of equal amount of proteins from the patient and the co-twin was labeled with Cy2 and used as an internal standard. After 30 min, the labeling reaction was stopped with 10 mM lysine. To avoid the possible effect of labeling efficiency, the dyes were swapped for each experiment using three gels. Labeled proteins were subjected to SDS-PAGE analysis, and the gels were scanned with the Typhoon 9400 scanner (GE Healthcare Bio-Sciences) at the wavelengths

Table 1. Characteristics of the subjects.

ID	Age	Sex	Diagnosis	Age at onset	Family history of bipolar disorder within first degree relatives	Medication
B1	40	F	Bipolar I disorder with psychotic features	24	Yes	Li, VPA, CBZ, HP, CP
B2	23	F	Bipolar I disorder	23	No	Li, VPA, PAR
B3	46	F	Bipolar I disorder	27	Yes	VPA, THY
B4	26	M	Bipolar I disorder, rapid cycling	24	Yes	Li
B5	38	M	Bipolar I disorder	35	No	VPA, OLA
B6	40	M	Bipolar I disorder	25	No (three had major depression)	Li, VPA
B7	27	F	Bipolar I disorder with psychotic features	24	No	Li, VPA
B8	45	M	Bipolar I disorder	20	No	Li
C1	36	M	-		No	-
C2	29	M	-		No	-
C3	34	M	-		No	-
C4	29	M	-		No	-
C5	27	F	-		No	-
C6	52	M	-		No	-
C7	52	M	-		No	-
C8	33	F	-		No	-

Li: lithium carbonate, VPA, sodium valproate, CBZ, carbamazepine, HP, haloperidol, CP, chlorpromazine, PAR, paroxetine, THY, levothyroxine, OLA, olanzapine.
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Table 2. Proteins differentially expressed in monozygotic twin discordant for BP identifed by LC-MS/MS.

			Exp.1		Exp.2		Exp.3		Exp.4	
			Fold change		Fold change		Fold change		Fold change	
Gene names	Acc. No.	Protein names	(BP/CT)	*p* – value	(BP/CT)	*p* – value	(BP/CT)	*p* – value	(BP/CT)	*p* – value
PSME1	Q06323	Proteasome activator complex subunit 1	−1.42	0.00013	−1.44	0.00018	−1.3	0.0079	−1.91	0.0056
RPLP0	P05388	60S acidic ribosomal protein P0	−1.34	0.00031	−1.39	0.03	−1.45	0.0053	−1.31	0.0059
TPI1	P00939	Triosephosphate isomerase	1.95	0.034	3.17	7.10E-05	1.68	0.015	1.3	0.015
ALDOC	P09972	Fructose-bisphosphate aldolase C	−1.47	0.0024	−1.35	0.0013	−1.26	0.0059		
ANXA4	P09525	Annexin A4	−1.96	0.00022	−1.62	0.00011	−1.35	0.00029		
PGAM1	P18669	Phosphoglycerate mutase 1	1.9	0.0023	1.59	0.0001			2.12	0.0035
WARS	P23381	Tryptophanyl-tRNA synthetase, cytoplasmic	1.6	0.00037	1.55	0.00023			1.28	0.00062
ACADS	P16219	Short-chain specific acyl-CoA dehydrogenase, mitochondrial					−1.56	0.00014	−1.37	0.0006
ALDH2	P05091	Aldehyde dehydrogenase, mitochondrial					−1.26	0.018	−1.29	0.01
ALDOA	P04075	Fructose-bisphosphate aldolase A			1.48	0.0023			1.29	0.0055
ANXA5	P08758	Annexin A5					−1.25	0.0046	−1.29	0.007
APOA1BP	Q8NCW5	Apolipoprotein A-I-binding protein	2.56	1.90E-06	2.16	0.00023				
ATP5A1	P25705	ATP synthase subunit alpha, mitochondrial	−1.61	0.00048			−1.33	6.70E-06		
C19orf10	Q969H8	UPF0556 protein C19orf10	1.66	0.001	2.16	0.0012				
CACYBP	Q9HB71	Calcyclin-binding protein			−1.99	0.00075	−11.74	0.00023		
CAPZB	P47756	F-actin-capping protein subunit beta	−1.56	0.00021	−1.36	7.10E-06				
CASP3	P42574	Caspase-3	1.49	0.00011	1.44	0.0054				
CMPK1	P30085	UMP-CMP kinase	2.9	1.40E-05	3.22	0.00029				
CORO1A	P31146	Coronin-1A	1.31	0.0013	1.36	0.0029				
DNAJB11	Q9UBS4	DnaJ homolog subfamily B member 11	1.37	0.00032	1.41	0.0052				
ECH1	Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.91	0.0014	1.74	0.00016				
ENO1	P06733	Alpha-enolase	−1.62	0.049	−1.58	0.012				
ETFB	P38117	Electron transfer flavoprotein subunit beta	1.27	0.019	1.36	0.00099				
GALE	Q14376	UDP-glucose 4-epimerase	−1.35	0.0051			−1.33	0.044		
GAPDH	P04406	Glyceraldehyde-3-phosphate dehydrogenase	−1.38	0.0038			−1.33	0.044		
HIST2H4B	P62805	Histone H4	2.07	0.0038	1.95	0.0014				
HNRNPM	P52272	Heterogeneous nuclear ribonucleoprotein M					1.46	0.0033	1.4	0.0023
HSPA5	P11021	78 kDa glucose-regulated protein	3.18	0.00012	3.02	5.50E-06				
HSPB1	P04792	Heat shock protein beta-1	1.58	0.0001	1.46	0.0071				
LDHA	P00338	L-lactate dehydrogenase A chain			1.26	0.035			5.6	0.0042
LGALS3	P17931	Galectin-3	−1.78	0.00033	−1.99	0.00075				
NANS	Q9NR45	Sialic acid synthase	1.46	0.0012	1.62	1.60E-05				
NDUFS3	O75489	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	1.58	0.0001	1.46	0.0071				
NPM1	P06748	Nucleophosmin	−1.53	0.00074	−1.42	0.0042				
OTUB1	Q96FW1	Ubiquitin thioesterase OTUB1	1.52	6.00E-05	1.47	0.0043				
P4HB	P07237	Protein disulfide-isomerase	1.47	0.00017	1.69	0.0029				
PACAP	Q8WU39	Plasma cell-induced resident endoplasmic reticulum protein	2	0.011	2.01	0.0077				
PCBP1	Q15365	Poly(rC)-binding protein 1	−1.47	0.0024	−1.35	0.0013				

