

Single Nucleotide Polymorphism of Interleukin-27 Gene: A Risk Factor of Recurrent Pregnancy Loss in Iraqi Women

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Research Article

Abstract

Background: Studies have investigated the association between a single nucleotide polymorphism (SNP) of interleukins and the recurrent pregnancy loss (RPL). However, different results have been found in different spots of the world. Studies on the association between the IL-27(-964 A>G) polymorphism and RPL are scarce. This is the first study shows the implication of IL-27(-964 A>G) polymorphism in RPL.

Objective: This study aims to investigate the association between RPL and interleukin-27(-964 A>G) polymorphism in Iraqi women.

Materials and methods: From September 2013 to September 2014, 100 women, as a control group, and 100 women (with three or more consecutive pregnancy loss), as a study group were chosen to investigate the association between the IL-27(-964 A>G) SNP and the RPL. The IL-27(-964 A>G) SNP was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Genotype and allele frequencies in study group were compared with those in the control group using Fisher tests. Results are considered to be statistically significant if p-value is less than 0.05.

Results: The age and the body mass index were not significantly different between the two groups. The frequencies of genotypes of IL-27 polymorphism in the RPL group were AG (60%), AA (31%) and GG (9%); while in control group were AG (21%), AA (68%) and GG (11%). The genotype frequencies of the -964 A>G SNP was significantly different between the study and the control group ($p=0.007$). Allele frequencies of this polymorphism were A (35%) and G (65%) in RPL group versus A (61%) and G

(39%) in the control group. The frequencies of A and G alleles were not significantly different between the two groups.

Conclusion: Our findings show that IL-27 (-964 A>G) polymorphism is considered a risk factor of RPL in Iraqi women which was different from what has been found previously; this might implicate other factors in the RPL.

Keywords: SNP; IL-27; Inflammation; Recurrent pregnancy loss; Iraqi women.

Introduction

Recurrent Pregnancy Loss (RPL), defined as three or more consecutive pregnancy losses before 20 weeks of gestation. This was found to affect approximately 1–5% of pregnant women with up to 50% of cases that do not have clear etiology [1,2]. However, studies have found that the genetic factors are highly associated with reproductive loss [3]. Successful pregnancy depends on immune balance, including immunotolerance, immune response and relative cytokines levels [4]. Additionally, inflammatory immune responses play a key role in early pregnancy loss. It has been found that the immune cells that reside at the interface between the placenta and the uterus is a superimposed layer of regulation by maternal immune cells, which not only foster placental development and function but also reduce the possibility of the placental attacking the fetus [5]. During implantation, Natural Killer (NK) cells migrate to the uterus and regulate the secretion of cytokines that limit the trophoblast invasion [6]. In normal pregnancy, a systemic or local inflammation is necessary; however, this might be disrupted during miscarriage [7,8]. Excessive or inappropriate recruitment of peripheral blood NK cells to the

uterus may lead to cytotoxic environment in utero, in which proliferation and differentiation of trophoblast is hampered. In normal pregnancy, an increase in Th2 cells and a decrease in Th1 cells type protect the allogeneic fetus from infiltrating cytotoxic T cells [6]. These immune cells/leukocytes secrete different types/levels of interleukins that play a role in placental immunoregulation [5]. Thus, interleukins and the corresponding immune cells/leukocytes work together to maintain the immune balance of mother and fetus.

Abnormal decidual leukocytes lead to RPL; for example, changing in the balance of Th cells will change the type and the levels of the interleukins secreted by these cells [9]. Variant alleles of interleukins have been studied and linked with the successful and the loss of pregnancies [10-13]. IL-6, IL-10 and IL-18 levels were found to be higher in plasma of women with successful pregnancy compared to women with RPL [10]. Single nucleotide polymorphism (SNP: a variation at a single nucleotide position in DNA sequence among individuals) can act as biological markers, helping in locating genes that are associated with disease [14]. SNPs could occur in non-coding regions (e.g. promoters) and coding regions such as gene body (at a total frequency of approximately every 200 ± 1000 bases). SNPs in promoters might affect the transcription factor binding which in turns could impact the production of the interleukin that associated with the RPL [15]. Although most SNPs have no impact on human health, some could alter an individual response to the environmental factors and increase the risk of developing a particular disease [14,16]. Therefore, if SNPs are high-risk candidate for a disease, they are important to investigate.

