Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи •
• Experimental and theoretical articles •

Biolog. Journal of Armenia, 1 (66), 2014

ASSOCIATION OF THE COMPLEMENT FACTOR H GENE VARIANTS AND ISCHEMIC STROKE IN ARMENIAN POPULATION

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Complement factor H (CFH) is a serum glycoprotein that regulates the function of the alternative complement pathway, sequence variations in the CFH gene are linked to many inflammatory and neovascular diseases. In the present study, we evaluated the potential association of the CFH rs800292, rs1061170, rs434535 single nucleotide polymorphisms (SNPs) with ischemic stroke (IS) in an Armenian population. In total, 136 patients with IS and 225 healthy subjects (controls) were involved in this study. Genomic DNA samples of ischemic stroke patients and controls were genotyped for CFH gene rs800292, rs1061170, rs434535 SNPs using polymerase chain reaction with sequence specific primers (PCR-SSP). Data were analyzed by Pearson's χ^2 test. There was a significant decrease in the frequencies of rs800292*A minor allele (p=0.01, pcorr=0.03) and carriage of this allele (p=0.006, pcorr=0.018) in IS patients compared with healthy controls. According to the results rs1061170*C and rs424535*A minor alleles frequencies were higher in patients than in controls (p=3.0E-6, pcorr=9.0E-6 and p=2.1E-5, pcorr=6.3E-5 respectively). Moreover, the carriers of these alleles were overrepresented in patients compared to controls (p=0.0002, pcorr=0.0006 and p=8.0E-6, pcorr=2.4E-5 respectively). The results obtained implicated CFH gene rs800292, rs1061170, rs434535 SNPs in pathogenesis of ischemic stroke. In particular, it was shown that the CFH rs1061170*C and rs424535*A minor alleles are positively associated with IS, rs800292*A minor allele is a protective factor for IS at least in Armenian population.

Ischemic stroke – complement factor H – single nucleotide polymorphism

Կոմպլեմենտի H գործոնը կոմպլեմենտի ակտիվությունն այլընտրանքային ճանապարհով կարգավորող սպիտակուց է: H գործոնի գենում եզակի նուկլեոտիդային պոլիմորֆիզմները ասոցիացված են տարբեր բորբոքային և նորանոթային հիվանդությունների հետ: Այս հետազոտության նպատակն էր բացահայտել հայկական պոպուլյացիայում H գործոնի գենի rs800292, rs1061170, rs434535 եզակի նուկլեոտիդային պոլիմորֆիզմների հնարավոր ասոցիացումը իշեմիկ կաթվածի հետ: Այդ նպատակով իրականացվել է նշված պոլիմորֆիզմների 136 իշեմիկ կաթվածով հիվանդների և 225 առողջների PCR-SSP մեթոդով ԴՆԹ նմուշների գենոտիպավորում: Ստացված տվյալների վիճակագրական վերլուծությունը կատարվել է Պիրսոնի χ^2 տեստով: Մասնավորապես ցույց է տրվել, որ հայկական պոպուլյացիայում կոմպլեմենտի H գործոնի գենի rs1061170*C և rs424535*A մինորային ալելները հանդիսանում են ռիսկի, իսկ rs800292*A մինորային ալելը պաշտպանիչ գործոններ իշեմիկ կաթվածի համար: Համաձայն ստացված ար□դյունքների H գործոնի գենի rs800292, rs1061170, rs434535 եզակի նուկլեոտիդային պոլիմորֆիզմները ներգրավված են իշեմիկ կաթվածի պատոգենեզում:

Իշեմիկ կաթված – կոմպլեմենտի H գործոն – եզակի նուկլեոտիդային պոլիմորֆիզմներ

Фактор Н системы комплемента представляет собой сывороточный гликопротеин, который регулирует альтернативный путь активации комплемента. С изменениями нуклеотидной последовательности в гене фактора Н связаны многие воспалительные и неоваску-

лярные заболевания. Целью данного исследования было изучение возможной ассоциации однонуклеотидных полиморфизмов rs800292, rs1061170, rs434535 гена фактора Н с ишемическим инсультом (ИИ) в армянской популяции. Для этой цели были генотипированы образцы ДНК 136 больных ИИ и 225 здоровых лиц армянской популяции. Генотипирование проводили методом полимеразной цепной реакции со специфичными к последовательности праймерами (PCR-SSP). Статистический анализ данных проводился согласно критерию χ^2 Пирсона. Согласно полученным результатам, rs800292, rs1061170, rs434535 полиморфизмы гена фактора Н вовлечены в патогенез ишемического инсульта. В частности, было показано, что rs1061170*C и rs424535*A минорные аллели положительно ассоциируют с ИИ, а rs800292*A минорная аллель является защитным фактором для армянской популяции.

