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# Association between Cytokine gene promoter polymorphisms of Interleukin IL-2 (-330 A/T) and TNF-α (-308 G/A) with recurrent pregnancy loss in Iraqi women

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**A B S T R A C T**

Pro-inflammatory and anti-inflammatory cytokines levels and polymorphisms of their genes have been described to be involved in the pathogenesis of recurrent spontaneous miscarriage(RSM). This study was designed to investigate the relationship of cytokines gene promoter polymorphism of Interleukin -2 and TNF- α with unexplained recurrent spontaneous miscarriage in Iraq women. The study enrolled 350 aborted women who had history of RSM as case group, in all cases full history and complete examinations including body weight, age and body mass index were done. In additionally, all of the patients were screened for various known causes of miscarriages. Based on the results screening 31.4 % of RSM cases are unknown cause, So out of 350 only 110 case were enrolled in this study to investigate the association between RSM and promoter Polymorphisms of cytokine genes, interleukin IL-2 (-330 A/T) and TNF-α (-308 G/A) and compared with 108 healthy controls. The frequency of the gene mutations was determined using (PCR-RFLEP). In addition, the ELISA was conducted to investigate cytokine (IL-2 and TNF- α) serum levels in women with RPA and healthy women. The result shown the estimated levels of serum IL-2 for recurrent pregnancy lose women, the difference was statistically not significant ($p > 0.05$) while the estimated levels of Mean serum TNF-α for patients group with RSM which was higher than the estimated level of the control group and the relationship is Statistically significant ($p < 0.01$) and to determine the association of The IL-2 Gene "-330 A/C" and TNF-α "-308 G/A" mutation with RSM, Results showed no significant difference in the prevalence of IL-2 Gene (-330 A/C) genotype and allele frequency among women with RSM and healthy controls, while the results showed significant difference in TNF-α (-308 G/A) genotype and allele frequency in Iraqi women with RSM.

## Introduction

Recurrent spontaneous misscariage is usually defined as three or more consecutive pregnancy losses before the 20th week of gestation, occurs miscarrige in 5% of clinically-recognized pregnancies, making it the most common complication of pregnancy(1,2) .RSM is a distressing problem, particularly to Iraqi families, the cause is elusive or multifactorial, and misinformation abounds, giving rise to frustration for the couple and the physician(3). Recurrent Spontaneous abortion

is one of the most common complications of pregnancy. Despite several well established etiologic factors of RSM, the cause of RSM cannot be determined in almost 50% of cases. It was suggested that these unexplained RSM might be due to immunologic factors(2).

Interleukin-2(IL-2), also named T-cell growth factor, plays an important role in T cell biology and can promote T-cell-dependent immune responses(4). IL-2 is an interleukin, a type of cytokine signaling molecule in the immune system. It is a protein that regulates the activities of white blood cells, often (lymphocytes) that are responsible for immunity. IL-2 is part of the body's natural response to microbial infection, and in discriminating between foreign "non-self" and "self". IL-2 mediates its effects by binding to IL-2 receptors, which are expressed by lymphocytes. Interleukin (IL)-2 may influence Th1/Th2 immune responsiveness and has been implicated association with RSM (5). Interleukin IL-2 also acts as a B-cell growth factor, stimulates antibody synthesis, and promotes proliferation and differentiation of NK cells to increase their cytolytic functions (6). IL-2 is a very important inflammatory cytokine. A lot of studies have been shown that the higher levels of circulating IL-2 were related to RSM (7) .

The chromosomal location of the gene controlling the secretion of The IL2 gene is located on the long (q) arm of chromosome 4 between positions 26 and 27 ( 4q26-q27) . IL-2 production is under genetic control in humans. It has been shown that different genotype at the site −330A/C (rs2069762) in the promoter region of the IL-2 gene is associated with the production of IL-2(8). Tumor necrosis factor (TNF) is a potent cytokine with a wide range of proinflammatory activities. It is mainly secreted by monocytes, natural killer (NK) cells ,macrophages, antigen-stimulated T cells although other cell types, such as T and B lymphocyte cells, also produce significant amounts(9,10). The TNF super family is involved in several physiological functions, such as immune response and morphogenesis. It has also been implicated in tumorigenesis, transplant rejection, septic shock and viral replication (11) .