Previous studies have demonstrated the association between SNPs in interleukins and the loss of pregnancies [6,11,13]. IL-27 (a family member of IL-12) plays a role in the development and the differentiation of T-cells [17,18]; however, very limited data was found on its impact on the loss of pregnancy [19]. It has been postulated that IL-27 promotes Th1 response and suppress Th17 differentiation [20]. Levels of Th1 cytokines are elevated in women with recurrent miscarriage compared to those with no history on miscarriage [21]. Additionally, when implantation occurs, Th1 and Th17 cells are actively participate in the pro-inflammatory immune response at the site of maternal-fetal interface, which could result in RPL [22,23].

Owing to the fact that SNPs in interleukins could alter the production of the proteins that involved in immune response to pregnancy and lead to RPL, the implication of environmental factors in the SNP's response to the risk of developing a specific disease, and that limited data on the association between SNPs in the interleukins on the RPL, we investigated the IL-27(-964 A>G) SNP to understand

its implication on RPL [14,24]. IL-27(-964 A>G) SNP in plasma of Iranian women showed no significant association with the RPL; therefore, this study aims to understand this association in Iraqi pregnant women to compare with and fill in the gap the information regarding the risk factor(s) of RPL.

Materials and Methods

Participants: A case control study of two groups of Iraqi pregnant women was carried out at AL-Yarmouk Teaching Hospital, Baghdad, Iraq. A control group of 100 healthy pregnant women with reproductive age and no history of miscarriage (or at least two successful pregnancy); and a case group of 100 women with RPL were investigated between September 2013 to September 2014. The inclusion criteria of the case group were characteristics that the women had no any previous viable pregnancies, no male factor cause or anatomical and endocrinal abnormality, the absence of anti-phospholipid, anti-cardiolipin and antinuclear antibodies, with a negative thrombophilia screen test. Patients with abnormal serum thyroid hormones, anti-thyroid peroxidase and anti-thyroglobulin and those with thyroid disorders were excluded from the study. Verbal consent from each woman before the examination was obtained. A complete evaluation for each pregnant woman including detailed history of age, number of miscarriage and number of previous pregnancies was also done. 5 ml blood samples were collected (from all the participants) in EDTA-treated tubes.

DNA extraction and IL-27 (-964 A>G) measurements: DNA was extracted from each (blood) sample using salting-out method (Promega, USA) [25,26]. Briefly, 3mL of blood was mixed with Triton lysis buffer (0.32 M sucrose, 1% Triton100, 5 mM MgCl₂, H₂O and 10 mM Tris-HCl, with pH 7.5). Leucocytes were spun down and washed with deionized H₂O. The pellet was then incubated with proteinase K at 56°C and salted out at 4°C using saturated NaCl solution. Precipitated proteins were then removed by centrifugation, and the extracted DNA was stored at -20°C until analysis.

Genotyping of -964 A>G in IL-27 was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method [27]. Forward and reverse primers were designed according to the published sequences in the Genome Database using the PRIMER3 software. Information on primer sequences is as follows: Forward: 5'-AACTAGGGTCAGGGCTGGAT-3', and Reverse: 5'-CCTCGGCAGATGTTGGTATT-3'. The PCR reaction was done in a 25 µl reaction volume containing 4 µl of DNA, 10 µl master mix, 8.5 µl distilled water and 1.25 µl of each specific reverse and forward primers. The PCR protocol was performed with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 45 s at 62°C and extension for 90 s at 72°C. The size of this product was 700 base

