Relation between *MTHFR* (Methylenetetrahydrofolate Reductase) gene functional polymorphisms and magnitude of Recurrent Pregnancy Losses (RPL) among women with low socio-economic status in Asian Indians

Dhatri Madduru[1], Supriya K[2], Kethora Dirsipam[3], Prasanna Latha Komaravalli[2], Syed Sayeeduddin[4] and Parveen Jahan[2, 4]

[1]Department of Biochemistry, Osmania University, Hyderabad, India

[2]Department of Genetics, Osmania University, Hyderabad, India

[3]Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, India

[4]Department of Zoology, School of Sciences, Maulana Azad National Urdu University, India

Corresponding author: Parveen Jahan

E-mail: dr.pjahan@gmail.com

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ABSTRACT.

The present study was intended to investigate the role of *MTHFR* gene functional polymorphisms, rs1801133 (C677T) and rs1801131 (A1298C) in the susceptibility and severity of Recurrent Pregnancy Losses (RPLs) in women with low socio-economic status among Asian Indians. We screened a total of 221 women that includes 102 RPL patients and 119 healthy controls. Following DNA isolation from whole blood, PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism) was carried out for genotyping the selected polymorphisms. Distribution of allele, genotype frequencies and Hardy-Weinberg equilibrium of patient and control groups were analyzed using statistical tools. Results exhibited that women with CC genotype of rs1801131 polymorphism were at 2.5 fold risk towards RPL. With respect to rs1801133 polymorphism we found no significant association

with RPL. However, stratified data based on the number of pregnancy losses (≤ 3 and >3) showed that patients with CT genotype at this locus and having more than 3 pregnancy losses were identified to be nearly at 4-fold increased risk for the disease. Additional analysis on combined genotype of both the polymorphisms revealed that subjects with CC-CC combination showed a 2.8-fold risk towards RPL, while patients with CT-CC combination were at 4.5-fold increased risk to experience >3 pregnancy losses. In conclusion, selected polymorphisms of *MTHFR* gene have different roles with respect to susceptibility and magnitude of RPL. To the best of our knowledge this is the first study emphasizing on the association of polymorphisms with increased magnitude of RPL in women with low socio-economic status among Asian Indians.

Keywords: Methylene tetrahydrofolate reductase (*MTHFR*); Recurrent pregnancy loss; Polymorphisms; Magnitude; Low socio-economic status.

INTRODUCTION

Recurrent pregnancy loss (RPL) is an obstetric complication observed in women which is characterized by two or more consecutive losses of fetal product before 20 weeks of gestation. It is affecting approximately 15-20% of clinically recognized pregnancies (Shiny et al., 2015). Pregnancy outcome is influenced by maternal genetic constitution and environmental factors such as lifestyle, nutritional status, inter-pregnancy interval, smoking, alcohol consumption, coffee consumption, stress, physical activity, socio-economic status etc. (Speroff et al., 1999; Seo and Sang, 2009). During pregnancy, fetal development is associated with rapid cell division and nucleic acid synthesis which is accomplished vitally by folate (vitamin B9) which also acts as a substrate for various enzymatic reactions involved in amino acid synthesis and vitamin metabolism (James et al., 2011).

Folate deficiency results in hyperhomocysteinemia, a condition where elevated levels of homocysteine that can be detected in plasma which is detrimental for fetus (Theresa and William, 2000). It could be due to low dietary folate intake or owing to defects in genes involved in folate metabolism such as Methylenetetrahydrofolate reductase (*MTHFR*) located in the short arm of chromosome 1 (1p36.3). This enzyme catalyzes the conversion of 5, 10-methylenetetrahydrofolate (5,10-MTHF) into 5-methyltetrahydrofolate (5-MTHF), which provides the single-carbon for homocysteine in methionine synthesis (Kobashi et al., 2005; Wu et al., 2012). The most common genetic polymorphisms of *MTHFR* gene are (rs1801133 (C677T) and rs1801131 (A1298C)) which have been extensively addressed in various human diseases such as neural tube defects (Steegers et al., 1992), Down's syndrome (Cyrus et al., 2009), cardio- vascular disorders (Kluijtmans et al., 1996), including Recurrent Miscarriages (Quere et al., 1998). The rs1801133 (C677T) is a non-synonymous substitution of alanine to valine in the protein as a result of transition in exon-4 that renders the enzyme, thermolability and decreased activity.