TNF-a Due to their pro-inflammatory and pro-apoptotic capacity, TNF-a was described to mediate several aspects of pregnancy complications, including preclampsia, miscarriage, and RM (12,13). TNF-a was also described to facilitate miscarriage indirectly by activating NK cells or macrophages (14). Several polymorphic gene variants of TNF-α were described, including the TNF-a -238G/A and -308G/A promoter single nucleotide polymorphisms (15).

The chromosomal location of the gene controlling the secretion of tumor necrosis factor (TNF)-α (Th1) is located on the short (p) arm of chromosome 6 at position 21.3( 6p21.3) Some of the polymorphisms that have been reported in this gene are at position −238; −308; and −863 located in the promoter region, and especially −308 G/A is known to cause an altered promoter activity, resulting in an increased production of TNF-α cytokine in blood (16).

Circulating levels of TNF alpha are higher in patients with pregnancy loss compared to those with a successful pregnancy, suggesting that this cytokine may be an etiologic factor in RSM (Jenkins *et al.*, 2000). Furthermore, functional polymorphisms at position -308 and -863 in the promoter region of the human TNF alpha gene have been reported to be associated with altered TNF- α activity . Though a few studies have reported these polymorphisms to increase the risk of RSM, others have found no association(17) .

## Materials and Methods

This study was carried out in the department of obstetrics and gynecology at Al-Elwiya and Al-yarmouk teaching hospitals in Baghdad, between October 2013 and June 2014. The patient group comprised 350 women with a history of at least three recurrent spontaneous abortions. The control group included 108 women who have previously had at least two normal pregnancies with no history of abortion, ectopic pregnancy, or stillbirth. All subjects were fully investigated for Routine analysis at the hospital laboratory were performed to exclude known causes of abortion including the Anticardiolipin antibodies (IgM) , antiphospholipid (IgM), Coagulation factors including protein C, protein S, Antithrombin 3, activated protein C resistance, and investigation of toxoplasmosis antibodies (IgM) and cytomegalovirus antibody (IgM), rubella antibody (IgM), as well as homocysteine level , TSH and progesterone hormones, also APTT. One hundred and ten cases of the recurrent pregnancy losses were recruited from the outpatient clinic after exclusion of the possible etiological factors. All selected patients with unexplained RM were measure cytokines IL-2 and TNF-α levels in the patient and control serum with used ELISA kit from Peprotech Company.

To determine of the TNF-a (−308G/A) promoter Polymorphism ,total genomic DNA from peripheral blood leukocytes was extracted by use the blood DNA isolation kit of promega company. Sequence amplification was performed by using polymerase chain reaction (PCR). The primers were **F:5′-**AGGCAATAGGTTTTGAGGGCCAT-3′ and R:5′-TCCTCCCT GCTCCGATTCCG-3′ .PCR was performed in a total volume of 25 µl ((12.5 µl ) of Green Master mix 1X, (9.5 µl) of distilled water D.W., (0.5 µl) of each primer and (2 µl) of genomic DNA). The cycling conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles at 94°C for30 sec, 55°C for 45 Sec and 72°C for 45 Sec with a final extension step of 5 min at 72°C in the last cycle. The PCR products and the ladder marker (25bp) were electrophoresed on 2% agarose gel for 2 h and gel viewed under ultraviolet transilluminator after staining with ethidium bromide stain. The PCR products were incubated at 37°C for 15 min with ***NcoI*** restriction enzyme (Biolabs Inc. company) Digestion products were run on 3% agarose gel electrophoresis for 2.5 hrs. and stained with ethidium bromide After that it was visualized under UV light , to detect the TNF-a alleles. Three genotypes were found in this our study: genotype A/A homozygous have one fragment 107 bp uncut fragments. genotype G/A heterozygous have three fragments 107bp, 87 and 20bp. G/G homozygous was represented by two fragment 87bp and 20bp.

