Original Article

Programmed Death Receptor 1 Gene Polymorphisms Influence the Occurrence of Recurrent Pregnancy Loss among Iraqi Women

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Abstract

Background: Programmed cell death protein 1 (PD-1) and PD ligand 1 (PDL-1) are speculated to have an important role in maintaining a normal pregnancy. **Objectives:** This study aims to compare the serum level of PD-1 and PDL-1 in recurrent pregnancy loss (RPL) with normal pregnancy and determine the impact of PD-1 gene polymorphism as a risk factor for RPL. **Materials and Methods:** This case–control study involved 57 females with RPL recruited from the gynecology and obstetrics department in Al-Emamain Al-Kadhmain Medical City Baghdad. The blood sample was processed into serum for PD-1 and PDL-1 measurement and whole blood for deoxyribonucleic acid extraction and PD-1 gene polymorphism detection using restriction fragment length polymorphism polymerase chain reaction. **Results:** Based on current findings, there were statistically significant increased serum PD-1 and PDL-1 proteins in the RPL group. The frequency of single-nucleotide polymorphisms rs36084323 of PD-1 was significantly higher in the RPL group than in the control group, with the risk allele (A) of rs36084323 at 23.7% in comparison with 13.2%, with odd ratio 2.05 for RPL chance in mutant allele A carriers. Both rs36084323 and rs7421861 gene polymorphisms were associated with statistically increased serum PD-1 and indirectly with DPL1 in the RPL group. **Conclusion:** Genetic variations PD-1 gene may be considered risk factors for RPL. The presence of the PD-1 risk allele had a predictive effect on the subsequent pregnancy outcome by abnormal increase in PD-1 and PDL-1 levels.

Keywords: Gene polymorphism, occurrence, programmed death, recurrent pregnancy loss

Introduction

The occurrence of two or three successive miscarriages before the 20th week of pregnancy is defined as recurrent pregnancy loss (RPL).[1] RPL affects roughly 5% of families who want to have a child and has a prevalence of 1–2%.[2]

Immune factors, endocrine factors, genetic factors, metabolic abnormalities, anatomic abnormalities, and other unexplained variables are among the causes of RPL that have been identified thus far. RPL is the outcome of complex interactions between the aforementioned elements. At least half of individuals who have completed a comprehensive evaluation are still unsure why they are unable to conceive.[3]

The role of immune checkpoint molecules could be of special interest. Programmed cell death protein 1 (PD-1) is a transmembrane receptor expressed by, for example, T cells, B cells, natural killer cells, and antigen-presenting cells.[4] PD-1 generates a strong inhibitory signal upon binding to its ligands PD-L1 and PD-L2, resulting in down-regulation of pro-inflammatory T-cell activity.[5,6] PD-L1 expression is increased by many pro-inflammatory factors: lipopolysaccharide (LPS), granulocyte

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Submission: 10-Jun-2025 **Accepted:** 10-Jun-2025 **Published:** 23-Jul-2025

How to cite this article: Sakran SM, Ghazi HF, Khazaali EAA. Programmed death receptor 1 gene polymorphisms influence the occurrence of recurrent pregnancy loss among Iraqi women. Med J Babylon 2025;22:S122-7.

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_589_25

macrophages colony-stimulating factor (GM-CSF), vascular endothelial growth factor, and cytokines (IFN-γ and TNF-α).[7,8] Although immunological acceptance of the fetus is primarily based on maternal tolerance mechanisms at the maternofetal interface (MFI) locally, it exerts a significant impact on systemic immunity as well,[6,8] with women compared to non-pregnant counterparts and soluble PD-L1 levels which increase throughout gestation.[9] In RPL, the decidual PD-L1 expression was significantly reduced on both microRNA (mRNA) and protein levels compared to healthy first-trimester decidua, while PD-1 expression by decidual lymphocytes showed no difference.[6] This study is designed to investigate the possible association of serum PD-1/PD ligand 1 (PDL-1) levels in RPL.

Materials and Methods

Study design and settings

This case–control study was conducted at AL-Imamain AL-Kadimain Medical City from December 2020 to October 2021. Fifty-seven women with RPL, 57 control pregnant women (age and gestational age-matched), and 52 non-pregnant women were enrolled in this study; their ages ranged from 20 to 40 years. They attended the gynecology outpatient clinic at Al-Imamain Al-Kadimain Medical City, the Gynecology Department Teaching Hospital, and Al-Karama Teaching Hospital in Baghdad.

Ethical approval was obtained from the institutional review board no: 202011149 dated February 9, 2021.

