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SHORT REPORT

Polymorphisms of plasminogen activator inhibitor-1, angiotensin converting enzyme and coagulation factor XIII genes in patients with recurrent spontaneous abortion

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Abstract

We investigated polymorphisms of plasminogen activator inhibitor-1 (PAI-1), angiotensin converting enzyme (ACE) and coagulation factor XIII (FXIII) genes and their association with recurrent spontaneous abortion (RSA) in Iranian patients and normal healthy controls. Ten (18.5%) patients were homozygote (4G/4G) for PAI-1 polymorphism, in contrast with two (2%) controls ($p = 0.001$). Patients with homozygote 4G mutation were significantly more prone to RSA in contrast to others (odds ratio: 11.0, 95% CI: 2.3–52.4). Nineteen (30.2%) patients and 25 (26.6%) controls were homozygote (DD) for ACE polymorphism. We observed only two patients and one control with homozygosity (34leu) for FXIII polymorphism. 4G/4G polymorphism for PAI-1 gene could be a thrombophilic mutation leading to abortion in Iranian population.

Keywords: *Recurrent spontaneous abortion, thrombophilia, plasminogen activator inhibitor-1, angiotensin converting enzyme, coagulation factor XIII*

Introduction

Recurrent spontaneous abortion (RSA) is among the most common complications of pregnancy, affecting more than 5% of women. It might cause considerable emotional distress in couples who desire to have children. The etiology of RSA remains unexplained in many patients, but about 55% of patients are suspected to be associated with thrombophilic defects [1].

Thrombophilia can be characterized as an increased tendency towards thrombosis through enhanced coagulation. It includes several rare inherited abnormalities, often leading to thrombosis in young people [2]. Thrombophilia is shown to be associated with venous thromboembolism, deep vein thrombosis, pulmonary embolism, myocardial infarction and cerebral vein thrombosis [1,2].

Pregnancy is stable when there is a balance between maternal coagulation and fibrinolysis. This balance prevents excess fibrin deposition in placental vessels and intravillous spaces, secures fibrin polymerization and stabilizes the placental basal plate [3]. Women with thrombophilic defects are at increased risk, not only for pregnancy associated thromboembolism, but also for other vascular complications, such as pre-eclampsia and fetal loss [2].

Homeostasis abnormalities could be due to heritable or acquired defects which lead to clinical thrombophilia. Inherited abnormalities include mutations of either the genes encoding natural anticoagulants like antithrombin III, proteins C and S [1], or clotting factors like prothrombin, factor V Leiden (FVL) and methylenetetrahydrofolate reductase (MTHFR) [4].

In addition to above genetic defects, polymorphisms of plasminogen activator inhibitor-1 (PAI-1), coagulation factor XIII (FXIII) and angiotensin converting enzyme (ACE) genes have been mentioned. These genes appear to be a cause of impaired fibrinolysis which could promote the development of RSA [5].

PAI-1 has a vital role in hypofibrinolysis and thrombotic complications. The gene expression is modulated by a 4G/5G polymorphism in the promoter region, which is located 675 bp upstream from the start site of transcription [6]. The 4G allele binds to transcription-regulating factors and produces more PAI-1 protein than 5G allele. High levels of PAI-1 concentration could result in increased thrombosis through the defect in fibrinolysis and coagulation. This can contribute to initiation of placental damage and thrombotic complication [7].

ACE regulates thrombosis through generating angiotensin II from angiotensin I [2]. In addition, ACE is a key component of the rennin–angiotensin system and affects homeostasis [3].The level of enzyme is related to polymorphism of ACE gene consisting of the insertion/deletion (I/D) of a 287-bp fragment in intron 16 [8].

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Blood FXIII plays an important role in fibrin stabilization and protection of fibrin from proteolytic degradation. It is a pro-transglutaminase of tetrameric structure (A2B2) consisting of two potentially active A subunits (FXIII-A) and two inhibitory/protective B subunits (FXIII-B) [7]. The Val34Leu polymorphism in exon 2 of the FXIII-A gene could have an antifibronolytic effect through the early cross-linking of fibrin fibers [9].

In this study, we have investigated the association of RSA and PAI-1 4G/5G, ACE D/I and FXIII Val 34 Leu polymorphisms in Iranian patients and healthy controls.

