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ALLELIC POLYMORPHISM OF *F2*, *F5* AND *MTHFR* GENES IN POPULATION OF UKRAINE

Analysis of F2, F5 and MTHFR genes SNPs allelic variants in population of Ukraine. Polymorphic variants were analyzed in 172 unrelated individuals using PCR followed by RFLP analysis. Following genotypes have been identified: GG (97 %), GA (3 %) for F2 gene G20210A SNP, GG (96.5 %), GA (3.5 %) for F5 gene G1691A SNP and CC (49.5 %), CT (43 %), TT (7.5 %) for MTHFR gene C677T SNP. Following combined genotypes have been detected. We observed 1.7 % heterozygous carriers of MTHFR gene 677T SNP which were heterozygous for one of the alleles of F5 1691A or F2 20210A genes. On the other hand, the 7.5 % MTHFR gene 677T SNP homozygous individuals carried wild type alleles only of F5 and F2 genes. None of the individuals was carrying F5 1691A and F2 20210A genes polymorphic variants simultaneously. The data about F2, F5 and MTHFR genes SNPs allelic frequencies in the population of Ukraine have been obtained. Thus, distribution of F2, F5 and MTHFR genotypes based on analysis of SNP in those three genes simultaneously has been detected.

Introduction. Taking into account the growing interest to the genetic aspects of complex diseases the era of whole genome association studies (WGAS) started in the last decade. Many single nucleotide polymorphisms (SNPs) have been discovered in different genes. Nevertheless there are contradictory conclusions regarding their contribution to the pathogenesis of different complex disorders. In different publications the newly described SNPs have been studied in many complex pathological conditions.

Methylenetetrahydrofolate reductase (*MTHFR*) is a key regulatory enzyme involved in folate metabolism, DNA synthesis and remethylation reactions [1]. Homocysteine is a metabolic product of remethylation and trans-sulphuration reactions involving methionine [1–4]. A common polymorphism in the *MTHFR* gene (C677T) results in a thermolabile phenotype associated with high levels of homocysteine [1–4]. It has been shown that homozygosity for the C to T substitution at nucleotide 677 of the *MTHFR* gene is associated with a 30–50 % reduction of this enzyme activity and is the most common inherited cause of moderate hyperhomocysteinemia [1, 2]. In the last publications there are evidences that the C677T *MTHFR* gene variation is a risk factor for ischemic stroke [2], infertility [1], primary closed-angle glaucoma (PCAG) [3], presence of anti-hepatitis B virus (HBV) antibodies [4], cytomegalovirus infection (CMV) [4], Huntington disease [5], cancer [6], coronary artery disease (CAD) [7], neural tube defects (spina bifida) and venous thrombosis [6, 8, 9], but the association of the C677T *MTHFR* gene variation and the risk of primary open-angle glaucoma (POAG) is still conflicting [3].

Factor V gene which encodes coagulation molecule responsible for blood coagulation through activation of protein C (APC) was discovered in 1987. APC is a serine protease with potent anticoagulant properties, which is formed in blood on the endothelium from an inactive precursor [10]. The point mutation of factor V (guanine is replaced by adenine in position 1691) led to the structural change in factor V molecule (*F5* Q506, or FV Leiden) that is not properly inactivated by APC and shifts the balance toward thrombosis in the clotting cascade [2, 9–15] and may increase the risk of systemic lupus erythematosus (SLE) [12], type 2 diabetes [16], recurrent pregnancy loss (RPL) [17–21], stroke [2, 22], venous thrombosis and myocardial infraction [2, 22].