Table 2. Cont.

			Exp.1		Exp.2		Exp.3		Exp.4	
			Fold change		Fold change		Fold change		Fold change	
Gene names	**Acc. No.**	**Protein names**	**(BP/CT)**	***p* – value**	**(BP/CT)**	***p* – value**	**(BP/CT)**	***p* – value**	**(BP/CT)**	***p* – value**
PDIA3	P30101	Protein disulfide-isomerase A3	1.98	8.60E-06	2.09	0.0017				
PDIA6	Q15084	Protein disulfide-isomerase A6	1.58	0.00042	1.75	1.20E-05				
PHGDH	O43175	D-3-phosphoglycerate dehydrogenase	2.05	0.003	3.13	0.0001				
PITHD1	Q9GZP4	PITH domain-containing protein 1	2.15	0.012	2.13	0.011				
PKM2	P14618	Pyruvate kinase isozymes M1/M2			1.36	0.0029			1.36	0.045
PNPO	Q9NVS9	Pyridoxine-5′-phosphate oxidase	−1.74	0.0085	−1.41	0.001				
POLR2E	P19388	DNA-directed RNA polymerases I, II, and III subunit RPABC1	1.65	0.00025	2.12	0.00013				
PRDX2	P32119	Peroxiredoxin-2	2.9	1.40E-05	3.22	0.00029				
PSMB1	P20618	Proteasome subunit beta type-1	−1.77	0.0093			−1.29	0.00032		
SARS	P49591	Seryl-tRNA synthetase, cytoplasmic	1.31	0.0013	1.36	0.0029				
SERPINB1	P30740	Leukocyte elastase inhibitor	1.37	0.00032	1.41	0.0052				
SSR4	P51571	Translocon-associated protein subunit delta	1.67	0.0059	1.77	0.00018				
STMN1	P16949	Stathmin	−2.02	6.90E-05	−1.53	3.70E-05				
UCHL1	P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	2.5	1.90E-05	2.16	0.00023				
VDAC1	P21796	Voltage-dependent anion-selective channel protein 1	1.43	0.0013	2.29	0.00023				

