Association of Endothelial Nitric Oxide Synthase and *MTHFD1* Polymorphisms with Idiopathic Recurrent Pregnancy Loss in Iranian Women

Research Article

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Abstract

Recurrent pregnancy loss (RPL) is a multifactorial disorder that remains idiopathic in 50% of the cases. The aim of this study was to investigate the association between *MTHFD1* as well as *eNOS* polymorphisms with idiopathic RPL. In a case-control study, 100 women with idiopathic recurrent pregnancy loss (PRL) and 50 controls referred to Noor Laboratory, Khoozestan, Iran, were evaluated. Genotyping of eNOSG894T and *MTHFD1* G1958A variants was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. Results were compared between the two groups. The allele and genotype frequency of *MTHFD1* G1958A variant as well as the allele frequency of eNOSG894G were not significantly different between the two groups while eNOSG894T genotype was significantly different between idiopathic RPL group and controls. Endothelial NOS G894T heterozygous (GT) genotype caused a 2.82 fold increase in the risk of idiopathic RPL compared to GG genotype. *eNOS* G894T variant is significantly associated with the risk of idiopathic RPL while there is no such association for *MTHFD1* G1958A variant in the Iranian population.

Key words: *eNOS*; *MTHFD1*; Polymorphism, Genetic; Idiopathic Recurrent Pregnancy Loss

Introduction

Recurrent pregnancy loss (RPL) is one of the most common and important complications of pregnancy that affects 1–5% of the couples (1). RPL is defined as two or more consecutive pregnancy losses before 20 weeks of gestation (documented by ultrasound or histopathological examination) by the American Society for Reproductive Medicine (ASRM) (2).

RPL is a multifactorial disorder and various factors including anatomic anomalies of the uterus or cervix, chromosomal abnormalities, hormonal and endocrinologic disorders, reproductive tract infections, immunologic causes such as thrombophilia and lifestyle factors as well as environmental factors have been known to contribute to the etiology of RPL (3, 4). These factors explain RPL causes only in 20 to 50% of the cases and RPL remains idiopathic with an unexplained etiology in 50% of the cases (5).

Folate and homocysteine are two key molecules of one-carbon metabolism that have an important role in maintaining pregnancy (6). Methylenetetrahydrofolate dehydrogenase (*MTHFD1*) has an important role in the metabolism of folate and homocysteine. Evidence suggests an association between *MTHFD1* polymorphisms and pregnancy loss (7, 8). The *MTHFD1* common variant of G1958A (rs2236225) (R653Q) located in exon 21 changes arginine in the

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synthetase domain to glutamine and reduces enzyme stability and impairs purine synthesis (9).

Nitric oxide (NO) is another molecule with an important role in preserving a normal pregnancy. NO is produced during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) enzymes. There are three isoforms of NOS enzymes; endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) (10). Endothelial NOS is the main enzyme required for NO synthesis in the vascular system. Endothelial NOS is expressed in terminal villous vessels and in the syncytiotrophoblast during pregnancy (11). Increased NO production in the endothelial cells may contribute to implantation, decidualization, and blood flow regulation in the placenta (12).

In the common variant of *eNOS* rs1799983 (G894T) located in exon 7, substitution of guanine with thymine in nucleotide 894 causes an amino acid change from glutamine to aspartic acid at position 298 (Glu-298Asp) (13). This variant has been associated with reduced NO production (14) that can impair placental perfusion as well as oxygen and nutrient supply to the fetus.

This study aimed to examine two polymorphisms of *MTHFD1* (G1958A) and *eNOS* (G894T) genes and their association with the increased risk of idiopathic RPL.

Materials and Methods

This case-control study was conducted on 100 women (20-60 years old) with idiopathic recurrent pregnancy loss (PRL) without live birth and 50 women (20-60 years old) with live birth as controls who were referred to Noor Laboratory, Khoozestan, Iran during the year 2014.

