Journal of Autism and Developmental Disorders
https://doi.org/10.1007/s10803-022-05818-2

ORIGINAL PAPER

Single-locus and Haplotype Associations of *GRIN2B* Gene with Autism Spectrum Disorders and the Demographic and Clinical Characteristics of Patients in Guilan, Iran

Donya Pouyan Mehr[1] · Niloofar Faraji[1,2] · Sajjad Rezaei[3] · Parvaneh Keshavarz[1]

Accepted: 5 November 2022

Abstract
Autism spectrum disorders (ASDs) are described as generalized developmental disorders, with an average age of onset of 36 months. Genetic and environmental factors may contribute to this multifactorial disorder. The present study aimed to investigate the association of three *GRIN2B* polymorphisms, including rs1019385, rs1024893, and rs3764028, with ASDs. Based on the results, there was a significant difference regarding the genotype frequency of rs3764028 polymorphism between the control and case (ASD) groups ($P = 0.027$). According to the recessive model, this variant was associated with ASDs ($P = 0.23$). None of the eight haplotype models with frequencies above 0.5 showed significant differences between the case and control groups in terms of allelic frequency. The present results showed that the rs376028 variant was directly related to the phenotypic symptoms of ASDs.

Keywords Autism spectrum disorder · GRIN2B · rs1019385 · rs1024893 · rs3764028

Introduction

Autism is a developmental disability that dramatically affects verbal and nonverbal communication and social interaction that usually occurs before the age of three. This disorder disrupts the academic performance of children, besides other related components, including repetitive activities and stereotyped movements, causing resistance to environmental changes, changes in daily life activities, and abnormal sensory experiences (Hus en Lord 2014).

Evidence suggests that gene mutations are one of the causes of autism (Kundu en Islam 2021; Manoli en State 2021). Generally, different genes are involved in the development of autism spectrum disorders (ASDs), such as *NF1* gene, with mutations in more than 45% of patients with different ASD phenotypes (Garg et al. 2013). According to some studies, five to 11 genes may be responsible for ASDs. On the other hand, some studies suggest that genes associated with ASDs cannot be detected, because ASDs are heterogeneous disorders that may involve tens or hundreds of genes, and some specific genes may be involved in the development of some ASD symptoms (Voineagu 2012; Rylaarsdam en Guemez-Gamboa 2019; Rodriguez-Fontenla en Carracedo 2021). Research on DNA of ASD patients and their families has found genes that are highly associated with the incidence of ASDs (Voineagu 2012). Besides, it has been shown that *de novo* mutations (gene mutations in a sperm or egg alone, with healthy genes in the parents) are more common in children with ASDs (Kong et al. 2012).

The N-methyl D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor, involved in neurotransmitter deficiencies in the mammalian central nervous system.

✉ Parvaneh Keshavarz
parvane.keshavarz.gene@gmail.com; keshavarz@gums.ac.ir

Donya Pouyan Mehr
donyapouyanmehr@gmail.com

Niloofar Faraji
niloofarfaraji.sci@gmail.com

Sajjad Rezaei
rezaei_psy@hotmail.com

[1] Cellular and Molecular Research Center, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran

[2] Gastrointestinal and Liver Diseases Research Center, Guilan University of Medical Sciences, Rasht, Iran

[3] Department of Psychology, University of Guilan, Rasht, Iran

Published online: 18 November 2022

NMDAR is associated with a higher brain function or cell apoptosis following cerebral ischemia (Lin en Lane 2019; Chen et al., 2022). It consists of three units, namely, GluN1, GluN2, and GluN3. The GluN2 subgroup is further classified into GluN2A, GluN2B, GluN2C, and GluN2D (Wyllie et al. 2013). The N-methyl-D-aspartate receptor gene (*GRIN2B*) is located on chromosome 12 and consists of 13 exons, encoding GluN2B. The *GRIN2B* mutations are associated with various psychiatric disorders, including schizophrenia, Parkinson's disease, bipolar disorder, ASDs, and cognitive-communication deficits associated with ASDs in childhood (Ohtsuki et al. 2001; Mori et al. 2011).

