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Common Variants in the *MKL1* Gene Confer Risk of Schizophrenia

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Genome-wide association studies (GWAS) of schizophrenia have identified multiple risk variants with robust association signals for schizophrenia. However, these variants could explain only a small proportion of schizophrenia heritability. Furthermore, the effect size of these risk variants is relatively small (eg, most of them had an OR less than 1.2), suggesting that additional risk variants may be detected when increasing sample size in analysis. Here, we report the identification of a genome-wide significant schizophrenia risk locus at 22q13.1 by combining 2 large-scale schizophrenia cohort studies. Our meta-analysis revealed that 7 single nucleotide polymorphism (SNPs) on chromosome 22q13.1 reached the genome-wide significance level ($P < 5.0 \times 10^{-8}$) in the combined samples (a total of 38 441 individuals). Among them, SNP rs6001946 had the most significant association with schizophrenia ($P = 2.04 \times 10^{-8}$). Interestingly, all 7 SNPs are in high linkage disequilibrium and located in the *MKL1* gene. Expression analysis showed that *MKL1* is highly expressed in human and mouse brains. We further investigated functional links between MKL1 and proteins encoded by other schizophrenia susceptibility genes in the whole human protein interaction network. We found that MKL1 physically interacts with GSK3B, a protein encoded by a well-characterized schizophrenia susceptibility gene. Collectively, our results revealed that genetic variants in *MKL1* might confer risk to schizophrenia. Further investigation of the roles of *MKL1* in the pathogenesis of schizophrenia is warranted.

Key words: schizophrenia/MKL1/genetic association/protein-protein interaction/*GSK3B*

Introduction

Schizophrenia is a severe mental disorder characterized by delusions, hallucinations, disorganized thinking, and cognitive deficits.[1,2] Accumulating evidence indicates that both genetic and environmental factors are involved in the pathogenesis of schizophrenia.[3–7] Though schizophrenia has a strong genetic component (with an estimated heritability of approximately 0.80),[8] the genetic architecture and etiology of schizophrenia remain largely unknown. To identify schizophrenia susceptibility genes, numerous genetic linkage and association studies have been conducted and many candidate genes and loci have been identified, including *COMT*,[9–11] *DISC1*,[12–14] *NRG1*,[15–18] *BDNF*,[19,20] MHC locus,[21,22] among others.[23]

With the dramatic increase in sample size and genotyping throughput, recent genome-wide association studies (GWAS) of schizophrenia have identified multiple convincing risk variants and loci that show robust association with schizophrenia.[21,24–30] However, these identified risk variants only explain a small proportion of schizophrenia heritability, because the effect size of these identified variants is relatively small, with most of them having an odds ratio (OR) less than 1.2.[24] Accumulating evidence suggests that common polygenic variation contributes to risk of schizophrenia,[21,24] implying that novel risk variants are likely to be discovered with the increase of sample size.

To identify additional genetic risk variants that might contribute to schizophrenia susceptibility, we analyzed 2 large schizophrenia cohort datasets. We found that common genetic variants in the Megakaryoblastic leukemia 1 (*MKL1*) gene were significantly associated with schizophrenia in both datasets. Further analyses in the combined sample (a total of 38 441 subjects) identified 7 SNPs

in *MKL1* with genome-wide significance ($P < 5.0 \times 10^{-8}$), and those SNPs were in high linkage disequilibrium (LD). Our results provide genetic evidence that supports the association of the *MKL1* gene with schizophrenia, suggesting that the *MKL1* gene might be involved in the pathogenesis of schizophrenia.

Materials and Methods

Schizophrenia Studies

The first study (case-control based, PGC + SWE samples) consisted of 13 833 cases and 18 310 controls and has been reported by Ripke et al.[24] This sample set is composed of a large-scale GWAS analysis of the Swedish national sample (SWE samples, including 5001 cases and 6243 controls) and independent samples from the schizophrenia Psychiatric Genomics Consortium (PGC samples, including 8832 cases and 12 067 controls). All of the samples were genotyped by Affymetrix, Illumina, or Perlegen high-throughput genotyping platforms. The association was performed by using logistic regression of imputed dosages with sample identifiers and 3 principal components as covariates. Briefly, genetic association information of 13 833 cases and 18 310 controls from the combined PGC + SWE samples were used in this study. Detailed information on sample ascertainment and diagnosis, genotyping quality control, genomic control, and statistical analyses can be found in the original work.[24]

The second study (family-based) contained 6289 individuals (including 3286 schizophrenia cases) from 1811 nuclear families and was reported by Aberg et al.[31] Three independent samples were recruited in this family-based replication sample: a European family-based sample (2740 individuals, 794 families, and 1420 schizophrenia cases), an Asian family-based sample (2296 individuals, 579 families, and 1222 schizophrenia cases), and an African family-based sample (1262 individuals, 438 families, and 644 schizophrenia cases). More detailed information regarding sample description (recruitment of schizophrenic patient and healthy controls and diagnosis), genotyping, quality control, population stratification analysis, and statistical analysis can be found in the original article.[31] There is no overlap between individuals from this family-based study and the subjects from the case-control based PGC + SWE sample (Ripke et al.[24]).

Extraction of Genetic Association Information and Meta-analysis

Because the family-based genetic association analysis is complementary to the case-control association study, we hypothesized that authentic schizophrenia risk variants may be identified if they simultaneously show significant association with schizophrenia in case-control and family-based samples. Moreover, with the increase of sample size, the statistical power would be expected to increase when family-based and case-control samples were combined. Based on this hypothesis, we first extracted all single nucleotide polymorphism (SNPs) from the family-based study.[31] Since our goal is to identify the promising genetic risk variants, only SNPs with a *P* value less than 0.01 from the study of Aberg et al.[31] were considered. As many of these SNPs are located in chromosomal regions that were reported by Ripke et al.,[24] we focused on chromosome 22, a chromosome where no genome-wide significant finding was reported by Ripke et al.[24] We then explored the association between these SNPs in PGC + SWE case-control samples. Finally, meta-analysis was performed with a fixed-effect model by using PLINK (a whole genome association analysis toolset, http://pngu.mgh.harvard.edu/~purcell/plink/)[32] or "Metafor" from the R package.[33] To test whether there is heterogeneity for the analyzed SNPs in case-control samples and family-based samples, we also performed heterogeneity tests by using PLINK and the Metafor package.[33]

Linkage Disequilibrium Analysis

To assess the LD of the SNPs located on chromosome 22, we downloaded the genotypic data of Europeans from the 1000 Genomes project.[34] The LD between these SNPs was then calculated and visualized by using the Haploview program.[35]