Institutional Review Board of Ahwaz University of Medical Sciences approved the study protocol and informed consent was obtained from the participants before enrollment.

RPL was defined by at least two consecutive pregnancy losses before 20 weeks' gestation, according to the ASRM definition (15).

Women with at least two consecutive pregnancy losses before 20 weeks gestation (RPL) referred to the laboratory for karyotyping were included in the study. Those with known anatomical, hormonal, infectious, immunological, or thrombotic causes for RPL and those with live births were excluded from the study as well as those with chromosomal disorders. Anatomic disorders such as septate uterus, intrauterine adhesion and uterine fibroids were ruled out by sonography, hysterosalpingography, hysteroscopy, computerized tomography (CT) scanning, or magnetic resonance imaging (MRI). Hormonal, immunological, and thrombotic disorders were evaluated with related proper assays. Chromosomal abnormalities were assessed by karyotyping.

Women with at least one live birth who were naturally conceived and lacked a history of pregnancy loss were selected randomly for the control group among those referred to Noor Lab for other causes.

In order to obtain DNA, 3-mL of peripheral blood was collected from the participants in EDTA-containing tubes and stored at -20ºC until DNA extraction. DNA was extracted from peripheral blood leukocytes using DNA Extraction kit (Bioneer Corporation, Korea) according to the manufacture instructions. The quality of the DNA was evaluated on the NanoDrop spectrophotometer (ASP-2680, ACTGene Inc., NJ, USA) at 260 and 280 nm.

Polymorphism genotyping of G894T in *eNOS* and G1958A in *MTHFD1* genes was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis.

Genomic DNA amplification was performed using the proper primers and appropriate time and thermal conditions. Amplification for *MTHFD1* G1958A was performed using a forward primer with the sequence of GTGTGATCCCACTTTGAAGC and a reverse primer of TCCATTCCAATGTCTGCTCC by the following PCR condition: 95°C, 5 min, 15 cycles (95°C, 30 s; 57.4°C, 30 s; 72°C, 30 s), 72°C, 5 min.

PCR reaction for amplification of *eNOS* G894T was performed by thermal condition of 95°C, 5 min, 40 cycles (95°C, 30 s; 63.7°C,30 s with 0.5°C reduction in each cycle ; 72°C, 30 s) and 15 cycles (95°C, 30 s; 58.7°C, 30 s; 72°C, 30 s), 72°C, 5 min using following forward and reverse primers: forward primer: CATGAGGCTCAGCCCCAGAAC and reverse primer AGTCAATCCCTTTGGTGCTCAC. The primers in this study were designed by the Primer3 online software.

Amplified products were subjected to digestion using MboI and MspI restriction endonucleases (Fermentas) at 37 ºC for 16 hours (according to the manufacture instructions) for G894T and G1958ASNPs respectively and DNA fragments were separated by an 8% polyacrylamide gel electrophoresis.

Digestion of DNA with MspI produced two fragments of 134 and 172 pb in the GG genotype, three fragments of 306, 134 and 172 bps in the GA genotype and only one fragment of 306 bp in the homozygote

AA genotype of rs2236225 (G1958A). DNA digestion of *eNOS* with MboI resulted in two fragments of 119 and 87 bps for the GG genotype, three fragments of 206, 119 and 87 bps for the GT genotype, and one fragment of 206 bp for the TT genotype of G894T.

Data Analysis

Data were analyzed by SPSS software version 17.00 for Windows. Data are presented as mean±SD for continuous variables and number and percentage for categorical variables. Chi- square was used to compare categorical data. The odds ratio and 95% confidence interval (CI) were also calculated to measure the strength of the association between different genotypes and risk of RPL. P value less than 0.05 were considered significant. Student t-test was used to examined the association with the risk of idiopathic RPL.

Results

DNA genotyping was performed in 100 women with idiopathic RPL and 50 healthy control women. The allele frequency of two SNPs has been shown in Table 1. The allele frequency of two variants was not significantly different between the two groups (P value>0.05) (Table 1).