The *GRIN2B* gene is majorly involved in neural pattern formation, synaptic plasticity, short-term memory, and language learning (Morisada et al. 2016). In the present study, three polymorphisms, namely, rs1019385, rs1024893, and rs3764028, were investigated regarding their relationship with ASDs. In a previous study, to examine the association of candidate NMDA receptor genes with ASDs, single-nucleotide polymorphisms (SNPs) of *GRIN2A* and *GRIN2B* genes were examined in 151 Korean families from three countries. Their results indicated a significant relationship between ASDs and haplotypes of *GRIN2B* gene and supported the possible role of *GRIN2B* as a candidate gene involved in ASDs (Yoo et al. 2012).

The rs1019385 polymorphism is located in the *GRIN2B* promoter region at the SP1 junction. A previous study reported a 30-fold increase in the activity of T alleles (Miyatake et al. 2002). In another study, the potential involvement of several *GRIN2B* polymorphisms in the risk of Alzheimer's disease was examined, and a significant association was found between rs3764028 polymorphism and Alzheimer's disease in a Chinese population (Jiang en Jia 2009). The third polymorphism selected in the present study (rs1024893) has not been studied yet. Therefore, the current study aimed to investigate the association of single-locus and haplotypes of *GRIN2B* gene with ASDs and to evaluate the demographic characteristics of these patients in Guilan, Iran. Besides other important genes involved in ASDs, the *GRIN2B* gene was selected according to the whole exome sequencing of children with ASDs, referred to our diagnostic laboratory, who showed *GRIN2B* mutations in ASDs. Also, we intend to investigate on other considerable genes in our future studies.

Materials and methods

Sampling and data Collection

In this case-control study, children with ASDs under the age of 18 years, who were referred to Guilan ASD Center, Guilan, Iran, were divided into two groups. The case group included children with ASDs (n = 62), while the control group (n = 101) consisted of healthy children without any symptoms of ASDs. The demographic characteristics of the participants, including age, sex, childhood diseases (e.g., jaundice, rubella, smallpox, and tics), comorbidities of ASDs (e.g., meningitis, brain abscess, epilepsy, mental retardation, attention deficit-hyperactivity disorder [ADHD], panic disorder, and schizophrenia), maternal hypertension and diabetes, smoking status of the parents, place of residence, family history of ASDs, and *intelligence quotient* (*IQ*) score, were recorded.

Moreover, to examine the demographic and clinical characteristics of the participants, the Gilliam ASD Rating Scale-2 (GARS-2) (Montgomery et al., 2008), which consists of four sections, namely, stereotyped behaviors, communication, social interaction, and developmental disorders, was used under the supervision of a psychologist. The exclusion criteria were as follows: fragile X syndrome, tuberous sclerosis, chromosomal abnormalities, and malformation features or any other neurological conditions suspicious of ASDs. In this study, 2 cc of peripheral blood was taken from the participants. Due to the non-cooperation of some children in the collection of salivary samples, 10–25 uprooted strands of hair were used.

Genotyping

DNA samples were extracted from whole blood, saliva, and hair, using a standard procedure (GenEx™, Germany) (Miller et al. 1999). The extracted DNA was genotyped using a polymerase chain reaction (PCR) assay, as described below.

Nested-PCR Assay

The nested-PCR assay is a powerful tool to increase the concentration of samples. The sequences of the corresponding polymorphisms were acquired from the NCBI website. Next, the primers for the nested-PCR reaction, above and below the three *GRIN2B* polymorphisms, were predesigned using Primer3 and Oligo (version 7) web services. The nested-PCR product length was designed for three polymorphisms, including rs3764028, rs1024893, and rs1024893, according to the primer position. The primers were diluted with the required amount of distilled water, according to the manufacturer's protocol (Ampliqon, Fermentas, USA) and then diluted to a ratio of 1:10 to prepare the PCR mixture. The final volume of the PCR mixture was considered to be 8 µL for each reaction.

Amplification-refractory Mutation System (ARMS)-PCR Assay

The ARMS-PCR assay was performed to detect mutations. The primers were diluted as described above. The amplification of a fragment by a mutant allele primer represented a mutation in the allele, while the amplification of a fragment by a wild-type allele primer indicated that there was no mutation in the sequence. The final volume of the PCR mixture was 8 µL for each reaction.

