Identification of a functional 339 bp *Alu* insertion polymorphism in the schizophrenia-associated locus at 10q24.32

DEAR EDITOR,

Genome-wide association studies (GWAS) have identified multiple single nucleotide polymorphisms (SNPs) or small indels robustly associated with schizophrenia; however, the functional risk variations remain largely unknown. We investigated the 10q24.32 locus and discovered a 339 bp *Alu* insertion polymorphism (rs71389983) in complete linkage disequilibrium (LD) with the schizophrenia GWAS risk variant rs7914558. The presence of the *Alu* insertion at rs71389983 strongly repressed transcriptional activities in *in vitro* luciferase assays. This polymorphism may be a target for future mechanistic research. Our study also underlines the importance and necessity of considering previously underestimated *Alu* polymorphisms in future genetic studies of schizophrenia.

Schizophrenia is a severe chronic psychiatric disorder with high heritability (Sullivan et al., 2003), and depicting the genetic architecture of schizophrenia is essential for understanding its pathophysiology. So far, GWAS have identified numerous risk loci (Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), and several studies have attempted to identify causative risk variations and underlying biological mechanisms from the massive tagged single nucleotide polymorphisms (SNPs) (Duan et al., 2014; Huo et al., 2019; Wu et al., 2017, 2019 Yang et al., 2018). However, one potential limitation of current GWAS platforms is that they have primarily focused on SNPs and small indels, ignoring other sequence variations that have also been implicated in the genetic risk of human disorders including schizophrenia (Payer et al., 2017; Song et al., 2018; Yang et al., 2019) and in non-human primates (Liu et al., 2018). For instance, Song et al. (2018) previously identified a functional human-specific tandem repeat in the *CACNA1C* gene as a potential causative variation for schizophrenia and bipolar disorder.

The chromosomal 10q24.32 region is a critical locus showing genome-wide significant associations with schizophrenia. For example, rs7914558 is reported to be the most significant SNP in the 10q24.32 region in the PGC1 GWAS of European populations (P=1.82×10^{-9}, n=51 695) (Schizophrenia Psychiatric Genome-Wide Association Study Consortium, 2011), and its association with schizophrenia has been further confirmed in subsequent GWAS with increased sample size (P=3.49×10^{-15}, n=79 845) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Intriguingly, according to data from a recent GWAS of East Asian populations, rs7914558 is also significantly associated with schizophrenia genome-wide (P=3.50×10^{-8}, n=58 140) (Lam et al., 2019). In the present study, through population genetic analyses, *in vitro* luciferase assays, and expression quantitative trait loci (eQTL) data, we identified a functional 339 bp *Alu* insertion polymorphism (rs71389983) within the 9th intron of the *AS3MT* gene in complete LD with rs7914558.

The study protocol was approved by the Institutional Review Board of the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). Informed consent was obtained before any study-related procedures were carried out. Genotyping of rs71389983 and rs7914558 was conducted using polymerase chain reaction (PCR) on 38 European and 39 Han Chinese subjects, with amplicons analyzed using

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agarose gel and Sanger sequencing to determine differences in alleles. The PCR primers were: 5'- ATGTAACTGGTATATCC ATCGCCT-3' (forward) and 5'- AGAAGACTCAAACAGATGAAC GGA-3' (reverse) for rs71389983; and 5'-CTCTACTTGCCCC CTTACAGC-3' (forward) and 5'-GAACCGTATCAGTAATCC AACAGA-3' (reverse) for rs7914558.

The HEK293T (human embryonic kidney 293T) and U87MG (human glioblastoma astrocytoma) cell lines used were originally obtained from the Kunming Cell Bank, KIZ, and the Cell Bank of Type Culture Collection of the CAS, respectively. Both cell lines were checked regularly for mycoplasma infection using PCR and microscopy. No cells were found to be contaminated during the study. The HEK293T cells were cultured in a humidified 5% CO_2 incubator at 37 °C in DMEM basic (Dulbecco's Modified Eagle's Medium) (Gibco, USA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin-streptomycin. The U87MG cells were cultured in a humidified 5% CO_2 incubator at 37 °C in MEM (Minimum Essential Medium) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 2.2 g/L $NaHCO_3$, and 1% penicillin-streptomycin.

