ORIGINAL ARTICLE
https://doi.org/10.30773/pi.2019.02.26.3
Print ISSN 1738-3684 / On-line ISSN 1976-3026
OPEN ACCESS

Polymorphisms of Ionotropic Glutamate Receptor-Related Genes and the Risk of Autism Spectrum Disorder in a Chinese Population

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Objective To evaluate the association of *GRIK2* and *NLGN1* with autism spectrum disorder in a Chinese population.

Methods We performed spatio-temporal expression analysis of *GRIK2* and *NLGN1* in the developing prefrontal cortex, and examined the expression of the genes in ASD cases and healthy controls using the GSE38322 data set. Following, we performed a case-control study in a Chinese population.

Results The analysis using the publicly available expression data showed that *GRIK2* and *NLGN1* may have a role in the development of human brain and contribute to the risk of ASD. Later genetic analysis in the Chinese population showed that the *GRIK2* rs6922753 for the T allele, TC genotype and dominant model played a significant protective role in ASD susceptibility (respectively: OR=0.840, p=0.023; OR=0.802, p=0.038; OR=0.791, p=0.020). The *NLGN1* rs9855544 for the G allele and GG genotype played a significant protective role in ASD susceptibility (respectively: OR=0.844, p=0.019; OR=0.717, p=0.022). After adjusting p values, the statistical significance was lost (p>0.05).

Conclusion Our results suggested that *GRIK2* rs6922753 and *NLGN1* rs9855544 might not confer susceptibility to ASD in the Chinese population.
Psychiatry Investig 2019;16(5):379-385

Key Words Autism spectrum disorder, Ionotropic glutamate receptors, *GRIK2*, *NLGN1*, Polymorphism.

INTRODUCTION

Glutamate ionotropic receptor kainate type subunit 2 (*GRIK2*) and neuroligin-1 (*NLGN1*) could play a part in the function of the ionotropic glutamate receptors (iGluRs) and thus, influence glutamate signaling and neuronal growth.[1-4] *NLGN1* are specific to excitatory synapses with the capacity to enhance excitatory synapses depending on Ca^{2+}/calmodulin kinase II (CaMKII), which robustly phosphorylates the T739 domain of *NLGN1*.[1] While *GRIK2* acts presynaptically to decrease glutamatergic transmission in the hippocampus.[2] Animal studies showed that the GluK2 receptor, one of the kainite receptors encoded by the *GRIK2* gene, regulates the maturation of synaptic circuits involved in learning and memory.[5] Family-based association study in the Korean trios found preferential transmission of the C allele at the rs3213607 of *GRIK2* in ASD.[6] For the European population, there was also a family-based association study identifying the *GRIK2* as an ASD candidate gene.[7] However, the family-based association study, combined with case-control study, based on an Indian population failed to find an association between *GRIK2* and ASD.[8] A *NLGN1* Pro89Leu (P89L) missense variant was found in ASD patients in the USA. Moreover, in knock-in P89L mice, the model mice showed abnormal social behavior.[9] Previous studies showed

Received: November 2, 2018 **Revised:** February 1, 2019
Accepted: February 26, 2019

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altered expression of *NLGN1* in the brain was observed in several mouse models for ASD, such as *Fmr1* and *Eif4ebp2* knockout mice.[10,11] However, there is little research focusing on the association between ASD and *GRIK2* and *NLGN1* among the Chinese population. Based on the roles of *GRIK2* and *NLGN1* in iGluRs and their inconsistent results, we think it is worthwhile to verify the association between ASD and *GRIK2* and *NLGN1* in the Chinese Han population.

Accumulating evidence suggests that ASD is likely a neurodevelopmental disorder.[12-14] Yuen et al.[15] found 61 ASD-risk genes with sequence-level mutations, which were enriched in synaptic transmission, transcriptional regulation and RNA processing functions. The genes associated with transcriptional regulation and RNA processing are more often expressed in the brain prenatally, while synaptic-function-related genes are expressed in brain throughout development. Based on the neurodevelopmental hypothesis, if the *GRIK2* and *NLGN1* genes are involved in brain development they may be expressed in developing human brain and fluctuate with brain development. We, therefore, explored the spatio-temporal expression pattern in the developing prefrontal cortex using publicly available expression data from Brainspan.[16] Furthermore, if the genes are true risk genes for ASD, the expression of the genes should be dysregulated in ASD. We obtained the publicly available expression data set GSE38322[17] and performed Student's t-test. Finally, in our case-control study, we investigated whether the *GRIK2* and *NLGN1* genes were associated with ASD risk in a Chinese population including 504 ASD patients and 1923 healthy controls.

