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ORIGINAL RESEARCH

Evaluating the Association Between Genetic Polymorphisms Related to Homocysteine Metabolism and Unexplained Recurrent Pregnancy Loss in Women

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Objective: To investigate the relationship between unexplained recurrent pregnancy loss (URPL) and polymorphisms of homocysteine metabolism-related genes in women.

Materials and Methods: A case–control study included 90 women with two or more consecutive unexplained pregnancy losses and 92 controlled women without miscarriage history; the female participants were in the age category of 18–35 years. The high-resolution melting technique was used to detect the single-nucleotide variants related to homocysteine metabolism disorder, namely *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphism.

Results: The *MTHFR* C677T polymorphism had significantly correlation with URPL. Indeed, the frequency of the677T allele and genotypes (677CT, 677TT) in the URPL group was significantly higher than that in the control group ($p < 0.05$). However, the allele, as well as genotype distribution of *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphisms showed no significant difference ($p > 0.05$). *MTHFR* 677CT-1298AC genotype combination led to a 9.0-fold increased risk of URPL (OR 9.0; 95% CI, 2.25–35.99; $p = 0.001$), while the risk increased 10.0-fold (OR 10.0; 95% CI, 1.8–55.53; $p = 0.008$) when participants had more than the 3 variant loci.

Conclusion: The *MTHFR* C677T polymorphism was a risk factor for URPL, and determining the *MTHFR* C677T polymorphism had a potential prediction of URPL risk. Moreover, the *MTHFR* C677T and *MTHFR* A1298C joint mutants might have a synergistic effect on URPL. Conversely, there is a lack of evidence suggesting the URPL risk of *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G polymorphisms.

Keywords: unexplained recurrent pregnancy loss, homocysteine, *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G

Introduction

According to the Practice Committee of the American Society for Reproductive Medicine, the term "recurrent pregnancy loss" (RPL) is defined as experiencing two or more spontaneous miscarriages occurring within 20 weeks of gestation.[1,2] By studying the incidence of RPL through various researches worldwide, this condition was reported to affect up to 1–5% of women of reproductive age seeking pregnancy, which leaves a significant adverse impact on couples and society. Recurrent pregnancy loss is multifactorial; the recognized diverse causes of RPL include parental chromosomal abnormalities, immunological factors, uterine anomalies, endocrine disturbances, and even lifestyle. However, the causes are unidentifiable in 50% of the patients who are classified as experiencing unexplained recurrent pregnancy loss (URPL).[3–5]

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Recently, it has been discovered that there exists an association between URPL and genetic polymorphisms related to inherited thrombophilic factors such as Factor V Leiden (FV), plasminogen activator inhibitor-1 (PAI-1), homocysteine metabolism, and so on.[6,7] Homocysteine (Hcy) is an amino acid made from methionine by losing its terminal methyl group, and the remethylation generating methionine requires folate and vitamin B12. Three main enzymes are involved in the metabolic pathway: methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and methionine synthase reductase (MTRR). Polymorphisms in the genes encoding for these enzymes have been suggested to be responsible for the altered enzyme activity that eventually accumulates homocysteine which may directly damage the endothelium and influence placental function as well as perfusion.[8,9] Hence, hyperhomocysteine during pregnancy was associated with recurrent pregnancy loss, preeclampsia, and placental abruption.[10–12] The human *MTHFR* gene contains 11 exons located on the short arm of chromosome 1p36.3. The gene encodes for methylenetetrahydrofolate reductase (MTHFR) that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The process provides the methyl group for the remethylation of homocysteine to methionine.[13] Although some mutations within the *MTHFR* gene were determined, the two most common polymorphisms demonstrated to be accountable for reducing enzyme activity were *MTHFR* C677T and *MTHFR* A1298C.[14,15] Indeed, *MTHFR* C677T has a replacement from C to T of nucleotide 677 (rs1801133) of the *MTHFR* gene, which can lead to amino acid substitution (Ala222Val). Concerning the *MTHFR* A1298C, it has a change in nucleotide 1298 (rs1801133) from A to C, which can result in amino acid substitution (Glu429Ala) in the structure of enzyme MTHFR. Recently, several studies have explored the association between these two polymorphisms and RPL, however, their contribution to the risk of RPL is controversial. In addition, MTRR is an enzyme that is required for the maintenance of the MTR active state; their combination takes part in the remethylation of homocysteine to methionine. Despite the crucial role of MTRR and MTR, few studies have explored the relationship between *MTR* A2756G (rs1805087, Asp919Gly), *MTRR* A66G (rs1801394, His595Try) polymorphism, and RPL.[16–19]

