Molecular Investigation of Association Among Common IL-6 Polymorphism with Cytomegalovirus(CMV) Infection and Recurrent Miscarriage in Iranian Women

Parisa Pourroostaei Ardakani

Pasteur Institute of Iran

Bahareh Rahimi

Iran University of Medical Sciences

Mohammad Panahi

Tabriz University of Medical Sciences

Babak Karimian

Pasteur Institute of Iran

Hamzeh Rahimi (✉ rahimi.h1981@gmail.com)

Pasteur Institute of Iran

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Abstract

Background: Recurrent pregnancy loss (RPL) is described as two or more spontaneous abortions. Until now, although various factors such as genetic, endocrinology, anatomy, immunology, and microbiology have been distinguished that affect abortions, the precise basic etiology in up to 50% of RPL cases are not determined. Human cytomegalovirus (CMV) infection and host genetic background, like IL-6 SNP polymorphisms play important roles in RPL etiology.

Objective: This study aimed to evaluate relationship among single nucleotide polymorphisms (-634C/G and -174 G/C) in the IL-6 gene with CMV infection and risk of RPL for early detection and treatment of RPL.

Materials and methods: This case-control study was carried on 80 Iranian females with RPL and 80 healthy females as control group. The extraction of DNA from samples and detection of CMV and IL6 SNPs were determined by Tetra ARMS-PCR. Finally, the statistical analysis for detection CMV and two polymorphisms roles in RPL were analyzed by Epi Info TM software by X2 test.

Results: Our results indicated an increased rate of CMV infection in RPL group (44%) versus the control group (25.45%). Also, the prevalence of IL-6 -634C/G genotype among RPL patients with CMV infection was 80%, while the frequency of this genotype among RPL patients without CMV infection was 50%. Furthermore, no substantial relation was found between IL-6 -174 G/C genotypes and RPL ($P \leq 0.0001$). Besides

Conclusion: This study not only indicated a significant role of CMV in RPL, but also showed CMV association with allele G in IL6 -634 among Iranian women. In addition, suggested the use of CMV and IL-6 -634 GG genotypes in RPL as diagnostic and prognostic biomarkers in Iranian population.

Introduction

Miscarriage is a severe pregnancy disorder which described as the loss of a pregnancy before 20th week. It usually occurs in 20 percent of all first pregnancies. genetic, endocrine, anatomic, immunologic factors, and various infections can lead to RPL. There is substantial confirmed evidence presents that preventable infections can cause up to 15% of early spontaneous abortions and up to 5% of all RPL [1]. Congenital infections are common vital dangers to the fetus's wellbeing, and it is noticeable that most of them are caused by viruses. Rubella, cytomegalovirus (CMV), and Toxoplasma are prevalent cause of infections in the human's uterus and can lead to various problems such as premature infant, abortion, stillbirth, and congenital anomalies [2, 3].

Human CMV, belongs to the viral family known as Herpesviridae, is one of the most prevalent causes of congenital infections [4, 5]. Over the last few decades, several studies were carried out on the pathogenesis and epidemiology of CMV-related infections, the most prevalent reason of congenital viral infections, in pregnant females. Congenital CMV, can develop during pregnancy as a result of reactivation

of formerly acquired CMV or primary infection [6, 7]. The effects of primary infection of CMV in pregnant women which can lead to the loss of pregnancy is still under study and analysis, and basic mechanisms are unknown [8–10]. Some investigations discovered the high level of CMV-related antigens in tissue from abortion [11] and some findings revealed higher seropositivity [12]. In addition, one study indicated an increased risk between pregnancy loss and CMV infection [13], though the findings of other related studies did not compatible with it [14]. Although some of these studies demonstrated the role of CMV-related infection in uncontrolled pregnancy loss, the effects of CMV infection on RPL is unknown.

Etiologic factor related with RPL are proposed as genetic, infectious, anatomical immunological, and environmental causes [15, 16].

On the other hand, the commitments of immunogenetic variables to the etiopathology of RPL have been recommended. Throughout the early phases of ordinary pregnancies, pro-inflammatory T aide (Th) 1 cytokine such as IL-6, TNF- α , and IL-10 are fundamental for fortifying vasculogenesis which is fundamental for effective embryonic implantation. While, drawn out presentation to Th1 cytokines can lead to a cell-mediated resistant reaction that's inconvenient to the baby, driving to pregnancy misfortunes [17, 18].

