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English version: SINGLE NUCLEOTIDE POLYMORPHISM OF -174G/C INTERLEUKIN-6 GENE IN PATIENTS OF DIFFERENT WEIGHT CATEGORIES WITH NON-LACUNAR STROKES AND ITS ASSOCIATIONS WITH CLINICAL AND FUNCTIONAL STROKE OUTCOMES*

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We studied the single nucleotid polymorphisms (SNP) distribution of -174 G/C interleukin-6 gene (IL-6) and determined its associations with functional outcomes after non-lacunar stroke in 33 patients with normal body weight and in 37 patients with abdominal obesity (AO) II class. The study has involved patients of similar age (56 to 65 years), with the similar clinical disease severity on admission (5-10 points according to NIHS scale), with relatively similar cerebral lesion volumes (from 10 to 20 cm^3), without diabetes mellitus and severe co-morbidity. Abdominally obese patients II class with non-lacunar strokes have significantly more common 174 C allele of the IL-6 gene compared with patients with normal body weight (65% versus 45%, p<0,05). In patients with normal body weight G/G genotype of -174 G/C gene IL-6 was significantly associated with more severe acute stroke an increased rate of late "disabling" functional states (values of modified Rankin scale more then 2 points) in 12 weeks after stroke. AO II class hasn't any patterns of clinical and functional outcomes distribution depending on SNP -174 G/C gene IL-6.

Key words: ischemic non-lacunar stroke, abdominal obesity, interleukin-6, gene, -174 G/C single nucleotide polymorphism.

This study is a fragment of the planned research activity of neurological department with neurosurgery and medical genetics "Ukrainian Medical Stomatological Academy" "Optimization and pathogenetic substantiation of diagnostic and treatment methods of neurovascular and neurodegenerative diseases with regard to clinical and hemodynamic, hormonal, metabolic, genetic and immuno-inflammatory factors" (state registration number 0111U006303).

Introduction

In recent years in scientific literature has emerged a lot of scientific reports which prove independent and significant influences of obesity on acute ischemic strokes and functional outcome. In particular, in previous works we demonstrated that obese patients (especially with abdominal obesity) had a more severe strokes, more frequent development of various post-stroke complications and, consequently, longer hospital stay and worse functional outcome [1].

Among the key factors that may determine the clinical severity of stroke, great importance has the intensity of local and systemic post-ischemic inflammatory response. Proinflammatory cytokine - interleukin-6 (IL-6) is one of the key regulators of post-ischemic inflammatory response intensity through the influence on the synthesis and secretion of acute-phase proteins, chemokines, adhesion molecules, etc. High serum IL-6 level in the first days after ischemic stroke onset is associated with the progression of secondary post-ischemic cerebral injury and is an independent predictor of acute stroke severity as well as early neurological complications and long-term functional outcome [10].

The degree of IL-6 blood elevation at acute stroke is closely correlated with the cerebral lesion volumes [10]. However, we had defined (the article in press), that under conditions of similar cerebral lesions volumes, abdominally obese patients (I-II obesity class) have significantly increased IL-6 blood levels at 1st as well as at 10th day after stroke onset compared with normal weight patients.

But the source of a significant IL-6 serum in abdominally obese patients under conditions of acute ischemic stroke still remains unknown.

Leptin deficient obese mice, compared with wild-type ones, have significantly elevated serum IL-6 after experimental ischemic stroke. Because brain tissue level of IL-6 was decreased in post-ischemic obese mice compared with wild-type animals, authors suggested that in

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acute stroke just adipose tissue is a major source of serum IL-6 [11].

