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Research article

POLYMORPHISM OF FOLATE CYCLE GENES AS A RISK FACTOR OF HYPERHOMOCYSTEINEMIA

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Hyperhomocysteinemia (HHc) is a new factor being considered at the moment that can cause damage to vessel walls. Its occurrence depends on genetic peculiarities of a body.

Our research goal was to estimate frequency of genetic polymorphisms (SNP) in folate cycle genes among people living in Perm region and its influence on homocysteine (Hc) concentration in blood serum.

We examined 189 women (32.2 ± 5.25). Hc concentration in blood serum was determined with immune chemiluminescent procedure. We examined frequency of SNP in folate cycle genes with pyrosequencing.

Homozygote state as per minor alleles in methylene tetrahydrofolate reductase (MTHFR) gene (rs 1801133 u rs 1801131) and MTR gene (rs 1805087) was registered 7.5, 5.4, and 13.75 times less frequently than homozygote state as per neutral alleles. Heterozygote state prevailed for genes of methionine synthase reductase and folate transport protein among examined SNP. Homozygotes as per minor allele SNP in MTHFR gene (Ala222Val; rs 1801133) had higher Hc concentration in blood serum that amounted to 8.476 ± 3.193 mmol/L and was 1.276 times higher than the same parameter in homozygotes as per neutral allele (p=0.0036). We didn't establish any influence on Hc contents in blood serum for the remaining 4 SNP in folate cycle genes (p> 0.1).

Examined SNP in MTHFR and MTR genes tended to have neutral alleles more frequently than minor ones. SNP in genes of other examined proteins belonging to folate cycle didn't have any differences in frequency of examined alleles. We didn't detect a combination of homozygote state as per two SNP in MTHFR gene or homozygote state as per one SNP and heterozygote state as per another one in a genome. Only SNP in MTHFR gene (Ala222Val, rs 1801133) authentically causes increase in homocysteine concentration out of all the examined SNP in genes of folate cycle enzymes and proteins.

Key words: *homocysteine, hyperhomocysteinemia, single-nucleotide polymorphisms, folate cycle genes, methylene tetrahydrofolate reductase, methionine synthase, methionine synthase reductase, folate transport protein.*

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Homocysteine (Hc) is a proteinogenic amino acid that is not used to synthesize proteins but instead is formed in intermediate metabolism of amino acids and their derivatives [1, 2]. Healthy people usually have rather low Hc concentration in their blood serum [3–5]. Its elevated concentration in blood serum (hyperhomocysteinemia or HHc) is a risk factor that can cause damage to vessels endothelium and endothelial dysfunction thus resulting in higher risks of thrombosis leading to disrupted blood supply to various organs and systems [4–7]. At present HHc is seen as a factor that participates in pathogenesis of multiple diseases [8–10]. HHc role in implantation defects and defects in fertilized ovum development has also been proven; it can result in infertility, miscarriage, and fetus development pathologies [11, 12]. Several researchers have drawn attention to a correlation between HHc and atherosclerotic damage to arteries, disrupted blood supply to the brain, and neurodegenerative diseases occurrence [13–17]. Besides, it has been proven that there is a correlation between elevated Hc contents in blood serum and diseases of other organs [18–20].

HHc occurs due to diseases of the liver when there are disorders in intermediate amino acids metabolism, or there is vitamin deficiency (vitamin B_6, vitamin B_c or folic acid, and vitamin B_{12}). The highest homocysteine contents in blood serum are detected in case a person suffers from hereditary hyperhomocysteinemia, a disease that is caused by congenital defects in the process of synthesizing enzymes that participate in Hc metabolism [21].