On the other hand, rs1801131 (A1298C) polymorphism is a result of missense mutation in exon-7 due to a transversion which lead to a substitution of glutamate with alanine, without overtly affecting enzyme activity (Van der put and Blom, 2000). Association of these polymorphisms in RPL have been reported previously from various population groups from India (Saraswathy et al., 2012), however, there were no studies in view of the severity or magnitude of RPL. Hence, the present study was intended to investigate the influence of *MTHFR* gene functional polymorphisms with respect to susceptibility and the magnitude of RPL among women with low socio-economic status.

MATERIALS AND METHODS

Study design

For the present study, a total of 221 subjects that include 102 patients and 119 controls belonging to low socio-economic status were enrolled. The diagnosis of women with RPL was made based on documented history of at least two or more spontaneous, consecutive miscarriages before 20 weeks of gestation. However, women with induced abortions and abortions due to any other known complications were excluded from the study (Shiny et al., 2015). Women having completed at least two successive pregnancies and no history of spontaneous miscarriages, were randomly selected as control group. The patients were recruited from Modern Government Maternity Hospital, Petlaburj, Hyderabad, India and Sri Venkata Sai (SVS) Hospital, Mahbubnagar, India whereas, the controls from health clinics. We recruited all the subjects following Udai Pareek revised scale (Singh et al., 2017).

The clinical and general characteristic aspects of the study group were collected using a structured questionnaire. The social aspects included consanguinity, nutrition (good and poor eating habits) and toddy consumption (intake and non-intake) (a locally available alcoholic drink). Clinical characteristics included hemoglobin (Hb) levels (≤ 11g/dl and >11g/dl), age at marriage (≤ 18years and >18 years), age at first conception (<18 years and ⩾18 years), menstrual status (regular and irregular), type of abortion (primary and secondary aborters), number of abortions (≤ 3 and >3) and Body Mass Index (BMI) (normal 18-23 kg/m2 and abnormal <18 &>23 kg/m2). The familial characteristics included family history of abortions and common health complications such as diabetes, hypertension etc. Blood samples were collected after obtaining written informed consent from the study participants. The study got approval from the Institutional Ethical Committee, Osmania University, Hyderabad, India.

MTHFR Genotyping

Genomic DNA was isolated from blood samples using standard established protocol in our lab (Tippisetty et al., 2011). PCR-RFLP was carried out for *MTHFR* gene polymorphisms using specific primers, FP (Forward Primer) 5'-TTTGAGGCTGACCTGAAGCACTTGAAGGAG-3' and RP (Reverse Primer) 5'-GAGTGGTAGCCCTGGATGGGAAAGATCCCG-3' for rs1801133 (Ramadevi et al., 2004) and FP 5'-CTTTGGGGAGCTGAAGGACTACTAC-3' and RP 5'-CACTTTGTGACCATTCCGGTTTG-3' for rs1801131 (Bioserve Biotechnologies, Hyderabad, India).

Amplification reactions were carried out in a Thermocycler (Eppendorf, USA Scientific Inc.) with a final volume of 25 µL reaction mixture, containing 100 ng of genomic DNA, 10pM of each primer, 2.0 mM of dNTPs (Lab pro, India), 1.5 mM of $MgCl_2$, 10X PCR buffer and 0.5U Taq DNA polymerase (Lab pro, India). PCR conditions comprised an initial denaturation step at 95ºC for 5 minutes, followed by 30 cycles of 94ºC for 30 seconds, annealing temperature of 65ºC (rs1801133) and 57ºC (rs1801131) for 45 seconds, and extension at 72ºC for 45 seconds. The final extension was at 72ºC for 5 minutes and holds at 4ºC.

The Oligonucleotide primers generated a PCR product of 173 base pair (bp) and 163 bp for rs1801133 and rs1801131 polymorphisms respectively. The PCR products were digested by using Hinf I for C677T and MboII for A1298C for one and a half hours in a dry bath (Genei, India). The digested products were then separated on 10% PAGE (Poly Acrylamide Gel Electrophoresis) and stained with ethidium bromide and observed under gel documentation system (Bio-Rad, India). For the C677T polymorphism, the substitution of T allele at nucleotide position 677 creates a Hinf I restriction site with subsequent cleavage of the original 173 bp PCR product into 125 and 48 bp fragments. The A1298C mutation abolishes the restriction site of MboII, resulting in the merger of 56 and 28 bp fragments into 84 bp fragment (Figures 1A and 1B).