In recent years, a number of investigations had shown that the degree of increase in IL-6 concentration in patients with ischemic stroke is largely determined by genetic factors. Expression of IL-6 is mainly regulated at the transcriptional level, gene promoter of IL-6 comprises several single nucleotide polymorphisms (SNP), which significantly affects the gene transcription. SNP in the promoter region of IL-6 gene as substitution of cytosine to guanine at position 174 (-174 G/C) is associated with changes in IL-6 blood concentration. There are conflicting scientific data about these associations with ischemic stroke, as well as about associations between genotype -174 G/C and clinical and functional features of ischemic stroke [3, 12]. Inconsistency of above mentioned results may reflect the regulatory processes' complexity in post-stroke inflammatory response, diversity of IL-6 physiology, may be a consequence of heterogeneity of the studied patients groups, as well as ischemic strokes, etc. Perhaps, the -174 G/C SNP of the IL-6 gene is one of the factors that could determine more severe non-lacunar strokes and more intensive post-ischemic inflammatory response in abdominally obese patients.

Purpose of the study was to indentify the distribution of -174 G/C SNP of the IL-6 gene and to determine associations between this SNP and clinical features, as well as functional outcomes of non-lacunar strokes in patients of different weight categories.

Materials and methods

In the study we have recruited 70 subjects of both genders (33 patients with normal body weight and 37 patients with class 2 abdominal obesity) with acute ischemic non-lacunar (atherothrombotic and cardioembolic subtypes) hemispheric strokes. All patients were admitted to Poltava city hospital not later than 24 hours after stroke onset.

Non-lacunar stroke subtype was verified by neurovisualization.

Peculiarities of acute ischemic stroke and post-stroke functional outcome are determined by numerous factors, among which the most important are the patient's age, stroke severity at admission (according to National Institutes of Health Stroke Scale (NIHSS)), the size of the cerebral lesion, previous strokes, and co-morbidities [14]. Therefore, to unify above mentioned conditions we had selected patients with relatively similar age (from 56 to 65 years), similar disease's severity at admission (from 5 to 10 points according to NIHSS), similar cerebral lesion size (from 10 to 20 cm^3). Patients didn't have diabetes mellitus and severe co-morbidities that could influence of neurological and functional recovery (oncological diseases, convulsive syndrome, hematological diseases, cardiac, liver, kidney and respiratory insufficiencies, progressive angina pectoris, acute myocardial infarction, vascular dementia, alcoholism, etc). Moreover, patients didn't have acute neurological episodes (according to medical records) and didn't have neuroimaging signs of previously unrecognized non-lacunar strokes.

Cerebral lesion volume was estimated by calculating the approximate volume of an ellipsoid on computed tomography brain scans or T-2 weighted magnetic resonance imaging brain scans.

Patient's body weight was determined with mechanical weights during hospitalization. In severe cases, body weight was measured after patient improvement, or according to patient's relatives. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Body weight categories were formed on the basis of BMI: normal body weight - BMI 20.0-24.9 kg/m^2, obesity class II – BMI >35.0 kg/m^2. Waist-to-hip ratio (WHR) was calculated as a measure of abdominal obesity. Waist circumference was measured with a soft tape midway between the lowest rib and the iliac crest. Hip circumference was measured over the widest part of the gluteal region. According to World Health Organization criteria, WHR >0.9 in men and >0.85 in women were denoted abdominal obesity.

In hospital stroke patients received uniform therapy (antiplatelet drugs for atherothrombotic stroke, anticoagulant drugs for cardioembolic stroke, hypotensive, metabolic, nootropic drugs, etc), physiotherapy, and massage. After hospital discharge patients took antihypertensive, antiplatelet drugs and continued outpatient rehabilitation services (physiotherapy, speech therapyy, etc.).

The cpicture of stroke linical has been analyzed by NIHSS score at the 10^{th} day of hospital stay. Post-stroke functional outcomes were evaluated by modified Rankin scale (mRS). We had dichotomized functional outcome: favorable – mRS ≤2 (patient able to look after own affairs without assistance) versus unfavorable – mRS>2 (patient requires assistance in daily life.

