DOI: 10.4149/BLL_2014_092

Bratisl Lek Listy 2014; 115 (8)
479 – 482

CLINICAL STUDY

IL-6 gene promoter polymorphisms: genetic susceptibility to recurrent pregnancy loss

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Abstract: Recurrent pregnancy loss (RPL) is defined as three or more pregnancy losses before 20 weeks. RPL is a multifactorial condition with several etiologic factors including genetic abnormalities of the parents, anatomical, endocrinological, hematologic and immunologic abnormalities, infections, nutritional and environmental factors. The causes of pregnancy loss in about half of the women with RPL even after extensive investigations remain unknown. We analyzed IL-6 -174 G/C, -572 G/C, -597 G/A, -1363 G/T, -2954 G/C promoter region polymorphisms in 113 RPL patients and 113 healthy subjects by using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay. The -174G/C genotypic and -174C allelic frequency and the -2954G/C genotypic and -2954C allelic frequency of IL-6 was higher in RPL patients than healthy controls and a significant association was found between RPL and -174G/C, -2954G/C polymorphisms ($p < 0.0001$, OR: 0.28, 95% CI: 0.15–0.51, $p < 0.034$, OR: 0.16, 95% CI: 0.01–1.12 respectively). We found remarkably similar frequencies in RPL patients compared to controls for IL-6 -572G/C,-597G/A and -1363G/T genotypes/alleles and no association was observed between RPL and these polymorphisms. Our study supported that IL-6 -174G/C and -2954G/C polymorphisms were associated with an increased risk of RPL in Turkish patients *(Tab. 3, Ref. 24)*. Text in PDF *www.elis.sk.*
Key words: IL-6, polymorphism, genetic susceptibility, recurrent pregnancy loss.

Recurrent pregnancy loss (RPL), defined as three or more pregnancy losses before 20 weeks (ectopic, molar and biochemical pregnancies not included), has been estimated to affect one in 300 pregnancies, and the etiology is unknown in at least 23–40 % of cases (1–3). RPL is a multifactorial condition with several etiologic factors including genetic abnormalities of the parents, anatomical, endocrinological, hematologic and immunologic and immunologic abnormalities, infections, nutritional and environmental factors (4, 5). The causes of pregnancy loss in about half of the women with RPL even after extensive investigations remain unknown.

Different studies have shown that cytokines play a major role in reproductive events. Th1 and Th2 cells are the major subsets of fully differentiated Th cells, with distinct functional properties. Th1 cells induce several cytotoxic and inflammatory reactions via interferon- γ (INF- γ), tumor necrosis factor-α (TNF-α), interleukin (IL)-1b and Th2 cells are associated with the production of IL-6, IL-10 cytokine that are related with B cells response and antibody formation (6, 7). For instance, IFN- γ and TNF-α inhibits trophoblast growth and differentiation, whereas IL-4, IL-10 and IL-13 may promote embryo development and placentation (8, 9, 10, 11). On the basis of these observations, a Th2-type dominant response has been associated with normal pregnancy, whereas a Th1-type response has been related to pregnancy failure (11, 12).

Several polymorphisms of IL-6 has been identified, polymorphisms at positions -572 G/C, -597 G/A, -1363 G/T, -2954 G/C reveal a cooperative impact on the IL-6 gene transcription. IL-6 is located in the 5' flanking region of the IL-6 gene, an important area in the regulation of gene expression, where a single nucleotide change from G to C at position -174, results in alterations of IL-6 transcription (13, 14). Also, the individual's IL-6 genotype may be relevant to conditions, such as juvenile systemic onset arthritis, development of Kaposi sarcoma and atherosclerosis (13, 15, 16). Clinical data like these suggest that carriage of the mutated IL-6 allele C confers protection against the development and course of inflammatory diseases.

In this study, we attempted to establish an association between the polymorphisms at positions -174 G/C, -572 G/C, -597 G/A, -1363 G/T, -2954 G/C in the promoter region of IL-6 and the occurrence of RPL in Turkish patients who attended to Gynecology and Obstetrics Clinic of Gaziosmanpasa University Faculty of Medicine in Tokat, Turkey.