METHODS

Spatio-temporal expression pattern analysis of risk genes

To explore the spatio-temporal expression of *GRIK2* and *NLGN1* genes in developing human brain, we downloaded the expression data (based on RNA sequencing) from the Allen Institute for Brain Science[16] (http://www.brain-map.org/) (access date: 10/16/2016) (n=42 individuals). We divided the prefrontal cortex into four sub-regions, including dorsolateral prefrontal cortex (DFC), ventrolateral prefrontal cortex (VFC), medial prefrontal cortex (MFC) and orbital prefrontal cortex (OFC).[18] The original expression values were linearly transformed using a min-max standardization method with the following function: x=(x-min)/(max-min), where x represents the original expression value.

Expression analysis in ASD cases and controls

To explore whether *GRIK2* and *NLGN1* genes are differentially expressed in ASD cases compared with controls, we obtained the publicly available expression data set GSE38322.[17] GSE38322 contains brain transcriptional [including cerebellum and occipital (BA19)] data of 18 ASD cases and 18 controls. We downloaded the raw expression values from GEO (https://www.ncbi.nlm.nih.gov/gds/) and performed Student's t-test.

Experiment in Chinese population

Subjects

Our study included 504 ASD patients and 1923 healthy controls. The ASD patients were recruited from the Maternal and Child Care Service Centre in Shenzhen city, Zhuhai city and Luohu district in China, Wuhan Mental Health Center in China and Special Children's Education Agency in Suzhou, Guangzhou and Wuhan in China between July 2010 and July 2016. ASD patients were diagnosed by professional neurologists based on the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV). The control data were selected from GWAS data for a healthy population without ASD, attention-deficit/hyperactivity disorder, mental retardation or other neurodevelopmental disorders in a Chinese population, and they were matched with ASD patients in gender. This case-control study was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology, China.

Identification of Candidate SNPs and Genotyping

The procedure for screening candidate SNPs that might be functional in *GRIK2* gene and *NLGN1* gene was as follows. First, we extracted the SNPs having possible functional effects of protein coding, splicing regulation, transcriptional regulation or post-translation from the F-SNP database (http://compbio.cs.queensu.ca/F-SNP). Second, these SNPs whose minor allele frequency (MAF) of Han Chinese in Beijing (CHB) are more than 5% were filtered from the HapMap database (http://hapmap.ncbi.nlm.nih.gov/). Third, we assessed the linkage disequilibrium (LD) among these SNPs using SNAP Pairwise (http://www.broadinstitute.org/mpg/snap/ldsearchpw.php); if the SNPs were in strong LD with each other ($r^2 \geq 0.80$), we considered reserving only one to analyze any further. As a result, there were two SNPs, which could be used to further selection, in *GRIK2* gene (rs6922753) and *NLGN1* gene (rs9855544).

Genomic DNA was extracted from oral swabs sample using TIANamp Swab DNA Kit DP080714 (Tiangen, Beijing, China) by reference to the manufacturer's instructions. DNA concentration and optical density were tested by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genotyping was performed at the BIO MIAOBIOLOGICAL Corporation (Beijing, China) with the Seque-

nomMassARRAY platform (San Diego, CA, USA) according to the manufacturer's protocol. The MassARRAY Assay Designer software (v3.1) was used to design PCR primers and termination mixes for multiplexed assays. The mass of extended primer was determined using a MALDI-TOF mass spectrometer and we analyzed the resulting genotype spectra using Mass ARRAY Type4.0 software.

Statistical analysis

The ggplot2 package (http://ggplot2.org/) in R (v3.2.5) was used to plot the spatial-temporal expression patterns of the risk genes. GEOquery (http://geoquery.org/) and ggplot2 package were used to analyze the expression pattern in ASD cases and controls from GED dataset. SPSS software v22.0 was used for statistical analyses in experiment of Chinese population. The Hardy-Weinberg equilibrium (HWE) for genotypes was analyzed by Goodness-of-fit χ^2 test in the healthy controls. Unconditional logistic regression (LR) using dominant, recessive and genotype models for each SNP were executed in association analysis. Odds ratios (OR) and 95% confidence intervals (95% CI) were adopted to assess the relative risk conferred by a possibly risk allele and genotype. To control for the false discovery rate (FDR), the Benjamin-Hochberg method was used to adjust the p values for multiple tests within the univariate LR analysis. The statistical power to detect the effects of the SNPs was calculated by Power v3.0.0. For example, for SNPs with minor allele frequency (MAF) of 0.312, and the prevalence of ASD in China was 2.00%, the power of the sample size to detect an OR of 1.50 was 88.4%. All p values were two-tailed with a statistical significant level set at 0.05.

