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ASSOCIATION OF GENE POLYMORPHYSMS OF FOLATE METABOLISM WITH SPERM ANEUPLOIDY IN MEN WITH LOW REPRODUCTIVE FUNCTION

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Aim. *The association of polymorphic variants of genes MTHFR (C677T, A1298C) and MTRR (A66G) with sperm aneuploidy in men with low reproductive function was investigated.* ***Methods.*** *SNPs determinations were performed by the real-time PCR technique. Sperm DNA fragmentation analysis was performed using the method of sperm chromatin dispersion. To detect aneuploidy in spermatozoa nuclei the method of fluorescent in situ hybridization (FISH) was used.* ***Results.*** *Polymorphic alleles in genes of folate metabolism are associated with sperm aneuploidy in men with low reproductive function. The link between the number of alternative alleles of polymorphic variants A1298C of MTHFR gene in genotype and the average level of aneuploidy in sperm chromosome 16 is proved.* ***Conclusion.*** *Aneuploid sperm is able to fertilize the oocytes, but the further formation of the blastocyst and embryo implantation may be blocked at various stages of development. Understanding the genetic basis of aneuploidy in sperm of men could reduce the reproductive losses in IVF practice.*

Keywords: *DNA fragmentation, sperm aneuploidy, MTHFR, MTRR, reproductive function.*

Introduction. In developed countries, 10–15 % of married couples encounter infertility. Up to 50 % of all infertility occurs in the male [1, 2]. In 32 % of cases the idiopathic infertility in men is caused by genetic factors [3, 4].

One of the possible reasons for the decline in fertility of men, according to some authors, are failures of compaction of chromatin in the nucleus of the sperm or DNA fragmentation [5–7]. Spermatogenesis failures at different stages can lead not only to a violation of the DNA integrity, but also to the chromosome abnormalities in sperm nuclei. According to the literature, aneuploidies of chromosomes 15, 18, 19, 21, X and Y are extensively studied in sperm. Aneuploidies of chromosomes 18, X and Y are the most frequently observed in male gametes [8–13]. Detection of the high content of aneuploidies in sperm is an indication for preimplantation genetic diagnosis (PGD) to select euploid embryos [14, 15].

It is known that polymorphic variants of individual genes controlling stages of spermatogenesis and metabolism of the organism might be associated with motility, morphology and fertility properties of sperm that are manifested in a range from mild abnormalities in sperm to complete absence of germ cells in the somniferous tubules (syndrome «only Sertoli cells»). A decrease in the functional activity of enzymes of folate metabolism is discussed as one of the possible reasons of the formation of the mature male gametes [16–18].

Folic acid deficiency and hypomethylation of DNA are associated with a high frequency of polymorphic alleles of the genes *MTHFR* and *MTRR* and may be the cause of nondisjunction. This is confirmed by the data presented in the literature about the association of the homozygous genotypes polymorphic alleles of genes of folate metabolism with increased risk of carcinogenesis (colorectal adenocarcinoma, breast cancer and ovarian cancer); cardiovascular disease (ischemic heart disease – coronary heart disease, myocardial infarction, atherosclerosis, atherothrombosis); pregnancy complications (placental insufficiency, premature detachment of normally situated placenta, late gestosis); fetal malformations

(cleft neural tube, anencephaly, deformities of the facial skeleton), children born with a chromosomal abnormality [19–24].

The aim of the present study was to analyze the relationship of polymorphic variants in genes of folate metabolism – C677T (Ala222Val), A1298C (Glu429Ala) of *MTHFR* gene and A66G (Ile22Met) of *MTRR* gene with aneuploidy of sperm in men with low reproductive function.

Materials and methods

Between June 2012 and November 2015, 245 couples with the male factor of infertility underwent the consultation of reproductologist in the Center for Human Reproduction «Clinic professor of Feskov A.M.» (Kharkiv, Ukraine). Basic sperm parameters (concentration, motility and morphology) showed a high variability among individual patients, ranging between a slight deviation from normal sperm parameters and severe oligoasthenoteratozoospermia and azoospermia. 39 patients with azoospermia were excluded from the studied group. The rest 206 men were undergone karyotype examination. The abnormal karyotype was found out among 13 patients (45,XY-,rob(13;21)(q10;q10) – 1 patient; 47,XXY46,XY – 4 patients; 47,XXY – 2 patients; 46,XY/45X – 2 patients; 46,XY-,inv(13)(p13q21) – 1 patient; 46,XY/46,XX – 1 patient; 45,XY,der(13;14)(p10;p10) – 2 patients). Only men with normal karyotype 46,XY were taken into the experimental group.