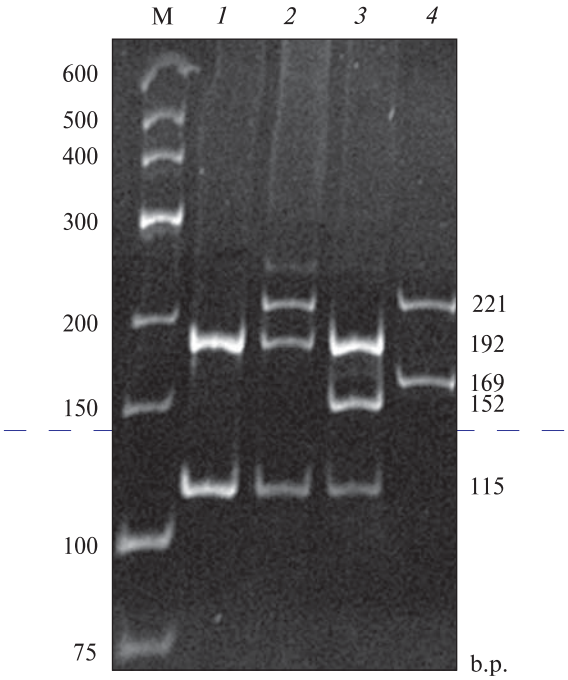

Fig. 1. RFLP analysis of *F5* (factor V Leiden G1691A) and *F2* (prothrombin G20210A) genes endonuclease restriction MnlI (7 % polyacrylamide gel electrophoresis): *1* – *F2* (GG – wild type) and *F5* (GG – wild type) genes; *2* – *F2* (GA – heterozygote) and *F5* (GG – wild type) genes; *3* – *F2* (GG – wild type) and *F5* (GA – heterozygote) genes; *4* – undigested PCR product of *F2* and *F5* genes, M – marker of molecular mass

Prothrombin is the precursor of the serine protease thrombin, a key enzyme in the processes of hemostasis and thrombosis, that exhibits procoagulant, anticoagulant, and antifibrinolytic activities [20, 23, 24]. The G to A transition at nucleotide position 20210, in the 3'-untranslated region of factor II (prothrombin) gene (*F2*) plays a regulatory role in gene expression [11, 20, 23]. It has been shown that *F2* gene G20210A SNP is associated with higher plasma prothrombin concentrations and augmented thrombin generation [11, 17, 20, 23, 24]. Patients carrying the 20210A allele have been demonstrated to be at risk for venous thrombosis [9, 11, 15, 17, 20], RPL and ischemic stroke [18–20].

Several studies argued that *F5* G1691A and *F2* G20210A were maintained at polymorphic frequencies among Caucasians, because they conferred an evolutionary advantage of reduced bleeding [25]. The high frequency of 677T allele *MTHFR* gene suggests that it may be of some benefit to the host. A survival advantage for homozygous fetuses is hypothesized in environment, in which folic acid consumption is adequate [25]. *F5* gene G1691A, *F2* gene G20210A and *MTHFR* gene C677T are three SNPs common among Caucasians. Therefore the study of their frequencies in various populations provides perspectives for both clinical medicine and population genetics.

This study aimed to analyze the *F5* gene G1691A, *F2* gene G20210A and *MTHFR* gene C677T alleles distribution in the population of Ukraine.

Materials and methods. The study subjects were recruited between 2007 and 2008. An informed consent was obtained from each participant prior to blood collection and DNA extraction. This study was approved by the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine Ethical Committee. We studied 172 unrelated individuals (men – 85, women – 87), having an age range of 25 to 45 years old, who were randomly selected from different regions of Ukraine from the general population.

Venous blood samples were collected into vacutainer tubes containing EDTA and stored frozen at –70 °C until using.

DNA was extracted from the peripheral blood leukocytes by standard phenol-chloroform extraction method [27] and stored at +4 °C until being used.

The *F5* gene G1691A, *F2* gene G20210A alleles were genotyped by multiplex polymerase chain reaction (PCR) with the use of specific oligonucleotide primers followed by MnlI restriction enzyme digestion as described by Koksal [9].

The PCR reaction was performed in a final volume of 15 μL containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 5 μM of each of the relevant primer (made on an Biosset ASM 800 DNA synthesizer), 0.2 units of Taq-DNA polymerase and 50–200 ng of the DNA template. The cycling conditions on the PCR-amplificator for alleles of *F5* and *F2* genes were as follows: initial denaturation at 95 °C for 5 min, 30 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec and a final elongation step at 72 °C for 5 min.

The *MTHFR* gene C677T allele was genotyped by PCR with the use of specific oligonucleotide primers followed by HinfI restriction enzyme digestion by Michael [3].