Multiplex PCR

Multiplex PCR Assay This method is commonly used to identify the loci of genes, where many types of mutations occur. After increasing the concentration of DNA extracted by nested-PCR, a multiplex ARMS-PCR assay was essential using the designed primers. Two tubes, including one tube with mutant forward primers and reverse primers and one tube with a mutant-forward primer, a wild type primer, and a reverse primer, were used. The final volume of the reaction mixture was 6.25 µL. The quantity and quality of PCR products were also confirmed by a NanoDrop spectrophotometer (OD, 260/280) and agarose gel (0.8%) electrophoresis (sharp single bands) (Lee et al., 2012; García-Alegría et al., 2020). The genotyping data were generated for more than 99% of sample DNA genomes. Additionally, genotyping of all duplicate quality-control assessments of the samples was performed with 100% consistency.

Haplotype Analysis

A haplotype analysis was carried out on the variants using the maximum likelihood method, and significant haplotypes with frequencies above 5% were selected. The permutation *P*-values were calculated based on 10,000 replications. Genetic linkage and genetic association were also evaluated based on the linkage disequilibrium (LD), using SNPAlyze Pro version 8.0 (Tokyo, Japan). Finally, the Hardy-Weinberg Equilibrium (HWE) and haplotype frequencies with 95% CIs were measured using the SNPAlyze tool (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) and the goodness-of-fit χ^2 test.

Statistical Analysis

To compare the frequencies of alleles between the two groups, ANOVA test, Chi-square test, and Pearson's correlation coefficient test were performed at a significance level of < 0.05 in SPSS version 16.

Table 1 Demographical data and clinical characteristics of participants by frequency

Variables		Case n (%)	Control n (%)
Gender	**Female**	9 (5.2)	40 (23.1)
	Male	53 (36.6)	61 (35.2)
Father with a history of smoking	Never	32 (18.5)	53 (30.6)
	Sometimes	9 (4.6)	14 (8.09)
	Always	9 (5.2)	24 (13.8)
Mother with a history of smoking	Never	39 (22.5)	17 (9.8)
	Sometimes	8 (4.6)	10 (5.7)
	Always	4 (2.3)	3 (1.73)
Family history of ASD		12 (6.9)	9 (5.2)
Habitat	Urban	64 (37)	90 (52)
	Rural	5 (2.9)	-
Childhood disease	Jaundice	9 (5.2)	10 (5.7)
	Rubella,	-	-
	Smallpox	5 (2.9)	-
	Tic	14 (8.1)	-
Disease comorbidity of ASD	Meningitis	-	-
	Brain abscess,	-	-
	Epilepsy	4 (2.3)	1 (0.57)
	Mental retardation	10 (5.8)	-
	ADHD	11 (6.4)	1 (0.57)
	Panic disorder	13(7.5)	-
	Schizophrenia	-	-
Mother with underlying disease	Hypertension	6 (3.5)	-
	Diabetes	2 (1.2)	-

*The differences between the total number of cases and controls are due to the data that are not applicable for variables

Attention deficit hyperactivity disorder (ADHD)

Autism spectrum disorder(ASD)

Results

Evaluation of Allelic and Genotypic Associations of rs1024893 Polymorphism

The demographic and clinical characteristics of children with ASDs and healthy individuals are presented in Table 1. There was a significant difference in terms of the allele frequency between the case and control groups ($P = 0.0493$). Considering genotypes in the dominant, recessive, and dominant hereditary models, no significant difference was observed between the case and control groups in terms of rs1024893 polymorphism ($P = 0.192$). Besides, the analysis of rs1019385, rs1024893, and rs3764028 variants showed no significant difference between the case and control groups considering the frequency of rs3764028 polymorphism ($P = 0.071$). However, there was a significant difference in the frequency of rs3764028 polymorphism genotypes between the two groups ($P = 0.027$). On the other hand, the frequency of G allele polymorphism was not significantly different between the two groups ($P = 0.6189$).

Similarly, the genotypic study of rs1019385 polymorphism between the two groups revealed no significant differences in the dominant, recessive, and dominant hereditary models ($P = 0.9307$) (Table 2).

Haplotype Analysis of GRIN2B Variants

According to the analysis of haplotypes among rs1024893, rs3764028, and rs1019385 polymorphisms, there were eight haplotypes with an overall frequency above 5%. Among these haplotypes, A-T-A, T-T-C, T-G-C, T-T-A, and T-G-A haplotypes were significantly different between children with ASDs and the healthy individuals (Table 3).

