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Genetic risk of clozapine-induced leukopenia and neutropenia: a genome-wide association study

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Abstract

Background: Clozapine is considered to be the most effective antipsychotic medication for schizophrenia. However, it is associated with several adverse effects such as leukopenia, and the underlying mechanism has not yet been fully elucidated. The authors performed a genome-wide association study (GWAS) in a Chinese population to identify genetic markers for clozapine-induced leukopenia (CIL) and clozapine-induced neutropenia (CIN).

Methods: A total of 1879 patients (225 CIL cases, including 43 CIN cases, and 1,654 controls) of Chinese descent were included. Data from common and rare single nucleotide polymorphisms (SNPs) were tested for association. The authors also performed a trans-ancestry meta-analysis with GWAS results of European individuals from the Clozapine-Induced Agranulocytosis Consortium (CIAC).

Results: The authors identified several novel loci reaching the threshold of genome-wide significance level ($P < 5 \times 10^{-8}$). Three novel loci were associated with CIL while six were associated with CIN, and two T cell related genes (*TRAC* and *TRAT1*) were implicated. The authors also observed that one locus with evidence close to genome-wide significance ($P = 5.08 \times 10^{-8}$) was near the *HLA-B* gene in the major histocompatibility complex region in the trans-ancestry meta-analysis.

Conclusions: The associations provide novel and valuable understanding of the genetic and immune causes of CIL and CIN, which is useful for improving clinical management of clozapine related treatment for schizophrenia. Causal variants and related underlying molecular mechanisms need to be understood in future developments.

Introduction

Schizophrenia is a severe mental disorder accompanied by considerable morbidity and mortality, which affects

~1% of the population worldwide¹. The etiology of schizophrenia is still not well understood. Environmental and genetic factors play important roles in the development of schizophrenia². The heritability of schizophrenia is estimated to be 60–80%^{2,3}. Antipsychotic medications are commonly used to treat patients with schizophrenia, but responses to these drugs vary widely⁴. Although antipsychotics can relieve symptoms of psychosis, ~75% of patients discontinue their therapy due to adverse effects in two years⁵.

Clozapine is considered to be the most effective antipsychotic medication for schizophrenia and has a

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distinctive pharmacological profile in treatment-resistant schizophrenia⁶. However, it is also associated with several adverse effects such as weight gain, metabolic dysfunction, cardiovascular disease and leukopenia. Leukopenia is defined as white blood cell (WBC) count less than 4,000 cells per microliter. Neutropenia and agranulocytosis are different types of leukopenia with an absolute neutrophil count (ANC) less than 1,500 and 500 cells per cubic millimeter (mm^{-3}), respectively. Clozapine-induced agranulocytosis (CIA) is a severe leukopenia that may be life-threatening, first reported in Finland in 1974^{7,8}. Among patients taking clozapine, the cumulative risk of neutropenia is 3.8% and for agranulocytosis is 0.9%⁹.

There is evidence of a genetic contribution in the onset of clozapine-induced leukopenia (CIL), neutropenia (CIN) and CIA¹⁰; however, the underlying mechanism remains unclear and may involve multiple genes. The HLA (human leukocyte antigen) system, which locates in the major histocompatibility complex (MHC) region, has been reported to be associated with CIL^{11–13}. HLA genes such as *HLA-B* and *HLA-DQB1* were reported to be associated with CIL by Clozapine-Induced Agranulocytosis Consortium (CIAC) and many other researchers^{11–13}. Previous genome-wide association studies (GWAS) and whole-exome sequencing studies have indicated that other genes such as *SLCO1B3*, *SLCO1B7*, *UBAP2* and *STARD9* also play important roles in the pathophysiology of CIN¹². Most studies of CIN have focused on individuals of European ancestry. To generalize the results, some recent studies have begun to investigate patients from different ethnic groups. Ancestry-based differences in alleles may be part of the reason for different prevalence of CIL; for example, Duffy-null genotype has been found to be associated with benign neutropenia in patients with African ancestry¹⁴, and some previously reported SNPs have not been replicated in individuals of Japanese ancestry^{12,15}.

Clozapine has been available for Chinese patients since the 1970s. It was once a commonly used antipsychotic drug in China, prescribed for ~25–60% of patients with schizophrenia^{16,17}, and the reported prevalence of CIL in China is 3.92% (0.21% for CIA)^{16,18}. In this study, we report what we believe to be the first GWAS of CIL in a Chinese population, seeking to identify genetic determinants and provide more information on possible underlying etiology.

Methods and materials

Study design and participants

We analyzed genome-wide data of patients with schizophrenia receiving clozapine treatment from a Chinese cohort. Patients were recruited for this study from Wuhu Fourth People's Hospital and Guangxi Longquan Mountain Hospital from 2007 to 2012. The clinical interviews were conducted by two independent psychiatrists.

All patients had diagnoses of schizophrenia according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. Those patients with treatment-resistant schizophrenia¹⁹ were prescribed clozapine and 1,983 patients were included in this study. Those who developed $\text{WBC} < 4,000 \text{ mm}^{-3}$ during treatment with clozapine were defined as clozapine-induced leukopenia (CIL) cases ($n = 242$). Of these, 46 cases also developed $\text{ANC} < 1,500 \text{ mm}^{-3}$, and were thus defined as clozapine-induced neutropenia (CIN) cases. All controls ($n = 1,741$) received clozapine without developing $\text{WBC} < 4,000 \text{ mm}^{-3}$ or $\text{ANC} < 1,500 \text{ mm}^{-3}$.

