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Genetic Risk Factor for Recurrent Pregnancy Loss: A Common Mutation (C667T) in *MTHFR* Gene

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ABSTRACT

Objective:

The study aimed to evaluate the correlation among methylenetetrahydrofolate reductase gene (C677T) polymorphism and Recurrent Pregnancy Loss in Andhra Pradesh women.

Methodology:

Blood samples were obtained from patients with three or more repeated abortions prior to 22 weeks of pregnancy (n = 128) against control (n = 144) who have no pregnancy complications. DNA was extracted through standard Phenol-chloroform method. PCR was done for gene annealing and samples were tested for *MTHFR* C677T polymorphism using the sanger's di-deoxy method. Functional significance of the observed C677T mutation was analyzed using computational tools.

Results:

The frequency of C677T mutation was CC (63%), CT (29%), TT (8%) in the patient group, and CC (79%), CT (19%), and TT (2%) in the control group (P=0.0025), which indicates significant association.

Conclusion: The outcome of the study recommends *MTHFR* mutation may be linked with RPL in the analyzed population.

KEYWORDS: Computational tools, In Silico analysis, Polymerase chain reaction, Sanger's dideoxy method, Single Nucleotide Polymorphism.

INTRODUCTION

Recurrent pregnancy loss (RPL) is a multi-factorial, complex issue and occurs in 1-2% of fertile women [1]. RPL defined as the loss of 2 or more fetal drops before 20 weeks of the gestational period [2]. The risk of miscarriage is higher in the earlier gestation, the majority occurring in the first trimester. The risk of miscarriages in subsequent pregnancies is 30% after 2 losses, compared with 33% after 3 losses among patients without a history of live birth. Pregnancy loss may be caused by numerous etiologies; either alone or in combination, has been proposed to contribute to. These include parental chromosomal abnormalities (5%), anatomical (15%), endocrinal disorders (20%), thrombophilia, hormonal (20%), antiphospholipid antibody syndrome, immunological factor (7%), infectious (6%) and environmental factors [3, 4] Even after the evaluation of these cases, more than 50% of cases will remain unexplained. Among the various etiologies, genetic factors can cause reproductive loss which is high in rate [5, 6]. Multiple miscarriages are affected by single gene defects, which cannot be detected by a karyotype

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[7]Among normal and rpl patients 30 genes are demonstrated at various levels of expression[8] Moreover, other studies have also recognized few genes that are expressed aberrantly in pregnancy failure. Methylenetetrahydrofolate reductase (*MTHFR*) is a vital enzyme in folate-homocysteine pathway. Catalization of 5, 10-methylenetetrahydrofolate to 5 methylenetetrahydrofolate mediated by *MTHFR* enzyme. So far numerous SNPs are reported in *MTHFR* gene but C677T variation is clinically significant [9, 10]

Several diseases/disorders such as—Down syndrome [11, 12] neural tube defects [13]orofacial clefts [14], type I diabetes [15], cardiovascular diseases [16, 17]male-female infertility [18, schizophrenia [19], RPL and cancer [20] caused by *MTHFR* (C677T) polymorphism and has been reported as a risk factor. The frequency of C677T polymorphism was described in numerous population studies, but the India has published very few case–control studies. Based on previous studies it is vital to know the frequency of particularly significant gene polymorphism in Indian population. Along with this the current study was to estimate the rate of recurrence of C677T polymorphism in RPL patients.

