

# Significant correlation of angiotensin converting enzyme and glycoprotein IIIa genes polymorphisms with unexplained recurrent pregnancy loss in north of Iran

Shokoufeh Fazelnia<sup>1</sup> M.Sc., Touraj Farazmandfar<sup>2</sup> Ph.D., Seyed Mohammad Bagher Hashemi-Soteh<sup>3</sup> Ph.D.

1. Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran.

2. Medical Cellular and Molecular Research Center, Golestan University of Medical Sciences, Gorgan, Iran.

3. Immunogenetic Research center, Molecular and cell Biology Research Centre, Mazandaran University of Medical Sciences, Sari, Iran.

## Corresponding Author:

Seyed Mohammad Bagher Hashemi-Soteh: Department of Genetics, School of Medicine, Mazandaran University of Medical Sciences, Farahabad Road., Sari, Iran. P.O. Box. 481751665  
**Email:** hashemisoteh@gmail.com  
**Tel:** (+98) 9111164034

Received: 13 September 2015

Accepted: 17 February 2016

## Abstract

**Background:** Spontaneous abortion is considered as the most complex problem during pregnancy. Thrombophilia is resumed as a cause of recurrent pregnancy loss (RPL). Glycoprotein IIIa (GPIIIa) gene is involved in thrombosis and abortion. Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II and is involved in thrombosis. The most common polymorphism in this gene is the insertion/deletion (I/D).

**Objective:** In this study, we analyzed the association between ACE I/D and GPIIIa c.98C>T polymorphisms in women with unexplained RPL from the north of Iran.

**Materials and Methods:** Sample population consisted of 100 women with unexplained RPL and 100 controls. The ACE I/D and GPIIIa c.98C>T polymorphisms were genotyped by TETRA-ARMS PCR. The association between genotypes frequency and RPL were analyzed using  $\chi^2$  and exact fisher tests. Associated risk with double genotype combinations was also investigated by binary logistic regression.

**Results:** There was significant association between ACE DD genotype and RPL (OR=2.04; 95% CI=0.94-4.44; p=0.036). ACE D Allele was also significantly associated with the RPL (OR=1.59; 95% CI=1.05-2.41; p=0.013). No significant association was observed between GPIIIa c.98C>T polymorphism and RPL.

**Conclusion:** ACE I/D polymorphism may probably be a prognostic factor in female family members of women with the history of recurrent abortion.

*Key words:* Angiotensin converting enzyme, Platelet glycoprotein IIIa, Recurrent abortion.

*This article extracted from M.Sc. thesis. (Shokoufeh Fazelnia)*

## Introduction

Recurrent pregnancy loss (RPL) appears a significant clinical problem affecting approximately 2% of women (1). RPL pathophysiology is poorly understood. Pregnancy loss may be caused by different reasons such as genetic factors, immune defect, infection and anatomical problem (2). However, even after more accurate investigations, as many as 50% of all cases remain unexplained (3). Thrombophilia has been presumed as a cause of RPL (4, 5). Many recent studies have examined the mutations incidence and variants in specific thrombophilic genes on women with unexplained pregnancy loss (4-10). Angiotensin converting enzyme (ACE) is a key component in rennin-angiotensin system

which converts angiotensin I to angiotensin II, a potent vasopressor. Many studies have been indicated that ACE affects hemostasis through different mechanisms, including platelet aggregation, blood clotting and fibrinolysis (11-14).

The human ACE gene contains variable polymorphic regions that can be used in genetic analysis. A well-known polymorphism is the insertion/deletion (I/D) of 287 base paired (bp) fragment in intron 16 which has been extensively investigated (1). Furthermore, it has been found that ACE D allele leads to increased expression in plasminogen activator inhibitor-1 (PAI-1) level, which can increase the angiotensin II production and enhance the thrombotic events risk (15, 16).

Past studies have been indicated that

thrombophilic defects affect pregnancy-associated thromboembolism such as preeclampsia and abortion (11). Few recent meta-analysis studies have demonstrated the role of *ACE* I/D polymorphism in increasing RPL risk (17, 18). Glycoprotein IIIa (*GPIIIa*) is one of thrombophilic genes involved in modulation of vascular thrombosis. The GPIIb/GPIIIa is an integrin complex in platelet aggregation as a fibrinogen receptor (19). The polymorphism c.98C>T in *GPIIIa* gene causes an amino acid substitution (p.L33P), leads to the creation of two distinct forms of GPIIb/IIIa antigen on platelets (20). This polymorphism has been associated with stroke in young Caucasian women and risk of premature acute coronary syndromes (21). This variation also results in spiral artery thrombosis and poor placental perfusion, which can explain the correlation of this polymorphism with RPL (22, 23).

