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ORIGINAL ARTICLE

The effect of single nucleotide genetic polymorphisms of folic acid cycle on the female reproductive system disorders

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Abstract

Currently the significant part of reproductive disorders such as refractory pregnancy loss (RPL), infertility, unsuccessful *in vitro* fertilization (IVF) are thought to be connected with different genetic factors. One of the main hereditary risk factors for obstetrical pathology development is the presence of polymorph alleles in several genes of folic acid cycle. The present study is dedicated to investigation of the effect of folic acid cycle polymorph variants MTHFR C677 T, MTR A2756G and MTRR A66G on the RPL development and unsuccessful IVF. The samples of peripheral blood of 138 women were tested and showed a statistically significant increase of pathologic genetic alleles of MTRR A66G and MTHFR C677T frequency in two groups of patients with reproductive disorders, i.e. RPL and IVF, versus the control group. Also the advantage of simultaneous analysis of three folic cycle genetic polymorphisms at once in women with reproductive function disorder was demonstrated in comparison with the analysis of isolated polymorphism MTHFR C677T. The combination of polymorph alleles has a significant influence on the pathology development and by many times increases the risk of RPL development and unsuccessful IVF.

Keywords

Folic acid cycle, IVF failure, miscarriage, SNP

History

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Introduction

Recently were was repeatedly noted the significant elevation of risk of obstetrical pathologies connected with the increased blood homocysteine level and several folic acid cycle genes pathological alleles presence in the genome [1].

Some authors emphasize the association between definite forms of reproductive pathologies, in particular, RPL and unsuccessful IVF, and polymorph variants of MTHFR, MTRR and MTR genetic alleles [2].

The current study aims to identify the effect of polymorph variants of folic cycle genes MTHFR C677, MTR A2756G and MTRR A66G on the development of refractory pregnancy loss (RPL) and unsuccessful in vitro fertilization (IVF).

The study objectives were as follows:

(1) To identify the frequencies of alleles and genotypes of folic acid cycle MTR A2756G, MTRR A66G and MTHFR C677T in healthy women and women with reproductive disorders (RPL and unsuccessful IVF).
(2) To combine the frequencies of alleles and genotypes of folic acid cycle MTR A2756G, MTRR A66G and MTHFR C677T in three groups of women: women with RPL, women with unsuccessful IVF and women of control group with one or more normal pregnancies.
(3) To estimate the mutual effect of polymorph genetic alleles MTR A2756G, MTRR A66G and MTHFR C677T combinations on the RPL and unsuccessful IVF risk.

In order to fulfill the objectives of the current study the frequencies of alleles and genotypes of three folic cycle genes polymorphisms were identified for all patients: MTHFR 677C > T, MTR 2756 A > G, MTRR 66 A > G.

Materials and methods

Clinical examination groups

In total 138 women were studied. The analysis material was represented by venous blood samples obtained after median cubital vein puncture and placed to the preservative tubes with anticoagulant EDTA.

The informed consent was obtained from all examined patients. During the study three groups were composed: (1) women with RPL, (2) women with regular unsuccessful IVF, (3) control group.

The first group (RPL) included 37 patients with not less than two spontaneous abortions or non-developing pregnancies in the first trimester in anamnesis.

The second group included 55 women with unsuccessful IVF resulted in non-registration of embryo implantation or miscarriage in the early stages of pregnancy after embryo transfer. Inclusion criteria were the anatomic abnormalities of the reproductive

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system, serious disorder of the endocrine system, acute infectious process.

The control group included 46 fertile women with not less than one healthy child. The examined women were between 22 and 42 years old. For all blood samples the karyotyping was performed which didn't show any chromosomal abnormalities. The frequencies of alleles and genotypes of three folic cycle genes polymorphisms were identified for all patients: MTHFR 677C > T, MTR 2756 A > G, MTRR 66 A > G.