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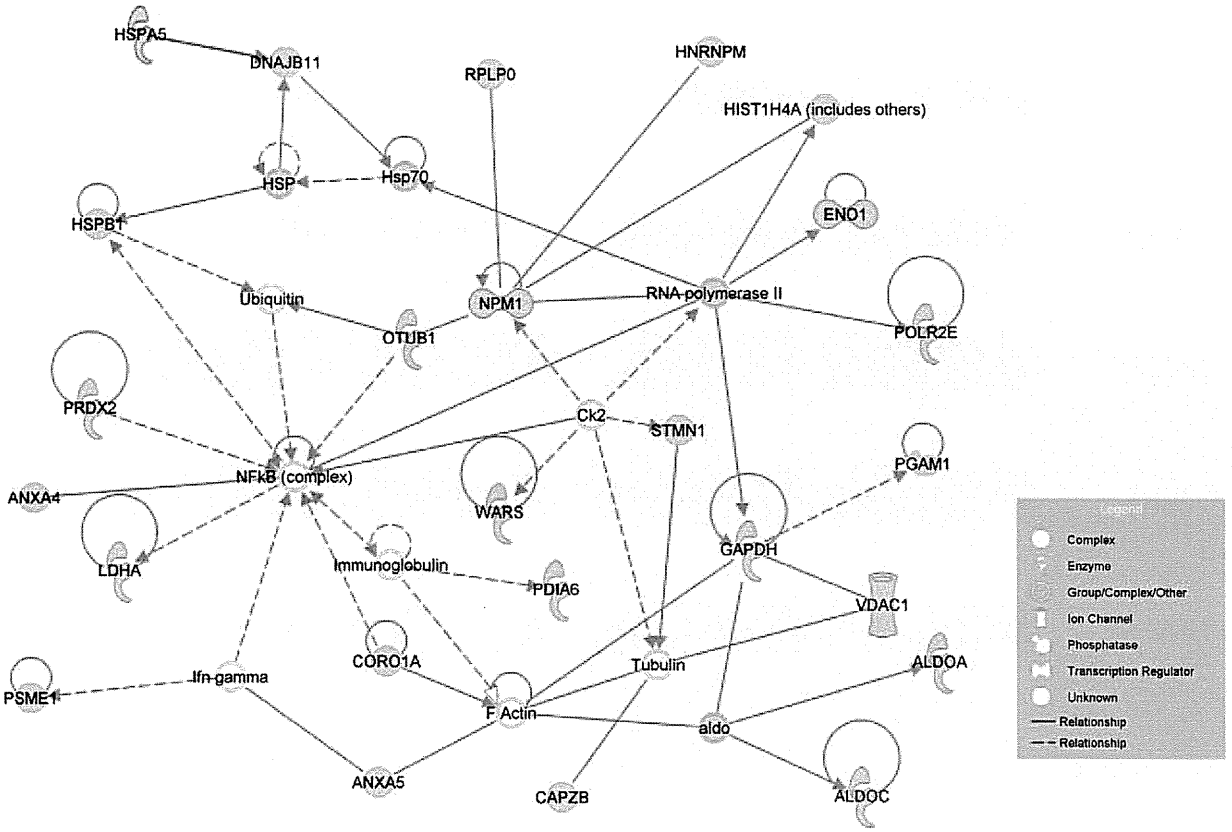
corresponding to each CyDye, namely 480 nm (Cy2), 532 nm (Cy3), and 633 nm (Cy5). A 50 μg portion of each Cy3-, Cy5-, and Cy2-labeled sample was combined. Nonlinear IPG strips (pH 3–10, 18 cm long; GE Healthcare Bio-Sciences) were rehydrated for 12 h at 50 mA per strip with the sample solution on an IPGphor isoelectric focusing unit (GE Healthcare Bio-Sciences). Three mixed samples were separated by isoelectric focusing on an IPGphor isoelectric focusing unit at 0.5 V·h, 0.8 V·h, 13.5 kV·h, and 21 kV·h at 20°C and a maximum current setting of 50 μA per strip. The second dimension was run on 12.5% acrylamide gels in a SE600 (GE Healthcare Bio-Sciences) at 45 mA per gel. To avoid artifacts, three gels of same condition were simultaneously run for each experiment. Gels were scanned directly between low-fluorescence glass plates with the Typhoon 9400 (GE Healthcare Bio-Sciences) scanner at the three wavelengths specific for the CyDyes. The resolution was approximately 100 μm. Determination of protein spot abundance was performed using the DeCyder 2D Ver. 6.0 software (GE Healthcare Bio-Sciences). Spots were automatically detected. Spot editing (separation of two spots) or deleting (artifacts) was performed manually. The three CyDye–labeled forms of each spot were co-detected within each gel. Ratios between sample and internal standard abundances were calculated for each protein spot with the Differential In-gel Analysis (DIA) module. Inter-gel variability was corrected by matching and normalization of the internal standard spot maps by the Biological Variance Analysis (BVA) module of the DeCyder software and incorrectly matched spots were manually eliminated or corrected if possible. During the spot detection, the estimated number of spots was set at 4000. Protein spots that showed a statistically significant intensity in Student's *t* test were accepted as being differentially expressed between the extracts under comparison among these. Protein spots showing at least 1.25-fold changes ($p<0.05$) in intensity were selected for next steps.

