Original Article

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

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Abstract

Background: Studies have been done to investigate the association between a single-nucleotide polymorphism (SNP) of interleukin-27 (IL-27) gene and the recurrent pregnancy loss (RPL). However, different results have been found in different spots of the world. Therefore, more studies are needed to understand the variation in these results. This is the first study that shows the implication of the SNP of IL-27 gene in RPL. **Objective:** This study aims to investigate the association between RPL and SNP of gene (−964 A > G) 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 recruited to investigate the association between the *IL-27(−964 A > G) SNP* and the PRL. The *IL-27(−964 A > G) SNP* was determined using polymerase chain reaction-restriction fragment length polymorphism technique. Genotype and allele frequencies were compared using Fisher test between the two groups. $P < 0.05$ is considered to be statistically significant. **Results:** The age and body mass index were both not significantly different between the two groups. The frequencies of genotypes of this polymorphism in the RPL group were AG (60%), AA (31%), and GG (9%), while these frequencies were AG (21%), AA (68%), and GG (11%) in the control group. The genotype frequencies of the −964 A > G polymorphism was significantly different between the study and the control groups ($P = 0.007$). The allele frequencies of this polymorphism were A (35%), G (65%) in the RPL group versus A (61%), G (39%) in the control group. The frequencies of A and G alleles in the both groups were not significantly different. **Conclusion:** IL-27 (−964 A > G) polymorphism is a risk factor for RPL in a sample of Iraqi women. However, this is different from what has been found in some studies which might implicate other factors in the RPL.

Keywords: Inflammation, interleukin-27, Iraqi women, recurrent pregnancy loss, single-nucleotide polymorphism

Introduction

Recurrent pregnancy loss (RPL), which is defined as three or more consecutive pregnancy losses before 20 weeks of gestation, was found to affect approximately 1%–5% of pregnant women,[1] with up to 50% of cases that do not have clear etiology.[2] However, studies have found that the genetic factors are highly associated with reproductive loss.[3] Studies have demonstrated that the successful pregnancy depends on immune balance, including immunotolerance, immune response, and relative cytokines levels.[4] Inflammatory immune responses play a key role in early pregnancy loss. The immune cells that reside at the interface between the placenta and the uterus were found to be a superimposed layer of regulation by maternal immune cells,[5] which not only foster placental development and function but also reduce the possibility of the placental attacking the fetus. 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 T helper (Th) 2 cells and a decrease in Th1 cells type protect the allogeneic fetus from infiltrating

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cytotoxic T-cells.[6] These immune cells/leukocytes secrete different types/levels of interleukins (ILs) that play a role in placental immunoregulation.[5] Thus, ILs 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 ILs secreted by these cells.[9] Variant alleles of ILs have been studied and linked with the successful pregnancy and the loss of pregnancy.[1-13] 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 noncoding 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[15] which in turns could impact the production of IL that associated with the RPL. 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 ILs and the loss of pregnancy.[6,11,13] While IL-27 (a family member of IL-12) found to play a role in the development and the differentiation of T-cells,[17,18] very limited data were 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] In addition, 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 ILs could alter the production of the proteins that involved in immune response to pregnancy which could led to RPL,[24] the implication of environmental factors in the SNP's response to the risk of developing a specific disease,[14] and that limited data were found on the association between SNPs in the ILs on the RPL in the Middle East, we investigated the IL-27 (−964 A > G) SNP to understand its implication on RPL in Iraqi women. 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 of 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 and 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 antiphospholipid, anticardiolipin, and antinuclear antibodies, with a negative thrombophilia screen test. Patients with abnormal serum thyroid hormones, antithyroid peroxidase, and antithyroglobulin, 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. About 5 ml blood samples were collected (from all the participants) in ethylenediaminetetraacetic acid-treated tubes.