Previous investigations state that nutritional inadequacies of Folic acid and vitamin B12 result in hyperhomocysteinemia. Nonetheless, the specific role of *MTHFR* C677T polymorphism in idiopathic recurrent miscarriages/ RPL has been as yet questionable till date because of two conflicting outcomes gathered over numerous years [21, 22,23]. However, a past report demonstrated that a homozygous *MTHFR* gene mutation augmented 3.3- fold risk of miscarriage in a population of 185 Caucasian women as compared to 113 mutation-free controls [24]. In addition, clinical and experimental data demonstrated the significance of the stable methylation of peri-centromeric DNA for chromosomal stabilization and segregation. Therefore, the 677C > T mutation is a risk factor for somatic chromosomal aberrations and RPL

Materials & Methods

Moral endorsement for this investigation was acquired from the INSTITUTIONAL ETHICS COMMITTEE (IEC), NRI medical college & General hospitals. (NRI Academy of sciences-S1. No. 373). All participants were read and signed on an informed consent form. The case-control study was undertaken with 128 RPL affected women and 144 as the control group (aged between 25 to 38 years). Patient screening criteria was based on three or more successive pregnancy loss prior to the 22 weeks of pregnancy, despite of previous live birth. Induced abortions, infection, systemic diseases and uterine structural anomalies were taken as Exclusion criteria. The methodology was designed in two ways, namely wet lab and *In silico* analysis.

Wet Lab Analysis

DNA extraction and molecular analysis was performed using EDTA added blood samples of female patients. DNA was extracted from whole blood, using standard protocol (phenol-chloroform method).

Molecular Diagnosis

To amplify the *MTHFR* gene, Primers [25, 26] were commercially synthesized at Barcode-Bioscience, Bangalore. Details of primers sequence tabulated in table-1

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Table 1: Primer sequences

Far word primer	5'TGAAGGAGAAGGTGTCTGCGGGA-3'
Reveres primer	5'AGGACGGTGCGGTGAGAGTG-3'

The total volume of PCR reaction was 50 µL, in which 35 µL of master mix, 5 µL of buffer (10X), 1.25 µL of MgCl2 (100 mM), 0.2 µLTaq DNA polymerase, 2 µLof template (150-200 ng) and 6.55 µL nuclease free water. PCRs were carried out with the followed conditions; an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. A final extension was carried out at 72°C for 7 min and hold at 4°C. Exo-SAP-IT was used to clean up the PCR products and 1.0 µl of the purified product was directly utilized as templates for sequencing by sanger's di-deoxy method. Both forward and reverse primers were utilized for sequencing [27]. Ethanol precipitation was done to purify the extended product and then run in sequencer (ABI 3730). Raw sequences were edited and analyzed with reference sequence by using codon-code aligner. By the utilization of user friendly soft ware tools Genetic Association, Hardy–Weinberg equilibrium and Chi-square test were completed.

In-silico Analysis

Pathogenic potential of significant mutation (C677T from RPL patients was predicted by seven different software's and figure 1 shown the brief protocol of *In silico* analysis. The regard and interest of this investigation are to find out the structure and function of C677T.

