J. Appl. Environ. Biol. Sci., 5(12s)918-927, 2015

ISSN: 2090-4274
Journal of Applied Environmental and Biological Sciences
www.textroad.com

Polymorphisms in the Promoter Region of Tumor Necrosis Factor-α Gene and Recurrent Pregnancy Loss in An Iranian Population

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Received: February 21, 2015
Accepted: May 29, 2015

ABSTRACT

Introduction: Recurrent pregnancy loss [RPL] is defined as three or more consecutive abortions. The tumor necrosis factor alpha [TNF-α] gene plays a crucial role in immunology and inflammation responses. TNF-α Polymorphisms are supposed to be associated with RPL. In the present study, the contribution of the -1031T/C, -863C/A, -857C/T, -376G/A, -308G/A and -238G/A polymorphisms in the promoter region of TNF-α gene to RPL were investigated. To our knowledge, it was the first study on the six polymorphisms of the TNF-α gene in an Iranian population.
Material and methods: The study participants consisted of 100 women with RPL from Iranian Azeri Turkish origin. The control group comprised 100 age and ethnically matched healthy women in the reproductive age. Genotyping of the six polymorphic sites in the promoter region of TNF-αgene was carried out by using polymerase chain reaction [PCR]-restriction fragment length polymorphism [RFLP] assay.
Results: Unlike the homozygous state, significantly higher frequency of -857 C/T variant were seen in RPL patients than control subjects [47% Vs. 30%, OR = 2.06, CI = 1.11–3.86, p = 0.02].Of note, significantly lower frequency of wild type genotype were observed in RPL patients than that of controls [45% Vs. 63%, OR = 0.48, CI = 0.26–0.87, p = 0.01]. Any association was found between the other TNF-α polymorphisms and RPL.
Conclusion: TNF-α-857 C/C variant might represent protective effect against RPL and the -857 C/T variant might be a genetic risk factor for the occurrence of RPL. However, further studies with a larger number of subjects from different ethnic groups are needed to confirm the findings.
KEYWORDS: Tumor necrosis factor-α, Recurrent pregnancy loss, Polymorphism

1.INTRODUCTION

Recurrent pregnancy loss [RPL] is a main complication among women at the reproductive age and explained as three or more consecutive abortions before 20 weeks of gestation [1].
It has been estimated 1-2% of healthy fertile women would experience this event [1-4]. Various causes are identified for RPL such as anatomic, autoimmune and endocrine [2] or chromosomal abnormalities [5], infectious agents [6], hyperprolactinemia and hyperhomocysteinuria [7] as well as coagulation factors mutations and immunological problems [8-10]. However, it has been proven that the reason is unknown for approximately 40-50% of patients [1, 11].
It is obvious that T helper 1 [Th1] and T helper 2 [Th2] are the major components of immune system and their functional balance is of great importance for a normal pregnancy [12-14]. Th2 type cytokines including Il-4, IL-5, IL-6 and IL-10 are involved in normal pregnancy. In contrast, Th1 produces proinflammatory cytokines containing IL-1, IFN-γ and TNF-αwhich are supposed to be related to poor pregnancy outcome [1, 12, 15].
Tumor necrosis factor alpha [TNF-α] is an important proinflammatory cytokine secreted by monocytes/macrophages, B cells, natural killer [NK] cells and antigen-stimulated T cells which is located in 6p21.3, the region of human leukocyte antigen [HLA] class III [1].
It has been described this cytokine is effective on the process of RPL [2, 11, 13]. Several mechanisms have been suggested for the effects of TNF-α on pregnancy outcome: inducing the procoagulant activity

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of the vascular endothelial cell thus triggering the thrombotic/inflammatory reactions and increasing the production of cytokines through positive feedback, prevention of trophoblast growth and differentiation, induction of apoptotic cascade in the membrane of embryo and therefore increasing its degradation, disruption in the development and function of the placenta, activation of NK cells and macrophages, fetal exclusion as a result of uterine contraction and necrosis of implanted embryo[1, 2, 8, 12, 13, 16].

TNF-α production, secretion and activity are influenced by eight polymorphisms which occur in the promoter region as follows: 1031T/C, 863C/A, 857C/T, 575G/A, 376G/A, 308G/A, 244G/A, and 238G/A [1, 2, 12, 16-19].

There are conflicting reports about the association of these polymorphisms and RPL in different population. In this regard, some literature showed a positive association [12, 20, 21] but a negative relationship was reported by others [11, 22, 23].