DNA purification was made out of peripheral blood leukocytes using the kit and protocol for DNA extraction ''DNA-express-blood'' of Litekh Research and Production Company, Moscow, Russia, in accordance with the manufacturer's recommendations. Concentration of genomic DNA, obtained during the above-mentioned process, was 20–100 ng.

Polymerase chain reaction (PCR) was accompanied by using the reagents kit for amplification ''SNP-express'' of Litekh Research and Production Company, Moscow, Russia. The amplification of the samples subject to analysis was carried out in 96-well plates (Hard-Shell, Bio-Rad, Hercules, CA) using the T100 Thermal Cycler, Bio-Rad. The amplification was performed according to the following: primary denaturation at 94° – 1 min, then for 35 cycles denaturation at 94° – 10 s, annealing of primers at 68° – 30 s, DNA synthesis for 20 s, and in conclusion DNA synthesis at 72° – 1 min.

Detection of amplification products was performed using the horizontal electrophoresis in 3% agarose gel (Helicon, Moscow, Russia) with 0.1% ethydium bromide in electrophoresis chamber (Sub-Cell GT Cell, Bio-Rad) with voltage of 130 V. The electrophoretic separation was controlled according to the dyed strip movement from starting point by 1.5–2 cm.

The electrophoresis results were visualized in the transilluminator (ECX-F15.C, Vilber Lourmat, Paris, France) using the UV irradiation (wave length of 310 nm). Fragments of the analyzed DNA appeared in the form of luminous orange-red strips.

Statistical processing of the results was carried out using Statistica 10, Microsoft Excel and ''Calculator for statistics of studies with case control'', submitted on the site of State Research Centre of the Russian Federation GENETIKA (http://www.gen-expert.ru).

Statistical analysis included the combination of two samples series according to one criterion. The criterion meant the existence (absence) of a definite allele, genotype or their combination in several genes.

Statistical analysis of accuracy of differences in distribution of the alleles and genotypes frequencies between the analyzed samples series and detection of genotype association with the disease development were performed using the standard method of Yates' chi-squared test (if $p < 0.05$ the results were considered statistically significant).

In order to describe the relative risk of disease the odds ratio (OR) was calculated.

OR = 1 was considered as association absence.

OR > 1 – was considered as positive association (increased risk for pathology development),

OR < 1 – was considered as negative association of allele or genotype with the disease (low risk for pathology development).

Results

The results are presented in tables.

The results showed that the proportion of polymorph allele 677T of MTHFR gene is reasonably higher in women with unsuccessful IVF and in the group of RPL and comprises 40% and 44%, but 18.5% ($p = 0.0003$, OR = 3.55 [1.77–7.14]) in the control group (Table 1).

Table 1. Frequencies of alleles folic acid cycle genes MTR A2756G, MTRR A66G and MTHFR C677T in women RPL, unsuccessful IVF and control group.

Allele	RPL $N = 37$	Control $N = 46$	p	OR Result	OR 95% CI
MTHFR 677C	55.4%	81.5%	**0.0003**	0.28	0.14–0.57
MTHFR 677T	44.6%	18.5%		3.55	1.77–7.14
MTR 2756*A*	66.2%	72.8%	0.36	0.73	0.38–1.42
MTR 2756*G*	33.8%	27.2%		1.37	0.70–2.66
MTRR 66*A*	40.5%	57.6%	**0.03**	0.50	0.27–0.93
MTRR 66G	59.5%	42.4%		1.99	1.07–3.71
Allele	IVF $N = 55$	Control $N = 46$	p	OR Result	OR 95% CI
MTHFR 677C	60.0%	81.5%	**0.0009**	0.34	0.18–0.65
MTHFR 677T	40.0%	18.5%		2.94	1.54–5.63
MTR 2756*A*	72.7%	72.8%	0.99	1.00	0.53–1.85
MTR 2756*G*	27.3%	27.2%		1.01	0.54–1.87
MTRR 66*A*	40.9%	57.6%	**0.02**	0.51	0.29–0.89
MTRR 66*G*	59.1%	42.4%		1.96	1.12–3.44

Bold values indicate statistically significant.

