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Original Research Article

Role of thrombophilia screening in recurrent pregnancy loss and poor pregnancy outcome

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ABSTRACT

Background: The pathophysiology of recurrent pregnancy loss is poorly understood and some factors have been implicated as causes of RPL including genetics, metabolic and infections. But still in majority of RPL cases, cause remains unexplained (around 40-50%). Recent few studies have shown that there is significant association between thrombophilia and RPL. The genetic predisposition to venous thrombosis such as factor V Leiden, factor V HR_2 haplotype, factor V HongKong/Cambridge and PAI-1 4G/5G promoter polymorphism have been reported to be associated with RPL. This study examined the specific prevalence of genetic thrombophilic markers in women with recurrent miscarriage.
Methods: A retrospective case-control study designed with 50 RPL cases and 50 healthy controls. Genotyping of the four thrompohilic mutation were performed by PCR-RFLP and AS-PCR methods.
Results: The frequencies of factor V HR_2 haplotype mutant heterozygous form (OR=1.46; p=0.758), PAI-1-675 4G/4G (OR=1.13; p=0.806) and PAI-1 -675 5G/5G (OR=1.24; p=0.815) were moderately higher in RPL patients than controls. While, the mutant form of factor V Hong Kong and factor V Cambridge were completely absent in this study population.
Conclusions: To our best knowledge, this is the first study to investigate the association of Factor V HR_2 haplotype, factor V Hong Kong/Cambridge and PAI-1 (-675 4G/5G) mutations with RPL in South Indian population. However, this study did not reveal any significant association between studied mutations and RPL due to small sample size.

Keywords: Factor V Cambridge, Factor V Hong Kong, Factor V HR_2 haplotype, Mutation, Recurrent pregnancy loss, Thrombophilia

INTRODUCTION

Recurrent pregnancy loss (RPL) poses a significant clinical problem in pregnant mothers. The ESHRE (European society of Human Reproduction and Embryology) guidelines refer to RPL as pregnancy loss of 2 or more pregnancies. RPL is a heterogeneous disorder which affects women of reproductive age.[1] Recently, The American Society of Reproductive Medicine has defined RPL as two or more than two failed pregnancies before the 20th week of pregnancy.[2] Overall, 1-5% of women during reproductive ages could be affected.[3] The pathophysiology of recurrent pregnancy loss is poorly understood and some factors have been implicated as causes of RPL including genetics, metabolic and infections. But still in majority of RPL cases, cause remains unexplained (around 40-50%).[4] Recent few studies have shown that there is significant association between thrombophilia and RPL.[5]

The pregnancy is already a hypercoagulable state where there is increased tendency to form blood clots. This state increases the risk of thrombophilia which is sustained by

some genetic factors such as polymorphisms that affect the coagulation system. When genetically susceptible women become pregnant, there is increased risk of thrombophilia (increased tendency to form abnormal blood clot) which in turn may lodge in feto maternal placental interface to cause miscarriage.[5,6]

A recently recognized polymorphism in factor V (FV) gene H1299R (also named HR_2) has been reported to be a possible risk factor for the development of thrombophilia. Similarly, factor $VThr^{306}$ also known as factor V Hong Kong/Cambridge is a mutation affecting the Arg^{306} activated protein C (APC) cleavage site and is the only mutation, other than factor V Leiden (Arg^{506}→Gln), that has been found in association with APC resistance causing thrombophilia.[7] Platelet activator inhibitor-1 (PAI-1) is also known as endothelial plasminogen activator inhibitor, family of serine protease inhibitor, which inhibits the activation of plasminogen thereby causing fibrinolysis. In case of polymorphisms related with plasminogen activator inhibitor-1 (PAI-1) results in unbalanced fibrin deposition. Unsuccessful implantation, PAI-1 controls maternal tissue during the trophoblast invasion and this polymorphism leads to an insufficient trophoblast invasion.[8]

Hence, the present study designed to investigate the association of thrombophilic gene polymorphisms (factor V HR_2 haplotype, factor V Hong Kong/Cambridge and PAI-1 4G/5G promoter polymorphism) with recurrent pregnancy loss and poor pregnancy outcome in South Tamil Nadu population.

METHODS

Study population

This was a retrospective study which was conducted at antenatal (AN) outpatient clinic in the Department of Obstetrics and Gynaecology, Government Rajaji Hospital, Madurai from November 2020 to October 2021. Study subjects comprised of 50 women with recurrent pregnancy loss or poor pregnancy outcome with IUGR/pre-eclampsia.

Inclusion criteria

Inclusion criteria of the cases include at least 3 miscarriages of unknown aetiology with the same partner, which occurred during the first trimester of gestation. The control group consisted of 50 healthy parous women with at least two live births and with no history of miscarriage, pre-eclampsia, ectopic pregnancy or preterm delivery. Controls were recruited following a routine check-up after an uncomplicated pregnancy and were matched to cases according to age and self-declared ethnic origin.