Methods

Patients

Patients with a history of spontaneous abortion and healthy women without history of abortion as control group were registered in Avicenna Infertility Clinic and Department of Obstetrics & Gynecology of Imam Hospital, Tehran, Iran. The inclusion criterion was having the experience of at least three unexplained consecutive spontaneous abortions before 25 weeks of gestation. We also studied the patients with two unexplained consecutive spontaneous abortions before 25 weeks of gestation. For all patients, we collected medical history as well as results of physical examination, routine laboratory, endocrinologic and immunological tests for autoantibodies from the database of above centers. We excluded patients with known risk factors for spontaneous abortion: anatomical abnormalities, endocrinologic dysfunctions, autoimmune diseases, urogenital infection and inflammatory pelvic disease. All patients and normal healthy controls were Iranian.

Approval from the Avicenna Research Institute's ethics & human rights committee was obtained for the use of blood samples and study protocol. Before study, all participants signed the informed consent form.

Determination of genotype

To analyze the D/I polymorphism in intron 16 of the ACE gene and the −675 4G/5G polymorphism in the promoter region of the PAI-1 gene and Val 34 Leu FXIII polymorphisms, 5 ml of blood samples were obtained. Genomic DNA was then extracted from leukocytes using salting out standard protocol. Genomic DNA was amplified by polymerase chain reaction (PCR) using gene-specific primers (Figure 1).

Each 25 μl of reaction contained 2.5 μl of 10 × PCR reaction buffer (100 mM Tris-HCl, 500 mM KCl pH: 8.3), 1.5 μl $MgCl_2$ (25 mM), 1 μl of forward and reverse primers (10 μM), 1U DNA polymerase (5U, ROCHE, Mannheim, Germany) and 150 ng of DNA. After denaturation at 94°C for 5 min, DNA fragments were either amplified for 35 cycles for FXIII (94°C for 30 s, 58°C for 30 s and 72°C for 30 s), 35 cycles for ACE (94°C for 30 s, 70°C for 40 s and 72°C for 1 min), and 32 cycles for PAI-1 polymorphisms (94°C for 30 s, 60°C for 30 s and 72°C for 1 min). PCR cycles were followed by final extension step at 72°C for 7 min.

For the amplification of PAI-1 gene, we designed a forward primer different from the native sequence in order to introduce a site for BseR I restriction enzyme. Originally, the 148 bp PCR product has no restriction site for enzyme, but combination of nucleotide substitution and genetic abnormality creates the enzyme cleavage site and cleaves the amplicon into two fragments (110 bp and 38 bp). The D/I genotype of the ACE gene was described by 190 bp PCR product length in deletion, as well as 490 bp in insertion. Finally, PCR product of FXIII Val 34 Leu polymorphism had a length of 192 bp with no restriction site for DdeI enzyme. The mutation in FXIII created a restriction site and produced two fragments (161 bp and 31 bp) after enzyme digestion.

PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining under UV light. Digested and undigested PCR products were separated by polyacrylamide gel electrophoresis (PAGE) and visualized using silver staining method.

Statistical methods

Patients were categorized according to their genotypes. Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS, version 16 for Windows, Chicago, IL). Results of the two groups were compared using Fisher's exact test or χ^2 test, as well as *t*-test for quantitative variables. An α level of 0.05 was used to indicate statistical significance. We firstly compared

Polymorphism	Primers	PCR Product	Restriction Enzyme
Factor XIII Val34Leu	CATGCCTTTTCTGTTGTCTTC TACCTTGCAGGTTGACGCCCCGGGGCACTA	192 bp (161, 31)	Dde I
PAI-1 -675 4G/5G	CACAGAGAGAGTCTGGACACGTGA TGCAGCCAGCCACGTGATTGTCTAG	148 bp (110, 38)	Bse RI
ACE intron 16 I/D	CTGGAGACCACTCCCATCCTTTCT GATGTGGCCATCACATTCGTCAGAT	Depends on genotype	287bp ins/del

Figure 1. Genotyping assays for PAI-1, ACE and factor XIII polymorphisms. Primer sequences are listed from 5′ to 3′, and the upper sequence represents the forward primer; the lower sequence, the reverse primer. The underlined bases were changed from the native sequence to introduce a restriction enzyme recognition sequence that would result in digestion of the PCR product to the fragments shown in parentheses.

patients with RSA (≥3 abortions) and controls. In the next step, the comparison was done for patients with two or more abortions and controls.