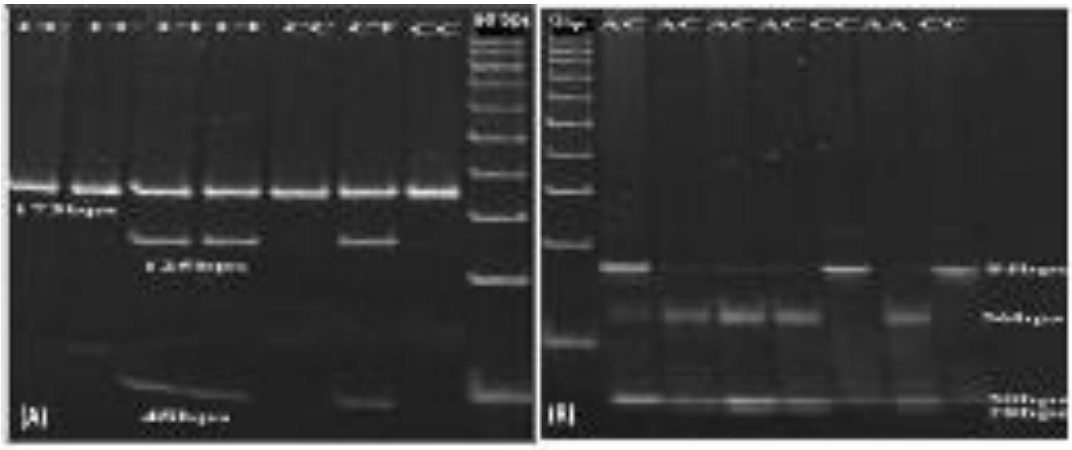


Figure 1: A) PCR products of *MTHFR* C677T fragment digested with Hinf- I restriction enzyme. C677T mutation analysis. Lane 1, 2, 5 & 7, CC (normal homozygote); lane 3,4 & 6, CT (heterozygote) and lane 8, 50 bp DNA ladder. B) PCR products of *MTHFR* A1298C fragment digested with Mbo- II restriction enzyme. A1298C mutation analysis. Lane 1, 50 bp DNA ladder; lane 2,3,4 & 5, AC (heterozygote); lane 6 & 8, CC (mutant homozygote) and lane 7, AA (normal homozygote).

Statistical Analysis

Distribution of allele, genotype frequencies and Hardy-Weinberg equilibrium (HWE) of patient and control groups were analyzed by $\chi 2$ test. Risk estimation was calculated by Odds ratio (OR) with 95% confidence intervals (CI) using online calculator. Data analysis was carried out by SPSS version 18.0 (spss.en.softonic.com), Chicago, USA wherever required. Statistical significance was defined as a two-sided p-value < 0.05. We also performed analysis on genotype combinations using online tools.

RESULTS

The present case-control study includes 102 women with RPLs and 119 normal healthy fertile women. All RPL women were in the age range of 17 to 40 years and the number of miscarriages ranging from 2 to 10. A total of 77 (76%) women were primary aborters i.e., those who have lost all previous pregnancies, and remaining were secondary aborters i.e., those who have had at least one successful pregnancy. Women in the control group were in the age range of 18 to 52 years with no history of miscarriage. The clinical and general characteristics of patient and control groups were summarized in the Tables 1 and 2 respectively. The mean BMI, age at marriage and age at first conception were significantly differed between the two groups ($p<0.05$). Ten percent of patients showed family history of abortions, however none from the control group. It can be highlighted that 13% of the patients were with higher magnitude of pregnancy losses (i.e., >3).

Table 1: Clinical features of the study group.