It has been shown that ordinary pregnancies are went with an in general higher level of Th2 cytokines whereas pregnancies with undesirable results are ordinarily related to an in general higher expression of Th1 cytokines such as interleukin 6 (IL-6) [19–22]. The IL-6 is a vital regulator of inflammation, has a remarkable role in the pro-inflammatory responses, and considers as an anti-inflammatory cytokine in the regulation of immune system. Several SNPs within the gene region of IL-6 could alter IL-6 expression and thence affect downstream target genes. Previous reports have suggested that -634C/G and -174 G/C SNPs may affect the IL-6 expression and is linked to the risk of RPL [23–26]. However, some studies display a negative affiliation between IL-6 polymorphism and RPL risk.

The aim of this study is to evaluate the associations between IL-6 polymorphism at position 634 and 174, CMV infection, and incidence of RPL in Iranian population. In order to achieve this purpose, we conducted a study through Tetra-ARMS PCR, PCR-RFLP, and statistical analysis (SPSS) methods. Our results demonstrated a significant association among CMV infection, IL-6 -634 G/C polymorphism, and RPL patient. We hope the findings of this study can shed light on molecular markers having significant roles in the ethology of the miscarriage.

Material And Methods

Participants

This study carried on two different groups as case-control study. The control group contained 80 women who experienced at least two successful pregnancies and no background of abortion. The case group consisted of 80 Iranian women with a history of RPL. The case samples involved women referred to Khatam-Ol-Anbia hospital, Tehran, Iran from April 2017 to June 2018. Written consent was received from

all the subjects to do molecular analyses. Detailed pedigree analyses and detailed assessment of the clinical reports was undertaken in all the subjects. Patients having acquired or hereditary thrombosis, anatomical abnormalities, hormonal diseases, such as abnormality in thyroid function, erythroblastosis fetal (Rh disease), chromosomal aberrations, hyperprolactinemia, and toxoplasmosis. Other infections, Rubella, HSV infections were excepted from the molecular study. To DNA extraction, under sterile conditions 5ml of peripheral blood were taken from all participants into tubes containing EDTA, consequently genomic DNA was extracted through standard guidelines, as previously explained [27]. The material of endocervical for CMV infection test was attained by a special swab. Then, care was taken with scraping and rotation to obtain adequate sample. Finally, the samples were kept frozen at -80° until CMV screening.

Dna Extraction And Cmv Detection

Total genomic DNA was isolated from the endocervical sample by viral DNA extraction kit (CinnaGen, Iran). The DNA from endocervical specimens was amplified by CMV primers (Oligo7 software was used for primer design). The PCR primers are described in Table 1. For detection of CMV, PCR amplification was done using two primers. The PCR reaction mixture underwent initial denaturation process at 95°C for 7 min, then 35 cycles at 95°C for 55 secs, 55°C for 45 secs, and 74°C for 59 sec. The final extension was performed at 74°C for 8 min in the thermocycler. The PCR fragments (280 bp) were run in 2% agarose gel and were visualized through cyber green staining.

Table 1
Nucleotide sequences of the primer sets for CMV detection

Name	Primer sequence	Length (bp)
CMV	5'-GTAGCTGGCATTGCGATTGGT-3' 5'-GCCAGGGCATAAAATGG-3'	280

IL-6 -174 G/c Genotyping By Tetra-arms Pcr Method

The genotypes of IL-6 -174 G/C polymorphism of the IL-6 gene were detected by the use of the Tetra-primer amplification refractory mutation system-polymerase chain (Tetra ARMS-PCR) procedure. The PCR was done with a total volume of 25 µl with 100 ng DNA template, 3.5 µl of 10X PCR buffer, 1 U of Taq DNA polymerase, 0.5 mM dNTPs and 0.5 µmol/l of each primer. The PCR conditions were 94°C for 7 minutes, 94°C for 58 secs, 60°C for 58 seconds, 72°C for 58 seconds, and 72°C for 10 minutes. Then, the obtained fragments were separated using electrophoresis on a 2.5% agarose gel and consequently visualized in three various patterns of fragments. The GG genotype made two fragments (326 and 205 bp), the CC homozygote made two fragments (326 and 176 bp) and the GC heterozygote produced distinct three fragments (326, 205 and 176 bp). The Tetra ARMS-PCR-primers are described in Table 2.