pairs (bp) which was confirmed with electrophoresis in a 1% agarose gel stained with ethidium bromide (EtBr). PCR product was digested by addition of *Ava*I restriction enzyme at 37°C for 60 min in a final volume of 30.2 µl under the following conditions; 10 µl PCR product, 2 µl buffer, 18 µl nuclease free water and 0.2 µl *Ava* I enzyme. This enzyme was used to recognize the nucleotide sequence of 5'...C↓Y C G R G...3' which can cut it at the desired location. The resulting fragments of the RFLP were analyzed using (2% gel agarose stained with ethidium bromide) electrophoresis. One duplicate was prepared per batch (10 samples) for quality control; duplicated were selected randomly.

Statistical Analysis

Statistical analysis was performed using SPSS version 18 (SPSS Inc., Chicago, IL, USA). The IL-27(-964 A>G) SNP was also evaluated for deviation from Hardy-Weinberg model for both RPL and control groups using Chi-square test. The genotype and allele frequencies of -964 A>G SNP were calculated by direct count. The differences in allele and genotypes frequencies between the study and the control group were determined using Chi-square test. P-value of <0.05 was considered statistically significant.

Results

Mean average ± Stdv of ages for control cases (range of 20 to 41 years) was 30.3 ± 4.4 years; and for study (RPL) cases (range of 22.0 to 42.0) was 31.6 ± 3.2 years. The Body Mass Index (BMI)

averaged to be 23.8 ± 3.9 and 22.3 ± 4.2 kg/m² for control cases versus study cases respectively. The menarche was averaged to be 13.4 ± 1.6 years for controls and 13.5 ± 1.6 years for study group (Table 1). Results of age, BMI, and menarche of study cases were not significantly different from those for control cases. However, the irregular menstrual history (%), number of successful pregnancies, and the number of miscarriage data in women with RPL was significantly different from those obtained for the control cases (Table 1).

Table 2 show genotype and allele frequencies of the -964 A>G polymorphism for IL-27 gene in women with RPL compared to controls. The frequencies of genotypes of this polymorphism in the RPL group were as follows; AG (60%), AA (31%) and GG (9%); and in control group were AG (21%), AA (68%), and GG (11%). Genotype frequencies of the -964 A>G polymorphism was significantly different between both groups (p=0.007). The frequencies of alleles of this polymorphism were A (35%), and G (65%) in the RPL group versus A (61%) and G (39%) in the control. The frequencies of alleles A and G in women with RPL were not significantly different from the control group (p>0.05) (Table 2). Calculation of odd ratios (ORs) and 95% CIs for the fixed effects model that presented in Table 2 show the association of IL-27 genotypes in women with RPL compared to controls; and the association of allele A and allele G in in women with RPL compared to controls. All genotypes of the IL-27 polymorphism were in accordance with the Hardy-Weinberg Equilibrium. The undigested PCR product size was 700 bp for SNP -964 A>G (Figure 1). Restriction digestion for

Table 1. The demographic data of the case-control study, with p-values of differences between the cases and the controls.

Characteristic	Cases	Controls	p-Value
	(Women with RPL)		
Mean age (years)	31.6 ± 3.2	30.3 ± 4.4	>0.05
Body mass index, BMI (kg/m ²)	22.3 ± 4.2	23.8 ± 3.9	>0.05
Menarche (years)	13.5 ± 1.6	13.4 ± 1.6	>0.05
Irregular menstrual history %	65	15	<0.01
Number of Successful pregnancies (n)	0	2.6 ± 1.38	<0.01
Number of Miscarriages (n)	2.7 ± 0.78	0	<0.001

Table 2. Genotype and allele frequencies of the SNP -964 A>G polymorphism for IL-27 gene in women with RPL and in the controls.