Features	Patients (102)	Controls (119)	p-value
BMI (kg/m^2)	21.64 ± 3.49	20.21 ± 2.65	< **0.01**
Hb (g/dl)	9.78 ± 1.23	9.43 ± 1.45	0.052
Age at marriage (years)	19.12 ± 3.76	16.76 ± 2.76	< **0.01**

Age at first conception (years)	20.09 ± 3.77	18.20 ± 2.82	**< 0.01**
No. of abortions	2.73 ± 1.05	-	-

Table 2: General Characteristics of the study group

Category	Patients (N=102)	Controls (N=119)
Hb levels (g/dl)		
≤ 11	92 (90%)	113 (95%)
>11	10 (10%)	6 (5%)
Age at marriage (in years)		
≤ 18	38 (37%)	66 (55%)
>18	64 (63%)	53 (45%)
Age at first conception (in years)		
< 18 (13-17)	23 (23%)	40 (34%)
≥ 18 (18-34)	79 (77%)	79 (66%)
Menstrual status		
Regular	91 (89%)	117 (98%)
Irregular	11 (11%)	2 (2%)
Consanguinity		
Yes	27 (26%)	31 (26%)
No	75 (74%)	88 (74%)
Nutritional status		
Good	88 (86%)	112 (94%)
Poor	14 (14%)	7 (6%)
Type of abortion		
Primary aborters	77 (76%)	0 (0%)
Secondary aborters	25 (24%)	0 (0%)
Number of abortions		
≤ 3	89 (87%)	0 (0%)
>3	13 (13%)	0 (0%)
Health complications		
Yes	9 (9%)	0 (0%)
No	93 (91%)	119 (100%)
Family history of abortions		
Yes	10 (10%)	0 (0%)
No	92 (90%)	0 (0%)
Social habits (toddy)		
Intake	10 (10%)	24 (20%)
Non- intake	92 (90%)	95 (80%)
BMI (kg/m^2)		
<18 and >23 (Low and high)	53 (52%)	45 (38%)
18-23 (Normal)	49 (48%)	74 (62%)

MTHFR gene polymorphisms

The genotype and allele frequencies for C677T (rs1801133) polymorphism did not differ significantly between patients and controls ($p>0.05$). On the other hand, for A1298C (rs1801131) polymorphism, the incidence of genotype and allelic classes displayed a significant variation between RPL patients and controls ($p<0.05$). The genotype distribution of both patient and control groups deviated from HWE ($p<0.05$) for C677T polymorphism whereas, it is in accordance with HWE ($p>0.05$) for A1298C polymorphism (Table 3).

Table 3: Allele and Genotype frequency distribution of *MTHFR C677T* and *A1298C* polymorphisms among RPL patients and controls

MTHFR C677T	**Control *vs.* Patient groups**		***MTHFR C677T***	**Control *vs.* Patient groups**
	Controls (N=119) n (%)	**Patients (N=102) n (%)**	**Comparison of groups**	**OR (95% CI)**
CC	76 (64)	68 (67)	***CC vs. CT***	1.13 (0.64-1.97)
CT	43 (36)	34 (33)		
TT	0 (0)	0 (0)	***CT vs. CC***	0.88 (0.50 –1.54)
X^2	0.19			
C	0.82	0.83	***C vs. T***	1.07 (0.516-2.223)
T	0.18	0.17		
X^2	0.03		***T vs. C***	0.93 (0.44-1.93)
HWE	**5.78***	**4.08***		
MTHFR A1298C	**Control *vs.* patient groups**		***MTHFR A1298C***	**Control *vs.* Patient groups**
	Controls (N=119) n (%)	**Patients (N=102) n (%)**	**Comparison of groups**	**OR (95% CI)**
AA	47 (40)	21 (21)	***AA vs. AC+CC***	**0.39* (0.21-0.72)**
AC	53 (44)	48 (47)		
CC	19 (16)	33 (32)	***AC vs. AA+CC***	1.10 (0.65-1.88)
X^2	**12.72***			
A	0.62	0.44	***CC vs. AA+AC***	**2.51* (1.32-4.78)**
C	0.38	0.56		
X^2	**6.50***		***A vs. C***	**0.48* (0.27-0.84)**
HWE	0.38	0.21	***C vs. A***	**2.08* (1.18-3.65)**

* p-value is statistically significant i.e., <0.05; NS: Not Significant.

Stratified data based on the magnitude of pregnancy losses (i.e., ≤ 3 or >3) revealed a significant difference with respect to genotype and allele frequencies of C677T polymorphism whereas, no association was observed for either genotypes or allelic classes of A1298C polymorphism (Table 4).