Nowadays hyperhomocysteinemia is considered to be a disorder with hereditary predisposition that can develop into an actual disease due to many other factors. It should be interesting to study influence exerted by single nucleotides replacements or so called *single nucleotide polymorphism or SNP* in folate cycle genes and their effects produced on homocysteine metabolism [22, 23]. Folate cycle is a complex cascade process controlled by enzymes that use folic acid derivatives as coenzymes. In some cases gene polymorphism that occurs due to SNP results in one amino acid being replaced with another. As a result, there are slight changes in the structure of a protein that is produced by a mutant gene. In some cases these changes can be adverse under certain conditions or they can bring about certain advantages for a person who carries such a gene in other circumstances. It is these mutations that provide basis for natural selection since mutations that are adverse under certain conditions can provide competitive advantages in other circumstances given changes in the environment an organism has to live in.

Interest a lot of researchers pay to genetic versions of genes that code folate cycle enzymes is mostly due to multiple publications dwelling on a correlation between various SNP and frequency of multiple different diseases. Thus, there are publications on a correlation between SNP in folate cycle genes and risks of vascular diseases, oncologic diseases, obstetric pathology, and infertility [16, 17, 24–31].

In relation to that it seems only natural to have interest in examining SNP in folate cycle genes among different population groups and its correlation with hyperhomocysteinemia occurrence. Over the last years there have been publications in the Russian Federation focusing on examining SNP in folate cycle genes among people living in Penza region and Altai region [32, 33]. However, we haven't been able to find any research on frequency of SNP in folate cycle genes in people living in Perm region; therefore, results obtained via such research are truly vital and they can be of significant interest for experts in the sphere.

Our research goal was to assess frequency of SNP in folate cycle genes in people living in Perm region; to analyze influence exerted by SNP in folate cycle genes as risk factors causing elevated Hc concentration in blood serum.

Data and methods. The study was accomplished in conformity with ethical principles for medical examinations with people participating in them fixed in the WHO's Helsinki Declaration. The study was also approved upon by the Ethical Committee of E.A. Vagner's Perm State Medical University of the RF Public Healthcare Ministry.

189 women who were in their fertile age took part in our research (32.2 ± 5.25; median 31 and interquartile range 28–36); they all were employed at enterprises located in Perm city.

Participants were included according to the following criteria:

– females;

– pregnancies in case history;

– all the examined women belonged to at least the 2nd generation living in Perm region.

Participants were excluded according to the following criteria:

– a woman was pregnant at the moment the study took place;

– pathology in the liver determined as per results obtained via examining activity of enzymes in blood serum (alanine aminotransferase; aspartate aminotransferase; gamma glutamyl transferase; alkaline phosphatase) and bilirubin concentration;

– a woman was taking sulfanamides, polyvitamins, or folic acid at the moment the study was accomplished;

– pancreatic diabetes, arterial hypertension, smoking, alcoholism.

Blood samples were taken in the morning on an empty stomach 12 hours after the last meal. We determined Hc concentration in blood serum with immune-chemical luminescent procedure performed with «Immulite-2000» immune-chemical analyzer (Siemens, Germany) and using original reagents kits. Genetic polymorphism in folate cycle genes was examined via pyrosequencing with the use of «AmpliSens® Pyroscreen» «FOLATE – screen» system for genetic analysis (The Central Scientific Research Institute for Epidemiology).

We examined frequency of the following SNP:

– mutation of methylenetetrahydrofolate reductase gene (MTHFR) (Ala222ValC>T, rs 1801133);

– mutation of methylenetetrahydrofolate reductase gene (MTHFR) (Glu429AlaA>C, rs 1801131);

– mutation of methionine-synthase gene (MTR) (Asp919Gly, A>G, rs 1805087);

– mutation of methionine-synthase reductase gene (MTRR) (Ile22Met, A>G, rs 1801394);

– mutation of folate transporter gene (SLC19A1) (His27Arg, A>G, rs 1051266).

Alleles frequency was calculated as per Hardy-Weinburg equation [34].

