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Association Study of Sequence Variants in Voltage-Gated Ca2+ Channel Subunit Alpha-1C and Autism Spectrum Disorders

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Abstract

Background: Autism spectrum disorders (ASDs) (MIM 209850) are a group of distinct neurodevelopmental disorders characterized by impaired social interactions and communication abilities and abnormal repetitive activities. Many genetic variants have been shown to be associated with ASD. Channelopathies are among putative culprits in the pathogenesis of many neurodevelopmental disorders, including autism. The calcium channel, voltage-dependent, L type, alpha 1C subunit gene (*CACNA1C*) encodes an alpha-1 subunit of a voltage-dependent calcium channel. Genetic variants within this gene have been associated with psychiatric disorders including Autism Spectrum Disorders (ASD). Our aim was to determine whether the SNPs rs1006737, rs4765905, and rs4765913 were associated with ASD in an Iranian population.

Methods: In the present case-control study we investigated the associations of rs1006737, rs4765905, and rs4765913 polymorphisms within *CACNA1C* and the risk of ASD in a population of 529 Iranian ASD patients and 480 age, gender, and ethnicity-matched healthy subjects

Results: None of these SNPs were associated with ASD risk in the assessed population. Although previous studies have shown an association between these polymorphisms and psychiatric disorders and an association between rs4765905 and ASD, we did not replicate those results in our study.

Conclusions: Our data indicate that these *CACNA1C* variants are not involved in the pathogenesis of ASD in the Iranian population.

Keywords: Autism Spectrum Disorder, *CACNA1C*, Channelopathy, polymorphism.

Introduction

Autism spectrum disorders (ASDs) (MIM 209850) are a group of diverse neurodevelopmental disorders characterized by impaired social interaction and communication abilities, and abnormal repetitive activities (1). Several susceptibility loci have been identified for this disorder (2, 3). Among hundreds of gene variants associated with autism, the risk effects are extremely variable. Notably, many distinct variants share biological pathways (4). Considering the role of cross-membrane anion passages in regulation of cell functions, including production of action potentials, gene expression, and cell morphology, channelopathies are putative culprits in the pathogenesis of many neurodevelopmental disorders, including autism (5). The calcium channel, voltage-dependent, L type, alpha 1C subunit gene (*CACNA1C*) encodes an alpha-1 subunit of a voltage-dependent calcium

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channel (6). Calcium channels have crucial roles in brain function, consequently their incorrect expression or dysfunction lead to a range of neurological impairments including pain, epilepsy, migraine, and ataxia (7). In particular, CACNA1C affects both neuronal firing and γ-aminobutyric acid (GABA) transmitting interneuron function influencing brain local activation and inter-regional communications (8). Imbalances in GABAergic and glutamatergic synapses have been demonstrated in autistic patients as a result of neuroinflammation (9). GABAergic signaling has been identified as a therapeutic target for this this kind of neurodevelopmental disorder (10). Genetic variance in *CACNA1C* has been associated with a range of neurologic disorders including depression, schizophrenia, and ASD, as well as alterations in brain function and structure in people with no diagnosable psychiatric disorder, which is in line with a chain of common neurobiological susceptibility among diverse neuropsychiatric diseases (11). Mutations in *CACNA1C* and other L-type calcium channels participate in the pathogenesis of Timothy and Fragile X syndromes, monogenic disorders with ASD-like symptoms (12). Genome-wide association studies (GWAS) have shown an association between the single nucleotide polymorphism (SNP) rs1006737 in this gene and bipolar disorder (13, 14). The same SNP has been shown to be associated with schizophrenia (15) and autism in a Chinese Han population (6). GWAS has also shown an association between rs4765905 and schizophrenia (16) and schizoaffective and bipolar disorders (17), and between rs4765913 and bipolar disorder (18). In addition, the risk allele of rs4765905 has been associated with autism in the Chinese Han population (6). Consequently, in the present research we performed a population-based association study to identify potential associations of *CACNA1C* genetic variants and haplotypes with ASD in an Iranian population.

