

MTR, MTRR and CBS Gene Polymorphisms in Recurrent Miscarriages: A Case Control Study from North India

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ABSTRACT

Background: According to various epidemiological studies, the aetiology of recurrent miscarriages (RMs) is multifactorial. The goal of this study is to learn more about the link between genetic polymorphisms and RM. **Aim:** To evaluate the association of 5-Methyltetrahydrofolate-Homocysteine Methyltransferase (MTR) A2756G, 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase (MTRR) A66G and cystathionine beta-synthase (CBS) 844INS68 genetic polymorphisms with RM and also to understand the combined effect of the selected genotypes. **Study Setting and Design:** This was a hospital-based, case-control, observational study. **Materials and Methods:** A total of 516 participants were recruited in the present study, of which 200 RM cases and 258 controls were included in the present study. Fasting blood sample (~5ml) was drawn from all the participants and were screened for genetic polymorphisms of MTR A2756G, MTRR A66G and CBS 844INS68. **Statistical Analysis:** The frequency, odd's ratio and Hardy-Weinberg equilibrium were evaluated. SPSS (version 21.0) was used for the data analysis. **Results:** MTR A2756G genetic polymorphism was not associated with the risk of RM. The ancestral allele of MTRR A66G and the mutant allele of CBS 844INS68 was causing an increased risk of more than two folds for RM. CBS 844INS68 in combination with MTR A2756G was found to pose an increased risk of more than two folds for RM. **Conclusion:** Genetic polymorphisms particularly MTRR A66G and CBS 844INS68 seems to be elevating the risk and hence making women susceptible for RM.

KEYWORDS: 5-methyltetrahydrofolate-homocysteine methyltransferase A2756G, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase A66G, CBS 844INS68, gene-gene interaction, genetic polymorphism, recurrent miscarriage

INTRODUCTION

The spontaneous loss of pregnancy before the foetus reaches viability at 24 weeks is defined as a miscarriage. The loss of three or more consecutive pregnancies is defined as recurrent miscarriage (RM) and it affects 1% of the couple trying to conceive Royal College of Obstetricians and Gynaecologists (RCOG) 2017 update, Green top guideline No. 17.^[1] It is a multifactorial disorder where various abnormal conditions intertwine together making the phenotype

adverse. Previous studies have reported that RM is caused by various factors like genetics, metabolic and hormonal disorders, lifestyle issues, thrombophilia, autoimmunity, uterine anomalies, antiphospholipid syndrome, infection and sperm quality,^[2-5] but there are no definitive conclusions for the aetiology of 50% of the RM cases.^[6,7]

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The significance of One-Carbon Metabolic Pathway (OCMP) in association with RM is quite evident from the literature.^[8-10] OCMP is guided both by biochemical as well as genetic polymorphisms. It is likely to be disturbed because of folic acid supplementation as folate acts as one of the critical cofactors playing a major role in the foetal epigenetic programming via OCMP.^[11] Mutations in the genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR), 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase (MTRR), 5-Methyltetrahydrofolate-Homocysteine Methyltransferase (MTR) and RFC-1; crucial to OCMP are found to be associated with low levels of folate and high levels of homocysteine among unexplained recurrent pregnancy loss/RMs cases.^[12] In addition, Cystathionine Beta-synthase (CBS) converts Hcy to H₂S and the cysteine precursor cystathionine and its genetic mutation is the most frequent cause of hyperhomocysteinaemia.^[13]

MTHFR is a widely studied genetic marker as compared to other genetic polymorphisms in RMs.^[8-10,14,15] Despite extensive studies in Obstetrics/Gynaecology clinics and *in vitro* fertilisation centres worldwide of this sporadic complication of early pregnancy, the aetiology of RM remains poorly understood.^[3,16]

Thus, the present study is an attempt to understand the association of the selected genetic markers with RMs. In the same cohort, we have already reported the association of MTHFR C677T polymorphism with RM.^[15] Hence, the aim of the present study is to evaluate the association of MTR A2756G, MTRR A66G and CBS 844INS68 genetic polymorphisms with RM and also to understand the combined effect of the selected genotypes.

SUBJECT AND METHODS

The total number of women recruited was 516, of which 200 RM cases and 258 controls were involved in the present study (aged 18–40 years) after obtaining the ethical clearance from the Institutional Ethical Committee of Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital, New Delhi, India. RM cases were the women with a history of three or more consecutive unexplained recurrent pregnancy losses before 24 weeks of gestation, whereas the controls were the women with one or more consecutive normal deliveries. Cases and controls were matched for their gestation, age, smoking and alcohol status. Data were collected after obtaining the informed written consent from all the participants.