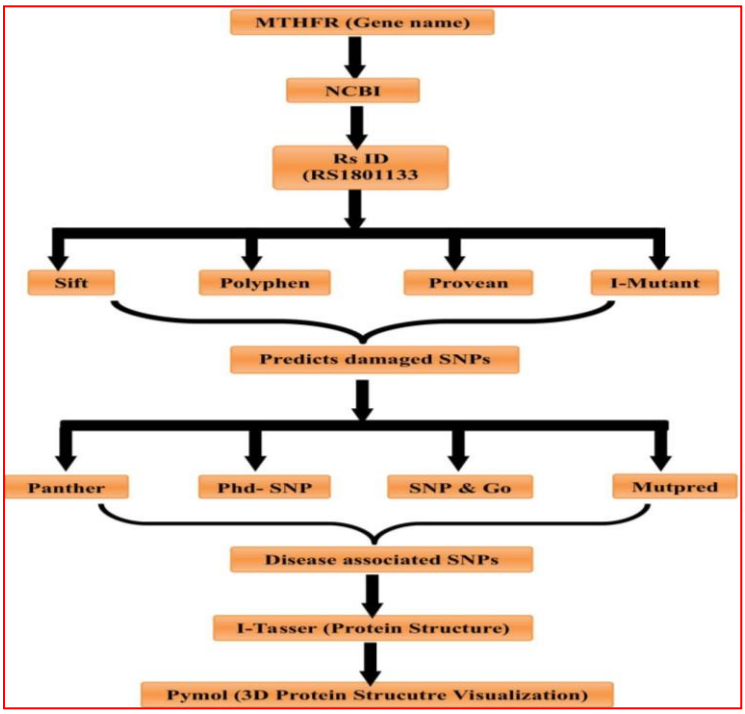

Figure 1: Protocol of *In Silico* work

SIFT (Sorting Intolerant from Tolerate) tool predicts intolerant from tolerant amino acid substitutions that affect protein function which is based on sequence homology [28]

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Polyphen(Polymorphism Phenotyping)possible impact on amino acid substitution based on structural and sequential aspects, the result classified as probably damaging, possibly damaging (≤0.5), and benign (≥0.51) [29]

Provean(Protein Variation Effect Analyzer) estimate the effects based on variation in protein sequences on protein function. If the score is ≤ -2.5, the protein variant is predicted to have deleterious effect, while score is ≥-2.5 it predicts as neutral [30]

I-mutant 3.0 allocating to predict changing mutated protein stability and classify into 3 parts. 1. Neutral mutation (-0.5) 2. Large decrease (≤0.5kcal/mol) 3.Large decrease (>0.5kcal/mol). I-mutant 3.0 is potentially predicting differences between changes in protein stability of mutant as well as wild protein with association of gibbs free energy [31]

Panther (Protein Analysis through Evolutionary Relationships) is a tool based on frequency occurrence of amino acid at a particular position in protein sequences comprehensively related on evolution. The threshold subpsec score of -3 has been assigned below which the predictions are considered as deleterious [32]

PHD-SNPpredicts whether the given amino acid substitution results in related or neutral diseases along with the reliability index score [33]. **SNP&GO** predicts the disease related mutations from protein sequence with a scoring accuracy of 82% and Mathews correlation co-efficient of 0.63. For disease associated mutation the probability score should be > 0.5 and if effects on parent protein function [34] **PMut** software predicts a mutation pathogenicity index of 0 to 1. If > 0.5 reveals pathological mutation while, < 0.5 suggests neutral [35] The protocol was depicted in figure-1

Statistical Analysis: SPSS version 20 was utilized to analyze statistical data ($P < 0.05$). To calculate the odd ratios with 95% confidence intervals researcher use Pearson's chi-square.

RESULTS

Wet Lab Analysis

Figure 2 and 3 shows gel picture of isolated DNA and PCR product from blood samples of RPL patients and controls. The widespread vogue of *MTHFR* mutation was figure out for 128 women with unidentified cause of RPL and nearly 144 women had a history of no complicated pregnancies. In RPL group the prevalence of C677T polymorphism was CC (62.5%), CT (29.68%), and TT (7.81%) and in control group CC (79.16%), CT (19.44%), and TT (1.38%) respectively. The results were tabulated in Table 2.

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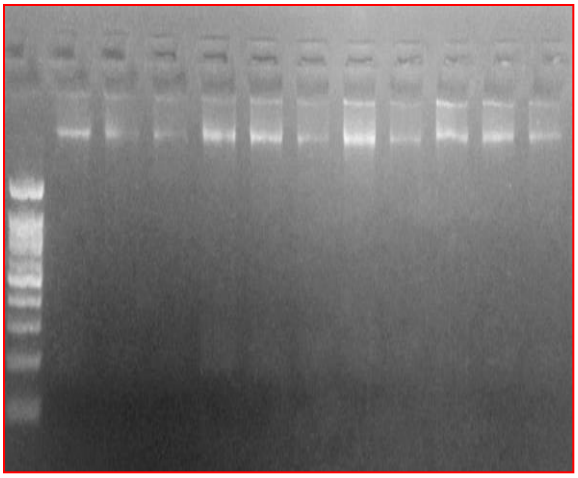