To determine the (−330 A/T) IL-2 gene promoter Polymorphism**,** total genomic DNA from peripheral blood leukocytes was extracted by use the blood DNA isolation kit of promega company. Sequence amplification was performed by using (PCR). The primers were designed by using Primer F:5'-TAT TCA CAT GTT CAG TGT AGT TCT-3' R:5'-CTC TTT GTT ACA TTA GCC CA-3'PCR was performed in a total volume of 25 µl ((12.5 µl ) of Green Master mix 1X, (8.5 µl) of distilled water D.W., (1 µl) of each primer and (2 µl) of genomic DNA). The cycling conditions were as follows: an initial denaturation at 95°C for 4 min, followed by 35 cycles at 95°C for 30 sec, 48°C for 30 sec, and 72°C for 40 sec, with a final extension step of 7 min at 72°C in the last cycle. The PCR products and the ladder marker (25bp) were electrophoresed on 2% agarose gel. The electrophoresis for 2 h and gel viewed under UV trans-illuminator after staining .The PCR

products were incubated at 37°C for 1hour with FspBI restriction endonuclease enzyme (Biolabs Inc. company) Digestion products were run on 3% agarose gel electrophoresis for time 2.5 hrs, and stained with ethidium bromide and after that it was visualized under UV light, to detect the IL-10 alleles. Three genotypes were found in our study: genotype A/A, have one fragment, 159 bp; genotype A/C, have three fragments, 159 bp, 23 bp and 136 bp; genotype C/C, have two fragments, 23 bp and 136 bp.

## Statistical analysis

Chi-square test was used to compare the genotype and allele frequencies. Means and standard deviations (SD) are presented for describing variables with continuous distribution. The odds ratio (OR) was used to estimate the ratio of the risk of RSM among patient group with various allele or genotype to the risk among control group. The 95% confidence interval (CI) for OR was calculated using confields' method.

## Results and Discussion

### The clinical characteristics of study participants

The clinical characteristics of patients and controls are listed in Table (1). There are no significant differences in mean age, Menarche (years), and mean Body mass index (BMI) ($kg/m^2$) between RPL cases and control ($p > 0.05$), while higher in irregular menstrual history (%) and number of pregnancies were seen in the RSM group. Although they did not constitute strong risk factors of RSM, they were selected as the covariates that were controlled for in subsequent analyses.The experimental group consisted of women with a history of three or more recurrent first trimester spontaneous abortions. These included 350 cases of RSM. The control group consisted of women with one or more live births and no history of first trimester spontaneous abortion and that included 108 normal women. This study applied for the detection of many of Immunological and coagulation assays for 350 women with RSM and the result were as shown in table (2).

Based on the results screening test 31.4 % of RSM cases are unknown cause, So out of 350 only 110 case were enrolled in this study to investigate the association between RSM and promoter Polymorphisms of cytokine genes,IL -2-330(A/C), and TNF-α-308(G/A) in Iraqi women and compared with 108 healthy controls. The frequency of the gene mutations in the patients and controls was determined using PCR-restriction fragment length polymorphism (PCR-RFLEP). In addition, the ELISA was conducted to investigate cytokines (IL-2 and TNF-a) serum levels in women with RSM and healthy women.

The estimated levels of serum IL-2 for recurrent pregnancy lose women were 55.7 ± 12.8 pg/ml (mean ± S.D) while that of healthy pregnant women was 48.3 ±11.3 pg/ml within first trimester. The difference was statistically not significant ($p > 0.05$).while the estimated levels of mean serum TNF-α for patients group with RPA was 285.23 ± 48.21 pg/ml (mean ±SD) which was higher than the estimated level of the control group 190.79 ± 53.74 pg/ml and the relationship is Statistically significant ($p < 0.01$).

