

Genetic association analysis of N-methyl-D-aspartate receptor subunit gene *GRIN2B* and clinical response to clozapine

Running Head: Association analysis of *GRIN2B* with response to clozapine

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Structured Abstract

Objective: Approximately 30% of schizophrenia patients fail to respond to antipsychotic therapy and are classified as having treatment resistant-schizophrenia (TRS). Clozapine is the most efficacious drug for TRS and may deliver its superior therapeutic effects partly by modulating glutamate neurotransmission. Response to clozapine is highly variable and may depend on genetic factors as indicated by twin studies. We investigated eight polymorphisms in the N-methyl-D-aspartate glutamate receptor (NMDAR) subunit gene *GRIN2B* with response to clozapine. Methods: *GRIN2B* variants were genotyped using standard TaqMan procedures in 171 European schizophrenia patients deemed resistant or intolerant to treatment. Response was assessed using change in Brief Psychiatric Rating Scale (BPRS) scores following six months of clozapine therapy. Categorical and continuous response was assessed using Chi-square test and analysis of covariance (ANCOVA), respectively. Results: No associations were observed between the variants and response to clozapine. A-allele carriers of rs1072388 responded marginally better to clozapine therapy than GG-homozygotes, however the difference was not statistically significant ($p=0.067$, uncorrected). Conclusions: Our findings do not support a role for these *GRIN2B* variants in altering response to clozapine in our sample. Investigation of additional glutamate variants in clozapine response is warranted.

Text

INTRODUCTION

Treatment resistant schizophrenia (TRS) occurs in approximately 30% (20-40%) of patients treated with antipsychotic (AP) drugs (Borgio et al. 2007, Elkis & Meltzer 2007, Solanki, Singh & Munshi 2009). TRS patients experience suboptimal response characterized by persistent positive symptoms that often result in long periods of hospitalization (McGlashan 1988) and chronic severe disability. Treatment resistance remains an immense burden to both patients and their families and is associated with annual healthcare costs ranging 3 to 11 times higher than schizophrenia (SCZ) patients who respond to treatment (\$66,360-\$163,795 vs. \$15,500-\$22,300 per year). TRS is also associated with poorer quality of life that is approximately 20% lower than patients with SCZ who achieve remission (0.61 vs. 0.75) (Kennedy et al. 2014).

The atypical AP drug clozapine (CLZ) is particularly effective for treating the persistent symptoms experienced by patients with TRS. A landmark clinical trial conducted by Kane et al. showed that approximately 30% of TRS patients responded to CLZ versus only 4% of patients given the prototypical AP chlorpromazine (CPZ) ($p < 0.001$) (Kane et al. 1988). As an atypical AP, CLZ differs from typical APs in particular dimensions, such as decreased dopamine (D2) receptor affinity (Altar et al. 1986) and lowered risk for adverse effects such as extrapyramidal symptoms (EPS) and tardive dyskinesia (TD) (Pickar et al. 1992, Tandon & Fleischhacker 2005). CLZ also has the unique ability to alter other neurotransmitter systems such as serotonin and glutamate (reviewed in Miyamoto et al. 2005).

A great deal of evidence suggests CLZ augments glutamate neurotransmission (reviewed in Heresco-Levy 2003). One pillar supporting this theory stems from observations that CLZ is capable of blunting the psychoto-mimetic effects of glutamate antagonists in SCZ patients (Malhotra et al. 1997). These antagonists, such as phencyclidine (PCP) and ketamine, bind to the glutamate N-methyl-D-aspartate receptor (NMDAR) and have been observed to elicit schizophrenia-like symptoms in healthy controls and exacerbate psychotic symptoms in SCZ patients (Luby et al. 1959, Luisada 1978, Javitt & Zukin 1991,

Lahti et al. 2001) – observations that form the basis of the “glutamate hypo-function hypothesis” of SCZ. Collectively, these findings suggest that blockade and underactivity of glutamate signaling contributes to clinical presentation of SCZ and that CLZ is able to offer therapeutic relief in part by augmenting glutamatergic signals.