We also performed a genome-wide meta-analysis of our Chinese sample with individuals of European ancestry from the Clozapine-Induced Agranulocytosis Consortium (CIAC)^{11,12}.

In accordance with the principles in Declaration of Helsinki, the study was approved by the Ethics Committee at the Bio-X Institutes of Shanghai Jiao Tong University and the written informed consent was obtained from each participant. It is confirmed that the research complied with the Guidance of the Ministry of Science and Technology (MOST) for the Review and Approval of Human Genetic Resources.

Procedures and statistical analysis

Details on genotyping and quality control procedures for the genome-wide analysis of Chinese samples are described in Supplementary Material.

We performed primary genome-wide association analysis for CIL. To minimize the effect for population stratification, we performed association analysis separately for two sample sets (according to genetic origin criteria), and then combined the summary in the meta-analysis. For the common variants (minor allele frequency [MAF] $\geq 1\%$), including both genotyped and imputed variants, association analyses were performed by logistic regression using imputed dosage files and the meta-analysis via METAL with a fixed-effects model. Heterogeneity among the sample collections in the meta-analysis was measured with the I^2 index and the p value, which was calculated with Cochran's Q test. For the rare variants, we restricted to the genotyped variants only, association and meta analyses were carried using PLINK with the Cochran–Mantel–Haenszel method.

We conducted secondary analyses on a subset of the more severely affected cases with $\text{ANC} < 1,500 \text{ mm}^{-3}$ (CIN). Controls were still those who received clozapine without developing $\text{WBC} < 4,000 \text{ mm}^{-3}$. Those who had a test result with $\text{ANC} \geq 1,500 \text{ mm}^{-3}$ and $\text{WBC} < 4,000 \text{ mm}^{-3}$ were excluded from the secondary analysis. All analyses performed were consistent with methods described above for CIL analysis.

We performed the trans-ancestry meta-analysis of our CIN results with data from the CIAC study^{11,12} for

common variants. We adopted a fixed-effects model implemented in RICOPIILI²⁰ for the analysis.

We annotated the genome-wide significant variants and their proxies ($r^2 \geq 0.8$ in EAS of 1000 Genomes Project Releases Phase 3) to explore putative causal variants and genes underlying these association signals. We also searched for evidence that the common variants were associated with expression of a particular gene in several expression quantitative trait locus (eQTL) datasets: the Genotype-Tissue Expression (GTEx, v7)²¹, immune cells²² and lymphoblastoid cell lines (LCLs)²³. Potential functional effects of the sentinel variants were predicted by the GWAVA (Genome Wide Annotation of Variants)²⁴, a web-based tool to prioritize the functional variations based on a wide range of annotations of non-coding elements (such as ENCODE/GENCODE, evolutionary conservation and GC-content).

Dual-luciferase reporter assays

An in vitro functional assay was used to investigate allele-dependent effects of the prioritized variants by GWAVA on gene transcription. Experiments were performed with human embryonic kidney (HEK293) cell line. Luciferase reporter constructs were cloned by using the pGL3-promoter vector (Promega, Madison, WI, USA) as a backbone. For each variant, two DNA fragments containing risk or nonrisk allele were produced and all constructs were verified via Sanger sequencing. The pGL3 reporter, risk and nonrisk vectors were transfected into HEK293 cells. Luciferase activities were assessed at 48-h post-transfection according to the manufacturer's protocols (Promega). The activity of firefly luciferase was normalized to that of Renilla luciferase to control for variations in the transfection efficiency between different wells. All assays were performed in three or more biological replicates in independent experiments, and two-tailed t-tests were performed to analyze statistical differences between experimental groups. Further details are provided in the Supplementary Material.