MKL1 Expression Analysis in Human Brains

We explored the expression pattern of *MKL1* in diverse human tissues using the gene enrichment profiler[36] and the genotype-tissue expression Portal (GTEx).[37] In addition, we examined the spatiotemporal expression pattern of *MKL1* in developing human brains using the BrainCloud.[38] To further characterize the expression profiling of *MKL1* in schizophrenia patients and healthy controls, we examined *MKL1* expression using the whole transcriptome sequencing (RNA-seq) from post-mortem human brain tissue.[39] Briefly, brain tissues [from the anterior cingulate cortex (Brodmann region 24)] of 35 schizophrenia patients, 35 bipolar disorder, and 35 healthy controls were obtained from the Stanley Medical Research Institute (SMRI; http://www.stanleyresearch.org/dnn/) for transcriptome sequencing. Total RNA was isolated by Trizol reagent (Invitrogen). RNA samples that passed the stringent quality control were used for deep sequencing using Illumine sequencer. More detailed about the dissection of brain tissues, RNA extraction, sequencing, and data analysis can be found in reference.[39]

MKL1 Expression Analysis in Developing and Adult Mouse Brains

We also investigated the expression of *MKL1* in developing and adult mouse brains using real-time quantitative PCR. In brief, C57BL/6J mice were used and embryos were designated as embryonic day 0.5 (E0.5) at noon on

the day at which vaginal plugs were observed. All animal procedures used in this study were approved by The University Committee of Animal Resources (UCAR) at the University of Rochester. Whole brains from 2 embryonic (E12.5 and E14.5) and 7 postnatal stages (P1, P2, P4, P7, P20, P40, and P90) were isolated and total RNA was extracted by Trizol reagent (Invitrogen). RNA was quantified and equal amount of total RNA (3 μg) was treated with DNase I (Fermentas) to remove the potential DNA contamination. DNase I treated RNA was then reversed transcribed by SuperScript III Reverse Transcriptase (Invitrogen) using oligo dT primers. The synthesized cDNA was used to conduct real-time quantitative PCR using SsoAdvanced SYBR Green Supermix (Bio-Rad) and the CFX Real-Time PCR Systems (BioRad). The expression of *MKL1* was normalized to the expression of *GAPDH* and the fold change in expression was calculated using the $\Delta\Delta C_t$ (threshold cycle) method. The primer sequences of *GAPDH* are: Forward: 5′-AGGTCGGTG TGAACGGATTTG-3′; Reverse: 5′-TGTAG ACCAT GTAGT TGAG GTCA-3′. The primers of *MKL1* are Forward: 5′- AGGAC CGAGGAC TATTTG AAAC G-3′; Reverse: 5′- CCACAAT GATAGCCTCCTTCAG-3′. For each stage, at least 3 animals were used and triplicate assays were performed.

Frequency Distribution of the Risk Variants in Global Populations

We examined the frequency distribution of the risk alleles of the identified risk variants in global populations using genotype information from the Human Genome Diversity Project (HGDP) selection browser (http://hgdp.uchicago.edu/cgi-bin/gbrowse/HGDP/),[40] which contains the allele frequency data of 53 worldwide populations.

Functional Prediction of the Significant SNPs

We performed bioinformatic analyses to predict the potential functional consequences of the significant SNPs using the Regulome DB (http://www.regulomedb.org).[41] Multiple types of data (eg, ChIP-seq, DNase-seq, and eQTLs) from the Encyclopedia of DNA Elements (ENCODE) project[42] were used by Regulome DB to annotate SNPs of interest.

Protein-Protein Interaction Analysis

Proteins often have their function through interacting with (eg, binding to) other proteins or other molecules in the cellular system. One protein interaction example is the protein complex—in schizophrenia, dysbindin, an essential component of the biogenesis of lysosome-related organelles complex 1 (BLOC-1), interacts with all 7 other components of BLOC-1.[43] Therefore, the malfunction of a member of the protein complex may lead to cascading functional consequences. Consistent with this, numerous studies have shown that disease-associated genes tend to interact more with each other than random proteins in the protein-protein interaction (PPI) network, and proteins located at the same genomic locus tend to interact within the PPI network.[44,45] Accumulating data have suggested that PPIs play pivotal roles in the identification and prioritization of schizophrenia candidate genes.[46,47] In fact, our recent study suggests that proteins encoded by schizophrenia susceptibility genes significantly and physically interact and encode a highly interconnected protein-protein interaction network.[48] Therefore, investigating the protein interaction may help to prioritize and identify schizophrenia risk genes.

To test whether MKL1 physically interacts with proteins encoded by other schizophrenia risk genes, we first extracted the known schizophrenia susceptibility genes using well-characterized databases of schizophrenia.[23,49–51] In addition, we also carefully curated high-confidence schizophrenia susceptibility genes, including genes identified by recent GWAS of schizophrenia and convergent functional genomics (CFG) studies of schizophrenia.[52] Finally, top-prioritized schizophrenia susceptibility genes from copy number variation studies of schizophrenia were also included. The detailed list of schizophrenia susceptibility genes is available in our previous study[48] and supplementary table S1. We then downloaded the well-defined PPI data from InWeb[53,54] and CytoScape[55] (iRefScape and GeneMANIA). To test whether proteins in the PPI network significantly interact with other proteins, a permutation test was performed using Disease Association Protein-Protein Link Evaluator (DAPPLE, http://www.broadinstitute.org/mpg/dapple/dapple.php)[56]

Results

Common Variants in MKL1 Gene were Significantly Associated with Schizophrenia

Under the hypothesis that true schizophrenia risk variants might be identified if they show significant association in both case-control and family-based schizophrenia samples, we obtained the genetic association data of the case-control PGC + SWE samples[24] and the family-based samples.[31] In total, 8107 SNPs were available from the family-based samples (see "Materials and Methods" section). Because we focused on the most promising risk variants, only SNPs with a *P* value less than 0.01 were considered for further investigation. A total of 117 SNPs with a *P* value less than 0.01 from the family-based sample were extracted (table 1). A detailed examination of these SNPs showed that most of them were reported by Ripke et al.[24] Interestingly, we noted that 28 of these SNPs were located on chromosome 22q13.1 (table 1), a chromosome region where no genome-wide significant finding had been previously reported by Ripke et al.[24]

We further explored the association between the 117 SNPs and schizophrenia in the case-control PGC + SWE