Table 4: Allele and Genotype frequency distribution of *MTHFR C677T* and *A1298C* polymorphisms in severity among RPL patients (≤ 3 and > 3)

MTHFR C677T	**≤ 3 *vs.* >3 RPL groups**		***MTHFR C677T***	**≤ 3 *vs.* >3 RPL groups**
	≤ 3 (N=89) n (%)	**>3 (N=13) n (%)**	**Comparison of groups**	**OR (95% CI)**
CC	63 (71)	5 (39)	***CC vs. CT***	**0.25* (0.07-0.86)**
CT	26 (29)	8 (61)		
TT	0 (0)	0 (0)	***CT vs. CC***	**3.87* (1.15-12.9)**
X^2	**5.33***			
C	0.85	0.69	***C vs. T***	**0.39* (0.19-0.78)**

T	0.15	0.31		
X^2	**7.23***		***T vs. C***	**2.54* (1.27-5.09)**
HWE	2.60	2.56		
MTHFR A1298C	**≤ 3 *vs.* >3 RPL groups**		***MTHFR A1298C***	**≤ 3 *vs.* >3 RPL groups**
	≤ 3 (N=89) n (%)	**>3 (N=13) n (%)**	**Comparison of groups**	**OR (95% CI)**
AA	18 (20)	3 (23)	***AA vs. AC+CC***	1.18 (0.29-4.75)
AC	45 (50)	3 (23)		
CC	26 (30)	7 (54)	***AC vs. AA+CC***	0.29 (0.07-1.13)
X^2	3.99			
A	0.46	0.35	***CC vs. AA+AC***	2.82 (0.86-9.22)
C	0.54	0.65		
X^2	2.51		***A vs. C***	0.63 (0.35-1.11)
HWE	0.03	3.12	***C vs. A***	1.58 (0.89-2.79)

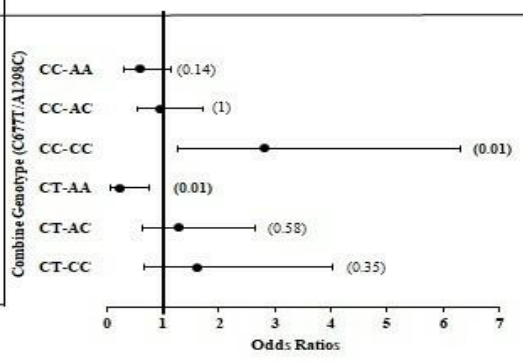
* p-value is statistically significant i.e. <0.05; NS: Not Significant.

Combined Genotype Analysis

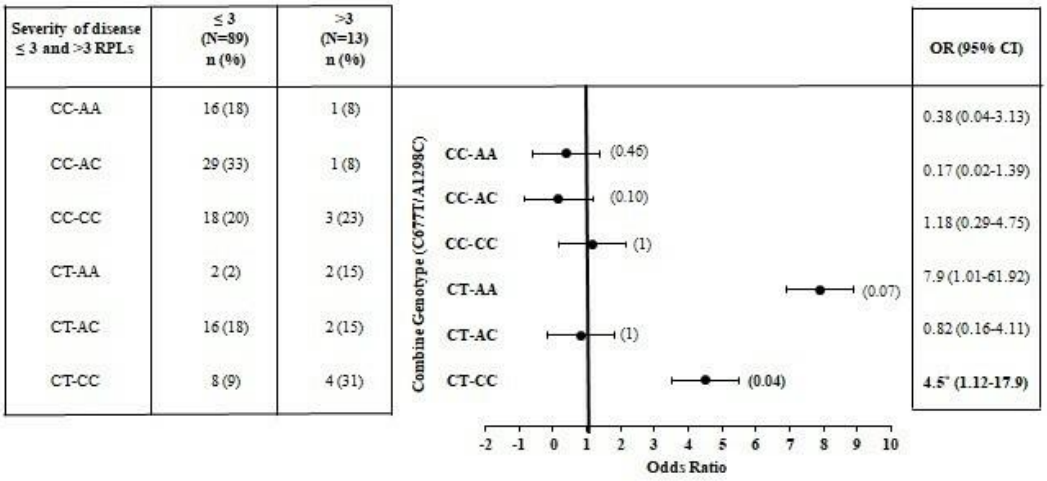
In the combined genotype analysis, CC-CC genotype emerged as a significant risk genotype combination (OR 2.82, 95% CI 1.26-6.32, p<0.05), while CT-AA genotype appeared to be an appreciable protective combination (OR 0.24, 95% CI 0.07-0.75, p <0.05) against RPL. Considerable elevated frequency of CT-CC genotype combination was seen in women with >3 pregnancy losses compared to women with ≤ 3 pregnancy losses (31% *vs.* 9%) (OR 4.5, 95% CI 1.12-17.9, p<0.05) (Figures 2A and 2B).