The frequency of different *MTHFD1* G1958A genotypes was not significantly different between idiopathic RPL and control groups (P value>0.05) (Table 1). There was a significant difference between the two groups regarding the genotypic frequency of *eNOS* G894T variant and the frequency of heterozygote genotype (GT) was significantly (p value <0.05) higher in the RPL group, while the homozygous (TT) genotype was not observed in the two groups (Table 1).

Data analysis showed that the AA genotype of G1958A decreased the risk of idiopathic RPL in comparison with the GG genotype with an odds ratio of 0.529 (95% CI: 0.192-1.475) (P value: 0.55). Also, the GA genotype of *MTHFD1* G1958A reduced the risk of idiopathic RPL by about 20% in comparison with the GG genotype with an odds ratio of 0.815 (95% CI: 0.243-1.246) (P value: 0.35). However, these odds ratios were not significant (Fig. 1).

The heterozygous genotype of *eNOS* G894T variant (GT) increased the risk of idiopathic RPL significantly as compared with the GG genotype with an odds ratio of 2.82 (95% CI: 1.19 -6.68) (P value: 0.018).

The A allele of *MTHFD1* G1958A variant versus the G allele was a protective allele with an odds ratio of 0.72 although the odds ratio was not significant (P value: 0.18). The T allele of *eNOS* G894T was identified as a risk allele that increased the risk of RPL with a significant odds ratio of 2.43 (P value: 0.03) (Fig. 2). The G894T genotype was significantly associated with the risk of idiopathic RPL woman (P value: 0.04).

Table 1: Allele and genotype frequency of two variants in *MTHFD1* and *eNOS* genein our study population and the frequency of these variants in Asian population from 1000 genomes

Variant Allele/genotype	Idiopathic RPL group n=100 n (%)	Control group n=50 n (%)	P value	Frequency in Asian population based on 1000 genome data	Odds ratio (p _value)
MTHFD1 **G1958A**					
A allele	84 (42)	(50) 50	0.35	0.23	0.72 (p= 0.18)
G allele	116 (58)	(50) 50	0.35	0.77	-
GG	34 (34)	(11) 22	0.13	0.57	-
GA	48 (48)	(28) 56	0.13	0.39	0.81 (p=0.35)
AA	18 (18)	(11) 22	0.26	0.038	0.52 (p=0.55)
eNOS **G894T**					
G allele	165 (82.5)	(92) 92	0.1	0.88	2.43 (p=0.03)*
T Allele	35 (17.5)	(8) 8	0.1	0.12	-
GG	65 (65)	(42) 84	0.01*	0.78	
GT	35 (35)	(8) 16	0.01*	0.20	2.82 (p=0.018)*
TT	(0) 0	(0) 0	-	0.014	-

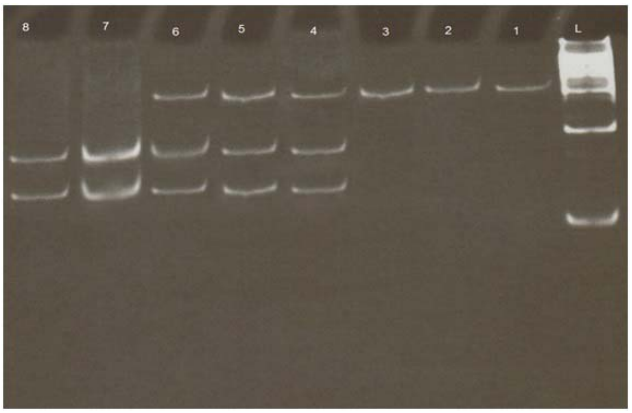

Figure 1: bands were observed after restriction enzyme in the MTHFD1 gene polymorphism on polyacrylamide gel electrophoresis. Rows 1,2, and 3 don't cut by *MspIenzyme* in 306 bp PCR products have genotypeAA. Rows 4,5and 6 cut fragments by *MspIenzyme*, have 306, 172 and 134 bp length and represents the genotype GA. Rows 7 and 8 cut fragments by *MspIenzyme*, have 172 and 134 bp length and represents the genotype GG. L:100bp DNA marker.