Protein identification by mass spectrometry and database search

The preparative gels were stained with a SYPRO® ruby (Invitrogen/Life Technologies Corporation) and scanned with the Typhoon 9400. Protein spots that showed differences in relative fluorescence were excised from the gel using the automated spot picker (GE Healthcare Bio-Sciences). The picked gel pieces were destained with 50% CH_3CN in a 50 mM NH_4HCO_3 solution. After removal of the supernatant, cysteine residues were reduced with dithiothreitol and carbamidomethylated with iodoacetamide. In-gel trypsin digestion was performed at 37°C overnight, using sequencing grade modified trypsin (Promega, Southampton, UK) reconstituted in 100 mM NH_4HCO_3. The trypsinized gel was rinsed three times in extraction buffer (5% trifluoroacetic acid in 50% CH_3CN and 50% H_2O). The trypsinized peptides solution was dried by speed vacuum, suspended in 2% CH_3CN with 0.1% trifluoroacetic acid, and analyzed by LTQ (Fisher Scientific, Waltham, MA) liquid chromatography/linear ion trap mass spectrometry (LC-MS/MS) system. Their corresponding proteins were searched using the program Mascot database-searching software (Matrix Science, London, UK), which accesses protein identification by matching mass spectroscopy data with the protein databases NCBI (http://www.ncbi.nlm.nih.gov) and UniProt (http://www.uniprot.org/uniprot). Identification criteria included a Mascot score >45 (selected based on a corrected *p*-value <0.05).

a



b

Category	*P*-value range	Molecules
Carbohydrate Metabolism	8.38E-10 - 3.27E-02	14
Cell Death	2.04E-05 - 4.72E-02	20

