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COMPLEMENT FACTOR H Y402H FUNCTIONAL POLYMORPHISM IN PATIENTS WITH ISCHEMIC STROKE

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The present study was focused on the assessment of Y402H functional polymorphism of the complement alternative pathway regulator, factor H, in ischemic stroke. For this purpose comparative determination of the blood serum levels of total factor H and its 402H and 402Y variants in 39 patients with ischemic stroke and the same number of age- and sex-matched healthy subjects was performed. In addition, the correlation between these parameters and blood level of C-reactive protein was assessed, and genotype frequency of Y402H polymorphism among patients with ischemic stroke and healthy individums were determined. The results obtained suggest implication of factor H, particularly its 402H variant, in postischemic inflammatory response.

Ischemic strok – factor H – Y402H polymorphism – C-reactive protein

Տվյալ աշխատանքը ուղղված է գնահատելու կոմպլեմենտի այլընտրանքային ուղու կարգավորիչի՝ H գործոնի Y402H ֆունկցիոնալ պոլիմորֆիզմը իշեմիկ կաթվածի ժամանակ: Այդ նպատակով իրագործվել է H գործոնի, նրա 402H և 402Y տարբերակների մակարդակների համեմատական որոշումը 39 իշեմիկ կաթվածով հիվանդների և նրանց սեռով ու հասակով համապատասխանող նույն քանակի առողջ անձանց արյան շիճուկում: Բացի դրանից, գնահատվել է նաև որոշված չափանիշների և արյան մեջ C-ռեակտիվ սպիտակուցի մակարդակների միջև համապատասխանությունը, որոշվել է Y402H պոլիմորֆիզմի հաճախականությունը հիվանդների և առողջների մոտ: Ստացված արդյունքները վկայում են, որ H գործոնը, մասնավորապես իր 402H տարբերակը, ներգրավված է հետիշեմիկ բորբոքային պատասխանի մեջ:

Իշեմիկ կաթված (H գործոն (Y402H պոլիմորֆիզմ (C-ռեակտիվ սպիտակուց

В настоящей работе дана оценка Y402H функционального полиморфизма регулятора альтернативного пути комплемента, фактора H, при ишемическом инсульте. С этой целью было проведено сравнительное определение уровней фактора H и его 402H и 402Y вариантов в сыворотке крови 39 больных ишемическим инсультом и такого же количества здоровых лиц, соответствующих больным по возрасту и полу. Кроме того, проведена оценка корреляции между определенными параметрами и уровнем С-реактивного белка, а также определены частоты Y402H полиморфизма у больных и здоровых лиц. Полученные данные свидетельствуют о вовлечении фактора H, в частности его 402H варианты, в постишемический воспалительный ответ.

Ишемический инсульт - фактор H - Y402H полиморфизм - С-реактивный белок

The inflammatory response plays a decisive role in pathophysiology of ischemic stroke (IS): on the one hand the response is pointed toward the removal of necrotic tissue from the ischemic area; whereas, on the other hand, it enlarges the ischemic area and the severity of disease [reviewed in 6]. In addition, the systemic inflammation also plays a central role in IS [reviewed in 6].

The complement system is a major mediator of inflammation. It is a cytotoxic host defense system that normally functions to eliminate foreign pathogens and to opsonize necrotic and apoptotic cells. Activation of complement by the classical, alternative and lectin pathways generates opsonins, anaphylatoxins, chemotaxins, and membranolytic complexes. Complement dysfunction, alterations in its regulatory mechanisms and undesirable activation, contributes to pathology of many human diseases by damaging tissue and promoting inflammation [4, 14, 24, 27].

In multiple animal models of stroke it has been shown that complement plays a key role in stroke outcome, and complement depletion improves neurological function after cerebral ischemia [reviewed in 6]. Few studies, including our own, demonstrated increased complement activities, levels of complement components and their activation products in the central nervous system and circulation after human IS onset [2, 9, 13, 20, 26]. It has been proposed that decreased expression of complement regulators is a possible mechanism of tissue damage during ischaemia [19, 21]. However, data on complement regulators in stroke are very limited.

Complement factor H (fH) is the major fluid-phase regulator of the alternative pathway of complement and plays a key role in controlling complement activation [4, 14, 24, 27]. Within the fH molecule there are binding sites for C-reactive protein (CRP) [7, 8, 11], systemic inflammatory marker [reviewed in 22], predictor of risk of coronary events [5, 18], and indicator of IS severity and outcome [reviewed in 6, 19]. A *common* T→C single nucleotide *polymorphism* (1277T>C) in exon 9 of the fH gene (rs1061170) resulting in *Tyr(Y)*-to-*His (H)* substitution at position 402 (Y402H) of the fH polypeptide chain, has been shown to associate with some diseased conditions characterised by altered inflammatory response, including atypical hemolytic uremic syndrome, age-related macular degeneration (AMD), and coronary heart disease [15, 17, 23, 30]. The Y402H polymorphism occurs in the SCR7 region of the gene which has the overlapping binding sites for CRP [7, 8, 11]. It has been shown that carriers of 402H have an increased risk for cardiovascular mortality and AMD, and the association of the Y402H polymorphism with increased CRP levels has been reported [12, 15, 17, 30]. Two reports were focused on association of Y402H polymorphism with IS, and the results obtained are controversial [23, 28].

