HYPERHOMOCYSTEINEMIA IN THE PATHOGENESIS OF VASCULAR DISORDERS: FROM HOMOCYSTINURIA TO MULTIFACTORIAL CHANGES (PRE-ECLAMPSIA AND ISCHEMIC HEART DISEASE)

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S u m m a r y. The subject of the present paper is the research of the main homocysteine remethylation regulators genotypes (HCys), and sulfur amino acids in the blood plasma of the patients suffering from the pre-eclamptic state and ischemic heart disease (IHD). The actual hyperhomocysteinemia among the patients with the pre-eclamptic state and the moderate hyperhomocysteinemia along with the actual hyperhomocysteinemia among patients with IHD were diagnosed. The genotypes prone to the development of atheromatosis changes were determined. There is some high probability of hidden hyperhomocysteinemia among patients with IHD, which is connected with the homocysteine thiolactone concentration increase due to the transsulfuration processes disturbance and methylation, which can involve endogens hydrogen sulfide biosynthesis disturbance.

K e y w o r d s: hyperhomocysteinemia, pre-eclampsia, ischemic heart disease *MTHFR* gene, *MTR* gene, *MTRR* gene,

INTRODUCTION

Hyperhomocysteinemia plays on important part in the pathogenesis of vascular disorders. At present hyperhomocysteinemia is said to be an independent factor causing the vascular wall damage and atherosclerosis development. It is commonly known that the homocysteine (HCys) concentration increase in blood by 5mM/L causes an increased atheromatosis risk, and affects clotting disturbances and lipid peroxidase.

The pathogenic HCys activity and its toxic effect on blood vessels was diagnosed during the monogenic homocystinuria disease. There are at least 6 monogenic diseases known these days [8, 13]. It has been found out that the hyperhomocysteinemia with the patients affected by the ischemia heart disease (IHD) and atheromatosis is an important factor leading to the myocardial infraction [18] and, with pregnant women, to serious complications including the fetus loss [21].

What focuses our attention is the issue of an objective risk prognosis of the complications in blood vessels caused by HCys. However, it is obvious that the traditional research of particular polymorphisms will be ineffective. The objective of this paper was the complex research of the genotypes division of the folic and methionine cycles and the profile description of sulfur amino acids in the blood plasma of the patients affected by IHD and among women with pregnancy complications.

MATERIALS AND METHODS

There were 71 male patients (research group) affected by IHD examined at the *municipal Alexander Hospital* in Kiev (the control group consisted of 153 people) and 55 female patients (research group) affected by the pre-eclampsia (PE) state examined at the Lviv Provincial Clinical Hospital (the control group consisted of 116 people). Patients with coronary heart disease (56 men and 15 women) ranged in age from 21 to 68 years. Genotyping and the rate determination of badly functioning alleles and genotypes were carried out according to the polymorphic loci: C677T and A1298C of the methylenetetrahydrofolate reductase gene (*MTHFR*), 2756AG of the 5-methyltetrahydrofolate-homocysteine methyltransferase gene (*MTR*), and 66AG of the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase gene (*MTRR*). For

the first time the complex genotypes research was performed according to all polymorphic *loci*, and complex genotypes (C677T/A1298C *MTHFR* and 2756AG/66AG *MTR/MTRR*). The molecular - genetic analysis was carried out by means of the DNA (PCR) polymerase chain reaction on a "Терцик" (ДНК-технологиа, Russia) with the use of oligonucleotide probes and restrictive enzymes (MBI Fermentase, Lithuania).

HCys concentration was determined by means of the immunoenzymatic test (ELISA) with the use of Axis-Shield reagent set (UK) on Stat Fax 2100 analyser (USA). The cysteine concentration in plasma was determined by means of Gaitonde M. K.'s modified method. The methionine determination was carried out by means of McCrthy-Sullivan's method.

The optic density measurement was performed with "Thermo Electron" spectrophotometer (USA) at l=560 and 545m wavelength for cysteine and methionine, respectively, and the concentration was calcutated on the basis of a calibration curve using cysteine hydrochloride and L-methionine (Sigma, USA). For the statistic analysis of the received results the following tests were used: c^2, t-student, and the correlation and regression analysis at the significance level of 5%.