Materials and methods

Patients and controls

The study group consisted of 113 women with a spontaneous abortion and 113 controls. The study group included 113 Turkish

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Tab. 1. Demographic data of women with idiopathic recurrent miscarriage and controls.

	Women with idiopathic recurrent miscarriage	Controls
No. of patients	113	113
Age (years)	27 (18–47)	28 (24–49)
Parity	1.06 (0–6)	2.2 (2–5)
No. of miscarriages	3.4 (3–6)	0
Menopausal status	0	0

women (mean age 27 years, range 18–47) with an unexplained RPL, consecutively referred. All of them had a history of at least three consecutive spontaneous miscarriages (mean 3.4, range 3–6). All cases were patients who attended Gynecology and Obstetrics Department of Gaziosmanpasa University Faculty of Medicine, Tokat, Turkey for spontaneous abortion. The following tests were performed to exclude known causes of abortion: serial ultrasound, prolactin dosage, glycemic curve, determination of thyroid hor-

Tab. 2. PCR programs, primer sequences and restriction enzymes for IL-6 polymorphisms.

Polymorphism	Primer Sequence	Restriction Enzyme	Product Size	Restriction Product Size	PCR program
IL-6 174 G/C	F:5′TTGTCAAGACATGCCAAAGTGCGGAG-3’ R:5′GTGCAATGTGACGTCCCTTAGCAT-3′	BseL-I	156 bp	139bp+17bp for G allele 117bp+22bp+17bp for C allele	95 °C 3 min 94 °C 30 s 56 °C 30 s 72 °C 1 min 72 °C 7 min } 40 cycle
IL-6 572 G/C	F: 5’-CAGCAGCCAACCTCCTCTAA-3’ R: 5’-CCAAGCCTGGGATTATGAAG-3′	BsrB-I	224 bp	150 bp +74 bp for G allele	95 °C 3 min 95 °C 30 sn 62 °C 30 sn 72 °C 1 min 72 °C 1 min } 40 cycle
IL-6 597 G/A	F: 5’-CAGCAGCCAACCTCCTCTAA-3’ R: 5’-CCAAGCCTGGGATTATGAAG-3′	Fok-1	224 bp	136 bp+88bp for A allele	
IL-6 1363 G/T	F: 5’-CGGTGAAGAATGGATGACCT-3’ R: 5’-AAACCAGACCCTTGCACAAC-3’.	Taq-I	205 bp	103 bp+102 bp g-for G allele	
IL-6 2954 G/C	F:5′-GCCAGTGATCCACAGAAACAA-3′ R: 5′-AGCTACTGGTGGCCAACGC-3′	BstU-I	192 bp	174 bp+18 bp for C allele	

Tab. 3. Genotypic and allelic frequencies in spontaneous abortion and controls for IL-6 gene.

	Spontaneous abortions n=113	Controls n=113	p	O.R (CI 95%)
IL-6 572 G/C			0.49	
GG	81 (71.5 %)	88 (78 %)		
GC	28 (25 %)	21 (18.5 %)		
CC	4 (3.5 %)	4 (3.5 %)		
Allelic frequency			0.176	
G	190 (84 %)	197 (87 %)		
C	36 (16 %)	29 (13 %)		
IL-6 597 G/A			0.106	
GG	96 (85 %)	87 (77 %)		
GA	16 (14 %)	26 (23 %)		
AA	1 (1 %)	0		
Allelic Frequency			0.105	
G	208 (92 %)	200 (88.5 %)		
A	18 (8 %)	26 (11.5 %)		
IL-6 1363 G/T			0.430	
GG	95 (84 %)	94 (83 %)		
GT	18 (16 %)	19 (17 %)		
TT	0 0			
Allelic Frequency			0.433	
G	208 (92 %)	207 (91.5 %)		
T	18 (8 %)	19 (8.5 %)		
IL-6 174 G/C			0.00001	
GG	72 (64 %)	100 (88.5 %)		
GC	36 (32 %)	11 (10 %)		
CC	5 (4 %)	2 (1.5 %)		
Allelic Frequency			0.0001	0.28 (0.15 to 0.51 %)
G	180 (80 %)	211 (93 %)		
C	46 (20 %)	15 (7 %)		
IL-6 2954 G/C			0.033	
GG	107 (94.5 %)	112 (99 %)		
GC	6 (5.5 %)	1 (1 %)		
CC	0	0		
Allelic Frequency			0.034	0.16 (0.01 to 1.12 %)
G	220 (97 %)	225 (99.5 %)		
C	6 (3 %)	1 (0.5 %)		