Figure 1. Gene networks showing interrelationships between differentially expressed genes in the twin with bipolar disorder.
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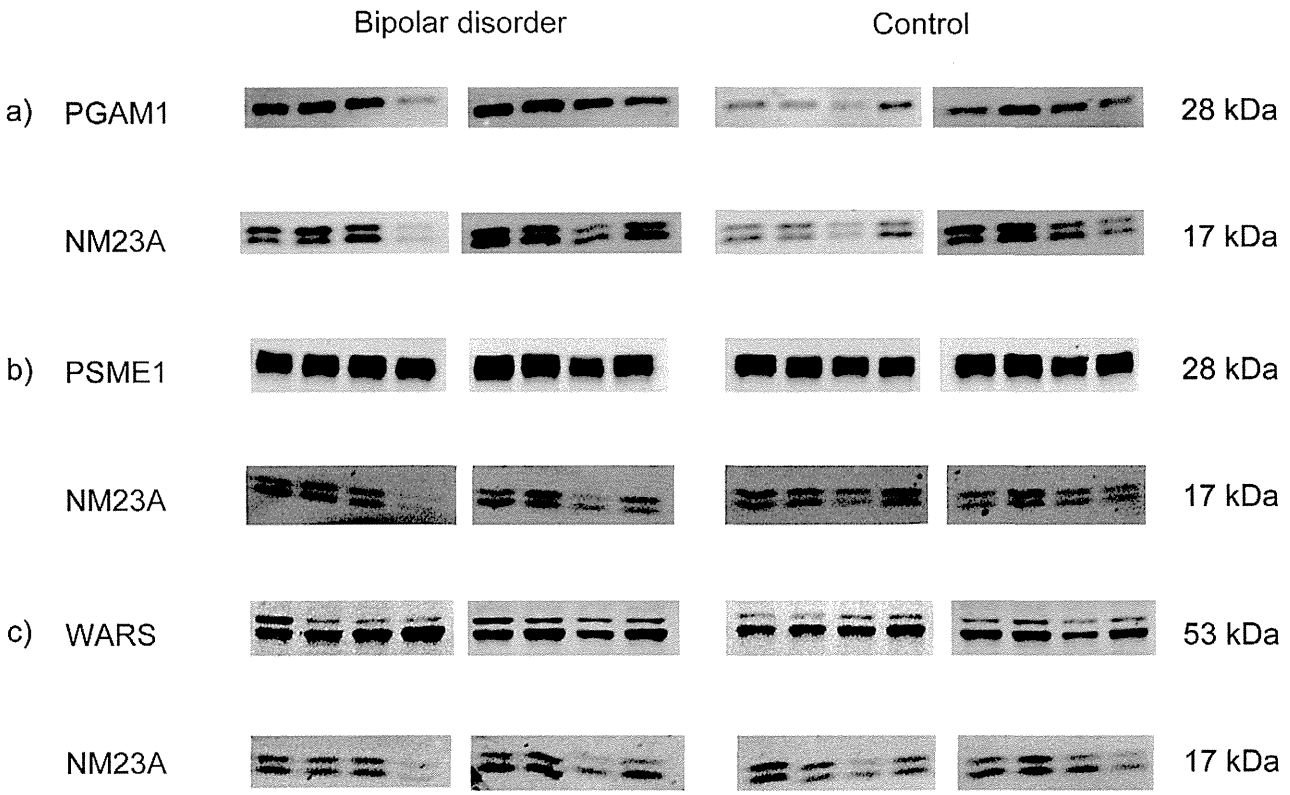
Functional grouping of altered proteins

The IPA software (Ingenuity Systems, Redwood City, CA) was used to identify the key biological relationships and functions of differentially expressed proteins and their interaction networks. For pathway analysis, Swiss-Prot and GenBank accession numbers were used to catalogue the identified protein into known interaction pathways based upon the Ingenuity Pathway Knowledge Base (IPKB). IPA classification data were derived from the published literature in a systematic way using a comprehensive ontology of functional annotations and protein–protein interaction data. The most significant interaction networks, biological functions and pathway associated with the differentially expressed proteins were identified. To confirm biological functions and gene ontology annotation between identified proteins, we used bioinformatics resources, PANTHER (http://www.pantherdb.org/) and DAVID (http://david.abcc.ncifcrf.gov/). DAVID program uses a modified Fisher's exact p value (EASE score) to rank gene clusters by statistical overrepresentation of individual genes, based on the co-occurrence/enrichment of the category within the gene list relative to all genes in the same category on the study.

Western blot analyses for validation

Lymphoblastoid cells in a case–control study were individually cultured. Total proteins were individually extracted using Q-proteome Mammalian Protein prep kit (Qiagen), and protein