Logistic Regression Analysis of Allelic and Genotypic Frequencies of GRIN2B Variants Between the Groups

There was a significant difference between the two groups in terms of rs3764028 genotype ($P = 0.027$). In other words, the genotyping frequency of rs3764028 variant was higher in a recessive genotyping model in the control group compared to the case group. The genotypic frequencies of rs1024893 and rs1019385 polymorphisms in the case and control groups represented no significant differences ($P > 0.05$). Moreover, the results of logistic regression analysis showed that none of the polymorphisms (rs1024893, rs3764028, and rs1019385) could significantly predict ASDs (Table 4).

Association of GRIN2B Variants with the Demographic and Clinical Features of Children with ASDs Based on the GARS test

Multiple comparisons of rs1024893, rs3764028, and rs1019385 polymorphisms in three dominant, recessive, and heterozygous models based on GARS scores revealed that the rs3764028 polymorphism had the least significant association in the recessive model ($P = 0.817$) and the most significant association in the dominant model ($P = 0.095$). Moreover, the GARS scores in the dominant and recessive models for rs1024893 and rs1019385 variants were not significantly different ($P > 0.05$). None of the aforementioned associations were significant (Table 5).

Discussion

Today, epigenetic processes and their complex mechanisms are the most important mediators of genome-environment interactions. Environmental factors can affect the quality and quantity of gene expression through epigenetic mechanisms, which mainly involve DNA methylation, histone protein changes, and expression of non-coding RNAs,

Table 2 Analysis of association of rs1019385, rs1024893, and rs3764028 variants in case and control groups

Variant	Study groups	Genotype n (%)				Allele n (%)			Dominant model		Recessive model		Co-dominant model	
		Major homozygote	**Heterozygote**	**Minor Homozygote**	***P***	**Major**	**minor**	***P***	**OR (95%CI)**	***p***	**OR (95%CI)**	***p***	**OR (95%CI)**	***p***
rs1024893	Case	13	7	41	0.192	89	33	0.049	0.451	0.070	0.674	0.268	0.72	0.125
		(21.3)	(11.5)	(67.2)		(72.9)	(27.1)		(0.19–1.08)		)0.33–1.36)		(0.47–1.10)	
	Control	11	14	76		6	66							
		(75.2)	(13.9)	(10.9)		(17.83)	(82.17)							
ra3764028	Case	24	19	18	0.027	55	67	0.071	0.891	0.0730	0.462	0.023	0.77	0.148
		(39.3)	(31.2)	(29.5)		(54.91)	(45.09)		(0.46–1.71)		(0.24–0.91)		(0.53–1.10)	
	Control	37	16	48		90	112							
		(36.5)	(15.9)	(47.6)		(44.56)	(55.45)							
rs1019385	Case	31	4	26	0.930	66	56	0.619	1.11	0.451	1.132	0.704	1.06	0.716
		(50.8)	(6.6)	(42.6)		(54.10)	(45.9)		(0.59–3.10)		(0.59–2.16)		(0.76–1.48)	
	Control	54	7	40		115	87							
		(53.5)	(6.9)	39.6)		(56.93)	(43.07)							

CI = Odds ratio, CI = Confidence Interval. OR and P-values of different hereditary models by SNPAlyze (ver. 8.1) and the Assotest web application were calculated

Table 3 Haplotype analysis of GRIN2B variants

Haplotype	Overall Frequency	Case Frequency	Control Frequency	Permutation *P* value
A-G-C	0.242	0.200	0.267	0.275
A-T-A	0.187	0.237	0.156	0.152
A-T-C	0.180	0.152	0.197	0.422
A-G-A	0.177	0.139	0.200	0.257
T-T-C	0.068	0.101	0.049	0.122
T-G-C	0.067	0.866	0.054	0.408
T-T-A	0.078	0.574	0.042	0.606
T-G-A	0.029	0.246	0.031	0.723

Three variants were used for haplotype analysis. Eight haplotypes with a frequency of more than 0.05% were found

Table 4 Logistic regression analysis of allelic and genotypic frequencies of GRIN2B gene variants between case and control groups