mone levels, investigation of toxoplasmosis, cytomegalovirus, rubella, AIDS, hepatitis B and C and bacterial vaginitis. The criteria for inclusion in the study were to be Turkish and to present with unexplained RPL after all the tests mentioned above. The control group consisted of 113 healthy Turkish women (mean age 28, range 24–49) who had at least one child, carefully matched fort the variables cited above. The study protocol was approved by the ethics committee of Gaziosmanpasa University Faculty of Medicine and a written informed consent was obtained from study participants. All women accepted the peripheral blood sampling for genotype analyses. Demographic data of women with idiopathic recurrent miscarriage and controls was given Table 1.

Genetic analysis

Genomic DNA was isolated from peripheral blood using Invitrogen DNA isolation kit. We used PCR based restriction fragment length polymorphism (RFLP) method for analyzing the polymorphisms of IL -6 (-174 G/C, -572 G/C, -597 G/A, -1363 G/T and -2954 G/C). The PCR was performed with a 25 µl reaction mixture containing 50 ng of genomic DNA, 0.8 nmol/ µl each primer, 10X reaction Buffer, 1.5 mM $MgCI_2$ 0.3 mM each dNTP and 1U Taq DNA polymerase (Fermentase). The PCR primers, PCR program and restriction enzymes were shown in Table 2. We used to electrophoresis in 3 % NuSieve agarose gel for analyzing the digested products.

Statistical analysis

Statistical analysis was performed by using Open Epi Info software package program. The distribution of IL-6 (-174 G/C, -572 G/C, -597 G/A, -1363 G/T and -2954 G/C) gene polymorphism between RPL patients and healthy controls were compared by using the χ^2 or Fisher's exact test. p values smaller than 0.05 were considered significant. Odds rations (ORs) and 95% confidence intervals (CIs) were also calculated whenever χ^2 or Fisher's exact test was significant. Goodness of fit χ^2 test was used to check Hardy-Weinberg equilibrium in the control population, Arlequin Software v. 2000 (University of Geneva, Geneva, Switzerland).

Results

Table 1 shows the demographic characteristics of RPL patients and healthy controls. No significant difference in the mean ages was observed between the two groups. The IL-6 -174 G/C, -572 G/C, -597G/A, -1363 G/T and -2954 G/C genotypic and allelic frequencies in patients with RPL and controls were shown in Table 3. For quality control, 10 % of the samples were randomly selected to be genotyped again by a different investigator and the results were 100 % concordant. The -174 G/C genotypic

-174 C allelic frequency and the -2954 G/C genotypic and -2954C allelic frequency of IL-6 was higher in RPL patients than healthy controls and a significant association was found between RPL and -174G/C, -2954G/C polymorphisms ($p < 0.0001$, OR: 0.28, 95% CI: 0.15–0.51, $p < 0.034$, OR: 0.16, 95% CI: 0.01–1.12 respectively). We found remarkably similar frequencies in RPL patients compared to controls for IL-6 -572G/C,-597G/A and -1363G/T genotypes /alleles and no association was observed between RPL and these polymorphisms. Also, IL-6 -1363 TT genotype was not found in either the patients or the control group.

Discussion

This study showed that patients who have recurrent pregnancy loss have impairment at the some polymorphisms of the gene IL6; RPL patients have statistically higher frequency of the IL 6 -174C and -2954C genotypes according to the control group. Some of gene polymorphism genotypes and the polymorphic allele frequency of IL6 as -572 G/C, -597 G/A, -1363 G/T are not related with recurrent pregnancy loss in our study. IL-6 -1363 TT genotype was not found in either the patients or the control group.