Informed consent and confidentiality

The experiments of the article was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology, China. Informed consent was acquired from the participants or participants' guardians. The patient's information was confidential. An ID was given to each participant. There were no real names, initials, or disclose information that might identify a particular person.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments.

RESULTS

Spatio-temporal expression pattern analysis of risk genes

The *NLGN1* gene showed higher expression levels at embryonic and fetal stages [8 pcw (post conception weeks) to 4 mos] compared with childhood and adulthood stages (8 yrs to 40 yrs) (Figure 1), suggesting this gene may have a role in neurodevelopment.[19] The *GRIK2* gene showed a trend of downregulated expression levels across age (Figure 1).

Expression analysis in ASD cases and controls

mRNA expression of *NLGN1* in ASD was significantly upregulated in the cerebellum ($p<0.001$). There was no significant change in the occipital ($p=0.145$) (Figure 2). mRNA expression of *GRIK2* did not show significant changes in the cerebellum ($p=0.088$) or occipital ($p=0.712$) in the GSE38322 data set (Figure 2). Considering that the trend of *GRIK2* expression level was downregulated across age in developing hu-

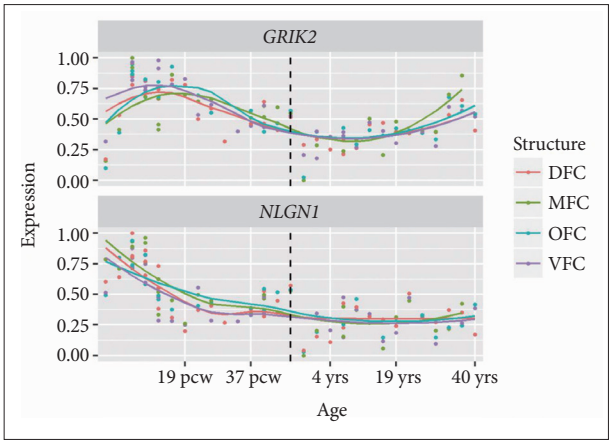

Figure 1. Expression patterns of the *GRIK2*, *NLGN1* genes in human frontal cortex. Expression level of the genes across the entire developing stages [from 8 post-conception weeks (pcw) to 40 years (yrs)] were depicted in the frontal cortex, which was divided into DFC, MFC, OFC and VFC. The expression data were downloaded from Brainspan. DFC: dorsolateral prefrontal cortex, VFC: ventrolateral prefrontal cortex, MFC: medial prefrontal cortex, OFC: orbital prefrontal cortex.

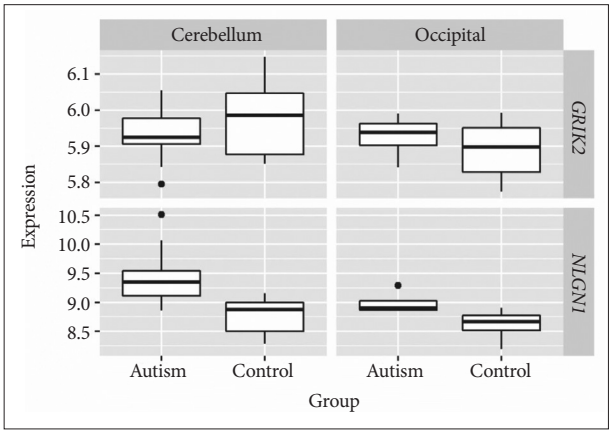

Figure 2. Dysregulation of *GRIK2*, *NLGN1* genes in ASD cases vs cases controls. The vertical axis represented the mRNA expression of *NLGN1* and *GRIK2* genes in the cerebellum and occipital in GSE38322 data set.

man brain from Brainspan[16] and Webster's study showed the same trend in schizophrenia,[20] we still included the *GRIK2* gene in the subsequent validation study in the Chinese Han population.

Experiment in Chinese population

Subjects' characteristics

In this case-control study, there were 504 ASD patients (441 males and 63 females, 8.23±3.15 years) and 1923 healthy controls (1683 males and 240 females, 61.38±8.51 years) for analysis. There was no statistically significant difference in the distribution of gender (χ^2=0.000, p=0.991) between cases and controls. The ASD and controls were matched according gender (male:female ratio of 7:1).