Table 1. Association of 7 SNPs in *MKL1* Gene with Schizophrenia

SNP	Chr	Position	A_{12}[c]	OR[a]			*P* value[b]		
				PGC + SWE[d]	Family-Based[e]	Combined[f]	PGC + SWE	Family-Based	Combined
rs10918670	1	167216210	GA	1.054	1.212	1.068	0.013	0.0032	0.0011
rs11584337	1	167248664	TG	1.054	1.213	1.068	0.013	0.0032	0.0011
rs2132303	1	2223258	TC	1.060	0.847	1.031	0.017	0.010	0.181
rs2296268	1	36603165	TC	1.029	1.152	1.048	0.14	0.0013	0.008
rs2636319	1	243501047	CA	0.932	0.882	0.926	4.15×10^{-5}	0.0083	2.03×10^{-6}
rs2636320	1	243501056	CA	0.931	0.882	0.925	3.6×10^{-5}	0.0083	1.57×10^{-6}
rs2643904	1	2236697	GA	0.943	1.341	0.978	0.018	6.54×10^{-5}	0.346
rs3006925	1	243609927	TC	0.905	0.825	0.898	1.42×10^{-6}	0.0045	5.13×10^{-8}
rs3767432	1	167383472	GA	1.060	1.239	1.075	0.0072	0.0017	4.5×10^{-4}
rs3767434	1	167371151	GA	1.060	1.235	1.074	0.0063	0.0024	4.6×10^{-4}
rs4915419	1	200251050	CA	0.943	1.127	0.966	6.3×10^{-4}	0.0064	0.031
rs589249	1	37162352	GA	1.062	1.138	1.073	9.7×10^{-4}	0.0040	3.6×10^{-5}
rs7532692	1	167250080	TC	1.053	1.196	1.067	0.015	0.0048	0.0014
rs10193188	2	152551210	GA	0.953	0.882	0.939	0.034	0.0059	0.0018
rs10445792	2	201078821	CA	1.107	1.179	1.116	8.09×10^{-6}	0.0047	2.19×10^{-7}
rs11687313	**2**	**201146399**	**GA**	**0.878**	**0.783**	**0.862**	$\mathbf{8.91\times10^{-8}}$	**0.00024**	$\mathbf{3.58\times10^{-10}}$
rs11688415	2	201143409	TC	0.876	0.788	0.865	4.98×10^{-8}	3.5×10^{-4}	2.14×10^{-10}
rs11694369	2	200741720	GA	0.885	0.796	0.871	1.29×10^{-8}	6.0×10^{-4}	1.19×10^{-10}
rs13010104	2	208369213	TC	1.117	NA	1.125	7.23×10^{-7}	NA	6.34×10^{-8}
rs13010676	2	208369294	GA	1.116	NA	1.095	8.29×10^{-7}	NA	2.78×10^{-5}
rs1861228	2	103208945	GA	0.971	0.898	0.961	0.091	0.015	0.013
rs4664494	2	152499580	TC	1.040	1.146	1.060	0.089	0.0030	0.0043
rs6713162	2	152496526	GA	1.041	1.121	1.056	0.082	0.040	0.008
rs6739563	**2**	**201134216**	**TG**	**1.114**	**1.189**	**1.125**	$\mathbf{1.77\times10^{-6}}$	**0.0016**	$\mathbf{1.95\times10^{-08}}$
rs1010471	3	180691092	GA	1.074	0.875	1.044	5.72×10^{-5}	0.0027	0.0093
rs12496073	3	25033188	GA	1.033	1.154	1.049	0.084	0.0019	0.0057
rs13076055	3	12341996	GA	0.963	1.129	0.986	0.048	0.0083	0.42
rs17203055	3	119084331	GA	1.126	NA	1.085	2.38×10^{-5}	NA	0.0024
rs4684846	3	12338849	GA	1.039	0.885	1.014	0.048	0.0078	0.43
rs6445062	3	172192239	TC	1.061	0.870	1.034	6.1×10^{-4}	0.0015	0.040
rs13120709	4	169435779	TC	1.027	1.121	1.040	0.14	0.012	0.021
rs17001561	4	77096118	GA	1.040	1.207	1.056	0.11	0.0072	0.017
rs29357	4	114209691	GA	1.021	NA	1.002	0.45	NA	0.95
rs566091	4	152852168	GC	0.973	0.855	0.953	0.25	0.0046	0.029
rs1986252	5	60754419	TC	0.931	0.879	0.925	2.1×10^{-5}	0.008	1.06×10^{-6}
rs4604142	5	60642595	TC	1.082	1.138	1.088	2.41×10^{-6}	0.0084	1.21×10^{-7}
rs6898746	5	60843706	TA	0.929	0.869	0.923	1.4×10^{-5}	0.0036	4.48×10^{-7}
rs7734879	5	60711632	CA	0.943	0.883	0.935	7.8×10^{-4}	0.010	4.94×10^{-5}
rs156743	6	27967089	TC	1.163	1.174	1.165	1.47×10^{-8}	0.0079	4.20×10^{-10}
rs16897515	6	27278020	CA	1.157	1.255	1.166	8.20×10^{-10}	0.0026	1.39×10^{-11}
rs2077580	6	32020844	GA	0.962	1.276	0.993	0.23	0.0086	0.82
rs2239523	6	31089507	GC	0.983	0.820	0.965	0.34	4.8×10^{-4}	0.046
rs2857605	6	31524851	GA	1.007	1.185	1.026	0.73	0.0039	0.20
rs3130287	6	32050544	TC	0.949	0.826	0.936	0.024	0.0085	0.0032
rs3130349	6	32147696	GA	NA	1.207	1.207	NA	0.0043	0.0043
rs3132935	6	32171075	GA	NA	0.832	0.832	NA	0.0013	0.0013
rs3132947	6	32176782	TG	NA	0.834	0.834	NA	0.0016	0.0016
rs3134605	6	32159956	GA	NA	0.858	0.858	NA	0.0043	0.0043
rs3134954	6	32071893	GA	1.057	1.217	1.071	0.018	0.0071	0.0021
rs354391	6	66319480	CA	1.030	1.137	1.093	0.13	0.0068	0.015
rs3800316	6	27256102	CA	0.894	0.831	0.888	1.85×10^{-8}	0.0033	4.20×10^{-10}
rs6923836	6	113393530	GA	1.045	0.873	1.021	0.011	0.0022	0.21
rs6940698	6	25821580	TC	0.867	0.700	0.853	3.54×10^{-6}	6.0×10^{-4}	6.06×10^{-8}
rs1229761	7	114223723	TG	1.03	0.827	1.013	0.11	0.0034	0.47
rs13274028	8	8729193	CA	1.041	0.850	1.016	0.025	7.1×10^{-4}	0.352
rs3739396	8	18388041	GA	1.092	1.184	1.104	2.6×10^{-4}	0.0058	1.05×10^{-5}

Table 1. (Continued)