The genotypes responsible for high and low IL-6 production are not completely defined in literature. The cytokine network is very sophisticated and regardless of the fact that the Th2 pathway seems to be responsible for a successful pregnancy, whereas the failure of pregnancy is rather associated with the predominance of Th1-type cytokines (18, 19).

Interleukin-6 is a pleiotropic cytokine produced by a variety of cell types, including lymphocytes, monocytes and endothelial cells. IL-6 gene has been mapped to chromosomal region 7p21 and two common SNPs at positions -174 and -634 of the promoter known to influence IL-6 expression (13, 17). This gene has a highly complex transcriptional regulation and the single nucleotide change from G to C could result in alteration of IL 6 transcription, as this nucleotide change creates a potential binding site for the transcription factor NF-1, which in HeLa cells was shown to be a repressor of gene expression (13). During the early 1990s, Wegmann et al. studied pregnancy success in rodents and he found a strong evidence about the predominant Th2 cytokine profile providing successful pregnancy (18). IL6 functions both in adaptive and innate immunity, it has proinflammatory characteristics and has implication in the pathophysiology of abnormal pregnancies (20). According to some in vitro observations, IL-6 stimulates growth, invasion and differentiation of the trophoblast (21). IL-6 is also related with angiogenesis, providing development of ovarian follicles and in the formation of the decidua following embryonic implantation (22). According to this information, the reduced cord serum levels of IL-6 has been found in women with preeclampsia, this situation may be related with placental insufficiency which is mainly caused by trophoblast function impairment (21–23). Our results showed that an increased frequency of the -174G/C genotypic and -174C allelic frequency and the -2954G/C genotypic and -2954C allelic frequency of IL-6 was higher in RPL patients. We appraise that for a successful pregnancy, IL-6 production must be higher than in women who have RPL, we could assume that the G/G genotype that was more frequent in controls is responsible for IL-6 high production according to Fishman et al 13). At the IL-6-1363 genotypic and allelic frequency G/G, G/T and T/T genotypes were similar in both groups. In English literature, there was no any similar study for comparison. Some authors who studied the relationship between IL-6 (-174 G/C) polymorphism and RPL, they did not find any association (11, 18). Von Linsingen R et al found that the frequency of -174 C/C genotype was significantly more common in the RPL

group in comparison with that observed in the control group (18 % versus 4 %) while -174 G/G was more common in the control group (54 % versus 37 %) (14). But in our study -174 CC genotype was more common in RPL group when compared to the control group (4 % versus 2 %) and -174 G/G genotype was more common in the control group according to the study group (88 % versus 64 %). Unfried et al found no association when they analyzed IL-6 gene polymorphism and RPL. (24). Maybe some hypothetical explanations for our opposite results could be due to the fact that in the study of Unfried et al, they investigated primary and secondary RPL and their control group were all postmenopausal women. In our study, we also selected primary and secondary RPL, but our control group was not postmenopausal or perimenopausal. In another study, which was performed by Daher et al., they did not find any association between RPL and IL-6 gene polymorphism (11). Their control group consisted of healthy individuals from the general population, but this population contained 26 males. In our study, to minimize biases in between the study and the control group, all individuals, were from the same ethno-geographic origin, age and social condition and all of them were women.

-174 G/C polymorphism varies with the ethnicity. Because of this, we cannot evaluate ethnicity and recurrent pregnancy loss relationship in between different countries; for example -174 G/C polymorphism is extremely rare in Chinese population. Also possible genetic linkage disequilibrium with other cytokine genes should be taken into consideration in the studies to elucidate the role of IL-6 polymorphisms affecting pregnancy outcome.

In summary, our data indicated that IL-6 -174G/C and -2954G/C polymorphisms were associated with an increased risk of RPL in Turkish patients. However, multiple cytokines may be able to show clinical impression of diseases including recurrent pregnancy loss. Because of that, possible genetic linkage disequilibrium with other cytokine genes must be thought over in future studies with larger sample sizes to compare the relationships better.

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Received March 11, 2013.
Accepted April 12, 2014.