DNA extraction and interleukin-27 (−964 A > G) measurements

DNA extraction and IL-27 −964 A > G measurements genomic were performed at the clinical laboratory located at AL-Yarmouk Teaching Hospital, Baghdad, Iraq. DNA was extracted each (blood) sample using the salting-out method,[25,26] and following the manufacturer's instructions (Promega, USA). Briefly, 3 mL of blood was mixed with Triton lysis buffer (0.32M sucrose, 1% Triton 100, 5 mM $MgCl_2$, H_2O, and 10 mM Tris–HCl, with pH 7.5). Leukocytes were spun down and washed with deionized H_2O. The pellet was then incubated with proteinase K at 56°C and subsequently salted out at 4°C using a saturated NaCl solution. Precipitated proteins were then removed by centrifugation. The extracted DNA was then stored at −20ºC 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, extension for 90 s at 72°C, followed by final at 72°C for 5 min. The size of this product was 753 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 AvaI 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 1 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) SNPs were also evaluated for deviation from Hardy–Weinberg model for both RPL and control groups by Chi-square test. The genotype and allele frequencies of SNP −964 A > G were calculated by direct count. The differences in allele and genotypes frequencies between cases and control groups were determined using Chi-square test. $P < 0.05$ was considered statistically significant.

Results

A 30.3 ± 4.4 years was the mean age of control group with range (20-41 years) and 31.6 ± 3.2 years for study (RPL) cases (range of 22.0–42.0). The body mass index (BMI) averaged to be 23.8 ± 3.9 and 22.3 ± 4.2 kg/m^2 for control cases versus study cases, respectively. The menarche in years 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 the study cases were not significantly different from those for control cases. However, the irregular menstrual history (%), number of pregnancies, and the number of miscarriage data in women with RPL were 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%); but for the control group, the frequencies of AG, AA, and GG were 21%, 68%, and 11%, respectively. The genotype frequencies of the −964 A > G polymorphism was statistically 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 when compared to control group ($P > 0.05$) [Table 2]. Calculation of odd ratios and 95% Cls for the fixed effects model that presented in Table 2 shows the association of genotypes of IL-27 gene in women with RPL compared to controls, and the association of allele A and allele G in the case study compared to control. All genotypes of the IL-27 polymorphism were in accordance with the Hardy–Weinberg Equilibrium (HWE). The undigested PCR product size was 753 bp for SNP-964 A > G [Figure 1]. Restriction digestion for the GG genotype generated 177, 205, and 37 bp fragments; AG genotype generated 177, 205, and 371 bp and 576 bp fragments, while AA genotype generated two bands (177 and 576 bp) [Figure 2].

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

Table 1: The demographic data of the case–control study, with *P* values of differences between the cases and the controls

Characteristic	Cases (women with RPL)	Controls	*P*
Mean age (years)	31.6±3.2	30.3±4.4	>0.05
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 pregnancies (*n*)	0	2.6±1.38	<0.01
Number of miscarriages (*n*)	2.7±0.78	0	<0.001

BMI: Body mass index, RPL: Recurrent pregnancy loss

Discussion

The susceptibility of the RPL to the genotypic and allelic variants in IL-27p28 was demonstrated in a sample of Iraqi pregnant women. A recent systematic review of previous studies was performed on 7 IL genes and 21 polymorphisms in a 9401 patients to understand the association of these ILs and polymorphisms with RPL; however, among those genes and polymorphisms, IL-1 β (-511 C/T) ($P = 0.02$, 95% confidence interval [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, ±819T/C) ($P = 0.01$,

Table 2: Genotype and allele frequencies of the single-nucleotide polymorphism −964 A > G polymorphism for interleukin-27 gene in women with recurrent pregnancy loss and in the controls

Parameter	Genotypes frequency (%)			Allele's frequency (%)	
	Homozygote wild-type A/A	Heterozygote A/G	Homozygote SNP type G/G	Allele A	Allele G
RPL cases (*n*=100)	31%	60%	9%	35%	65%
Controls (*n*=100)	68%	21%	11%	61%	39%
χ^2	0.26	0.15	0.23	0.20	1.31
P	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)