For the reporter gene assays, DNA fragments encompassing rs71389983 with either allele were amplified from human genomic DNA using primers 5'-GGCTGCCAGGTTCAAGTAAT- 3' (forward) and 5'-CACACTGGAATACTATTCAGACTT-3' (reverse). The sequences were then cloned into the pGL3-promoter vector (Promega, USA) upstream of the SV40 promoter. The recombinant clones were verified through Sanger sequencing to ensure they only differed at the rs71389983 locus. The pGL3-promoter reporters were transiently co-transfected into cells together with the pRL-TK plasmid (Promega, USA) using Lipofectamine 3000 (Thermo Fisher Scientific, USA). All plasmids were accurately quantified and equal amounts were used for transfection. All transfection procedures lasted 36–48 h, and the cells were then collected to measure luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, USA). The activity of firefly luciferase was normalized to that of Renilla luciferase to control for variations in transfection efficiency. All assays were performed with at least three biological replicates in independent experiments, and statistical analyses were performed by two-tailed *t*-tests.

We also examined the impacts of risk SNPs on gene mRNA expression using two public RNA-seq brain eQTL datasets, i. e., BrainSeq Phase 2 (http://eqtl.brainseq.org/phase2/eqtl/) and GTEx (https://www.gtexportal.org/) (Collado-Torres et al., 2019; GTEx Consortium et al., 2017). Briefly, from the BrainSeq dataset, we obtained eQTL data of the dorsolateral prefrontal cortex (DLPFC) from 397 individuals, which were calculated using linear regression by covarying diagnosis, gender, genotyping principal components, and expression principal components. From the GTEx dataset, we retrieved the eQTL association results from the frontal cortex (BA9) of 175 subjects, which were calculated using linear regression by covarying genotyping principal components, gender, genotyping platforms, and additional covariates.

Recent study has shown that a subset of *Alu* insertion polymorphisms exhibit moderate to strong LD (r^2>0.7) with GWAS risk SNPs of complex illnesses (Payer et al., 2017). We therefore examined whether there were *Alu* insertion polymorphisms within the 10q24.32 region. Using public genomic variation databases (i.e., UCSC, http://genome.ucsc.edu/) followed by Sanger sequencing of target regions, we identified an *Alu* insertion polymorphism (339 bp) rs71389983 in intron 9 of *AS3MT*, which was in complete LD with rs7914558 in the Han Chinese and European populations (both r^2=1.00, Figure 1A). The presence of the *Alu* insertion at rs71389983 was linked with the schizophrenia risk G-allele at rs7914558, and therefore may be associated with increased risk of schizophrenia. We note that the frequency of rs71389983 (and rs7914558) showed divergence between the two populations (frequency of *Alu* insertion at rs71389983: 0.423 in Han Chinese vs. 0.605 in Europeans). We also compared the LD structures of the 10q24.32 region between Europeans and East Asians using genotype data from the 1000 Genomes Project (Genomes Project Consortium et al., 2015), and found that the LD structures were relatively similar across distinct populations, despite showing tiny differences (Figure 1A), in agreement with the significant associations of this genomic area in both populations.

The DNA sequence covering rs7914558 is conserved across humans and non-human primates, whereas the *Alu* polymorphism rs71389983 appears to be human-unique. We thus performed bioinformatics functional prediction of rs7914558 using the HaploReg v4.1 dataset (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) (Ward & Kellis, 2012). However, we found that it was unlikely located at any DNA segments showing open-chromatin peaks or directly binding to transcription factors or histone markers (e.g., H3K4me1, H3K4me3, H3K9ac, and H3K27ac). On the other hand, *Alu* insertions have been found to affect both transcription and post-transcriptional processes (Häsler & Strub, 2006). Considering that rs71389983 was found in intron 9 of *AS3MT*, we hypothesized that it may be within the enhancer/repressor region of the genome. To test this, we amplified the DNA fragments spanning rs71389983 from individuals carrying different homozygotes (PCR product length: presence of Alu insertion: 589 bp; absence of Alu insertion: 250 bp), and then sub-cloned them into the pGL3 promoter vector. These plasmids were then transfected into the human HEK293T and U87MG cell lines, and reporter gene assays were carried out to examine their regulatory effects. In the HEK293T cells, the transcriptional activity of the pGL3 promoter containing the *Alu* insertion at rs71389983 was significantly lower than that of the promoter without the allele (P<0.000 01, Figure 1B) and that of the empty vector (P<0.000 01). In the U87MG cells, this trend was reproduced and the presence of the *Alu* insertion at rs71389983 corresponded to significantly lower activity of the pGL3 promoter compared with that of the pGL3 promoter without the allele (P<0.000 01, Figure 1B) and the empty vector (P<0.000 01).

