Relationship of the Protein Z Intron F G79A and IL6 C634G Gene Polymorphisms With the Risk of Recurrent Pregnancy Loss in Egyptian Women

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Purpose: To investigate the relationship between recurrent pregnancy loss and single nucleotide polymorphisms in the protein Z (PZ) intron F G79A and the promoter region of the *IL6 C634G* genes in Egyptian women.

Procedures: Single nucleotide polymorphisms in the PZ intron *F G79A* gene and the promoter region of the *IL6 C634G* gene were studied in 70 Egyptian women; 40 patients and 30 healthy and parous volunteers using the polymerase chain reaction–restriction fragment length polymorphism technique.

Results: Regarding the PZ intron F G79A polymorphism; a higher prevalence of the A allele in the controls (53.3%) compared with the cases (22.5%) was found, and the difference proved to be statistically significant ($P = 0.008$). As for the IL6 C634G polymorphism, the frequency of the G allele was higher in the controls (100%) than in the cases (95%), but the difference did not prove to be statistically significant ($P = 0.503$). A statistically significant difference between the prevalence of the IL6 C634G (95%) and the PZ intron F G79A (22.5%) was detected ($P \leq 0.001$).

Conclusion: A statistically significant difference of the frequency of the A allele of the PZ intron F G79A polymorphism was found with a higher prevalence of the A allele among the controls compared with the patients, suggesting a lower risk of recurrent pregnancy loss among the studied patients, but the IL6 C634G polymorphism did not prove to have an equivalent effect.

Key Words: recurrent pregnancy loss, protein Z intron F G79A polymorphism, IL6 C634G polymorphism, PCR-RFLP

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Spontaneous abortion is defined as an expulsion of an embryo or fetus weighting 500 g or less from the mother at a gestational age of 20 to 22 weeks or less.[1] It occurs in 10% to 20% of all clinically recognized pregnancies.[2] For a successful pregnancy outcome, a balance between the coagulation factors and the fibrinolytic system of the mother is required. Pregnancy is one of the common prothrombotic conditions that precipitate a primary hypercoagulable state,[3] so recurrent pregnancy loss (RPL) may be associated with some thrombophilic risk factors, such as the deficiencies of antithrombin, protein C, and protein S as well as factor V Leiden mutation, the prothrombin gene mutation G20210A, and the antiphospholipid syndrome.[4,5]

The Protein Z (PZ) is a vitamin K–dependent plasma glycoprotein that has an important anticoagulant property through inhibition of the activated factor X (Xa) by serving as a cofactor to a plasma proteinase inhibitor,[6] as well as a procoagulant activity through enhancing the synthesis of thrombin. Many polymorphisms of the PZ gene have been investigated. The well-studied is probably the G79A polymorphism in intron F. The PZ deficiency has recently been reported in women with unexplained early fetal losses.[7] Lower plasma PZ level is usually associated with the carriers of the A allele; however, the homozygous G allele has been reported as an independent risk factor for stroke by some[8] but not all investigators.[9]

Interleukin 6 (IL-6) is a Th2-type pro as well as anti-inflammatory cytokine secreted by several tissues and cell types including the immune cells, fibroblasts, endothelial cells, skeletal muscle, and the adipose tissue.[10] The human IL-6 is a single glycoprotein chain.[11] The gene for IL-6 is located on chromosome 7p2l and consists of 4 introns and 5 exons.[12] Different functional gene polymorphisms of the IL-6 may or may not be associated with the risk and/or the pathogenesis of repeated spontaneous abortion.[13] −174 G/C and −634C/G are 2 common polymorphisms of the IL6 that are well studied. The former polymorphism affects the transcriptional rate of the gene and in turn the plasma concentrations of the circulating IL-6,[11] whereas the later one did not prove to influence the IL-6 production in circulation.[14]

Interleukin 6 favors the success of pregnancy. Women with 3 or more pregnancy losses have a more severe condition of RPL and a poorer reproductive outcome in a subsequent pregnancy than do women with 2 pregnancy losses. Thus, the causal association of the deviation of the IL-6 −634C→G genotype with RPL was suggested further.[13]

SUBJECTS AND METHODS

Subjects

In this study, 70 Egyptian female patients were recruited: 40 patients with a history of 2 consecutive or more than 3 nonconsecutive early (8th–12th week of gestation) pregnancy losses (ages ranged between 18 and 38 years, with a mean value of 27.95 ± 5.9 years) and 30 unrelated apparently healthy and parous volunteers matched for age and ethnic background, with no history of pregnancy loss selected as controls (ages ranged between 19 and 32 years, with a mean value of 26.3 ± 4.87 years). The patients were selected from the outpatient clinic of the Obstetric and Gynecology unit of El Kasr El-Aini Hospital.

