



CBS 844INS68 AND MS D919G POLYMORPHISM: EARLY PREGNANCY LOSS RISK

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Abstract: Cystathionine beta synthase (CBS) and Methionine synthase (MS) maintains the level of homocysteine in blood. Our objective was to study the genetic association of CBS 844ins68 and MS D919G polymorphism with early pregnancy loss (EPL) and Recurrent pregnancy loss (RPL). We investigated 174 EPL patients in which 130 were RPL patients and 180 healthy controls. Genotyping was done through PCR-RFLP for MS D919G polymorphism whereas that for CBS 844ins68 was performed in duplex PCR reaction. Age adjusted odds ratios (AORs) were calculated by logistic regression analysis. The CBS 844ins68 was found to have protective effect rather than having risk for RPL (AORs = 0.49, 95% CI=0.22-1.08, p=0.07). The MS D919G polymorphism also did not differ significantly between patients and controls. This is the first study to see the association of CBS and MS with EPL and more study in ethnically different population is needed to confirm its association with EPL.

Keywords: Cystathionine beta synthase, Hyperhomocysteniemia, Methionine synthase, Recurrent pregnancy loss.

INTRODUCTION

Folate deficiency increases the risk of fetal loss through defective chorionic villi vascularization¹. Hyperhomocysteniemia in plasma is caused either by genetic defect in the enzyme involved in homocysteine metabolism or by nutritional deficiency². Hyperhomocysteniemia leads to autooxidation and hydrogen peroxide generation which damages the endothelium and causes vascular injury and is considered as risk factor for recurrent pregnancy loss³⁻⁵.

Homocysteine is a sulfhydryl amino formed by the catabolism of methionine. Homocysteine can either be converted to cystathionine by the enzyme CBS (the transsulfuration pathway), or it can be methylated to form methionine by the enzyme MS (the remethylation pathway), using 5, methyltetrahydrofolate as a methyl donor, which is converted from 5, 10 methylene tetrahydrofolate by the enzyme 5, 10 methylene tetrahydrofolate reductase (MTHFR). Transsulfuration of homocysteine catalyzed by CBS lead to the production of cystathione.

The CBS 844ins68 polymorphism was firstly reported as a novel mutation in an Italian patient with classic homocystinuria due to CBS deficiency⁶. The patient was heterozygous (I/N) for the mutation. Insertion (I allele) previously thought to cause premature termination codon in the CBS mRNA, is now known to have normal size mRNA^{6,7}. Moreover, I allele

is found to be associated with reduced level of plasma homocysteine and subsequently lowered risk of developing atherosclerotic and/or thrombotic diseases^{8,9}. MS maintain the adequate intracellular methionine and normal homocysteine concentration by remethylating homocysteine to methionine. D919G a missense mutation in the MS gene is found to be common in the general population and leads to mild hyperhomocysteinaemia¹⁰. Folate metabolizing genes play an imperative role in early pregnancy establishment and maintenance. Hence we hypothesized that nucleotide variations in CBS and MS may affect the outcome in pregnant women, particularly the risk of miscarriage.

We had previously studied the effect of the MTHFR C677T polymorphisms in an Indian cohort and found that the homozygosity for the MTHFR C677T polymorphism confers a 6.3-fold increased risk of idiopathic RPL¹¹. In the present study we investigated CBS 844ins68 and MS D919G polymorphisms of folate metabolic pathway both alone and in combination to see its association with early pregnancy loss.

MATERIALS AND METHODS

Early pregnancy loss is defined as miscarriage before twelve weeks of gestation and recurrent pregnancy loss is defined as three or more consecutive early pregnancy losses (before 12 weeks' gestation) after conceiving from the same partner. In a hospital based case-control study, we recruited 191 EPL women