While analyzing the polymorph alleles frequencies of other genes of the folic cycle the accurate differences in frequency of allele 66G of MTRR gene between the groups were observed. In the RPL and unsuccessful IVF groups it comprised 59.5% ($p = 0.03$, OR = 1.99 [1.07–3.7]) and 59.1% ($p = 0.02$, OR = 1.96 [1.12–3.44]), but 42.4% in the control group.

According to the presented results the frequency of favourable allele C of MTHFR gene in the control group prevails over the frequency of allele T (81.5% in the control group and 55.4% and 60.0% in the RPL and unsuccessful IVF groups, respectively).

The proportion of the polymorph allele 677T of MTHFR gene is accurately higher, the frequency of its occurrence in women with unsuccessful IVF and RPL comprises 40% ($p = 0.0003$, OR = 3.55 [1.77–7.14]) and 44.6% ($p = 0.0009$, OR = 2.94 [1.54–5.63]) versus 18.5% in the control group.

There were no significant differences in frequencies of alleles 2756A and 2756G of gene MTR in the studied groups.

The next stage included the analysis of genotype distribution by the studied polymorphisms (Table 2). The following was investigated: which mutation form (homozygous or heterozygous) and to which extent can influence the RPL development and the unsuccessful IVF.

The results showed that the favourable homozygous genotype of gene MTHFR was less frequent in the RPL and unsuccessful IVF groups (C/C) (36.4%, 32.4%) versus the control group (67.4%).

The polymorphism of MTHFR gene C677T was significantly more frequent in women with RPL and unsuccessful IVF versus the control group. In the samples series of the patients with unsuccessful IVF these differences were characteristic for both homozygous and heterozygous forms.

The proportion of heterozygous form comprised 47.5% in ($p = 0.049$, OR = 2.28 [0.99–5.23]) and 28.3% in the control group. Homozygous form was detected in 21.6% ($p = 0.016$, OR = 5.9 [1.2–30.6]) of women with RPL and in 16.4% ($p = 0.051$, OR = 4.3 [0.88–21.04]) of women with unsuccessful IVF versus 4.3% in the control group.

The comparative analysis of frequencies of genotypes A66G of polymorphism of MTRR gene in the blood samples of healthy women and patients with different reproductive disorders showed that in the groups of patients with reproductive problems the favourable homozygous genotype (A/A) was accurately less frequent. The genotype A66G was found in the RPL group in

Table 2. Genotype distribution in genes MTHFR, MTR and MTRR in women RPL group, unsuccessful IVF group and in control group.