Variants	ASD n = 62 (%)	Control n = 101 (%)	χ^2 test	Logistic regression OR(95%CI)
rs1024893 A T	33 (27.1) 89(72.9)	36(17.83) (17.82)166	3.863	0.584 (0.343–1.027)
rs1024893 AA AT TT	(21.3)13 (11.5)7 (67.2)41	(10.9)11 (13.9)14 (75.2)76	3.294	0.42 (0.13–1.42) 1.08 (0.4–1.89) 0.46 (0.19–1.11)
rs3764028 A C	(54.91)67 (45.09)55	(44.55)90 (55.44)112	3.270	0.659
rs3764028 AA AC CC	39.3))24 (31.1)19 (29.5)18	37(36.5) 16(15.8) 48(47.5)	7.222	1.83(0.79–4.24) 0.32 (0.013–0.74) 0.58(0.27–1.22)
rs1019385 G T	(54.91)66 (45.09) 56	56.93)) 115 43.7))87	0.247	1.121 (0.723–1.812)
rs1019385 GG GT TT	(50.8)31 (6.6)4 (42.6)26	53.5))54 6.9)) 7 39.6)) 40	0.143	1 (0.27–3.67) 1.14 (0.3–4.28) 1.13 (0.58–2.2)

Results of Pearson (χ2) tests and bidirectional logistic regression using SPSS software version 18

Table 5 Multiple Comparison of GARS total score for polymorphisms of BRIN2B

Variants	Models in pairwise comparison	Mean Differences	Standard Error	*P*-value	95% CI
rs1024893	**Dominant Heterozygote**	-5.044	10.458	0.880	-30.248-20.160
	Recessive	-3.723	7.192	0.863	-21.057-13.610
	Heterozygote Dominant	5.044	10.458	0.880	-20.160-30.248
	Recessive	1.320	9.194	0.989	-20.839-23.480
	Recessive Dominant	3.723	7.192	0.863	-13.610-21.057
	Heterozygote	-1.320	7.194	0.989	-23.480-20.839
ra3764028	**Dominant Heterozygote**	10.285	6.885	0.302	-6.308-26.879
	Recessive	14.619	6.885	0.095	-1.975-31.213
	Heterozygote Dominant	-10.285	6.885	0.302	-26.879-6.308
	Recessive	4.333	7.145	0.817	-12.887-21.553
	Recessive Dominant	-14.619	6.885	0.095	-31.213-1.975
	Heterozygote	-4.333	7.145	0.817	-21.553-12.887
rs1019385	**Dominant Heterozygote**	5.0333	11.776	0.904	-23.348-33.414
	Recessive	-5.553	6.131	0.639	-20.231-9.223
	Heterozygote Dominant	-5.033	11.776	0.904	-33.414-23.348
	Recessive	-10.587	11.985	0.653	-39.472-20.331
	Recessive Dominant	5.553	6.131	0.639	-9.223-20.331
	Heterozygote	10.587	11.985	0.653	-18.298-39.472

According to the observed criterion, the error means field (error) = 497.673

without altering the DNA sequence or extension to the next generation of cells and organisms. Therefore, exposure to destructive environmental factors, affecting the expression of evolutionary genes, is about 2.59 times higher in children compared to their fathers (in the age range of 24–25 years), and these children are at an increased risk of developing ASDs (Maccari et al. 2003; Foley et al. 2009; Eshraghi et al. 2018).

Spontaneous mutations in germ cells and changes in DNA methylation can cause general epigenetic changes in the expression of genes involved in neurodevelopment and impair genomic imprinting of sperm, thereby increasing the risk of neurological disorders, such as ASDs (Marcho et al. 2020; Escher et al., 2021). Since environmental factors are believed to contribute to natural variation in gene expression, population studies show that the heritability of ASD is approximately 52.4%, which is mostly related to common genetic variants or their interactions with environmental factors (Rylaarsdam en Guemez-Gamboa 2019).

The *de novo* mutations have been identified separately in various genes, including *GRIN2B* gene. In this regard, Tarabeux et al. identified three *de novo GRIN2B* mutations in a patient with ASD (Tarabeux et al. 2011). Moreover, in a study by O'Roak et al., the advancing age of fathers was associated with the relative risk of ASDs in their children.

The number of *de novo* mutation events in *GRIN2B* was significantly higher than expected, based on the predicted mutation rate for each gene (O'Roak et al. 2012). Also, the disruption of NMDARs causes abnormal pathogenesis, besides imbalanced stimulus and inhibitory currents, which are important in the pathogenesis of ASDs (Tzang et al. 2019).