Parameter	Genotypes Frequency (%)			Allele's frequency (%)	
	Homozygote	Heterozygote	Homozygote	Allele A	Allele G
	Wild Type A/A	A/G	SNP Type G/G		
RPL cases (n=100)	31%	60%	9%	35%	65 %
Controls (n=100)	68%	21%	11%	61%	39%
X ²	0.26	0.15	0.23	0.20	1.31
p-Value	0.033	0.007	0.71	0.67	0.58
OR (95%)	1.23 (0.47-2.34)	0.92 (0.62-1.87)	0.87 (0.45-2.84)	0.88 (0.61-1.45)	0.78 (0.56-1.2)

the GG genotype generated 177, 205 and 37 bp fragments; AG genotype generated 177, 205 and 371bp and 576 bp fragments, while AA genotype generated two bands (177 and 576 bp) (Figure 2).

Allele and genotypes frequencies were calculated using Hardy-Weinberg Equilibrium (HWE). If the observed frequencies of genotype are close to the expected genotype frequencies, then the population is in Hardy-Weinberg Equilibrium and allele combinations are likely to be independent of each other. The genotypes and alleles frequencies for single nucleotide polymorphism in interleukin-27 gene were concordant with Hardy-Weinberg Equilibrium in both unexplained RPL and control groups with no significant differences in the distribution of IL-27-964 A>G genotypes ($P>0.05$) (Table 3).

Discussion

The susceptibility of the RPL to the genotypic and allelic variants in IL-27 gene was demonstrated in Iraqi pregnant women. A recent systematic review of previous studies was performed on seven interleukin genes and 21 polymorphisms in a 9,401 patients to understand the association of these interleukins and polymorphisms with RPL; it has been found that genes and polymorphisms including IL-1 β (-511C/T)

($P=0.02$, 95% CI 0.77 [0.62, 0.96]), IL-6 (-634C/G) ($P<0.001$, 95% CI 2.91 [2.01, 4.22]), IL-10 (-1082G/A, $\pm 819T/C$) ($P=0.01$, 95% CI 0.80[0.67, 0.96]; $P<0.01$, 95% CI 0.66[0.49, 0.89]) and IL-18 (-137G/C, -105G/A) ($P<0.01$, 95% CI 1.69 [1.24, 2.31]; $P<0.01$, 95% CI 1.41 [1.17, 1.70]), were associated with RPL [24]. However, IL-27 gene and its association with RPL were not included in that review due to limited data on this gene. Results from this study show significant association between the IL-27 (-964 A>G) polymorphism and the RPL in Iraqi women. One study was performed to understand the implication of IL-27 (-964 A>G) polymorphism in RPL; that study was performed in Iran, where no association was observed between IL-27 (-964 A>G) polymorphism and the RPL [19]. However, in Nematollahi et al. [19], study, the frequencies of genotypes of IL-27 (-964 A>G) polymorphism in the study group (women with RPL) were as follows, AG (49.3%), AA (40%) and GG (10.7%); while in the control group were AG (43.3%), AA (48.7%) and GG (8%). Those results showed no significant difference in genotype frequencies of the -964 A>G polymorphism between the study and the control group ($p=0.23$) [19]. Our study showed that the frequencies of genotypes of IL-27 (-964 A>G) polymorphism in Iraqi women with RPL were significantly different from control group ($p=0.007$)

