Identification of IL6 as a susceptibility gene for major depressive disorder

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Our previous work implied that interleukin 6 (IL6) may be a biological marker for major depressive disorder (MDD). In this study, we performed a comprehensive genetic study to determine the association between the gene encoding IL6 (IL6) and MDD in Han Chinese. There were 50 drug-naive MDD patients and 50 healthy controls undergoing an mRNA expression study. A sample of 772 patients with MDD and 759 healthy controls were used for genetic analysis. Next, we performed an eQTL analysis to identify whether risk SNP(s) is associated with IL6 expression in brain. Our results showed that patients with MDD have higher levels of IL6 than healthy controls (P = 0.008). The SNP rs1800797 has a significant association with MDD (P = 0.01) in a dominant model. The eQTL analysis showed a marginally significant association between the rs1800797 and IL6 expression in the frontal cortex (P = 0.087). Our preliminary findings are suggestive of an association between rs1800797 and the risk of MDD. Further investigations are required to evaluate this association in larger samples to increase statistical power, and to examine the correlation between rs1800797 and IL6 methylation patterns.

Major depressive disorder (MDD) is a severe mental disorder, typically characterized by a cluster of emotional and somatic symptoms at psychological level1, and abnormal brain connectivity and functioning at physical level2. MDD is prevalent in excess of 17% among Han Chinese3 and also becoming a leading cause of disability and mortality in the worldwide. Thus, it is one of the most urgent challenges for current psychiatric research to understand the pathogenesis of MDD.

It has been confirmed that the heritability of MDD is estimated up to 70%4 and recent studies into its genetic etiology have detected a number of susceptibility genes and chromosomal regions implicated with immune system5,6. Clinical studies have widely demonstrated aberrant inflammation profiles of MDD patients in either central neural system (CNS) or peripheral tissues7,8. Therefore, one emerging hypothesis for this association is that chronic low-grade activation of inflammation and the immune system likely contribute to some of the biological mechanisms in the development of MDD9.

Our previous work using whole-genome cRNA microarrays found that genes associated with MDD were enriched in interleukin 6 (IL6) -mediated signaling events10. Similar results were also reported in the Netherlands11. IL6 is a multifunctional cytokine that regulates the growth and differentiation of various tissues, and plays an important role in the immune response and acute phase reactions12. Goldsmith et al.13 performed a meta-analysis of blood cytokines in 18 studies for MDD. The authors observed enhanced level of IL6 in acutely MDD patients, and following treatment for acute phase, IL6 level significantly decreased in patients with MDD. In CNS, IL6 acts as a neurotrophic cytokine expressed in both neurons and glia14, whose level is also reported to be increased in the cerebrospinal fluid of MDD patients15. Such findings provided suggestive evidence for the role of IL6 in the pathophysiology of MDD.

At the molecular level, the human gene encoding IL6 (IL6) is mapped to chromosome 7p15, a candidate region previously implicated in MDD16. Unsurprisingly, genetic variations of IL6 have been reported to modulate the chronic stress exposure in the development of depressive symptoms17, and increase the risk of interferon-induced depression18. Based on this premise, it is plausible that IL6 is likely to be a promising candidate gene for MDD susceptibility.

In this study, we hypothesized that IL6 may be a susceptibility gene for MDD. First, we analyzed the IL6 mRNA expression difference between drug-naive MDD patients and normal controls. Given the relevance of genetic variations of IL6 to depressive symptoms, we subsequently investigated whether IL6 is genetically associated with MDD among a Chinese Han population. As a third aim, we performed an eQTL (expression quantitative trait loci) analysis via an available database to investigate the potential role of the risk SNP in IL6 mRNA expression in brain.
of being reduced to a normal level after antidepressant treatment as the depressive symptoms improved.23,24. Taken together, the above evidence implies that increased functional polymorphism in the promoter of IL6 is associated with a number of human diseases, such as asthma, type II diabetes and leprosy that were reported to have a high morbidity prevalence with MDD.29–31. SNP rs1800797 presents in the promoter region of IL6. Variations in this region may lead to functional alteration of IL6expression in frontal cortex that those without A allele.