To date, few studies have established on the relationship between thrombophilic genes and RPL in Iranian population (24-26). We, therefore, determined the association between of *ACE* I/D and *GPIIIa* c.98C>T polymorphisms in women from the northern of Iran with unexplained RPL.

## Materials and methods

### Subjects

This case-control study was done carried out during the 2013-2014 year's in Sari, Iran. A total of 100 women with unexplained RPL aged 20-40 yrs and 100 healthy controls aged 27-44 yrs, with at least two live births and no history of abortion, infertility or endometriosis were comprised. Prior to enrollment, all patients were given an explanation of study nature, and written informed consent was obtained from all individuals. The study protocol was approved by the Clinical Research Ethics Committee in Mazandaran University of Medical Sciences, Sari, Mazandaran. RPL was defined as two or more spontaneous consecutive abortions at 5-20 wks of gestation. Miscarriage history of women with unexplained RPL was examined and cases with anatomic, chromosomal, hormonal, autoimmune or infectious causes were excluded from this study. There was no pregnancy-related problems such as hypertension, diabetes, thyroid abnormalities, etc. in none of cases.

### Genotyping

Genomic DNA was isolated from whole blood by a modified Nucleon BACC II method from whole blood (Tepnel Life Sciences, Manchester, UK). The *ACE* I/D polymorphism was genotyped using two primers and *GPIIIa* c.98C>T polymorphism was genotyped using a TETRA-ARMS PCR method (27, 28). The *GPIIIa* c.98C>T polymorphism primers were designed by Gene Runner software (version 3.05) (Table I). The polymerase chain reaction (PCR) was performed using standard PCR methods with 100-200 ng of DNA template and 5 picomol of each primer and PCR materials (Cinnagen, Tehran, Iran) in thermal cycler (Eppendorf, Hamburg, Germany) as reported previously (29).

PCR conditions for *ACE* genotyping included one step initial denaturation (94°C for 3 min), 35 cycles (94°C for 45 sec, 60.5°C for 50 sec, and 72°C for 40 sec) and a final extension at 72°C for 5 min. PCR conditions for *GPIIIa* genotyping also included one step initial denaturation (94°C for 3 min), 35 cycles (94°C for 35 sec, 58°C for 40 sec, and 72°C for 40 sec) and a final extension at 72°C for 5 min. Then, PCR products were electrophoresed in a 1.5% agarose gel (Fermentas, Sankt Leon-Rot, Germany).

### Statistical analysis

Association between genotypes frequency and RPL were analyzed using  $\chi^2$  and Fisher's exact tests. The risk associated with the double genotype combinations was also investigated by binary logistic regression. Data were analyzed by SPSS software (version 17.0) and  $p < 0.05$  were considered significant.

## Results

The *ACE* I/D Alleles were determined based on Product size-band; 490 bp for the I allele and 190 bp for D allele (Figure 1A). The *GPIIIa* c.98C>T Alleles were also determined by bands; 395 bp for the C allele (by primers of reverse outer and forward inner), 200 bp for T allele (by primers of forward outer and reverse inner) and 560 bp as control (by primers of outer) (Table I) (Figure 1B). The *ACE* I/D and *GPIIIa* c.98C>T genotypes in women with RPL and control group was in accordance with Hardy-Weinberg equilibrium. A few samples from each genotype were

confirmed by sequencing. The Genotypic and allelic frequencies of ACE I/D and GPIIIa c.98C>T polymorphisms in women with RPL and control group were shown in tables II and III. The ACE II genotype was observed in 23% of cases, ACE ID genotype in 33% and ACE DD genotype in 44%. The GPIIIa c.98C>T wild-type genotype (TT) was observed in 84% of cases, whereas 16% were heterozygous (TC) and no CC genotype was observed. Our results showed that, there is a significant difference regarding ACEDD genotype

between cases and control groups (OR=2.04; 95% CI=0.94-4.44; p=0.036) (Table II).

Our results also indicated that D Allele is statistically associated with RPL (OR=1.59; 95% CI=1.05-2.41; p=0.013) (Table III). No significant difference was observed between genotypic and allelic frequencies of GPIIIa c.98C>T polymorphism and RPL in case and control groups. In combination analysis, there was no significant association between combination of ACEDD genotype and GPIIIa TT genotype with RPL (Table IV).