Molecular genetic analysis was performed in a laboratory «Gentrees LTD» (Poltava). DNA was extracted from white blood cells using ion exchange resin Chelex-100 [13]. Determination of allelic status of-174 G/C SNP of the IL-6 gene (rs1800795) was performed as it had been described by Spanish scientists [4]. Polymerase chain reaction (PCR) was performed in the thermocycler Tertsik (DNA-Technology, Russia).

To amplify the fragment of IL-6 gene, which contains the polymorphic site 174 G/C, oligonucleotide have been used primers – direct IL6174F: TGACTTCAGCTTTACTCTTTGT and reverse IL6174R: AATAGGTTTTGAGGGCCATG. Restriction of the amplification products was performed using endoculease *Lwe*I (*Sfa*NI) (MBI Fermentas, Lithuania). Restriction materials were analyzed by electrophoresis in 2% agarose gel in 1xTBE. As molecular weight marker it had been used DNA pUC19, which was hydrolysed by endonuclease *Msp*I (MBI Fermentas, Lithuania). Visualization of amplification and restriction products was performed by gel dyeing with ethidium bromide and photographing in ultraviolet light transilluminator.

Statistical processing of the results was performed by means of software package Statistika Excel. Distributions of allele frequencies in different weight body groups, equality of the actual distribution of genotypes, as well as the actual and theoretical ones (according to Hardy-Weinberg equilibrium) were analyzed using the criterion χ^2. Comparison of functional outcomes rates depending on the genotype was calculated using Fisher's exact test. Quantitative data (NIHSS score) is shown as mean (M), standard error of mean (m). Data from patients were analyzed using the Mann-Whitney *U* test for the comparison of continuous variables. P-value less 0.05 was taken to indicate statistical significance.

Results and discussion

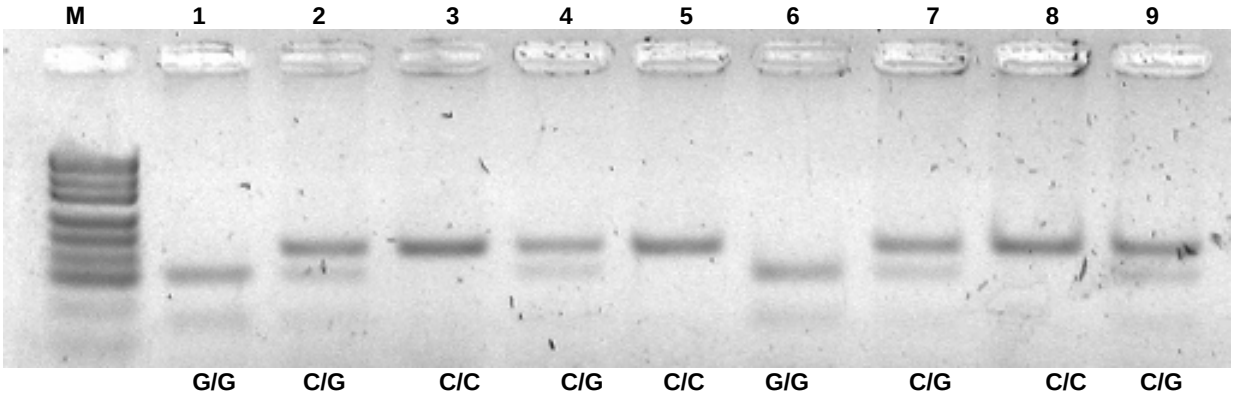

Fig. 1. Electrophoretogram of PCR products genotyped by SNP -174 G/C IL-6 gene: M - molecular weight marker DNA pUC19, hydrolyzed by endonuclease MspI.; 1-9 - DNA of patients with non-lacunar stroke and different genotypes.

Restriction fragment of 164 nucleotides pairs matches 174 C allele, the two restriction fragments of 112 and 52 nucleotides pairs matches 174 G allele of the IL-6 gene.