Fig. 2: Agarose gel picture of isolated DNA

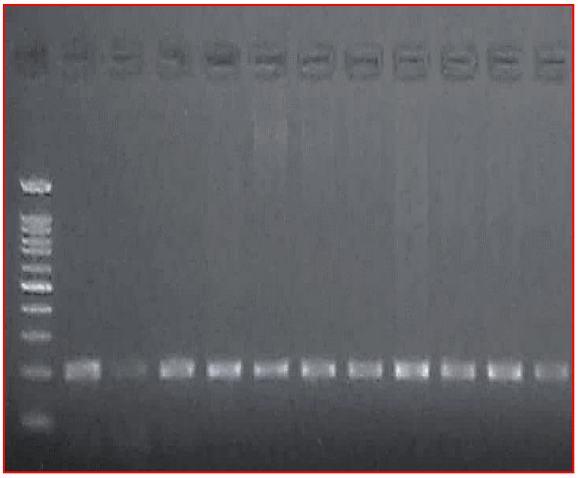

Fig.3: Agarose gel picture of PCR product

Table 2: Genotypic and allelic frequency distribution between RPL cases and controls

Group	***Genotype frequency***					***Allelic frequency***			
	CC	CT	TT	TOTAL		C	T	TOTAL	
RPL cases	80 (62.5%)	38 (29.68%)	10 (7.81%)	128 (100%)	**P = 0.0025**	196 (0.767)	60 (0.234)	253	P=0.0002
Control	114 (79.16%)	28 (19.44%)	2 (1.38%)	144(100 %)		256 (0.870)	32 (0.129)	288	

Statistical analysis indicated a significant association (P =0.0025).The percentages of *MTHFR* C677T heterozygote (CT) were higher in cases as compared to controls. Besides, T allele occurrence was seen as higher in cases, though mutant homozygote's (TT) were more in cases. Though, the genotypes distribution among cases and controls uncovered a critical affiliation. Figure 4 shows the sequences of homozygous and heterozygous variations.

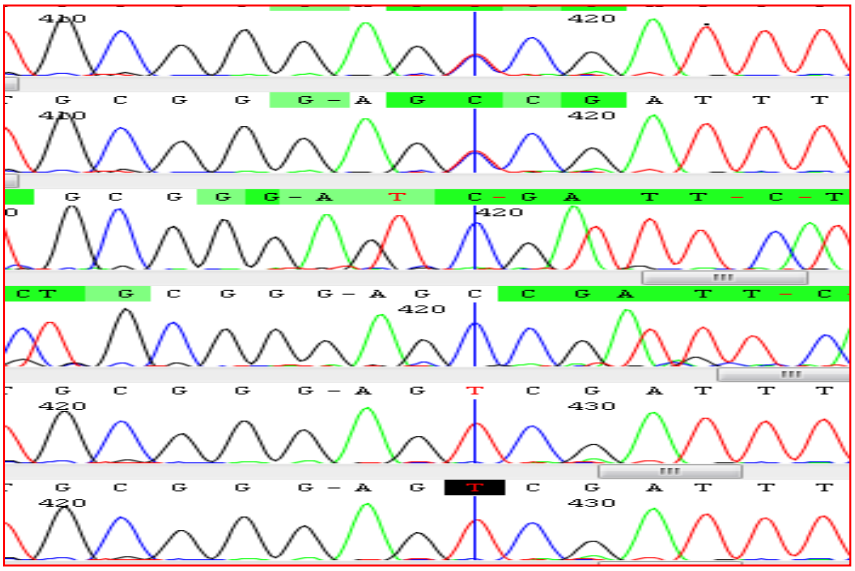

Figure 4: Electro-gram showing wild and mutant variations at position C677T

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The frequencies in between cases and controls of *MTHFR* 677TT were respectively 7.81% and 1.38%, while various genotypes distribution of *MTHFR* C677T comprehensively shows significant difference in patients with frequent unexplained natural abortions and healthy control subjects (P = 0.0025). Table 3 explored the summarized results.Four genetic replicas (dominant, co-dominant, recessive, and over-dominant) were utilized to determine the risk of the mutant alleles for RPL. T allele was discovered to be a risk factor. on the other hand, in co-dominant and recessive models TT genotype confirmed as 7.13 and 6.02 fold increased risk of disease (OR= 7.13, P= 0.0043, co-dominant model; recessive model OR= 6.02, P= 0.0108) (Table 3).