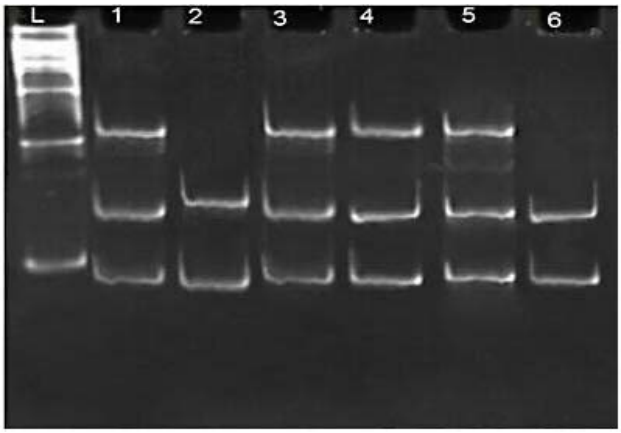

Figure 2: bands were observed after restriction enzyme in the eNOS gene polymorphism on polyacrylamide gel electrophoresis. Rows 2and 6cut by MboIenzyme in 206 bp PCR products and have 119 and 87 bp length have GGgenotype. Rows 1,3,4 and 5 cut fragments by *MboIenzyme*, have 206, 119, 87 bp length and represents the genotype GT.L:100bp DNA marker.

Discussion

The study results showed no significant association between the *MTHFD1* G1958A variant and idiopathic RPL but found a significant association between *eNOS* G894T variant and idiopathic RPL. However, the AA genotype of G1958A decreased the risk of idiopathic RPL by q factor of 0.529 as compared with the GG genotype (wild allele) although this odds ratio was not significant. The risk of RPL increased by 2.82-folds in women with heterozygous GT genotype of *eNOS* G894T as compared with those with homozygous GG genotype and this odds ratio was significant (P value: 0.018).

The association between *MTHFD1* G1958A and the risk of pregnancy loss was first reported by Parle-McDermott et al. in 2005. They genotyped G1958A variant in 125 Irish pregnant women and found that the AA genotype versus the AG/GG increased the risk of unexplained second trimester pregnancy loss by a factor of 1.64 (P value: 0.03) (16). Their reported odds ratio is different from our study finding probably due to the different race. Their study showed that A allele versus G allele increased the risk for idiopathic RPL by 1.23 folds (P value: 0.14) while we found that it was a protective allele (odds ratio: 0.72, P value: 0.18) (31). Eight years later, a study on 353 Korean women with idiopathic RPL and 226 controls showed no significant association between *MTHFD1* G1958A and idiopathic RPL (Table 2) (17). Although similar to our study, this study also showed that AA genotype versus AG and GG genotypes and A allele versus G allele were protective against RPL with odds ratios of 0.91 and 0.84 respectively, the odds ratios were not significant (P value: 0.79 and 0.29, respectively) like our odds ratios (Table 2) (17). How-

Table 2: Comparison of previous studies about the studied polymorphisms in *MTHFD1* and *eNOS*

Author	Year	Country/Race	Sample size	Variant
Parle-McDermott A, et al (16)	2005	Dublin, Ireland/Irish	125	MTHFD1 G1958A
Kim JH, et al (17)	2013	Korea/Korean	579	MTHFD1 G1958A
Hefler LA, et al	2002	USA/white Cucasian	197	eNOS G894T
Karvela M, et al (26)	2008	Greece/Greek	256	eNOS G894T
Zammiti W, et al (25)	2008	Tunisia/Tunisian	550	eNOS G894T
Shin SJ, et al (21)	2010	Korea/Korean	455	eNOS G894T
Parveen F, et al (22)	2011	India/north Indian	500	eNOS G894T
Ozturk E, et al (24)	2011	Turkey/ Turkish	120	eNOS G894T
Luo L, et al (20)	2013	China/ Chinese	393	eNOS G894T
Seyhon I, et al	2014	Iran/Iranian	180	eNOS G894T

ever, we could not explain the cause of such similarity despite different race. We found no further study that evaluated *MTHFD1* G1958A variant in women with idiopathic RPL in the literature. However, other studies have shown the association of this variant with an increased risk of congenital heart and neural tube defects (9, 18) that may be considered as a cause of RPL.