Furthermore, not many comprehensive studies on the homocysteine metabolic pathway have been carried out concerning the single nucleotide polymorphisms (SNPs) in all three genes together; various studies focused on the *MTHFR* mutations.[20,21] Consequently, concerning the different genotypes of women affected with recurrent pregnancy loss, this prospective case-control study aimed to examine the association between polymorphisms in homocysteine metabolism-related genes: *MTHFR, MTR*, and *MTRR* with RPL in a Vietnamese population.

Materials and Methods

Subjects

Our case-control study enrolled a total of 182 Vietnamese women without lifestyle habits (smoking and alcohol use), the female participants were in the age category of 18–35 years; they were treated in IVF center - Military institute of clinical embryology and histology from May 2019 to May 2021. Ninety patients who had experienced two or more spontaneous abortions were all diagnosed with URPL after excluding chromosomal abnormalities, anatomical disorders, endocrine disorders, autoimmune disorders, or any uterine infections by a questionnaire survey of the obstetric history, and standardized clinical and laboratory examinations. The control group included 92 women who had at least one normal full-term pregnancy without any history of miscarriage or other gestational complications. This study was approved by the Ethical Review Committee of Vietnam Military Medical University.

Genotyping

Genomic DNA was extracted from peripheral blood with anticoagulant with the E.Z.N.A.® Blood DNA Mini Kit (Omega BIO-TEK, USA – Lot No. D339218115-42, Exp. Date 09/01/2022) following the protocol provided by the manufacturers. DNA samples were stored at −20°C until use.

The polymorphisms of *MTHFR, MTR*, and *MTRR* were detected by high resolution melting technology with the Folate Metabolism REAL-TIME PCR Genotyping Kit (DNA-Technology, Russia - LOT No. F0906U-2P, Exp. Date 06/09/2021). The reactions were carried out on the DTprime 5M1 real-time system, with the reaction conditions set to 80°C in 2 min, 94°C in 5 min, followed by 5 cycles of 94°C 30 s and 67°C 15 s and 45 cycles of 94°C 5 s and 67°C 15 s then finished with a ramping of temperature from 25°C to 75°C, each cycle lasts 30 s with $\Delta = 1$°C. The fluorescence for gDNA control, intake

control, and melting temperature were collected and then analyzed by the DTprimer. As a result, the genotypes for each gene would be annotated then used for statistic measurements.

Statistical Analysis

General clinical characteristics were presented as mean - standard deviation, the crude odds ratio (OR) at 95% confidence intervals (95% CIs) were calculated to appreciate the associations between alleles as well as genotypes and RPL by logistic regression. Differences in variables were statistically analyzed with the Student's *t*-test, Mann–Whitney *U*-test, and Chi-square test, when appropriate. All data analyses were performed using SPSS software (version 26; IBM SPSS, NY, USA), the difference was considered statistically significant when P<0.05.

Result

The mean age was 30.20 ± 2.82 and 30.70 ± 2.44 in the URPL group and the control group, respectively. Otherwise, the height, weight, and body mass index of all participants were in the normal range.