RESULTS AND DISCUSSION

The homocysteine metabolism/homocysteine remethylation into methionine depends on the folic acid and other vitamins, with results in the fact that the examination of not individual but complex genotypes formed by the various alleles variants of all the genes taking part in this biosynthesis is more appropriate. We have carried out, for the first time, the analysis of the complex genotypes of the folic acid and methionine cycles, talking into account some possible combinations of alleles with four polymorphic *loci* of *MTHFR*, *MTR* and *MTRR* genes in the combined genotype: *MTHFR* C677T_A1298C/*MTR* A2756G/*MTRR* A66G.

In the female group with pre-eclampsia states, 25 out of 81 possible combinations of the examined *loci* (in the control group - 29) were found. Statistically among the patients, the carriers of genotypes CT_AA/AA/GG(12,73% and 2,41%, respectively c2=5,78, p<0,01), CC_AC/AA/AG (10,91% and 2,41%, respectively c^2=4,38, P<0,05) and CC_AA/AA/AG (5,45% with the lack of carries in control group II c^2=4,63, P<0,05) dominated. The above mentioned genotypes constituted 29,09% of all the genotypes in the female group with PE state at 4,82% in the control group (c^2=27,6; P<0,001), which justifies the opinion that they might act as the genes responsible for the proneness for the PE states development during pregnancy.

In the control group, the following genotypes: CT_AA/GG/AG, CC_AA/AA/AA, CC_AA/AA/GG, CC_CC/GG/AG, CT_AC/AA/AA were not found contrary to the test group with PE states. However their rare occurrence in the population (1,82%) does not allow to attribute any aggressive effect to them.

In the group of female patients with PE states, the HCys levels fluctuations from 4,48 to 31,97 mM/L, were observed in the control group (normal pregnancy)- 4,96-12,53 mM/L. In the group with the PE states the mean HCys concentration was within 8,93± 1,88 mM/L [in the control group (t=0,026, P<0,05)], which statistically proves the hiperhomocysteinemia occurrence in them. Applying the HCys level standard deviation data in the control group the variations of normal HCys levels in blood plasma among the women with standard pregnancy: 5,74-9,06 mM/L. The interval top limit of the obtained factors is at the bottom level of the HCys border value in a healthy individual. This phenomenon is due to the homocysteinuria decrease during physiological pregnancy.

In order to determine the border interval of HCys compactness, which can indicate the HCys occurrence danger, connected which the PE state, the analysis and cases division in the test and control groups were performed.

Table 1. Cases rate with various Hey concentration in blood plasma among pregnant women (%).

Test groups	N	HCys level						
		>6	>7	>8	>9	>10	>11	>12
Groups with PE state	52	96,15	73,08	61,54	40,38	28,85	25,00	15,38
Control group	31	83,87	51,61	29,03	34,78	22,58	3,33	0
χ2			3,79	3,94	8,21	1,82	0,8	6,57
P			>0,05	<0,05	<0,001	>0,05	>0,05	=0,01

The above results show the significant difference in the division of cases of various HCys concentration: in the test (with PE states) and control (standard pregnancy) groups. Soon after going beyond 7 mM/L level, the percentage of women with PE states exceeded the control group rates (73,08% and 51,61%, $\chi 2=3,94$, $P<0,05$), and with the HCys concentration over 8 mM/L the difference was significant - 61,54% and 29,03% respectively ($\chi 2=8,21$, $P<0,001$).

However the most frequent HCys concentrations in the control group - below 8 mM/L (70,97% pregnant women) were found only among 38,46% of women with PE states. Going beyond level 11 mM/L one was detected among 13 female patients and only in one patient in the control group (25% and 3,3%, respectively $\chi 2=6,57$, $P=0,01$), but the rates exceeding 12 mM/L were noticed only among women with PE states (15,38%, $\chi 2=5,28$, $P<0,05$).

