

**ASSOCIATION BETWEEN CYTOKINE GENE POLYMORPHISM AND
RECURRENT PREGNANCY LOSS AMONG BASRA PROVINCE WOMEN**

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ABSTRACT: *Forty cases of women sever from recurrent pregnancy loss (RPL) compared with 40 normal women for determining the cytokine gene polymorphism in the promoter region of : tumor necrosis factor-alpha TNF- α (-308 G/A), interleukin IL-6 (-634 G/C) and IL-10 (-592 C/A) in aborted women of Basra /Iraq. The results indicated that there was an association between RPL and the polymorphisms in inflammatory cytokines (IL-6, TNF- α), while IL-10 gene did not showed any effect on both cases of RPL and normal cases.*

KEYWORDS: Cytokine Genes, Recurrent Pregnancy, Loss, TNF- α , IL-10, IL-6

INTRODUCTION

Successful pregnancy depends on the induction of local maternal tolerance toward foreign fetus tissue actually there are multiple mechanisms like : angiogenesis, cytokine and hormonal balance, genetic and epigenetic as well as environmental factors influence pregnancy outcomes (Hunt et al., 2005, Piccinni., 2005, Salamonsen *et al.*, 2007, Van and Oudejans 2013, Sharma 2014).

Miscarriage is one of the pregnant complications may be a consequence of implantation failure or secondary to inappropriate humoral or cellular immunological response to the implanting embryo (Li *et al.*, 2002).

Some studies referred to an increase in the production of pro-inflammatory cytokines (Th-1 type) and reduced production of anti-inflammatory cytokines (Th-2 type) in women with recurrent pregnancy losses which is suggesting (Th-1 bias in pregnancy failure and Th-2 bias in successful pregnancy) (Wegmann *et al.*, 1993 and Raghupathy, 1997, Piccinni., 2005, Walia *et al.*, 2008).

Some of the earliest *in vitro* studies showed that the trophoblast antigens activate lymphocytes of recurrent pregnancy loss (RPL) susceptible women to produce embryotoxic cytokines i.e. TNF- α , IFN- γ and IL-2 (Yamada *et al.* 1994 and Hill. 1995).While the study of Clark and Chaouat in 1989 documented that the anti-inflammatory cytokines (IL-4, IL-6 and IL-10) are favorable to maintain the successful pregnancy and deficiency of these cytokines will be lead to poor placentation, subnormal growth and even sometimes fetal death.

In fact the productions of both Th1 and Th2 cytokines are genetically controlled and there are many genetic polymorphisms are associated with (high, intermediate or low) production of

these cytokines like TNF- α , and IL-10 and IL-6 (Wilson *et al.* 1997, Alkhuriji *et al.* 2013, Paryeen *et al.* 2013 and Liu *et al.* 2015).

In TNF- α , the polymorphism (-308 G/A) in promoter region was known to cause an alteration in activity, resulting in an increase production of this cytokine in blood among humans in sever disease (Wilson *et al.* 1997). Also Alkhuriji *et al.* (2013), found the same position (-308) could be a genetic predisposing factor for unexplained RPL in Saudi women.

On the other hand, there are many single -nucleotide polymorphisms (SNPs) are reported in the proximal (-1082A/G; -819T/C; -592A/C) and distal region of the IL-10 gene involved in transcription rate and affecting its production level (Mormann *et al.* 2004).

Kamali-Sarvestani *et al.* (2005) found in the study on RPL in Iranian women there was an association between IL-10 (592A/C) and RPL as well as the same association was reported by Alkhuriji *et al.* (2013) in Saudi women and that may be due to the role of IL-10 as it acts as an immunosuppressive cytokine by keeping a balance of pro- and anti-inflammatory signals that coordinate the satisfactory development of pregnancy, placental growth, and remodeling for favorable pregnancy outcome (Parveen *et al.* 2013)

Also some researchers found that IL-6 (-634C/G) polymorphism may be associated with RPL like Ma *et al.* (2012) in Chinese women; Alkhuriji *et al.* (2013) in Saudi and Liu *et al.* (2015) also in Chinese women.

Aim of present study: - To investigate the relationships between recurrent pregnancy loss (RPL) and single nucleotide polymorphisms in the promoter region of 2 inflammatory and 1 anti-inflammatory cytokines respectively: - tumor necrosis factor-alpha TNF- α (-308 G/A), interleukin IL-6 (-634 G/C) and IL-10 (-592 C/A) in women of Basra /Iraq in order to determine their critical role in regulation, balance, and maintenance of successful pregnancy.