Ишемический инсульт – Н фактор комплемента – однонуклеотидный полиморфизм

Complement system is one of the key factors generating and maintaining inflammation in the arterial intima and plays a critical role in post-ischemic inflammation [2, 3, 13, 25]. CFH is a serum glycoprotein that regulates the function of the alternative complement pathway in fluid phase and on cellular surfaces. It binds to three sites on C3b and destabilizes the alternative pathway convertase C3bBb, also acts as a cofactor for complement factor I. CFH protein consists of 20 short consensus repeats (SCR). The SCRs contain binding sites for C3b, heparin, sialic acid, and C-reactive protein (CRP). The CFH gene is located in chromosome 1 (1q32), sequence variations in the CFH are linked to many inflammatory and neovascular diseases, such as age-related macular degeneration (AMD), hemolytic uremic syndrome (HUS), Alzheimer's disease (AD), diabetic retinopathy (DR), coronary heart disease (CHD), IS [5, 15, 18, 22, 23, 24].

I62V (rs800292) and Y402H (rs1061170) are coding-region nonsynonymous SNPs in CFH gene. G>A transversion in exon 2 of the CFH gene results in a change in the amino acid sequence from Val to Ile at position 62 of the CFH polypeptide in SCR2, which includes a C3b binding site. A CFH Ile62 variant display increased binding to C3b compared to CFH Val62, and is also a more efficient cofactor for factor I in the proteolytic inactivation of C3b [20]. CFH SNP Y402H characterizes a T>C substitution in exon 9, which results in a Tyr to His change in SCR7 domain, which binds heparin and C-reactive protein. CFH 402H variant showed reduced binding to CRP, compared with 402Y variant [12]. Rs424535 is one of tag SNPs in the CFH gene and leads to A>T substitution in intron 17.

In the present study, we evaluated the potential association of the CFH rs800292, rs1061170, rs434535 SNPs with IS in an Armenian population.

Materials and methods. *Study population*. In total, 136 patients with first episode IS (males/females: 71/65; 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 subtype was classified according to TOAST definitions [1]. Stroke severity was scored using the National Institutes of Health Stroke Scale. Among IS patients involved in this study 30 had cardioembolic stroke, and 106 - large vessel atherothromboembolic stroke. Fifty-three patients presented anatomically relevant CT hypodense areas in cortical-subcortical parts of cerebral right hemisphere, 78 - in left hemisphere and 5 - in brain stem. In this trial, patients with a moderately severe baseline strokes (median score of 17) were involved; lesion volume ± SD 174.9±156.7 cm3. 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 5 smokers), and 34 were alcohol consumers; 62 patients had positive family history of IS (39 -maternal heredity, 22 - paternal heredity, 1 - 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 (MH) of RA and had no history of previous ischemic cerebrovascular event. Healthy controls had no serious medical disorder, 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 healthy subjects group. 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 [16].

Genotyping of CFH rs800292, rs1061170, rs434535 SNPs

All DNA samples were genotyped for CFH rs800292, rs1061170, rs434535 SNPs using PCR-SSP under earlier described conditions [4]. All primers for the PCR-SSP were designed using the genomic sequences in the GenBank (http://www.ncbi.nlm.nih.gov). The primer sequences for three mentioned SNPs were as follows:

1. CFH rs800292: reverse 5'CCTTCCTGCATACCATTATTAC for G allele, reverse 5'CCTTCCTGCATACCATTTTAT for A allele, constant forward 5'GACCTGTGACTGTCTAGGC;
2. CFH rs1061170: reverse 5'CCCTGTACAAACTTTCTTCCATG for C allele, reverse 5'CCCTGTACAAACTTTCTTCCATA for T allele, constant forward 5'GTTAGTAACTTTAGTTCGTCTTCAG;
3. CFH rs424535: forward 5' GAGAACAGCAGCAGAGGAAA for T allele, forward 5'GAGAACAGCAGCAGAGGAAT for A allele, constant reverse 5'GCCTGGTAAACAATGCCTCT.

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 rs800292, rs1061170, and rs424535 SNPs were checked for correspondence to Hardy–Weinberg equilibrium. To reveal a potential association of these SNPs with IS, their genotype, allele, and phenotype frequencies (carriage rates) in patients and controls were compared. The significance of differences between allelic and phenotype frequencies in 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. All tests were two-sided with 95% significance level ($p \leq 0.05$). P values adjusted by Bonferroni multiple correction approach and ≤ 0.05 were considered significant.

Results and Discussion. To assess potential association between CFH rs800292, rs1061170, rs424535 SNPs and IS, genomic DNA samples of patients and controls were genotyped for the selected polymorphisms. All genotype frequencies of the three selected SNPs followed the Hardy-Weinberg equilibrium in all subjects ($p>0.05$). Estimated genotype and allele frequencies of CFH rs1061170 and rs424535 SNPs were similar and of CFH rs800292 were different to those reported for European population in public genetic database (http://www.ncbi.nlm.nih.gov/SNP). The distribution of CFH rs800292, rs1061170, rs424535 variants in both groups is presented in tab. 1. Regarding CFH rs800292, there was a significant decrease in the frequencies of A minor allele ($p=0.01$, $pcorr=0.03$) and carriage of this allele ($p=0.006$, $pcorr=0.018$) in IS patients compared with healthy controls. According to the results rs1061170*C and rs424535*A minor alleles frequencies were higher in patients than in controls ($p=3.0E-6$, $pcorr=9.0E-6$ and $p=2.1E-5$, $pcorr=6.3E-5$ respectively). Accordingly, the carriers of these alleles were

overrepresented in patients compared to controls (p=0.0002, pcorr=0.0006 and p=8.0E-6, pcorr=2.4E-5 respectively).