Findings from preclinical models and functional studies also support this theory. CLZ administration reverses PCP-induced psychosis-like behavior such as hyper-locomotion (Freed, Bing & Wyatt 1984, Sun, Hu & Li 2009, Zhao, Sun & Li 2012), social deficits (Corbett et al. 1995), enhanced immobility (Noda et al. 1995) and pre-pulse inhibition (PPI) deficits (Linn et al. 2003). Functional studies in both preclinical models and SCZ patients observe that CLZ alters glutamate receptor subunit expression (Fitzgerald et al. 1995, Meshul et al. 1996), binding (Giardino et al. 1997, Pilowsky et al. 2006), and density (Ossowska et al. 1999, Schmitt et al. 2003). CLZ also appears to alter glutamate concentrations and activate excitatory glutamate neurotransmission in different brain regions as exhibited by: microdialysis studies in rodents (Daly & Moghaddam 1993, Yamamoto & Cooperman 1994), increased amplitude of excitatory post-synaptic potentials (EPSPs) in neuronal cell cultures (Kubota et al. 2000, Banerjee et al. 1995, Arvanov et al. 1997, Ninan, Jardemark & Wang 2003, Kargieman et al. 2007), and increased serum glutamate levels in patients switched to CLZ (Evins et al. 1997). Taken together, these results provide evidence for CLZ’s ability to alter glutamate signaling – a factor that may distinguish CLZ as the most efficacious drug for treatment resistant patients.

Clinical response to CLZ is highly variable (Bleehen 1993, Davis, Chen & Glick 2003) and an estimated 30-60% of TRS patients fail to respond to CLZ therapy {{336 Kane,J. 1988; 378 Lieberman,J.A. 1994}}. Because early intervention strategies with AP drugs attenuates disease prognosis, the ability to predict response prior to drug administration would have important clinical utility (Gunduz-Bruce et al. 2005, Perkins et al. 2005, Emsley et al. 2007, Wyatt & Henter 2001). A portion of the inter-individual variability in CLZ response is thought to be caused by genetic variation (Mata et al. 2001, Horacek et al. 2001, Theisen et al. 2005, Hoyer et al. 2010, Vojvoda et al. 1996). Quite a few pharmacogenetic (PGx)

studies investigating genetic variability and CLZ response have been conducted to date (reviewed in Kohlrausch 2013), however, no predictive test for AP response utilizing this genetic information is currently used in the clinic. The most notable gene variants investigated for association with CLZ response include those related to the dopamine, serotonin and to a lesser degree, glutamate neurotransmitter systems.

Glutamate is becoming increasingly popular in recent years due to novel SCZ therapies that target this neurotransmitter system (Heresco-Levy et al. 1996, Pinard et al. 2010, Hopkins 2011, Patil et al. 2007). Additional advancements in SCZ genetics have also yielded promising genome-wide (GWAS) hits for glutamate genes in risk (GAIN Collaborative Research Group et al. 2007, Jia et al. 2012, Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). Genome-wide significant associations with schizophrenia were observed in the glutamate system genes *GRM3*, *GRIN2A*, *GRIA1*, and *GRIN2B*. The *GRIN2B* gene (138252 [MIM]) codes for subunit 2B of the NMDAR and is of particular interest. This gene contains 13 exons and is mapped to 12p12 (Mandich et al. 1994, Endeley et al. 2010). Single nucleotide polymorphisms (SNPs) in *GRIN2B* have been investigated for association with several neuropsychiatric disorders, including drug response phenotypes (**Table 1**). A small number of *GRIN2B* variants have also been studied in CLZ response – while no associations between rs1806201 (2664C/T) (Hong et al. 2001, Chiu et al. 2003) or rs1019385 (-200T/G) (Hwang et al. 2011) were observed, the rs1805502 variant (T5988C) predicted negative symptom response during CLZ therapy (Martucci & Kennedy 2010). Associations between additional *GRIN2B* polymorphisms and CLZ response have not yet been investigated. This study examined for associations between eight *GRIN2B* variants and response to CLZ monotherapy in a sample of treatment resistant or intolerant patients.