Overall results reflect that, *MTHFR C677T* polymorphism has no significant effect on disease vulnerability but were predisposed to have higher magnitude of pregnancy losses (>3) with nearly a five-fold risk. In contrary, A1298C polymorphism has a link with RPL susceptibility but not with respect to magnitude of the RPL.

Risk Association	Controls (N=119) n (%)	Patients (N=102) n (%)	OR (95% CI)
CC-AA	30 (25)	17 (16.5)	0.59 (0.3-1.15)
CC-AC	36 (30)	30 (29)	0.96 (0.53-1.71)
CC-CC	10 (9)	21 (20.5)	**2.82* (1.26-6.32)**
CT-AA	17 (14)	4 (4)	**0.24* (0.07-0.75)**
CT-AC	17 (14)	18 (18)	1.28 (0.62-2.64)
CT-CC	9 (8)	12 (12)	1.62 (0.65- 4.04)



(A) Forest plot representing the distribution of genotype combination effect of both *MTHFR* C677T and A1298C polymorphisms in Recurrent Pregnancy Loss (RPL) Cases and controls towards susceptibility to the disease.



(B) Forest plot representing the distribution of genotype combination effect of both *MTHFR* C677T and A1298C polymorphisms in patients with ≤ 3 and >3 Recurrent Pregnancy Losses (RPLs) contributing towards severity of the disease.

* p-value is statistically significant i.e. <0.05; NS: Not Significant.

Figure 2: Graphical representation of distribution of combined effect of both *MTHFR* C677T and A1298C polymorphisms in Recurrent Pregnancy Loss (RPL) Cases and controls and severity of disease.

DISCUSSION

MTHFR is a critical enzyme for the production of 5-MTHF, a circulating form of folate that is vital for re-methylation of homocysteine to methionine and finally to S-adenosyl methionine (SAM), a major methyl group donor to biomolecules such as DNA which is synthesized at a high rate during embryonic development. These methyl groups are essential for appropriate methylation patterns, failing which can adversely affect embryogenesis, leading to developmental malformations like defective chorionic villous vascularization. Nutritional basis of folate deficiency or the genetic mutations in the *MTHFR* gene or both the factors may cause the elevated homocysteine, a risk factor for thrombosis in placenta and impaired endothelial vasomotor function consequently disquieting implantation leading to early pregnancy loss. It shows that methylation is sensitive to both varying environment and genetic changes or modified by gene-environment interactions which may significantly contribute to embryonic development (Mohamed et al., 2014; Wael et al., 2016).Two most studied polymorphisms i.e. *C677T* (NCBI SNP (Single Nucleotide Polymorphism) ID: rs1801133) and A1298C (NCBI SNP ID: rs1801131) were well linked with the activity of *MTHFR* which were shown to impact several diseases including adverse pregnancy outcomes. However, there is a scantiness of reports with respect to these polymorphisms and magnitude of pregnancy losses particularly among women with low socio-economic status. Hence, the present case-control study is an attempt to investigate the relationship between *MTHFR* gene polymorphisms, *C677T* and *A1298C* with the severity of RPL in Asian Indians. Our study showed lack of association of *C677T* polymorphism with RPL, which corroborated with reports from India as well as Polish and Chinese populations (Ren and Wang, 2006; Seremak et al., 2010; Saraswathy et al., 2012).

Very low frequency of mutant genotype (TT) was reported from different parts of India (Anil Kumar et al., 2018; Vandana Rai 2016; Seerat et al., 2012; Saraswathy et al., 2012) however, the complete absence of this genotype (TT) was observed in the present study suggesting the severe pathogenic nature of this polymorphism in homozygous condition. In contradiction to the above reports, Wang et al., (2004) reported a significant decrease in

the frequency of CC genotype and increase in TT genotype in unexplained recurrent spontaneous abortion patients compared to controls in Chinese population (Wang et al., 2004). The difference in the results could be due to different environmental factors and ethnicity.