To make a better decision about the association of TNF-α polymorphisms in RPL, in the present study, we compared the presence of six TNF-α polymorphisms at the positions -1031T/C,-863C/A, -857C/T ,-376G/A, -308G/A and -238G/A in 100 RPL patients with 100 healthy women at childbearing ages from the Iranian Azeri Turkish origin.

MATERIALS AND METHODS

A total of 100 patients and 100 healthy were included in this retrospective case control study. Subjects were women aged 21-45 years who had experienced at least three continuous abortion before 20 weeks of conception. The patients' karyotypes and the structure of uterine were normal, no infection related miscarriages were detected and any other identifiable causes were figured out. So, the events were classified as unexplained pregnancy loss. The age matched control subjects were selected from healthy fertile women with at least two live births and no history of pregnancy loss. In order to prevent the epidemiological bias, all recruited subjects had same ethnicity and belong with Iranian Azeri Turkish origin. All participants were informed about the study and signed a consent form.

Blood samples [5 ml] from antecubital vein were collected into tubes containing EDTA as an anticoagulant. Genomic DNA was extracted using the proteinase K method […]. Nanodrop instrument was employed to determine the quality and quantity of each DNA sample and electrophoresis on the 1% agarose gel was performed to confirm the results. The extracted DNA samples were stored at20- °C until analyzed. DNA samples were amplified and investigated for six polymorphisms in the promoter region of TNF-α gene using polymerase chain reaction [PCR]. They were -1031T/C ,-863C/A, -857C/T ,-376G/A, -308G/A and -238G/A. The following protocol was applied for PCR:

1 cycle of initial denaturation in 94°C for 5 minutes, followed by 35 cycles of denaturation [94°C for 1 minutes], annealing [for 45"], extension [72°C for 45"] and a final extension at 72°C for 5 minutes. This protocol was used for all polymorphisms but the annealing temperature for each pair of primers were different [Table 1]. The PCR products were electrophoresed on1.5% agarose gel stained by ethidium bromide. Following amplification reaction, the PCR products were digested through restriction fragment length polymorphism [RFLP] analysis using the appropriate restriction endonucleases [Table 2]. Then, electrophoresis of the digested products was performed on 3% agarose gel stained by ethidium bromide. The size of bands were estimated by using a 50 base pairmolecular weight marker. A gel documentation instrument was used for visualizing the bands of PCR and digested products of RFLP analysis. All figures of PCR and RFLP analysis are depicted in below.

The odds ratio [OR] was used as a measure of the strength of the association between allele frequencies and RPL. All P values were two-tailed and 95% confidence intervals [CI] were calculated. P values <0.05 were considered statistically significant.

Table 1. Primer sequences used for detection of TNF-α gene polymorphisms

Polymorphism	Primer Sequences	Annealing Temperature	Size of amplified product
TNFα-1031	F-TATGTGATGGACTCACCAGGT R-CCTCTACATGGCCCTGTCTT	55	**264**
TNFα-863	F-GGCTCGAGGATGGGTTAC R-CTACAGCGCTCTTCGTTAG	57	**125**
TNFα-857	F-GGCTTGAGGTATGGTTAC R-CGCTACGTCGCTCTCTCTAC	56	**128**
TNFα-376	F-CCCCGTTTTCTCTCCCTCAA R-TGTGGTCTGTTTCCTTCTAA	52	**105**
TNFα-308	F-AGGCAATAGGTTTTGAGGGCCAT R-GGGACACACAAGCATCAAG	55	**147**
TNFα- 238	F-AAACAGACCACAGACCTGGTC R-TCACACTCCCCATCCTCCCGGATC	64	**309**

Table 2. Restriction fragment length polymorphism [RFLP] analysis.

Polymorphism	Size of amplified fragment	Restriction enzyme	Genotype	Size of digested fragments
-1031 T/C	264	BbsI	TT	264
			TC	264-193-71
			CC	193-71
-863 C/A	125	TaiI	CC	125
			CA	125-104-21
			AA	104-21
-857 C/T	128	TaiI	CC	109-19
			CT	128-109-19
			TT	128
-376 G/A	105	Tsp5091	GG	105
			GA	105-83-22
			AA	83-22
-308 G/A	146	NcoI	GG	125-21
			GA	146-125-21
			AA	146
-238 G/A	154	BamHI	GG	129-25
			GA	154-129-25
			AA	154