METHODS

Subjects

One hundred and seventy-five patients meeting Diagnostic and Statistical Manual of Mental Disorders (DSM)-III-R or DSM-IV criteria for schizophrenia or schizoaffective disorder were included in this study

(American Psychiatric Association 1987, American Psychiatric Association 1994). Patients were recruited from three clinical sites: Case Western Reserve University in Cleveland, Ohio (**HY Meltzer, n=74**), **Hillside Hospital in Glen Oaks, New York (JA Lieberman, n=73)**, and **the University of California at Irvine (SG Potkin, n=28)**. All patients were considered European based on self-reported ancestry. Informed consent was obtained from each participant prior to study enrollment in accordance with the Ethical Principles for Medical Research Involving Human Subjects at the Centre for Addiction and Mental Health (CAMH) and with the Helsinki Declaration of 1975, as revised in 1989 (World Medical Association 2013).

The majority of patients met criteria for TRS defined as failure to respond to two or more AP drug trials in the previous five years (involving drugs from two different chemical classes, with doses ≥ 1000 mg chlorpromazine equivalents for four to six weeks), accompanied by no period of good functioning within the preceding five years (Kane et al. 1988). The remaining participants (<15%) met criteria for treatment intolerance defined as the presence of moderate to severe TD and/or extreme EPS making treatment with therapeutic dosages intolerable (Lieberman et al. 1994). Before beginning CLZ treatment, **patients underwent a two to four week wash-out period which involved no administration of pharmacotherapy unless clinically necessary.** Following the washout period, all patients were treated with CLZ monotherapy, with mean dosages of 453 mg/d, for a period of six months or longer. Benzodiazepines were administered intermittently during the titration period. **Throughout treatment, CLZ serum levels were monitored to ascertain adherence to the medication.**

Response Measures

Baseline Brief Psychiatric Rating Scale (BPRS) (Overall & Gorham 1962) scores were obtained at time of study enrollment **I would recommend adding references that support that BPRS is a reliable rating method for treatment outcome in psychotic patients.** Following CLZ administration, response was evaluated after six months using two BPRS scoring methods. The first was a categorical responder/non-responder response measure that classified responders as individuals who experienced a $\geq 20\%$ decrease in

BPRS total score. The second scoring method was a quantitative response measure that divided the BPRS items into three subcategories [positive (BPOS), negative (BNEG), and general (BPRS)], and calculated the percent score reduction using the following equation: $[(6 \text{ month score} - \text{baseline score}) / (\text{baseline score})] \times 100$.

SNP Selection

A literature search was conducted on PUBMED for the following terms: “*GRIN2B*”, “NMDAR”, “N-methyl-D-aspartate receptor”, “clozapine” and “pharmacogenetics.” Literature pertaining to genetic association studies in psychiatric disorders and psychotropic drug response was reviewed to identify *GRIN2B* SNPs of interest. Variants previously investigated for an association with CLZ response or other psychiatric phenotypes were considered for inclusion in our study. Altogether, eight polymorphisms were selected: rs1072388, rs12826365, rs1806191, rs2284411, rs3764030, rs7301328 (366G>C), rs1806201 (2664C>T), and rs890 (Table 1). These eight variants were assessed for potential functionality using the National Institute of Environmental Health Sciences (NIEHS) Functional SNP Prediction (FuncPred) database (Xu & Taylor 2009). Variants were deemed functional if alternate alleles were thought to have differential effects on gene transcription, translation, or splicing.

Variant rs1072388 and rs12826365 were selected from the Genetics Association Information Network (GAIN) schizophrenia Genome Wide Association Study (GWAS) as lowest *p*-value *GRIN2B* SNPs from the European and African samples, respectively (Ayalew et al. 2012). Variant rs1806191 was previously investigated in SCZ risk and obsessive compulsive disorder (OCD) (Di Maria et al. 2004, Alonso et al. 2012), while variant rs2284411 has been tested for associated with attention deficit hyperactivity disorder (ADHD) (Dorval et al. 2007, Park et al. 2013). The promoter variant rs3764030 has been previously studied in autism spectrum disorder (ASD) (Yoo et al. 2012) and may affect *GRIN2B* expression by altering transcription factor binding (TFBS). Both rs7301328 and rs1806201 have been investigated in a number of psychiatric phenotypes, including response to lithium ((Szczepankiewicz et al. 2009) and CLZ (Hong et al. 2001). These SNPs lie near intron-exon borders (within two base-pairs, specifically) and may

regulate mRNA splicing. Lastly, rs890 has been investigated in SCZ risk (Di Maria et al. 2004), treatment-resistant depression (TRD) (Zhang et al. 2014), CLZ-induced OCD (CI-OCD) (Cai et al. 2013), and response to lithium (Szczepankiewicz et al. 2009). This variant lies in a micro RNA (miRNA) binding site at the 3' untranslated region (UTR) of *GRIN2B* and may alter translation initiation.