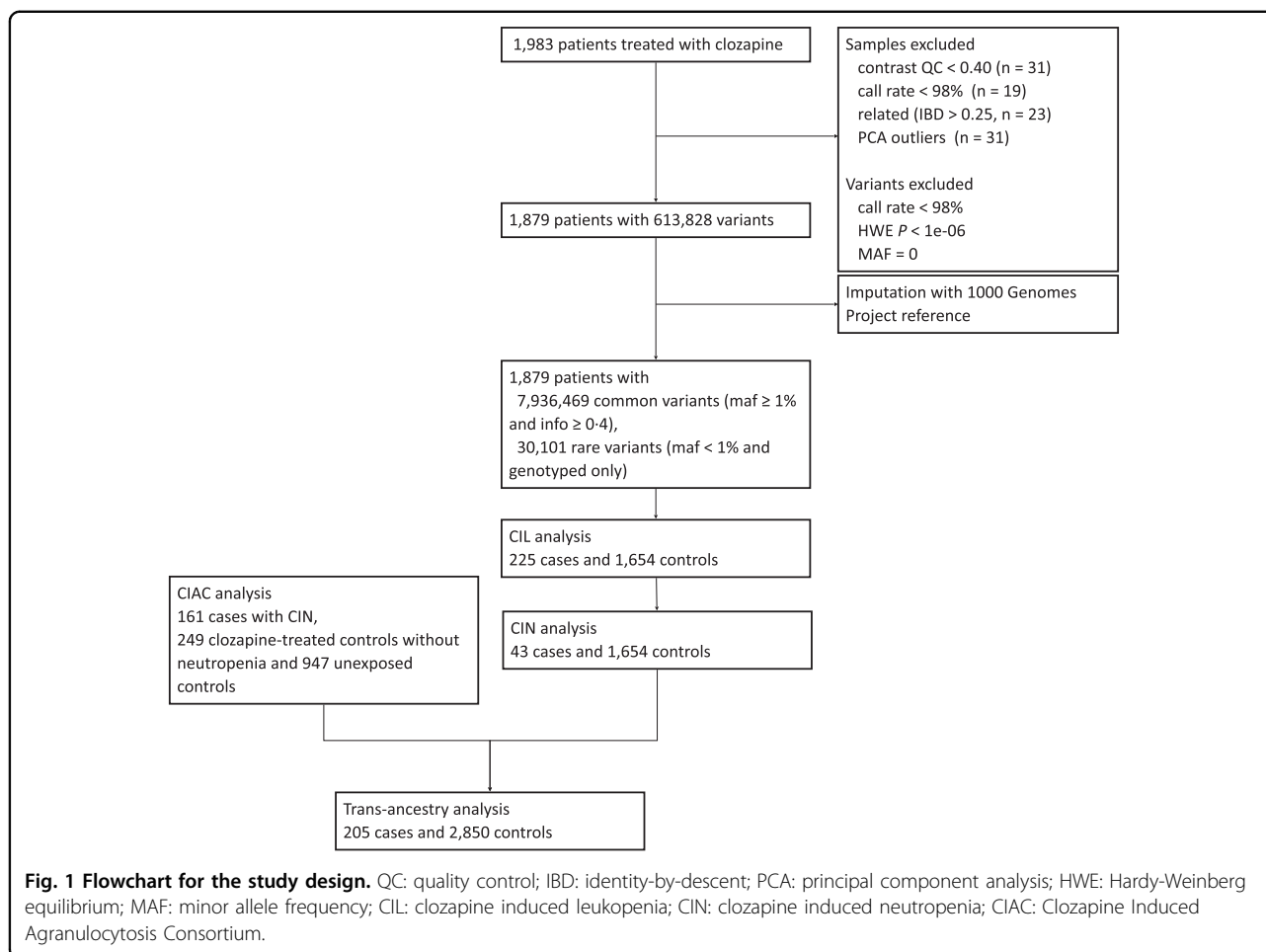
Results

From Jan 1, 2007 to Dec 31, 2012, 1,983 patients with treatment-resistant schizophrenia, who were prescribed clozapine, were enrolled from two Mental Health Centers in China for genome-wide genotyping; 104 (5%) failed quality control (see Supplementary Material), of whom 31 did not meet array QC metrics criteria, 19 did not meet call rate criteria, 23 did not meet identity-by-descent criteria, and 31 were excluded as population outliers after a principal component analysis (see Supplementary Fig. 1). The analyzed sample thus comprised 225 CIL cases (mean [\pm SD] age, 39.3 \pm 12.7 years), 43 CIN cases (37.7 \pm 13.3 years) and 1,654 controls (36.2 \pm 12.7 years)(see Supplementary Table 1). The sample was drawn from two

datasets: Dataset 1: Central Chinese, 155 CIL cases (including 18 CIN cases) and 1,355 controls; Dataset 2: Southern Chinese, 70 CIL cases (including 25 CIN cases and 299 controls). 613,828 genotyped SNPs passed the predefined genotyping quality-control criteria for further analysis (see Fig. 1, Supplementary Material). After imputation and quality control, genotypes for 7,966,570 variants were available for downstream analyses. We performed genome-wide association analysis for 7,936,469 common (MAF \geq 1%, both genotyped and imputed, see Fig. 2) and 30,101 rare (MAF < 1%, genotyped only, see Supplementary Fig. 2) variants.

In the association analysis of 225 CIL cases and 1,654 controls for common variants, one locus near *TRAC* on chromosome 14 was identified to be associated with CIL genome-wide significantly (five variants, $P < 5 \times 10^{-8}$, see Fig. 2), and the strongest association was at rs377360 (MAF = 18.9%, OR = 2.19, 95% CI = 1.66–2.89, $P = 2.58 \times 10^{-8}$, see Table 1, Fig. 3 and Supplementary Table 2). As shown in the Q-Q plot, the genomic inflation factor ($\lambda = 0.976$) indicated good control of population stratification (see Supplementary Fig. 3). None of the genome-wide significant variants and their proxies ($r^2 \geq 0.8$ in EAS of 1000 Genomes Project Releases Phase 3) fell within coding regions, except rs3701 which overlaps a 3' untranslated region (3'-UTR) of *TRAC* (see Supplementary Table 3). Based on our eQTL analysis, most were associated with gene expression levels of multiple genes ($P < 0.05$) in whole blood and immune cells (see Supplementary Table 4). The target genes included T cell receptor alpha and delta variable and constant segments coding genes (such as *TRAC*), and also other genes (such as *DADI*), as shown in Supplementary Table 4. For the rare variants, we identified five genome-wide significant variants from two regions (see Supplementary Fig. 2), which were indexed by rs4773794 in *DCT* on chromosome 13 (MAF = 0.18%, OR = 12.79, $P = 3.01 \times 10^{-10}$), and rs10512698 in *EEFSEC* on chromosome 3 (MAF = 0.88%, OR = 4.79, $P = 1.09 \times 10^{-8}$). All these lead variants showed significant associations in both datasets ($P < 0.05$, see Supplementary Table 2) and no evidence for heterogeneity across datasets were observed (Het $P > 0.05$, see Fig. 4 and Supplementary Fig. 4).