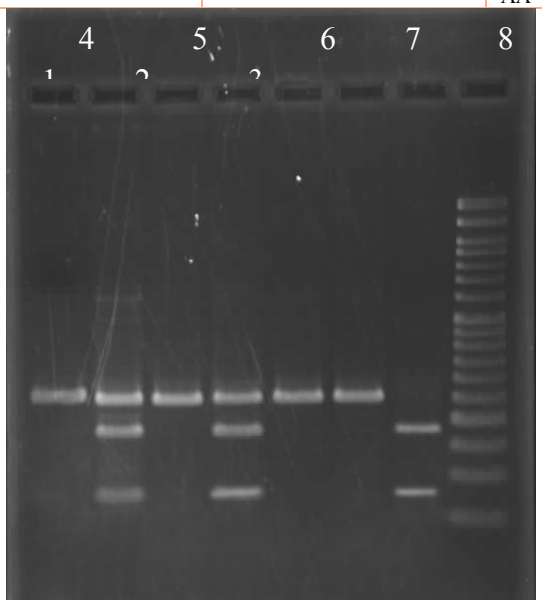

Figure1 1. PCR products before RFLP/ 3, 5, 6. Normal/ 7. Homozygote/ 2, 4. Heterozygote/ 8. 50 bp DNA ladder

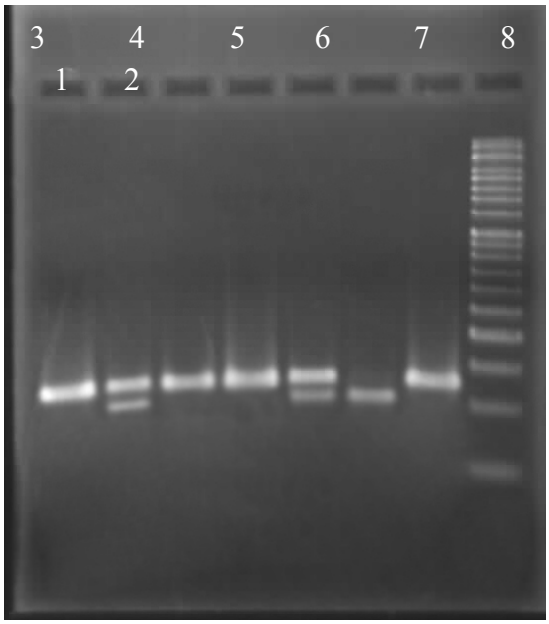

Figure 2. 1. PCR products before RFLP/ 3, 4, 7. Normal/ 6. Homozygote/ 2, 5. Heterozygote/ 8. 50 bp DNA ladder

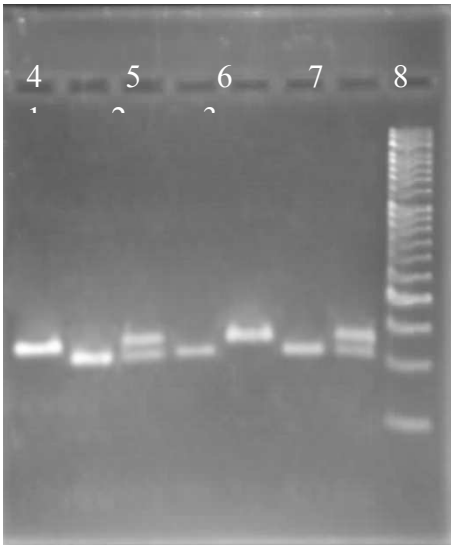

Figure 3. 1. PCR products before RFLP/ 2, 4, 6. Normal type/ 3, 7. Heterozygote/ 5. Homozygote/ 8. 50 bp DNA ladder

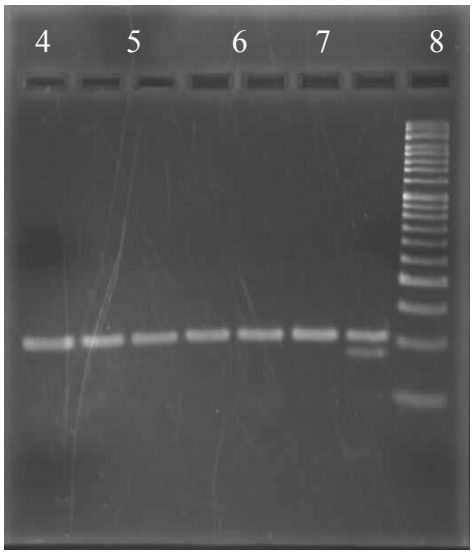

Figure 4. 1. PCR products before RFLP/ 2-6. Normal/ 7. Heterozygote/ 8. 50 bp DNA ladder