In order to prove statistically the PE state occurrence risk in the dangerous cases of HCys concentrations, the probability ratio was used (LR - the rate determining the disease occurrence chances in a patient). It has turned out that exceeding level 8 mM/L results in the risk increase up to 3,9 times (LR= 3,91, 95%/D; 1,5-10,17; $P<0,05$), and HCys concentration over 11 mM/L indicates that the PE state occurrence risk increases 10 times (LR=10,95%, D:1,24-80,76, $P<0,05$).

The occurrence rate of mutated alleles carriers in MTHFR/MTR/MTRP combined genotypes according to the HCys concentration level in blood plasma. The carriers of 4 mutated alleles attracted our special attention. There were 23,68% of female patients among the carriers with the HCys concentration level over 7 mM/L. However, in the control group, there were no carriers ($\chi 2=4,55$, $P<0,05$).

Similarly, in the cases where the concentration level exceeded 8 mM/L: 25% of female patients were then carriers of 4 mutated alleles with the lack of such cases in the control group ($\chi 2=5,91$, $P<0,01$).

So, the HCys concentration over 8 mM/L among pregnant women should be assumed as the dangers of the HCys occurrence and PE state related to it.

If such HCys concentrations appear with a pregnant woman - the carrier of 4 mutated alleles of the folic cycle that is: MTHFR (677C®T, 1298A®C), MTR (2756A®G) and MTRR (66A®G), then, such a situation should be regarded as the PE state occurrence danger. These women should be under constant medical observation and they should apply preventive and therapeutic procedures in case of the occurrence of the clinical and laboratory symptoms of the PE state. The HCys concentration over 11 mM/L in a pregnant women shows the high risk of the PE state occurrence, and requires immediate treatment.

The above mentioned studies revealed the structure differences of the following combined genotypes: MTHFR/MTR/MTRR. In the control group, there were no CC_AC/AG/GG and CT_AC/AG/GG genotype carriers, but in the control group, there were 13% of them (7,84% and 5,23% accordingly). The division difference of the above-mentioned genotypes proved to be statistically significant ($\chi 2=8,24$, $P<0,01$), which allows to assume that they act as a protective factor against the tendency to the IHD occurrence. On the other hand among the test group members, some genotype carriers were never found or found in less quantity in the representative control group: CT_AA/AA/GG (10,53% compared with 1,31% in the control group, $y2=9,63$, $P<0,01$), CC_AA/AA/GG (7,02% with no carriers in the control group, $\chi 2=10,95$, $P<0,01$), CT/AC/AA/GG (7,02% and 1,31 respectively, $\chi 2=4,88$, and OC_AC/GG/GG and CC_AA/AA/GG (after 3,51% with the complete lack of them in the control group, $\chi 2=5,42$, $P<0,05$). The LR calculation showed that the carrier state of the genotypes mentioned above increases the IHD occurrence risk at least 5 times, especially: CT_AA/AA/GG - 8,9 times (LR=8,88,95% ID; 1,74-75,4, $P<0,01$), CC_AA/AA/GG - 25,8 times (LR25,82 ,95% ID; 1,37-487,61, $P<0,001$), CT_AC/AA/GG - 5,7 times (LR-5,7, 95% ID; 1,1-24,68, $P<0,05$).

Thus, the received data confirm the usefulness of the genetic examination of the folic and methionine genes polymorphism of the cycles (MTHFR 677C→T, MTHFR 1298A→C, MTR 2756A→G, MTRR 66A→G) to the IHD occurrence. It must be pointed out that CT-AA/AA/GG proved to be common and the most widespread genotype both in reference to IHD and PE states (10,53% and 12,73% of patients respectively) opposite to other proneness genes with which the selective connection with only one disease was ascertained. We rather think that the sex difference of the examined groups (PE states and IHD) accounts for the above phenomenon: in the group affected by IHD, there were three-times fewer women than men.

