The association of -511 C/T and -31 C/T polymorphisms in the interleukin 1 beta (Il-1β) gene with unexplained recurrent pregnancy loss in an Iranian Azeri Turkish population

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ABSTRACT
Objective: Proinflammatory cytokines have been indicated to be involved in the pathogenesis of unexplained recurrent pregnancy loss (RPL). The association between the polymorphisms in the promoter region of the interleukin-1 beta (IL-1β) gene (-31C/T and -511C/T) and RPL was investigated. To our knowledge, this was the first study on the effect of IL-1β gene polymorphisms on RPL among an Iranian population.
Materials and Methods: Subjects were comprised of 100 women with a history of three or more consecutive pregnancy losses before 20 weeks of gestation and 100 age-matched healthy controls with at least two successful pregnancies and no history of pregnancy loss. All participants were from Iranian Azeri Turkish origin. Genotype determinations were performed using PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis. **Results:** Allele and Genotype frequencies of IL-1β -31T (rs1143627) and -511T (rs16944) polymorphisms were not significantly different between the case and control groups (all P>0.05). **Conclusion**: Invariant differences in the prevalence of these polymorphisms between RPL patients and the control group suggest that IL-1β variants may not play a role in the women's tendency to develop RPL.
KEYWORDS: interleukin-1 β, polymorphism, unexplained recurrent pregnancy loss, Iranian Azeri Turkish population

INTRODUCTION

Recurrent pregnancy loss (RPL) is defined as three or more consecutive abortions before
20 weeks of gestation (1, 2) and occurs in 1-2% of fertile women (3-5). To date, several causes have been introduced for RPL such as genetic, anatomical, chromosomal, endocrinological and environmental factors (6). Furthermore, hyperprolactinemia and hyperhomocysteinuria (5) as well as immunological problems (7) have been identified. According to these heterogeneous causes, the appropriate evaluation of patients is necessary to explore the exact underlying pathophysiologic mechanisms; thereby the cases experiencing this event would receive a better treatment (5, 8).
As a result of increase in the anti-inflammatory immune response during normal pregnancy, Th1 type pro-inflammatory cytokines, IL-1, TNFα and IFNγ, are produced (9). It has been suggested that IL-1 family has great importance in inflammatory reactions and regulate the production of Th1/Th2 cytokines. Thus, the implantation and trophoblast growth and invasion would be affected (10-12). Two pro-inflammatory (IL-1α, IL-1β) and one anti-inflammatory cytokines (IL-1 receptor antagonist: IL-1RA) are included in this family (13-17) which places within 430 kb region on chromosome 2 (2q13-21) (16, 18). The human IL-1β is a 30 kDa polypeptide produced by monocytes, macrophages and epithelial cells, and the encoding gene has been determined within a 40 kb region on the band q14.2. A decrease in IL-1β levels has been reported in endometrium of women experiencing recurrent miscarriage; therefore, it is assumed that IL-1β is involved in the pregnancy outcome (19).
IL-1β is known as a crucial factor for reproductive functions. Interestingly, it seems that cell proliferation, differentiation and apoptosis are controlled by IL-1β. Moreover, the secretion of this cytokine leads to the production of other pro-inflammatory cytokines which trigger an inflammatory cascade (5, 19, 20). Due to its pro-inflammatory essence, it is not illogical to hypothesize a role for IL-1β gene in RPL(20).
IL-1β -511 C/T (rs16944) and IL-1β -31 C/T (rs1143627) are two common polymorphisms which have been considered to be related with RPL (10, 20). IL-1β production and individuals' tendency to inflammatory disorders as well as some defined infectious and malignant diseases would be influenced by these polymorphisms which are located in the promoter region of IL-1β gene (10, 12, 20, 21). In addition, an association between elevated serum IL-1β protein and a polymorphism has been found (5).
To our knowledge, there have been no reports on the frequency of these in the IL-1β gene and their

relationship with RPL in Iranian population. Furthermore, conflicting findings have been mentioned in different studies. Hence, to shed new insight on the mechanisms of the RPL, allele frequencies and genotype distributions of these two IL-1β gene polymorphisms and their association with RPL were investigated in cases and the results were compared with those of the age and ethnically matched healthy fertile subjects from Iranian Azeri Turkish population.