Table 1 shows the alleles and genotype prevalences of tested polymorphisms in the URPL group and the control group. The frequencies in the T mutant allele of the *MTHFR* C677T and G mutant allele of the *MTR* A2756G were statistically significantly higher in the URPL than that in the control group, moreover, statistical analysis of these alleles using logistic regression showed the URPL risk increased 2.35-fold and 1.88-fold (OR 2.35; 95% CI, 1.43–3.86; P = 0.001 and OR 1.88; 95% CI, 1.08–3.24; P = 0.02), separately. Conversely, no different prevalences of *MTHFR* A1298C and *MTRR* A66G mutant allele were found between the two groups (P > 0.05). Concerning the genotypes, only *MTHFR* C677T polymorphism had significantly higher prevalences of 677CT and 677TT genotypes compared with the controls (OR 2.49; 95% CI, 1.32–4.71; P = 0.004 and OR 4.68; 95% CI, 1.19–18.32; P = 0.02, respectively). The genotype distributions of the three remaining variants were similar among women with unexplained recurrent spontaneous abortions and controls (p > 0.05).

Table 1 Association Between Genetic Polymorphisms Related to Homocysteine Metabolism and the URPL

Gene	Locus	Group			P value
		Case (n = 90)	Control (n = 92)	OR (95% CI)	
MTHFR 677C>T	T	58 (32.2)	31 (16.8)	2.35 (1.43–3.86)	0.00
	C	122 (67.8)	153 (83.2)		
MTHFR 1298A>C	C	55 (30.6)	51 (27.7)	1.15 (0.73–1.80)	0.55
	A	125 (69.4)	133 (72.3)		
MTR 2756A>G	G	41 (22.8)	25 (13.6)	1.88 (1.08–3.24)	0.02
	A	139 (77.2)	159 (86.4)		
MTRR 66A>G	G	59 (32.8)	53 (28.8)	1.21 (0.77–1.88)	0.41
	A	121 (67.2)	131 (71.2)		
MTHFR 677C>T	CC	41 (45.6)	64 (69.6)	1	
	CT	40 (44.4)	25 (27.2)	2.49 (1.32–4.71)	0.00
	TT	9 (10.0)	3 (3.3)	4.68 (1.19–18.32)	0.02
MTHFR 1298A>C	AA	9 (10.0)	7 (7.6)	1	
	AC	37 (41.1)	37 (40.2)	1.09 (0.59–2.01)	0.78
	CC	44 (48.9)	48 (52.2)	1.4 (0.48–4.08)	0.53

(*Continued*)

Table 1 (Continued).

Gene	Locus	Group			P value
		Case (n = 90)	Control (n = 92)	OR (95% CI)	
MTR 2756A>G	AA	54 (60.0)	68 (73.9)	1	
	AG	31 (34.4)	23 (25.0)	1.69 (0.89–3.24)	0.14
	GG	5 (5.6)	1 (1.1)	6.29 (0.71–55.5)	0.09
MTRR 66A>G	AA	45 (50.0)	49 (53.3)	1	
	AG	32 (35.6)	36 (39.1)	0.97 (0.52–1.81)	0.92
	GG	13 (14.4)	7 (7.6)	2.02 (0.74–5.52)	0.16

Note: Values are number (percent) unless specified otherwise.
Abbreviations: CI, confidence interval; OR, odds ratio.

Table 2 shows the number of mutations occurring on loci C677T and A1298C of the *MTHFR* gene, and the synergistic effect causing URPL of the two loci was analyzed by logistic regression. Firstly, the prevalence of linkage heterozygous genotype (677CT/1298AC) was significantly higher in the patients than that in the controls (13.3% versus 4.3%, P = 0.001). Moreover, patients carrying the compound heterozygous genotype were 9.0-fold (OR 9.0; 95% CI, 2.25–35.99) greater in risk of URPL than participants with the wild-type homozygous genotype (677CC/1298AA).

Table 3 illustrates a certain number of loci containing the mutations among four reported loci and their association with the URPL. Although some enrolled women had 1 or 2 changed loci in total evaluated polymorphisms, their URPL risk did not increase significantly (OR 2.93; 95% CI, 0.58–14.78 and OR 4.0; 95% CI, 0.79–20.27, respectively). However, individuals who carried three or four mutant loci had a remarkable URPL risk which was 10.0 times greater than that in non-mutant women.