IL6, a key proinflammatory cytokine, has been reported in the development of MDD.21 It is suggested that increased IL6 expression may have a regulatory effect on IL6 expression in the frontal cortex and individuals with A allele of rs1800797 have higher IL6 expression in frontal cortex and ultimately influence the occurrence of MDD.20 Higher levels of IL6 expression in MDD group than that in control one. This is in line with previous reports.22,23 On the other hand, the elevated level of IL6 in MDD patients can be reduced to a normal level after antidepressant treatment as the depressive symptoms improved.23,24. Taken together, the above evidence implies that increased IL6 expression is possibly involved in the etiology of MDD.

In the first step, we tested the levels of IL6 mRNA expression in drug-naïve patients with MDD and healthy controls. Our results showed a significant higher level of IL6 expression in MDD group than that in control one. This is in line with previous reports.22,23. On the other hand, the elevated level of IL6 in MDD patients can be reduced to a normal level after antidepressant treatment as the depressive symptoms improved.23,24. Taken together, the above evidence implies that increased IL6 expression is possibly involved in the etiology of MDD.

Figure 1. Expression levels of IL6 mRNA in peripheral blood in drug-naïve patients with major depressive disorder and healthy controls. IL6 mRNA was normalized to that of GAPDH. MDD, major depressive disorder patients (n = 50); CTL, control controls (n = 50).
resonance imaging (MRI) to investigate the alterations in the cortical surface of MDD, and our findings suggest that frontal cortical alteration is a vulnerability to MDD during earlier neurodevelopmental process. A functional MRI experiment showed that MDD patients exhibit abnormal long distance connectivity and dysregulation of large-scale neural networks in medial prefrontal cortex. A number of positron emission tomography (PET) studies have repeatedly identified a decrease in metabolic activity in the prefrontal cortex in patients with MDD. Hence, abnormalities in prefrontal cortex may be important for investigations of the pathophysiology of MDD. Furthermore, Setiawan et al. applied PET to examine a marker of neuroinflammation, translocator protein (TSPO) binding in vivo, in order to determine the neuroinflammatory hypothesis of MDD. Their results showed that the magnitude of TSPO density elevation was 26% in the prefrontal cortex of patients with MDD than that of controls. This suggests that neuroinflammation activation leading to abnormal function in prefrontal cortex may contribute to the development of MDD. As such, it is remain unknown whether rs1800797 polymorphism is responsible for the MDD-related neuroinflammation in frontal cortex, and this will subsequently need to be investigated in future. Meanwhile, literature indicated that gene expression of IL6 is regulated by DNA methylation of its promoter region. The region from positions −666 to −426 relative to the transcription start site in IL6 may be the potential binding sites for methylation. SNP rs1800797 consists of a G to A substitution at the −597 site. We speculated that DNA methylation may explain the underlying mechanism of rs1800797 in the etiology of MDD. This also requires for further clarification.

There are two limitations to the present study. As known that MDD is a mental disorder that originates from brain dysfunction, we measured the peripheral level of IL6 mRNA expression in this study. Thus, further analyses using brain tissues are needed to validate our results. Second, our results showed that the A allele of rs1800797 was found in 4 out of 759 controls. Although the HWE P value for this SNP in controls is 0.94, such a low