For evaluation of the common causes for RPL or intrauterine fetal death, none of the women had any gynecologic or hormonal reasons for RPL. No cases or controls had experienced any arterial or venous thrombotic event. The women were not taking any medications including oral contraceptive pills that could possibly affect any of the measured parameters. All women gave their informed consents for participating in this study and were subjected to the following investigations: clinical examination, analysis of complete blood count, antiphospholipid antibodies (lupus anticoagulant), protein C, protein S

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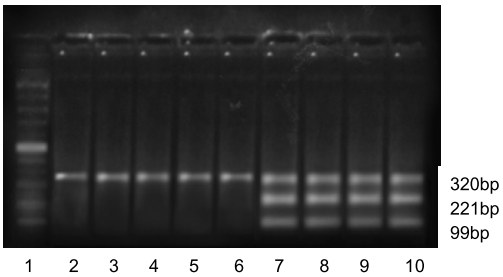

FIGURE 1. Polymerase chain reaction–restriction fragment length polymorphism results for PZ G79A polymorphism among patients. Lane 1: DNA ladder (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps). Lanes 2 to 6: homozygous GG (1 band of 320 bp). Lanes 7 to 10: heterozygous GA (3 bands of 320, 221 and 99 bp).

and antithrombin III assays as previously described,[15] and a special laboratory investigation including the study of the PZ intron F G79A polymorphism according to the method described by Dossenbach-Glaninger et al.[16] and the *IL6 C634G* gene polymorphism according to the method described by Saijo et al.[13] using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique.

Methods

Five milliliters of venous blood were collected from each patient and each control subject by a sterile venipuncture and divided as follows: 1 ml of venous blood for the complete blood count analysis; 2 ml for the lupus anticoagulant, protein C, protein S, and antithrombin III assays; and 2 ml for the study of PZ intron F G79A and IL6 C634G genes polymorphisms by PCR-RFLP assay.

Genetic Analysis

Study of the PZ intron F G79A and the *IL6 C634G* genes polymorphisms by PCR-RFLP assay:

I. DNA extraction

The genomic DNA was extracted from the cells using QIAamp blood DNA isolation kits (Qiagen, Crawley, UK) according to the manufacturer's protocol.

II. PCR reaction

Polymerase chain reaction followed by the enzymatic digestion of the PCR products was used for genotyping of the polymorphisms.

A mixture 25-μL reaction consisted of 5 μL genomic DNA, 12.5 μL of the PCR master mix, 2 μl of each primer, and 3.5 μL DW (Qiagen) were prepared.

For PZ G79A polymorphism, the following primers (Qiagen) were used:

PZ G79A F 5′-TAA CAC CAT AGA CAG AGT CCG ATA TTC GC -3′

PZ G79A R 5′-ATG AAC TCG GCA TTA GAA CAT GGT TGG AA -3′

For IL-6 C634G polymorphism, the following primers (Qiagen) were used:

IL-6 C634GF 5′-GAG AGG CCT TGA AGT AAC TG -3′ and

IL-6 C634GR 5′-AAC CAA AGA TGT TCT GAA CTG A -3′

The following cycles were used:

For the PZ G79A polymorphism, there was an initial heat-activation step at 94°C for 4 minutes followed by 32 cycles with the following program: denaturation at 94°C for 60 seconds, annealing at 57°C for 60 seconds, and finally extention at 74°C for 60 seconds.

For the IL-6 C634G polymorphism, denaturation at 95°C for 45 seconds followed by annealing at 48°C for 45 minutes, then extention at 72°C for 60 seconds for a total of 40 cycles ending with a final extention at 72°C for 10 minutes.

The samples were then run in parallel on 2% agarose gel using gel electrophoresis (electro-4, Thermal Hybaid, from Promega) and visualized on an UV transilluminator (wave length, 312) for the detection of the DNA bands.

III. Digestion of the PCR products by a specific restriction enzyme for the detection of the PZ G79A and the IL-6 C634G polymorphisms

For the PZ G79A polymorphism, after amplification, the PCR product (320 bp) was digested with 10 U HpaI enzyme (Fermentas Life Sciences, USA) in the manufacturer's buffer at 37°C overnight, resulting in 1 band at 320 bp for the homozygous GG genotype; 3 bands at 320, 221, and 99 bp for the heterozygous GA allele; and 2 bands at 221 and 99 bp for homozygous AA allele.