			RPL			Control			OR	
Allele	Genotype	*n*	*N*	%	*n*	*N*	%	*p*	Result	95% CI
MTHFR C677T	C/C	12	37	32.4	31	46	67.4	**0.002**	0.2	0.09–0.59
	C/T	17	37	45.9	13	46	28.3	0.096	2.2	0.87–5.37
	T/T	8	37	21.6	2	46	4.3	**0.016**	5.9	1.2–30.6
	C/T + T/T	25	37	67.6	15	46	32.6	**0.002**	4.3	1.7–10.8
MTR A2756G	A/A	17	37	45.9	22	46	47.8	0.865	0.9	0.39–2.21
	A/G	15	37	40.5	23	46	50.0	0.266	0.7	0.28–1.64
	G/G	5	37	13.5	1	46	2.2	0.056	7	0.78–63.1
	A/G + G/G	20	37	54.1	24	46	52.2	0.687	1.1	0.45–2.57
MTRR A66G	A/A	6	37	16.2	18	46	39.1	**0.029**	0.3	0.11–0.87
	A/G	18	37	48.6	17	46	37.0	0.372	1.6	0.67–3.9
	G/G	13	37	35.1	11	46	23.9	0.332	1.7	0.66–4.49
	A/G + G/G	31	37	83.8	28	46	60.9	**0.029**	3.3	1.16–9.55
			IVF							
MTHFR C677T	C/C	20	55	36.4	31	46	67.4	**0.003**	0.28	0.12–0.63
	C/T	26	55	47.3	13	46	28.3	**0.049**	2.28	0.99–5.23
	T/T	9	55	16.4	2	46	4.3	**0.051**	4.3	0.88–21.04
	C/T + T/T	35	55	63.6	15	46	32.6	**0.003**	3.62	1.58–8.26
MTR A2756G	A/A	27	55	49.1	22	46	47.8	0.899	1.05	0.48–2.3
	A/G	26	55	47.3	23	46	50.0	0.843	0.9	0.41–1.96
	G/G	2	55	3.6	1	46	2.2	1.000	1.7	0.43–2.08
	A/G + G/G	28	55	50.9	24	46	52.2	0.899	0.95	0.43–2.08
MTRR A66G	A/A	9	55	16.4	18	46	39.1	**0.013**	0.3	0.12–0.77
	A/G	27	55	49.1	17	46	37.0	0.221	1.64	0.74–3.66
	G/G	19	55	34.5	11	46	23.9	0.280	1.68	0.7–4.03
	A/G + G/G	46	55	83.6	28	46	60.9	**0.013**	3.29	1.3–8.31

Bold values indicate statistically significant.

16.2% ($p = 0.029$) of cases, in the unsuccessful IVF group – in 16.4% ($p = 0.013$) of cases, while in the control group it was observed in 39.1% of women. The frequency of heterozygous forms (A/G) in the studied groups had no accurate differences.

There were no accurate differences in frequencies of alleles of MTRR gene between the RPL and unsuccessful IVF groups. As far as the polymorphism A2756G of MTR gene is concerned, the statistically significant differences between the genotype frequencies in the studied groups were not found. The frequencies of A/A and A/G genotypes were practically equal in all studied groups. G/G genotype was very rare.

All studied enzymes MTR, MTRR and MTHFR are together involved in the cycle of folic acid and therefore the intergenic interaction is highly possible. Some investigators have demonstrated the significant increase of risk of different pathology forms associated with elevated blood homocysteine, which is accompanied by low-function alleles in several genes of folic cycle, in comparison with existence of such allele only in one gene.

For adequate analysis of mutual effect of several mutations on the RPL development and the outcome of IVF we studied different combinations of polymorph alleles of genes MTHFR, MTR, MTRR.

More often the combination of two low-function alleles 677T and 2756G of MTHFR and MTR genes was observed in women with RPL versus the control group 27% versus 8.7% in the control group, $p = 0.027$, OR = 3.89 [1.11–13.66], as well as the combination of alleles 677T and 66G of MTHFR and MTRR genes (40.5% versus 19.6%). The existence of such mutations presupposes that the risk of RPL is about 3.3 times higher (OR = 2.8 [1.05 up to 7.47]).

The combination of three unfavourable alleles 66G, 677T and 2756G ($p = 0.038$) is accurately more frequent in women with RPL (21.6% and 6.5%) and risk of miscarriage is about four times higher (OR = 4.1 [1–16.77]).

The unsuccessful IVF group versus the control group showed the accurate increase of the similar observed combination variants, which contained polymorph alleles identical to alleles found in the RPL group.

There were no accurate differences in frequencies of polymorph alleles combinations between the RPL and unsuccessful IVF groups (Table 3).

Discussion

After the study of peripheral blood samples of women out of the RPL group, unsuccessful IVF group and control group the frequencies of alleles of three folic cycle genes.

In the control group the frequency of allele 677C of MTHFR gene prevailed over the frequency of allele 677T. The proportion of polymorph allele 677T of MTHFR gene was accurately higher in women with unsuccessful IVF and RPL.