All the results were statistically processed with STATISTIC Av. 7 software package (StatSoft Inc., the USA). We calculated descriptive statistic parameters for each data array such as simple mean (M), standard deviation (SD), median (Me), and interquartile range (LQ; UQ), as well as minimum (min) and maximum (max) value. All the obtained results were estimated with Shapiro-Wilk test and it allowed us to reject a zero hypothesis that all the obtained results were distributed normally. Given that, we used Kruskal – Wallis non-parametric test H to make comparisons.

Maximum permissible probability of type I error (p) was taken at statistical significance level equal to or lower than 0.05.

Results and discussion. We analyzed genotypes of SNP in various folate cycle proteins and enzymes in the examined group and detected significant discrepancies. Results are given in Table 1 and shown in Figure (Table 1, Figure).

Most examined SNP in MTHFR gene (rs 1801133 и rs 1801131) and MTR gene (rs 1805087) were characterized with homozygous state as per variants of allele that prevailed among the examined population. Homozygous state as per minor alleles was registered 7.5, 5.4 and 13.75 times less frequently that homozygous state as per neutral alleles (Table 1).

Methionine-synthase reductase enzyme and folate transporter protein predominantly had heterozygous state for the examined SNP and a number of homozygous state genotype cases, both for traditional alleles and minor ones, was almost the same (Figure). Homozygous state frequency as per minor and neutral (wild type) allele was practically the same for folate transporter; as for methionine-synthase reductase, homozygous state here occurred 1.44 times more frequently as per minor allele than as per neutral (wild type) one.

Table 1

Distribution of relative frequency (%) of examined genetic polymorphisms in folate cycle genes in women living in Perm region (n = 189)

No	SNP in folate cycle genes	Alleles combinations		
		Homozygous as per neutral allele	Heterozygous	Homozygous as per minor allele
1	Methylenetetrahydrofolate reductase (MTHFR) (Ala222ValC>T, rs 1801133)	105 (55.6%)	70 (37%)	14 (7.4%)
2	Methylenetetrahydrofolate reductase (MTHFR) (Glu429AlaA>C, rs 1801131)	81 (42.9%)	93 (49.3%)	15 (8 %)
3	Methionine-synthase (MTR) (Asp919GlyA>G, rs 1805087)	110 (58.2%)	71 (37.6%)	8 (4.2%)
4	Methionine-synthase reductase (MTRR) (Ile22Met, A>G, rs 1801394)	43 (22.8%)	84 (44.4%)	62 (32.8%)
5	Folate transporter (SLC19A1) (His27Arg, A>G, rs 1051266)	46 (24.3%)	97 (51.4%)	46 (24.3%)

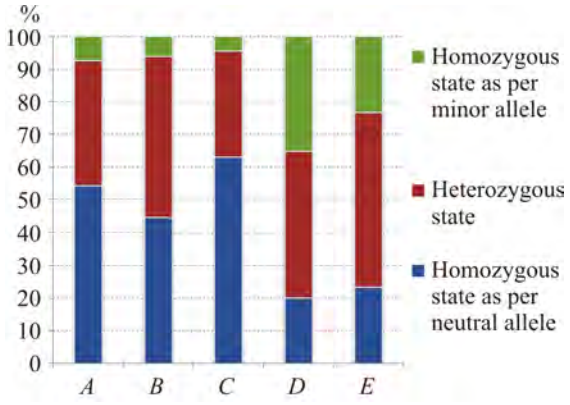

Figure. Folate cycle proteins genes: frequency (%) of genotypes (A – Methylenetetrahydrofolate reductase; B – Methylenetetrahydrofolate reductase; C – Methionine-synthase; D – Methionine-synthase reductase; E – folate transporter)

Table 2 contains results obtained via calculating neutral and minor alleles frequency; the calculations were performed according to Hardy-Weinburg formula (Table 2).