SNP	Chr	Position	A_{12}[c]	OR[a] PGC + SWE[d]	OR[a] Family-Based[e]	OR[a] Combined[f]	*P* value[b] PGC + SWE	*P* value[b] Family-Based	*P* value[b] Combined
rs10115971	9	101024887	GA	1.187	0.843	1.136	0.021	0.0026	0.493
rs12343574	9	129440840	CA	1.079	0.825	1.043	0.0081	0.010	0.12
rs1004467	**10**	**104594507**	**TC**	**1.157**	**1.126**	**1.149**	$\mathbf{3.00 \times 10^{-7}}$	**0.016**	$\mathbf{1.77 \times 10^{-8}}$
rs10883757	10	104400133	TC	0.894	0.892	0.894	2.2×10^{-4}	0.021	1.31×10^{-5}
rs11190913	10	103059038	GA	0.941	1.110	0.959	0.0013	0.051	0.018
rs11191499	10	104764271	TC	1.203	1.158	1.193	5.20×10^{-10}	0.0064	1.32×10^{-11}
rs11191514	10	104773364	TC	0.828	0.865	0.837	2.64×10^{-10}	0.007	8.29×10^{-12}
rs11191548	10	104846178	TC	1.209	1.139	1.193	2.68×10^{-10}	0.02	2.49×10^{-11}
rs11191560	10	104869038	TC	1.209	1.143	1.194	2.05×10^{-10}	0.016	1.70×10^{-11}
rs11594111	10	14945406	GA	0.990	NA	1.007	0.70	NA	0.76
rs11818043	10	104391627	GA	0.888	0.887	0.888	7.77×10^{-5}	0.014	3.47×10^{-06}
rs12221064	10	104677126	TC	0.834	0.866	0.841	9.83×10^{-10}	0.0075	2.93×10^{-11}
rs12413046	10	104871204	GA	0.826	0.879	0.838	1.68×10^{-10}	0.020	1.63×10^{-11}
rs17094683	10	104851301	TC	0.827	0.868	0.836	2.07×10^{-10}	0.0088	8.58×10^{-12}
rs17114803	10	104386934	TC	1.125	1.122	1.124	8.62×10^{-5}	0.018	4.81×10^{-6}
rs2298278	10	104390303	GA	1.126	0.893	1.056	8.09×10^{-5}	0.019	0.034
rs3740387	10	104849468	TC	0.897	0.905	0.898	2.26×10^{-10}	0.018	1.29×10^{-11}
rs3740390	10	104638480	GA	1.195	1.161	1.187	2.21×10^{-9}	0.0054	4.59×10^{-11}
rs4409766	**10**	**104616663**	**TC**	**1.159**	**1.156**	**1.158**	$\mathbf{2.26 \times 10^{-7}}$	**0.0035**	$\mathbf{2.82 \times 10^{-9}}$
rs4919666	10	104384029	GA	1.126	1.124	1.125	8.71×10^{-5}	0.017	4.45×10^{-6}
rs7897654	10	104662458	TC	1.113	1.121	1.114	6.52×10^{-9}	0.0085	1.72×10^{-10}
rs7923415	10	104418656	TC	0.972	0.879	0.959	0.11	0.0053	0.013
rs1059440	11	63991801	GA	1.052	1.141	1.065	0.025	0.011	0.0022
rs11231727	11	64011854	TC	0.961	0.895	0.952	0.022	0.011	0.002
rs11605738	11	64013406	GA	0.933	0.879	0.924	0.0021	0.013	0.00014
rs3219243	12	109542392	TC	0.961	0.868	0.945	0.062	0.0041	0.0043
rs17065458	13	44704662	TC	1.073	1.177	1.091	0.010	0.0047	4.1×10^{-4}
rs2036130	13	82606614	TC	0.952	0.805	0.925	0.13	0.0029	0.0096
rs1261117	18	52949657	TC	1.153	1.225	1.166	9.3×10^{-4}	0.026	8.21×10^{-5}
rs1564483	18	60794654	GA	1.038	1.150	1.054	0.069	0.0045	0.0056
rs4892046	18	70484496	GA	1.021	1.151	1.036	0.21	0.0025	0.027
rs12611334	19	40229409	GC	1.038	1.152	1.048	0.037	0.0087	0.005
rs16939	19	46276056	CA	1.031	1.113	1.041	0.076	0.0147	0.011
rs10483204	22	40870794	TC	1.134	1.156	1.138	5.90×10^{-6}	0.0093	1.91×10^{-7}
rs10483205	22	40883599	TC	0.877	0.856	0.874	3.99×10^{-6}	0.014	1.86×10^{-7}
rs12159787	22	40870699	GA	0.883	0.861	0.878	7.51×10^{-6}	0.0062	1.56×10^{-7}
rs138866	22	50205903	GA	0.933	0.865	0.925	0.0010	0.015	8.94×10^{-5}
rs138880	22	50218611	CA	1.072	1.187	1.086	0.0010	0.0029	4.01×10^{-5}
rs16985899	22	40963402	TC	0.874	0.854	0.871	1.67×10^{-6}	0.010	5.64×10^{-8}
rs17001977	22	40880213	GA	1.139	1.163	1.143	4.81×10^{-6}	0.014	2.27×10^{-7}
rs17001993	22	40900077	GA	1.139	1.168	1.144	4.24×10^{-6}	0.012	1.64×10^{-7}
rs17001997	22	40905072	GA	1.139	1.168	1.144	4.54×10^{-6}	0.012	1.84×10^{-7}
rs17002024	22	40975268	TC	0.878	0.853	0.873	2.46×10^{-6}	0.0065	5.81×10^{-8}
rs17002026	22	40982581	TC	0.878	0.854	0.873	2.21×10^{-6}	0.0067	5.34×10^{-8}
rs17002027	**22**	**40984571**	**TC**	**1.145**	**1.178**	**1.151**	$\mathbf{1.40 \times 10^{-6}}$	**0.0076**	$\mathbf{3.98 \times 10^{-8}}$
rs17002030	**22**	**40989374**	**TC**	**1.147**	**1.173**	**1.151**	$\mathbf{9.35 \times 10^{-7}}$	**0.0096**	$\mathbf{2.92 \times 10^{-8}}$
rs17002034	**22**	**40996367**	**TG**	**0.873**	**0.850**	**0.869**	$\mathbf{1.40 \times 10^{-6}}$	**0.0082**	$\mathbf{4.08 \times 10^{-8}}$
rs17002038	22	41000964	TC	0.874	0.853	0.870	1.53×10^{-6}	0.0099	5.39×10^{-8}
rs3827381	22	40881402	TC	1.131	1.154	1.136	1.55×10^{-5}	0.010	5.26×10^{-7}
rs3827382	22	40881403	GA	1.138	1.163	1.143	7.94×10^{-6}	0.014	3.61×10^{-7}
rs5749683	22	34252374	TC	0.924	0.868	0.915	2.2×10^{-4}	0.0053	7.11×10^{-6}
rs5995867	22	40888136	TG	1.135	1.160	1.140	5.37×10^{-6}	0.0076	1.37×10^{-7}
rs5995871	22	40922332	GA	0.879	0.850	0.873	2.87×10^{-6}	0.0054	6.31×10^{-8}
rs5995886	22	41033801	GA	1.102	1.139	1.108	4.02×10^{-6}	0.0076	1.21×10^{-7}
rs6001912	22	40828361	TC	1.138	1.158	1.142	4.74×10^{-6}	0.0092	1.4×10^{-7}
rs6001930	22	40876234	TC	1.133	1.154	1.137	6.84×10^{-6}	0.010	2.23×10^{-7}
rs6001931	22	40877514	GA	1.133	1.155	1.138	6.88×10^{-6}	0.0099	2.24×10^{-7}
rs6001946	**22**	**40903421**	**GA**	**0.882**	**0.876**	**0.880**	$\mathbf{6.38 \times 10^{-6}}$	**0.0009**	$\mathbf{2.04 \times 10^{-8}}$