Table 3: Odds ratios and confidence interval (CI) of four different inheritance models

	Dominant	*Co-dominant*	*Recessive*	*OVER-Dominant*
Cases	48/80	38/80;10/80	10/118	38/90
Controls	30/114	28/114;2/114	2/142	28/116
Odds ratio	2.28	1.93;7.13	6.02	1.75
CI	1.33-3.91	1.1-3.4/1.52-33.4	1.29-28	1-3.06
p-value	0.0024	0.0212;0.0043	0.010	0.049

***In silico* Analysis**

To prognosticate the structural and ascetic consequences of C677T SNP we implemented different algorithms that are computer friendly. **SIFT** score was 0.06 tolerant, PolyPhen score 0.998 representing probably damaging the Protein function, **Provean** predicts as deleterious with the score of -3.760, **I-Mutant** ΔΔG is -0.77 this negative ΔΔG value indicate the low stability of the mutated C677T, and finally, **PhD-SNP, SNP&GO, PMut** represents as disease associated with score of 0.885, 0.655, 0.73 respectively which indicates a deleterious effect on protein function. All together tools score (Table 4) suggesting that this polymorphism may impact on protein function and associated with RPL. All seven software's scores indicated as it was damaged, pathogenic and disease-associated SNP. It's changing the amino acid Alanine to valine (A222V). By using I-TASSER, I modeled the protein structure [36] and variations of wild and mutant protein structures showed by Pymol tool and depicted in Figure 5.

Table 4: Computational tools and their results

Gene	Rs id	A.A change	Sift	Polyphen	Provean	I-Mutant	PhD-Snp	Snp&go	pmut
MTHFR	Rs1801133	A222V	0.06	0.998	-3.760	-0.77	0.885	0.655	0.73

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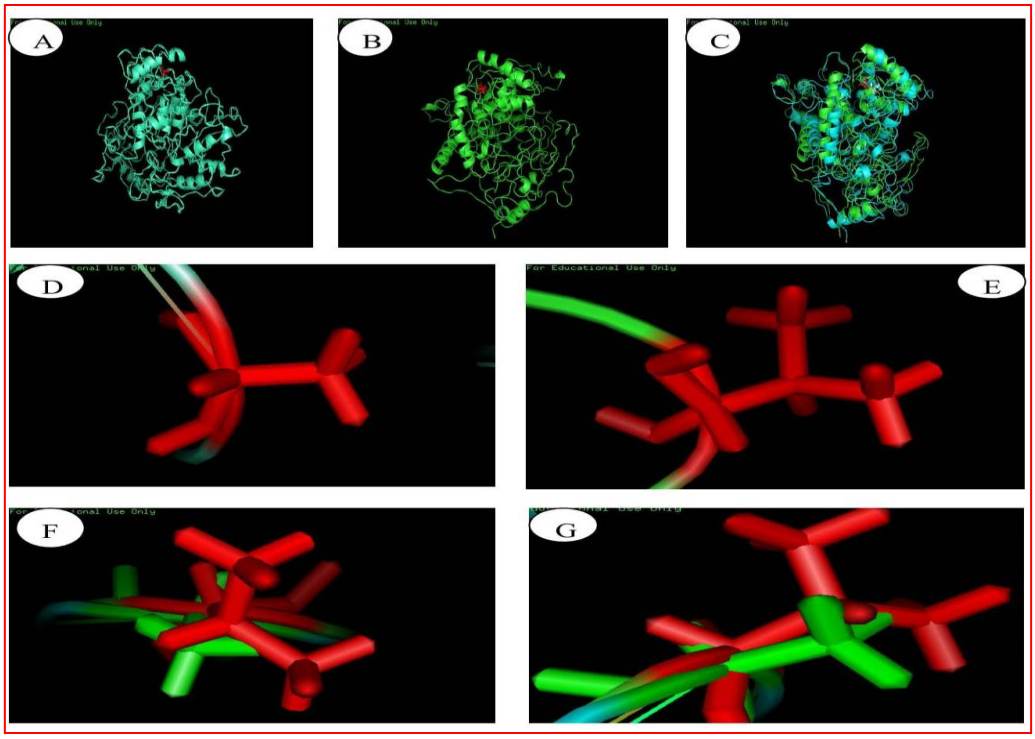