Inclusion and exclusion criteria

Inclusion criteria included pregnant females with a history of two or more consecutive pregnancy loss. Exclusion criteria included pregnant females with any autoimmune disease, thyroid disease, diabetes mellites, thrombophilia, or chromosomal abnormalities.

This study included 57 pregnant women; their gestational ages and ages were matched with the normal pregnant group, which was considered a control, and compared with the non-pregnant group. All of them received no treatment with no complaint of autoimmune disease, thyroid disease, diabetes mellites, thrombophilia, or chromosomal abnormalities, and their age range was 20–40 years.

Methods

Sandwich enzyme-linked immunosorbent assay was used for the measurement of PD-1 (abx251891), PD-L1 (ELISA kit abx252917), and Abbexa®, UK, according to the manufacturer's instructions.

Polymerase chain reaction requirements

DNA was extracted from whole blood samples using a ready kit Blood Genomic DNA Miniprep System abx098078 (Extraction kit)/Abbexa–UK, according to the manufacturer's instructions as follows:

Gene amplification by conventional polymerase chain reaction

Two sets of primers were used in this study to amplify the corresponding targets of the PD-1 gene (rs36084323) and (rs7421861). The sequence of primers was obtained from the study[8,10]: rs36084323, F: ATCTGGAACTGTGGCCATGGTG, R: ATTCTGTCGGAGCCTCTGGGAG, 206 bp; and rs7421861, F: CACCCAGACGAGTTACAC, R: AGAGATGGAGAGAGGTGAG, 398 bp.

Polymerase chain reaction (PCR) was carried out in a 20 µL reaction containing 0.5 µL of 10 pmol/µL of each primer and 3 µL of DNA template. The volume was completed to 20 µL using nuclease-free water. The tubes were transferred to the thermocycler which is previously programmed with the protocol below according to the gene to be amplified as follows: thermocycling programs rs36084323—initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 7 min. The reaction was carried out in a 20 µL reaction containing 20 µL of PCR product, 1 µL of HPAll restriction enzyme mix, and 5 µL of 1 X NE Buffer. The volume was completed to 50 µL using nuclease-free water. Eppendorf tubes were incubated for 15 min at 37°C in a PCR thermocycler.

For rs7421861, the PCR conditions are as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 35 s, annealing at 61°C for 35 s, extension at 72°C for 45 s, and final extension at 72°C for 7 min. The reaction was carried out in a 50 µL reaction containing 20 µL of PCR product, 1 µL of ASPLE1 restriction enzyme mix, and 5 µL of 1 X NE Buffer. The volume was completed to 50 µL using nuclease-free water. Eppendorf tubes were incubated for 60 min at 37°C in a PCR thermocycler.

Statistical analysis

All statistical analyses were conducted using Statistical Package for Social Science (SPSS) software version 20. A $P \leq 0.05$ (exact two-sided) was accepted as the level of significance. Continuous variables were subjected to a normality test. Non-normally distributed data either the Mann–Whitney *U* test or the Kruskal Wallis test was used for comparison between the different medians. Therefore, these variables were expressed as the mean ± standard deviation or median (range). Binomial variables were expressed as numbers and percentages and analyzed with chi-square. Odd ratio (OR) and the corresponding 95% confidence intervals (CIs) were calculated to assess the association between the different genotypes and alleles of polymorphisms with the risk of RPL.

Results

Socio-demographic and clinical characteristics of the study population

The results showed that the median age of RPL was 30 years, normal pregnancy controls were 28 years, and non-pregnant females were 29 years. There were no statistically significant differences in the median age of the study group ($P = 0.788$), as shown in Table 1.

The median number of gravida was five in RPL, two in the normal pregnancy group, and a similar number in non-pregnant control. The median number of parity was two in the RPL group, one in each normal pregnancy control group, and one in non-pregnant control. Median miscarriage was two (2–3) in the RPL group. The median gestational age was 10 weeks in both the RPL group and the normal study group.

Serum levels of immunoinhibitory molecules

Serum PD-1 was measured using the sandwich ELISA method. However, the high statistically significant difference among study groups ($P < 0.001$) in which the RPL group was 1.45 ng/mL, the normal pregnancy group was 0.76 ng/mL, and the non-pregnant group was 0.46 ng/mL. Serum PDL-1 was measured using the sandwich ELISA method. However, the high statistically significant difference among study groups ($P < 0.001$) in which the RPL group was 1.73 ng/mL, the normal pregnancy group was 0.97 ng/mL, and the non-pregnant group was 0.40 ng/mL. Serum PD-1/PDL-1 was calculated. The results reported a high statistically significant difference among study groups ($P < 0.001$) in which the RPL group showed a low ratio (0.83), the normal pregnancy group was 0.99, and the non-pregnant group was high (1.17) [Table 2].