For the IL-6 C634G polymorphism, after amplification, the PCR product (180 bp) was digested with 10U BsrBI enzyme (Fermentas Life Sciences) in the manufacturer's buffer at 37°C overnight, resulting in 1 band at 180 bp for the homozygous CC allele; 3 bands at 180, 120, and 60 bp for the heterozygous CG allele; and 2 bands at 120 and 60 bp for the homozygous GG allele.

IV. Detection of the PCR products

The bands of the PZ and *IL-6* genes were identified by using 2% agarose gel. Ethidium bromide staining was used to reveal the fragments under an ultraviolet light transillumination.

A DNA molecular weight marker (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 bps) was used (Figs. 1–4).

Statistical Analysis of the Data

The quantitative values were expressed as mean ± SD and were compared using the *t* test for the 2 groups. The qualitative data were compared using the χ^2 test. The odds ratio (OR) and 95% confidence intervals (CIs) were calculated using the logistic regression analysis. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant. SPSS 12 statistical package was used for analyses.

RESULTS

Comparison between the patients and the control group regarding the demographic (age and clinical data) and the laboratory characteristics is presented in Table 1.

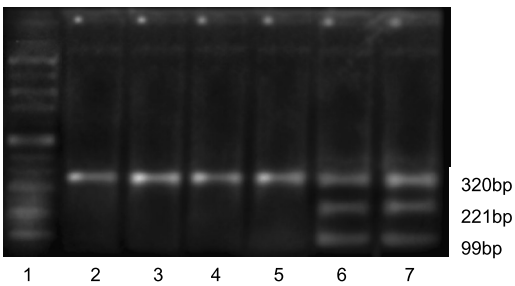

FIGURE 2. Polymerase chain reaction–restriction fragment length polymorphism results for PZ G79A polymorphism among controls. Lane 1: DNA ladder (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps). Lanes 2 to 5: homozygous GG (1 band of 320 bp). Lanes 6 & 7: heterozygous GA (3 bands of 320, 221 and 99 bp).

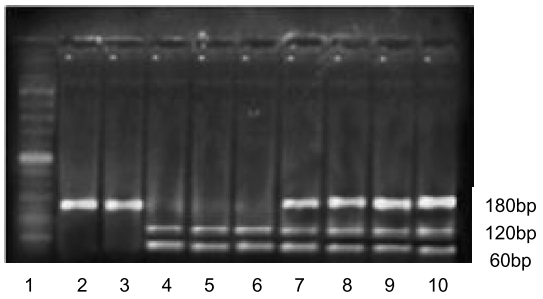

FIGURE 3. Polymerase chain reaction–restriction fragment length polymorphism results for IL-6 C634G polymorphism among patients. Lane 1: DNA ladder (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps). Lanes 2 & 3: homozygous CC (1 band of 180 bp). Lanes 4 to 6: homozygous GG (2 bands of 120 & 60 bp). Lanes 7 to 10: heterozygous CG (3 bands of 180, 120 & 60 bp).

TABLE 1. Demographic, Clinical, and Laboratory Data of Patients and Control Group

Items	Patients (n = 40)	Controls (n = 30)	*P*
Age (yr)	27.95 ± 5.90	26.76 ± 4.04	0.31 (NS)
No. abortions	4.78 ± 2.27	0.00 ± 0.00	<0.001 (S)
No. living offspring	0.00 ± 0.00	2.50 ± 0.682	<0.001 (S)
Lupus anticoagulants (s)	32.05 ± 4.93	32.40 ± 5.23	0.77 (NS)
Protein C (%)	76.75 ± 8.95	77.33 ± 9.26	0.79 (NS)
Protein S (%)	77.50 ± 8.39	78.66 ± 9.37	0.59 (NS)
Antithrombin III (%)	77.12 ± 8.15	78.5 ± 8.92	0.50 (NS)

Values are expressed as mean ± SD.

NS indicates nonstatistically significant difference; S, statistically significant difference.

Regarding age, the difference was statistically insignificant between both groups ($P = 0.31$). The number of abortions was significantly higher in patients because there were no recorded abortions in the controls ($P < 0.001$). The number of living children was significantly higher in the controls because there were no recorded living children in the patients ($P < 0.001$). Regarding the lupus anticoagulants, protein C, protein S, and antithrombin III assays, the differences were statistically insignificant between both groups ($P = 0.79$, 0.59, 0.50, and 0.77 respectively).