For the CIN analysis of 43 cases and 1,654 controls, we identified three associated common variants, having MAF between 1% and 5%, that achieved genome-wide significance. These are from two regions and indexed by rs116982346 (MAF = 1.90%, OR = 19.96, $P = 2.15 \times 10^{-8}$) on chromosome 3 and rs73482673 (MAF = 1.10%, OR = 12.05, $P = 3.30 \times 10^{-8}$) on chromosome 9 (see Fig. 3 and Table 1). All these variants showed significant associations in both datasets ($P < 0.05$, see Supplementary Table 2) and no evidence for heterogeneity across datasets was observed (Het $P > 0.05$, see Fig. 4). All the genome-wide significant variants and their proxies



are in intergenic regions, and the nearest coding genes are *TRATI* (rs116982346) and *ELAVL2* (rs73482673) (see Fig. 4). One of them was associated with gene expression level of *ELAVL2* in lymphoblastoid cell lines at $P < 0.05$ (see Supplementary Table 4). We also found several CIN associated rare variants (see Supplementary Fig. 2), which were indexed by rs9808117 in *HECW2* on chromosome 2 (MAF = 0.64%, OR = 9.10, $P = 9.82 \times 10^{-9}$), rs373695 in *F13A1* on chromosome 6 (MAF = 0.06%, OR = 74.83, $P = 1.36 \times 10^{-8}$), rs7501702 ear *MFAP4* on chromosome 17 (MAF = 0.33%, OR = 14.95, $P = 1.76 \times 10^{-8}$) and rs8024434 in *NEO1* on chromosome 15 (MAF = 0.36%, OR = 19.39, $P = 4.83 \times 10^{-8}$) (see Supplementary Table 2). Some proxies of the genome-wide significant variants are variants in coding regions, such as 3'-UTR in *EPN2* and a missense variant in *B9DI* for rs7501702, 3'-UTR in *REC114* and a synonymous variant in *HCN4* for rs8024434 (see Supplementary Table 3).

To explore the effects of power and heterogeneity, we performed a trans-ancestry GWAS meta-analysis of CIAC (161 cases and 1,196 controls) with CIN analysis

(43 cases and 1,654 controls) for common variants. The combined GWAS meta-analysis identified rs11753309 (MAF > 0.05) near *HLA-B* on chromosome 6 as close to genome-wide significant (OR = 2.95, 95% CI = 2.00–4.36; $P = 5.08 \times 10^{-8}$, Table 1) and no evidence for heterogeneity across ancestries was observed ($P > 0.05$) (see Fig. 4).

Five prioritized variants (rs377360 and rs4773794 for CIL; rs9808117, rs8024434 and rs7501702 for CIN) by their highest GWAVA scores (see Supplementary Table 5) were tested for transcriptional activity using dual-luciferase reporter assay (see Supplementary Table 6). Luciferase assays showed the DNA segment containing rs377360-T (risk for CIL) increased transcriptional activity in HEK293 cells (49% increase [SD 16.6] comparing to nonrisk allele, $P < 0.001$, Fig. 5). Consistent with GWAVA prediction, we found rs4773794-G (protective for CIL) was associated with an increase of transcriptional activity in HEK293 cells most significantly (50% increase [SD 27.2] comparing to risk allele, $P < 0.001$, Fig. 5). For the CIN associated variants, mild or little affections on transcriptional activity were observed (see Fig. 5).