### Genotyping results of the Interleukin- 2 Gene "-330 A/C" Promoter Polymorphism

Results from Table (3) illustrate the genotype frequencies of the IL-2"-330 A/C" polymorphism among RSM patients compared with healthy group. The frequency of the wild type AA was 64.54.0%, the frequency of the heterozygote AC was

27.027% ,and the frequency of the homozygotes for the polymorphic allele CC was 8.18% in RSM patients, whereas in healthy women the genotype frequency of the wild type AA was 70.37%, the frequency of the heterozygote AC was 25.30% and the frequency of the homozygotes for the polymorphic allele CC was 83.33%. On the other hand results illustrates also alleles frequency of the IL-*2* Gene "-330 A/C" polymorphism among RSM patients and controls.

The allele frequency for A allele in RSM cases and controls was (78.18)% and (83.33)% and that of C was (21.81) %and (16.66)% respectively.

There was no preferential prevalence of a particular allele, and thus no statistically significant difference ( P-value = 0.66) in genotype distribution of allele frequency in recurrent miscarriage patients and controls. Statistical analysis revealed that (IL-2) Gene "-330 A/C" promoter polymorphism in the present sample (35.18%) and the frequency of the homozygotes for the polymorphic allele AA was (20.37%) .The observed allele frequency for G allele in RSM cases and controls was (20%) and (62.03%) and that of A was (80 %) and (37.96%), respectively.

There was preferential prevalence of a particular allele, and thus statistically significant difference in genotype distribution of allele frequency in recurrent miscarriage cases and controls. Statistical analysis revealed that TNF-α-308G/A promoter polymorphism in the present sample is associated with recurrent miscarriages.

Occurred with the allele A of the risk of recurrent miscarriage was significantly increased ($\chi 2$ = 15.6, p = 0.003, OR = 0.73, 95% CI: 0.77-2.89).

IL-2 is a very important inflammatory cytokine which produced by Th1 cells. It can induce T cells proliferation and differentiation; promote the regulatory T cells in playing immunosuppressant function [(18)] moreover, the role of IL-2 also involved enhancing the role of cytotoxic T cells induced by IL-2 receptor expression, promoting cell migration, enhancing the contact between the cells and inducing cytokine secretion. Studies have shown that IL-2 levels of abnormal not only associated with certain T cell 19mediated autoimmune diseases , but also with some of the endocrine system diseases and tumor [(19,20)]

During pregnancy, IL-2 activate T cells to promote secretion of HLA-Class II molecules and a variety of cytokines, and also activate natural killer (NK) cells, which can enhance the secretion of inflammatory factors, make the embryo being rejected [(7).] Women with normal pregnancy have a greater Th2-type basis, whereas women with a history of RSM have a basis towards Th1-type reactivity [(21)] .

Another study indicated that the IL-2 -330A/C , allele A may be considered as risk factor for RSM C→A mutation is may associated with RSM in Ningxia Han people [(5)].

Abbas Rezaei's study observe that the levels of IL-2 were significantly higher in the patient group than in the control group and that contradiction with our study [(21)] ,while in another study has indicated that the levels of IL-2 mRNA were significantly higher in the patient RSM group than in the control group and that also contradiction with our study [(5)] .

**Table.1** The demographic data of the unexplained RPL patient and controls

| Characteristic | Cases | Controls | P value |
|---|---|---|---|
| Mean age (years) | 30.23±4.76 | 29.89±5.22 | p>0.05 |
| body mass index BMI(Kg/m$^2$) | 22.37±4.24 | 23.78±3.88 | p>0.05 |
| Menarche (years) | 13.46±1.62 | 13.35±1.57 | p>0.05 |
| Irregular menstrual history % | 65 | 15 | p<0.01 |
| Number of pregnancies | 4.37±1.22 | 2.6±1.38 | p<0.01 |
| Abortion | 3.7±0.78 | 0 | p<0.001 |