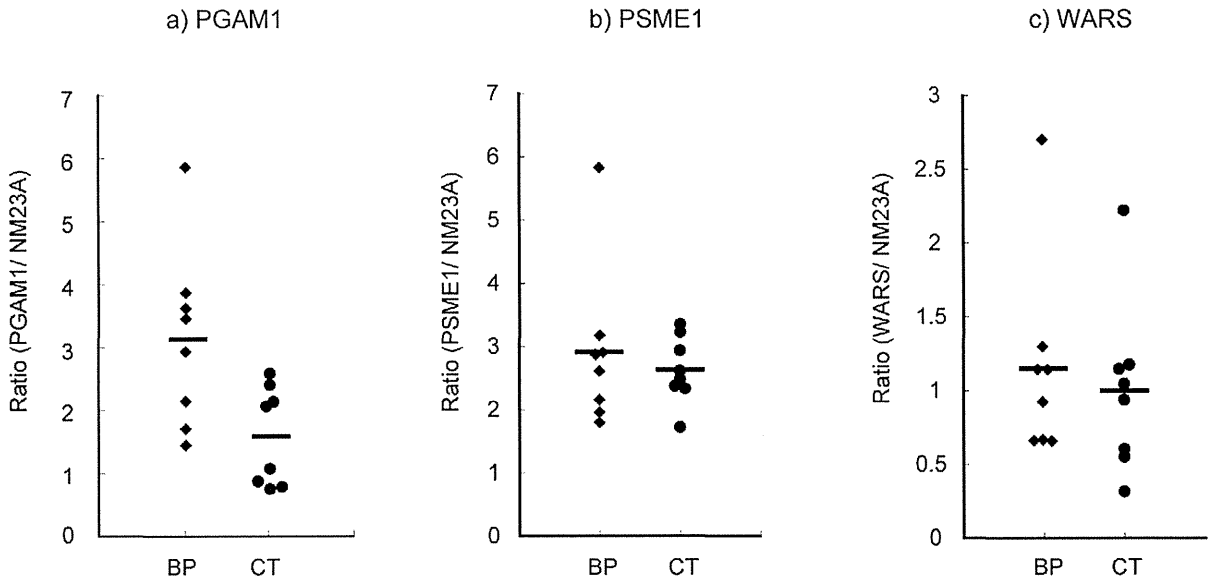


Figure 2. Protein expression validation by Western blot analysis with an internal standard, NM23A. Proteins from each of the eight pairs of samples were separated by SDS-PAGE and transferred to PVDF membranes. Proteins were immunodetected using the respective primary antibodies and fluorescent secondary antibodies. Signals were captured with FX and signal intensity and shown by the. a) PGAM1, b) PSME1, c) WARS. Scatter plots show the ratio of each protein to an internal standard protein, NM23A, measured by densitometric scanning of the band

intensities. The *p* values were calculated using a *t* test in all proteins. Number of the subjects is 8 for bipolar disorder and 8 for controls, respectively. The absolute band intensity for the PGAM1 was also significantly higher in patients with bipolar disorder (0.93+/−0.23 [mean +/− standard deviation] [arbitrary unit]) than control subjects (0.39+/−0.18, $p<0.0005$).
doi:10.1371/journal.pone.0053855.g002

concentrations were determined by the methods mentioned above. Equal concentration (5 μg per lane) of proteins from control and case samples were separated by 12% or 4–15% SDS-polyacrylamide gel electrophoresis and transferred onto Immun-Blot PVDF membranes (Bio-Rad) using a mini Trans-blot Cell (Bio-Rad). After transfer, the blotted membrane were blocked with 4% w/v ECL Advance Blocking Reagent (GE Healthcare Bio-Sciences) in phosphate-buffered saline containing 0.1% Tween20 (PBST) (MP Biomedicals Inc., Santa Ana, CA) at 4°C overnight and incubated with primary antibody in PBST with 4% w/v skim milk for 1 h at room temperature. PSME1, WARS, and PGAM1 were chosen for quantification by Western blot analysis. As an internal control, NM23A was used because there was no significant difference of protein levels of NM23A between patient and the co-twin in 2D-DIGE. Primary antibodies were as follows: rabbit antibody against human PSME1 (Calbiochem, La Jolla, CA), mouse antibody against human WARS (Abnova, Taipei City, Taiwan), goat antibody against human PGAM1 (Novus Biologicals, Littleton, CO), and rabbit monoclonal and polyclonal against human NM23A (Abcam, Cambridge, MA). The blotted membranes were washed in TBST and incubated at room temperature with each secondary antibodies, Alexa Fluor 488–labeled donkey anti-goat IgG antibody (Molecular Probes/Life Technologies Corporation), Cy5-conjugated affiniPure donkey anti rabbit IgG antibody and Cy3-conjugated affiniPure donkey anti mouse IgG antibody (Jackson Immuno Research, West Grove, PA). The membranes were directly scanned with the Molecular Imager FX (Bio-Rad). Protein bands were analyzed to give a quantitative estimation of intensity change using the Quantity One Software (Bio-Rad) adapted to the Molecular Imager FX. To estimate the relative molecular weight of each protein, molecular markers, Dual Color Precision Plus Protein Standards (Bio-Rad) and ECL Plex Fluorescent Rainbow Markers (GE Healthcare Bio-Sciences) were used. Preliminary experiments indicated that amounts of these proteins in the lysates of lymphoblastoid cells were within the linear range of detection.