Materials and methods

Subjects

The proper sample size for the current research was calculated using suppositions defined formerly (2). Presumed proportion of controls with exposure to risk allele was defined as 0.3 based on the dbSNP database for rs4765905. Briefly, this case–control study included 529 Iranian ASD patients and 480 age, gender, and ethnicity-matched healthy subjects. All patients were evaluated by psychiatrists to meet the criteria expressed by the Diagnostic and Statistical Manual of Mental Disorders 5th edition (19) and the Autism Diagnostic Inventory-Revised (ADI-R) (20). Potential subjects with any genetic syndromes or metabolic disorders were excluded from the study. Control group subjects were volunteers who were also evaluated to rule out neurological disorders. All participants signed informed consent forms. The study was approved by the local ethics committee.

Sample Collection and DNA Extraction

DNA was extracted from buccal epithelial cells in mouthwash samples as described previously (2) using a GeneAll Exgene Cell SV mini DNA kit (Cat. No. 106-152). A WPA Biowave II UV/Visible Spectrophotometer (Serial No. 80-3003-75) was used to estimate the purity and concentration of extracted DNA by computing the ratio of the absorbances at 260 and 280 nm (A260/280) and the A260, respectively.

Genotyping of rs1006737, rs4765905, and rs4765913

The tetra primer-amplification refractory mutation system-PCR (4P-ARMS-PCR) technique was applied to genotype the *CACNA1C* rs1006737, rs4765905, and rs4765913 variants. The primer sequences and melting temperatures are listed in Table 1. The PCRs were performed in 25 µl mixtures containing 100 ng of genomic DNA, 12.5 µl of Taq DNA Pol 2X Master Mix Red (Ampliqon, Denmark), and 0.5 µl of four primers for each SNP.To verify the results of the 4P-ARMS-PCR method, 10% of each samples was sequenced using an ABI 3730xl DNA analyzer (Macrogen, Korea).

Statistical Analysis

Allele and genotype frequencies and their compliance with Hardy-Weinberg equilibrium were assessed by the $\chi 2$ test using SNPStats (21). The associations of rs1006737, rs4765905, and rs4765913 polymorphisms with ASD risk were

analyzed using recessive, dominant, codominant, and overdominant inheritance models through calculation of odds ratios (ORs) and 95% confidence intervals (CIs). Haplotype frequencies for *CACNA1C* were measured using the SNPStats online program (21) and Haploview 4.2 (22). The latter was used to assess the pairwise linkage disequilibrium (LD) between the SNPs by calculating D' and r parameters. D' was defined as the quotient of the unstandardized coefficient divided by its maximal/minimal value. To reduce false-positive results permutation tests were applied for multiple testing corrections of the haplotype analysis (n=10,000). The level of significance was defined at *P* value of <0.05.

Table 1. Primer sequences and melting temperatues.

Genetic polymorphism	Primer sequence	T_m	Annealing temperature	PCR product size (bp)
rs1006737	Forward inner primer (G allele): 5′-ATAAGTTCCATTCCATCTCAGCCCGCAG	71°C	65 °C	199 bp (G allele)
	Reverse inner primer (A allele): 5′-CACTGTGAGGTCTCCCGCTCTGAAAAAAT	71°C		139 bp (A allele)
	Forward outer primer: 5′-TATCGACATTTGCTTCTGGAGCTGGACC	71 °C		281 bp (two outer primers)
	Reverse outer primer: 5′-CACACTGACATTACCTGGGAGCTTGCTG	71 °C		
rs4765905	Forward inner primer (G allele): 5′-GATTTGGATAGCATTTTAGCAATCTTGTG	65 °C	58 °C	209 bp (G allele)
	Reverse inner primer (C allele): 5′-TGTCTTCACACATCACAGACCCCTAG	65 °C		143 bp (C allele)
	Forward outer primer: 5′-TTTCCCCCTATTTAGAAAAACAAAGACGT	65 °C		298 bp (two outer primers)
	Reverse outer primer: 5′-ATCTTATGAAATGTCTCACCCCTCCAG	65 °C		209 bp (G allele)
rs4765913	Forward inner primer (T allele): 5′-CACAGGGTTCTTTCATTCTGTGGACT	65 °	62 °C	211 bp (T allele)
	Reverse inner primer (A allele): 5′-GCATCTCACATGCCCAGAGAACTAGT	65 °C		162 bp (A allele)
	Forward outer primer: 5′-TCTGAAGAGGGAAACAACAAGGTAGGTA	65 °C		321 bp (two outer primers)
	Reverse outer primer: 5′-CTGTCTTCCTTTTTCTACCCCTCAACTC	65 °C		

Results

Descriptive characteristics of the SNPs are presented in Table 2. The genotype distribution of all SNPs in each study group was in compliance with Hardy–Weinberg equilibrium (*P*>0.05).