Analysis of the PZ intron F G79A and the IL6 C634G polymorphisms in the RPL patients and the control groups is shown in Table 2.

For the PZ intron F G79A polymorphism, the frequency of the homozygous GG genotype was higher than the A allele, heterozygous GA and homozygous AA genotypes, in the cases (77.5% vs 22.5%, 0.0%), whereas in the controls, the A allele, heterozygous GA genotype was higher than the homozygous GG genotype and the homozygous AA genotype (53.3% vs 46.7%, 0.0%), respectively. The frequency of the A allele was lower in the cases than in the controls (11.25% vs 26.7%). The difference of the observed genotypes' frequencies proved to be statistically significant ($P = 0.008$).

For the IL6 C634G polymorphism, the frequency of the G allele, homozygous GG genotype, was higher than the heterozygous CG and the homozygous CC genotypes in both cases and the controls (82.5% vs 12.5%, 5.0%) and (90.0% vs 10.0%, 0.0%), respectively. The frequency of the G allele was lower in the cases than in the controls (88.75% vs 95%). The difference between the observed genotypes' frequencies did not prove to be statistically significant ($P = 0.503$).

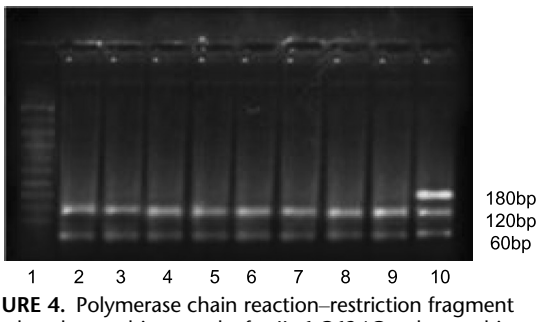

FIGURE 4. Polymerase chain reaction–restriction fragment length polymorphism results for IL-6 C634G polymorphism among controls. Lane 1: DNA ladder (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps). Lanes 2 to 9: homozygous GG (2 bands of 120 & 60 bp). Lane 10: heterozygous CG (3 bands of 180, 120 and 60 bp).

Statistical comparison between the patients and the control subjects as regard the risk of RPL is presented in Table 3.

The OR value of the PZ intron F G79A polymorphism is 0.254 (95% CI, 0.090–0.713). The difference proved to be statistically significant ($P = 0.008$). The OR value of the A allele is 0.349 (95% CI, 0.142-0.857). The difference proved to be statistically significant ($P = 0.018$).

The OR value of the IL6 C634G polymorphism could not be calculated because the observed frequencies did not allow its calculation. The difference of the observed genotypes' frequencies did not prove to be statistically significant ($P = 0.503$). The OR value of the G allele is 1.392 (95% CI, 0.517–3.752). The difference did not prove to be statistically significant ($P = 0.316$).

Comparison between the frequencies of the IL6 C634G gene polymorphism and the PZ intron F G79A gene polymorphism is shown in Table 4.

The frequency of the C634G polymorphism (95%) was higher than the frequency of the FG79A polymorphism (22.5%). The difference proved to be statistically significant ($P \leq 0.001$).

DISCUSSION

Our thesis studied 2 markers in 2 different systems, coagulation and immune systems, because of the strong link between them. The immune and the blood coagulation systems are simultaneously activated in many inflammatory systemic disorders, such as systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel diseases.[17] Interleukin 6 is a proinflammatory cytokine that induces the expression of the tissue factor that, in turn, stimulates the expression of various proinflammatory cytokines that have been associated with thrombosis in a number of settings. This cross-link between inflammation and coagulation initiates and maintains the activation of both systems.[18]

Recurrent pregnancy loss is defined as 2 or more spontaneous losses (abortions) of the fetus before the 20th week of gestation.[19] Approximately 1% of the population have recurrent spontaneous abortion.[20]

The PZ is a vitamin K–dependent factor, synthesized by the liver, and serves as an anticoagulant and procoagulant factor.[21,22] PZ level increases as pregnancy progresses and returns to the

TABLE 2. Comparison Between RPL Patients and Control Groups Regarding Frequency of PZ Intron F G79A and IL6 C634G Genes Polymorphisms