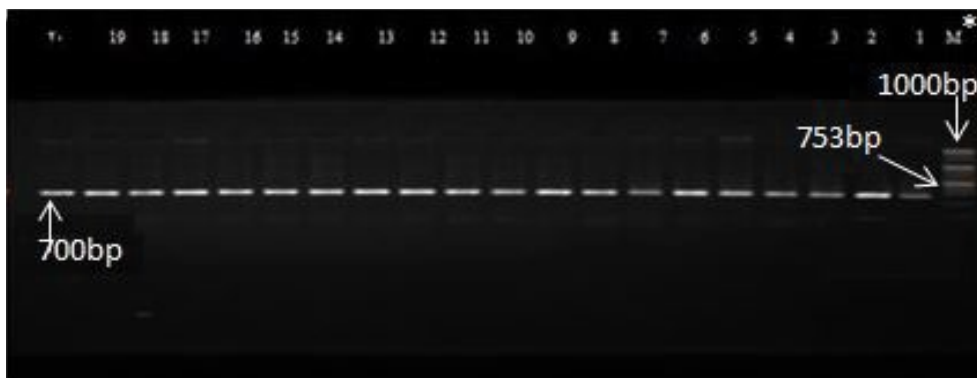


Figure 1. Graph shows the electrophoresis of PCR product of IL-27 gene (700 bp) and the ladder marker (1000 bp). The electrophoresis was undertaken at 70 V and gel viewed under UV trans-illumination after staining with ethidium bromide; *M: Marker; control sample (lanes 1-5); RPL sample (lane 6-20)

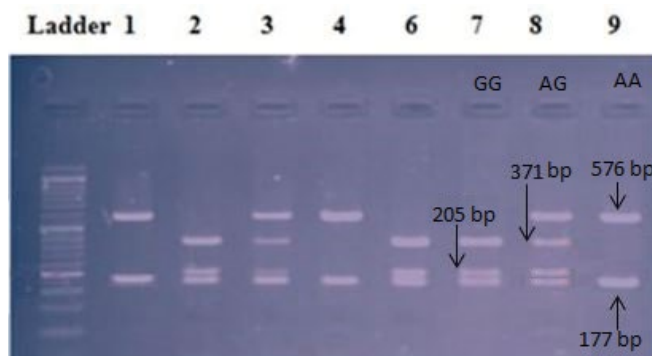


Figure 2. Graph shows the restriction fragment length polymorphism (RFLP) for the SNP-964 A>G of IL-27 gene in women with RPL. GG genotype generated 177, 205 and 371 bp fragments; AG genotype generated 177, 205, 371 and 576 bp fragments; and AA genotype generated 177 and 576 bp

Table 3. Observed and expected number of genotype of the SNP -964 A>G polymorphism for IL-27 gene in women with RPL and in the control group.

Genotype		A/A	A/G	GG	Hardy-Weinberg Probability
RPL cases	Observed	31	60	9	>0.05
	Expected	37	48	15	
Controls	Observed	68	21	11	>0.05
	Expected	62	33	5	

(Table 2). SNPs could occur in coding regions, such as the gene body, and non-coding regions such as promoters. SNPs that occur in promoters could affect transcription factor binding and may in turn influence interleukin production and thus be associated with RPL [15].

IL-27 has been associated with the inflammatory and autoimmune diseases; however, interleukins that are secreted by different cells play distinct roles at different tissues [28,29]. Additionally, studies have demonstrated that a successful pregnancy depends on immune balance, including immunotolerance, the immune response and relative cytokines levels [4]. Previous studies showed that IL-1 β is a pro-inflammatory cytokine that assists in B cell proliferation and maturation, natural killer (NK) cell activation and T cell stimulation [30]; the IL-1 β (-511C/T) polymorphism leads to an increase in IL-1 β production and the NK cell proportion of the lymphocytes population [30,31]. These alterations are observed in women with RPL as a larger number of peripheral CD16+ CD15+ NK cells [32]. Also, IL-10 takes part in the immunosuppressive response in congestion; and high maternal IL-10 levels are associated with successful pregnancy and vice versa, but the IL-10 (-819) polymorphism alters IL-10 secretion [33]. Although, IL-27 gene is identified as a suppressor of inflammation during pregnancy, the IL-27 (-964 A>G) polymorphism could be a genetic marker of the adverse effect (i.e., RPL) in pregnancy.

Conclusion

Our results showed that IL-27 (-964 A>G) polymorphism is considered a risk factor of RPL in Iraqi women. These findings are in contrast with a previous study in Iran [19]. This suggest the sensitivity of IL-27 (-964 A>G) polymorphism to other factors including genetic (the location of the SNP), environmental and ethnic factor [14,16,32]. Therefore, future studies might include these factors to demonstrate the risk of IL-27 (-964 A>G) polymorphism in RPL.

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