Risk of PD-1 gene polymorphism on recurrent pregnancy loss

The results in Table 3 showed that the frequency of homozygous mutant genotype AA was 6 (10.5%) in the RPL group and only 1 (1.8%) in the normal pregnancy group, while heterozygous genotype GA was 15 (26.3%) in RPL group and 13 (22.8%) in normal pregnancy group. However, the frequency of the mutant allele was 27 (23.7%) in the RPL group and 15 (13.2%) in the normal pregnancy group. The OR was 2.05 risk of induction pregnancy loss. The results of other single-nucleotide polymorphism (SNP) showed that the frequency of homozygous mutant genotype AA was 3 (5.3%) in the RPL group and only 1 (1.8%) in the normal pregnancy group, while heterozygous genotype TA was 14 (24.6%) in RPL group and normal pregnancy group. However, the frequency of the mutant allele was 20 (17.5%) in the RPL group and 16 (14%) in the normal pregnancy group. No statistically significant association was found [Figures 1 and 2].

Impact of PD-1 genes polymorphisms on serum PD-1, PDL-1, and PD-1/PDL-1

The association between two SNPs and serum levels of PD-1 among RPL is described in Table 4. However, females with rs36084323 mutant genotype have a much higher median serum level (11.43 ng/mL) than heterozygous genotype (1.41 ng/mL) and homozygous wild genotype (1.36 ng/mL),

Table 1: Descriptive statistics of age clinical parameter recurrent pregnancy loss (RPL), pregnant controls, and non-pregnant controls

	Study groups			*P* value
	Recurrent pregnancy loss $N = 57$	Normal pregnancy controls $N = 57$	Non-pregnant $N = 52$	
Age (years)	30 (19–40)	28 (19–39)	29 (20–40)	0.788[NS]
Gravida	5 (3–6)	2 (1–3)	2 (1–3)	0.077[NS]
Parity	2 (1–3)	1 (0–2)	1 (0–2)	0.673[NS]
Miscarriage	2 (2–3)	–	–	–
Gestational age	10 (8–12)	10 (8–12)	–	1.000[NS]

[NS]Non-statistical significant difference ($P > 0.05$)

Table 2: Median serum PD-1, PDL-1, and their ratio levels of RPL woman, controls, and non-pregnant weman

	Study groups			*P* value
	Recurrent pregnancy loss	Normal pregnancy	Non-pregnant	
PD-1 (ng/mL)	1.45 (0.44–14.33)	0.76 (0.19–9.44)	0.46 (0.35–0.74)	<0.001[**]
PDL-1 (ng/mL)	1.73 (0.40–13.26)	0.97 (0.31–12.87)	0.40 (0.32–0.51)	<0.001[**]
PD-1/PDL-1	0.83 (0.45–2.58)	0.99 (0.03–1.56)	1.17 (1.00–1.49)	<0.001[**]

[**]High statistically significant difference ($P < 0.001$)

Table 3: Frequency of different genotypes and alleles of PD-1 polymorphisms in RPL patients and controls

		Study groups		*P* value	Odd ratio (CI)
		Recurrent pregnancy loss	Normal pregnancy		
rs36084323 Genotype	AA	6 (10.5%)	1 (1.8%)	0.013*	
	GA	15 (26.3%)	13 (22.8%)		
	GG	36 (63.2%)	43 (75.4%)		
rs36084323 Allele	A	27 (23.7%)	15 (13.2%)	<0.001**	2.05 (1–4.07)
	G	87 (76.3%)	99 (86.8%)		
rs7421861 Genotype	AA	3 (5.3%)	1 (1.8%)	0.350NS	
	TA	14 (24.6%)	14 (24.6%)		
	TT	40 (70.2%)	42 (73.7%)		
rs7421861 Allele	A	20 (17.5%)	16 (14.0%)	0.293NS	1.3 (0.63–2.63)
	T	94 (82.5%)	98 (86.0%)		