**Table.2** Results of screening tests for abortion cases and control

| Parameter | Prevalence of Positive cases % | Seroprevalence PRL cases | Seroprevalence Control | Normal value |
|---|---|---|---|---|
| TOXO(IgM) | 4.28 | 27.51±2.5 | 7.33±1.3 | <12 mIU/L |
| CMV(IgM) | 6.85 | 21.4±2.12 | 6.21±1.3 | <12 mIU/L |
| Rubella (IgM) | 4.00 | 18.1±2.4 | 4.43±1.1 | <11 mIU/L |
| Cardiolipin (IgM) | 7.42 | 16.2±2.8 | 5.67±1.8 | <12 mIU/L |
| Phospholipid (IgM) | 7.14 | 23.1±2.9 | 4.15±1.8 | <12 mIU/L |
| Protein C deficiency | 6.00 | 67.85 ± 17.21 | 83.41 ± 9.62 | 70-130 % |
| Protein S deficiency | 4.85 | 68.63 ± 17.4 | 93.35 ± 1.6 | 65-140 % |
| Homocysteine | 8.85 | 18.9±6.8 | 7.31±2.21 | 7.1±2.2mg/dl |
| TSH | 3.14 | 7.21±1.8 | 3.8±1.4 | 0.5-5. mIU/L |
| Progesterone | 4.85 | 5.8±1.92 | 31.81±2.9 | 30±2. ng/ml |
| APCR | 5.14 | >2 | <2 | <2 |
| APTT | 2.00 | 19 ±21 | 14±2 | 13±2 sec |
| Antithrombin III | 4.00 | 67 ± 13 | 87±33 | 75-145 ng/ml |

**Table.3** Genotype and Allele frequencies of the −330A/C polymorphism in the IL-2 gene promoter in women with recurrent spontaneous abortions and in the control group

| IL2 -330 (A/C) | Genotypes frequency (%) | | | Allele's frequency (%) | |
|---|---|---|---|---|---|
| **Parameter** | **Homozygote Wild type A/A** | **Heterozygote A/C** | **Homozygote Mutant type C/C** | **Allele (A)** | **Allele (C)** |
| **Patients(N:110)** | **71 (64.54)%** | **30 (27.27)%** | **9 (8.18)%** | **172(78.18)%** | **48(21.81) %** |
| **Control N:108)** | **76 (70.37)%** | **28 (25.29)%** | **4 (3.70)%** | **180 (83.33)%** | **36 (16.66)%** |
| **$\chi 2$** | **0.26** | **0.15** | **0.23** | **0.20** | **1.31** |
| **P value** | **0.63** | **0.76** | **0.71** | **0.67** | **0.58** |
| **OR(95%CI)** | **1.23(0.472.34)** | **0.92(0.62-1.87** | **0.87(0.45-2.84)** | **0.88(0.61-1.45** | **0.78 (0.56-1.2)** |

**Table.4** Genotype and Allele frequencies of −308 G/A polymorphism in the TNF –α gene promoter in women with recurrent spontaneous abortions and in the control group

| TNF- α -308 (G/A) | Genotypes frequency (%) | | | Allele's frequency (%) | |
|---|---|---|---|---|---|
| **Parameter** | **Homozygote Wild type G/G** | **Heterozygote G/A** | **Homozygote Mutant type A/A** | **Allele (G)** | **Allele (A)** |
| **Patients(N:110)** | **8 (7.27%)** | **28(25.45%)** | **74 (67.27%)** | **44 (20%)** | **176 (80%)** |
| **Control(N:108)** | **48 (44.44%)** | **38 (35.18%)** | **22 (20.37%)** | **134(62.03%)** | **82 (37.96%)** |
| **$\chi 2$** | **17.8** | **11.8** | **13.6** | **14.4** | **15.6** |
| **P value** | **0.002** | **0.001** | **0.002** | **0.001** | **0.003** |
| **OR(95%CI)** | **0.76(0.56-2.6)** | **0.81(0.44-2.2)** | **0.95(0.33-2.7)** | **0.88(0.81-1.78** | **0.73 (0.77-2.89** |

Our result agree well with that reported by Trajkov (22) who indicated no significant association between the "-330 A/C" polymorphism in the promoter region of the human IL-2 gene and occurrence of unexplained RSA. In addition some studies have shown comparative the normal population; women with recurrent miscarriage have lower IL-2 levels *in vivo.* IL-2 not only in the clones of T cells play a key role also involved in the activation of natural killer cells and increased B cell growth and immunoglobulin production (5) .