Table 2
Tetra ARMS-PCR-primers

Primers	Primers sequence
Forward inner primer (G allele)	5'-TTTCCCCCTAGTTGTGTCTTCCG-3'
Reverse inner primer (C allele)	5'-TGCAATGTGACGTCCTTTAGCTTG-3'
Forward outer primer	5'-TGTCAAGACATGCCAAAGTGCTG-3'
Reverse outer primer	5'-GGGCAGAATGAGCCTCAGACAT-3'

IL-6 -634c/g Genotyping By Pcr-rflp

To detection of IL-6 -634C/G genotypes was done by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism PCR-RFLP. PCR amplification was done using 100 ng of DNA, extracted from samples with primers and PCR amplification conditions (Table 3). All PCRs were started by a 10-min denaturing step at 94°C and consequently completed by a 7-min extension step at 72°C. The following temperature cycles were used for different types of PCRs: 35 cycles of 55 seconds at 95°C, 58 seconds at 56°C and 72°C for 60 seconds. (PaqLab, Germany). For IL-6 -634C/G genotyping, in addition to PCR, C and G alleles were identified by BsrBI restriction enzyme digestions of the 180 bp product amplified by PCR. Amplified samples were identified with the 2.5% agarose gel electrophoresis. BsrBI cleaves the GG into 2 fragments (120bp and 60bp), CC into one fragment (180) and GC into 3 fragments (120bp, 80bp and 60bp).

Table 3
RFLP-PCR-primers for IL-6 -634C> G genotyping

Primer	Sequence (5'-3')	Size (bp)
FW	5'- GAG AGG CCT TGA AGT AAC TG-3'	180
RW	5'- AAC CAA AGA TGT TCT GAA CTG A-3'	

Statistical Analysis

In order to statistical analysis of data, the statistical package for the social sciences (SPSS) software was used. Relations between genotypes, virulence markers, and clinical disorders were assessed. Statistical analyses for detection of common IL-6 polymorphism and CMV roles in RPL were carried out by χ^2 (Chi-square test) and p-values below 0.05 were statistically significant. Anticipated genotype prevalence were calculated from the allele frequencies according to the Hardy–Weinberg equilibrium.

Results

Results Of Cervical Lesions

In total, there were two classifications including RPL group and control group. The RPL patient group included 80 endocervical samples and the control group (healthy group) contained 80 endocervical samples. The range of age among the study sample was from 20 to 35 years. The mean mother’s age was 25.9 years and that of controls was 26.4 years. The mean miscarriage age was 2.4 months. Mean gestational age was 3.7 months. Patients with acquired or hereditary thrombosis, hormonal disorders, anatomical abnormalities, chromosomal aberrations, and other TORCH infections were excluded in genotyping.

Detection Of Cmv

According a standard protocol, CMV was detected in all samples with PCR after DNA extraction. In patients, CMV positivity was found in 35 (44%) while it was present in only 20 (25%) of controls, which was detected to be significant (P-value = 0.002) (fig,1).

Il-6 -174 G/c Genotyping

In RPL, the frequency of GG, CG and CC genotypes from IL-6 -174 polymorphism were 0.64, 0.32 and 0.04, respectively, but in the control group, they were 0.58, 0.38 and 0.04, respectively (Table 4). Interestingly, no considerable relationship was observed between IL-6 -174 G/C genotypes and RPL ($P \leq 0.0001$) (Fig. 2).

Table 4
Frequency of IL-6 -634C> G genotyping in CMV (+) patients and CMV (+) controls

Genotypes	Patients with CMV (+) (n=35)	Controls with CMV (+) (n=20)	Odds Ratio
CC	2 (6%)	3 (15%)	1.00
GC	5 (14%)	7 (35%)	1.00
GG	28 (80%)	10 (50%)	4.00

The allele frequency of the IL-6 -634 G/C gene polymorphisms were also calculated. Results reveal substantial variations between cases and controls. In addition, the frequencies of G and C alleles were 81% and 19% in patients and 67% and 33% in controls, respectively. Both A and G allele of IL-6 -634 had a statistically noticeable association with the disease ($P \leq 0.005$) (Fig. 3).