NSNon-statistical significant difference ($P > 0.05$),

*Statistical significant difference ($P \leq 0.05$),

**High statistically significant difference ($P \leq 0.001$)

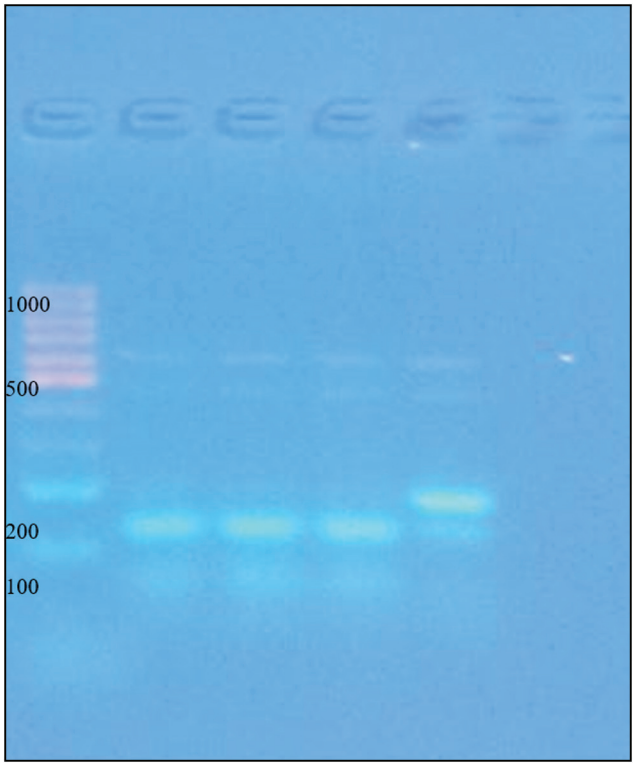

Figure 1: Gel electrophoresis for PD-1 rs36084323 PCR products visualized under UV light after staining with green star dye. AA, 277 and 29 bp; GG, 206 bp; GA, 206, 277, and 29 bp; MW: 100–1000 bp ladder

with a highly statistically significant difference. Moreover, females carrying the mutant allele A have 7.41 ng/mL than females carrying the G allele 1.36 ng/mL ($P < 0.001$).

Similarly, in females with rs7421861, median PDL-1 in mutant homozygous genotype AA was 10.37 ng/mL, heterozygous carrier genotype TA was 3.42 ng/mL, and homozygous wild-type carrier was 1.07 ng/mL, with high statistically significant difference with genotype. Like this, 7.6 ng/mL in mutant allele carriers of females with

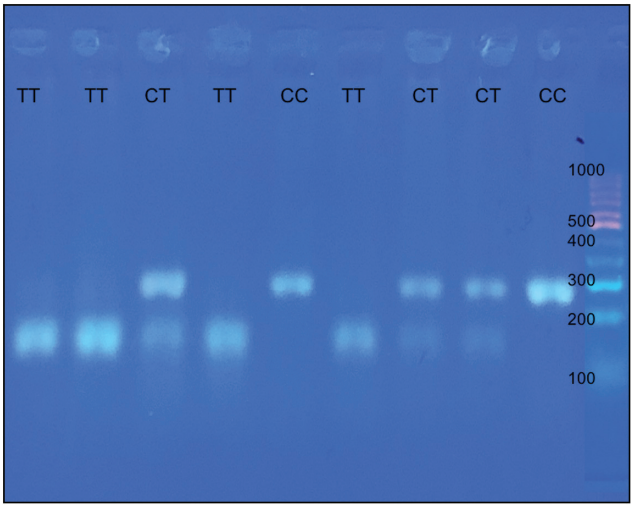

Figure 2: Gel electrophoresis for PD-1 rs7421861 PCR products visualized under UV light after staining with green star. CC, 278 bp; CT, 278, 140, and 84 bp; M: 100–1000 bp ladder

recurrent abortion and wild-type allele has 1.39 ng/mL with a high significant difference.

Discussion

The hypothesis was that safe pregnancy required continuous immunological tolerance against fetal allogenic antigens via the immunoinhibitory activity of PD-1 and PDL-1 pathways. This study found the inverse hypothesis that RPL has elevated serum PD-1 and PDL-1 than normal pregnancy group and non-pregnant females. A number of studies have connected paradixal involvement in RPL with PD-1/PDL-1 pathway action in humans.[11-13]

PD-1/PD-L1 axis participates in T-cell regulation by suppressing T-cell activation and differentiation, modifying molecular secretion patterns, and inducing cell death and exhaustion of T cells.[14,15]

Table 4: The influence of PD-1 serum value by rs36084323 and rs7421861 PD-1 polymorphism in RPL patients

		Serum PD-1 (ng/mL)	*P* value
rs36084323	AA	11.43 (2.89–16.99)	<0.001**
	GA	1.41 (0.42–16.26)	
	GG	1.36 (0.44–9.45)	
	A allele	7.41 (0.47–16.99)	<0.001**
	G allele	1.39 (0.44–10.02)	
rs7421861	AA	10.37 (5.74–16.99)	<0.001**
	TA	3.42 (0.70–16.26)	
	TT	1.07 (0.43–8.72)	
	A allele	7.60 (0.71–16.99)	<0.001**
	T allele	1.39 (0.44–12.50)	