Figure 5: 3D structure of wild and mutant proteins and its alignments

(A. wild protein structure, B. mutant protein structure, C. alignment of wild & mutant protein structure, D E F G shows the structural variation in different angles)

DISCUSSION

In the current investigation, we found a relationship between the most common *MTHFR* gene polymorphism and RPL of Andhra Pradesh (southeast) women. C677T is a significant SNP for the *MTHFR* gene that may influence the level & the activity of *MTHFR* enzymes in the blood, clinically hyperhomocysteinemia brought transformation related with coronary artery disorder, venous thrombosis, as well as complexity in pregnancy [37, 38] along these lines, it was important to explore a link between *MTHFR* with RPL. As RPL was correlated with placental infarctions [39] As per the literature, countless studies have explored the relationship between the *MTHFR* gene (C677T) polymorphism and the risk of RPL but with conflicting results [40, 41, 42,43]The Meta-analysis of 16 studies has indicated that *MTHFR* homozygous (677T/T) and heterozygous (677C/T) genotypes multiplied significantly the risk of RPL in the Chinese population [44] A similar exploration completed in Northern Indian region women population reported that RPL was associated with the genotype of the *MTHFR* variation [45]As well as it says the presence of *MTHFR* gene polymorphism three folds increases the risk of RPL. Nonetheless, in an extreme research conducted in Palestinian women population, and found no such statistical variations among the allele disbursement and therefore the genotype frequencies among case and control group ($p>0.05$) [46,47] Correspondingly, during Meta-analysis between Dutch women homozygosity for a typical 677CT mutation within the *MTHFR* gene brought about a two to threefold high hazard of RPL [48] Hereafter homozygosity for the mutated *MTHFR* gene in general is indeed a risk factor for RPL.

Our outcomes additionally exhibited that the recurrence of homozygous genotype (T/T) in women with RPL (7.81%) was more noteworthy than the regulation (1.38%) group and this variation was significant (p=0.0002). our findings advanced that for RPL, *MTHFR* (C677T) gene polymorphism become a potent risk factor. Moreover, the frequency of heterozygous genotype has a statistically significant differ in our patients.

Our *In silico* studies elucidate that C677T mutation changes its amino acid from alanine to valine at codon position 222. In view of scores from seven different computational tools, the targeted SNP is disease-associated and at the end of structural level obtained residue is shorter than wild type. Due to variation in the hydrophobicity between wild and mutant type residue may leads to cause improper functioning of protein and also causing failure of outward interactions.

CONCLUSION

It concludes that, this is the primary report that reveals the genetic susceptibility due to polymorphisms (C677T) in the *MTHFR* gene in women leading risk to RPL of Andhra Pradesh (Southeast). Our investigation supports that *MTHFR* C677T mutation, T allele, and CT + TT genotypes were more frequent in RPL patients, and the presence of T allele could be a risk element for RPL. Nevertheless, C677T polymorphism in the Andhra Pradesh women population is associated with RPL.

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