Results

After excluding the patients with known risk factors of spontaneous abortion, we investigated 63 patients and 114 healthy women of the control group for the PAI-1, ACE, and FXIII gene polymorphisms. Mean age at registration was 32.5 (95% CI: 31.1–34.0) years for patients and 32.9(95% CI: 31.4–34.5) years for controls. The difference between ages in patient population *versus* the control group was not statistically significant (*T*-Test, $p = 0.01$). Mean frequency of abortion among 63 patients was 4.6 (95% CI: 4.0–5.3) with a range of 3–15. Neither physical examination nor laboratory tests were significantly different among patient and control groups.

The incidence of the PAI-1 4G/5G polymorphism in patients was 10 (18.5%) for homozygous 4G, 23 (42.6%) for heterozygous 4G/5G and 21 (38.9%) for homozygous 5G *versus* 2 (2.0%), 66 (66.7%) and 31 (31.3%) in controls, respectively. The prevalence of polymorphism (4G/4G) in patients was significantly higher than controls (χ^2 test: $p = 0.001$). Patients with homozygote 4G/4G polymorphism were significantly more prone to RSA in contrast to controls (OR: 11.0, 95% CI: 2.3–52.4) (Table I). Interestingly, the frequency of 4G polymorphism was higher among the participants with two or more abortions rather than controls (χ^2 test: $p = 00.1$). We found that this polymorphism could result in the higher risk of having two or more abortions in our study population (OR: 8.2, 95% CI: 1.8–36.5) (Table II).

The frequency of ACE D/I polymorphism in patients was 14 (22.2%) for homozygous II, 30 (47.6%) for heterozygous DI and 19 (30.2%) for homozygous DD; whereas the frequency in the control group was 22 (23.4%), 47 (50%) and 25 (26.6%), respectively. The rate of mutation in patients was not statistically higher than control group.

FXIII 34Val, Val 34Leu, and 34Leu genotypes were observed in 71.9%, 24.6% and 3.5% of patients, respectively. There was no significant difference between patients and controls (Figure 2).

We also combined the results of polymorphisms of above three genes to find any probable differences between patients and controls. However, we were unable to find any significant associations. More details of results are shown in Tables I and II.

Table I. Frequency of homozygous mutations in patient group and controls.

	Patients		Controls			OR
	No.	Fr.	No.	Fr.	*p* value	(CI 95%)
PAI-1	10	18.5	2	2.0	0.001	11.0 (2.3–52.4)
ACE	19	30.2	25	26.6	0.626	1.2 (0.6–2.4)
FXIII	2	3.5	1	0.9	0.277	3.9 (0.3–43.5)
PAI-1&ACE	2	3.3	0	0	0.125	NS
PAI-1&FXIII	0	0	0	0	NS	NS
ACE&FXIII	0	0	0	0	NS	NS
PAI-1&ACE&FXIII	0	0	0	0	NS	NS

OR, odds ratio; CI, confidence interval; Fr., frequency; NS, not significant.

Table II. Frequency of homozygous mutations in participants with two or more spontaneous abortions and controls.

	Patients		Controls			OR
	No.	Fr.	No.	Fr.	*p* value	(CI 95%)
PAI-1	16	14.4	2	2.06	0.001	8.2 (1.8–36.5)
ACE	38	29.5	25	26.6	0.639	1.2 (0.7–2.1)
FXIII	2	1.7	1	0.9	1	1.8 (0.2–20.1)
PAI-1&ACE	3	2.4	0	0	0.25	NS
PAI-1&FXIII	0	0	0	0	NS	NS
ACE&FXIII	0	0	0	0	NS	NS
PAI-1&ACE&FXIII	0	0	0	0	NS	NS

OR, odds ratio; CI, confidence interval; Fr. Frequency; NS, not significant.

Discussion

Polymorphism analysis of genes involved in thrombophilia should be investigated in different ethnics. Detection of those polymorphisms with RSA can help clinicians to design better therapeutic strategies.

In this study, we observed the significant increase of PAI-1 (4G/4G) polymorphism in patients with RSA.