**Table I.** Primers used for genotyping

Primer	Sequence (5'→3')	Genbank accession No.
ACE I/D		NG_011648.1
Forward	CTGAGACCACTCCCATCCTTCT	
Reverse	GATGTGGCCATCACATTCGTCAGAT	
GPIIIa c.98C>T		NG_008332.2
Forward outer	CCTTTCTGTACAACGGTCTCT	
Reverse outer	CAGATCTTCTGACTCAAGTCCT	
Forward inner (C)	CTTACAGGCCCTGCGTCC	
Reverse inner (T)	CACAGCGAGGTGAGCACA	

**Table II.** Genotype frequencies of ACE I/D and GPIIIa c.98C>T polymorphisms in women with RPL. The risk of I/I versus (I/D + D/D) and (I/I + I/D) versus D/D for RPL was evaluated in dominant and recessive models (n=100)

	Case	Control	OR (95% CI)	p-value
ACE I/D				
II	23	31	1.00	
DI	33	40	1.11 (0.51 - 2.40)	0.455
DD	44	29	2.04 (0.94 - 4.44)	0.036 <sup>§</sup>
DI + DD	77	69	1.50 (0.76 - 2.97)	0.104
DD	44	29	1.00	
DI	33	40	0.54 (0.26 - 1.10)	0.097
II	23	31	0.48 (0.22 - 1.06)	0.071
DI+II	56	71	0.51 (0.28 - 0.93)	0.131
GPIIIa c.98C>T				
TT	84	80	1.00	
TC	16	20	0.76 (0.36 - 1.58)	0.469
CC	0	0	-	-
TC + CC	16	20	0.76 (0.36 - 1.58)	0.469

OR: odds ratio CI: confidence interval  
<sup>§</sup> significant p-values

**Table III.** Allelic frequencies of ACE I/D and GPIIIa c.98C>T polymorphisms in women with RPL

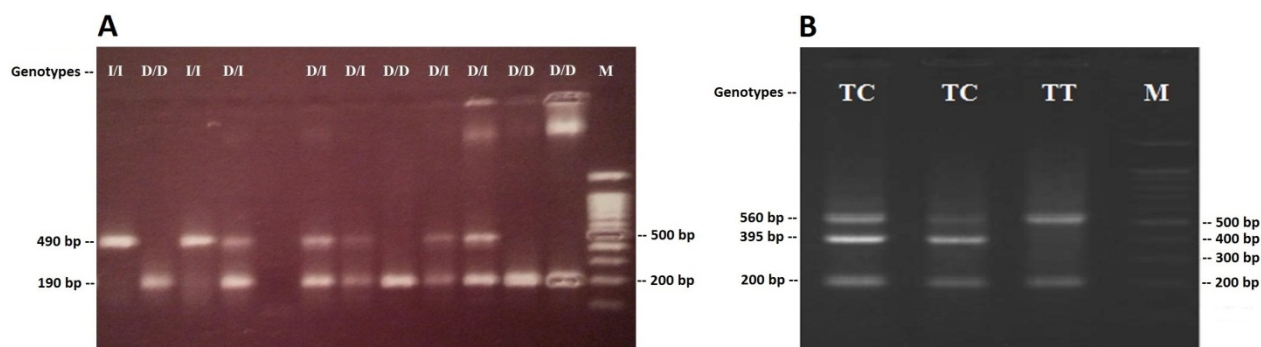
	Case	Control	OR (95% CI)	p-value
ACE I/D				
I	79 (39.5%)	102 (51%)	1.00	
D	121 (60.5%)	98 (49%)	1.59 (1.05 - 2.41)	0.013 <sup>§</sup>
GPIIIa c.98C>T				
T	184 (92%)	180 (90%)	1.00	
C	16 (8%)	20 (10%)	0.78 (0.38 - 1.56)	0.491

Data presented as n (%).  
<sup>§</sup> Significant p-values  
 OR: odds ratio CI: confidence interval

**Table IV.** Combination analysis of ACE I/D and GPIIIa c.98C>T polymorphisms in women with RPL

	Case	Control	OR (95% CI)	p-value
ACE/ GPIIIa				
II/TT	20	26	1.00	
II/TC	3	5	0.78 (0.10 - 4.59)	0.389
DI/TT	28	30	1.21 (0.51 - 2.84)	0.316
DI/TC	5	10	0.65 (0.15 - 2.52)	0.255
DD/TT	36	24	1.95 (0.83 - 4.57)	0.067
DD/TC	8	5	2.08 (0.50 - 9.29)	0.136

OR: odds ratio CI: confidence interval



**Figure 1.** Electrophoresis pattern of PCR products for detection of polymorphisms. (A) ACE I/D polymorphism. Band of 490 bp and 190 bp are as I allele and D allele respectively. (B) GPIIIa c.98C>T polymorphism. Band of 395 bp and 200 bp are as C allele and T allele respectively. Band of 560 bp is as control.