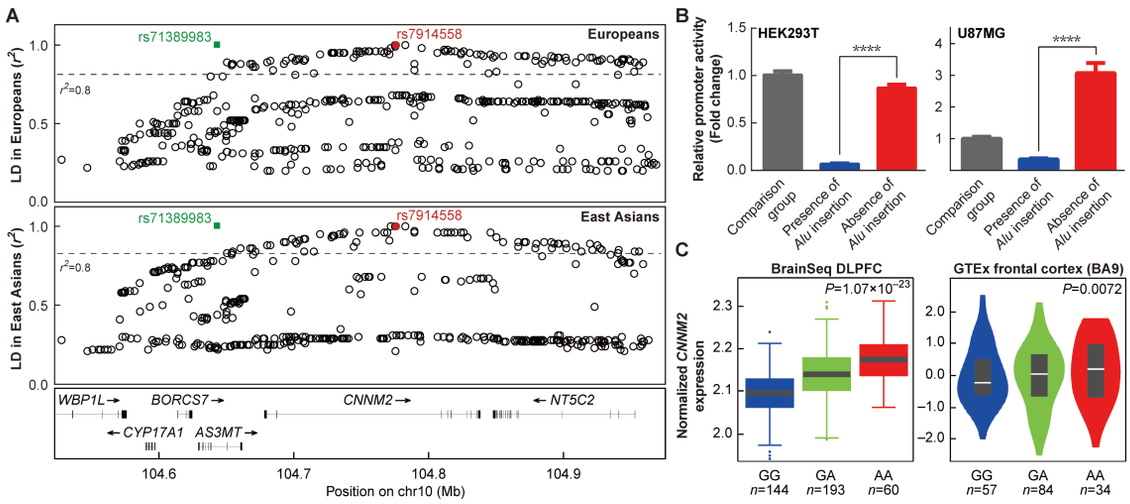

Figure 1 Linkage disequilibrium (LD) analysis of rs7914558 and nearby variations (including rs71389983) in European and East Asian populations (A); Reporter gene assay testing regulatory activity of rs71389983 in HEK293T and U87MG cells (B); Expression quantitative trait loci (eQTL) analyses of rs7914558 with *CNNM2* mRNA in BrainSeq and GTEx datasets (C)

Rs7914558 is located in intron of *CNNM2*, and *Alu* polymorphism rs71389983 is located in intron 9 of *AS3MT*. Both variations are in complete LD in both Europeans and East Asians. Effects of rs71389983 allele variation on pGL3 promoter activity in HEK293T and U87MG cells are shown. "Comparison group"in figure represents empty pGL3 promoter. Values represent fold change in luciferase activity relative to control pGL3 vector. Means and standard deviations of at least three independent experiments are shown. ****: P<0.000 01.

Therefore, rs71389983 is likely a functional variation and the *Alu* insertion at this locus likely exerts repressive effects on transcription. In addition to the consistent trend of the effect of the rs71389983 *Alu* insertion on both cell lines, a slight difference between the HEK293T and U87MG cell results was observed, as the pGL3 promoter carrying the "absence of *Alu* insertion" at rs71389983 showed higher transcriptional activity than the empty vector in the U87MG cells, but lower activity than the empty vector in the HEK293T cells. This inconsistency could be explained by the different genetic and physiological backgrounds between the different cell lines.

To further confirm the regulatory effects of the *Alu* polymorphism (rs71389983) on gene expression, we examined two public RNA-seq eQTL datasets (i.e., BrainSeq and GTEx-brain) in human brains (Collado-Torres et al., 2019; GTEx Consortium et al., 2017). As rs71389983 is not genotyped in those eQTL databases, we used rs7914558 as an index SNP. In the BrainSeq dataset, which included DLPFC tissues from 397 individuals, the schizophrenia risk G-allele at rs7914558 was significantly associated with increased gene expression of *BORCS7* (P=9.28×10^{-27}), as well as decreased mRNA expression of *CNNM2* (P=1.07×10^{-23}, Figure 1C) and *CYP17A1-AS1* (P=7.98×10^{-4}). In the 175 frontal cortex (BA9) tissues of the GTEx dataset, the G-allele at rs7914558 was also strongly associated with increased gene expression of *BORCS7* (P=1.00×10^{-9}) and decreased mRNA expression of *CNNM2* (P=0.007 2, Figure 1C), but not with the expression of *CYP17A1-AS1* (P=0.94).