Exclusion criteria

The women with other co-morbidities like diabetes mellitus, thyroid dysfunction, heart disease or uterine anomalies were excluded.

This study was approved by the ethics committee of Madurai Medical College, Madurai and performed as per the standards laid down by the Declaration of Helsinki for medical research involving human subjects. Written informed consent to participate in this study was obtained from all the individuals.

DNA extraction and genotyping

Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The presence of four thrombophilia gene mutations [factor V HR_2 haplotype, factor V Hong Kong, factor V Cambridge and PAI-1 (-675 4G/5G)] were detected by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and allele specific PCR respectively.[9,10] The primer sequences, specific restriction enzymes used for mutational analysis and the fragment size before and after digestion were listed in Table 1.

Table 1: Primer sequences and restriction enzymes used for detection of four thrombophilia gene mutations.

Mutation	Primers	Enzyme	PCR product (bp)	RFLP products (bp)
Factor V HR_2 haplotype	F: 5′-CAAGTCCTTCCCCACA GATATA-3′ R: 5′-AGATCTGCAAAGAGG GGCAT-3′	RsaI	703	Normal (R_1R_1): 703 heterozygous (R_1R_2): 703, 492, 211 mutant (R_2R_2): 492, 21
Factor V Hong Kong/ Cambridge	F: 5′-TCCCACCTCTTCATGT GCCGCCTCTG-3 R: 5′-CCAAACTAAAATGTTCAAAAATTGCCTGG GCATTA-3′	Hong Kong: HpaII Cambridge: MvaI	252	For Cambridge: normal: 173, 53 heterozygous: 226, 173, 53 mutant: 226 For Hong Kong: normal: 252

Continued.

Mutation	Primers	Enzyme	PCR product (bp)	RFLP products (bp)
				heterozygous: 252, 198, 54 mutant: 198, 54
PAI-1 -675 4G/5G	F: IC: 5`-AAGCTTTTACCATGGTAACCCCTGGT-3` 4G: 5`- GTCTGGACACGTGGGGA-3` 5G: 5`- GTCTGGACACGTGGGGG-3` R: Co: 5`-TTTCCCCCAGGGCTGTCCA-3`	AS-PCR	340 231/232	IC: 349 4G: 231 5G: 232

PCR: polymerase chain reaction, RFLP: restriction fragment length polymorphisms, bp: base pair, AS-PCR: allele specific - polymerase chain reaction

Statistical analysis

Numerical data were expressed as mean and standard deviation. Qualitative data were expressed as frequency and percentage. Genotypic and allelic frequencies were determined by gene counting. Chi square tests (χ^2) were used to analyse and compare the genotypic and allelic distribution between the patients and controls. Risk assessment was evaluated by calculating Odds ratio (OR) with 95% confidence interval (CI). P values <0.05 were considered statistically significant. All statistical analysis were done using statistical software Epi info version 7.

RESULTS

Patient characteristics

The demographic and clinical characteristics of the study subjects are shown in Table 2. The present study consists of 50 RPL patients (mean age of 26.7±4.2 years) and 50 healthy controls (mean age of 24.5±4.1 years). All patients (100%) showed regular menstrual cycles just as in controls (96%). Further, the percentage of consanguinity was 28% in patients and 16% in controls.

Prevalence of thrombophilia gene mutations

The frequency of four thrombophilic gene mutations [factor V HR_2 haplotype, factor V Hong Kong, factor V Cambridge and PAI-1 (-675 4G/5G)] in the study population and its association with RPL are summarized in Table 3. The frequency of factor V HR_2 haplotype mutant heterozygous form was found to be moderately high in RPL patients (OR=1.46; p=0.758) when compared to controls. While, the homozygous mutant form of factor V HR_2 haplotype was not observed in both RPL patients and controls. Similarly, the mutant form of factor V Hong Kong and factor V Cambridge were completely absent in the study population.

The homozygous frequencies of PAI-1 -675 4G/4G (OR=1.13; p=0.806) and PAI-1 -675 5G/5G (OR=1.24; p=0.815) were moderately higher in RPL patients than controls. Similarly, increased frequency of PAI-1 -675 5G allele (OR=1.04; p=0.887) was observed in RPL patients when compared to controls. However, the studied polymorphisms did not show statistically significant difference between RPL patients and controls in the study cohort.

Table 2: Demographic and clinical characteristics of RPL patients and controls.

Characteristics	Patients (N=50)	Controls (N=50)	P value
Age, years (mean±SD)	26.7±4.2	24.5±4.1	0.009
Number of abortions	2.8±1.3	0	NA
Menstrual cycle (regular)	50 (100%)	48 (96%)	0.475
Consanguineous marriage	14 (28%)	8 (16%)	0.227

SD: Standard deviation

Table 3: Distribution of four thrombophilia gene mutations in RPL patients and controls.