Increased expression of *eNOS* has been shown in the endometrium of women with repeated miscarriage (19). Various studies have examined the association between *eNOS* polymorphism and idiopathic RPL. Someof these studies (20-23), similar to our study, confirmed a significant association between G894T and idiopathic RPL while most of them did not report such an association (Table 2) (13, 21, 24-26).

In a study by Zammiti et al in Tunesia, TT genotype of G894T variant versus GT and GG genotypes increased the risk of RPL by 1.6 folds but the odds ratio was not significant (25). Karvela et al also did not find any significant difference in the frequency of TT genotype in G894T variant between RPL group and controls in a Greek population (26). Suryanarayana et al showed that the T allele versus the G allele in G894T variant increased the risk of early RPL in Indian women with an odds ratio of 1.3 (95%CI: 0.6-2.8) (23). However, another study in north Indian women identified the G allele of G894T polymorphism as the risk allele that increased the risk of RPL by 3.58 folds (P value <0.0001).

In a study by Luo et al, the TT genotype of G894T variant was identified as a protective genotype with an odds ratio of 0.43 in Chinese women (P value: 0.001) (20). Shin et al showed a 2.39-fold increase in the risk of RPL in women with GT and TT genotypes versus those with the GG genotype (P value: 0.008) in Korean women (21). Also, Öztürk et al showed that GT and TT genotypes versus the GG genotype of G894T variant increased the risk of RPL by 2.7 folds in Turkish women (P value: 0.055) (24).

Hefler et al studied G894T variant in white Caucasians in the USA and found that the 894TT genotype increased the risk of RPL by a factor of 1.2 (P value: 0.03) (13). A recent study in Iran did not find any association between various genotypes of G894T of *eNOS* and RPL (27).

These differences between the results of various studies and our findings may be explained by different genetic background of different races and ethnicities in various studies. Most of our study participants were Arabs. Despite these results, two separate recent meta-analyses reported a significant association between *eNOS* G894T polymorphism and idiopathic RPL with odds ratio of 1.5 and 1.9 which are lower than odds ratios (2.82) (28, 29). In one of these two meta-analyses, when the data were stratified by ethnicity, the significant association of eNOSG894T variant and idiopathic RPL was observed only in East Asians (28).

We did not observe the TT genotype in *eNOS* G894T variant in our study, neither in cases nor in controls. After comparison with similar studies that evaluated this polymorphism in normal individuals in Iran and in a European ancestry, we found that the distribution of this genotype was lower than two other genotypes and was less in females than males (30, 31). Therefore, it seems that both the small sample size and distribution of our study population (only women) were the reason for these results. However, the frequency of *MTHFD1* G1958A variant in our study was similar to a previous study in Iran (in another condition) (32) but different from a study in Jordan (33).

The main limitation of present study is that we did not consider positive and negative controls confirmed by direct sequencing. The relatively small sample size, and lack of the measurement of serum levels of NO and folate were the other limitations of our study.

In conclusion, according to our results, *eNOS* G894T, but not *MTHFD1* G1958A, contributes to idiopathic RPL in the Iranian population. Therefore, it may be a candidate variant to be used as a clinical marker to assess the risk of idiopathic RPL.

However, before including these genetic tests in the clinical practice of RPL management, further case-control studies with larger sample sizes in our region and in diverse ethnic populations are necessary to clarify and confirm the association of this and other *eNOS* and *MTHFD1* polymorphisms with idiopathic RPL.

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