DNA Isolation and Genotyping

Venous blood samples were collected from study participants and sent to CAMH in Toronto, Canada where genomic DNA was isolated using the high salt method (Lahiri & Nurnberger 1991). *GRIN2B* genotypes were determined using TaqMan allele specific single tube assays, the ABI Prism®7500 Sequence Detection System and the ABI allelic discrimination software according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The reaction mixture consisted of 20ng of genomic DNA, 2X TaqMan® Universal Master Mix and 40X SNP Genotyping Assay for a final reaction volume of 10 µL. The polymerase chain reaction (PCR) protocol consisted of a denaturation step for 10 mins at 95°C, followed by 50 cycles of amplification that consisted of denaturation (92°C for 15 s) and annealing (60°C for 1 min). Genotype calls were confirmed by two independent researchers and 10% of the total sample was re-genotyped to ensure genotyping accuracy. Discordant genotypes were set as missing in the statistical analysis.

Statistical Analyses

Quality control (QC) was carried out using PLINK software v1.07 (Purcell et al. 2007). To pass filtering, SNPs were required to have a $\geq 90\%$ genotyping rate and a minor allele frequency (MAF) of ≥ 0.05 . Samples with a genotype success rate less than 75% across all markers (samples producing genotypes for 5 SNPs or less) were also excluded from analysis. All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) v20 (IBM Corp, Armonk NY, USA) and PLINK software.

Descriptive statistics were obtained for each clinical site. Mean age and baseline BPRS, BPOS, and BNEG subscale scores across sites were compared using Student's t-test or analysis of variance

(ANOVA), while frequency counts for gender and response rates were compared using Pearson's Chi-square test (χ^2 -test). Genotype and allele frequencies in the responder/non-responder (R/NR) groups were compared using χ^2 -test or Fisher's exact test (for cell counts less than 5). Differences in percent score reduction in BPRS, BPOS, and BNEG subscales between genotype groups were compared using analysis of covariance (ANCOVA), co-varying for age and baseline scores.

Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) among SNPs were determined using Haploview v4.2 using the Solid Spine of LD to construct LD blocks (**Figure 2**) (Barrett et al. 2005). Haplotype analysis was carried out in UNPHASED v3.1.5 (Dudbridge 2008). Haplotypes with less than 5% frequency were removed from analysis. (Add a few sentences to clarify why a haplotype was constructed from only 3 SNPs)

Power calculations were conducted in Quanto v1.2.4 (Gauderman 2002, Gauderman & Morrison 2006). Correction for the effective number of independent markers was performed using the Nyholt method and resulted in a statistical significance threshold of $p < 0.0076$ (Nyholt 2004).

RESULTS

Sample Characteristics

Of the eight SNPs genotyped, seven met QC criteria and were included in subsequent association analyses. **The genotype distribution for variant rs7301328 significantly deviated from HWE ($p < 0.05$) and therefore this SNP was excluded from future analyses.** The remaining SNPs had a mean genotyping rate of 99.1%. Regarding study participants, 18 patients were removed due to low genotyping efficiency (<75% of markers successfully genotyped). The remaining sample (**n=157**) had a mean genotyping efficiency of 98.9%. (Mention ppl were removed due to noncompliance?)

Following QC, 157 European treatment resistant and intolerant patients were included in the study (Table 2). Our sample had over 80% power to detect an odds ratio (OR) as low as 2.00 (unmatched case-control design: $n=157$, non-responder frequency=48.4%, MAF=30.5%, $\alpha/2=0.05$) and down to 8.5% of

the variance in the quantitative response variable (continuous design: n=85). **The average minor allele frequency across the seven *GRIN2B* variants (excluding rs7301328)** was used for power calculations. Patients between clinical sites did not differ in gender, categorical response rates, or BPRS/BPOS/BNEG baseline scores. The sites did, however, differ in mean age ($F(2,157) = 7.10, p = 0.001$). *Post hoc* analysis showed that the average age of the HY Meltzer sample was significantly lower than the SG Potkin sample. In order to combine samples for analysis, age and baseline BPRS/BPOS/BNEG scores were added as covariates to account for any disparities. **Following QC, the final combined sample (n=157) consisted of 76.4% men and had a total mean age of 35.06 years (SD 8.1). Approximately half (51.6%) of the sample were considered CLZ “responders” (n=81).** Quantitative response measures were available for a subset of patients in the BPRS (n=86), BPOS (n=84), and BNEG (n=85) subscales.