Then we analyzed the distribution of genotypes by polymorphisms studied in women with reproductive disorders and in women of the control group. In the group of reproductive disorders the favourable homozygous genotype of MTHFR gene (C/C) was accurately less frequent. The frequency of homozygotes in allele 677T (T/T) in the groups of RPL and unsuccessful IVF was much higher than the similar parameter in the control group. The frequency of heterozygotes (C/T) was accurately different from the control parameters only in the group of unsuccessful IVF. The complex of genotypes with polymorph allele 677T in homo- and heterozygous form (C/T +T/T) was also more often observed in women with reproductive disorders versus the control group.

Based on the obtained data we may assume that the existence of allele 677T of gene MTHFR increases the risk of miscarriage and negative outcome of IVF. The observed tendencies correlate with the results of the previous works on studying the association between polymorphisms of folate metabolism genes and human embryogenesis disorders.

According to literature there are explicit contradictions concerning the association between polymorphism C677T of

Table 3. Combination of polymorphic alleles folic cycle genes MTR A2756G, MTRR A66G and MTHFR C677T in women unsuccessful IVF group and in RPL group.

	IVF		RPL			OR	
Combination of polymorphic alleles	$N = 55$	%	$N = 37$	%	p	Result	95% CI
677T + 2756G	18	32.7	10	27.0	0.647	1.31	0.52–3.29
677T + 66G	25	45.5	15	40.5	0.673	1.22	0.53–2.84
677T + 2756G + 66G	16	29.1	8	21.6	0.468	1.61	0.6–4.29
2756G + 66G	25	45.5	14	37.8	0.523	1.37	0.58–3.2

MTHFR gene existence and reproductive disorder. Some investigators found the association between MTHFR and RPL [3–5], while the others didn't show any evidence [6–8] and thereby demonstrated the possible effect of the environment on the manifestation of MTHFR action. Some investigators confirmed that the allele 677T of MTHFR gene existence increases the risk of RPL in 4–10 times. Moreover, in one study the association between polymorphism C677T of MTHFR gene and even one case of spontaneous abortion in the early stages of pregnancy was demonstrated. At the same time, the investigators of other countries didn't find the similar association.

Series of studies showed the association between polymorphism C677T of MTHFR gene and female infertility of unclear genesis [9].

Based on the previously obtained data the miscarriage is associated with allele 677T of MTHFR gene existence not only in a mother, but also in a fetus [10]. According to the investigation of abortive material the alleles MTHFR 677T and/or 1298C in homo- or heterozygous form increase the risk of miscarriage almost in 14 times. Elevation of blood homocysteine is often accompanied by secondary autoimmune reactions and currently is regarded as one of the possible reasons for antiphospholipid syndrome (APS). Thus, in the study of Brazilian scientists the frequency of allele 677T (40.3%) was accurately higher in women with RPL in anamnesis and APS versus women in the control group [5]. Particularly, this allele is considered to be contributing to thrombosis during RPL.

It is unknown how the MTHFR gene mutations can cause the pregnancy complications, but there is an assumption that the high concentration of homocysteine contributes to endothelium damage, which causes venous thromboembolism and placental insufficiency.

The frequencies of genotypes and alleles of polymorphism A66G of MTRR gene in patients with non-developing pregnancies in anamnesis, unsuccessful IVF, and in women with regular reproductive function of the control group, were accurately different in the frequency of favourable homozygous genotype (A/A), which was rarely observed in women with reproductive disorders. Also the complex of genotypes with polymorph allele 66G was accurately more often observed in the groups of RPL and unsuccessful IVF.

There were no significant differences in the frequencies of alleles 2756A and 2756G for gene MTR in the studied groups. Also there were no statistically significant differences in the frequencies of genotypes in the studied groups.

The role of polymorphism MTR A2756G is under investigation currently and its effect on the reproductive disorders is not clear yet. The increased frequency of allele 66A of MTRR gene and allele 2756G of MTR gene in women with premature delivery in anamnesis (after 22nd week of gestation) was shown [11].