Table 1. (Continued)

SNP	Chr	Position	A_{12}[c]	OR[a] PGC + SWE[d]	OR[a] Family-Based[e]	OR[a] Combined[f]	*P* value[b] PGC + SWE	*P* value[b] Family-Based	*P* value[b] Combined
rs6001974	**22**	**40979164**	**GA**	**0.877**	**0.850**	**0.872**	**2.18×10^{-6}**	**0.0053**	**4.16×10^{-8}**
rs6001980	**22**	**41004384**	**TC**	**1.140**	**1.172**	**1.146**	**2.02×10^{-6}**	**0.0061**	**4.60×10^{-8}**
rs6001981	**22**	**41017425**	**TC**	**0.876**	**0.854**	**0.872**	**1.79×10^{-6}**	**0.0069**	**4.66×10^{-8}**

[a]The OR is based on reference alleles.
[b]Two-tailed *P* values.
[c]A_{12}, reference and alternative alleles.
[d]PGC + SWE case-control samples consisted of 13 833 cases and 18 310 controls.
[e]The family-based samples are from 3 different subsamples with a total of 6283 subjects.
[f]Meta-analysis results for the case-control and family-based samples. Meta-analysis was performed based on a fixed-effects model. SNPs reached genome-wide significance level in PGC + SWE case-control sample are in italics. Genome-wide significant *P* values ($P < 5.0 \times 10^{-8}$) in the combined samples are shown in bold.

samples (13 833 cases and 18 310 controls). The results from the family-based and case-control PCG + SWE samples were highly concordant, with 88% (103 out of 117) of the OR having the same direction of effect (table 1). Among the 117 SNPs, 15 (marked by *underline* in table 1) reached genome-wide significance level ($P < 5.0 \times 10^{-10}$) in the case-control PGC + SWE samples and have been reported by Ripke et al.[24] Intriguingly, we found that all of the 28 SNPs from chromosome 22 showed moderate association with schizophrenia in the PGC + SWE samples (table 1). More importantly, we noticed that the effect direction (risk allele) of these 28 SNPs in the family-based sample (from Aberg et al.'s study)[31] were the same as in the case-control samples (PGC + SWE) (table 1), strongly suggesting that these SNPs may be authentic risk variants.

Next, we evaluated the association between the 117 SNPs and schizophrenia in the combined samples from the 2 studies (a total of 38 441 subjects) by using meta-analysis, which was performed with a fixed-effect model using PLINK[32] or "metafor" package implemented in R.[33] In addition to the 15 genome-wide significant SNPs (*underlined* in table 1) reported by Ripke et al.,[24] we identified 11 new SNPs (marked in bold in table 1) that reached genome-wide significance level ($P < 5.0 \times 10^{-8}$) in the combined samples (table 1). Among the 11 newly identified SNPs, rs11687313 and rs6739563 are located on chromosome 2, rs1004467 and rs4409766 are located on chromosome 10, and the remaining 7 SNPs are located on chromosome 22. We further tested if the 11 newly identified SNPs represent independent association signals by using LD information (Europeans) from the 1000 Genomes project.[34] We found that rs11687313 and rs6739563 are highly linked with rs11688415 ($r^2 = 1$ and 0.79, respectively, supplementary figure 1), a SNP that has been reported by Ripke et al.[24] In addition, rs1004467 is highly linked with rs11191514 ($r^2 = 0.82$, supplementary figure 2), a SNP reached genome-wide significance level in study of Ripke et al.[24] These results suggested that the significant SNPs from chromosome 2 and 10 were likely due to the linkage of these SNPs with the genome-wide significant SNPs reported by Ripke et al.[24]

Intriguingly, the meta-analysis revealed that 7 out of the 28 SNPs on chromosome 22q13.1 reached the genome-wide significance level ($P < 5.0 \times 10^{-8}$) (table 1) in the combined samples. Among them, SNP rs6001946 had the most significant association with schizophrenia ($P = 2.04 \times 10^{-8}$) (table 1). To determine the genomic location of the 7 SNPs that reached the genome-wide significance level ($P < 5.0 \times 10^{-8}$), we mapped these SNPs to the human reference genome (hg19). All 7 of these significant SNPs are located in the *MKL1* gene: rs6001981, rs6001980, and rs17002034 reside in intron 1; rs17002027, rs17002030 and rs6001974 are located in intron 2; and rs6001946 is located in intron 3 of the *MKL1* gene (figure 1A). Despite the fact that these 7 SNPs reached genome-wide significance level, it should be noted that the OR of these 7 SNPs are relatively small, with all of them having an OR less than 1.2.

Genome-Wide Significant SNPs in the MKL1 Gene are Highly Linked

We analyzed the LD pattern among the 7 SNPs (in *MKL1*) that reached genome-wide significance level by using the genotypic data (Phase I) of Europeans from the 1000 Genomes project.[57] The LD analysis revealed that the 7 genome-wide significant SNPs from chromosome 22 are in high-LD. In fact, we found that all 7 significant SNPs are located in 1 haplotype block (figure 1B). Because all the significant SNPs are located in introns of *MKL1* gene, we therefore explored whether these SNPs have potential functional consequences, eg, influence transcription factor binding or gene expression. We found that 4 SNPs have RegulomeDB scores (supplementary table S2). Of note, the RegulomeDB score of rs6001974 is relatively high and transcription factor USF2 binds to the region containing SNP rs6001974. These results suggested that these SNPs may have potential functional consequences.