Table 2. Descriptive characteristics of the studied SNPs.

SNP	Position	Minor Allele	MAF	MAC	Type
rs4765905	chr12:2240418	C	0.30	1507	intron variant
rs4765913	chr12:2310730	A	0.14	702	intron variant
rs1006737	chr12:2236129	A	0.30	1510	intron variant

Abbreviations: MAF: minor allele frequency; MAC: minor allele content

Allele and genotype frequencies of all polymorphisms are shown in Table 3. After application of the permutation tests, none of the SNPs were found to be associated with ASD in the assessed population. In addition, haplotype analysis exhibited no significant association between haplotype blocks and ASD. Eight anticipated haplotype blocks originated from the mentioned SNPs and their frequencies are shown in Table 4. No strong pair-wise linkage disequilibrium was detected between these three SNPs (Table 5).

Table 3. Allele and genotype frequencies of all polymorphisms.

Gene	SNP	Model		Sample size (%)		Fix-effect model	
				ASD cases Number (%) n=529	Controls Number (%) n=480	OR	P value
CACNA1C	rs4765905	Allele	C vs G	283 (27) 775 (73)	266 (28) 694 (72)	0.95 (0.78-1.15)	0.63
		Co-dominant	CC vs GG CG vs GG	33(6.2) 217 (41)	35 (7.3) 196 (40.8)	1.01 (0.78-1.31) 1..19 (0.72-1.97)	0.8
		Dominant	CC+CG vs GG	250 (47.3) 279 (52.7)	231 (48.1) 249 (51.9)	1.04 (0.81-1.33)	0.78
		Recessive	CC vs GG+CG	33 (6.2) 496 (93.8)	35(7.3) 445 (92.7)	1.18 (0.72-1.93)	0.51
		Over dominant	CG vs C+GG	217 (41) 312 (59)	196 (40.8) 284 (59.2)	0.99 (0.77-1.28)	0.95
	rs4765913	Allele	A vs C	79 (0.07) 974 (93)	85 (0.09) 859 (91)	0.82 (0.59-1.13)	0.22
		Co-dominant	AA vs TT AT vs TT	34 (6.4) 239 (45.2)	29 (6) 251 (52.3)	0.87 (0.51-1.47) 0.85 (0.66-1.10)	0.46
		Dominant	AA+AT vs TT	273(51.6) 256 (48.4)	229 (47.7) 251 (52.3)	0.86 (0.67-1.10)	0.22
		Recessive	AA vs AT+TT	34 (6.4) 495 (93.6)	29 (6) 451 (94)	0.94 (0.56-1.56)	0.8
		Over dominant	AT vs TT+AA	239 (45.2) 290 (54.8)	200 (41.7) 280 (85.3)	0.87 (0.68-1.11)	0.26
	rs1006737	Allele	G vs A	234 (22) 824 (78)	246 (26) 714 (74)	1.34 (0.96-1.86)	0.09
		Co-dominant	GG vs AA AG vs AA	28 (5.3) 178 (33.6)	40 (8.3) 166 (34.6)	1.10 (0.84-1.43) 1.68 (1.01-2.80)	0.12
		Dominant	GG+AG vs AA	206 (38.9) 323 (61.1)	206 (42.9) 274 (57.1)	1.18 (0.92-1.52)	0.2
		Recessive	GG vs AG+AA	28 (5.3) 501 (94.7)	40 (8.3) 440 (91.7)	1.63 (0.99-2.68)	0.05
		Over dominant	AG vs GG+AA	178 (33.6) 351 (66.3)	166 (34.6) 314 (65.4)	1.04 (0.80-1.35)	0.75

Table 4. Haplotype association analysis between *CACNA1C* and ASD.