| SNP      | Genotype | P    | OR (95% CI) | P   | n   | Allele | OR (95% CI) | P   |
|----------|----------|------|-------------|-----|-----|--------|-------------|-----|
| rs1800797| AA       | 0.002| 4.76 (1.61–14.07) | 0.002 | 1544 | A      | 1525 (98.8) | 4.72 (1.60–13.89) | 0.002 |
|          | AG       |      |             |     |     | G      |             |     |
|          | GG       |      |             |     |     | C      |             |     |
| Control  | 759      |      |             |     |     |        |             |     |
|          | 0        | 0    | 753 (97.5)  | 0.002 | 1544 | 19 (1.2) | 4 (0.3)     | 1518 |
|          | 4 (0.5)  | 755 (99.5) | NA     | NA  | 0.94 | 1518 |
|          | 4 (0.3)  | 1514 (99.7) |       |     |     |
| rs1800796| GG       | 0.48 | 1.06 (0.87–1.30) | 0.57 | 1544 | 458 (29.7) | 1086 (70.3) | 1.01 (0.86–1.18) | 0.93 |
|          | GC       |      |             |     |     | G      |             |     |
|          | CC       |      |             |     |     | C      |             |     |
|          |          |      |             |     |     |        |             |     |
| Case     | 759      |      |             |     |     |        |             |     |
|          | 0        | 0    | 756 (97.9)  | 0.06 | 1544 | 16 (1.0) | 752 (99.1) | 2.26 (0.93–5.51) | 0.07 |
|          | 7 (0.9)  | 752 (99.1) | NA     | NA  | 0.90 | 7 (0.5)     | 1518 |
|          | 4 (0.3)  | 1514 (99.7) |       |     |     |
| rs2069837| GG       | 0.70 | 1.06 (0.86–1.30) | 0.60 | 1544 | 313 (20.3) | 1231 (79.7) | 1.03 (0.86–1.23) | 0.76 |
|          | GA       |      |             |     |     | G      |             |     |
|          | AA       |      |             |     |     | A      |             |     |
|          |          |      |             |     |     |        |             |     |
| Case     | 759      |      |             |     |     |        |             |     |
|          | 0        | 0    | 481 (62.3)  | 0.68 | 1544 | 470 (30.4) | 1074 (69.6) | 1.00 (0.86–1.17) | 1.00 |
|          | 22 (2.8) | 360 (46.6) | 1.04 (0.85–1.27) | 0.72 | 1544 | 313 (20.3) | 1231 (79.7) | 1.03 (0.86–1.23) | 0.76 |
| rs1524107| CC       | 0.86 | 0.86 (0.86–1.54) | 0.66 | 1518 | 301 (19.8) | 1217 (80.2) | 1.00 (0.86–1.17) | 1.00 |
|          | CT       |      |             |     |     | C      |             |     |
|          | TT       |      |             |     |     | C      |             |     |
|          |          |      |             |     |     |        |             |     |
| Case     | 759      |      |             |     |     |        |             |     |
|          | 0        | 0    | 483 (63.6)  | 0.27 | 1544 |
|          | 7 (0.9)  | 752 (99.1) | NA     | NA  | 0.90 | 7 (0.5)     | 1518 |
|          | 4 (0.3)  | 1514 (99.7) |       |     |     |
| rs1800797| GG       | 0.88 | 0.61–1.28 | 0.51 | 1518 |
|          | GA       |      |             |     |     | A      |             |     |
|          | AA       |      |             |     |     | C      |             |     |
|          |          |      |             |     |     |        |             |     |
| Control  | 759      |      |             |     |     |        |             |     |
|          | 0        | 0    | 361 (47.6)  | 0.28 | 1518 |
|          | 64 (8.4) | 462 (30.4) | 1056 (69.6) | 1.00 (0.86–1.17) | 1.00 |
|          | 334 (44.0) | 361 (47.6) | 0.88 (0.61–1.28) | 0.51 | 1518 |

Table 1. Comparison of genotype and allele frequencies of IL6 SNPs between MDD and control groups.

aP values in dominant model. bP values in recessive model. cP values for Hardy-Weinberg equilibrium in control group.
frequency in a small sample size could potentially bias the HWE and dilute the statistical power. Taking it into consideration, our findings should be considered only preliminary.

In conclusion, we performed a comprehensive analysis to detect the role of IL6 on the pathophysiology of MDD in Chinese Han population. Our preliminary findings are suggestive of an association between rs1800797 and the risk of MDD. Further investigations are required to evaluate this association in larger samples to increase statistical power, and to examine the correlation between rs1800797 and IL6 methylation patterns.

Methods

Participants. For the expression study, there were 50 drug-naïve MDD patients, and 50 healthy controls recruited from the Division of Mood Disorders, Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine. Demographic data on age, gender, smoking status, BMI, alcoholic abuse, duration of illness prior to admission, number of episode, family history of mood disorders was collected. Assessments of the Hamilton Rating Scale for Depression – 17 (HRSD-17) were conducted independently by two experienced psychiatrists (intrarater reliability, kappa = 0.84)44.

For the genetic study, we enrolled the MDD samples from our previous clinical trials: the "OPERATION" (OPTimized trEatment stRAtgeries for Treatment-resIstant depressiON) study42,43 and the "CARE-SSD/MDD" (Construct An Rough Evaluation index system for subsyndromal symptomatic depression and major depressiv disorder) study: All patients were diagnosed with MDD strictly according to The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. Standard diagnostic assessments were supplemented with clinical information obtained by a review of medical records and interviews with family informants. Patients were excluded on the following criteria: (1) those with a lifetime diagnosis of bipolar disorder, schizoaffective disorder, schizophrenia, or another psychotic disorder; as well as (2) female patients who were pregnant, planning to become pregnant, or breast-feeding during the study period.