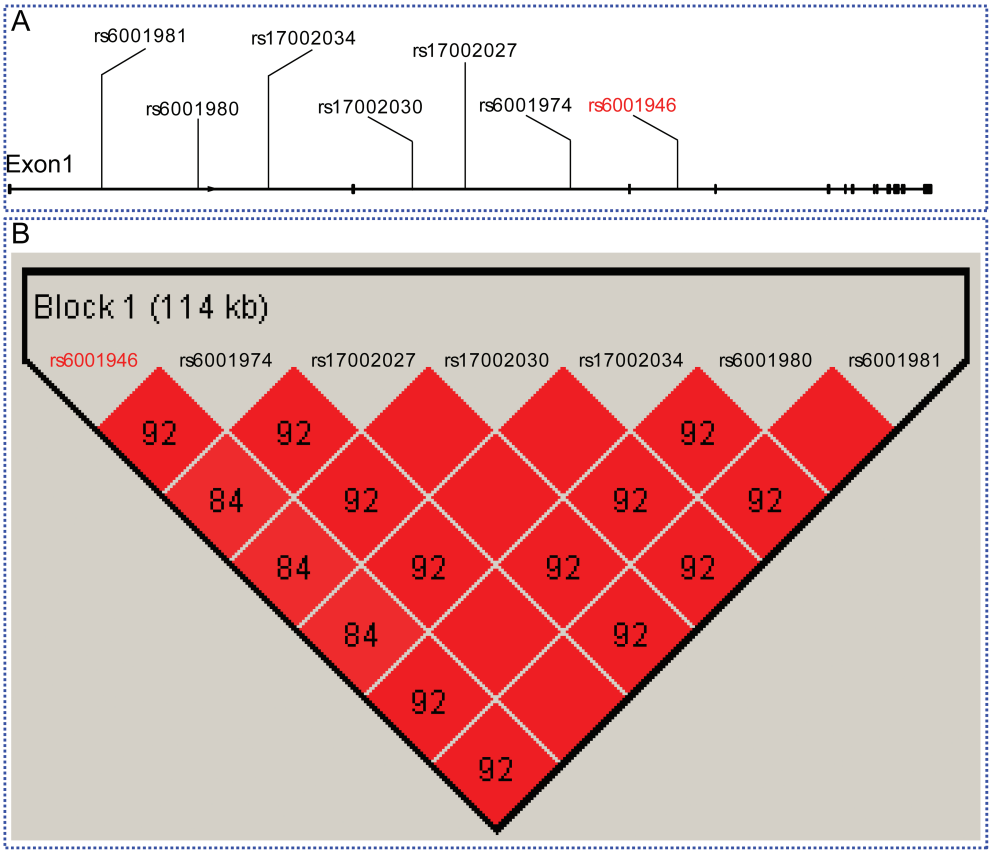

Fig. 1. Genomic locations and linkage disequilibrium of the 7 genome-wide significant single nucleotide polymorphism (SNPs) identified in this study. (A) All of these 7 SNPs are located in introns of the *MKL*1 gene. Of note, rs6001946 (marked by red), which showed the most significant association with schizophrenia, is located in intron 3 of *MKL*1. (B) These 7 SNPs are highly linked in Europeans based on the genotype data from the 1000 Genomes project. Linkage disequilibrium values (r^2) are showed in the red rectangles.

However, further work is needed to test whether these SNPs can regulate or affect the expression of *MKL1*.

Expression Profiling of the MKL1 Gene in the Human Brain

Schizophrenia is a mental disorder that mainly originates from the dysfunction of brain. If *MKL1* contributes to schizophrenia risk, it might be interesting to explore whether it expresses in brain tissues. To characterize the expression pattern of *MKL1*, we examined the expression profiling of *MKL1* in diverse human tissues using the gene enrichment profiler.[36] We found that *MKL1* is widely expressed in human brain tissues, with the highest expression in trigeminal ganglion (figure 2A). Using RNA sequencing-based expression data from the GTEx, we confirmed that *MKL1* is highly expressed in the human brain (figure 2B). We also explored the temporal expression pattern of *MKL1* in developing and adult human brains using the BrainCloud. The expression of *MKL1* is relatively low in prenatal human brains; however, *MKL1* is expressed at a high level in human brains after birth (figure 2B). Finally, we investigated whether *MKL1* is differentially expressed in schizophrenia patients and healthy controls using brain tissues (the anterior cingulate cortex (Brodmann region 24)) from the Stanley Medical Research Institute (see Materials and Methods).[39] We found that *MKL1* is highly expressed in human brains compared with all other human genes (figure 3A, B). In addition, expression of *MKL1* is slightly higher in schizophrenia brain tissue samples than others (control or bipolar disorder tissue samples) (figure 3A). Nevertheless, *MKL1* did not show significant differential expression in schizophrenia cases and healthy controls. These collective results indicated that *MKL*1 is widely expressed in brain at high level, suggesting that it may also be involved in brain development and schizophrenia pathogenesis.

MKL1 is Highly Expressed in Developing and Adult Mouse Brain

We further investigated *MKL1* expression in developing and adult mouse brain using real-time quantitative PCR. Consistent with the findings from human brain, *MKL1* is highly expressed in mouse brain at different developing stages (figure 3C, D). At E12.5, expression of *MKL1* is

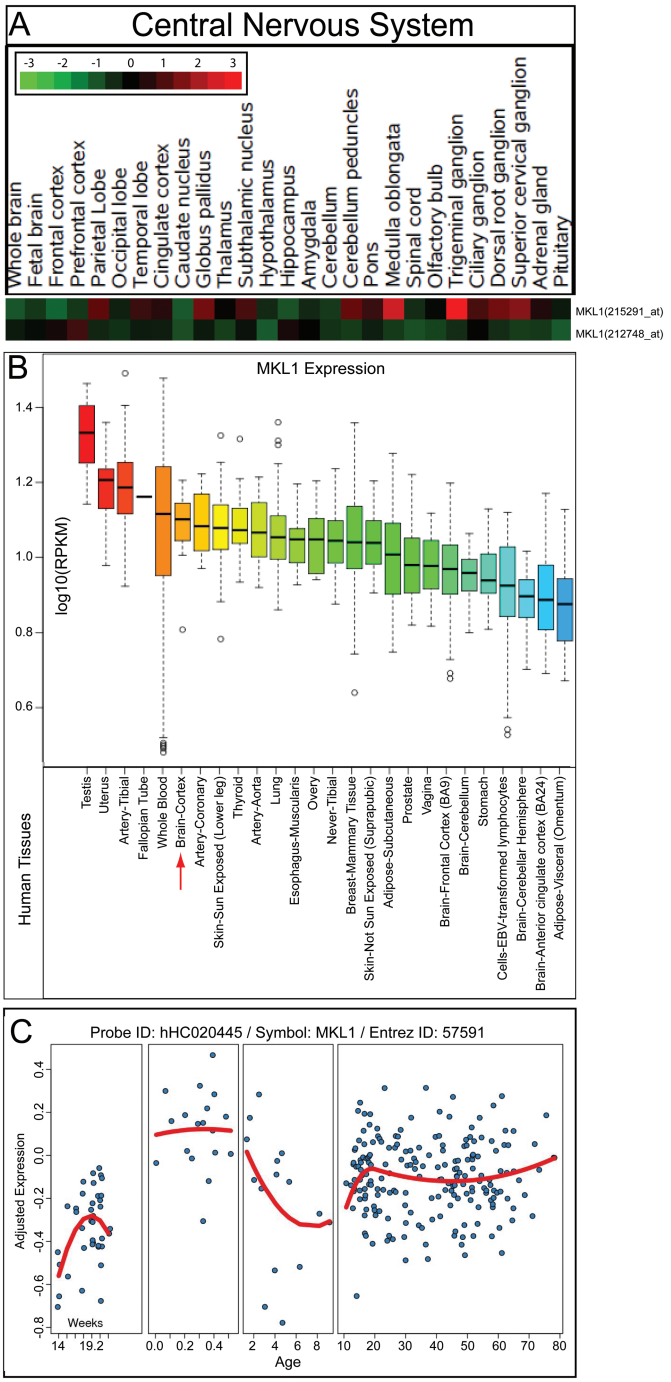

Fig. 2. Spatiotemporal expression pattern of *MKL1* in human brain. (A) *MKL1* is widely expressed in human brain tissues, with the highest expression level in the trigeminal ganglion. (B) RNA sequencing-based expression data showed that *MKL1* is highly expressed in human cortex. (C) Temporal expression profiling of *MKL1* in the prefrontal cortex of humans. The expression level of *MKL1* is relatively low in the early developmental stage. After birth, the expression level of *MKL1* is increased in the human brain.

relatively low. *MKL1* is abundantly expressed in mouse brain from E14.5, with the highest expression level at P40 (figure 3D). These results further confirmed that *MKL1* is highly expressed in central nervous system, suggesting that it may play a role in brain development and schizophrenia pathogenesis.