## Discussion

This study was designed to determine the association between certain polymorphisms in *ACE* and *GPIIIa* genes and RPL in Mazandaran province, northern Iran. Changes in renin-angiotensin-aldosterone system are involved in pathogenesis of pregnancy complications such as preeclampsia and fetal loss (30, 31). Previous studies elucidated the role of *ACE* I/D polymorphism in susceptibility to pregnancy loss (32-36).

*ACE* alters hemostasis through some mechanisms, including platelet aggregation, influence on fibrinolysis. Some previous studies have reported an association between the *ACE* D allele and increased risk of thrombosis (15, 37). Data by Fatini *et al* demonstrated that the *ACE* I/D polymorphism may be an important risk factor for RPL (35). Buchholz *et al* also reported that the *ACE* DD genotype leads to increased PAI-1 concentration and thus correlated with an increased risk of RPL in Caucasians (14). However, Vettriselvi *et al* showed no significant association between the frequencies of deletion allele and RPL (1). In this study, we found an association between *ACE* I/D polymorphism and RPL in our population. This study demonstrated that the DD genotype was more prevalent in RPL cases (44%) than in controls (29%), and D allele by itself might be a risk factor for RPL in this population.

Concerning the *GPIIIa* c.98C>T polymorphism, it seems to increase susceptibility to premature acute coronary syndrome and risk of stroke in young Caucasian women (21). T allele frequency was low in all the past studies which is in

contrast with this study (1%-17%), this may be due to small sample size in our study (36, 38, 39). Yenicesu *et al* reported that heterozygous mutations of *GPIIIa* c.98C>T (CT) were associated with RPL (38). Goodman *et al* showed that *GPIIIa* L33P can identify women at risk for RPL (7). Coulam *et al* also determined the frequency of *GPIIIa* L33P in women with RPL history (40). Our results indicated that the *GPIIIa* c.98 C>T polymorphism was not significantly correlated with RPL. These results are in agreement with the study's results of, Ozdemir *et al*, Torabi *et al*, Pihusch *et al*, Coulam *et al*, Goodman *et al* and Hohlagschwandtner *et al* (7, 26, 39-42).

## Conclusion

The major finding of this study shows that *ACE* D allele may increase susceptibility to RPL. *ACE* I/D polymorphism could probably be investigated as one of the prognostic factors in women with RPL history among the family members.

## Acknowledgments

We wish to thank members of research staff in Novin Genetic laboratory and all the patients who participated in this study.

## Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References

1. Vettriselvi V, Vijayalakshmi K, Paul SFD,

- Venkatachalam P. ACE and MTHFR gene polymorphisms in unexplained recurrent pregnancy loss. *J Obstet Gynaecol Res* 2008; 34: 301-306.
2. Carp H, Salomon O, Seidman D, Dardik R, Rosenberg N, Inbal A. Prevalence of genetic markers for thrombophilia in recurrent pregnancy loss. *Hum Reprod* 2002; 17: 1633-1637.
  3. Kutteh WH, Triplett DA. Thrombophilias and recurrent pregnancy loss. *Semin Reprod Med* 2006; 24: 54-66.
  4. Sarig G, Younis JS, Hoffman R, Lanir N, Blumenfeld Z, Brenner B. Thrombophilia is common in women with idiopathic pregnancy loss and is associated with late pregnancy wastage. *Fertil Steril* 2002; 77: 342-347.
  5. Wrambsy ML, Sten-Linder M, Bremme K. Primary habitual abortions are associated with high frequency of factor V Leiden mutation. *Fertil Steril* 2000; 74: 987-991.
  6. Brenner B, Sarig G, Weiner Z, Younis J, Blumenfeld Z, Lanir N. Thrombophilic polymorphisms are common in women with fetal loss without apparent cause. *Thromb Haemost* 1999; 82: 6-9.
  7. Goodman CS, Coulam CB, Jeyendran RS, Acosta VA, Roussev R. Which thrombophilic gene mutations are risk factors for recurrent pregnancy loss? *Am J Reprod Immunol* 2006; 56: 230-236.
  8. Kovalevsky G, Gracia CR, Berlin JA, Sammel MD, Barnhart KT. Evaluation of the association between hereditary thrombophilias and recurrent pregnancy loss: a meta-analysis. *Arch Intern Med* 2004; 164: 558-563.
  9. Jivraj S, Rai R, Underwood J, Regan L. Genetic thrombophilic mutations among couples with recurrent miscarriage. *Hum Reprod* 2006; 21: 1161-1165.
  10. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet* 2003; 361: 901-908.
  11. Kempf Haber M, Klimek M. [Thrombophilia in pregnancy and its influence on venous thromboembolism and recurrent miscarriages]. *Przegl Lek* 2005; 62: 164-168. (In Polish)
  12. Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JCM. ACE polymorphisms. *Circ Res* 2006; 98: 1123-1133.
  13. Ueda S, Elliott HL, Morton JJ, Connell JM. Enhanced pressor response to angiotensin I in normotensive men with the deletion genotype (DD) for angiotensin-converting enzyme. *Hypertension* 1995; 25: 1266-1269.
  14. Buchholz T, Thaler CJ. Inherited thrombophilia: impact on human reproduction. *Am J Reprod Immunol* 2003; 50: 20-32.
  15. Fatini C, Gensini F, Sticchi E, Battaglini B, Prisco D, Fedi S, et al. ACE DD genotype: an independent predisposition factor to venous thromboembolism. *Eur J Clin Invest* 2003; 33: 642-647.
  16. Kim DK, Kim JW, Kim S, Gwon HC, Ryu JC, Huh JE, et al. Polymorphism of angiotensin converting enzyme gene is associated with circulating levels of plasminogen activator inhibitor-1. *Arterioscler Thromb Vasc Biol* 1997; 17: 3242-3247.
  17. Su M-T, Lin S-H, Chen Y-C, Kuo P-L. Genetic association studies of ACE and PAI-1 genes in women with recurrent pregnancy loss: a systematic review and meta-analysis. *Thromb Haemost* 2013; 109: 8-15.
  18. Su MT, Lin S-H, Chen Y-C. Genetic association studies of angiogenesis- and vasoconstriction-related genes in women with recurrent pregnancy loss: a systematic review and meta-analysis. *Hum Reprod Update* 2011; 17: 803-812.
  19. Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. *Lancet* 2005; 365: 224-232.
  20. Newman PJ, Derbes RS, Aster RH. The human platelet alloantigens, PIA1 and PIA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J Clin Invest* 1989; 83: 1778-1781.
  21. Carter AM, Catto AJ, Bamford JM, Grant PJ. Platelet GP IIIaPIA and GP Ib variable number tandem repeat polymorphisms and markers of platelet activation in acute stroke. *Arterioscler Thromb Vasc Biol* 1998; 18: 1124-1131.
  22. Yilmaz S, Bayan K, Tüzün Y, Batun S, Altıntaş A. A comprehensive analysis of 12 thrombophilic mutations and related parameters in patients with inflammatory bowel disease: data from Turkey. *J Thromb Thrombolysis* 2006; 22: 205-212.
  23. Ruzzi L, Ciarafoni I, Silvestri L, Semeraro ML, Abeni D. Association of PLA2 polymorphism of the ITGB3 gene with early fetal loss. *Fertil Steril* 2005; 83: 511-512.
  24. Jeddi-Tehrani M, Torabi R, Mohammadzadeh A, Arefi S, Keramatipour M, Zeraati H, et al. Investigating Association of Three Polymorphisms of Coagulation Factor XIII and Recurrent Pregnancy Loss. *Am J Reprod Immunol* 2010; 64: 212-217.
  25. Jeddi-Tehrani M, Torabi R, Zarnani AH, Mohammadzadeh A, Arefi S, Zeraati H, et al. Analysis of plasminogen activator inhibitor-1, integrin beta3, beta fibrinogen, and methylene tetra hydro folate reductase polymorphisms in Iranian women with recurrent pregnancy loss. *Am J Reprod Immunol* 2011; 66: 149-156.
  26. Torabi R, Zarei S, Zeraati H, Zarnani AH, Akhondi MM, Hadavi R, et al. Combination of thrombophilic gene polymorphisms as a cause of increased the risk of recurrent pregnancy loss. *J Reprod Infertil* 2012; 13: 89-94.
  27. Joung CI, Park YW, Kim SK, Uhm WS, Kim TH, Yoo DH. Angiotensin-Converting Enzyme Gene Insertion/Deletion Polymorphism in Korean Patients with Systemic Sclerosis. *J Korean Med Sci* 2006; 21: 329-332.
  28. Javaheri-Kermani M, Farazmandfar T, Ajami A, Yazdani Y. Impact of hepcidin antimicrobial peptide on iron overload in tuberculosis patients. *Scand J Infect Dis* 2014; 46: 693-696.
  29. Hashemi-Soteh SMB, Sarzare F, Merat F, Salehifar E, Shiran MR. Frequencies of three CYP2D6 nonfunctional alleles (CYP2D6\*3, \*4, and \*6) within an Iranian population (Mazandaran). *Genet Test Mol Biomark* 2011; 15: 821-825.
  30. Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 1999; 180: 499-506.
  31. Irani RA, Xia Y. The functional role of the renin-angiotensin system in pregnancy and preeclampsia.