Association Cmv With Il-6 -634c/g Polymorphism

The frequency of IL-6 -634 GG genotypes among RPL with CMV infection was 80% and without CMV infection was 50% ($P < 0.0005$). Individuals carrying the IL-6 -634 GG homozygous genotype with CMV infection have a 4-times greater risk of presenting with RPL than an individual with the CC homozygous genotype ($P \leq 0.0005$). Additionally, the presence of RPL was significantly associated with family history

and ages ($P \leq 0.004$). The Frequency of IL-6 -634 genotypes in RPL samples with CMV (+) and controls with CMV (-) are shown in (Table 4).

Discussion

RPL is a frequent disorder among women and shows a remarkable concern for reproductive problem cause approximately one in 300 pregnancies [1, 28]. Until now, while various criteria such as anatomic, genetic, endocrine, immunologic, and microbiologic have been acknowledged that affect miscarriage, the exact etiology of RPL cases in up to 50% is unknown [2, 3, 29]. The occurrence of RPL can be controlled by genetic-related factors such as genetic polymorphisms that have been connected with poor pregnancy. Some studies have led to the consciousness that cytokines were considered as key factors in the preservation of pregnancy by regulation the immune responses [17]. It is also found that embryo loss can be caused by improved uterine expression of some pro-inflammatory cytokines like (tumor necrosis factor (TNF)- α , interferon-gamma (IFN- γ), and interleukin (IL)-1 β) [18–20, 30]. In addition, anti-inflammatory cytokines such as IL-6 and IL-10 considered as crucial factors for maintaining a normal pregnancy. Among potential co-factors, functional SNPs in which alter the expression of IL-6 play a decisive role at various stages of RPL development. The main findings of this study were an important positive association between IL-6 SNP with RPL risk. The IL-6 -634C/G polymorphism is located at the 634 base pair upstream of the IL-6 gene promoter. It might be able to control IL-6 transcription or regulates posttranscriptional modification. Genetic predisposing factors may influence the likelihood of, sensitivity to or persistence of CMV infection, as well as the rate of RPL development [31, 32].

The literature review reveals that the large number of studies investigated the role of IL-6 in the risk of RPL, focused on this SNP, with little evidence of relation [21, 26, 33]. In Iran, several studies on the relationship among various cytokine polymorphism and RPL were reported. In one study, Torabi et al. reported that there are no substantial variations between RPL cases and controls, when they investigated the role of [-592A/C, -819C/T, -1082A/G (IL-10)], -174C/G (IL-6), and -197G/A (IL-17) in the risk of RPL [24]. But, Nematollahi et al. revealed the efficacy of the -964 A/G polymorphism in IL27 gene in the RPL etiology, especially in Iranian populations, suggesting that IL-17F (rs 763780) can have a significant role in RPL susceptibility [24]. Besides, several studies and investigations failed to discover the connection between the prevalent polymorphisms in the IL-6 SNP and RPL risk [23, 24, 32, 34].

However, our results present a substantial association between RPL risk and G allele in the IL-6 -634 among Iranian women. This is in line with Nasiri et al. study that indicated a remarkable association between allele G and the risk of RPL among Iranian population [35]. We obtained favorable results from our cases, as the allele G is substantially linked to incidence of RPL in Iranian women. However, it is in contrast to Saijo et al. [33] and Ma et al. [36] who reported a relation between the decreased risk of RPL and allele G, in Japanese and Chinese women, respectively. It is likely that the variation of results among these studies were attributed to ethnic differences or patients enrollment criteria impact. Besides, based on several studies we suggested that this polymorphism (IL-6 -634C/G) has different affect in RPL among different populations.

In this study, we also indicated that the IL-6 -174 G/C polymorphism has no association with RPL risk. This is in line with other studies. To evaluate the effect of the IL-6 174 on incidence of RPL, Daher et al. reviewed all five research reported in Europe [37]. In none of the studies, no variations in IL-6 genotype frequencies were found between controls and RPL patients, even in Japanese women not a allele was identifies neither in control nor in the patients [37]. Since the race had a significant impact on findings, they could not be applied to all people, because they were not comprehensive. In comparison to other populations, the Caucasian Northern Ireland people is the only one with a lower frequency of IL-6 174 allele [38]. One possible scientific reason that can explain these findings could be that -634C/G polymorphism is more effective than -174 G/C on IL-6 gene expression and translation. As a result, -634C/G polymorphism conceivably contributing to RPL risk among Iranian women.