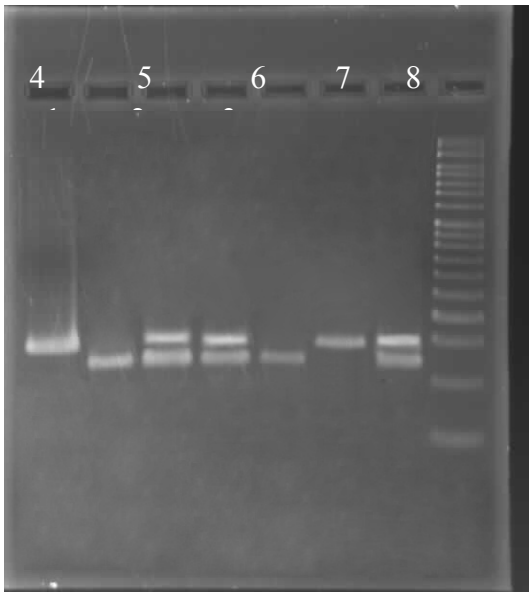

Figure 5. 1. PCR products before RFLP/ 2, 5. Wild type/ 3, 4, 7. Heterozygote/ 8. 50 bp DNA ladder

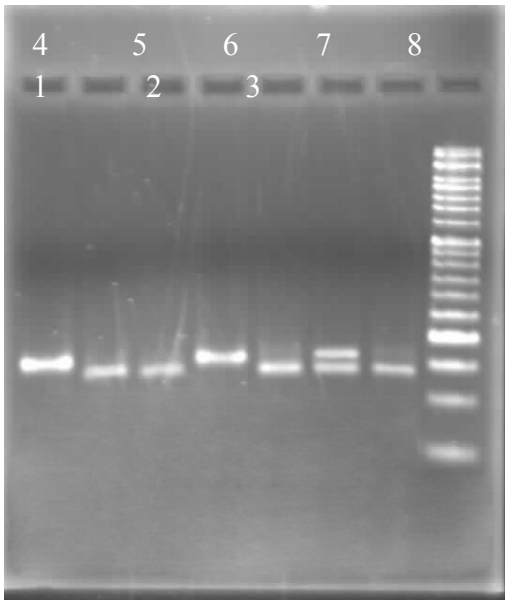

Figure 6. 1. PCR products before RFLP/ 2, 3, 7. Wild type/ 4. Homozygote/ 6. Heterozygote/ 8. 50 bp DNA ladder

RESULTS

100 patients with at least three unexplained RPL [mean 5, range 3-7]and 100 age and ethnically matched controls who had at least two successful delivery from Iranian Azeri Turkish origin were screened for the TNF-α 1031T/C, 863C/A, 857C/T, 376G/A, 308G/A, and 238G/A gene polymorphisms.

TNF-a allele frequency and genotypes distribution in RPL patients and control subjects are shown in tables 3 and 4, respectively. With exception of -857 C/T variant, there were no significant association between the genotype prevalences of TNF-α polymorphisms in the case and the control groups. According to TNF-α -857 variant, there was a lower frequency of CC genotype in RPL patients than that of controls which was statistically significant [45% Vs. 63%, OR = 0.48, CI = 0.26–0.87, p = 0.01]. In contrast, the frequency of CT genotype across the cases was significantly higher than controls [47% vs. 30%, OR = 2.06, CI = 1.11–3.86, p = 0.02]. However, no significant differences were displayed for TNF-α -857T [mutant] allele [31.5% Vs. 22%, OR = 1.63, CI =1.01–2.61, p = 0.42]and homozygous [TT] genotype [8% Vs.7%, OR = 1.15, CI =0.36–3.78, p = 1.00] among the case and control groups, respectively. Hence, it seems that being heterozygous for TNF-α -857 variant may lead to an increase in susceptibility to the RPL.

Table 3. TNF-α polymorphisms allele frequencies among cases and control subjects.

Polymorphism	Minor Allele	Cases [%]	Controls[%]	Pvalue	OR [95% CI]
-1031 T/C	C	15/5	20/5	0/24	**0/71 [0/41-1/22]**
-863 C/A	A	15	18/5	0/42	**0/77 [0/44-1/36]**
-857 C/T	T	31/5	22	0/42	**1/63 [1/01-2/61]**
-376 G/A	A	0/5	1	1/00	**0/49 [0/01-7/04]**
-308 G/A	A	54	48	0/27	**1/27 [0/84-1/92]**
-238 G/A	A	2	2	1/00	**1/00 [0/20-4/82]**

Table 4. Distribution of TNF-α genotypes among cases and control subjects.