Frequency of various allele variants was significantly different in the examined population for SNP in MTHFR gene (rs 1801133 и rs 1801131) and MTR gene (rs 1805087) (Table 3). The said SNP were characterized with neutral allele being much more frequent than minor one. Thus, the discrepancy amounted to 2.86 times for SNP in MTHFR gene (rs 1801133) and 2.07 times for SNP in MTHFR gene (rs 1801131). The greatest discrepancies were detected for SNP in MTR gene (rs 1805087) where minor allele occurred 3.34 times less frequently than a variant that was prevailing in the examined population.

Table 2

Relative frequency (%) of neutral and minor alleles in folate cycle genes in women living in Perm region (n = 189)

No	A type of SNP in folate cycle gene	Alleles frequency	
		neutral	minor
1	Methylenetetrahydrofolate reductase (MTHFR) (Ala222ValC>T, rs 1801133)	0.7408	0.2592
2	Methylenetetrahydrofolate reductase (MTHFR) (Glu429AlaA>C, rs 1801131)	0.6746	0.3254
3	Methionine-synthase (MTR) (Asp919GlyA>G, rs 1805087)	0.7698	0.2302
4	Methionine-synthase reductase (MTRR) (Ile22Met, A>G, rs 1801394)	0.4497	0.5503
5	Folate transporter (SLC19A1) (His27Arg, A>G, rs 1051266)	0.5	0.5

Table 3

Frequency of SNP in MTHFR gene among all the examined women

Parameter		SNP MTHFR (Glu429Ala A>C, rs 1801131)		
		Homozygotes as per neutral allele	Heterozygotes	Homozygotes as per minor allele
SNP MTHFR (Ala222Val C>T, rs 1801133)	Homozygotes as per neutral allele	17.07 %	30.84 %	6.29 %
	Heterozygotes	20.06 %	18.26 %	–
	Homozygotes as per minor allele	7.48 %	–	–

Table 4

Homocysteine concentration (μmol/L) in blood serum of healthy women (n = 189) with different variants of SNP in folate cycle genes

Single nucleotide polymorphism (SNP) types	Genotype as per single nucleotide polymorphism (SNP)			p
	Homozygous as per neutral allele	Heterozygous	Homozygous as per minor allele	
Methylenetetrahydrofolate reductase (MTHFR) (Ala222ValC>T, rs 1801133)	6.642 ± 2.242 6.21 (5.09–7.54) 2.57–16.8	7.656 ± 2.885 6.915 (6.05–8.47) 3.6–21.2	8.476 ± 3.193 7.095 (6.74–9.46) 5.03–15.5	0.0036 (H = 11.27)
Methylenetetrahydrofolate reductase (MTHFR) (Glu429AlaA>C, rs 1801131)	7.271 ± 2.576 6.85 (5.57–8.2) 2.57 – 16.8	6.998 ± 2.745 6.33 (5.24–7.63) 3.84 – 21.2	7.479 ± 2.245 6.86 (5.61–9.24) 4.43 – 11.6	0.27 (H = 2.64)
Methionine-synthase (MTR) (Asp919GlyA>G, rs 1805087)	7.275 ± 3.009 6.435 (5.37–8.03) 2.57 – 21.2	6.916 ± 1.901 6.79 (5.45–7.76) 3.6 – 12.9	7.59 ± 2.745 6.985 (5.745–8.745) 4.67 – 13.1	0.85 (H = 0.32)
Methionine-synthase reductase (MTRR) (Ile22Met, A>G, rs 1801394)	7.019 ± 2.395 6.38 (5.1–8.24) 4.14 – 14.7	6.802 ± 2.281 6.585 (5.325–7.595) 2.57 – 16.7	7.723 ± 3.125 7.14 (5.61–8.77) 3.6 – 21.2	0.16 (H = 3.72)
Folate transporter (SLC19A1) (His27Arg, A>G, rs 1051266)	7.009 ± 1.996 6.825 (5.37–7.83) 3.84–12.4	7.171 ± 2.989 6.37 (5.43–7.6) 2.57–21.2	7.261 ± 2.41 6.905 (5.61–9.15) 3.6–13.1	0.66 (H = 0.84)

Note:

Numerator is $M \pm SD$, denominator is Me (LQ – UQ), minimum and maximum values are given under each fraction; p is determined as per H values of Kruskal – Wallis criterion.