The cycling conditions for *MTHFR* gene C677T allele were as follows: initial denaturation at 95 °C for 5 min, 30 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 66 °C for 30 sec, extension at 72 °C for 30 sec and a final elongation step at 72 °C for 3 min. Electrophoresis of the PCR products was performed in a 1.8 % agarose gel with EtBr followed by visualization under UV. PCR products 169, 221 and 294 bp long correspondently were digested and run on 7 % PAGE.

The G to A transition in position 1691 of exon 10 *F5* gene results in a disappearance of restriction site for MnlI endonuclease. With a purpose to detect G to A transition in position 20210 of the *F2* gene 3'-untranslated region detection, a single mismatch nucleotide has been introduced in the reverse primer [9]. This mismatch results in the generation of a new MnlI restriction site in the wild type allele. The C to T transition in position 677 of exon 4 *MTHFR* gene creates a restriction site for HinfI endonuclease.

Digestion was performed in 15 μL reaction volume containing 1 × reaction buffer, 0.5 units of the restriction enzyme and 10 μL of purified PCR product, incubated at 37 °C for 3 hours or overnight. The digested products with 100 bp leader were electrophoresed on 7 % PAGE gel with EtBr and photographed under UV.

Statistical analysis was performed by χ^2 test, using GENEPOP [28] package.

Results and discussion. In wild type alleles MnlI digestion of *F5* and *F2* genes amplicons yielded fragments of 17, 37 and 115 bp for *F5* gene (Fig. 1), the 17 bp fragment was a result of an invariant MnlI site. For digested *F2* gene amplicon 29 and 192 bp fragments were observed (Fig. 1). In wild type allele HinfI digestion of *MTHFR* gene amplicons yielded fragment of 294 bp (Fig. 2).

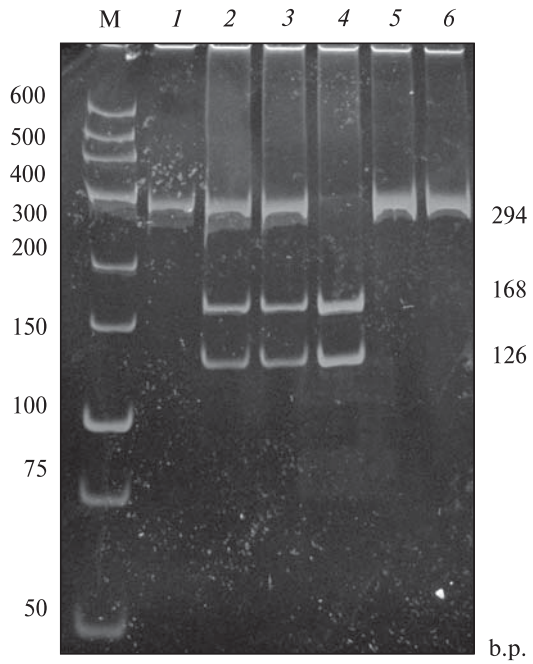

Fig. 2. RFLP analysis of MTHFR (C677T) gene endonuclease restriction HinfI (7 % polyacrylamide gel electrophoresis): *1* – undigested PCR product of MTHFR gene; *2*, *3* – CT (heterozygote); *4* – TT (mutant variant); *5*, *6* – CC (wild type); M – marker of molecular mass

Digestion of the *F5* gene G1691A SNP homozygote gave fragments of 17 and 152 bp, and the *F2* gene G20210A SNP homozygote yielded fragment of 221 bp (Fig. 1). Digestion of *MTHFR* gene C677T SNP homozygote yielded fragments of 126 and 168 bp (Fig. 2). The small 17, 29 and 37 bp fragments has run out from the gel (Fig. 1).

Table 1

Genotype and allele frequencies distribution of *F5* G1691A, *F2* G20210A and *MTHFR* C677T gene variants in Ukraine

SNPs	Homozygote (wild type)		Heterozygote		Homozygote (mutant allele)		Mutant allele frequency
	n	%	*n*	%	*n*	%	
F2 G20210A	167(GG)	97	5(GA)	3	0(AA)	0	0.015
F5 G1691A	166(GG)	96.5	6(GA)	3.5	0(AA)	0	0.017
MTHFR C677T	85(CC)	49.5	74(CT)	43	13(TT)	7.5	0.29

Note. *n* – group size.