The selected molecular marker,IL-2 −330A/C are the candidate gene for the recurrent spontaneous abortions and the manifestation of the disease vary in different ethnic groups as they are largely influenced by the surrounding genetic environment, lifestyle factors ,mating patterns, and other environmental factors, which are sample specific. The polymorphisms in different genes have been investigated. In most studies the linked between a polymorphism and RPL is negative, has not been replicated in follow-up studies or demonstrate opposite results

between studies. Inconsistencies may arise due to (1) differences in study design, definitions of RSM and control group; (2) focus on RSM women instead of couples or placenta, (3) low statistical power due to small sample size, (4) ethnic difference in risk variants, population-specific low-impact gene variants increasing RM risk in consort, (5) contribution of life-style and environmental factors on the pregnancy course, (6) secondary pathways affecting protein translation/metabolism leading to discrepancies between genotype and respective protein levels, e.g., Factor XII, Protein Z (23-25) .

These strategies in the present study used to examine associations between maternal carriage of cytokine polymorphisms and RSM patients by investigating -308 (G/A) polymorphism in the promoter region of TNF-α gene in Iraqi females. The TNF-α is a potent cytokine with a wide range of pro-inflammatory activities. Prevalent levels of TNF-α are higher in patients with a posterior miscarriage compared to those with a successful pregnancy, suggesting that this cytokine may be an etiologic factor in recurrent spontaneous abortions (26-28). The bi allelic G/A polymorphism at position −308 of the TNF-a promoter has an influence on the TNF-a production: the allele A at position −308 is associated with an increased protein serum concentration, therefore enhancing its proinflammatory activity and susceptibility to fetal loss. It is expected, therefore that association studies will reveal an association between the A genotype and RSM.

Results of this work indicated a significance association of allele or a particular genotype of TNF-a promoter -308 (G/A) with RSM as shown in table 4. The distribution indicated higher frequency of AA genotype among the RSM cases compared with the normal controls in TNF-α-308G/A polymorphism and the difference was statistically significant. For that our results are contradiction with those reported by (29-31) ,while results of the present study in agreement with studies done by Daher (32). These studies provided evidence for the linked of AA genotype with RSM, and our result agree with(22,33) which indicated a significant association between the -308 G/A polymorphism in the promoter region of the human TNF-α gene and occurrence of unexplained RSM. Also Previous studies, which investigated the association of RSM and TNF-α polymorphisms reported contradictory results. Babbage (12) observed that the -308A TNF-α polymorphism was not associated with RSA in a Caucasian population of 43 women with RSM, while Reid confirmed that an increased risk of RSM for carriers of the TNF-α -308A allele., about this, our results are in line with the results of Reid (17).

It was suggested that Th1, but not Th2, cytokine gene polymorphisms were linked with RSM, an indication that heightened Th1 cytokines (TNF-α) adversely affected pregnancy outcome. This was exemplified by the findings that the TNF-α -308(G/A) polymorphisms were associated with RSM(33) .Also several studies have identified an association between TNF-a gene polymorphisms and this pregnancy pathology, and higher concentrations of (TNF-α) are detected in women with RSM than in women with successful pregnancy (34-36).TNF-α is known to cause fetal expulsion due to uterine contraction or may even cause necrosis of implanted embryo [(37)] and TNF-α is also reported to act along with the hormones and causes thromboses in the placenta during pregnancy resulting in miscarriage and its production is enhanced at the onset of labor and spontaneous abortion(32).The study suggested polymorphism Cytokine gene promoter polymorphisms of Interleukin IL-2 (-330 A/T) and TNF-α (-308 G/A) were a risk factor with recurrent pregnancy loss in Iraqi women

## Conflict of interest

The authors declare that they have no conflicts of interest. The authors are responsible for the content and writing of the paper.

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