Discrepancies in allele variants frequency detected for folate transporter protein (SLC19A1) and MTRR enzymes were not so drastic as opposed to significantly asymmetric distribution of alleles frequency detected for SNP in MTHFR and MTR enzymes (Table 2). Thus, neutral and minor allele frequency was practically the same for folate transporter protein, and as for methionine-synthase reductase, minor allele here was 1.22 times more frequent that neutral one.

We examined 189 women and analyzed the results; there was not one case when an examined woman has a combination of homozygous state as per SNP for two minor alleles in MTHFR gene (Table 3).

Table 4 contains the results obtained via examining Hc concentration in blood serum

depending on a type of genetic polymorphism in the examined genes.

We analyzed dependence between Hc concentration in blood serum and SNP in folate cycle and revealed statistically significant discrepancies only for SNP in methylenetetrahydrofolate reductase gene (MTHFR) (Ala222ValC>T, rs 1801133). Hc concentration in blood serum of homozygotes as per minor allele was 1.276 times higher than in that of homozygotes as per neutral allele ($H = 11.27$; $p = 0.0036$); average Hc concentration in blood serum of heterozygous women was somewhere in between values obtained for women with homozygous state (as per traditional and minor allele) of the examined genetic polymorphism.

As for the rest examined single nucleotide polymorphisms in folate cycle genes, we didn't establish any statistically significant effects produced by them on Hc concentration in blood serum ($p > 0.1$).

Regularities which we detected in frequency of alleles in folate cycle genes are typical for population living on the examined territory. We analyzed relative frequency of the examined alleles and established that their distribution was quite typical for people living in the European part of Russia.

Discrepancies in frequency of alleles in genes caused by SNP probably determine their influence on adaptability (advantages) their carriers have. Thus, more frequent neutral alleles probably determine certain advantages that their carriers have in specific conditions in comparison with minor allele carriers. At the same time practically the same frequency of alleles indicates that their carriers don't have any advantages. This conclusion is the most probable for the examined SNP in folate transporter protein gene and SNP in methionine-synthase reductase gene.

It is especially interesting to note that there was no homozygous combination of two SNP in the genome of the same protein. Having examined 189 women, we didn't detect any case in which an examined woman had homozygous state as per SNP for two minor alleles in MTHFR gene. Replacement of one nucleotide in the genome is probably accompanied with replacement of one amino acid and it has insignificant influence on functions performed by a coded protein. Combination of two SNP, each producing insignificant effects on functional activity of a protein molecule, probably results in synthesis of a defect protein with gravely distorted properties. In homozygous state such a combination may lead to disorders in body vital capacity. We also didn't detect any states of genotypes in MTHFR genes in which there would be a combination of homozygous SNP state as per one SNP with heterozygous state as per another SNP.

Hc concentration in blood serum depends on multiple factors that could be rather conditionally divided into non-modifiable and modifiable ones depending on impacts exerted on damage to vessel walls.

Modifiable factors that make for hyperhomocysteinemia are factors that can be adjusted, for example, deficiency of group B vitamins (B_6, B_c and B_{12}), metabolic disorders caused by liver and kidneys diseases, nutrition habits, or hormonal background.

Sex, age, and genotype peculiarities are non-modifiable risk factors that can cause hyperhomocysteinemia.

Reference range for Hc concentration in blood serum is 5–15 μmol/L. Hc concentration in blood serum equal to 15–30 μmol/L is considered moderate increase in homocysteine contents; values within 30–100 μmol/L are seen as average hyperhomocysteinemia. Hc concentration in blood serum being higher than 100 μmol/L means there is grave hyperhomocysteinemia.