Table 2

Frequencies of *F5* G1691A, *F2* G20210A and *MTHFR* C677T genes alleles in different countries

Population	Gene/mutant allele						Reference
	*FII**A	*n*	*FV**A	*n*	*MTHFR**T	*n*	
Russia	–	–	–	–	0.259	599	7, 26
Japan	–	–	0	386	–	–	21
Turkey	0.013	311	0.041	387	0.325	1885	25
Italy	0.0199	1429	0.0187	1415	0.432	1504	25, 26, 19
Greece	0.022	160	0.025	160	0.353	160	25
Cyprus	0.039	90	0.072	90	0.400	90	25
Jordan	0.010	200	0.085	200	0.160	200	25
Lebanon	0.014	697	0.079	697	0.304	697	25
Tunisia	0.013	313	0.035	313	0.292	313	25
Bahrain	0.005	191	0.016	191	0.126	191	25
Saudi-Arabia	0.003	884	0.007	902	0.148	884	25
Iran-west	0.017	180	0.014	180	0.278	180	25
Morocco	0.012	124	0	159	0.289	162	25
Palestinians	0.033	107	0.082	581	0.322	284	25
Pakistan	–	–	–	–	0.0928	70	3
China	–	–	0	99	0.5707	99	2
Brazil	0.0052	384	0.00826	484	–	–	20, 14
Indian	–	–	–	–	0.4	200	1
Canadian	–	–	0.0266	356	–	–	14
United States	–	–	0.0298	704	–	–	14
Finland	–	–	0.0198	303	–	–	14
Germany	0.0098	102	0.0274	510	–	–	21, 18, 14
Australia	–	–	0.0198	126	–	–	14
United Kingdom	–	–	0.0227	352	0.186	94	14, 26
France	–	–	0.0133	300	–	–	14
Azerbaijan	0.0136	110	–	–	–	–	24
Spain	–	–	–	–	0.545	33	26
Ireland	–	–	–	–	0.286	947	8, 26
Netherlands	–	–	0.0156	641	0.270	318	14, 26
Sweden	–	–	–	–	0.338	6644	26

Note. *n* – group size; *FII**A – 20210A; *FV**A – 1691A; *MTHFR**T – 677T.

Genotypes and allele frequencies of the three polymorphisms are presented in Table 1. Of the 172 analyzed samples from the present study, we have found 5 to have *F2* gene 20210A (mutant allele frequency 0.015), 6 to have *F5* gene 1691A (mutant allele frequency 0.017) and 87 to have at least one *MTHFR* gene 677T allele (mutant allele frequency 0.29). The genotype distribution of the three analyzed SNPs is in Hardy-Weinberg equilibrium $\chi^2 = 0.0374$, $P > 0.05$, $\chi^2 = 0.0542$, $P > 0.05$ and $\chi^2 = 0.3222$, $P > 0.05$ for *F2* G20210A, *F5* G1691A and *MTHFR* C677T genes SNPs correspondently. Expected heterozygosity for the studied loci corresponded to the observed one.

To our knowledge this is the first published data regarding research of *F5* gene G1691A SNP, *F2* gene G20210A SNP and *MTHFR* gene C677T SNP combined genotype distribution in the general population of Ukraine.

The frequency of allelic variants of *F2* gene 20210A, *F5* gene 1691A and *MTHFR* gene 677T in our study is in agreement with the published data obtained in other countries (Table 2). The exception appears to be the Japanese population in which the *F5* gene G1691A and *F2* gene G20210A were not detected yet [21]. The expected frequency of homozygotes in our study is 0.017 % for *F2* gene 20210A and 0.026 % for *F5* gene 1691A.

Table 3

Combined genotypes frequencies of *F5* G1691A, *F2* G20210A and *MTHFR* C677T genes variants in Ukraine

Genotype			*n*	%
FII	*FV*	*MTHFR*		
GG	GG	TT	13	7.5
GG	GA	CT	1	0.5
GA	GG	CT	2	1.2
GG	GG	CT	71	41.3
GG	GA	CC	5	2.9
GA	GG	CC	3	1.8
GG	GG	CC	77	44.8

Note. *n* – group size; *FII*: A – 20210A; G – 20210G, *FV*: A – 1691A; G – 1691G, *MTHFR*: T – 677T, C – 677C.