Control subjects were enlisted from the hospital staff and students of the School of Medicine in Shanghai that were interviewed by a specialized psychiatrist with SCID-P. Subjects with any psychiatric disorder and chronic physical disease were excluded from our analysis44,45.

All the patients and control subjects were of Han Chinese origin from Shanghai. All procedures were reviewed and approved by Institutional Review Boards of Shanghai Mental Health Center and other participating institutions. This study was performed in accordance with the guidelines laid out in the Declaration of Helsinki as revised in 1989. All subjects were of Han Chinese origin and provided written informed consent before any study-related procedures were performed.

RNA preparation and Quantitative real-time polymerase chain reaction (qRT-PCR). On admission, 20 ml peripheral blood of fasting patients and healthy controls were collected between 07:00 am and 09:00 am, to avoid potential diurnal influence. RNA preparation was carried out as previously described46.

Relative IL6 mRNA expression levels were assessed by qRT-PCR with commercially available TaqMan gene expression assays for target gene IL6 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as reference gene (Applied Biosystems, CA, USA). All experiments were conducted as referring to our previous studies45,47,48. In each sample, the expression of IL6 was normalized to the expression of the reference gene GAPDH. Results were reported in fold change using $2^{-\Delta \Delta Ct}$.

SNP selection and Genotyping. We retrieved CHB data from the HapMap database (http://www.hapmap.org) and defined linkage disequilibrium (LD) blocks using Haploview 4.2 (Broad Institute, Cambridge, MA, USA) to set inclusion criteria for tagging SNPs. Haplotype-tagging single nucleotide polymorphisms (htSNPs) with $r^2$ cutoff > 0.8 and minor allele frequency (MAF) > 0.1 were selected. In total, there are two tag SNPs (rs1524107 and rs2069837) of IL6 selected for genotyping. Three functional SNPs (rs1800797, −597G/A, rs1800795, −174G/C) within IL6 were also examined in this study, because the activity of the promoter region of IL6 is affected by the polymorphisms49. Detailed information for these selected SNPs is shown in Supplementary Table S3.

Genomic DNA was isolated from whole blood using a Tiangen DNA isolation kit (Tiangen Biotech, Beijing, China). The five SNPs were detected using multiplex PCR and the SNaPshot assay. The detailed experiment procedures were described in our previous publication50. All of the sample call rates exceeded 99.7%. Of the collected samples, 10% were repeated for the genotyping assay to ensure quality-control, and the results were 100% concordant.

Brain eQTL analysis. Converging evidence suggests that MDD originates from abnormal brain functions51, and brain samples are presumably appropriate for eQTL analysis of risk SNP(s). Here, we performed an eQTL analysis to identify whether risk SNP(s) is associated with IL6 expression in brain, using the brain eQTL database (http://caprica.genetics.kcl.ac.uk/BRAINEAC/), a large exon-specific eQTL data set covering ten human brain regions. More detailed information can be found in the original study52.

Statistical analysis. The statistical differences in the characteristics between groups were compared using chi-square test or t test. For the expression analysis, ANCOVA was carried out with age, gender, BMI and smoking status as covariates controlled in the model, to minimize the potential effect of these factors on the expression levels of IL6 mRNA53. For the genetic analyses, HWE testing, genotype and allele frequency analyses were conducted using SHEsis (http://analysis.bio-x.cn)54. Pairwise linkage disequilibrium of all pairs of SNPs was assessed using Haploview 4.2 (Broad Institute, Cambridge, MA, USA)53, and the extent of linkage disequilibrium (LD) was measured by the standardized $D'$ and $r^2$. Odds ratios (ORs) and the corresponding 95% confidence intervals
(CIs) were used to measure the association of the selected SNPs with MDD in dominant and recessive models, respectively. Calculations were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). To adjust for multiple testing, the level of significance was corrected via Bonferroni correction. All tests were two-tails, and the significance level was set at 0.05.

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Author Contributions
C.Z. and Y.F. designed the study. C.Z., Z.W., G.Z. and F.W. acquired the data, which all of the authors analyzed. C.Z. drafted the manuscript. Y.F. supervised this work and edited the manuscript. All the authors critically reviewed the manuscript and gave final approval for its publication.

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