Sample size calculation

Considering the power of the study to be 80% and with a precision of 5%, the sample size was calculated based on the MTHFR gene polymorphism of RM.^[17] The sample size was calculated to be 258, each for RM cases and controls. After recruiting, there was a higher dropout rate from the case group, and even from whom the blood samples were collected, because of the poor DNA quality, further analysis was not possible. Hence, only 200 cases could be involved in the present study; however, all the 258 controls recruited were included in the study.

To rule out for RM cases with explained reasons, all the women with recurrent pregnancy losses were subjected to glucose tolerance test, antiphospholipid antibodies workup, lupus anticoagulants, β -microglobulin test, ultrasonography for ruling out for uterine anomalies, polycystic ovaries, antral follicle count for ovarian reserve, hysterosalpingography/hysteroscopy for ruling out the uterine anomaly and intrauterine adhesions. Premenstrual endometrium biopsy for ruling out tuberculosis was performed. Further, dilatation test for ruling out cervical incompetence, hormonal profile including day 2 follicle-stimulating hormone, luteinising hormone, prolactin and thyroid profile test were performed. Furthermore, we have ruled out the women with abnormal karyotype.

Ethical policy and institutional review board statement

The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2013. Data were collected after obtaining the informed written consent from all the participants.

Ethics committee approval number: IEC-60/2011.

Genetic analysis

In the present study, 5 ml of overnight fasting intravenous blood sample was drawn from the cases and controls from whom demographic and clinical data had already been collected. DNA was extracted from the blood samples using the salting-out method.^[18] The genetic markers MTR A2756G, MTRR A66G and CBS 844INS68 were analysed on all the cases and controls. Genotypes of the two polymorphisms, i.e., MTR A2756G and MTRR A66G, were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis using specific primers already designed,^[19,20] while the polymorphism of CBS 844INS68 was ascertained through allele-specific PCR amplification using specific primer designed

and used.^[13] About 10% of the total samples were re-genotyped (randomly selected), for quality control and no discrepancy in the genotypes were found.

Statistical analysis

Hardy-Weinberg equilibrium analyses were performed to compare the observed and expected genotype frequencies using the Chi-square test. The distributions of the genotypes among the cases and controls were evaluated using the Chi-square test. Odds ratios (ORs) were calculated and are presented within the 95% confidence intervals (95% CIs). SPSS (IBM SPSS Statistics, Version 22) was used for data analysis.

RESULTS

Distribution of the genetic polymorphisms among RM cases and controls

The distribution of MTR A2756G, MTRR A66G and CBS 844INS68 polymorphisms were analysed for cases and controls. The frequencies of the three genotypes with respect to MTR A2756G genetic polymorphism were found to be similar both among cases and controls. The genotypic frequency distribution of MTRR A66G genetic polymorphism normal homozygous genotype AA and heterozygous AG genotypes was found to be significantly higher among cases as compared to controls, whereas the frequency of GG genotypes was significantly higher among controls as compared to cases ($P = 0.049$). The cases and controls were found to deviate from the Hardy-Weinberg equilibrium with respect to both MTR A2756G and MTRR A66G polymorphisms ($P < 0.05$). In the case of CBS 844INS68 polymorphism, the controls were found to be having a significantly higher frequency of NN genotype as compared to cases, whereas cases were found to be having a significantly higher frequency of NI genotype as compared to controls ($P = 0.016$). Further, the mutated allele frequency (which allele) was found to be higher among cases. Both cases and controls were found to follow Hardy-Weinberg equilibrium with respect to CBS 844INS68 polymorphism ($P < 0.05$) [Table 1].

Association of MTR A2756G, MTRR A66G and CBS 844INS68 polymorphisms with recurrent miscarriage

The OR analysis with respect to MTR A2756G polymorphism revealed that, AG, GG and AG + GG genotypes were found to pose a decreased risk for RM as compared to AA genotype (although no statistical significance). The OR with respect to MTRR A66G polymorphism revealed that AG, GG and AG + GG genotypes are causing a decreased risk for RM as compared to AA (not significant). The OR calculated to understand the risk of AA in RM revealed that AG and

AG + AA genotypes were posing a significant increased risk of more than 2.5 folds on RMs as compared to GG genotype. Furthermore, AA genotype was found to be posing an increased risk of 2.72 folds on RMs as compared to GG genotype although no statistical significance. The OR with respect to CBS 844INS68 genetic polymorphism was calculated considering the risk of NI heterozygotes against normal homozygotes, which was found to pose a significantly increased risk of 2.22 folds for RMs [Table 2].