Collection of primary information and laboratory tests were carried out at the Center for Human Reproduction «Clinic professor of Feskov A.M.» (Kharkiv, Ukraine). All participants gave their informed consents to participate in the study, which was approved by Institutional Ethics Committee. As a result the samples of peripheral blood of 136 men with low reproductive function with mean age 37.3±5.9 years old were used for the molecular genetic study. The patients were divided into 2 groups. Group I consisted of 112 men with the mean age 37.0±6.1 years old. A level of DNA fragmentation in sperm in the ejaculate was determined for patients in Group I. Group II consisted of 24 men with the mean age 38.6±5.1 years old. The level of aneuploid sperm in the ejaculate was determined in Group II.

Using standard techniques DNA extraction was performed with extraction kits (Macherey- Nagel, NucleoSpin® Blood, Germany) [25]. Real-time PCR was done with the ABI PRISM 7500 real-time PCR system (USA), and SNPs determinations in the *MTHFR* and *MTRR* genes were performed with Applied BioSystem kits (USA). The literature data with the results of genetic testing of Ukrainian population were taken as a control group [26–28]. The examination of karyotype was done for the patients of both experimental groups. Chromosomes samples for cytogenetic analyses were obtained from peripheral blood lymphocytes via Giemsa banding (G-banding, GTG), and the results were presented following the International System of Human Cytogenetic Nomenclature [29].

Sperm DNA fragmentation analysis was performed using the method of sperm chromatin dispersion (SCD, Halotech, HaloSperm, Spain), and the data were collected and analyzed with the software Lucia FISH (LIM, Czech Republic).

To detect aneuploidy in spermatozoa nuclei on chromosomes 13, 16, 18, 21, X, Y the method of fluorescent *in situ* hybridization in (FISH) was used. The following DNA probes were used: CEP Y (DYZ3) Satellite DNA SpectrumOrange, CEP X (DXZ1) Alpha Satellite DNA SpectrumGreen, LSI 13 (13q14) SpectrumGreen, CEP 16 (D16Z3) Satellite II DNA SpectrumGreen, CEP 18 (D18Z1) Alpha Satellite DNA SpectrumAqua, LSI 21 (loci D21S259, D21S341, D21S342, region 21q22.13-q22.2) SpectrumOrange (Vysis-Abbott, USA).

Since distribution of frequencies in data sets for alternative alleles did not follow a Gaussian distribution, non-parametric methods were utilized for statistical analyses. Frequency differences in genotypes were compared by Fischer's test. Relationships between variables were examined by correlation analyses. Verifications of associations of alleles/genotypes and of equality of distributions were accomplished by the Chi-square test (χ^2 test) at significance levels of P=0.05, P=0.01 and P=0.001. To compare the quantitative characteristics of the different groups the Mann-Whitney test was used [30].

Results and discussion

The level of DNA fragmentation for men in Group I ranged from 1.5 % to 75.0 %. According to the recommendations of the European Association of Urology, the content of sperm with fragmented DNA in the ejaculate normally should not exceed 20.0 % [31]. The level of sperm aneuploidy for patients in Group II ranges from 0.2 % to 4.3 %. Normally, the content of aneuploid sperm in the ejaculate should not exceed 1.3 % [32].

The frequency of alleles and genotypes of studied polymorphisms for *MTHFR* and MTRR genes are calculated for the men of both groups. For studied SNP C677T of *MTHFR* gene the frequencies of alleles for patients in group I and in group II were as follows: P_C=0.71; Q_T=0.29.