Translating the GWAS risk associations of complex disorders into biological mechanisms remains an urgent task (Barešic et al., 2019; Birnbaum & Weinberger, 2017; Edwards et al., 2013; Forrest et al., 2018; Gandal et al., 2016). However, most genetic risk loci are located in noncoding regions, which may affect transcription factor binding affinities, gene expression, or even cellular physiological processes (Duan et al., 2014; Forrest et al., 2017; Li et al., 2011; Roussos et al., 2014). We identified a 339 bp *Alu* insertion polymorphism (rs71389983) in the 10q24.32 locus, and reporter gene assays showed that different alleles of rs71389983 exhibited significantly different regulatory activities. The promoter carrying the "absence of *Alu* insertion" at rs71389983 exhibited more than 10-fold higher transcriptional activity than the promoter carrying the "presence of *Alu* insertion", suggesting that the *Alu* insertion sequence likely confers function as a gene silencer. Although this effect is usually caused by certain epigenetic modifications such as DNA methylation or noncoding RNA, the current genome-wide sequencing technologies do not provide ideal tools for answering this question. For example, the ENCODE datasets are mostly based on short DNA reads (≤250 bp) (Encode Project Consortium, 2012), and such methods are not able to precisely map retrotransposons, like *Alu* regions, as *Alu* elements contain multiple highly similar sequences (>300 bp) across the genome. Thus, it is difficult to identify the epigenetic or regulatory markers at rs71389983 (as reflected in the UCSC browser, which shows no ChIP-seq data at the rs71389983 locus). To resolve this problem, long-read sequencing technologies should be applied.

We found that in both the BrainSeq and GTEx-brain tissues, the schizophrenia risk allele at rs71389983 (i.e., its complete linked SNP rs7914558) predicted lower expression of *CNNM2*, consistent with the results of our *in vitro* luciferase assays.

Therefore, *CNNM2* is likely a schizophrenia risk gene, in agreement with previous study (Thyme et al., 2019). However, the present results do not necessarily mean that rs71389983 directly regulates *CNNM2* expression, unless further functional studies (e.g., CRISPR/Cas9 genome editing) are carried out. The significant association of risk SNPs (e.g., rs7914558) at 10q24.32 with *BORCS7* expression is also consistent with earlier research (Duarte et al., 2016; Li et al., 2016a).

Previous studies have demonstrated that *Alu* insertion polymorphisms are significantly associated with multiple complex human disorders and traits, including multiple sclerosis, obesity, height, Alzheimer's disease, breast cancer, and blood pressure (Payer et al., 2017). Our recent study also identified a functional *Alu* polymorphism at 3p21.1 affecting DNA regulatory activity, which was significantly associated with increased risk of psychiatric disorders (e.g., schizophrenia, bipolar disorder, and major depressive disorder) and cognitive disfunctions (Yang et al., 2019). Combined with the present data, these studies suggest that such types of sequence variations may play essential roles in shaping phenotypes during primate or human evolution (Deininger, 2011; Häsler & Strub, 2006). However, our previous study showed that the *Alu* insertion sequence at 3p21.1 increased regulatory activity (Yang et al., 2019), and herein the *Alu* insertion sequence at 10q24.32 reduced transcriptional activities. Although the majority of the *Alu* sequences across the genome show high similarity, their functional regulatory effects may be distinct.

In summary, we discovered a human-unique *Alu* insertion in strong LD with the schizophrenia GWAS risk SNP at 10q24.32. Schizophrenia is hypothesized to be specific to or dominant in humans, and its evolutionary mechanism may be related to unique human variations. For example, we previously identified a human-specific allele rs13107325 at *SLC39A8* undergoing Darwinian natural selection, which enabled humans to adapt to cold environments in Europe, but simultaneously also increased the risk of schizophrenia (Li et al., 2016b). The schizophrenia risk allele at *SLC39A8* is also significantly associated with cognitive function and brain structures in human populations (Davies et al., 2018; Elliott et al., 2018; Luo et al., 2019; Savage et al., 2018). Assuming that the human-unique *Alu* insertions play pivotal roles in shaping humanity, such as development of the dorsolateral prefrontal cortex and higher order human features (e.g., higher cognitive processing) (Wang & Arnsten, 2015), they may also deliver some susceptible or deleterious effects to human health, such as predisposition to schizophrenia. Investigations of such human-unique variations in non-human primates or other species (such as tree shrews) that are evolutionarily close to humans or in human-induced pluripotent stem cells (hiPSC) or reprogrammed cells *via* genome editing, may provide novel insights into the pathophysiology of schizophrenia and other human-dominant disorders (Falk et al., 2016; Hoffman et al., 2019; Luo et al., 2016; Xiao et al., 2017; Xu et al., 2013; Yao, 2017).

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.H.Y., Y.L., and M.L. designed the study and interpreted the results. Z.H.Y., N.Q., and X.C. performed DNA extraction, Alu genotyping, population genetic analysis, and primary experiments. L.J.Z., B.L.Z., S.F.Z., J.C., B.X., H.Y.J., D.Y.Z., W.L., H.C., and X.X. contributed to design and helped in the experiments. Z.H.Y., X.X., and M.L. drafted the manuscript.

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