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who had miscarriages before 12 weeks of gestation as cases and 180 healthy fertile women with successful pregnancy outcome and no history of pregnancy related complications as controls. The patients were enrolled between July 2009 and March 2012. Patients and controls were recruited from Out Patient Department of the University Hospital, Department of Obstetrics and Gynaecology, Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, India. Questionnaire was filled up for each patient to record details of their lifestyle, habits and family history. Informed consent was obtained from all participants before their enrollment in the study. Women having prior miscarriage were investigated by a routine work up, including testing for chromosomal aberrations, uterine abnormalities, hormonal status, positive lupus anticoagulant or anticardiolipin antibodies or TORCH. Patients who experienced miscarriage for first time were analyzed only for chromosomal abnormality. 17 patients due to anatomic, hormonal, chromosomal, infectious, autoimmune, or thrombotic causes were excluded after standardized clinical and laboratory evaluation. Among seventeen patients, four represented polycystic ovaries, three were anticardiolipin antibodies positive, one was both lupus anticoagulant and anticardiolipin antibodies positive, four were TORCH positive, one with arcuate uterus, one with septate uterus and one had hypothyroidism. Karyotype analysis revealed two patients with abnormal numerical chromosome complement. Two patients had Mosaic (46, XX and 45, XO) karyotype. After exclusion of 17 patients with above mentioned known causes of abnormality, genotype analysis was conducted on remaining 174 patients and all 180 controls. Among 174 cases thus selected, 130 had at least three prior miscarriages (RPL group), 33 had two prior miscarriage and 11 experienced miscarriage for the first time. All the subjects included in this study belong to the eastern Uttar Pradesh province of North India and fall within the same ethnic group. Characteristic of patients and controls are presented in Table.1. Approval of the University's Ethical Committee for research on human material was obtained.

Table.1: Characteristic of RPL patients and Controls group

S.No	Parameters	Patients	Controls
1	Mean age	26.52(±4.17)	30.64 (±4.56)
2	Ethnicity	North Indian	North Indian
3	Previous miscarriages	Mean 4 (3-7)	N.A
4	No. of live births	0	2
5	Smokers	None	None
6	Alcohol consumers	None	None

Genotyping for CBS844ins68, D919G MS polymorphisms:

Genotyping of MS D919G was done using polymerase chain reaction (PCR) for amplification

followed by restriction fragment length polymorphism (RFLP) whereas of CBS 844ins68 was performed in duplex PCR reaction systems. In each reaction, 50 ng of genomic DNA was amplified in 1X of PCR buffer containing 200µM dNTPs and their respective forward and reverse primers containing 0.5U of Taq DNA polymerase. All reactions were conducted in an oil-free thermal cycler (Thermal Cyclers, BioRad Inc.) The amplified products were electrophoresed on 2% agarose gels containing ethidium bromide and the product bands were visualized under ultraviolet light. The presence of functional CBS 844ins68 gene was determined by a band of the expected size. Variant allele of MS was characterized by a gain of restriction site upon digestion with HaeIII. (Figure.1).

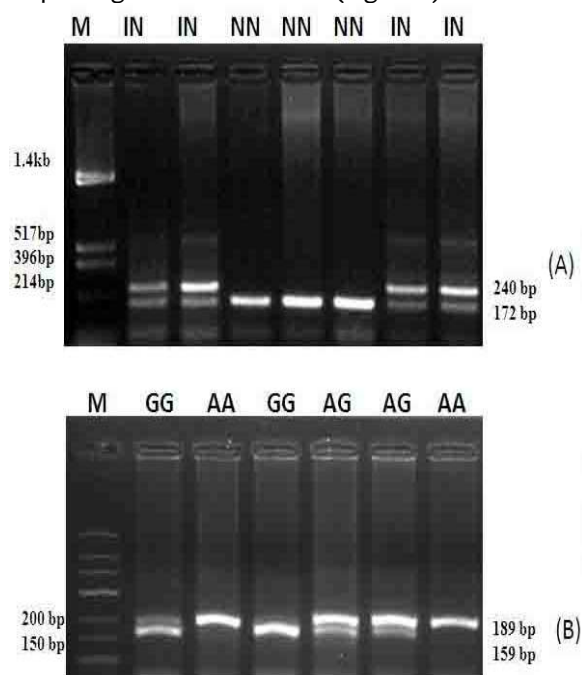


Figure.1. Genotype patterns of the four polymorphisms displayed by agarose gel electrophoresis. M - pUC/HinfI marker for A, 100 bp DNA ladder for B (A) Genotype patterns of the CBS 844ins68 polymorphism. Lane 2, 3, 7 & 8, I/N genotype; lanes 4 to 7, N/N genotype. (B) Genotype patterns of the MSD919G SNP. Lane 3 & 7, A/A genotype; lane 5 & 6, A/G genotype; and lane 2 & 4, G/G genotype.

Statistical analysis:

Statistical significance of the differences in the frequency of genotypes using the chi-square test, crude odds ratio (ORs) at 95% confidence interval (95 % CI) were calculated to assess the relative risk conferred by the mutant genotype. In addition, unconditional logistic regression was done to calculate age adjusted odds ratios (AORs). Logistic regression analysis and calculation of confidence intervals was done using logistic regression online statistical calculator (www.statpages.org/logistic.html). Power of study was calculated using G* power¹². All statistical

tests were two-sided, and $P < 0.05$ was considered statistically significant. The allele frequencies of all genotypes were in Hardy-Weinberg equilibrium.