Association analysis between individual SNPs and ASD risk

The two SNPs conformed to Hardy-Weinberg equilibrium (p>0.05). The MAFs of the two SNPs were similar to those in the 1000 Genomes Project of Han Chinese in Beijing, China. The statistical power for detecting the effects of the SNPs were 88.4% and 90.8% (Table 1). As shown in Table 2, the two SNPs were significantly associated with ASD risk. The T allele and the TC genotype of the rs6922753 polymorphism in *GRIK2* were significantly associated with decreased risk of ASD (respectively: OR=0.840, 95% CI=0.722−0.976, p=0.023; OR=0.802, 95% CI=0.651−0.988, p=0.038), as was the dominant model (OR=0.791, 95% CI=0.649−0.963, p=0.020). The *NLGN1* rs9855544 polymorphism for the G allele and GG genotype played a significant protective role in ASD susceptibility (respectively: OR=0.844, 95% CI=0.732−0.973, p=0.019; OR=0.717, 95% CI=0.539-0.954, p=0.022). However, after adjusting p values, the statistical significance was lost (p>0.05).

DISCUSSION

In our study, we explored the spatio-temporal expression pattern in the developing prefrontal cortex and mRNA expression in ASD cases compared with controls of *GRIK2* and *NLGN1* genes using the publicly available expression data. The results suggested that the genes may have a role in the human brain and contribute to the risk of ASD.[19] In the subsequent validation study in the Chinese Han population, we found that rs6922753 in *GRIK2* and *NLGN1* rs9855544 polymorphisms were unlikely to be associated with ASD. The sta-

Table 1. Basic information of SNPs in study

Gene	SNP	MA	MAF*	MAF†	MAF‡	p§	Power (%)
GRIK2/GLUR6	rs6922753	T	0.342	0.312	0.385	0.851	88.4
NLGN1	rs9855544	G	0.458	0.418	0.462	0.082	90.8

*the minimum allele frequency of the SNPs in the control group, †the minimum allele frequency of the SNPs in the 1000 Genomes Project in the Han Chinese in Beijing, China, ‡the minimum allele frequency of the SNPs in the 1000 Genomes Project in the Japanese in Tokyo, Japan, §Hardy-Weinberg equilibrium test. MA: minor allele, MAF: minor allele frequency

Table 2. Association analysis between individual SNP and ASD risk

SNP	Genotype	Case (%)	Control (%)	OR (95%CI)	p*	FDR-P
rs6922753	C	696 (69.6)	2530 (65.8)	1.00		
	T	304 (30.4)	1316 (34.2)	0.840 (0.722−0.976)	0.023	0.058
	CC	246 (49.2)	834 (43.4)	1.00		
	TC	204 (40.8)	862 (44.8)	0.802 (0.651−0.988)	0.038	0.076
	TT	50 (10.0)	227 (11.8)	0.747 (0.533−1.046)	0.090	0.113
	Dominant model			0.791 (0.649−0.963)	0.020	0.100
	Recessive model			0.830 (0.601−1.147)	0.259	0.259
rs9855544	A	574 (58.3)	2083 (54.2)	1.00		
	G	410 (41.7)	1763 (45.8)	0.844 (0.732−0.973)	0.019	0.190
	AA	171 (34.8)	583 (30.3)	1.00		
	GA	232 (47.2)	917 (47.7)	0.863 (0.690−1.078)	0.194	0.216
	GG	89 (18.1)	423 (22.0)	0.717 (0.539−0.954)	0.022	0.073
	Dominant model			0.817 (0.662−1.007)	0.058	0.097
	Recessive model			0.783 (0.608−1.009)	0.059	0.084

*p values were calculated by binary logistic regression, adjusted by gender. OR: odds ratio, CI: confidence interval, FDR: false discovery rate

tistical significance was lost after controlling for the false discovery rate (FDR). Studies with larger sample sizes are needed.

Synaptic transmission underlies every aspect of brain function. Excitatory synapses, which release the neurotransmitter glutamate, are the most numerous type of synapse in the brain. Furthermore, the trafficking of glutamate receptors to and from these synapses controls the strength of excitatory synaptic transmission.[21] Glutamate receptors and synapses are shown to be related to disrupted synapse development and homeostasis in ASD. iGluRs are integral membrane proteins composed of four large subunits that form a central ion channel pore. Disruption in synaptic transmission often implicates reduced AMPA- and NMDA-dependent glutamatergic transmission.[22] Weak NMDA antagonists may be effective in treating ASD, predicting that NMDA hyperfunction has a role in ASD.[23] Downregulation of AMPARs is related to the reduction of plasticity and post-synaptic excitatory potentials, which are strongly associated with learning and memory, and disruption of these may underlie intellectual disabilities in ASD.[22]