Items	Patients, n (%)	Controls, n (%)	*P*
PZ intron F G79A polymorphism			
Homozygous (G/G) genotype	31 (77.5%)	14 (46.7%)	
(A) allele genotype	9 (22.5%)	16 (53.3%)	0.008 (S)
Heterozygous (G/A)	9 (22.5%)	16 (53.3%)	
Homozygous (A/A)	0 (0%)	0 (0%)	
No. alleles	80	60	
Frequency and percentage of A allele	9 (11.25%)	16 (26.7%)	0.018 (S)
Frequency and percentage of G allele	71 (88.75%)	44 (73.3%)	—
IL6 C634G gene polymorphism			
Homozygous (C/C) genotype	2 (5%)	0 (0%)	
(G) allele genotype	38 (95%)	30 (100%)	0.503 (NS)
Heterozygous (C/G)	5 (12.5%)	3 (10%)	
Homozygous (G/G)	33 (82.5%)	27 (90%)	
No. alleles	80	60	
Frequency and percentage of G allele	71 (88.75%)	57 (95%)	0.316 (NS)
Frequency and percentage of C allele	9 (11.25%)	3 (5%)	—

Values are expressed as number and percentage (n [%]).
NS indicates nonstatistically significant difference; S, statistically significant difference.

nonpregnant level in approximately 6 to 12 weeks after delivery. The *PZ* gene is present on chromosome 13 at band q34 and spans 389bp.[23] Many polymorphisms of the *PZ* gene have been detected. The most studied one affects the intron F and is designated G79A polymorphism.[9]

Interleukin 6 is a proinflammatory and an anti-inflammatory cytokine. It is secreted from the T-helper 2-type cells as a humoral immune response (Th2). During pregnancy, the secretion of the IL-6 and IL-10 increases.[24] During normal labor, the IL-6 level increases in the maternal serum and the different fluids compared with the nonlabor state.[24] Different polymorphisms of the IL-6 have been encountered and associated with the pathogenesis as well as the risk of many diseases. These polymorphisms include the IL-6 G174C and IL-6 C634G, where the former polymorphism is frequently found in whites and the latter in the Japanese.[13]

Our data demonstrated that the PZ intron *F G79A* gene polymorphism has a lower frequency among the patients with RPL compared with the controls. In cases, the frequency of the A allele of the intron F G79A was lower than that in the controls (11.25% vs 26.7%), suggesting a lower risk of RPL among the patients (OR, 0.349; 95% CI, 0.142–0.857). The observed frequency of the intron F G79A genotypes between the cases and the controls proved to be statistically significant ($P = 0.008$). This is in accordance with Dossenbach-Glaninger et al.,[16] who also demonstrated a significantly lower frequency of pregnancy loss in the carriers of the less common A allele of the intron F G79A. The prevalence of the PZ intron F G79A polymorphism in patients (carried at least 1 A allele) was 28.6% compared with 50% in the control subjects; this difference was significant, indicating a reduction of the relative risk of RPL (OR, 0.4; 95% CI, 0.2–0.9; $P = 0.038$). Also, our results were in agreement with the data by Lichy et al.[22] and Staton et al.[9] who described a reduction of the relative risk for cerebral ischemia in the carriers of at least 1 A allele. In contrast, Santacroce et al.[25] found only little impact of the PZ plasma levels and of the PZ gene polymorphisms for the occurrence of DVT. This may indicate that in peripheral venous disease, the protective effect of the PZ 79A allele is less pronounced. Similarly, Lichy et al.[22] reported a decreased relative risk in the carriers of the PZ 79A allele for cerebral venous thrombosis (OR, 0.77), but these results were not statistically significant. In contrast, Topalidou et al.[26] could not establish a significant difference in the frequency of carriers of the A allele between the cases with unexplained early pregnancy loss (39.2%) and the healthy controls (40.4%). However, they established that PZ plasma levels were significantly lower

TABLE 3. Statistical Comparison Between Patients and Control Subjects as Regards Risk of RPL

Gene Polymorphism	Cases, n (%)	Controls, n (%)	OR	95% CI	*P*
PZ Intron F G79A genotypes					
Homozygous GG genotype	31 (77.5%)	14 (46.7%)	0.254	0.090–0.713	0.008 (S)
A allele (GA &AA genotypes)	9 (22.5%)	16 (53.3%)	0.349	0.142–0.857	0.018 (S)
IL6 C634G genotypes					
Homozygous CC genotype	2 (5%)	0 (0%)	—	—	0.503 (NS)
G allele (CG and GG genotypes)	38 (95%)	30 (100%)	1.392	0.517–3.752	0.316 (NS)

Values are expressed as number and percentage (n [%]).
NS indicates nonstatistically significant difference; S, statistically significant difference.