MATERIALS AND METHODS

In this study an association between RPL and IL-1β polymorphisms, IL-1β -511 C/T and IL-1β -311 C/T, were investigated. For this purpose, 100 women aged 21-45 years who had experienced at least three consecutive abortions before 20 weeks of conception were included. There were no identifiable causes for RPL. In this regard, the patients' karyotype and their uterine anatomical structure were normal and no infections related miscarriages were found. Altogether, there were no certain causes for RPL and the events were thus categorized as unexplained pregnancy loss. The control group consisted of 100 age matched healthy women in childbearing ages with at least two successful gestations and no history of pregnancy loss. All subjects in both groups were of the Iranian Azeri Turkish origin who attended the educational hospitals related to Tabriz university of medical sciences between 2011 and 2012. The same ethnicity of the subjects would prevent its confounding effect. All participants were informed about the study and the consent forms were signed. In DNA extraction stage, 5 ml of peripheral blood samples were collected into tubes and EDTA was used as an anticoagulant. The proteinase K method was applied for DNA extraction from whole blood. The quality and quantity of DNA samples were determined by a nanodrop instrument. Finally, the extracted DNA stored at -20 °C until analyzed. Then, Polymerase chain reaction (PCR) was used to amplify the extracted DNA samples. The applied oligonucleotide primers are shown in table 1. The PCR conditions encompassed 1 cycle of initial denaturation in 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 1 minutes), annealing (52°C for 45 seconds), extension (72°C for
45 seconds) and a final extension at 72°C for 5 minutes. This protocol was used for both polymorphisms with exception the annealing step of IL-1β -511C/T which was 55°C for 45 seconds. Next, electrophoresis on the 1.5% agarose gel stained by ethidium bromide was performed and a 50 bp size marker was also loaded onto the gel. The gel was placed in the gel documentation instrument to take photos. To determine the IL-1β polymorphisms, restriction fragment length polymorphism (RFLP) analysis was accomplished by using AvaI (-511 C/T) and AluI (-31 C/T) as the restriction enzymes. IL-1β -31 C/T and IL-1β -
511 C/T samples were incubated in 37°C for 5 and 15 minutes, respectively. This was followed by electrophoresis on 3% agarose gel stained by ethidium bromide to define the size of amplified PCR products and the genotypes of the polymorphisms.

The chi-square test was used to analyze differences in the IL-1β genotype distribution and allele frequencies between the case and control groups (SPSS software version 20). 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. All figures are included in supplementary 1.

RESULTS

The allele frequency and genotype distribution of the IL-1β -511C/T and IL-1β -31C/T in 100 women with at least three unexplained recurrent pregnancy losses were compared with those of the control group consisting of 100 women in childbearing ages who delivered at least two healthy, term infant without any history of previous abortion among the Iranian Azeri Turkish women. The quality and quantity of DNA samples were determined by a nanodrop instrument and the results were confirmed by electrophoresis on the 1% agarose gel (Figure 1). -31 C/T and -511 C/T polymorphisms in promoter region of IL-1β gene amplified using specific 234 and 305 bp primers, respectively (Figure 2 and 3). The sizes of the wild type (CC), heterozygous (CT) and homozygous (TT) alleles for IL-1β -511 C/T variant were 305 bp, 305-190-115 bp and 190-115 bp, respectively. The 190-bp and 115-bp fragments represented the “T” allele and the 305-bp fragment represented the “C” allele. Considering IL-1β -31 C/T variant, these were 234 bp, 234-150-84 and150-84 bp, respectively. The 150-bp and 84-bp fragments illustrated the “T” allele and the 234-bp fragment illustrated the “C” allele (Figures 4 and 5).

The genotype frequencies of IL-1β-511C/T variant in the case group were 35% CC, 43% CT and 22% TT, while the frequencies in the control group were 40% CC, 42% CT and 18% TT, respectively (Table 2). Of note, similar findings obtained for IL-1β-31C/T variant in which the frequencies in cases and controls were 37% CC, 44% CT and 19% TT and 38% CC, 42% CT and 20% TT, respectively (Table 3). On the basis of statistical analysis, no significant solidarity between patients and controls were perceived concerning IL-1β genotype frequencies. The allele frequencies for each polymorphism of IL-1β in both groups of subjects were also deliberated (Table 4). Accordingly, there were no meaningful differences in the prevalence of IL-1β

variants between the women with RPL and their healthy controls. Finally, it was assumed IL-1β polymorphisms are not related to RPL.

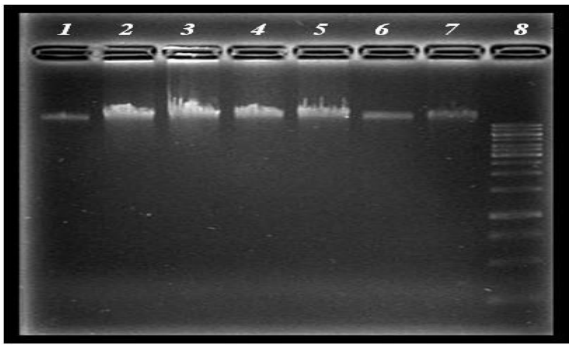

Figure 1: DNA Electrophoresis on 1.5% agarose gel.
1. Negative control
2-7. patient samples that selected randomly
8. 250 bp DNA ladder