RESULT

Association of CBS 844ins68, MS D919G, polymorphism with RPL:

For 68 base pair insertion CBS polymorphisms, Insertion allele was found to be more in controls compared to cases however no significant association was found with EPL or RPL group. There was no homozygous state of the insertion allele (I/I genotype) for the CBS 844ins68 polymorphism in the 354 individuals tested. As the study population groups differed in age, AORs was calculated. The calculated AORs of the CBS 844ins68 versus the wild NN

genotypes is given in Table 2. In contrast, MS D919G the frequency of homozygote GG were 6.9% and 7.22%, and that of heterozygote was 42.53% and 38.89% in patients and controls respectively in EPL group. The comparison of genotype distribution and allele frequencies showed no significant difference between EPL & RPL patients and the controls (Table 2). The genotype frequencies for all the polymorphisms were in agreement with the Hardy-Weinberg equilibrium in patients as well in controls. Combined analysis of CBS 844ins68 and MS D919G also found no significant association with EPL & RPL. No statistically significant interaction of genotype with the confounding factors age was observed (data not shown).

Table.2: Genotype and allele frequencies of the CBS 844ins68 and MS D919G polymorphisms among the cases and controls and their associations with risk of EPL & RPL

		Cases	Controls	Crude Odds Ratio (95%CI)	p-value	Adjusted Odds Ratio (95%CI)	p-value	
EPL	CBS 844ins68	NN	154/174	154/180	1.0			
		NI	20/174	26/180	0.77 (0.41-1.43)	0.40	0.78 (0.41-1.48)	0.45
		RPL	CBS 844ins68	NN	120/130	154/180	1.0	
NI	10/130	26/180		0.51 (0.25-1.04)	0.06	0.49 (0.22-1.08)	0.07	
EPL	MS D919G	DD	88/174	97/180	1.0			
		DG	74/174	70/180	1.16 (0.75-1.79)	0.49	1.18 (0.75-1.85)	0.45
		GG	12/174	13/180	1.01 (0.44-2.34)	0.96	1.09 (0.46-2.57)	0.83
		RPL	MS D919G	DD	64/130	97/180	1.0	
DG	57/130	70/180		1.17 (0.73-1.88)	0.38	1.21 (0.74-1.96)	0.43	
GG	9/130	13/180		1.00 (0.40-2.47)	0.91	1.07 (0.42-2.69)	0.87	

The difference in frequencies between the case and control groups was analyzed for statistical significance at the 95% confidence interval using chi square test. Odds ratios (ORs) were calculated and reported within the 95% confidence limits. Unconditional logistic regression was done to adjust for age #Adjusted for age groups, *statistically significant ($P < 0.05$).

DISCUSSION AND CONCLUSION

Hyperhomocysteinemia is an independent risk factor for recurrent pregnancy loss¹. Hyperhomocysteinaemia leads to the generation of reactive oxygen species and damages the vascular endothelium resulting in placental vasculopathy and endothelial dysfunction¹³⁻¹⁵. Studies both in vivo and in vitro have found alter activity of many clotting proteins on the endothelial cell surface^{16,17}. In our previous study, we found significant association of *MTHFR* 677 T mutant allele with RPL¹¹, which is supported by several others^{18,19} including a meta-analysis²⁰. In the present study, we investigated case-control association of CBS 844ins68, MS D919G, gene polymorphism and risk of pregnancy loss. This is the first study to see the

association of CBS 844ins68 and MS D919G with pregnancy loss.

CBS and MS act in concert with *MTHFR* and are the key enzymes regulating plasma homocysteine level. In the present study, the I allele for the CBS 844ins68 polymorphism was only present as the I/N heterozygous genotype in the 474 individuals. A previous study showed that the insertion was found to be more in controls compared to patients in Portland population²¹. However, the difference was not statistically significant hence no association with the coronary artery disease was found. Since CBS I allele is found to have reduced level of plasma homocysteine level it might be having protective effect rather than

having risk^{6,22}. As previously studied in both American and Japanese population MS D919G mutation was found to be common and in our population also it seems to be prevalent^{10,23}. Thus apparently it seems to be unaffected by ethnic differences.

CONCLUSION

The present study signifies the CBS 844ins68 as protective effect for RPL however the difference was statistically non-significant. This is the first study to see the association of CBS and MS with pregnancy loss risk and further studies in ethnically different population is needed to validate its role with EPL.

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