TABLE 4. Comparison of the Prevalence of FG97A and C634G Gene Polymorphisms Among Patients

Items	PZ Intron F G79A, n (%)	IL6 C634G, n (%)
No. cases	40	40
Frequency and percentage of homozygous GG and homozygous CC genotypes	31 (77.5%)	2 (5%)
Frequency and percentage of A allele (GA and AA) and G allele (CG and GG)	9 (22.5%)	38 (95%)
P	<0.001 (S)	

S indicates statistically significant difference.

in cases than in controls ($P \leq 0.001$) and also in the carriers of the A allele of intron F G79A compared with the GG homozygous subjects ($P = 0.044$), concluding that the intron F G79A polymorphism was unrelated to the unexplained early pregnancy loss but associated with significantly lower PZ levels, which is possibly considered a novel risk factor for the unexplained recurrent miscarriage or fetal death.

Van Goor et al.[8] found in their study some contradictory data as well as some data in accordance with the abovementioned studies. They found that reduced levels of the PZ were independently associated with an increased risk of first ischemic stroke. However, they did not observe an association between the *PZ G79A* gene polymorphism and the risk of ischemic stroke. They also found a relationship between the PZ level and the presence of the A allele of the PZ G79A gene polymorphism; they demonstrated that the highest PZ levels were found in subjects with the homozygous GG genotype and the lowest in subjects with the homozygous AA genotype in both patients and controls. The PZ-lowering effect of the A allele seems to be dominant rather than additive because there were no statistically significant differences in PZ levels between the heterozygous GA and the homozygous AA genotypes.[8]

To date, it is unknown how the PZ intron F G79A polymorphism may mediate its protective effect. Lichy et al.[22] discussed that a direct regulatory role of this polymorphism is possible, but an indirect effect due to linkage with other published or yet unknown polymorphisms seems more likely. It was already shown that, although a number of polymorphisms in the PZ gene have been described, the haplotype structure does not show linkage disequilibrium between the G79A polymorphism and possible functional variants controlling the production of PZ.[8]

In our study, the prevalence of the G allele of the IL6 *C634G* gene polymorphism in patients was 12.5% for the heterozygous CG and 82.5% for the homozygous GG versus 10% and 90%, respectively, in the control subjects. The homozygous CC genotype was only found in 5% of the cases. The difference in the IL6 C634G genotypes' frequencies between the cases and the controls did not prove to be statistically significant ($P = 0.503$) (OR, 1.392; 95% CI, 0.517–3.752). However, the higher prevalence of the G allele in the controls (95%) compared with the cases (88.75%) might suggest a protective effect of this allele against RPL. Saijo et al.[13] demonstrated a statistically significant difference in the C634G genotype frequency (CC vs CG/GG) between women with RPL and controls ($P = 0.026$). The risk of RPL was lower in the carriers of the G allele than in the subjects with the homozygous type (CC) (OR, 0.46; 95% CI, 0.24–0.91). Also, Ota et al.[14] reported, in their study, similar results to Saijo et al.[13] with the same conclusion of the protective role of the G allele against RPL. The striking finding in our study was that the percentage of the G allele among the controls was 100% compared with 39.8% among the controls of the Saijo et al.[13] study which was carried on Japanese women. Also the study done by Ota et al.[14] on Japanese women also showed almost the same results of prevalence of the G allele among controls as Saijo et al.,[13] indicating that population bias definitely exists.

This study is the first study which established an association between the PZ FG79A polymorphism and the IL6 C634G polymorphism among subjects with early RPL. In cases, the frequency of the IL6 C634G gene polymorphism (95%) was higher than the frequency of the PZ FG79A gene polymorphism (22.5%). The difference proved to be statistically significant ($P \leq 0.001$). This demonstrates that the presence of the A allele of the PZ FG79A polymorphism has a higher protective effect against RPL than the presence of the G allele of the IL6 C634G polymorphism.

In conclusion, a statistically significant difference of the frequency of the A allele of the PZ intron F G79A polymorphism was found with a higher prevalence of the A allele among the controls compared with the patients, suggesting a lower risk of RPL among the studied patients, but the IL6 C634G polymorphism did not prove to have an equivalent effect. Because we could only include a small number of participants in the present study, the obtained results need confirmation in a larger series of probands.

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