The *GRIK2* gene codes for the kainate receptor subunit 2. It has been suggested as a candidate gene for ASD because of its localization in the autism specific region on chromosome 6q21 and the involvement of the receptor protein in cognitive functions like learning and memory.[8,24] Recently, a chromosomal microarray (CMA) analysis describing a 19-year old patient showed two de novo microdeletions that spanned 10 genes including *GRIK2*.[25] Mutation screening revealed several SNPs, including one nucleotide variation changing the protein (M867I) of *GRIK2*, which may be functionally relevant to the development of ASD.[26] Shuang's family-based association study in Chinese Han trios demonstrated that *GRIK2* rs2227281 and rs2227283 showed preferential transmission and revealed an association between the GluR6 locus and ASD.[27] However, our study suggests that the rs6922753 in *GRIK2* is unlikely to confer susceptibility to ASD in the Chinese population. Studies using larger samples, which are representative of all of the Chinese population, are needed. In addition, our study results are consistent with family-based studies in the Indian population, which also failed to show evidence of genetic association of *GRIK2* with ASD.[8]

Neuroligins are postsynaptic cell adhesion molecules that are important for synaptic function. *NLGN1* is localized predominantly to excitatory synapses and plays a pivotal role in brain glutamatergic transmission and cognition.[1] NMDARs are dispensable for synapse formation and connect to *NLGN1* in that both bind to PSD-95, which is proven to be associated with ASD in a previous study.[28] *NLGN1*-mediated synaptic potentiation is diminished after chronic blockade of NMDARs, suggesting that the function of *NLGN1* is influenced by NMDAR signaling.[29] Previous studies have indicated that *NLGN1* is associated with ASD,[9] schizophrenia,[30] Alzheimer's disease,[31] depression[32] and post-traumatic stress disorder.[33] A novel *NLGN1* Pro89Leu (P89L) missense variant was found in two ASD siblings, which led to the impairment of spine formation and changes in protein degradation and cellular localization. The results were validated in an experiment of knock-in P89L mice.[9] Although it has been reported in previous studies, we found the significance was lost between *NLGN1* and ASD after FDR. One of the reasons for this discrepancy may be the heterogeneity of ASD and the difference between Chinese and other races. Our inability to detect an association with the SNPs does not imply that *NLGN1* is not a candidate gene for ASD in the Chinese population. It is necessary to explore the possibility that other polymorphic variants of *NLGN1* gene act as risk alleles for ASD before coming to a conclusion on its involvement in ASD.

To the best of our knowledge, this is the first study to explore a correlation between polymorphisms in these two iGluRs-related genes and ASD in a Chinese Han population combined with the analysis of publicly available datasets. We got the negative findings about the two SNPs. As we known, the ASD is a polygenic complex disease. Genome-wide association studies have identified some important single nucleotide polymorphisms, but none has a large enough effect to be deemed causal.[34] However, up to 40% of simplex families and 60% of multiplex families (in which more than one individual has autism) could have several single nucleotide polymorphisms that, when combined, have an additive effect on risk.[35] These results demonstrate that a myriad of common variants of very small effect impacts ASD liability. In our study, the logistic regression showed the significant p value before FDR, which may show the minor effect of the two genes. The limitations of this study were that we did not exactly match our controls to cases. We used public controls from an exon chip genotyping database for a healthy population. The studies were based on the hypothesis that human genotypes generally do not change with age, and the differences that come from age could be diluted by the large sample size of controls. For another, there are many case-control studies in genetic studies.[36-39] The common application of case-control research proves its rationality to some extent. However, we could not ignore the advantages of other study designs, such as family-based and twin studies, as the ASD is high familial. Further analyses based on rigorous case-control studies or family-based studies along with substantially larger sample size are required.

In summary, the analysis of the publicly available datasets indicated that *GRIK2* and *NLGN1* may have a role in the development of human brain and *NLGN1* gene was dysregulated in the cerebellum of ASD cases. The following experiment in the Chinese Han population implied that the single poly-

morphisms of rs6922753 in *GRIK2* and rs9855544 in *NLGN1* were unlikely to demonstrate an association with the development of ASD.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81872636) to S.R. and Deep Science and Technology Innovation Fund from Shenzhen (JCYJ20160428095110571). The authors are very grateful to all participants.

Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Author Contributions

Designed the experiments: Xinyan Xie, Fang Hou, Ranran Song, Jianhua Gong, Li Li. Performed the experiments: Xinyan Xie, Fang Hou, Huaiting Gu, Lingfei Liu, Xiu Luo, Xin Li. Analyzed the data: Xinyan Xie, Fang Hou, Jiajia Zhang. Contributed the materials: Li Li, Yanlin Chen, Jianhua Gong. Writing—original draft: Xinyan Xie, Fang Hou. Writing— review & editing: Ranran Song, Jianhua Gong.

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