Tab. 1. Genotype and allele frequencies of CFH polymorphisms in IS patients and controls

SNP ID	Genotype distribution (%)		Allele distribution (%)		*P*value (*P*corr)	Odds ratio (95% CI)	Carriage (%)		*P*value (*P*corr)	Odds ratio (95% CI)
	IS (n=136)	Controls (n=225)	IS	Controls			IS	Controls		
rs800292	GG 117(86) GA 17(12.5) AA 2(1.5)	GG 166(73.8) GA 55(24.5) AA 4(1.7)	G 251(92.3) A 21(7.7)	G 387(86) A 63(14)	0.01 (0.03)	0.52 (0.3-0.86)	19(14)	59(26.2)	0.006 (0.018)	0.47 (0.26-0.8)
rs1061170	TT 32(23.5) TC 67(49.3) CC 37(27.2)	TT 96(42.7) TC 104(46.2) CC 25(11.1)	T 131(48.2) C 141(51.8)	T 296(65.8) C 154(34.2)	3.0E-6 (9.0E-6)	2 (1.52-2)	104(74.5)	129(57.3)	0.0002 (0.0006)	2.42 (1.5-3.7)
rs424535	AA 49(36) AT 57(42) TT 30(22)	AA 35(15.6) AT 113(50.2) TT 77(34.2)	A 155(57) T 117(43)	A 183(40.7) T 267(59.3)	2.1E-5 (6.3E-5)	0.52 (0.38-0.7)	87(64)	190(84.4)	8.0E-6 (2.4E-5)	0.33 (0.2-0.54)

Many studies demonstrated involvement of CFH in pathomechanisms of IS [3, 13, 25]. It is clear from in vitro and in vivo studies that complement activation implicate end-organ damage in CHD and stroke [13, 19]. CFH rs800292 and rs1061170 SNPs lead to structural changes affecting the ability of CFH to bind C3b and CRP respectively and so changes the activation of the alternative pathway [13, 19]. This study explored the potential contribution of the CFH rs800292, rs1061170, rs424535 SNPs to IS. The results obtained indicated implication of these polymorphisms in pathogenesis of IS.

CFH rs800292 associated with a decreased risk for AMD, significant decreases in the frequencies of A allele and AA genotype for rs800292 were observed in DR patients compared with diabetic controls (pcorr = 0.04, OR = 0.72; pcorr = 0.015, OR = 0.51, respectively) [9, 22]. The present study demonstrated CFH rs800292*A minor allele negative association with IS and protective effect at least in Armenian population.

Recent findings indicated CFH rs1061170 association with AD, HUS, AMD, associations with CHD risk showed conflicting results, thus, in a prospective study with 5,520 men and women from the Netherlands, the CFH Y402H polymorphism was associated with an increased risk for myocardial infarction [6, 10, 11, 24]. However, these results have not been consistent in several case–control studies [14, 17]. Also, Volcik et al., evaluated the association of the Y402H polymorphism with CHD, IS, and carotid artery wall thickness (intima-media thickness), and found that the CFH 402H allele was associated with an increased risk for incident CHD and IS in whites [21]. In the present case-control association study there was a significant increase in the frequencies and carriage of CFH rs1061170*C minor allele (402H) in IS patients compared with healthy controls in Armenian population.

Only a limited number of studies have explored potential association between GFH rs424535 and diseased conditions. Thus, GFH rs424535 showed an allelic association with HUS [7]. In case-control study with 225 schizophrenia patients and the same number healthy subjects of Armenian ancestry the CFH rs424535*A minor allele was associated with schizophrenia [8]. Our study also revealed rs424535*A minor allele of CFH as a risk factor for IS in Armenian population.

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 (136 patients and 225 controls).

Our findings suggest CFH rs1061170*C and rs424535*A minor alleles as a risk factors, rs800292*A minor allele as a protective factor for IS at least in Armenian population.

Acknowledgments

I express my sincere thanks to the administration and medical staff of the Medical Clinic N2 of the Yerevan State Medical University and to my supervisor Prof. Anna Boyajyan.

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List of abbreviations
AD Alzheimer's disease
AMD age-related macular degeneration
CFH complement factor H
CHD coronary heart disease
CI confidence interval
CRP C-reactive protein
DR diabetic retinopathy
HUS hemolytic uremic syndrome
IS ischemic stroke
OR odds ratio
PCR-SSP polymerase chain reaction sequence-specific primer
SNP single nucleotide polymorphism

Received 05.09.2013