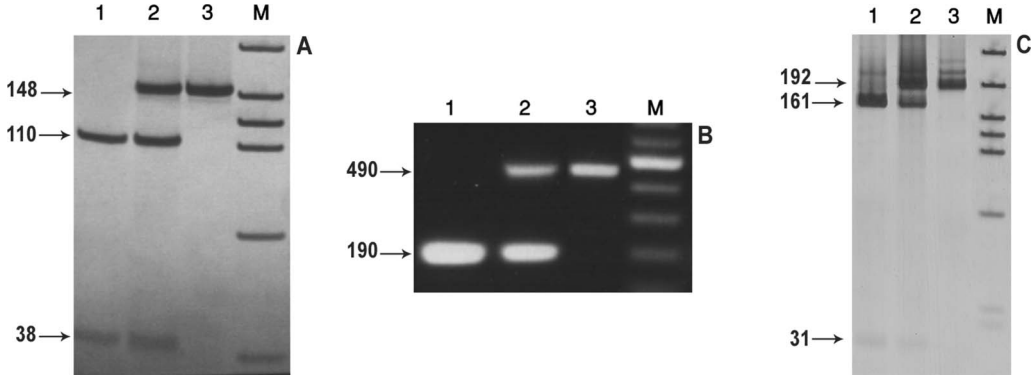

Figure 2. PCR products of thrombophilic mutations among patients with RSA. M: marker, 1: homozygote patient, 2: normal heterozygote, 3: normal homozygote (A: PAI-1, B: ACE, C: FXIII).

We also found this polymorphism as a risk factor for spontaneous abortion. Three-fold elevations in the level of functionally active plasminogen have been reported as a risk factor for pregnancy disorders and fetal damage [6]. In addition, Kohler has described higher concentrations of PAI-1 as well as increased frequency of PAI-1 4G/4G genotype in patients with myocardial infarction [10]. Their data suggested that the PAI-1 4G and FXIII 34Leu variants synergistically contribute to impaired fibrinolysis because of reduced activity of the fibrinolytic system and increased resistance of the fibrin network to fibrinolysis [7,10]. Our data support parts of their suggestion that PAI-1 4G variant might be a risk factor for recurrent abortion, but does not support the role of FXIII 34Leu. On the other hand, our findings are supported by other studies which reported the elevated plasminogen activator inhibitor type 1 (PAI-1) and PAI-1 polymorphisms [7] in patients with RSA and thrombotic homeostasis defects. This may promote the development of early pregnancy loss in affected women by insufficient trophoblast invasion and unbalanced fibrin deposition in the early placental circulation.

We considered three or more spontaneous abortions as the definition of RSA in this study. However, we found that the PAI-1 4G polymorphism is even more frequent among participants with two or more abortions over the controls. This finding highlights the important role of 4G polymorphism as a potential risk factor of spontaneous abortion. We recommend the screening of this polymorphism not only for women with RSA, but for women with the history of two or more spontaneous abortions.

We demonstrated that homozygosity for the ACE alleles is not a risk factor for RSA in our population. The difference of patterns of I/D polymorphism in ACE gene is suggested to be an important risk factor for cardiovascular morbidity and mortality in Western and Asian individuals. This might be relevant in explaining differences of thromboembolic diseases in different populations. ACE D allele is demonstrated to be in association with increased level of PAI-1 and susceptibility to fibrinolysis [8]. We could not find any significant association between ACE polymorphism as well as combination of polymorphisms and RSA in our study. This finding could be due to the differences between ethnicity of participating women in studies. The strong association between the ethnicity of the study population and the polymorphism frequencies is possible and should be considered [11].

We previously analyzed the frequency of FVL (G1691A), MTHFR (C677T), and FII (G20210A) mutations in Iranian patients who suffered from unexplained infertility or RSA and compared the results with healthy controls. Those data showed a skew towards higher mutation frequencies of FVL and MTHFR in the patients [4]. Investigation of the cumulative risk associated with a combination of those three mutations and sequence variants of the PAI-1, ACE and FXIII genes in our patients revealed no association between above six polymorphisms as a risk factor for pregnancy loss.

This study along with our previous published data emphasize that analysis of PAI-1 (4G/4G) polymorphism, FVL (G1691A) and MTHFR (C677T) mutations should be included in the routine work-up of patients with RSA. The clinical implications of these data need to be addressed in a prospective study to answer the question whether or not homozygous individuals should be treated with heparin to prevent RSA.

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