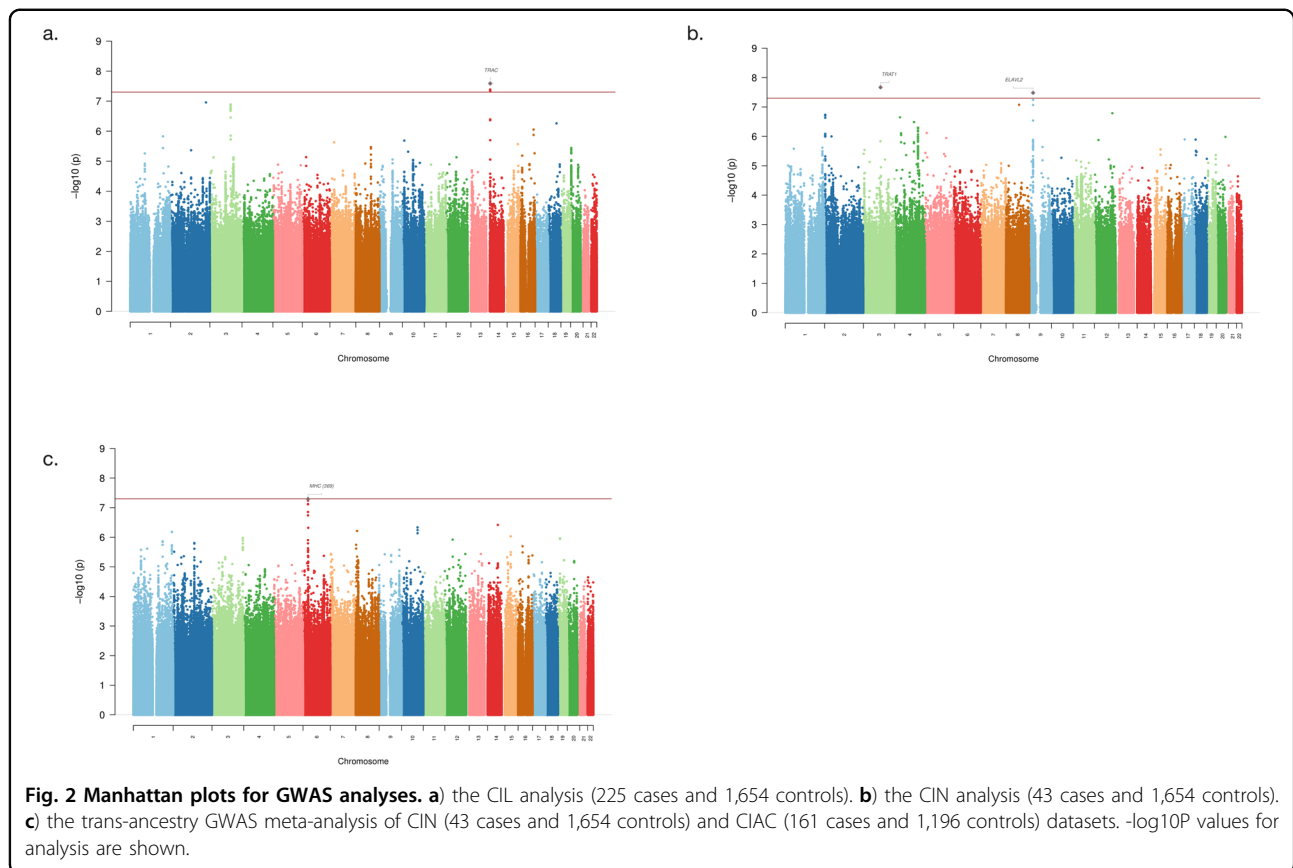


Table 1 Association results for the identified risk loci from the common variant analyses.

Analysis	CHR	SNP	BP	A1/A2	OR [95% CI]	P	Nearby gene
CIL	14	rs377360	23022276	A/T	2.19 [1.66–2.89]	2.58E–08	<i>TRAC</i>
CIN	3	rs116982346	108586107	C/G	19.96 [7.00–56.90]	2.15E–08	<i>TRAT1</i>
CIN	9	rs73482673	24121611	A/G	12.05 [4.98–29.15]	3.30E–08	<i>ELAVL2</i>
TRANS	6	rs11753309	31320645	A/C	2.95 [2.00–4.36]	5.08E–08	<i>HLA-B</i>