Table 2 The Synergistic Effect Causing URPL of the Two Loci on the *MTHFR* Gene

No. of Mutations	Gene Combination	Group			P value
		Case (n = 90)	Control (n = 92)	OR (95% CI)	
0	CC/AA	8 (8.9)	24 (26.1)	1	
1	CC/AC	25 (27.8)	33 (35.9)	2.27 (0.88–5.9)	0.08
	CT/AA	28 (31.1)	21 (22.8)	4.0 (1.5–10.66)	0.00
2	CC/CC	8 (8.9)	7 (7.6)	3.43 (0.94–12.48)	0.06
	CT/AC	12 (13.3)	4 (4.3)	9.0 (2.25–35.99)	0.00
	TT/AA	8 (8.9)	3 (3.3)	8.0 (1.69–37.67)	0.01
3	CT/CC	0 (0.0)	0 (0.0)	NE	NE
	TT/AC	0 (0.0)	0 (0.0)	NE	NE
4	TT/CC	1 (1.1)	0 (0.0)	NE	NE

Note: Values are number (percent) unless specified otherwise.
Abbreviations: CI, confidence interval; OR, odds ratio; NE, not estimable.

Table 3 Association Between the Number Changed Loci (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, *MTRR* A66G) and the URPL

No. of Changed Loci	Group			P value
	Case (n = 90)	Control (n = 92)	OR (95% CI)	
0	2 (2.2)	8 (8.7)	1	
1	30 (33.3)	41 (44.6)	2.93 (0.58–14.78)	0.30
2	33 (36.7)	33 (35.9)	4.0 (0.79–20.27)	0.09
≥ 3	25 (27.8)	10 (10.9)	10.0 (1.8–55.53)	0.00

Note: Values are number (percent) unless specified otherwise.
Abbreviations: CI, confidence interval; OR, odds ratio.

Discussion

Recurrent pregnancy loss is a frustrating medical condition and shows high risks for developing depression and anxiety which affect both men and women.[22] In recent years, various studies focusing on the causes of RPL have demonstrated that it may be multifactorial in many pregnancies; however, little has been understood about the underlying causes of approximately 50% of patients.[23,24] Consequently, the emerging prevalence for RPL has brought much attention to the study of its possible risk factors, such as advanced maternal age, obesity, unhealthy lifestyle habits (including smoking, alcohol consumption), and especially genetic polymorphisms.[25–27]

Genetic polymorphisms have been intensively evaluated. Their findings suggested an essential role of the risk in the pathogenesis of recurrent pregnancy loss. More than 37 genes with 53 polymorphisms were discovered, including *MTR, MTHFR, PAI-1, HLA-G, TNF, IL-6, FII, FV, FXIII, ITGB3, NOS3, KDR, TP53, VEGFA, CYP17CYP2D6, ANXA5*, and so on. The four major causes of URPL were thrombophilia, disordered placental function, abnormal immunological responses, and metabolic regulation disturbance, but their results conflicted, especially when conducted in variant populations.[6] This study focused on inherited thrombophilia of the Vietnamese population by examining the association between polymorphisms in homocysteine metabolism-related genes: *MTHFR, MTR*, and *MTRR* with RPL. Other risk factors were excluded to reduce the bias. Maternal age is a primary confounding matter strongly associated with recurrent pregnancy loss. Many studies reported that recurrent miscarriages often occur more frequently with increasing maternal age, the age threshold is usually defined as 35 years.[28] Indeed, all participants were in the age category of 18–35 years and did not have significant differences between the mean ages between the two groups.