These frequencies are similar to the frequencies p_C=0.721, q_T=0.279 obtained for the population of Kharkiv region and given earlier by the authors [26]. The frequencies of alleles of polymorphic variants of *MTHFR* gene A1298C accounted for the total group of men were P_A=0.91 and Q_C=0.09; for Group I – p_A=0.95 and q_C=0.05; for Group II – p_A=0.71 and q_C=0.29, respectively. We identified allele frequencies comparable with data for the Ukrainian population reported by other authors – P_A=0.65 and q_c=0.35, respectively [28]

As for studied SNP A66G for *MTRR* gene the allele frequencies were as follows: P_A = 0.72 q_G=0.28 for total experimental goup, for Group I – P_A=0.79 and q_G=0.21, and for Group II – P_A=0.42 and q_G=0.58 respectively. These allele frequencies are similar to those obtained by other authors:– p_A=0.44, q_G=0.56 [28].

The alleles' frequencies of analyzed genes do not differ significantly for men with elevated levels of DNA fragmentation of sperm (Group I) and increased levels of aneuploidy (Group II). The frequencies of genotypes for the polymorphic variant C677T in *MTHFR* gene in total experimental group were 52.2 %, 36.8 % and 11.0 %. These data are comparable with those ones given for the Ukrainian population previously by us and by other authors. The genotype frequencies of polymorphic variants of the C677T in *MTHFR* gene were 54.0 %, 41.0 % and 5.0 %; 54.7 %, 34.7 % and 10.5 %; and 50.0 %, 42.0 %, 8.0 % respectively [26–28]. There was no statistically significant difference between the frequencies of the genotypes for patients of each group and the data for the Ukrainian population [26]. The obtained genotype frequencies of polymorphic variants A1298C of *MTHFR* gene in patients with impaired sperm DNA fragmentation (Group I) are significantly different from the frequency distribution among the Ukrainian population: 89.3 %, 10.7 % and 0.0 % vs. 43.0 %, 45.0 % and 12.0 % (df=2, χ^2_{actual} =49.33, $\chi^2_{critical}$ =9.21, p<0.01) [28]. The number of wild type homozygous alleles in this group of patients was twofold higher comparing with the control group. The number of patients with the heterozygous genotype in Group I was fourfold lower comparing with the control data.

Analysis of the genotype frequencies of polymorphic variants A66G in *MTRR* gene showed that for patients with high level of DNA fragmentation in sperm (Group I) also was established significant differences in frequencies of genotypes of the data for Ukrainian population: 68.8 %, 19.6 %, 11, 6 % vs. 26.0 %, 37.0 % and 37.0 % (df=2, χ^2_{actual} =37.95, $\chi^2_{critical}$ =9.21, p<0.01). The number of wild type homozygous alleles in this group of patients was twofold higher comparing with the control group. The number of patients with the heterozygous genotype in Group I was twofold lower comparing with the control data [28]. At the same time, there was no statistically significant difference between the frequencies of genotypes of polymorphic variants A1298C of *MTHFR* gene and A66G of *MTRR* one in patients with high content of aneuploid spermatozoa in ejaculate (Group II) and for men in total experimental group.

A statistically significant difference is found out for genotype frequencies of polymorphic variant A1298C of MTHFR gene (df=2, χ^2_{actual} =24.74, $\chi^2_{critical}$ =9.21, p<0.01) and A66G of MTRR one (df=2, χ^2_{actual} =22.33, $\chi^2_{critical}$ =9.21, p<0.01) between Groups I and II. In patients with a high content of aneuploid sperm in the ejaculate (Group II) genotype frequencies of polymorphic alleles AC and CC are 41.7 % and 8.3 %; AG and GG – 50.0 % and 33.3 %, whereas the same data for patients with high level of DNA fragmentation of sperm (Group I) were – 10.7 % and 0.0 %, 19.6 % and 11.1 % (in 2-4-fold less, respectively). It is known that the presence of genotypes that lead to violations of folate metabolism, disturbed the normal chromosome segregation in gametogenesis. Thus, according to C.A. Hobbs (2000), a polymorphic T allele of *MTHFR* gene is two times more common in mothers of children with Down syndrome than among women in the control group. When the mother is a carrier of the genotype G/G (*MTRR*) then having a child with Down syndrome increases 2.5 times. When a combination of 2 genotypes aforementioned risk of fetal Down syndrome increases four times [19].