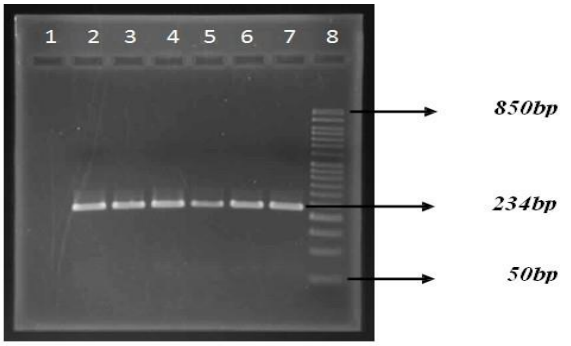

Figure 2: Electrophoresis of PCR products for IL-1b -31 C/T polymorphism on 1.5% agarose gel.
1. Negative control
2-7. 234 bp band
8. 50 bp DNA ladder

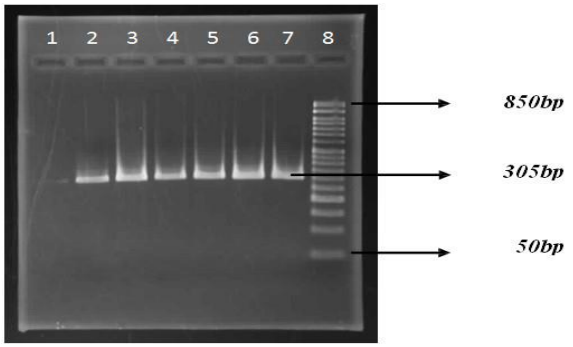

Figure 3: Electrophoresis of PCR products for IL-1b -511 C/T polymorphism on 1.5% agarose gel.
1. Negative control
2-7. 305 bp band
8. 50 bp DNA ladder

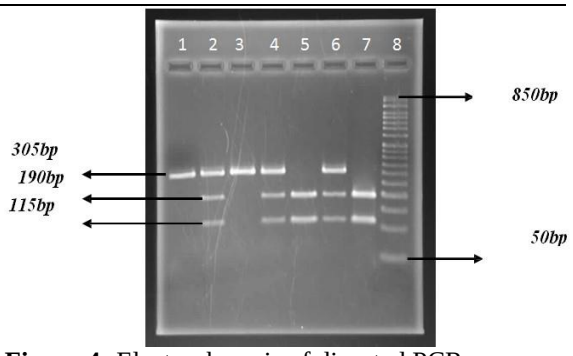

Figure 4: Electrophoresis of digested PCR products for -511 C/T polymorphism on 3% agarose gel.
1. PCR products before RFLP
2, 4, 6. Heterozygote
3. Homozygote
5, 7. Wild type
8. 50 bp DNA ladder

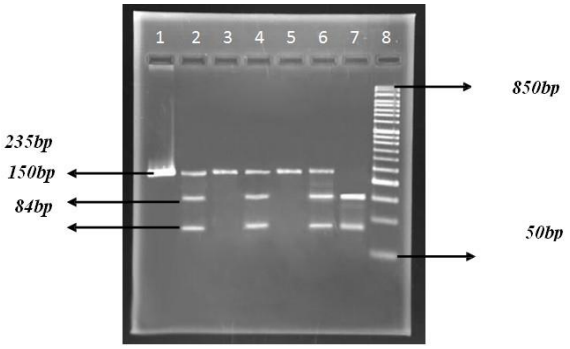

Figure5: Electrophoresis of digested PCR products for -31 C/T polymorphism on 3% agarose gel.
1. PCR products before RFLP
2, 4, 6. Heterozygote
3, 5. Normal
7. Wild type
8. 50 bp DNA ladder

Table1: Primer sequences used for detection of IL-1β gene polymorphisms

Polymorphism	Primer sequence	Melting temperature	Size of amplified product
IL-1β (-31C/T)	F: TCTTTTCCCCTTTCCTTTAACT R: GAGAGACTCCCTTAGCACCTAGT	49/1 54/3	234
IL-1β (-511C/T)	F: TGGCATTGATCTGGTTCATC R: GTTTAGGAATCTTCCCACTT	50/00 46/6	305

Table 2: Genotype frequencies of the IL-1β -511 C/T polymorphism among Iranian RPL patients and healthy fertile women

		(n=100)	(n=100)		(95% CI)
	CC	35	40	1/00	0/000 (0/000-17/447)
	TT	22	18	0/59	1/042 (0/572-1/898) 1/285 (0/607-2/728)

Table 3: Genotype frequencies of the IL-1β -31 C/T polymorphism among Iranian RPL patients and healthy fertile women