Combined effects of the selected genetic polymorphisms using binary logistics in the causation of recurrent miscarriages

In the present study, the three genetic markers MTR A2756G, CBS 844INS68 and MTRR A66G (risk of AA) screened were analysed. CBS 844INS68 in association with MTRR A66G was posing a significantly increased risk of 2 folds for RMs ($P = 0.016$). Furthermore, CBS 844INS68 in association with MTR A2756G was found to pose 1.998 folds increased risk for RM, with suggestive P value (95% CI - 0.67–1.43, $P = 0.06$) [Table 2].

DISCUSSION

Despite years of research, miscarriage, especially RM continues to pose a medical challenge as its aetiology is still unclear. In the present study, the impact of MTR A2756G, MTRR A66G and CBS 844INS68 individually as well as in association with each other on RM is evaluated.

MTR A2756G and MTRR A66G polymorphisms were not following Hardy-Weinberg equilibrium for both RM cases and controls. This might be due to nonrandom collection of the samples with specific selection criteria.

Mutation in the MTR A2756G gene could lead to increase in the plasma homocysteine levels.^[21] MTR A2756G is a maternal risk factor and is found to be associated with recurrent pregnancy loss, birth of a child with Down syndrome and is a risk factor for breast cancer.^[22-25] A number of studies have reported the association of MTR A2756G polymorphism with RM.^[26] However, in the present study, the AG and GG genotypes of MTR A2756G polymorphism were not found to be associated with RM and this is in concordance with earlier studies.^[27,28] The variability in different results point towards the variability of the presence of the MTR A2756G polymorphism in different geographical regions.^[29]

It is worth noting that the MTHFR C677T gene polymorphism, which is an important genetic marker

Table 1: Distribution of methylenetetrahydrofolate-homocysteine methyltransferase A2756G, methylenetetrahydrofolate-homocysteine methyltransferase reductase A66G and cystathionine beta-synthase 844INS68 polymorphisms among recurrent miscarriage cases and controls

Genetic marker	Genotypes	Cases, n (%)	Controls, n (%)	Chi-square test
MTR A2756G (cases=200; controls=258)	AA	108 (54)	136 (52.71)	$\chi^2=0.03, P=0.986$
	AG	90 (45)	118 (45.74)	
	GG	2 (1)	4 (1.55)	
	HWE (P)	0.0003	0.0001	
	Allele frequency			
	A	0.76	0.76	
	G	0.24	0.24	
MTRR A66G (cases=198; controls=258)	AA	7 (3.54)	8 (3.10)	$\chi^2=5.99, P=0.049$
	AG	182 (91.92)	222 (86.05)	
	GG	9 (4.54)	28 (10.85)	
	HWE (P)	<0.0001	<0.0001	
	Allele frequency			
	A	0.49	0.46	
	G	0.51	0.54	
CBS 844 INS 68 (cases=194; controls=258)	NN	158 (81.44)	234 (90.70)	$\chi^2=8.24, P=0.016$
	NI	36 (18.56)	24 (9.30)	
	II	-	-	
	HWE (P)	0.15	0.43	
	Allele frequency			
	N	0.91	0.95	
	I	0.09	0.05	

*Source- The authors

Notes- Level of significance $P \leq 0.05$ **Table 2: Odds ratio w.r.t. methylenetetrahydrofolate-homocysteine methyltransferase A2756G, methylenetetrahydrofolate-homocysteine methyltransferase reductase A66G and cystathionine beta-synthase 844INS68 polymorphisms and the combined effects of the selected genetic polymorphisms in recurrent miscarriage**

Genetic polymorphism	Genotype	Cases, n (%)	Controls, n (%)	OR (95% CI)	P
MTR A2756G	AA	108 (54)	136 (52.71)	Reference	
	AG	90 (45)	118 (45.74)	0.96 (0.66–1.39)	0.82
	GG	2 (1)	4 (1.55)	0.63 (0.11–3.50)	0.60
	AG or GG	92 (46)	122 (47.29)	0.95 (0.66–1.38)	0.78
MTRR A66G (taking AA as referent)	AA	7 (3.54)	8 (3.10)	Reference	
	AG	182 (91.92)	222 (86.05)	0.94 (0.33–2.63)	0.90
	GG	9 (4.54)	28 (10.85)	0.37 (0.10–1.30)	0.12
	AG or GG	191 (96.46)	250 (96.90)	0.87 (0.31–2.45)	0.80
MTRR A66G (taking GG as referent)	GG	9 (4.54)	28 (10.85)	Reference	
	AG	182 (91.92)	222 (86.05)	2.55 (1.17–5.54)	0.02
	AA	7 (3.54)	8 (3.10)	2.72 (0.77–9.62)	0.12
	AG or AA	189 (95.45)	230 (89.15)	2.56 (1.18–5.55)	0.02
CBS 844INS68	NN	158 (81.44)	234 (90.70)	Reference	
	NI	36 (18.56)	24 (9.30)	2.22 (1.28–3.87)	0.0048