On the other hand, the cytokine IL-6 has a protective role in immune responses against infections that caused by different bacteria and viruses. To cause an inflammatory reaction, pathogenic agents need access the intrauterine surroundings of the fetal tissues or endometrium, which can result in miscarriage [3, 39]. Prasad et al. investigated the role of Th1/Th2/Th17 cytokines in the immunopathogenesis process of miscarriage in Chlamydia trachomatis (Ct)-positive first-trimester aborters [19, 40]. They found that some cytokines such as IL-6, IFN- γ , IL-2, TNF- α , and IL-17A were noticeably increased in spontaneous abortion (SA) group and recurrent miscarriage(RM) group (Ct-infected) versus controls. Moreover, TNF- α , IL-17A, IL-6, and IFN- γ cytokines were considerably elevated in Ct-positive RM group compared to Chlamydia-infected SA group. Williams et al. demonstrated the TNF-alpha was produced in vivo during C. trachomatis infection and has a prime role in host defense [41].

In this study, our findings indicated a remarkable association between RPL with CMV positive patients and allele G in IL-6 -634 among Iranian women. Besides, the individuals with allele G in IL-6 -634 and CMV infection have greater risk of RPL in comparison with C allele. These findings are compatible with Edmunds et al. study, reported that the anti HCMV-IgM was identified in 94% of the Iranian women with the abortion background [29, 42]. Also, in the other study which conducted by Janan et al. on Iranian women with spontaneous abortion, results indicated a remarkable variation in the frequency of anti-Rubella, Cytomegalovirus, and Toxoplasma antibodies between RPL cases and healthy women [43]. They detected that the frequency of antibodies against Cytomegalovirus (CMV-IgM and CMV-IgG) in the patient group was noticeably higher than in the control group, and found that the high titer of CMV-IgM and CMV-IgG play the remarkable role in RPL [43]. In addition, Ebadi et al. discovered an important association between abortion and CMV infection in Iranian population [44]. According to these results we suggested that allele G in IL-6 -634 has both directly and indirectly effects on RPL in Iranian women. Directly, it can significantly increase risk of RPL, and indirectly it can increase CMV infection, which in turn might play effective roles in the incidence of RPL among Iranian women.

Conclusion

This study showed significant associations among IL-6 -634C/G polymorphism with RPL risk and CMV infection, may contribute to susceptibility of RPL, in Iranian patients. In particular, IL-6 -634GG genotypes

and CMV infection are associated with increased RPL risk. Detection of CMV and IL-6 -634GG genotypes can play important roles for early detection and early treatment of RPL in Iranian women. As IL-6 -634GG frequencies vary among ethnic groups, large and different population-based genetic studies are required to approve our results and suggestions.

Declarations

Authors' contributions

Parisa Pourroostaei Ardakani Methodology, Conceptualization, and Writing original draft. Bahareh Rahimi and Mohammad Panahi methodology, editing, and writing some sections of draft. Hamzeh Rahimi supervision, project administration, review and edit the manuscript. All authors read and approved the final draft.

Data availability statement

Input data for the analyses are available from the corresponding authors on request.

Acknowledgment

We would like to appreciate all the patients and their families for their contribution in this study.

Conflict of interest

The authors declare no conflict of interest

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

CMV PCR products. well 1 was positive control, well 2, 4,5,6 were positive CMV, wells 3 and 7 were negative CMV, wells 8 was negative control. add product size based on size marker

Figure 2

Electrophoresis of Tetra-amplification-refractory mutation system (Tetra-ARMS) polymerase chain reaction products for IL-6 -174 genotyping. Lane M, 100-bp ladder; lane 1 , 3, 4 and 8 positive for GG genotypes; lane 2,5 and 6 positive for CC genotypes; lane 7 positive for GC genotypes.

Figure 3

Electrophoresis of RFLP-PCR for IL-6 -634 genotyping. Lane M, 100-bp ladder; lane 1 positive for CC genotypes; lane 2 positive for GC genotypes; lane 3 positive for GG genotypes.