The HCys concentration variations among the patients affected by IHD are as follows: 3,41-20,12mM/L, in the control group - 4,13-17,52 mM/L. The mean HCys concentration rates among IHD patients and in the control group does not differ significantly and is 56±8,76 ±1,90 mM/L respectively (Table 2). While the patients with IHD there were much more cases of the increased HCys concentration – 11 mM/L: 30,91% and 10,71% ($\chi 2$= 4,14, P<0,05), and with every fifth patients this factor went beyond 13 mM/L (18,18% with the complete lack of it concentration in the control group, $\chi 2y2$= 5,79, P<0,05). The moderate HCys (within 15-30 mM/L) occurred only with 10,91% of patients and with 3,57% of the control group patients (Table 2).

Table 2. Sulfuric amino acids concentrations in blood plasma among the people with IHD symptoms in the control group.

Group	N	Homocysteine mM/L	Methionine mM/L	Aysteine mM/L
Control	28	8,76±1,90	17,74±2,78	270,93±38,28
IHD	55	9,56±2,54	19,89±3,95	239,35±47,38
	t	0,30	0,07	0,034
	p	>0,05	>0,05	<0,05

The above data differ significantly from the results of the similar examinations of IHD patients [2], which exceed them (17,00 ± 0,7 and 9,56 ± 2,54 mM/L respectively). The difference of the patients mean age (46,9 ±7,5 compared with 58,9 ± 0,8 years) [2], and the non-homogeneity (considering sex) of the IHD patients of young age where male patients outnumber females a lot (3,7 : 1), count for this phenomenon. It is well known that the HCys concentration in blood plasma increased with age, but it happens earlier among male patients, which might have had some influence on the results divergence.

The IHD patients division results according to the HCys concentration in blood plasma turned out contradictory. Our researches prove that the most representative group was the patient group with the rates lower than 10 mM/L (67,27%), while according to data [2] there were only 175 of such cases, though the moderate hyperhomocysteinemia was found among 55% of the patients (HCys with in 15-30 mM/L) among our patients there were 10,91% of such cases. The groups of normally high HCys concentration proved to be practically comparable (10-15 mM/L): 22% and 28% respectively. The rates analyses in the control group revealed the data comparability among the patients of normal HCys concentration (71% and 79% in accordance with data [2]), though in our researches in the group of IHD patients, there were twice as many normally high HCys concentration rates (25% and 10,5% respectively) and three times fewer cases of moderate HCys concentration (3,5% and 10,5% respectively) than those presented in paper [2].

A little increased methionine concentration among IHD patients was not confirmed at 95% of credibility (Table2), and the dependence between the HCys concentration and methionine did not have the linear correlation character: the highest significance of the correlation rate (factor) occurred with low credibility at 95% of probability (P=0,18, P=0,,098, 0,05<P0,10).

The HCys concentration increase is the first result of increased methionine and the lack of their rates linear correlation can be explained by the weak impact of hipermethioninemia of alimentary origin on the hiperhomocysteinemia development among the people of both test and control groups.

The important differences among the IHD patients and control group patients were revealed during the cysteine concentration rates comparison.

The reliable cysteine concentration decrease among the IHD patients blood plasma was found when compared to the control group rates: 239,35 ± 47,38 and 270,93 ± 38,29 mM/L (P<0,05) Table 2). The probable mean rates difference of the IHD patients cysteine levels were observed along with the increased low rates cases- below 216 mM/L (41,8% to 3,60% in the control group, P<0,01).

Contrary to our data, the results [3] show credible HCys among IHD patients: 342,2 ± 5,73 to 285,0 ± 4,3 mM/L in the control group. The cysteine compactness rates of the control group in our test and test [3] do not differ, and the division according to the cysteine concentration in blood plasma proved alike , too: 78,57% and 78% (<300 mM/L) 14,29% and 12% (300-350 mM/L), 7,14% and 10%, (>350 mM/L) respectively. However, the IHD patients rates structure proved different: 81,82% and 28% (<300 mM/L) 12,7% and 32% (300-350 umol/l), 7,27%-40% (>350 mM/L).

The revealed differences results might be the different measurement methods of cysteine concentration in blood plasma, though the control group data do not approve this. Another possible reason might be the patients age difference. Our attention was focused on examining the young people's IHD cases, which is justified by the

significant age difference among the patients in our test and test [3].

