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ASSOCIATION OF THE COMPLEXIN-2 GENE RS1366116 POLYMORPHISM WITH ISCHEMIC STROKE

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Glutamate-induced excitotoxicity is considered one of the major mechanisms involved in the pathogenesis of ischemic stroke (IS), a severe acute neurological complex disorder with polygenic inheritance. However, molecular level alterations responsible for IS-associated excitotoxicity are yet unclear. Complexins represent a family of proteins contributing to the modulation of neurotransmitter release and maintenance of synaptic plasticity. Recent study demonstrated upregulation of complexin-2 in the ischemic brain that may suggest its implication to synaptic malfunction in IS. In the present work we evaluated the potential association of the complexin-2 gene (*CPLX2*) rs1366116 and rs3892909 single nucleotide polymorphisms (SNPs) with IS. For this purpose, genomic DNA samples of 172 patients with IS and 225 healthy subjects of Armenian nationality were genotyped for the selected *CPLX2* gene SNPs using polymerase chain reaction with sequence-specific primers. Data were analyzed by Pearson's $\chi 2$ test. The obtained results demonstrated positive association of the *CPLX2* gene rs1366116 SNP and IS and absence of any association between this disorder and the rs3892909 SNP of the *CPLX2* gene. Our finding suggests that T minor allele of the rs1366116 SNP of the *CPLX2* gene may be considered as a risk factor of IS.

Ischemic stroke - complexin-2 - single nucleotide polymorphisms - genotyping

Իշեմիկ կաթվածը (ԻԿ) պոլիգեն, սուր նյարդային համալիր ծանր հիվանդություն է, որի կարևորագույն պատոմեխանիզմներից է հանդիսանում գլյուտամատով խթանված էքսայտոտոքսիկությունը: Վերջինիս համար պատասխանատու խանգարումների մոլեկուլային մեխանիզմները դեռևս հայտնի չեն: Կոմպլեքսինները սինապտիկ պլաստիկության կարգավորիչ սպիտակուցներ են, որոնք մասնակցում են նեյրոմիջնորդանյութերի արտազատմանը: Համաձայն վերջին շրջանի հետազոտությունների արդյունքների, ԻԿ ժամանակ ուղեղի հյուսվածքներում բարձրանում է կոմպլեքսին-2 սպիտակուցի մակարդակը, ինչը վկայում է այս հիվանդությանը բնորոշ սինապտիկ թերֆունկցիայի մեջ նշված սպիտակուցի հնարավոր ներգրավվածության մասին: Մեր հետազոտության խնդիրն էր հանդիսացել ուսումնասիրել կոմպլեքսին-2 սպիտակուցի գենի (CPLX2) rs1366116 և rs3892909 եզակի նուկլեոտիդային պոլիմորֆիզմների հնարավոր ասոցիացումը ԻԿ հետ հայկական պոպուլյացիայում: Այդ նպատակով կատարվել է 172 հիվանդների և 225 առողջների PCR-SSP մեթոդով ԴՆԹ նմուշների գենոտիպավորում՝ ըստ նշված պոլիմորֆիզմների: Ստացված տվյալների վիճակագրական վերլուծությունը կատարվել է Պիրսոնի $\chi 2$ թեստով: Համաձայն ստացված արդյունքների, CPLX2 գենի rs1366116 պոլիմորֆիզմը դրական ասոցիացված է ԻԿ հետ, իսկ CPLX2 գենի rs3892909 պոլիմորֆիզմի և ԻԿ հետ ասոցիացիան բացակայում է: Տվյալ հետազոտության արդյունքները վկայում են, որ CPLX2 գենի rs1366116 պոլիմորֆիզմի T մինորային ալելի ժառանգումը բարձրացնում է ԻԿ-ի զարգացման ռիսկը:

Իշեմիկ կաթված - կոմպլեքսին-2 - եզակի նուկլեոտիդային պոլիմորֆիզմներ - գենոտիպավորում

Глутаматная эксайтотоксичность рассматривается как важнейший патогенетический механизм ишемического инсульта (ИИ) - тяжелого острого комплексного неврологического заболевания с полигенным типом наследования. Однако до настоящего времени неясно какие нарушения на молекулярном уровне ответственны за ассоциированную с ИИ эксайтотоксичность. Комплексины представляют семейство белков, участвуют в модуляции высвобождения нейротрансмиттеров и поддержании синаптической пластичности. Согласно литературным данным в недавно проведенных исследований было продемонстрировано сверхпродукция комплексина-2 в ткани головного мозга при ишемии, что может свидетельствовать о его вовлечении в синаптическую дисфункцию при ИИ. Целью настоящей работы было изучение возможной ассоциации однонуклеотидных полиморфизмов rs1366116 и rs3892909 гена комплексина-2 с ИИ. С этой целью образцы геномной ДНК 172 больных ИИ и 225 здоровых лиц армянской национальности были генотипированы методом полимеразной цепной реакции со специфичными к последовательности праймерами (PCR-SSP). Статистический анализ данных проводился согласно критерию χ^2 Пирсона. Проведенное исследование показало наличие положительной ассоциация между rs1366116 полиморфизмом гена *CPLX2* и ИИ и отсутствие какой-либо ассоциации между этим заболеванием и rs3892909 полиморфизмом гена *CPLX2*. Результаты настоящего исследования свидетельствуют о том, что наследование Т минорной аллели rs1366116 полиморфизма гена *CPLX2* повышает риск развития инсульта.