The second polymorphism A1298C was shown to be allied with altered distribution of intracellular folate metabolites and its concentration (Cande et al., 2007). With respect to this single nucleotide variant, the AA genotype and A allele showed a significant protective role against RPL. Whereas, the homozygous CC genotype and C allele revealed a 2.5- and 2.08-fold increased risk towards RPL respectively. A meta-analysis conducted based on electronic data base by Nair et al., (2013) reported an association of both alleles and genotypes of A1298C variant with RPL susceptibility (Nair et al., 2013). Similar studies from North India and other populations also show a link between A1298C polymorphism and pregnancy losses (Mtiraoui et al., 2006;Amira et al., 2008;Fatemeh et al., 2013; Farah et al., 2013). However, studies from Chinese, Egyptian and Iranian populations showed no significant association of A1298C polymorphism with RPL (Wang et al., 2004; Seremak et al., 2010; Settin et al., 2011; Yunlei et al., 2012; Amin et al., 2014).

When we investigated the joint effect of the two SNPs (Figures 2A and 2B), the wildtype CC of C677T and the mutant homozygote CC of A1298C, CC-CC frequency was extremely high among the patients compared to controls (20.5% *vs.* 9%) thus was contributing to nearly 3-fold elevated risk to develop the disease phenotype. This can be explained by the dominant effect of CC of A1298C polymorphism over CC of C677T polymorphism in genetic predisposition of RPL. On the other hand, the frequency of CT-AA is higher among controls than the patients suggesting the protective role of this combined genotype against RPL. In support of our results, Zetterberg et al., showed a strong protective role of CC-AA and CT-AA genotype combinations against RPLs (Zetterberg et al., 2002).

Studies conducted on the plasma levels by Lynn and Jesse reported that there is up to 70% reduction in enzyme activity in homozygous mutants of C677T variant, while only 35% reduction for heterozygotes compared to wild type. They further showed that the C677T variant was associated with elevated plasma homocysteine concentrations and interact with plasma folate, which was not observed in the case of A1298C polymorphism (Lynn and Jesse, 1999). It was suggested that activity of compound heterozygote (CT-AC) is as similar to that of the activity of TT homozygote of C677T polymorphism (Van der put et al., 1998). In addition, studies reported that individuals with heterozygous genotype (CT-AC) at both loci experience an intermediate loss of enzyme activity i.e. 40–50% (Muralidhara Rao et al., 2010). Though the present study did not have results on enzyme activity, the 4% increase in the frequency of CT-AC combination in patients compared to controls might be in support of above results. On the other hand, interesting observations were made when magnitude of disease was evaluated in relation to genotypes of the two SNPs. The CT genotype of C677T polymorphism exhibited a 4-fold (OR 3.87, 95% CI 1.15-12.9, p-value <0.05) increased risk for RPL women to experience >3 abortions which suggests an increase in the magnitude of pregnancy losses.

Though there is no significant risk association was found in the case of A1298C polymorphism, the frequency of mutant variant CC is raised to 54% among women with >3 pregnancy losses over women with less than 3 pregnancy losses. With regard to the magnitude of pregnancy losses, patients with CT-CC combination were significantly at higher risk for more than 3 pregnancy losses. Our observations were similar to the studies conducted by Saraswathy et al., who explained the selective disadvantage of presence of more than two mutant alleles in the fetal viability (Saraswathy et al., 2012).

As both the *MTHFR* polymorphisms are associated with reduced function and activity, the detrimental effect of mutations can be more pronounced in individuals with nutritional deficiency of folic acid and vitamin B12 and they may experience susceptibility to RPL. To resolve this contention, folic acid /homocysteine levels have to be measured in individuals who experiences RPL with mutant genotype which was the limitation of our study. Overall, it is not the solitary role of genetic polymorphisms in the establishment of the disease, gene and environmental interaction in the form of metabolic needs and dietary adequacy which is the main perpetrator in increasing the magnitude of RPLs.

CONCLUSION

Though there is literature exists establishing the association of these polymorphisms with the genetic predisposition to RPL, this is the first study in the direction of evaluating the data for magnitude of pregnancy loss particularly in low socio-economic group. The information acquired in the study is relevant to the better understanding and management of the condition. It is speculated that individuals carrying these polymorphisms with nutritional deficiency of folic acid and vitamin B12 may be at the higher risk for severe RPL as a result of gene and environmental interaction. To further validate, studies need to be focused on these lines in large sample size and by estimating the levels. In addition, an extended study in comparison with women belonging to high socio-economic status might present a clear idea on the potential role of gene and environmental factors (dietary) on RPL.

CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this article.

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