It is not unlikely that the early clinical IHD manifestations have a different printing structure of genetic proneness, and respectively some different pathogenic pathways of metabolism violation.

While examining the control group we ascertained the high linear correlation rate between the HCys and cysteine concentration levels (P=0,74, P<0,020 indicating a standard process course of homocysteine transsulfuration in order to normalize its concentration in blood plasma. We did not observe such a phenomenon in the group of IHD patients: the dependence between the homocysteine and cysteine rates does not have a linear character (p=0,17, P>0,74). Besides, a negative linear correlation between methionine and cysteine (p= 0,33, P<0,02) was found among these patients, which was not revealed in the control group.

The above results account for the multidirectional changes of sulfuric amino acid metabolism among IHD patients. Such changes result in disturbing the transsulfuration and HCys methylation processes. It is well known that the moderate hyperhomocysteinemia which was observed among patients is a usual phenomenon to human multifactorial diseases and can result in serious pathogenic results [1, 2, 3, 14, 18, 19]. Even short-term hyperhomocysteinemia during the methionine load test causes oxidation stress [23].

80% of HCys is connected with proteins, and the remain 20% appears mainly in the shape of homocysteine or homocysteine disulfide with other thiols. The above fact makes it impossible to determine HCys objectively by means of ELISA test basing on S-adenosyl-L- homocysteine identification [19]. This test allows to determine only free HCys, but not the HCys concentrations connected with proteins, thus the total HCys content was not given our attention to.

It is well known that the HCys methylation process inhibition leads to its metabolite formation - homocysteine thiolactone (HTL) which results in haemostatic protein modifications (N- and S-homocysteinilation [17]). It has been found out that the most reactive homocysteine form is thiolactone. This may be the main cause of their biotoxination: haemostatic disorders, circulatory system diseases, and some phenomena taking part in the inflammatory process, HCys can induce and intensify this process [4, 9, 17, 20, 24]. HTL can undergo non-enzymatic hydrolysis, though most frequently it is removed by the homocysteine thiolactone hydrolase which also goes by the name of homocysteine thiolactose (HTLaza) or paraoxonase (PON1). It is worth mentioning that HCys stars the oxidation process of the lipoprotein fraction of low density [LDL], which then increases their atherogenesis [4, 17]. The atherogenic homocysteine can reduce the HCys content level in blood plasma to the moderate level, thus masking hyperhomocysteinemia. Almost all the clinic tests confirm the strong connection between the increased homocysteine level and the cardiovascular system diseases.

Some hyperhomocysteinemia cases can explain some genetically conditioned disorders of the remethylation process. The homocysteine remethylation is a reversible process, which intensifies in methionine deficiency states. This reaction takes place with the help of the methionine synthesis (MS) whose cofactor is vitamin B12 (methylcobalamine). The methylene group donor is 5 methyltetrahydrolofolate (folic acid derivative), originating during the reaction catalyzed by 5, 10-methylenote-tetrahydrofololate reductase. The above enzyme indirectly, but significantly influences the remethylation process. Most frequently, the genetically conditioned reason of moderate hiperhomocysteinemia is MTHER coding gene polymorphism. It is the point mutation consisting in changing cytosine into thymine at 677 (C677T position [14]. The *MTR* and *MTRR* importance (and other genes) in the Heys methylation process is not precisely known, but our and other scientists researches allow to maintain that they are the most credible factors of the cardiovascular system diseases [1, 2, 3, 6, 7, 10, 12, 15, 16, 22].

The franssulfuration process affects the HCys concentration normalization in blood plasma. On the pathway homocysteine joins serine during the reaction catalyzed by the cystationine ß-synthesis (CBS). Then, the received cystationine breaks down due to y-cystationase (CTS). The test group patients did not have any hiperhomocysteinemia symptoms, though they can be the heterozygotic carriers of CBS/CSE genes mutations. Such a possibility was proved with knockout type animals and in the clinical researches of moderate hiperhomocysteinemia cases [9, 18]. The occurrence rate of the heterozygotic carrier state of CBS and CSE mutations in the populations of Norway and the Czech Republic is 1: 6,400 and 1: 15,500 respectively.