Ишемический инсульт - комплексин-2 - однонуклеотидные полиморфизмы - генотипирование

Introduction.

Stroke is a leading cause of death and adult disability in the developed world, and there is no effective treatment for this disorder. Ischemic stroke (IS) is a polygenic complex disorder caused by gene-environmental interactions, which induce different pathophysiological processes [6]. Identification of whole complex of genetic variations associated with IS could sufficiently enlarge our knowledge on molecular targets for stroke therapy and prevention of its complications.

Glutamate-induced excitotoxicity is considered one of the major mechanisms involved in the pathogenesis of IS. An increase in intracellular calcium following over-activation of glutamate receptors leads to excitotoxicity and tissue injury [8]. Ca^{2+}-dependent glutamate release is a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein-dependent synaptic vesicle exocytosis process [7]. However, molecular level alterations responsible for IS-associated *excitotoxicity* are yet unclear. Complexins represent a family of proteins contributing to the modulation of neurotransmitter release and maintenance of synaptic plasticity [11]. These proteins have been characterized as being responsible for the regulation of SNARE-mediated fusion [10]. Promising studies have indicated that complexins bind in an antiparallel α-helical conformation to the groove between synaptobrevin and syntaxin and stabilizes the interface between these two SNAREs that bears the repulsive forces between the apposed membranes [4]. Recently it has been reported that inhibition of the SNARE pathway attenuated damage after stroke suggesting that complexin-2 is a central target molecule that links NADPH oxidase-derived reactive oxygen species to glutamate-mediated neuronal excitotoxicity in IS [13]. Also, upregulation of complexin-2 in the

ischemic brain has been demonstrated [13] that provides further evidence on implication of this protein to synaptic malfunction in IS.

The present study was designed to investigate potential association of the functional single nucleotide polymorphisms (SNPs) of gene encoding complexin-2 (CPLX2) with IS in an Armenian population.

Materials and Methods.

Study population. In total, 172 patients with first episode IS (males/females: 90/82; mean age ±SD: 50±9.7 years) and 225 healthy subjects (males/females: 154/71; mean age ±SD: 42.6±9.2 years) were enrolled in this study. All subjects were unrelated Caucasians of Armenian ancestry. Patients were hospitalized in the Medical Clinic N2 of the Yerevan State Medical University. Diagnosis of IS was based on clinical history and neurological examination and was confirmed by brain computer tomography (CT) imaging and basal laboratory tests. Stroke severity was scored using the National Institutes of Health Stroke Scale. Among IS patients involved in this study 38 had cardioembolic stroke, and 134 - large vessel atherothromboembolic stroke. Among IS patients 89 had hyperlipidemia, 70 had arterial hypertension, 32 had atrial fibrillation, and 53 had coronary artery disease; 63 patients were nicotine-dependent (cigarette smokers), and 34 were alcohol consumers; 86 patients had positive family history of IS (54 -maternal heredity, 30 - paternal heredity, 2 - both). Healthy subjects (controls) without family history of IS and myocardial infarction were recruited among the blood donors of the Erebouni Medical Center of the Ministry of Health of the Republic of Armenia (MH RA) and had no history of previous ischemic cerebrovascular event. Controls had no serious medical disorders, including coronary artery disease, atrial fibrillation, arterial hypertension, and hyperlipidemia, or treatment during the past 12 months. At the time of blood sampling they do not have symptoms of IS or a transient ischemic attack. No special studies have been performed to assess the progress of atherosclerotic process in controls. Exclusion criteria for all subjects include past or present history of neuropsychiatric disorders, metabolic disorders, myocardial infarction, oncological and immune system diseases. All subjects or their legal representatives gave their informed consent to participate in the study, which was approved by the Ethical Committee of the Institute of Molecular Biology NAS RA (IRB #00004079).

Collection of blood samples and extraction of genomic DNA. About 5 ml of venous blood was collected from each study participant by venipuncture and transferred to EDTA-containing tubes. Blood samples of IS patients were collected on days 1-4 of stroke onset. Genomic DNA was isolated from fresh blood samples according to the standard phenol-chloroform method and stored at -30°C until further use [12].