The *MTHFR* gene encodes the enzyme methylenetetrahydrofolate reductase (MTHFR), which is responsible for production of 5-methyltetrahydrofolate. Then 5-methyltetrahydrofolate provides the methyl group for the remethylation of methionine from homocysteine.[13] Two predominant single-nucleotide polymorphisms in the *MTHFR* gene are 677C>T (rs1801133) and 1298A>C (rs1801131), reducing MTHFR enzyme function, especially in the homozygous recessive genotype. The prevalence of people carrying at least one of these variants was reported at 60–70%, while our figure was 75.56% in controls.[29,30] The *MTHFR 677T* and *1298C* allele frequencies depend on populations which were 16.8% and 27.7%, respectively, in controls of this study.[31,32] Moreover, the *MTHFR 677T* allele frequency was higher in the URPL than in the control group; statistical analysis showed the URPL risk of women carrying the allele increased 2.35-fold. Homozygote and heterozygote genotype (MTHFR 677TT, MTHFR 677CT) increased the risk of URPL significantly to 4.68 and 2.49, separately. This was caused by reducing MTHFR enzyme function, carriers of the homozygote and heterozygote genotype retained approximately 30% and 65% of enzyme activity.[15] Numerous studies have reported the associations between *MTHFR* C677T polymorphism and URPL; however, the results have been controversial. Indeed, a majority of the studies supposed *MTHFR* C677T SNP was the risk factor for the RPL, and others argued that there was no association between *MTHFR* C677T and the disease.[33–35] The 677CT and 677TT genotype of *MTHFR* and composite heterozygote genotype (677CT/1298AC) were risk factors for URPL. The prevalence of linkage heterozygous genotype (677 CT/1298 AC) was associated with a 9.0-fold increase in the risk of RPL over individuals

with homozygous wild-type genotype (677 CC/1298 AA). Our obtained result was consistent with other previously published papers with modest fluctuation in OR (95% CI) and produced one similar conclusion that the compound heterozygosity may be responsible for miscarriage risks.[33,35,36]

In variant *MTR* A2756G, a higher figure in women with recurrent pregnancy loss was observed in the frequency of both G allele and 2756 GG genotype ($p < 0.05$), proving that this polymorphism was related to the rising risk of URPL. In fact, this glycine substitution for aspartic acid at the 919 position of the protein might lead to the structural impairment and eventually cause the elevation of plasma homocysteine concentration which contributed to clinical conditions such as spontaneous miscarriage.[37] Our conclusion was consistent with the previously published paper conducted in the Iranian population.[38] Conversely, the *MTR* A2756G and *MTRR* A66G polymorphisms in RPL patients have been studied in the Korean population since 2013; the finding suggested that *MTR* A2756G and *MTRR* A66G polymorphisms were not possible predisposing markers for URPL.[18]

This report was the first to reveal the genetic susceptibility due to the SNPs (*MTHFR* C677T; *MTHFR* A1298C; *MTR* A2756G; *MTRR* A66G) in Vietnamese women and the risk of experiencing URPL. Participants with more than three changed loci demonstrated a remarkably high risk of experiencing URPL compared with control groups (OR 10.0; 95% CI; 1.8–55.53). This indicated that more aspects and correlations between these polymorphisms should carefully be investigated to shed light on the underlying risk of recurrent pregnancy loss and single-polynucleotide polymorphisms.

Conclusion

In conclusion, the *MTHFR* C677T polymorphism was a risk factor for URPL, and identifying the *MTHFR* C677T polymorphism had a potential prediction of URPL risk. Moreover, the *MTHFR* C677T and *MTHFR* A1298C joint mutants might have a synergistic effect on URPL. However, there was a lack of evidence suggesting the URPL risk of MTHFR A1298C, MTR A2756G, and MTRR A66G polymorphisms. Consequently, further genetic research needs to be conducted on a bigger sample size to investigate the correlation between these subjects.

Ethical Statements

All participants signed informed written consent to be part of the study. Our protocol was appropriate to the Declaration of Helsinki, and approved by the Ethical Review Committee of Vietnam Military Medical University (No. 1068/2019/VMMU-IRB).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

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