Results

Detection of spots differentially expressed between monozygotic twins discordant for bipolar disorder by 2D-DIGE

First, we extracted total protein from cultured lymphoblastoid cells derived from a pair of monozygotic twins discordant for bipolar disorder. The total protein for each twin was separately labeled with different CyDyes (Cy3 or Cy5), and dyes were swapped between gels. A mixed sample composed of an equal amount of proteins from the patient and the co-twin was labeled with Cy2 and used as an internal standard. These processes minimized gel-to-gel variation and improved protein spot statistics at the analysis stage. These labeled proteins were mixed and analyzed by 2D-DIGE. To detect robust differences between the patient and the co-twin, we performed 2D-DIGE. To avoid artifacts, four gels of same condition were simultaneously run for each experiment. Three gels were used as analytical gels to detect differentially expressed spots between the patient and the co-twin, and the remaining gel was used as preparative gel for picking out differentially expressed spots. We performed 2D-DIGE and liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses in quadruplicate experiments using protein samples independently extracted from different aliquots of cell culture.

Using 2D-DIGE and DeCyder Ver.6.0 image analysis software, approximately 3200 protein spots were separated (3220±51 [mean ± SD]). The observed spot pattern images in each gel were very similar among quadruplicate experiments. The protein spots were selected if the intensity difference between the affected and nonaffected twin was larger than 1.25-fold (absolute value >1.25 or absolute value <-1.25). Approximately 200 spots (211±115, quadruplicate) were found to be significantly differentially expressed ($p<0.05$, Student's *t* test) between the twins per experiment.

Identification of spots by LC-MS/MS

The proteins in preparative gels were stained by SYPRO Ruby after electrophoresis, and the preparative gels were performed matching to analytical gels. The largest 100 spots out of the 200 differentially expressed protein spots were picked from each preparative gel and were considered suitable for subsequent analysis by LC-MS/MS. The spots were chosen sequentially from those with a large absolute value fold change. The 68 spots, averaged for quadruplicate values, were successfully identified as unique proteins through LC-MS/MS and are listed in Table 2. Since Epstein-Barr virus–transformed lymphoblastoid cells were used in this study, all immunoglobulin and B-cell–related proteins were removed from the analysis. Moreover, we also removed keratin and trypsin-related proteins because of the possibility of experimental contamination. Table 2 shows only proteins identified by LC-MS/MS in common with each experiment. Proteins identified twice or more with Mascot search results (ion scores of higher than 45) included PSME1, RPLP0, TPI1, ALDOC, ANXA4, PGAM1, and WARS. Fifty-three proteins had high ion scores and were identified at least twice in four experiments.

Functional grouping of altered proteins

To explore the biological function (protein-interaction network) of the 53 differentially expressed proteins, we performed protein classification using the Ingenuity Pathway Knowledge Base software. A data set containing the gene symbols by The HUGO Gene Nomenclature Committee was uploaded into the application. Each protein identifier was converted to its gene identification and mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base. These genes were overlaid onto a global molecular network developed from information contained in the Ingenuity pathways analysis (IPA) knowledge base, which is based entirely on findings reported in the literature. Networks of these focus genes were algorithmically generated based on their connectivity. One of the 53 proteins was omitted because it was not included in the database, and the remaining 52 proteins were mapped onto mainly three networks. The largest network, having the highest score, is associated with carbohydrate metabolism, neurological disease, and skeletal and muscular disorders (Fig. 1A). This network includes ALDOA, ALDOC, ANXA4, ANXA5, CAPZB, CORO1A, DNAJB11, ENO1, GAPDH, HIST1H4A (includes others), HNRNPM, HSPB1, LDHA, NPM1, OTUB1, PDIA6, PGAM1, POLR2E, PRDX2, PSME1, RNA polymerase II, RPLP0, STMN1, VDAC1, and WARS. Next, the 53 proteins were classified according to biological function and canonical