In the groups of patients with reproductive system disorders we observed the increase in proportion of the following genotype combinations: accurately more often the combinations 677T + 2756G, 677T + 66G were noted, as well as the combination of three unfavourable alleles 677T + 2756G + 66G. Common peculiarity of these combinations is expressed by polymorph variant of MTHFR gene existence. Thus, it was demonstrated that a mutation gene polymorphism MTHFR C677T plays a significant role in the infertility development, as well as its combination with polymorph variant of MTRR gene A66G.

Based on the received results the accurate differences in frequencies of combinations between the groups of RPL and unsuccessful IVF versus the control group were demonstrated. However, in the present study there were no accurate differences in frequencies of alleles combinations 2756G and 66G of MTR and MTRR genes in women with reproductive system disorders and women of the control group, and it more likely indicates the insignificance of negative influence of these alleles and their combination on the folate cycle. It correlates with the literature data [11]. The tendency to increase of frequency of genotypes with low-function alleles was observed in women with reproductive problems. After comparison of the cumulative proportions of homozygous and heterozygous mutation forms, in the groups of RPL and unsuccessful IVF versus the control group the significant increase in proportion of genotypes containing polymorph alleles of MTHFR and MTRR genes was identified. In our study, the low-function alleles of folate metabolism genes were accurately more frequent in women with unsuccessful IVF. The existence of low-function alleles of folate metabolism genes leads to the alteration of cell DNA methylation profile, disruption of chromosome disjunction in the process of gametes formation and appearance of fetal aneuploidy. Deficit of methyl groups in the quickly dividing embryo cells leads to increased inclusion of an uridylic nucleotide in synthesizable DNA chain instead of a thymidylic one. As a result the abnormally easy fragmented DNA is formed, its synthesis abruptly decelerates and that leads to the disruption of quickly dividing embryo cells cycle and possibly contributes to triggering the apoptosis mechanisms. In case of folate metabolism disorder besides the above-mentioned mechanisms of embryogenesis disorder the disruption of some strands of implantation occurs. In people with ''functionally weakened'' genotypes of MTHFR and MTRR genes the hyperhomocysteinemia develops, and it leads to the endothelium dysfunction accompanied by development of atherosclerotic vascular disease, desynchronization of fibrinolysis and fibrin formation processes, vasoconstriction, and possibly contributes to disruption of ovum nidation and trophoblast invasion.

Based on the results of the study we can assume that the existence of homozygous or heterozygous form of MTHFR gene mutation (genotype 677C/T and 677T/T), and the combination of any form of this mutation with the mutations of MTRR and MTR genes are clearly associated with elevation of risk of RPL and decrease in IVF efficiency, and that is confirmed by the literature data. In case of ''functionally unfavourable'' polymorph alleles in several genes of folic acid cycle the risk of spontaneous abortion significantly increases.

MTR gene mutation has a negative influence only in combination with mutations of two other genes. It correlates with the literature data [12], which states that some low-function alleles has a significant influence (677T, 66G) on the pathology

development in comparison with others (2756G), and their combination in many times increases the risk of RPL.

All patients with pregnancy complications, spontaneous abortions in the early stages of pregnancy, and patients before IVF are recommended to perform a test for genetic polymorphisms of folic acid cycle genes. Timely detection of genetic defects while preparing for IVF procedure will contribute to the increase of ART methods efficiency.

Thus, the differences in frequencies of alleles, genotypes and their combinations, which decrease the enzyme activity, revealed in the studied groups, correlate with the literature data on the role of folic acid cycle genes, and polymorphisms MTHFR C677T and MTRR A66G in particular, in RPL development and unsuccessful outcome of IVF.

Declaration of interest

The authors have no relevant financial, personal, political, intellectual or religious interests. The authors have had full control of all primary data and they agree to allow the Journal to review their data if requested.

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