Probably, the same effect occurs during spermatogenesis, whereby in men with high level of aneuploid sperm in the ejaculate genotype frequencies polymorphic alleles folate metabolism are 2-4 times higher than in men with impaired DNA fragmentation. We have analyzed the relation between the level of DNA fragmentation of sperm with the number of polymorphic alleles of the genes studied in men Groups I, and did not find significant correlation (rs=0.108, rcritical=0.2, p>0.05). The failures of DNA fragmentation of sperm is probably more related to polymorphic variants of other genes that control the different stages of spermatogenesis, such as the gene for FSH receptor [33].

The correlation between the level of aneuploidies in sperm and the patients' age has been studied for men in Group II. A statistically significant correlation between the age of patients and the level of aneuploidy in sperm hasn't been identified. However, separately the group of men under the age of 35 years (n=10) was considered, as our previous studies have shown the effect of age on the

Table 1. The level of aneuploidy in sperm nuclei for patients with different genotypes of polymorphic variants C677T and A1298C of *MTHFR* gene

Genotypes		CTAC*	CTAA*	CCAA**	CCAC*	TTAA**	CCCC**
Number of patients		5	5	5	5	2	2
Sperm aneuploidy, %	13	0.53±0.16	0.60±0.25	1.02±0.61	0.66±0.17	1.06±0.22	0.67±0.33
	16	0.52±0.13	0.63±0.41	1.17±0.95	0.31±0.13	0.81±0.60	0.72±0.04
	18	0.62±0.34	0.64±0.26	1.91±0.99	1.16±0.37	0.45±0.25	0.79±0.11
	21	0.96±0.33	0.73±0.24	2.46±1.55	0.64±0.41	0.98±0.34	1.40±0.40
	X, Y	1.05±0.36	1.21±0.29	1.92±1.15	1.30±0.24	1.62±0.79	1.27±0.13
Statistics	*, ** $U_{empiric}$=0, $U_{critical}$=0, p≤0,05						

level of aneuploidy in sperm nuclei in men older than 35 years.

For patients with increased levels of aneuploidy in sperm (Group II), the analysis of the relationship between the number of polymorphic alleles of genes MTHFR and MTRR and the level of aneuploid sperm in the ejaculate was carried out. In homozygotes CCAA, TTAA, CCCC for single nucleotide polymorphisms C677T and A1298C in MTHFR gene the level of aneuploidy in sperm for chromosome 16 was significantly higher than in heterozygotes CTAC, CTAA, CCAC ($U_{empiric}$=0, $U_{critical}$=0, p≤0,05) (Table 1). For heterozygotes A66G in gene *MTRR* the average level of aneuploidy in sperm is significantly lower than homozygotes A66A ($U_{empiric}$=1.0 $U_{critical}$=4.0 p<0.05) for all analyzed chromosomes (Table 2).

Protective heterozygous genotypes for the genes of folate metabolism represented in the literature for certain pathologies, including the violation in cell division. Thus, according to M.R. Lozinskaya, a higher frequency of heterozygotes C677T in patients with colorectal cancer of women compared to men is predictive sign on the incidence of colon cancer – among home patients with colon cancer female heterozygotes CT – 60.0 %, men – 32.4 %. This provides better survival rate and lower mortality in female patients versus male patients [34–36].

The protective effects in oncological processes in heterozygotes were shown for psoriasis [37]. Studies have shown higher proportion of heterozygous C677T in psoriasis patients comparing with distribution of genotypes in Ukrainian population: 50.6 % versus 33.7 %. Probably hyperhomocysteinemia in heterozygotes C677T and in diheterozygotes C677T / A1298C [38], including in patients with psoriasis can be one of protective effects in oncological processes for patients [38–41].

According to E. Strauss et al., heterozygotes CTAC are less likely to develop left ventricular systolic dysfunction (LVSI), that although the contribution of known polymorphic alleles of *MTHFR* gene in the development of cardiovascular disease. The authors conclude that this protective genotype may be an example of molecular heterosis [42].