The HCys transsulfuration process sluggishness not only maintains its high content in blood (hiperhomocysteinemia) but also causes

biosynthesis disorders, first of all - cysteine production. A cysteine particle contains the (-SH) thiol due to which it is capable to form sulfide bonds - one of the factors affecting the tertiary protein structure, and it enters into the composition of glutathione tetrapeptide (L-gamma-g*lutamyl*-L-*cysteinyl-glycine)*.

Both CBS and CSE can synthesize hydrogen sulfide at the sufficient cysteine concentration. The hydrogen sulfide loosens blood vessels, and affects cellular signals transmission.

It either stimulates or inhibits the ERK kinase regulating a signal transmission (extracellular signal-regulated kinases), stimulates ATP- dependent potassium canals and increases the sensitivity of N-methyl-D-aspartate receptors (NMDA) which stimulate capsaicin-sensitive nervous transmitters [9, 11, 25, 28]. At present the clinical examinations of hydrogen sulfide are held during the IHD patients treatment after the aortic-coronary bridging (Clinical Trials. Gov) [11]. It has been ascertained that the hiperhomocysteinemia development proceeds along with the hydrogen sulfide level decrease in blood plasma and along with the activity of liver enzymes, including CBS and g-*cystationase* [CGL] producing it.

Connecting hiperhomocysteinemia with the endogenic hydrogen sulfide production decrease might be the genetically determined disturbance result of the transsulfuration process for example, with the heterozygotic CBS and CBL genes mutation carrier state.

However, the possibility of the homocysteine thiolactone epigenetic impact on the after translation modification course of the above mentioned enzymes should not be ruled out. This can result in the acquired decreased individual enzymes activity. The obtained results show the heterogeneous of the genetic proneness for vascular disorders already at the methionine metabolism levels - the hiperhomocysteinemia transsulfuration and methylation processes disturbance, the intensive homocysteine thiolactone synthesis and decreased endogenic hydrogen sulfur. Such a situation induces new methods and diagnostic markers elaboration and initiation and prognostic factors specification Applying the molecular-genetic researches, beyond a doubt, is necessary to determine the genotypes of the folic and methionine polymorphism of the cycles (*MTHFR, MTR, MTRP*) and the activity of the enzymes taking part in the transsulfuration processes (CBS and CSE). One can use the data referring to the credible genotypes of the proneness for IHD or resistance to IHD. Considering the significance of the inherited methionine metabolism defects, it is worth analyzing the possibility of diagnosing the methionine adenosyltranpherase I/III deficiency, glycine N- methyltransferase (MAT I-III), *S-adenosylhomocysteine hydrolase,* cobalamin metabolism enzymes, and methyltetrahydrofolate metabolism disorders (methyl-THF).

Taking into consideration the results, not identical in meaning, of the polymorphic changes of some phenotype realizations, the genetic examination data should be confirmed by biochemical tests. The selective analysis of the HCys concentration in blood plasma fails because even a slight increase of its concentration level can cause significant results. This results in the necessity of broadening the biochemical hiperhomocysteinemia markers spectrum including determining folates and metabolically bound sulfuric amino acids (methionine and particularly cysteine) levels, carrying out methionine load tests, and determining the homocysteine thiolacton and hydrogen sulfide concentration.

CONCLUSIONS

1. Among 81 possible complex MTHFRC677T_A1298S/MTR A2756G/MTRR A66G genotypes it has been ascertained that there are 6 types whose carrier state increases the circulatory system disease risk five times: PE state (CT_AA/AA/GG, CC_AC/AA/AG, CC_AA/AA/AG) and IHD (CT_AA/AA/GG, CC_AA/AA/GG, CT_AC/AA/GG).

2. The credible sulfuric amino acids profiles changes in the blood plasma of IHD and PE state patients indicate some significant discords of HCys transsulfuration and remethylation processes in the vascular disturbance pathogenesis typical for this disease.

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