RPL: Recurrent pregnancy loss, OR: Odds ratio, SNP: Single-nucleotide polymorphism, SNP: Single-nucleotide polymorphism

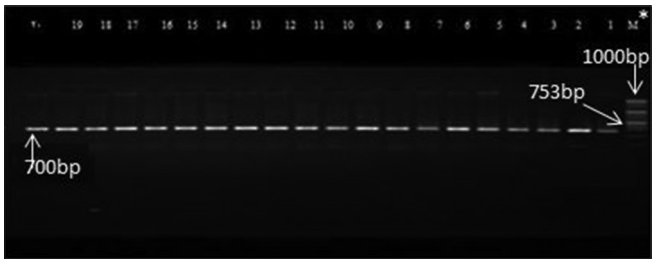

Figure 1: Graph shows the electrophoresis of polymerase chain reaction product of IL-27 gene (753 bp) and the ladder marker (1000 bp). The electrophoresis was undertaken at 70 V and gel viewed under UV transilluminator after staining with ethidium bromide; *M: Marker; control sample (lanes 1–5); recurrent pregnancy loss sample (lane 6–20)

Table 3: Observed and expected number of genotype of the single-nucleotide polymorphism −964 A > G polymorphism for interleukin-27 gene in women with recurrent pregnancy loss 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	

RPL: Recurrent pregnancy loss

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 found to be associated with RPL.[24] IL-27 gene and its association with RPL were not included in that review due to limited data on this gene. Our results showed significant association between the IL-27 (−964 A > G) and the RPL in a sample of Iraqi women. The same association (between IL-27 (−964 A > G) and the RPL) was performed in Iranian women; however, that study showed no association between IL-27 (−964 A > G) and the RPL in Iranian women (Nematollahi *et al*. 2015). IL-27 has been associated with the inflammatory and autoimmune diseases; however, ILs that are secreted by different cells play distinct roles at different tissues.[28,29] In addition, 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-1β is a pro-inflammatory cytokine that assists in B-cell proliferation and maturation, NK cell activation, and T-cell stimulation;[1] 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] Furthermore, 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

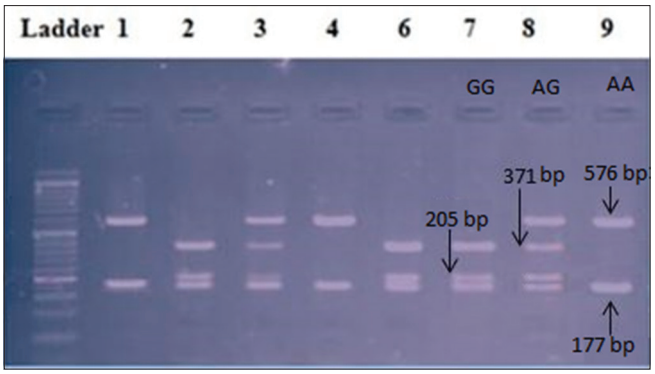

Figure 2: Graph shows the restriction fragment length polymorphism for the SNP −964 A > G of IL-27 gene in women with recurrent pregnancy loss. GG genotype generated 177, 205, and 371 bp fragments; AG genotype generated 177, 205, 371, and 576 bp fragments; and AA genotype generated 177 and 576 bp

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. Our results showed that IL-27 (−964 A > G) polymorphism is an important genetic marker of RPL in Iraqi women. However, these findings are in contrast with findings reported by Nematollahi *et al*.[19] where IL-27 (−964 A > G) polymorphism was not considered as a risk factor for RPL in Iranian women.[19] These different observations could implicate other factors such as the environment, ethnicity, and lifestyle; therefore, studying the IL between the SNPs-related RPL and these factors are important to better understand the population that is at risk of RPL.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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