**High statistically significant difference ($P \leq 0.001$)

This study observed that the level of serum PD-1 among RPL cases was higher than in the control group and non-pregnant. This finding suggests that case activity, at least in some RPL, may be correlated with PD-1 serum levels. Normal pregnant individuals showed a low level of serum PD-1 in comparison with RPL, and this may be due to a healthy immune system because PD-1 can inhibit leukocyte infiltration at the site of the uterus, interfere with mediators of inflammatory response, and suppress humoral immune responses. PD-1 reduces inflammatory reactions by limiting capillary dilatation and permeability of the vascular structures. These compounds restrict the accumulation of polymorphonuclear leukocytes and macrophages.

Studies have observed high levels of serum PD-1 in patients with RPL; also other studies reported elevated in PD-1 mRNA levels and high levels of PD-1 mRNA protein produced by B, T, and macrophages in females with RPL.[11]

Regarding the comparison between RPL, normal pregnancy, and non-pregnant, the results revealed that the frequencies of PD-1, PDL-1, and AA genotypes were more frequent in RPL than in normal and non-pregnant. The risk of AA genotype to RPL was higher among pregnant carrying AA genotypes than among those carrying GG and GA genotypes.

These results illustrate that the A allele was responsible for the development and the responsiveness to RPL, as it was highly expressed in RPL than normal and non-pregnant females.

In the case of the PD-1 promoter SNPs, some reports concluded that the A allele yielded an increase in transcription in reporter assay, so the presence of A allele was associated with higher levels of PD-1 expression.

This study included groups of RPL and normal pregnancy. SNPs in the promoter region of the PD-1 genes can influence the activity of the gene promoter and its output quantity. The current results showed that −606 G > A polymorphic site was associated with higher PD-1 serum levels among the RPL group.

Considering this finding, studies on other diseases have argued this finding. It was argued by Ishizaki *et al*. (2010) found that the level of the PDCD1 gene expression has been associated with the PDCD1 gene polymorphisms −606 G > A.[16] Similarly, Silva *et al*. investigated the relationship between alleles/genotypes with the higher PD-1 mRNA expression and protein level.[17] Liu *et al*. found that rs36084323 exhibited increased messenger (m) RNA expression levels of PD-1, and variant genotype/allele was considered a risk factor for developing rheumatoid arthritis.[18]

The functional influence of PD-1 gene polymorphisms has been suggested to drive or modulate the immune response in RPL patients. It could explain that the increased level of PD-1 might related to post-transcriptional regulation of transcriptional elements that may differentially target gene polymorphic sites, the UCE-2 transcription regulator, which modulates the PDCD1 −606 G > A polymorphic site.[19]

The mRNA research which involves the regulation of the PD-1/PD-L1 immune checkpoint has been reviewed by Wang *et al*.[20] The hsa-MiR-6771-5p binds to the −606 G alleles with high affinity and weakly to the mutant A allele, and it is involved in mutant allele A increased transcriptional activity.[21] Similarly, the hsa-MiR-204-3p targets the wild PDCD1 −606G allele.[22-24] This functional abnormality might lead to positive regulation by cytotoxic lymphocyte activity patients with a mutant allele.[25,26] PD-1 or Tim-3 molecules did not negatively regulate Th1-mediated immunity.[27] This might explain why abnormal high level of PD-1 is associated with RPL in this study.

The local MFI might be different in their relative PD-1/PDL-1 mRNA and protein. The study by Hayashi *et al*. showed increased PDL-1 protein expression in the RPL group in comparison with controls, while PD-1 protein was non-significant. However, they mentioned that PD-1 polymorphisms have been identified as risk factors for disease. However, this controversy might be occurring due to epigenetic control or post-translation modification of protein (as discussed above).

A study by Atwan *et al.* showed an increased PD-1 immunoreactivity rather than PDL-1 in the placental tissue of *Toxoplasma gondii*-infected aborted females.[28] The increased ratio of PD-1/PDL-1 proteins might be related to the immunoregulatory role occurring in chronic infection or reactivation such as infections in pregnancy.[29] The abnormal increasing or inhibitory molecules might be due to imbalance of cell cycle regulatory proteins in interferon gamma-dependent way; this will activate or promote cell death.[30-32]

Conclusion

This study concluded that serum PD-1 level is significantly increased in the RPL group than in the control group and in the non-pregnant group. This provides strong evidence that PD-1 may play a role in RPL. The genotype for rs36048323/AA SNPs showed a significant association as a risk factor for RPL development, and the PD-1 SNPs did significantly elevate PD-1 protein levels in RPL.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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