Our previous studies demonstrated alterations in the alternative complement pathway [2, 3] in IS. In the present study we performed comparative determination of the blood levels of total fH and its 402H and 402Y variants in patients with IS and healthy subjects. In addition, the analysis of correlation between these parameters and blood level of CRP was performed, and genotype frequency of fH Y402H polymorphism among IS-patients and healthy subjects was studied.

Material and Methods. Thirty nine patients (females 27; mean age ± SD 67±12 years) with acute IS symptoms developed within 24 hours and the same number of sex- and age-matched healthy controls entered the study. The patients were recruited from "St. Gregory the Illuminator" Scientific Medical Center of the Ministry of Health of Armenia. Stroke subtype was classified according to TOAST definitions [1]. Eight patients had cardioembolic stroke, and 31 - large vessel atherothromboembolic stroke. Stroke severity was scored using the US National Institutes of Health Stroke Scale. In this trial, patients with moderately severe baseline strokes (median score 17; lesion volume ± SD 65541±63745mm^3) were involved. Diagnosis of IS was based on clinical history and neurological examination and was confirmed by brain computed tomography (CT) imaging and basal laboratory tests. Thirty two patients presented anatomically relevant CT hypodense areas in subcortical parts of cerebral hemispheres and 7 in brain stem. Twenty eight

patients had hyperlipidemia, 18 had hypertension, 8 had arterial fibrillation, 10 had coronary artery disease, and 8 had diabetes mellitus; 19 patients were current smokers, and 9 were alcohol consumers. Three patients previously had stroke and 13 patients had positive family history of IS (6 - maternal heredity, 7 - paternal heredity). Thirty nine age- and sex-matched healthy volunteers without family history of IS, myocardial infarction, and diabetes mellitus served as a control group. No special studies have been performed to assess the progress of atherosclerotic process in the healthy subjects group. Subjects with concurrent diseases or conditions interfering with the aim of this study, such as inflammatory, infectious, or autoimmune diseases, vasculitis, myocardial infarction, cancer, hematological diseases, severe renal or liver failure, gynecologic or urologic diseases, any surgical interventions within the previous 12 months, and those on immuno-modulator drugs, were not included in the study. All subjects were Armenians living in Armenia. Informed consent was obtained from all study participants or their legal representatives. The Ethics Committee of the Institute of Molecular Biology has approved the study.

Blood sampling of IS-patients was performed at admission, before any medication was applied. Practically fasting blood samples were collected by venipuncture at 9:00-10:00 a.m. (first sampling) in appropriate tubes and kept on ice for 60 min. After that the blood was centrifuged at 3000g for 15min at 4^0C to separate serum from blood corpuscles. The obtained serum samples were stored in aliquots at -30^0C and thawed immediately prior to use.

Concentrations of total fH and fH 402H variant in the serum of study subjects were measured by in-house enzyme-linked imunosorbent assay (ELISA) as described earlier [9] on "Stat Fax 3200" (Awareness Technology Inc., USA) apparatus in 96-well microplates at 492 nm. Concentration of fH 402Y variant was calculated by subtraction of fH 402H variant level from total fH concentration and expressed in milligrams per 1 liter of serum (mg/L). The ratio of 402H and 402Y levels was used to identify genotype frequency of Y402H polymorphism as described earlier [9].

Concentration of CRP was measured by immunonephelometric assay on a BN II analyzer (Dade Behring, Germany), using commercially available kits (Dade Behring, Germany), as described by the manufacturers. Concentration of CRP was expressed in milligrams per 1 liter of serum (mg/L).

SNPanalyzer web-based software [29] was used to perform essential statistical analyses of genotype and allelic frequency data. The distributions of genotypes for all studied SNPs were checked for correspondence to the Hardy-Weinberg (H-W) equilibrium. Allelic (gene) and phenotype frequencies in the patients and control groups were compared. Allelic and phenotype frequencies were calculated according to the observed number of genotypes. The significance of differences between phenotype and allele frequencies in both groups was calculated using Pearson's Chi-square test.