At the same time it is known that carriage of allele T at position 677 and C allele at position 1298 of the *MTHFR* gene in the homozygous state is shown as an essential component of the high risk of vascular and reproductive disorders, cancer and precancerous states, i.e. violations connected with the violation of cell division and chromosome segregation [32, 43–45]. For example, it was found that the presence of allele 677T and 66G in the homozygous state increases the risk of recurrent miscarriage in 7 times [43]. These data are consistent with our results.

We found a positive correlation between the number of polymorphic alleles of single nucleotide polymorphism A1298C of the *MTHFR* gene and the level of aneuploidy for chromosome 16 in patients younger then 35 years old (r_s=0.86±0.21, $R_{critical}$=0.72, p<0,05). The highest level of aneuploidy is observed in homozygous polymorphic alleles CC. A statistically significant correlation between the level of aneuploidy of chromosome 16 for the patients with impaired level of aneuploidy in total group and the number of polymorphic alleles of single nucleotide polymorphism A1298C of the *MTHFR* gene was not found.

The link of the presence of the polymorphic variants of the genes and reduce the functional activity of enzymes of folate metabolism with the aneuploidy of chromo-

Table 2. The level of aneuploidy in sperm nuclei for patients with different genotypes of polymorphic variant A66G in *MTRR* gene

Genotypes		AA*	AG**
Number of patients		4	12
Sperm aneuploidy, %	13	1.01±0.60	0.65±0.25
	16	1.19±1.07	0.44±0.17
	18	1.49±0.71	0.71±0.37
	21	1.78±1.36	0.95±0.40
	X, Y	1.63±0.54	1.16±0.54
Statistics	*, ** $U_{empiric}$=1.0 $U_{critical}$=4.0, P<0.05		

some 16 is probably due to the structural features of this chromosome. Chromosome 16 is a metacentric chromosome with a secondary constriction in the long arm with the large block of heterochromatin near the centromere. It is known that the violations in the processes of DNA methylation in centromeric areas may violate the divergence of homologous chromosomes. According to T.S. Beskorovainaya et al., decreased activity of the processes of methylation is associated with folate metabolism enzyme insufficiency or deficiency of methyl groups, which are changing the methylation of centromeric regions of chromosomes and disruption of chromosome segregation in oogenesis [43]. The literature provides data on the association of changes in the DNA methylation with the violation of chromosomes 18, 21 [32, 43, 46, 47]. Probably violation of folate cycle in men can also cause a violation of chromosome segregation during the process of spermatogenesis.

Since the frequency of alleles and genotypes of folate metabolism genes in different populations and ethnic groups varies, and for certain pathologies the reducing the activity of folate metabolism is shown affecting chromosome segregation during cell division, successfully adjusted dietary folate [48–50], we should expect ambiguity presented by various authors as the data on the effect of different genotypes of folate metabolism genes in the development of certain pathologies, and the contribution of chromosome aneuploidies in 16 stages of human development [51, 52]. Thus, according to F. Vogel, trisomy 16 are incompatible with the early stages of embryonic development, and fully lethal [53]. At the same time, according to Petracchi F., the most common trisomy on autosomes, diagnosed in studying karyotype in the case of non-viable pregnancy in humans is trisomy of chromosome 16 (more than one per cent of cases pregnancy fading) [54]. The consequence of this is trisomy spontaneous miscarriage in the first trimester of pregnancy. According Yong P.J. an extra copy of chromosome 16 has a maternal origin, and the main reason for such violations is considered to be the woman's age [55]. As for monosomy of chromosome 16 in embryos, according to the literature data [56] autosomal monosomy is the result of errors in mitosis after the stage of zygote. According to Artukhova V.G, autosomal monosomies have a somatic origin. Determination of autosomal monosomy in embryos is possible only during the preimplantation genetic diagnosis (PGD) [56].

The link between the level of sperm aneuploidy for other chromosomes and the number of polymorphic alleles for the examined men hasn't been identified. The correlation coefficients between the level of aneuploidy in sperm and the number of alternative alleles for studied polymorphic variants of *MTHFR* and *MTRR* genes range from -0.22 to 0.274 ($p>0.05$).