Therefore, the absence of homozygous individuals in our study is not surprising. From all the 27 possible combinations of *F2* 20210A, *F5* 1691A and *MTHFR* 677T genes SNPs combined genotypes, we observed only 7 (Table 3). None of the individuals was carrying *F5* gene 1691A and *F2* gene 20210A alleles simultaneously. We observed 3 heterozygous individuals for *MTHFR* gene 677T allele who at the same time carried one of the *F5* gene 1691A or *F2* gene 20210A alleles. On the other hand, the *MTHFR* gene 677T allele homozygous individuals carried *F5* and *F2* genes wild type alleles only. The obtained allelic frequencies in the current study were in the lower range frame of the world distribution. Obtained results from our study add to the world distribution data concerning *F2* 20210A, *F5* 1691A and *MTHFR* 677T genes allelic variants. Although further studies based on population data as controls are required to clarify their involvement in different complex disorders, increased awareness of this genetic risk factors is warranted.

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АЛЛЕЛЬНЫЙ ПОЛИМОРФИЗМ ГЕНОВ *F2*, *F5* И *MTHFR* СРЕДИ НАСЕЛЕНИЯ УКРАИНЫ

Проанализировано распределение полиморфных вариантов генов по SNP *F2* G20210A, *F5* G1691A и *MTHFR* C677T среди населения Украины. Аллельные варианты SNP анализировали среди 172 неродственных индивидуумов методами ПЦР- и ПДРФ-анализа. Выявлены следующие генотипы: GG (97 %), GA (3 %) для гена *F2* G20210A, GG (96,5 %), GA (3,5 %) для гена *F5* G1691A и CC (49,5 %), CT (43 %), TT (7,5 %) для гена *MTHFR* C677T. Идентифицированы следующие комбинированные генотипы: 1,7 % гетерозиготных носителей 677T аллеля гена *MTHFR* одновременно были носителями одного из полиморфных аллелей генов *F2* 20210A или *F5* 1691A. Вместе с тем 7,5 % гомозиготных носителей 677T аллеля гена *MTHFR* были гомозиготами по аллелям дикого типа генов *F2* и *F5*. Ни у одного из индивидов в генотипе не выявлены исследуемые аллели 20210A и 1691A генов *F2* и *F5* одновременно. Получены данные о частоте аллельных вариантов SNP по генам *F2*, *F5* и *MTHFR* среди населения Украины. На основании комплексного анализа SNP этих трех генов было установлено распределение комбинированных генотипов.

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АЛЕЛЬНИЙ ПОЛІМОРФІЗМ ГЕНІВ *F2*, *F5* ТА *MTHFR* СЕРЕД НАСЕЛЕННЯ УКРАЇНИ

Проаналізували розподіл алельних варіантів генів за SNP *F2* G20210A, *F5* G1691A та *MTHFR* C677T серед населення України. Алельні варіанти SNP аналізували серед 172 неспоріднених індивідів методом ПЛР- та ПДРФ-аналізу. Виявлено наступні генотипи: GG (97 %), GA (3 %) для гена *F2* G20210A, GG (96,5 %), GA (3,5 %) для гена *F5* G1691A та CC (49,5 %), CT (43 %), TT (7,5 %) для гена *MTHFR* C677T. Було ідентифіковано наступні комбіновані генотипи: 1,7 % гетерозиготних носіїв 677T алеля гена *MTHFR* водночас виявились носіями одного з поліморфних алелів гена *F2* 20210A чи гена *F5* 1691A. Разом з тим 7,5 % гомозиготних носіїв 677T алеля гена *MTHFR* були гомозиготами за алелями дикого типу генів *F2* та *F5*. У жодного з індивідів в генотипі не виявлені досліджувані алелі 20210A та 1691A генів *F2* та *F5* одночасно. Отримано дані про частоти алельних варіантів SNP за генами *F2*, *F5* та *MTHFR* серед населення України. На основі комплексного аналізу SNP цих трьох генів було встановлено розподіл комбінованих генотипів.

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