Various studies on *GRIN2B* gene polymorphisms and some mental disorders, such as Alzheimer-related obsessive-compulsive disorder (OCD) and ASDs, have demonstrated a significant association between the mutations of *GRIN2B* gene and these disorders (Jiang en Jia 2009). Additionally, in a study by Takasaki et al., five rare missense mutations were identified through mutation screening of *GRIN2B* gene (Takasaki et al. 2016). Also, Yongcheng et al., investigating the association of *GRIN2B* variants with ASDs, found that common variants and related haplotypes of *GRIN2B* were associated with the ASD risk, while some rare variants of *GRIN2B* were not associated with the ASD risk (Pan et al. 2015).

According to previous studies, males are more frequently diagnosed with ASDs compared to females. The prevalence ratio is approximately four males for every one female diagnosed This finding is consistent with the results of the present study, in which 53 out of 62 autistic individuals were male (Thapar en Rutter 2015). In this regard, a Swedish twin study showed that ASD was highly heritable and that three quarters of its genetic variance was shared with ADHD; also, genetic factors contributed to the overlap between ASDs and learning problems, motor coordination problems, and tic disorders (Lichtenstein et al. 2010; Ghirardi et al. 2018).

In the present study, 14 (8.1%), 4 (2.3%), 10 (5.8%), 11 (6.4%), and 13 (7.5%) autistic individuals had tic disorders, epilepsy, mental retardation, ADHD, and panic disorder, respectively; in the control group, only one child with epilepsy and one child with ADHD were identified. Moreover, in the autistic group, 6 (3.5%) mothers had hypertension complications, and 2 (1.2%) mothers had diabetes. Additionally, the results of a study on maternal diabetes and hypertensive disorders indicated an association between conditions marked by high blood pressure and ASDs; nevertheless, no significant association was found between conditions marked by high blood sugar and ASDs (Cordero et al. 2019). Also, the relationship between the haplotypes of *BRIN2B* as a candidate gene and ASDs has been reported in the literature (Yoo et al. 2012; Pan et al. 2015), although more comprehensive research is needed.

Due to lack of research on three variants of *GRIN2B*, that is, rs1024893, rs3764028, and rs1019385, in children with ASDs, the current study aimed to evaluate these three variants due to their important role in neurodevelopmental diseases. The genetic analysis confirmed significant differences in the genotype ($P=0.029$) and allele ($P=0.010$) frequencies of rs3764028 between the case (patients with Alzheimer's disease) and control groups, which can lead to impairment in learning, memory, and information processing (Jiang en Jia 2009). Also, some other studies have reported dysregulation of NMDARs as a risk factor for ASD (Kim et al. 2016; Takasaki et al. 2016). In the present study, the frequency of rs3764028 polymorphism genotype was significantly different between children with ASDs and healthy individuals.

A study on *GRIN2B* variants and their association with Alzheimer's disease indicated no significant differences in the distribution of allele and haplotype frequencies of rs1019385, rs1806201 and rs890 variants between patients with Alzheimer's disease and healthy control individuals (Seripa et al. 2008). Another study on *GRIN2B* gene polymorphism in OCD and its symptoms revealed that T allele and TT genotypes were significantly associated with OCD. These preliminary results demonstrated that the *GRIN2B* gene may, to some extent, confer susceptibility to OCD and its symptoms (Kohlrausch et al. 2016). The OCD in children with ASD varies depending on the individual's mental and chronological age, and the etiology of ASD (Jacob et al. 2009).

Based on the present results, the genotype association and frequency of rs1024893 polymorphism as a *GRIN2B* variant, which has not been studied in the literature, were not significantly different between children with ASDs and the control group, and further investigation on a larger population is essential.

Conclusion

The present results indicated that rs3764028 polymorphism of *GRIN2B* gene might be associated with an increased risk of ASDs. However, further research is needed to evaluate the possible relationship between *GRIN2B* and ASD polymorphisms on a larger sample size and appraise the phenotypic features and alternative population sites and SNPs.

Acknowledgements We thankfully express our thanks to all those blood donors of the survey subjects for their contribution to the DNA samples.

Funding No funding.

Declarations

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Statement All procedures performed in studies involving human participants were by the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication The authors affirm that human research participants provided informed consent for publication of the study.

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