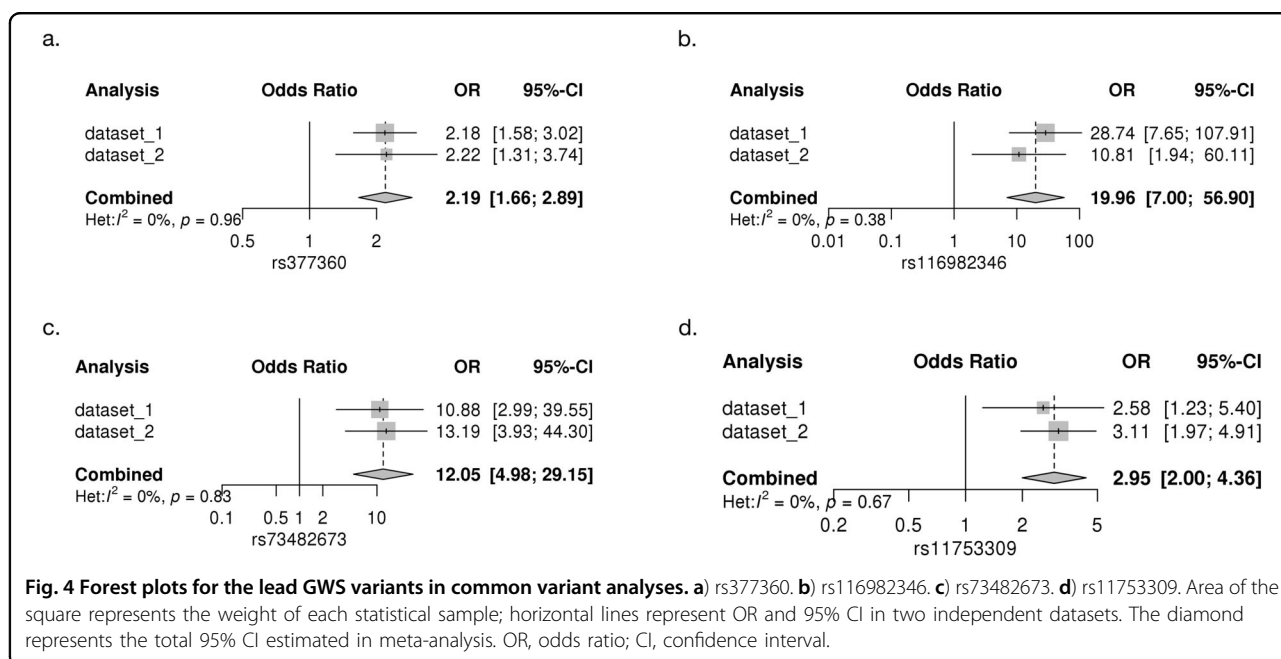
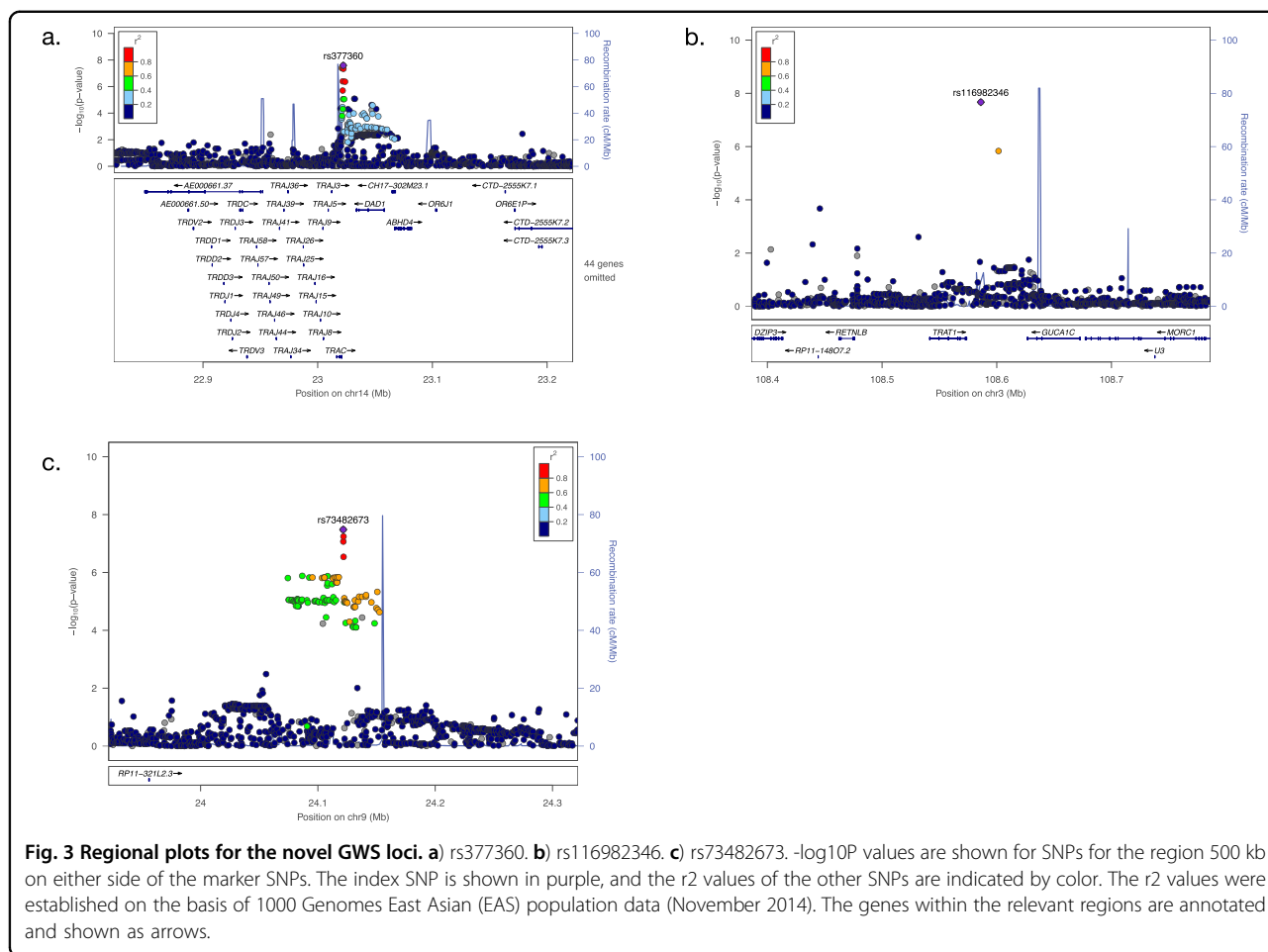
The CIL analysis is for 225 cases and 1,654 controls (dataset 1: 155 cases and 1,355 controls; dataset 2: 70 cases and 299 controls). The CIN analysis is for 43 cases and 1,654 controls (dataset 1: 18 cases and 1,355 controls; dataset 2: 25 cases and 299 controls). The TRANS analysis is for the trans-ancestry GWAS meta-analysis of CIN analysis (dataset 1: 43 cases and 1,654 controls) and CIAC (dataset 2: 161 cases and 1,196 controls). rs11753309 was close to genome-wide significance. *CHR* chromosome, *SNP* rs number, *BP* base position based on hg19, *A1/A2* effect allele/other allele, *OR* odds ratio, *95% CI* 95% confidence interval.

Discussion

To our knowledge, our study is the first GWAS of CIL and CIN in individuals of Chinese ancestry and identified nine novel genome-wide significant loci. Five of the nine index variants identified in our GWAS mapped to the intron of a protein-coding gene, and all except one of the others had a protein-coding gene within 15 kb. The trans-ancestry meta-analysis of the Chinese and European ancestry identified one risk locus which was close to genome-wide significance.