Our analysis of SNP in folate cycle genes allowed revealing that some of them were associated with elevated homocysteine concentration and risks of hyperhomocysteinemia occurrence. In particular, homozygous state of single nucleotide replacement in methylenetetrahydrofolate reductase gene (MTHFR) (Ala222ValC>T, rs 1801133) should be treated as an independent risk factor that might cause HHc occurrence.

Despite there is slight increase in Hc concentration in blood serum (by 26.7% against

values obtained for women with homozygous state as per neutral allele), this effect can result in clinical manifestations of various diseases and can be considered an independent risk factor that might cause hyperhomocysteinemia occurrence. Over recent years there have been publications where it is stated that Hc concentration in blood serum within 12–15 μmol/L, or the upper limit of the reference range, should be considered mild HHc for people older than 50 and not a physiologically normal state [35].

Elderly people didn't take part in the present research; despite that, all the obtained data allow assuming that statistically significant HHc occurs in patients with homozygous state as per single nucleotide replacement in methylenetetrahydrofolate reductase gene (MTHFR) (Ala222ValC>T, rs 1801133).

Other examined polymorphisms in folate cycle genes do not have any associations with statistically significant increase in homocysteine concentration in blood serum and can't be considered risk factors that might cause hyperhomocysteinemia occurrence.

Bearing in mind that genetic shapers are non-modifiable risk factors for patients with homozygous state of minor allele in methylenetetrahydrofolate reductase gene (MTHFR) (Ala222ValC>T, rs 1801133), it is necessary to pay great attention to control over modifiable risk factors that can cause hyperhomocysteinemia.

Folate cycle is a complex metabolic process that is aimed at turning sulfur-containing acid, or homocysteine, into methionine catalyzed by enzymes with their co-enzymes being vitamin B_c (folic acid) derivatives. A key role in folate cycle belongs to such an enzyme as 5,10-methylenetetrahydrofolate reductase (MTHFR) [36]. Mutation in a gene that codes MTHFR is the most frequent enzyme defect that is related to elevated homocysteine contents. At present there are several known mutations in MTHFR gene located in the locus 1p36.3. The most frequent one is replacement of C677T nucleotides (alanine being replaced with valine in MTHFR protein) that becomes apparent via their thermal lability and a 60% decrease in MTHFR enzyme activity [35, 36]. Another possible polymorphism in MTHFR gene is adenine being replaced with cytosine in position 1298. It results in glutamine acid residue being replaced with alanine residue in the regulatory domain of the enzyme and it is usually accompanied with a slight decrease in enzyme activity. People who are homozygous as per A1298C mutation tend to have 35 % lower activity of their MTHFR gene than the physiological standard. This decrease can probably result in elevated homocysteine contents but there have been no significant changes in Hc concentration detected in practice.

Conclusions. Women living in Perm region tend to have different frequency of SNP alleles in folate cycle genes. SNP in MTHFR and MTR genes usually have prevailing neutral allele against minor one. We haven't been able to detect any significant discrepancies in the examined alleles frequency for SNP in methionine-synthase reductase gene and folate transporter gene.

We haven't either detected a combination of homozygous states as per two SNP in MTHFR gene or homozygous state as per one SNP and heterozygous state as per another.

Our analysis of the examined SNP in genes of folate cycle enzymes and proteins allowed establishing that only SNP in MTHFR gene resulted in authentic decrease in homocysteine concentration; when this SNP occurs, it can be considered an independent risk factor causing hyperhomocysteinemia. People who are homozygous as per SNP in MTHFR (Ala222ValC>T, rs 1801133) tend to have elevated average homocysteine concentration that is by 27.6 % higher than in people who are homozygous as per neutral allele. Other examined polymorphisms were not accompanied with elevated homocysteine concentration in blood serum and they can't be considered risk factors that might cause an increase in homocysteine concentration in blood serum.

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