- Placenta* 2008; 29: 763-771.
32. Aarabi M, Memariani T, Arefi S, Aarabi M, Hantoosh Zadeh S, Akhondi MA, et al. Polymorphisms of plasminogen activator inhibitor-1, angiotensin converting enzyme and coagulation factor XIII genes in patients with recurrent spontaneous abortion. *J Matern-Fetal Neonatal Med* 2011; 24: 545-548.
  33. Bagheri M, Abdi Rad I, Omrani MD, Nanbaksh F. Polymorphisms of the angiotensin converting enzyme gene in Iranian Azeri Turkish women with unexplained recurrent pregnancy loss. *Hum Fertil* 2010; 13: 79-82.
  34. Buchholz T, Lohse P, Rogenhofer N, Kosian E, Pihusch R, Thaler CJ. Polymorphisms in the ACE and PAI-1 genes are associated with recurrent spontaneous miscarriages. *Hum Reprod* 2003; 18: 2473-2477.
  35. Fatini C, Gensini F, Battagliani B, Prisco D, Cellai AP, Fedi S, et al. Angiotensin-converting enzyme DD genotype, angiotensin type 1 receptor CC genotype, and hyperhomocysteinemia increase first-trimester fetal-loss susceptibility. *Blood Coagul Fibrinolysis* 2000; 11: 657-662.
  36. Valdez-Velazquez LL, Quintero-Ramos A, Perez SA, Mendoza-Carrera F, Montoya-Fuentes H, Rivas F et al. Genetic polymorphisms of the renin-angiotensin system in preterm delivery and premature rupture of membranes. *J Renin Angiotensin Aldosterone Syst* 2007; 8: 160-168.
  37. Mello G, Parretti E, Gensini F, Sticchi E, Mecacci F, Scarselli G, et al. Maternal-fetal flow, negative events, and preeclampsia: role of ACE I/D polymorphism. *Hypertension* 2003; 41: 932-937.
  38. Yenicesu GI, Cetin M, Ozdemir O, Cetin A, Ozen F, Yenicesu C, et al. A prospective case-control study analyzes 12 thrombophilic gene mutations in Turkish couples with recurrent pregnancy loss. *Am J Reprod Immunol* 2010; 63: 126-136.
  39. Ozdemir O, Yenicesu GI, Silan F, Köksal B, Atik S, Ozen F et al. Recurrent pregnancy loss and its relation to combined parental thrombophilic gene mutations. *Genet Test Mol Biomark* 2012; 16: 279-286.
  40. Coulam CB, Wallis D, Weinstein J, DasGupta DS, Jeyendran RS. Comparison of thrombophilic gene mutations among patients experiencing recurrent miscarriage and deep vein thrombosis. *Am J Reprod Immunol* 2008; 60: 426-431.
  41. Pihusch R, Buchholz T, Lohse P, Rübsamen H, Rogenhofer N, Hasbargen U, et al. Thrombophilic gene mutations and recurrent spontaneous abortion: prothrombin mutation increases the risk in the first trimester. *Am J Reprod Immunol* 2001; 46: 124-131.
  42. Hohlagschwandtner M, Unfried G, Heinze G, Huber JC, Nagele F, Tempfer C. Combined thrombophilic polymorphisms in women with idiopathic recurrent miscarriage. *Fertil Steril* 2003; 79: 1141-1148.