Mechanisms underlying CIL and CIN have not yet been fully elucidated, although an immune reaction has been

hypothesized for a long time as implicated¹⁰. We identified two T cell related genes in this study, *TRAC* (T cell receptor alpha constant) and *TRAT1* (T Cell Receptor Associated Transmembrane Adaptor 1). The *TRAC* gene locating on 14q11-12 encodes the constant region of the T cell receptor (TCR) alpha chain. The alpha loci are synthesized by variable, joining, and constant segments. TCR recognizes antigen fragments as peptides bound to MHC molecules, which are important for thymic selection. Deficiency of MHC may cause abnormal immune function and result in a lack of maturation of the corresponding T cells²⁵. Diseases associated with *TRAC*



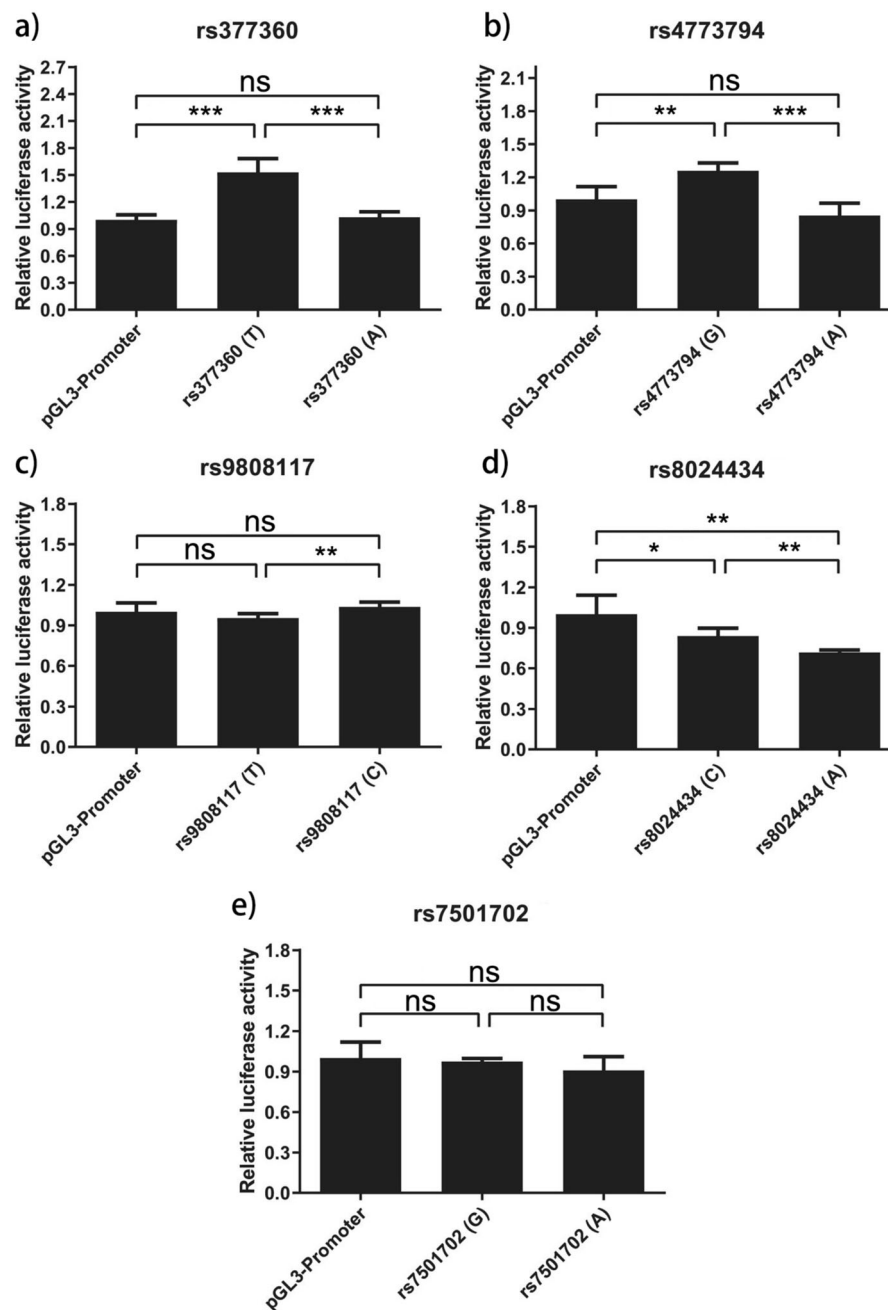


Fig. 5 Luciferase reporter assays comparing transcriptional activation with pGL3-Promoter or variant alleles of genetic variants. **a)** rs377360. **b)** rs4773794. **c)** rs9808117. **d)** rs8024434. **e)** rs7501702. HEK-293 cells were transfected with luciferase reporter constructs for both alleles of each variant. Each construct was transfected five times and assayed in quintuplicate in each experiment. The luciferase activities, normalized with a cotransfected Renilla activities, were expressed by taking the normalized luciferase activity of the vector (pGL3-Promoter) to be 1. Data represent means \pm SDs. P values were determined by using the t-test. Significance indicators will be written above the bars: *** if $p < 0.001$, ** if $p < 0.01$, * if $p < 0.05$, ns otherwise.

include TCR alpha/beta deficiency. It has been reported that *TRAC* mutations cause a human immunodeficiency disorder characterized by a lack of TCR $\alpha\beta$ + T cells²⁶. *TRAT1* also plays an important role in the immune process. Among the predominantly downregulated cell

surface antigens, *TRAT1* along with ZAP70, LCK and TCR associated tyrosine kinases are involved in TCR activation, and were all simultaneously downregulated²⁷.