GRIN2B SNP and Haplotype Association Analyses

Genotype and allele frequencies for each of the seven *GRIN2B* variants were not significantly different between CLZ responder and non-responder groups or as measured by the quantitative percent score reduction in BPRS, BPOS, and BNEG subscales (Table 3). Variant rs1072388 A-allele carriers (AA and AG) responded marginally better to CLZ therapy than GG-homozygotes, however this difference was not statistically significant ($p_{\text{uncorrected}} = 0.067$) (Figure 3). Haplotype association analyses confirmed allele and genotype findings with no association reported across all haplotypes tested (Table 4).

2.5 DISCUSSION

We report no significant associations among the seven *GRIN2B* variants analyzed and response to CLZ in our sample of patients. Despite A-allele carriers of the intronic variant rs1072388 responding marginally better to CLZ than G- homozygotes ($p_{\text{uncorrected}} = 0.067$, genotype), this observation was statistically non-significant ($p_{\text{corrected}} = 0.440$, assuming 6.56 independent tests). **The rs1806201 marker has previously been investigated in two other studies** (Hong et al. 2001, Chiu et al. 2003). **To our knowledge, this study is the first reported study to investigate associations of the remaining seven SNPs (rs1072388, rs3764030, rs12826365, rs2284411, rs1806191, rs890 and rs7301328) with CLZ response.** Each

aforementioned variant was selected because of previous investigation in CLZ response or other psychiatric phenotypes. After inclusion, the potential functionality of these variants was assessed using the NIEHS Functional SNP Prediction Database (Xu & Taylor 2009).

Our negative finding for rs1806201 (2664C/T) is in accordance with previous reports that this variant is not associated with CLZ response (Hong et al. 2001, Chiu et al. 2003). These studies did report a marginally higher mean CLZ dosage for patients carrying the CC genotype of rs1806201. Due to the inter-individual variability in CLZ metabolism and absorption, future studies may benefit from measuring serum CLZ levels as opposed to dosage in order to predict response. An optimal threshold for CLZ serum levels has already been established (≥ 450 ng/mL) and may be of clinical utility to identify non-responders {{488 Potkin,S.G. 1994; 420 Lindenmayer,J. 1996}}.

As a measure of genotyping quality, we tested for HWE to ensure genotype proportions observed in the study population coincided with those expected under equilibrium conditions (Hardy 1908, Weinberg 1908). The potentially functional variant rs7301328 deviated from HWE ($p < 0.05$, data not shown). This deviation may have been caused by a number of reasons including: systematic genotyping errors that mistype heterozygotes as homozygotes or vice versa (Gomes et al. 1999, Hosking et al. 2004), stochastic variation, or because of the study population's biological characteristics. The biological explanation posits that patients deviate from HWE because of the effect an allele has on a disease phenotype, from which the sample was non-randomly selected (Xu & Taylor 2009, Wittke-Thompson, Pluzhnikov & Cox 2005). The cause for deviation from HWE in our sample appears to be genotyping error: no homozygous recessive genotypes were observed for either the rs7301328 responder or non-responder groups, even though this variant has a MAF of 23% and therefore, **was expected to have a homozygous recessive count of approximately eight people out of 157**. Because many genetic tests such as χ^2 -test require HWE to be assumed, rs7301328 was removed from subsequent analyses {{427 Sasieni,P.D. 1997}}. Retyping of rs7301328 with a newly designed assay remains a priority for future work.