Table 1

Allele frequencies of SNP -174 G/C of the IL-6 gene in patients of different weight categories with non-lacunar stroke

Patients group	Totally	Genotype			Allele frequencies	Statistics
		G/G	G/C	C/C		
normal body weight	33	13	10	10	p_G=0,55; p_C=0,45	χ^2=4,57; df=1; χ^2_{st}=3,84; p<0,05
abdominal obesity 2 class	37	6	14	17	p_G=0,35; p_C=0,65	

n – the number of patients; p_G u p_C –frequencies of G-allele and C-allele; χ^2 u χ^2_{st} – actual and threshold criterion value; df – the number of steps of freedom; p – significance level.

The table 1 shows, in normal weight patients with non-lacunar stroke G-allele is major allele, but in abdominally obese patients major allele is C-allele; and this difference has statistical significance. In literature there are contradictory data about relations between -174 G/C SNP of the IL-6 gene and risk of stroke. In mixed population of US patients with cardiovascular and cerebrovascular pathology, presence of the −174C allele was associated with risk of «silent» MRI infarcts (odds ratio 1.5) [7]. Among Italian patients with stroke history, 174 G allele was major (66.5%), whereas in the control group the frequency of this allele was only 47.3% [5]. According to Austrian researchers, there are no links between-174 G/C SNP of the IL-6 gene and risk of ischemic stroke [6]. The significant increase of allele 174 C frequency in a abdominally obese patients may be a manifestation of its selectivity towards stroke risk, and maybe (that is more likely) a manifestation of the general population patterns – C-allele is associated with obesity. In particular, in French and Swedish populations 174 C allele of IL-6 gene was significantly more common in overweight and obese people [15].

Table 2

Ratio of -174 G/C IL-6 genotypes in patients of different weight categories with non-lacunar stroke

Patients group	G/G		G/C		C/C		Statistics
	n	%	n	%	n	%	
normal body weight	11	39,4	10	30,3	10	30,3	χ^2=8,73; df=2; χ^2_{st}=5,99; p<0,05.
abdominal obesity 2 class	6	16,2	14	37,8	17	46,0	

n – the number of patients; χ^2 u χ^2_{st} – actual and threshold criterion value; df – the number of steps of freedom; p – significance level.

Table 2 shows the significant increase of C/C homozygotes and heterozygotes frequency and, thus reducing the proportion of G/G homozygote among abdominally obese patients with non-lacunar strokes in comparison with normal weight patients. In the analysis of this phenomenon it is necessary to take into account the peculiarities -174 G/C SNP of the IL-6 gene in obese humans. In particular, C/C genotype had significantly associations with abdominal obesity in French and German populations [2, 8]. According to Swedish scientists, there were significant odds ratios for the association of CC (2.13) and GC (1.76) genotypes with overweight (BMI>25 kg/m^2) in healthy humans [15]. Genetically determined individual differences in production of IL-6 may be relevant for the regulation of body fat mass. Based on these observations, the authors concluded that genetically determined individual differences in production of IL-6 may be relevant for the regulation of body fat mass.

Table 3

Structure of the population of patients with different weight and non-lacunar strokes according to -174 G/C SNP of the IL-6 gene

Patients group	The distribution of genotypes	Genotype, %			Statistics	
		G/G	G/C	C/C		
normal body weight	actual	39,4	30,3	30,3	χ^2=15,31; p<0,05.	df=2; χ^2_{st}=5,99.
	theoretical	29,7	49,7	20,6		
abdominal obesity 2 class	actual	16,2	37,8	46,0	χ^2=2,78; p>0,05.	
	theoretical	12,4	45,4	42,2		

% – percentages; χ^2 и χ^2_{st} – фактическое и пороговое значения критерия; df – число степеней свободы; p – уровень значимости.

Table 3 shows that the distribution of genotypes in abdominally obese patients corresponds to the Hardy-Weinberg equilibrium and doesn't significantly deviate from it. Whereas in normal body weight patients distribution of genotypes is significantly deviates from this equilibrium to a lack of heterozygotes. Perhaps, in the Ukrainian population the heterozygous state of -174 G/C IL-6 genes in normal body weight patients is a protective factor regarding the non-lacunar strokes. However, this assumption needs to be further confirmed in a more extensive group of patients.