Although we did not identify susceptibility loci reaching the level of genome-wide significance after the trans-

ancestry meta-analysis, we found a near-significant risk locus for CIN near *HLA-B*. *HLA-B* is a paralog of *HLA* class I heavy chain, which is anchored in the membrane and plays an important role in the immune system by presenting peptides derived from the lumen of the endoplasmic reticulum. *HLA-B* was reported as a susceptibility gene of CIA in Caucasian population¹¹, while *HLA-B*59:01* was suggested as a risk factor for CIA in the Japanese population²⁸. Most studies of CIL have been conducted in Caucasian populations, and it may not be possible to replicate identified variants across different races and populations for many reasons. However, associations of variants from GWAS have generally added evidence for immune-related mechanisms.

The biological activation or the conversion of stable metabolites to chemically reactive nitrogen ions could represent another causal pathway^{29–31}. However, hypotheses of toxicity and immune regulation are not necessarily mutually exclusive. The combination of these reactive metabolites can result in CIL, CIN or CIA through direct toxicity or via initiation of immune mechanisms or both. Clozapine may potentially be oxidized into toxic nitrenium ions, which bind to neutrophils and cause an autoimmune reaction or induce cell death.

In this study, we also found several other compelling candidate genes. *NEO1* at 15q24.1 encodes a cell surface protein composed of four N-terminal immunoglobulin-like domains, which may be involved in cell growth and differentiation and cell adhesion. *Neo1* contributes to the inflammatory response and resolution mechanisms³². Genetic deletion or functional inhibition of *Neo1* leads to reduced neutrophil recruitment and shortened the neutrophil lifespan by increasing apoptosis³³. *DCT* at 13q32.1 encodes dopachrome tautomerase and participates in melanocytes pigment biosynthesis with tyrosinase and tyrosinase-related protein 1³⁴. Meanwhile it also plays a role in oxidative stress and apoptotic stimuli responsiveness^{35,36}. An experiment in mice found that the spatiotemporal distribution of *DCT* expression was related to cortical neurogenesis during embryonic development³⁷. Diseases associated with *DCT* include microphthalmia and melanoma. Previous studies have proved that a large portion of chromosome, spanning 68 Mb from 13q12 to 13q34, could be linked to susceptibility to schizophrenia^{38,39}. *EEFSEC* located at 3q21.3, encoding selenocysteine-tRNA specific eukaryotic elongation factor, which is essential for the synthesis of selenoproteins⁴⁰. Selenoproteins contain several proteins with different functions, some of which play important roles in antioxidant defense preservation and anti-inflammatory regulation⁴¹. Polymorphisms or mutations in *EEFSEC* could account for a variety of disorders, including neuropsychiatric disorders and immune dysfunctions⁴². We hypothesize that *EEFSEC* may participate in the mechanism of CIL through selenoproteins. Many

selenoproteins participate in redox signaling, redox homeostasis, and antioxidant defense by the action of glutathione peroxidase⁴³. The amount of selenoproteins and glutathione peroxidase decreases under oxidative stress conditions, and the activity of selenium-dependent glutathione peroxidase has been found to be lower in patients treated with clozapine⁴⁴. Activation of clozapine and/or its metabolites can produce electrophilic nitrenium ions, which might either covalently bind to neutrophils such as glutathione to cause cell death or cause oxidative stress-induced neutrophil apoptosis^{45,46}.

We feel it is reasonable to assume that the risks of CIL and CIN are genetically complex, involving several genes. The application of GWAS and other modern genomic technologies gives us a better understanding of the genetic basis of CIL and CIN. Our study is the first GWAS of CIL and CIN in individuals of Chinese ancestry and identified several novel genome-wide significant loci. However, there are still some limitations in our study. First, many SNPs detected were noncoding variants with unknown effects, which is a common problem with the GWAS approach. There may also be potential Winner's curse bias in estimating the average genetic effect of a set of rare variants and identifying statistically significant associations. Therefore, further functional studies are needed to verify the related biological mechanisms. Second, since some conditions such as circadian rhythm can affect the amount of peripheral blood neutrophils in patients, other related factors should also be taken into account. Finally, an important challenge is that there is relatively rare incidence of CIN and even rarer incidence of CIA in the Chinese population. In fact, only six CIA cases met the criteria in our study, which may be due to the success of the monitoring system, since once leukopenia is detected, clozapine will be discontinued. It is therefore impossible to know which patients would continue to develop CIA. The rare incidence limits the availability of suitable patients and thus the sample size for research. Therefore, replication of larger sample sizes is critical for clinical applications.

Clozapine is the only effective medication for treatment-resistant schizophrenia, but its use is limited due to the risk of leukopenia, particularly agranulocytosis. Genetic and immune factors are likely to play an important role in determining risk for such adverse reactions. Our results provide novel and valuable understanding of the genetic and immune causes of CIL and CIN. Causal variants and related underlying molecular mechanisms need to be understood in future developments, which will be key to improving diagnostic and therapeutic capabilities in treatment-resistant schizophrenia.

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Conflict of interest

The authors declare no competing interests.

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