Genotyping of the CPLX2 gene rs1366116 and rs3892909 SNPs. All DNA samples were genotyped for *CPLX2* gene rs1366116 and rs3892909 SNPs using polymerase chain reaction with sequence-specific primers (PCR-SSP) under earlier described conditions [3]. All primers for the PCR-SSP were designed using the genomic sequences in the GenBank database (www.ncbi.nlm.nih.gov). The primers sequences were as follows:

1. *CPLX2* rs1366116: reverse 5′-ATG-TGT-AGG-AAA-ATG-GCT-TCG-3′ for standard C allele, reverse 5′-ATG-TGT-AGG-AAA-ATG-GCT-TCA-3′ for minor T allele, and constant: forward 5′- CAA-TGG-CCT-CTG-ACT-GGT-G-3′;
2. *CPLX2* rs3892909: forward 5′- GGT-GAG-GCT-GCT-GTC-TGC-3′ for standard C allele, forward 5′-GGT-GAG-GCT-GCT-GTC-TGT-3′ for minor T allele, and constant: reverse 5′-CTG-CTT-CAT-GAC-GAA-GTC-CA-3′.

The presence/absence of allele-specific amplicons was visualized by electrophoresis in 2% agarose gel stained with ethidium bromide.

Statistical analysis. Distribution of genotypes for the rs1366116 and rs3892909 SNPs were checked for correspondence to Hardy–Weinberg equilibrium. To reveal a potential association of these SNPs with IS, their genotype, allele, and minor allele carriage frequencies in patients and controls were compared. The significance of differences in the mentioned parameters between the study groups was determined using Pearson's $\chi 2$ test. The odds ratio (OR), 95% confidence interval (CI), and Pearson's p value were calculated. Statistical power of the present study was assessed as described earlier [9]. All tests were two-sided with 95% significance level ($p<0.05$). P

values <0.05 after adjustment by Bonferroni multiple correction approach were considered significant.

Results.

To assess potential association between the *CPLX2* gene rs1366116 and rs3892909 SNPs and IS, genomic DNA samples of patients and controls were genotyped for the selected polymorphisms. The genotype frequencies of the selected SNPs followed the Hardy-Weinberg equilibrium (p>0.05). Estimated genotype and allele frequencies of the *CPLX2* gene rs1366116 and rs3892909 SNPs in controls were similar to those reported for European population in public genetic database (www.ncbi.nlm.nih.gov/SNP). The distributions of the rs1366116 and rs3892909 variants in both study groups are presented in Table 1. Regarding the rs1366116 SNP, there was detected a significant increase in the frequency of T minor allele (p=8.8E-5, p_{corr}=1.7E-4, OR =1.8, 95% CI 1.343-2.434) and carriage of this allele (p=0.015, p_{corr}=0.03, OR=0.6, 95% CI 0.4-1) in patients compared with controls. Accordingly, the carriers of *CPLX2* rs1366116*T minor allele were overrepresented in patients compared to controls. Statistical power of this study, indicating the difference in the allele frequency of the rs1366116 SNP between the patients and controls, was 99.54%.

No association between the rs3892909 SNP and IS was found.

Table 1. Genotype, allele and minor allele carriage frequency distributions (FD) of the *CPLX2* gene rs1366116 and rs3892909 SNPs in IS patients and controls.

SNP ID	Genotype (FD, %)		Allele (FD, %)		$P_{nominal}$ $P_{corrected}$	Carriage (FD, %)		$P_{nominal}$ $P_{corrected}$
	IS (n=172)	Controls (n=225)	IS	Controls		IS	Controls	
rs1366116	CC (41) CT (36) TT (23)	CC (53) CT (38) TT (9)	C (59) T (41)	C (72) T (28)	8.8E-5 1.7E-4	(59)	(47)	0.015 0.030
rs3892909	CC (23) CT (49) TT (28)	CC (20) CT (52) TT (28)	C (47) T (53)	C (46) T (54)	0.7 1.4	(77)	(80)	0.52 1.04

Discussion.

Recent report have indicated that gene silencing of complexin-2 ameliorated cerebral injury as evidenced by reduced infarction volume, neurological deficit, and neuron necrosis accompanied by decreased glutamate levels [13]. The present study demonstrated that the *CPLX2* rs1366116*T minor allele is positively associated with IS in Armenian population. Interestingly, previous studies performed in our laboratory demonstrated associated of this SNP with schizophrenia and posttraumatic stress disorder in Armenian population [1, 2], which like IS are characterized by defects in synaptic plasticity [5]. All together these observations emphasize the important role of complexin-2, in particular, and genetic factors, in general, in development of pathogenic alterations relative to synaptic-plasticity.

Since the present observation refers to one given population (Armenian), the results should be replicated in other populations/ethnic groups. Another limitation of the present study is relatively small sample size (172 patients and 225 controls).

Conclusion.

Our finding nominates the minor T allele of the *CPLX2* gene rs1366116 SNP as a risk factor for IS at least in Armenian population.

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Abbreviations

CPLX2 - complexin-2
IS - ischemic stroke
MH RA - Ministry of Health of the Republic of Armenia
NAS RA - National Academy of Sciences of the Republic of Armenia
OR - odds ratio
PCR-SSP - polymerase chain reaction with sequence-specific primers
SNARE - soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNP - single nucleotide polymorphism