The Risk Variants in MKL1 Showed Dramatic Allele Frequency Difference in World-Wide Populations

Recent studies suggest that genetic variants conferring risk of psychiatric disorders show significant frequency differences among worldwide populations.[58,59] Thus, we examined the allele frequency distribution of the risk variants identified in this study in global populations. We utilized the genotype data from the HGDP selection database.[40] Because the 7 significant SNPs are in high LD, we first focused on rs6001946, which showed the most significant association with schizophrenia (table 1). Nevertheless, rs6001946 was not found in the HGDP database. Therefore, we checked rs6001974, which is highly linked with rs6001946 (r^2 = 0.92) (figure 1B). We found that the risk allele (A allele) of rs6001974 showed drastic frequency differences in worldwide populations (figure 4A). Interestingly, the risk allele (A) of rs6001974 is common in most world populations (eg, fixed in some populations, red arrows in figure 4A), while the frequency of A allele is relatively low in some populations (blue arrows in figure 4A).

MKL1 Physically Interacts with GSK3B

Our recent studies have shown that proteins encoded by schizophrenia susceptibility genes tend to interact with each other.[48,60] If *MKL1* confers risk to schizophrenia, then it may physically interact with other proteins encoded by schizophrenia susceptibility genes. Using high-confidence protein-protein interactions from the well-characterized PPI databases, we investigated the interactions between MKL1 and other proteins encoded by schizophrenia risk genes. We found that MKL1 physically interacts with GSK3B (figure 4B), a protein encoded by a promising schizophrenia susceptibility gene, *GSK3B*.[61–64] Several lines of evidence suggest that *GSK3B* may represent a promising schizophrenia candidate gene. First, previous studies have shown that genetic variants in *GSK3B* were significantly associated with schizophrenia.[61–63] Second, AKT1-GSK3B signaling was impaired in schizophrenia.[61] Third, expression of *GSK3B* was down-regulated in schizophrenia patients compared with healthy controls.[64] Fourth, a recent study revealed that *GSK3B* expression-correlated genetic variation is associated with prefrontal cortical thickness, prefrontal physiology and schizophrenia.[62] Fifth, *GSK3B* is the target of lithium,[65] a mood-stabilizing agent, ie used as an adjunctive treatment to antipsychotic for schizophrenia.[66,67] Finally, *GSK3B* plays pivotal role in brain development.[68,69] These lines of evidence suggested that *GSK3B* may represent a promising schizophrenia candidate gene. Considering the importance of

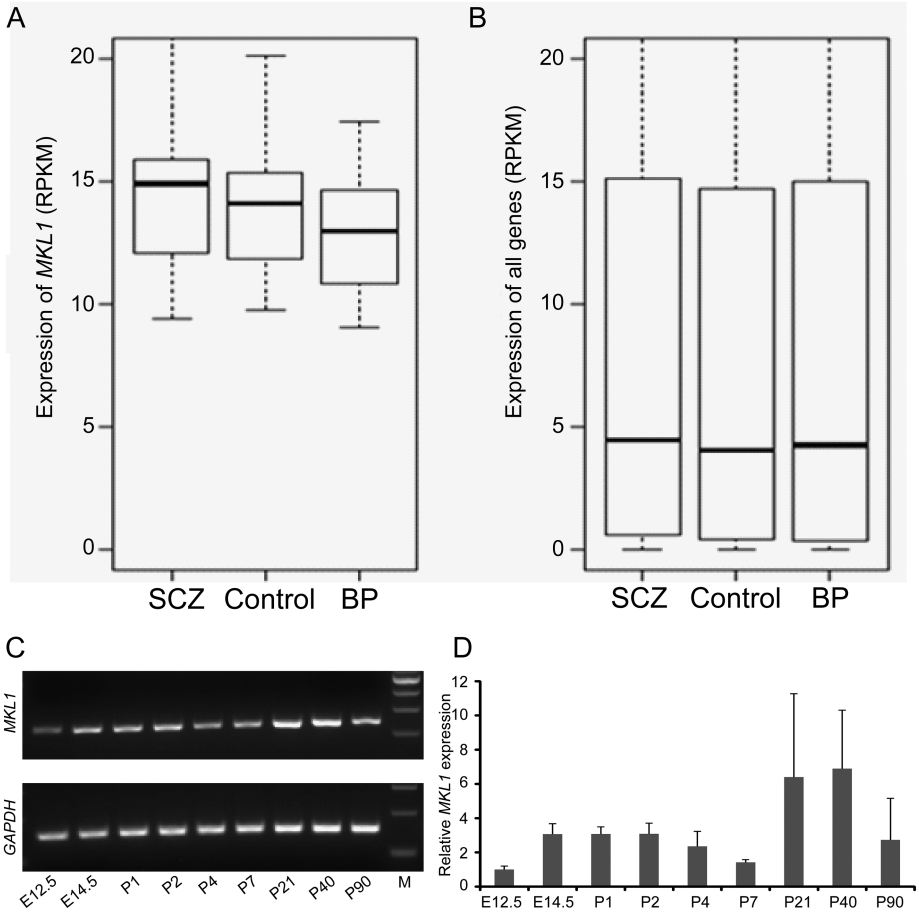

Fig. 3. *MKL1* is highly expressed in human and mouse brains. (A, B) Expression profiling of *MKL1* in the anterior cingulate cortex of schizophrenia patients, bipolar disorder patients and healthy controls. Compared with all other human genes (average RPKM value < 5) (B), *MKL1* showed high expression level in human brains (RPKM > 10) (A). In addition, *MKL1* expression level in the schizophrenia patients is slightly higher than in healthy controls (A). (C, D) *MKL1* is highly expressed in developing and adult mouse brain. (C) RT-PCR revealed the expression of *MKL1* in developing mouse brain from E12.5 to adult (P90). (D) Real-time quantitative PCR showed that the expression of *MKL1* peaks at P21-P40. The *GAPDH* was used as the internal control. Data are expressed as mean ± SD (n = 3). RPKM: reads per kilobase per million reads, a standard measure of gene expression in RNA-seq data. BP, bipolar disorder; M, DNA marker; SCZ, schizophrenia.

GSK3B signaling in brain development[68–70] and schizophrenia pathogenesis, the interaction between MKL1 and GSK3B further supports the role of *MKL1* gene in schizophrenia pathogenesis.