Combined effects of the selected genetic polymorphisms using binary logistic in the causation of RMs

Genetic polymorphism	MTR A2756G	CBS 844INS68	MTRR A66G
MTR A2756G	-	1.998 (0.67–1.43); $P=0.06$	0.983 (0.67–1.43); $P=0.932$
CBS 844INS68	1.998 (0.67–1.43); $P=0.06$	-	2.004 (1.13–3.53); $P=0.016$
MTRR A66G	0.983 (0.67–1.43); $P=0.932$	2.004 (1.13–3.53); $P=0.016$	-

*Source- The authors

Notes- Level of significance $P \leq 0.05$

in the one-carbon metabolic pathway, yielded a similar result of no association with RM in the same cohort.^[15]

In the case of MTRR A66G polymorphism, the frequency of heterozygotes AG was found to be the highest. The frequency of the MTRR A allele (wild type) is found to be 49% among cases and 54% among controls. Various studies have reported A as the ancestral allele and G as the risk mutant allele.^[20,30] The OR for the risk of GG genotype (present study) revealed that there is no association of the variant genotype with RM, which is in agreement with various studies.^[27,28,31,32] Although A is the ancestral allele, it was reported that the mutant allele was found to be reaching almost the same frequency as the ancestral allele and even going beyond in various studies done on the Indian population.^[33,34] The frequency of G allele is found to be more than that of A allele, thus showing some selective advantage for G allele and some selective disadvantage of A allele.

In addition, it was observed that the AG and AA + AG genotypes are posing a significant risk of about 2.5 folds for RM which is in agreement with the study by Popp *et al.*, 2009 in the European population.^[35] As the North Indians are reported to show a gradient of proximity to West Eurasians,^[36] the results above are quite justified in the case of the RM cases and controls belonging to the North Indian population. Further, the maximum number/frequency of heterozygotes observed could be attributed to selective advantage to the heterozygotes (AG) and the minimum number/frequency of ancestral genotype seems interesting and should be explored further.

In the present study, as well as various other studies, the homozygous mutant genotype of CBS 844 INS68 genetic polymorphism was absent.^[13,27] One of the reasons might be that the II genotype is lethal that it could not survive in the population. The heterozygotes NI was found to be causing an increased risk of more than 2 folds for RM. Very few studies have been conducted on the association of this polymorphism with RMs. No association between the insertion allele and RMs was reported among the Caucasian population^[13] and the South Korean population.^[27] On the contrary, a study conducted on the North Indian population found that the insertion allele was protective for recurrent pregnancy losses.^[37] This discrepancy in the association of insertion allele with RM could be attributed to differences in the ethnic backgrounds of the participants and also the sample size. Further, this is the first study to report the association of CBS 844INS68 genetic polymorphism with RM.

The combined effects of these genotypes revealed that insertion of CBS 844INS68 mutation was found to pose an increased risk for RM in combination with MTR

A2756G and MTRR A66G. Considering the genetic markers individually, MTRR A66G and CBS 844INS68 are causing a significantly increased risk for RM as against MTR A2756G polymorphism which was found to be posing no risk at all. Interestingly, CBS 844ins68 (I allele) mutation in association with MTRR A66G was found to pose about 2 folds significantly increased risk for RMs. Further, CBS 844ins68 (I allele) in association with MTR 2756G allele was elevating the risk by 2 folds for RM. Thus, CBS 844INS68 and MTRR A66G both independently as well as in association with each other were risk factors for RM.

CONCLUSION

The genetic polymorphisms, particularly MTRR A66G and CBS 844ins68 were found to make women susceptible for RM in the given North Indian population. However, large sample size studies are warranted to have a clinical significance of the selected markers for RM.

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Conflicts of interest

All the authors of this manuscript declare no conflict of interest regarding any issue.

Data availability statement

The data that support the findings of the study are available on request from the corresponding author.

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