Table 4

Dynamics of the NIHSS score at acute non-lacunar strokes in patients with different weight and -174 G/C SNP of the IL-6 gene

Patients group		Abdominal obesity 2 class			Normal body weight		
Genotype		G/G	G/C	C/C	G/G	G/C	C/C
Day after stroke	1st	7,5±0,5	7,1±0,5	7,9±0,7	6,8±0,6	7,6±0,5	7,5±0,4
	10th	5,5±0,6	5,5±0,4	5,4±0,3	4,9*±0,5	3,5±0,3	3,4±0,3

** - significant difference in comparison with other genotypes carriers among normal body weight patients at 10th post-stroke day (p <0,05).*

Table 4 shows that in patients with abdominal obesity 2 class there is no difference in the clinical features of acute stroke, depending on the genotype. In normal body weight patients with -174 G/C SNP of the IL-6 gene there is a more severe clinical course of the disease. This fact is consistent with results of the Austrian study that 174 G/G genotype carriers have a significantly worse NIHSS score on admission and at 1st week after stroke onset [6]. Although in the Indian population genotype 174 G/G, on the contrary, had been characterized by a significantly milder strokes at admission and more favorable course of the acute stroke [3], and authors had concluded that G/G genotype has protective effects against ischemic stroke [3]

Table 5

The rate of "disabling" states according to mRS after non-lacunar strokes depending on the weight categories and genotype of -174 G/C IL-6 gene

The observation period		at hospital discharge				12 weeks after stroke			
Patients group		abdominal obesity 2 class		normal body weight		abdominal obesity 2 class		normal body weight	
mRS		≤2	>2	≤2	>2	≤2	>2	≤2	>2
Genotype	G/G	1	5	3	10	2	4	3*	10*
	G/C	3	11	4	6	6	8	8	2
	C/C	3	14	5	5	4	13	9	1

** - significant difference in comparison with other genotypes carriers in normal body weight patients at 12 weeks after stroke (p <0,05).*

Table 5 shows that in normal body weight patients there is a certain structuring of functional consequences depending on the genotype: G/G homozygous state of -174 G/C IL-6 gene was significantly associated with an increased incidence of "disabling" functional outcomes. A similar pattern was recorded by Austrian authors, carriers of 174 G/G genotype had significantly more often "poor" functional outcome (as mRS ≥ 4) at 3 months after stroke [6]. At the same time, in the Indian population, carriers of G/G genotype, by contrast, had «complete» recovery of functional ability (according mRS and index Barthel) at 3 and 6 months after stroke [3].

Perhaps the lack of post-stroke functional recovery in normal-weight and -174 G/G homozygous patients are realized through the levels of IL-6 expression, and, possibly through other pathophysiological mechanisms which are associated with this genotype. The absence of any regularity between functional outcomes rate and genotype in abdominally obese patients may be explained, at least partially, by too little effects of -174 G/C SNP of the IL-6 gene on the clinical stroke features: lipotoxicity (for example, at the basal state, adipose tissue produces more than a third of the whole IL-6 amount in the body [9]) eliminates the modulating clinical effect of this SNP. Under conditions of abdominal obesity, proinflammatory factors associated with adipose tissue probably negate the effect of the genetic component.

Conclusions

1. Abdominally obese patients 2 class with non-lacunar strokes have significantly more common 174 C allele of the IL-6 gene (increase of heterozygous and homozygous states) compared with patients with normal body weight.

2. In patients with normal body weight G/G genotype of -174 G/C IL-6 gene was significantly associated with more severe acute stroke, and an increased rate of late "disabling" functional states (values of mRS more 2 points) in 12 weeks after non-lacunar stroke.

3. Abdominally obese patients 2 class do not have any patterns of clinical and functional outcomes distribu-

tion after non-lacunar stroke depending on -174 G/C SNP of the IL-6 gene.

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