Discussion

MKL1 is a major coactivator of the serum response factor (SRF),[71] an important transcription factor (TF) that regulates activity-driven gene expression in neurons.[72] It has been well-established that SRF is a multifaceted TF in the brain, and it plays essential roles in neurodevelopment and brain function,[73] including in neuronal migration,[72,74] neuronal circuit assembly,[75] hippocampal lamination and dendrite development,[76] learning and memory,[77] axon outgrowth and projection,[78,79] and axon regeneration.[80] Interactions with other coactivator, such as MLK1, is necessary for the function of SRF.[71] As one of the most important coactivators of SRF, *MKL1* has been reported to play important roles in brain function and neurodevelopment. *MKL1* is widely expressed in diverse tissues, with the highest expression levels in the testis and brain.[81] Inactivation of *MKL1* leads to neuronal migration defects and aberrant neurite outgrowth during development.[74] In addition, the inhibition of *MKL1* by RNAi causes a decreased number of dendritic processes and dendritic length.[71] Intriguingly, recent studies have also shown that *MKL1* can regulate neuronal plasticity through influencing the expression of BDNF,[82–84] a protein, ie encoded by a well-studied schizophrenia susceptibility gene.[85–87] These lines of convergent evidence strongly suggest that *MKL1* plays a pivotal role in brain development. Considering that accumulating evidence supports that schizophrenia is a neurodevelopmental disorder,[88–97] our results suggest that *MKL1* might be involved in schizophrenia pathogenesis through influencing brain development. Further investigation is thus warranted.

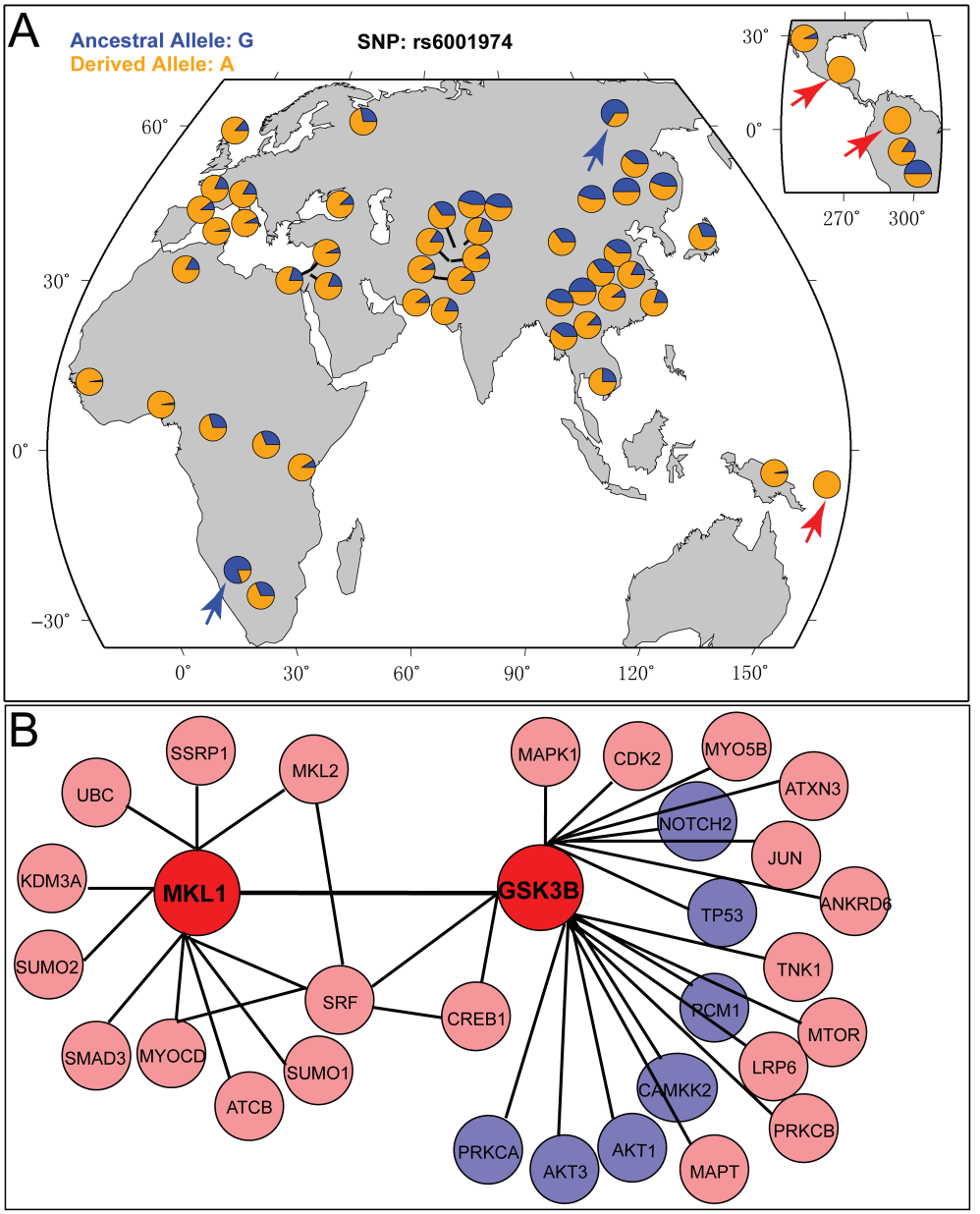

Fig. 4. Frequency distribution of the risk allele of SNP rs17002034 in global populations and protein-protein interaction (PPI) analysis. (A) The risk allele (T) of rs6001974 showed drastic differences in its frequency among global populations. Of note, the A allele (risk allele) is fixed in some populations. (B) MKL1 physically interacts with GSK3B, a protein encoded by a well-known schizophrenia risk gene. Circles represent proteins and edges represent PPIs. MKL1 and GSK3B are marked by red circles. Blue circles represent proteins encoded by schizophrenia susceptibility genes.

Our findings provide genetic evidence of common variants in the *MKL1* gene conferring the risk of schizophrenia. First, given that 2 large complementary samples were used (ie case-control and family-based samples) in this study, the significant association between SNPs in the *MKL1* gene and schizophrenia in both samples support that the *MKL1* gene likely represents a novel risk gene for schizophrenia. Second, the risk alleles of these 7 SNPs in the family-based study are the same as those in the case-control study (PGC + SWE); this striking feature provides further evidence that these identified variants might represent authentic schizophrenia risk variants. Third, expression data indicates that *MKL1* is highly expressed in the human brain, implying its potential roles in neurodevelopment and cognitive function. Fourth, protein-protein interaction analysis further confirmed the potential role of the *MKL1* gene in the pathogenesis of schizophrenia. Collectively, our study supports that *MKL1* is likely a novel, promising schizophrenia susceptibility gene. Our results also support the common polygenic model of schizophrenia,[21,24] which further suggests that

additional risk variants are likely to be identified with the increase of sample size and better analysis strategies. Further work is needed to replicate our results and to elucidate the role of *MKL1* in the etiology of schizophrenia.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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