Polymorphism	Genotype	RPL Cases[100]	Controls[100]	P value	OR
-1031 T/C	TT	71	63	0.29	1.43
	TC	27	33	0.44	0.75
	CC	2	4	0.68	0.79
-863 C/A	CC	72	68	0.64	1.21
	CA	26	27	1.0	0.95
	AA	2	5	0.44	0.38
-857 C/T	CC	45	63	0.01	0.48
	CT	47	30	0.02	2.06
	TT	8	7	1.00	1.15
-376 G/A	GG	99	98	1.00	2.02
	GA	1	2	1.00	0.49
	AA	0	0	1.00	-
-308 G/A	GG	4	9	0.25	0.42
	GA	84	86	0.84	0.85
	AA	12	5	0.12	2.59
-238 G/A	GG	96	97	1.00	0.74
	GA	4	2	0.68	2.04
	AA	0	1	1.00	0.00

DISCUSSION

According to the heterogeneous entity of pregnancy loss, different factors have been considered to be related with this unpleasant event including coagulation factor gene polymorphisms[24, 25], HLA-G polymorphisms [26], anatomical problems, chromosomal abnormality[27] and some other reasons[5, 7]. Among these varied causes, immunological problems are of great importance and the vast studies have been conducted to clarify the role of immune system in RPL [12, 28-32].It has been shown that Th1 cells play a crucial role in an inflammatory response and through the production of pro-inflammatory cytokines, IL-1, IFN-γ and TNF-α; they will affect the pregnancy outcome. It has been mentioned Th1 type cytokines are associated with poor pregnancy outcome, while the secreted cytokines from Th2 cell are involved in a normal pregnancy[33-35]. Besides the proinflammatory function of TNF-α, this cytokine participates in cell survival, growth and differentiation. Additionally, TNF-α levels are also elevated in both mother and her embryo which is related with preterm parturition [1, 36]. Considering the vital effect of TNF-α on pregnancy outcome and according to the varied reports on the association of different polymorphisms and the occurrence of RPL, we focused on six TNF-α gene polymorphisms.

Liu et al. [13] reported a positive association for TNF-α 238 polymorphism and unexplained recurrent spontaneous abortion, but a negative association was shown for TNF-α 308 polymorphism [13].

Zammiti et al. [2] claimed a similar result and Zhang et al. Also found that the -238G/A polymorphism might be related with the risk of recurrent spontaneous abortion [37]. However, there are some studies in which TNF-α-308 has been known to be associated with pregnancy loss [1, 2, 21]. In a study among Iranian women, no significant association between TNF-α-308 and RPL was observed [38]. TNF-α −1031T/C, −863C/A, −857C/T, −376G/A, −308G/A, −238G/A, and +488G/A single nucleotide polymorphisms were investigated across Bahraini Arabs by Finnan et al. Among these variants, −1031T/C, −376G/A, and −238G/A were determined to be independently associated with recurrent miscarriage [9]. Base on another report, TNF-α-863 variant was not also related to RPL in Caucasian women [11]. Babbage et al. Investigated the predisposing effect of TNF-α, IFN-γ and IL-10 polymorphisms on the occurrence of RPL and no relation were found[23]. Since various ethnic groups were selected in different studies, for instance, Caucasian [6, 11, 39], Iranian [38], Chinese [13], Indian [1] or Arabs [9]; it is not illogical to relate the differences in the results of these studies to the effect of different ethnicity of study population. Besides, sample size, selected cases and selection criteria [40], geographic differences [41] and the other involved genes [42] might be attributed to the conflicting results.

Apart from TNF-α-857 variant, our results were in concordance with previously mentioned studies. In this regard, it was found that TNF-α -857CT genotype was highly associated with RPL in Iranian Azeri Turkish women, while no significant association was detected for other five polymorphisms. Thereby, suggests TNF-α -857 C/C variant might have a protective effect against RPL, but the -857 C/T variant is probably a genetic risk factor for the occurrence of RPL. Therefore, it is suggested that for the future studies, the effect of -857C/T polymorphism on the predisposition to RPL should be considered with more attention. It should be noted that RPL is a multifactorial condition and the lack of relation between

these polymorphisms and RPL must not preclude us to discard the importance of these genetic factors in the pathogenesis of RPL. To confirm the results and to identify the exact effects of cytokine gene polymorphisms on the occurrence of RPL, further assessments are needed with the larger study populations from different ethnic groups. Furthermore, to obtain the comprehensive results, other cytokines should be included in the studies. To our knowledge, it was the first study on Iranian population in which six TNF-α - gene polymorphisms were investigated.
Finally, in the present study, an association was found just for TNF-α -857 gene polymorphism and no remarkable differences were observed for the other TNF-α variants when compared to normal controls.

Acknowledgements
This work was supported by research grants from the Ahar Branch, Islamic Azad University for research with title: The effects of tumor necrosis factor-α, IL-1 receptor antagonist and Interleukin-1β genes polymorphisms on recurrent abortion in Azari women.

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