Conclusion

Studies have shown that polymorphic alleles in genes of folate metabolism are associated with sperm aneuploidy in men with low reproductive function. The link between the number of alternative alleles of polymorphic variants A1298C of *MTHFR* gene in genotype for patients younger than 35 years and the average level of aneuploidy in sperm chromosome 16 is proved. It was found that the level of the sperm aneuploidy for chromosome 16 is higher for homozygotes CCAA, TTAA, CCCC for single nucleotide polymorphisms C677T and A1298C *MTHFR* gene comparing with heterozygotes CTAC, CTAA, CACC. As for the polymorphism A66G of the of *MTRR* gene the average level of aneuploidy in sperm nuclei is lower at heterozygous comparing with homozygotes A66A for all analyzed chromosomes. It is known that aneuploid sperm is able to fertilize the oocytes, but the further formation of the blastocyst and embryo implantation may be blocked at various stages of development. Understanding the genetic basis of aneuploidy in sperm of men will lower the reproductive losses during *in vitro* fertilization programs [57, 58].

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АСОЦІАЦІЯ ПОЛІМОРФНИХ ВАРІАНТІВ ГЕНІВ ФОЛАТНОГО ОБМІНУ З АНЕУПЛОЇДІЄЮ У СПЕРМІ У ЧОЛОВІКІВ ЗІ ЗНИЖЕНОЮ РЕПРОДУКТИВНОЮ ФУНКЦІЄЮ

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Мета. Дослідити асоціацію поліморфних варіантів генів *MTHFR* (C677T, A1298C) та *MTRR* (A66G) з якісними показниками сперми у чоловіків з порушеннями репродуктивної функції. **Методи.** Визначення однонуклеотидних генетичних поліморфізмів SNPs проведено методом ПЛР у реальному часі. Рівень фрагментації ДНК у спермі виявлено за допомогою методу хроматинної дисперсії. Анеуплоїдію в спермі досліджено методом флуоресцентної гібридизації *in situ* (FISH). **Результати.** Поліморфні алелі генів фолатного обміну асоційовані з анеуплоїдією в спермі у чоловіків із порушеною репродуктивною функцією. Доведено зв'язок між кількістю поліморфних алелів A1298C гену *MTHFR* у генотипі та рівнем анеуплоїдії хромосоми 16 в спермі. **Висновки.** Анеуплоїдна сперма здатна до запліднення ооцитів, але подальше формування бластоцист та імплантація ембріонів можуть бути блоковані на різних стадіях розвитку. Розуміння генетичних причин анеуплоїдії сперми дозволить зменшити репродуктивні втрати у практиці ЕКЗ.

Ключові слова: фрагментація ДНК, анеуплоїдія сперми, *MTHFR*, *MTRR*, репродуктивна функція.

АССОЦИАЦИЯ ПЛИМОРФНЫХ ВАРИАНТОВ ГЕНОВ ФОЛАТНОГО ОБМЕНА С АНЕУПЛОИДИЕЙ В СПЕРМЕ У МУЖЧИН СО СНИЖЕННОЙ РЕПРОДУКТИВНОЙ ФУНКЦИЕЙ

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Цель. Исследовать ассоциацию полиморфных вариантов генов *MTHFR* (C677T, A1298C) и *MTRR* (A66G) с качественными показателями спермы у мужчин со сниженной репродуктивной функцией. **Методы.** Определение однонуклеотидных генетических полиморфизмов проведено методом ПЦР в реальном времени. Уровень фрагментации ДНК спермы определен методом хроматинной дисперсии. Анеуплоидия в сперме исследована методом флуоресцентной гибридизации *in situ* (FISH). **Результаты.** Полиморфные аллели генов фолатного обмена ассоциируются с анеуплоидией в сперме у мужчин с нарушенной репродуктивной функцией. Доказана связь полиморфных аллелей A1298C гена *MTHFR* в генотипе и уровнем анеуплоидии хромосомы 16 в сперме. **Выводы.** Анеуплоидная сперма способна оплодотворять ооциты, но дальнейшее формирование бластоцист и имплантация эмбрионов могут быть блокированы на разных стадиях. Понимание генетических причин анеуплоидии спермы позволит снизить репродуктивные потери в ЭКО.

Ключевые слова: фрагментация ДНК, анеуплоидия спермы, *MTHFR*, *MTRR*, репродуктивная функция.