For analysis of the total fH, its variants and CRP levels "Graphpad Prism" (GraphPad Software Inc., USA) software was applied. Group statistics, otherwise indicated, was expressed as mean±SD. The unpaired two-tailed Student's t-test was used for evaluation of intergroup differences in the levels of total fH, its 402H and 402Y variants, and CRP. Pearson's correlation coefficient (r) was calculated to assess relationships between measured parameters. P values less than 0.05 were considered significant.

Results and Discussion. We observed a significantly increased serum levels of total fH in IS-patients compared to controls (294.1±44.8 vs. 265.6±28.4; p=0.0042). According to the results obtained, genotype frequencies of fH Y402H polymorphism in IS-patient and control subjects did not differ significantly from each other (p>0.5). Further analysis showed that the levels of 402H variant were significantly higher in heterozygous IS-patients compared to control subject (p=0.024), whereas no difference in 402Y variant levels between IS-patients and control subjects was observed (p>0.05). In case of patients, we found no difference between total fH levels of mutant variant carriers (YH+HH) and standard variant homozygotes (YY) (296.6±50.4 vs 293.0±41.9; p>0.05), whereas in control subjects the total levels of fH in YH+HH were significantly lower than YY (255.0±24.4 vs 283.2±27.32; p=0.05). The results obtained are summarized in Table 1 and Figure 1.

The data are expressed as whisker box plots; the box represents the 25th–75th percentiles, the median is indicated by a horizontal bar across the box, the whiskers on each box represent the 10th–90th percentiles.

Table 1. Genotype, allele, and carriage frequency of fH Y402H polymorphism among IS-patients and healthy controls

Target		Controls	Patients	P
		Absolute number (Relative number, %)	Absolute number (Relative number, %)	
Genotype	YY	14 (0.36)	13 (0.33)	>0.05
	HY	22 (0.56)	22 (0.57)	
	HH	3 (0.08)	4 (0.10)	
Allele	Y	50 (0.64)	48 (0.61)	>0.05
	H	28 (0.36)	30(0.39)	
Carriage	Y	36 (0.92)	35 (0.90)	>0.05
	H	25 (0.64)	26 (0.67)	

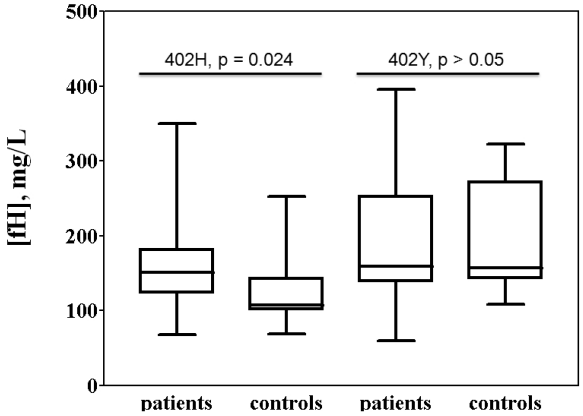

Fig. 1. The levels of fH 402H and fH 402Y variants in the blood of heterozygous IS-patients and control subjects.

We found significantly higher serum CRP levels in IS-patients compared to controls (23.3±17.6 vs. 4.25±2.2, p=0.00002). The levels of CRP in the serum of fH 402 heterozygous IS-patients correlated with the levels of fH 402H variant (r=0.52, p= 0.002), but not with the fH 402Y variant levels (r = -0.06, p=0.731). No correlation was observed between the levels of CRP and fH variants in control subjects (402H: r=0.02, p = 0.97; 402Y: r =0.35, p=0.28).

In this paper we focused our attention on Y402H functional polymorphism of complement alternative pathway regulator, fH, in IS by evaluating the levels of total fH, and its 402H and 402Y variants in the blood of patients with IS and healthy subjects. The data obtained demonstrated that in IS-patients the levels of total fH are higher than in controls due to increased levels of its 402H mutant variant, which may be responsible for alterations in the alternative complement cascade demonstrated in our previous studies [2, 3].

As it was mentioned in the introduction, the data relevant to association of fH Y402H genetic polymorphism with IS, presented only in two reports, are controversial [23, 28]. This discrepancy may reflect population differences of study subjects. Our present data, indicating no association between Y402H polymorphism and IS in Armenian population, are consistent with the one of this reports [23]. However, the *sample size of our study* does not allow definite conclusion.

Our data also demonstrated positive correlation between the levels of CRP in the blood of fH 402 heterozygous IS-patients with the levels of fH 402H variant. That may reflect diminished binding of denaturized CRP to fH 402H variant [10], since decreased level of blood Ca2+ in IS [16] may promote CRP denaturation [10].

Thus, the present findings clearly demonstrated the involvement of fH, particularly its 402H variant in the development of systemic postischemic inflammatory response.

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