Polymorphism					
-31C/T	TT	19	20	1/00	/ 0/938 (0/440-1/998)
	TC	44	42	0/88	1/085 (0/596-1/975)
	CC	37	38	1/00	0/958 (0/519-1/769)

Table 4: Allelic frequencies of the IL-1β polymorphisms among Iranian RPL patients and healthy fertile women

Polymorphism	Polymorphic Allele	Cases (%)	Controls (%)	P value	Odds Ratio (95% CI)
C/T 31-	T	41	41	1/00	1/000 (0/658-1/520)
C/T 511-	T	43.5	39	0/47	1/204 (0/792-1/830)

DISCUSSION

In this case control study, we decided to clarify whether IL-1β -511C/T or IL-1β -31C/T polymorphisms play a role in RPL among Iranian population. Several investigations have up to now been performed to identify the association of RPL and different polymorphisms in a variety of factors such as factor V Leiden and factor II G20210A(22), TNF-α and IL-10 (23), HLA-G (24), methylenetetrahydrofolate reductase (MTHFR) (25) and some other immunological studies (26-30). Unlike the T helper 2 type cytokines, T helper 1 type ones (proinflammatory cytokines), IL-1 and IFN-γ, are associated with pregnancy loss (31-33). This hypothesis is on the basis of the fact that Th1 cells have great importance in acute allograft rejection, while immune tolerance has been known to be related to Th2 cells (34). It has been postulated the occurrence of polymorphisms in the IL-1β gene play a role in some other disorders like gastric adenocarcinoma (35), inflammatory bowel diseases (36, 37), febrile seizures (21), helicobacter pylori infection (38) and susceptibility to bacteremia in kidney transplant recipients(39).

Some of the bi-allelic polymorphisms in Th1 and Th2 type cytokines were investigated in a study on Iranian RPL patients and healthy controls. Among them, a significant association was only found between the presence of CC genotype of IL-10−592C→Apolymorphism and the occurrence of RPL. So, IL-10 polymorphism at this position was introduced as a genetic factor for RPL (40).

This was the first study on the association of IL-1β polymorphisms and RPL in an Iranian population. Based on our results, no association was ascertained. Therefore, IL-1β polymorphisms are not probably involved in the pathogenesis of RPL in Iranian population from Azeri Turkish origin, so normal pregnancy will not be influenced. These are in line with some previously reported data. According to the proinflammatory property of IL-1, Hefler et al, demonstrated that idiopathic recurrent miscarriage (IRM) is not affected by the IL-1β polymorphism, but they investigated the polymorphism in the exon 5 of IL-1β and their subjects were from Caucasian population (5). In another study, Hefler et al, revealed -511C/T polymorphism in the promoter region of IL-1β gene, like the foregone polymorphism, was not correlated with recurrent miscarriage in white Caucasian women (20). Similarly, Linjawi et al, reported no association between IL-1β -511 C/T gene polymorphism and recurrent miscarriage in European Caucasian population (12). Against these literatures, Wang ZC et al. proposed that Caucasian women who had suffered from three or more spontaneous abortions and were homozygous for IL-1β -511C or IL-1β -31T might be more susceptible to RPL. They explained that such an association is probably arises from the role of IL-1β in the regulation of the Th2 cytokine production or trophoblast growth and invasion during early gestation (10). The predisposing effect of TNF-α, IFN-γ and IL-10 polymorphisms for the occurrence of RPL were investigated by Babbage et al. They found RPL is not influenced by these cytokines which is probably due to the minor effect of these genetic factors on cytokine production during gestation (41).

Wang et al, studied on the association of CD46 gene polymorphisms and IL-1β -511C/T variant with RPL in women with or without Th1 immunity to trophoblast antigens. They concluded that women who were homozygous for both alleles were at a higher risk of developing RPL-Th1(+) (42).

Differences in reported results by different studies might be due to the other involved genes (18), geographic differences (21), sample size and selection bias (11), the presence of more than one predisposing factors (43), ethnic heterogeneity (18, 44, 45) and the different environmental factors (46).

Although multiple studies have been surveyed on the association of different factors and RPL in Iranian population (40, 47-51), for the first time in Iran, we illustrated no association between RPL and the -511C/T or -31C/T polymorphisms in the promoter region of the IL-1β gene. Our findings showed more details of the unexplained RPL as a multifactorial event and suggested that IL-1β polymorphisms seem not to be related gene and play no functional role in RPL. Nevertheless, to reach the better understanding about RPL, in view of its multigenetic background, identification of gene variants would determine the treatment strategies of the subjects (17, 18, 43, 52, 53); therefore, it is irrevocable to design greater studies in different ethnicity groups.

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