	rs4765905	rs4765913	rs1006737	Total Frequency	Frequency in Cases	Frequency in Controls	OR (95% CI)	*P* value
1	G	T	A	0.47	0.49	0.48	1.00	—
2	G	T	G	0.16	0.14	0.15	1.13 (0.83 - 1.53)	0.44
3	C	A	A	0.15	0.14	0.12	0.86 (0.63 - 1.18)	0.36
4	G	A	A	0.08	0.09	0.08	0.84 (0.56 - 1.27)	0.41
5	C	T	A	0.06	0.06	0.07	1.19 (0.77 - 1.82)	0.43
6	C	A	G	0.04	0.05	0.06	1.32 (0.83 - 2.10)	0.25
7	C	T	G	0.02	0.02	0.03	1.23 (0.57 - 2.68)	0.6
8	G	A	G	0.02	0.01	0.01	0.91 (0.30 - 2.70)	0.86

Global haplotype association *P* value: 0.47

Abbreviations: OR: Odds Ratio; CI: confidence interval

Table 5. Pair-wise linkage disequilibrium values for SNPs as assessed for D' (a) and r (b) values.

a. D' statistics

	rs4765905	rs4765913	rs1006737
rs4765905	.	0.5593	0.0844
rs4765913	.	.	0.0151
rs1006737	.	.	.

b. r statistics

	rs4765905	rs4765913	rs1006737
rs4765905	.	0.5483	0.0771
rs4765913	.	.	0.0135
rs1006737	.	.	

Discussion

L-type calcium channels have been shown to participate in the function of almost all cells that generate action potentials. Their contribution in brain diseases such as Parkinson disease, febrile seizures, and neuropsychiatric disorders have been shown recently. Consequently, suppression of brain L-type channel isoforms by certain drugs might be therapeutic in these disorders (23). In the current study, we investigated the possible association of previously identified *CACNA1C* variants and ASD in a population of Iranian patients. We found no association between rs1006737, rs4765905, and rs4765913 SNPs with ASD in our population. Based on the results of our single locus tests and haplotype analyses, we conclude that these variants are not associated with ASD in this Iranian population. Recently, rs1006737 has been shown to affect *CACNA1C* transcript levels in a way that its risk allele (A) is associated with lower *CACNA1C* expression in the superior temporal gyrus (24). This result is consistent with that of the Gomez-Ospina et al. study of autopsy brain specimens, which demonstrated an association of this allele with decreased *CACNA1C* expression in the cerebellum but not in the parietal cortex (25). However, Yoshimizu et al. showed an association between the A allele of this SNP and elevated *CACNA1C* expression in induced neuron (iN) cells (26). These inconsistent results might be due to dissimilarities between distinct brain regions, which may demonstrate the significance of precise *CACNA1C* expression regulation in the central nervous system (24). Specific brain areas linked with clinical phenotypes of ASD are the frontotemporal lobe, frontoparietal cortex, amygdala, hippocampus, basal ganglia, and anterior cingulate cortex (27). However, in schizophrenia and bipolar disorder patients the main deficits are in medial and right dorsolateral prefrontal, ventrolateral prefrontal and insular cortical areas, left superior temporal cortex, and minor medial parietal and parietooccipital areas (28). This distinct pattern of brain involvement between ASD patients and those with schizophrenia or bipolar disorder might be reflected in the lack of association between the SNPs and ASD in our study despite the association with the latter disorders observed previously (15). The data presented above indicates that the mechanism of *CACNA1C* participation in ASD might be different from that in bipolar disorder or schizophrenia, or another variant of this gene might be the main culprit in the pathogenesis of ASD in our population. The other assessed SNP in our study was rs4765905. The risk allele (C) of this SNP has been consistently associated with reduced*CACNA1C* expression in SK-N-SH cells, but in HEK293 cells, the direction was not consistent (24). These results demonstrate complex *CACNA1C* regulation and the possibility of the role of unknown subtle variables in the control of activity of these sequences (24).

In brief, although previous studies have shown associations between these polymorphisms and psychiatric disorders and rs4765905 with ASD, we did not replicate their results in Iranian ASD patients. Further studies in independent sample sets from Iranian population are needed to confirm the results of our study. In addition, broad fine-mapping and resequencing are necessary to identify the main contributing genetic factor(s) in the pathogenesis of ASD.

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