Mutation	Patients (50) N (%)	Controls (50) N (%)	OR (95% CI)	P value
Factor V HR_2 haplotype				
Mutant form (R_2R_2)	0	0		
Heterozygous form (R_1R_2)	07 (14)	05 (10)	1.46 (0.43-4.97)	0.758
Wild type (R_1R_1)	43 (86)	45 (90)	0.68 (0.20-2.31)	0.758

Continued.

Mutation	Patients (50) N (%)	Controls (50) N (%)	OR (95% CI)	P value
Factor V Hong Kong				
Mutant form	0	0		
Wild type	50 (100)	50 (100)		
Factor V Cambridge				
Mutant form	0	0		
Wild type	50 (100)	50 (100)		
PAI-1 -675				
4G/4G	11 (22)	10 (20)	1.13 (0.43-2.95)	0.806
4G/5G	26 (52)	29 (58)	0.78 (0.35-1.73)	0.688
5G/5G	13 (26)	11 (22)	1.24 (0.50-3.13)	0.815
4G allele	48 (48)	49 (49)	0.96 (0.55-1.67)	0.887
5G allele	52 (52)	51 (51)	1.04 (0.60-1.81)	0.887

OR: odds ratio; CI: confidence interval

DISCUSSION

Globally**,** the genetic variants of thrombophilic gene mutations have been extensively associated as risk factors for the development of RPL. It is a well-known fact that the genetic variants associated with various diseases depends on different frequencies and hence the ethnicity match studies are desirable to ascertain the precise role of these mutations in disease pathogenesis. Therefore, the main objective of this study was to determine frequencies of four thrombophilic gene mutations [factor V HR_2 haplotype, factor V Hong Kong, factor V Cambridge and PAI-1 (-675 4G/5G)] and to associate their existence with RPL in South Indian population.

The protein C pathway is a key anticoagulant process that down-regulates the prothrombin- intrinsic factor X (FX) activating complexes through inactivation of their respective cofactors, activated factors V (FVa) and VIII (FVIIIa). Cofactor inactivation follows through limited proteolysis of FVa at amino acid positions (306, 506, and 679) and FVIIIa at amino acid positions (336, 562, and 740).[11-13] These reactions are catalysed by the serine protease activated protein C (APC) and stimulated by the APC cofactor protein S.[14] Functional defects of the protein C pathway, due to inherited or acquired conditions, decide a plasma phenotype known as APC resistance, which is significant risk factor for the development of RPL.[15] To date, few FV gene mutations have been reported to be associated with APC resistance either by reducing the predisposition of FVa to APC-mediated inactivation or by interfering FVIIIa inactivation via the APC cofactor activity of FV. Among which factor V Hong Kong/Cambridge are two mutations located in activated protein C cleavage site of factor V at Arg^{306}. Factor V Cambridge mutation is caused by G to C transversion at nucleotide position 1091 resulting in an Arg^{306}~Thr substitution and factor V Hong Kong mutation is defined by an A to G transition at nucleotide position 1090 resulting in an Arg^{306}~Gly substitution.[16,17] Another important mutation termed as factor V HR_2 haplotype is caused by an A to G transition at nucleotide position 4070 is located in exon 13 of FV resulting in His1299Arg substitution named R_2.[18]

In this study population, we did not observe a single mutant type (homozygote or heterozygote) of factor V Hong Kong/Cambridge mutations in either RPL patients or controls. Similarly, various studies reported very low frequency of factor V Hong Kong/Cambridge mutations in other Asian populations. However, factor V HR_2 Haplotype revealed an occurrence rate of 14% and 10% of R_1R_2 heterozygous genotype in RPL patients and controls respectively in this population. So, a large sample size is essential to determine the impact of these mutations in the development of RPL.

Fibrinolysis plays a crucial role in maintaining blood homeostasis by resolving the blood clots. Plasminogen activator inhibitor-1 (PAI-1) regulates fibrinolysis by inhibiting tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) responsible for activating plasmin that dissolve blood clots. Elevated levels of PAI-1 is associated with a wide range of diseases including malignant tumors, pregnancy related complications, cardiovascular and thromboembolic diseases.[19] PAI-1 gene is located in the chromosome 7 (q21.3–q22) and a presence of 4G/5G polymorphism (rs72578597) at 675 bases upstream from the transcription site is associated with altered levels of PAI-1.[20] Inheritance of homozygous 4G/4G has been associated with elevated PAI-1 levels and consequently with increased risk of RPL.[21] Our results showed that there is a moderately increased frequency of 4G/4G homozygous genotype in RPL patients which was not significant. A meta-analysis reported that PAI-1 4G/5G polymorphism was significantly associated with RPL risk in Caucasian population but not in Asians.[22] These contradictory findings are suspected due to small sample size and various ethnic differences.

CONCLUSION

To our best knowledge, this is the first study to investigate the association of factor V HR_2 haplotype, factor V Hong

Kong, factor V Cambridge and PAI-1 (-675 4G/5G) mutations with RPL in South Indian population. However, this study did not reveal any significant association between studied mutations and RPL due to small sample size. Hence, further studies with large sample size are needed to understand the role of these mutations in the pathogenesis of RPL.

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