(Add a large paragraph or two discussing the importance of GRIN2B and glutamate in CLZ response). Mention physiological significance on the NMDAR, how its malfunction may correspond to the symptoms observed in patients w/ SCZ. Have to brainstorm for this section. Mention briefly GRM2&CLZ may be one proposed mechanism of action (See Thesis Discussion for this). Look online for papers on GRIN2B/NMDAR and CLZ.) (One done, transfer to thesis chapter)

This study has several limitations. The sample size is relatively small and may not have had sufficient power to detect the effect sizes of these genes. To increase statistical power in the future, it will be necessary to collect larger CLZ response samples. One promising sample is being collected by the CRESTAR consortium in Europe (CRESTAR 2011). Sample heterogeneity may also have limited our findings. Patients were collected from three clinical sites that could have differed in methods and populations. Population ancestry was also based on self-report and in the future, a principle components analysis (PCA) on the study sample may provide more accurate ancestry profiles (Price et al. 2008, Paschou et al. 2008, Liu et al. 2011).

Phenotypic heterogeneity regarding psychotic diagnosis between SCZ and SA disorder patients may have also increased variation in our sample. Several differences exist between SCZ and SA disorder. For instance, these disorders have different clinical presentations: SA disorder is characterized as periods of psychosis with concurrent symptoms meeting criteria for a Major Mood Episode, while in comparison, SCZ is characterized by less frequent/prominent depressed or manic mood states {{288 American Psychiatric Association 2013}}. As well, SA disorder patients tend to experience higher rates of response and better clinical outcomes than patients with SCZ {{448 Harrow,M. 2000}}. Taken together, these differences suggest SCZ and SA disorder may differ in their underlying biology {{455 Abrams,D.J. 2008; 288 American Psychiatric Association 2013; 449 Cosgrove,V.E. 2013}}. In regard to our findings, the propensity of SA disorder patients to

respond more favourably to treatment may have increased our CLZ response rate, and it is possible that combining these two disorders into a single sample for analysis may have limited our ability to detect causal variants contributing to response.

Inter-study variability may also limit comparison of results across investigations. We evaluated treatment response after six months of CLZ therapy, while previous studies evaluated response after shorter periods, e.g. two months of treatment (Hong et al. 2001, Chiu et al. 2003). Difference in treatment length is particularly problematic because response to CLZ may take five months or longer in a subset of patients (Lieberman et al. 1994, Meltzer & Okayli 1995) and thus patients deemed non-responders after a two month treatment period would have the potential to be reclassified as responders following a longer trial of CLZ therapy. This disparity makes comparing results across studies problematic. Consensus among duration of treatment would benefit researchers and make meta-analyses more efficient in the future.

(Add paragraph on noncompliance, and the benefits of blood monitoring for patients taking CLZ). See Thesis Discussion. Incomplete outcome data

(Add a paragraph highlighting the importance of multiple gene systems (ie glutamate/serotonin interaction)

Environmental factors (diet, stress) on the complexity/variability in PGx studies). See Thesis Discussion. In addition, two environmental factors with the potential to alter CLZ metabolism were not controlled in our study: cigarette smoking and caffeine consumption.

Cigarette smoking induces the Cytochrome P450 hepatic enzyme *CYP1A2* that is primarily responsible for CLZ metabolism {{437 Bertilsson,L. 1994; 438 Ghassabian,S. 2010}}. As a result, smokers are observed to have lower CLZ serum levels than non-smokers {{436 Seppala,N.H. 1999; 435 Palego,L. 2002}} and therefore require higher CLZ dosages to achieve similar plasma levels as patient who do not smoke. In contrast, caffeine inhibits *CYP1A2* and increases both CLZ plasma

concentrations and the risk of developing side effects linked to toxicity {{434 Odom-White,A. 1996; 433 Hagg,S. 2000}}. Future studies incorporating these environmental factors may help identify more accurate predictors of response and develop dosage guides that are tailored to the individual patient. ADD SENTENCES ON STRESS → (Real et al, 2013)

To conclude, we report no association between seven *GRIN2B* markers and CLZ response in our sample. Response to CLZ therapy is complex and likely to depend on multiple gene systems and environmental factors. Therefore, investigation of additional glutamate variants and variants within related neurotransmitter systems is warranted for future studies. Even though conclusive predictive markers remain elusive thus far, continued research to identify such markers remains of high interest to improve clinical response and treatment outcomes.

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JLK, DLT, DJM, JK, and GR designed the study. HYM, JAL, and SGP provided the samples. DLT collected the genotype data, managed the literature searches and analyses. DLT and AKT

undertook the statistical analysis. DLT wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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