MATERIALS AND METHODS

Samples collection

Three ml of venous blood were collected by vein puncture in EDTA tube from 80 women during a period from November 2014 - September 2015. The women participated in this study were Iraqi women lived in Basra province in the south of Iraq and their age ranged from (18-40) years. They attended to the obstetrics and gynecology private clinic with vaginal bleeding and no fetal heart tone were found. They were followed up by the same gynecologist doctor who referred them to the emergency unit in Basra Maternity and Children Hospital for evacuation.

40 of enrolled women were suffering from recurrent pregnancy loss in the first trimester with at least (two previous consecutive miscarriages or more) of gestational age (between 9- 12 weeks). All the women with RPL were previously subjected to the different tests to exclude other causes of RPL. All women with RPL had normal levels of serum progesterone in the luteal phase (>10 ng/ml), normal thyroid function (T3 between 0.9 and 2.5 nmol/L; T4 between 60 and 120 nmol/L) and normal glucose titration test. As well as; they had negative results for all of the antiphospholipid antibodies, antinuclear antibodies, anticardiolipin antibodies and TORCH (toxoplasmosis, rubella, cytomegalovirus, herpes simplex virus).

The control groups consist of 40 healthy pregnant women who were at the same gestational age and had previously at least one normal pregnancy with no history of miscarriage, ectopic pregnancy, pre-term delivery or stillbirth and they attend the same private clinic.

The data were collected from the studied women depending on a special questionnaire designed for the purpose of the present study and they were all provided with written informed consent.

Genomic DNA was extracted from all blood samples using DNA extraction kits from Promega company and determined by using 0.8 % agarose gel electrophoresis and stored frozen until used (figure 1).



Figure (1): 0.8% agarose gel electrophoresis of genomic DNA for normal women and RPL cases. (Lane: 1-3 DNA of normal women; lane: 4-6: DNA of RPL cases)

Polymerase chain reaction PCR:

Conventional PCR were used for amplification of DNA samples by using specific sequences of primers described in table (1) according to Alkhuriji *et al* (2013). PCR mixture were performed in 20 μ l tube which contain 5 μ l of master mix, 5 μ l of DNA template, 1 μ l of forward primer (10 μ M/ μ l), 1 μ l of reverse primer (10 μ M/ μ l) and 8 μ l of deionized water.

Three programs of amplification were selected for the promoter region of TNF- α , IL-6, and IL-10 genes; the cycling conditions were done as follow: an initial denaturation at 95C $^{\circ}$ for 15 minutes, followed by 36 cycles at 95C $^{\circ}$ for 30 seconds and 60C $^{\circ}$ for 30 seconds. The final extension step was at 72 C $^{\circ}$ for 10 minutes (Alkhuriji *et al* 2013).

Table (1) The primer sequences for the three gene used during this study

TNF-α (-238G/A,-308 G/A)		
Forward	5' CAGGCCTCAGGACTCAACAC3'	430
Reverse	5' AAGGAAGTTTCCG3'	
IL-6 (634G/C)		
Forward	5' AGGCAAACCTCTGGCACA3'	400
Reverse	5' TTC AGCCTGTTAATGGTCAC3'	
IL-10 (-592C/A)		
Forward	5' CTGTGCCTCAGTTTGCTCAC3',	420
Reverse	5' GTCTTGGGTATTCATCCCAGG3'	

Statistical Analysis

All the statistical analysis were performed using the statistical package for the social sciences (SPSS) software version 13.0 for Microsoft windows to be compared between normal pregnant women and RPL.

RESULTS

Table (2) summarized the demographic characteristics of the studied women participating in this study. The mean age were (29.75 ± 5.67 and 25.53 ± 5.61) in each RPL cases and normal pregnant women respectively, and there were no significant differences (0.81) between the age of RPL when compare with normal pregnant women but there were significant differences in both number of live babies (0.000) and the number of previous pregnancy (0.031).

Table (2): The demographic characteristics of this study participants

characters	RPL (40)		Normal pregnancy (40)		t-test
	Mean± Deviation	Std.	Mean± Deviation	Std.	
Age	29.75 ± 5.67		25.53 ± 5.61		0.8
No. of live Baby	0.35 ± 0.48		2.26 ± 1.2		0.000
No. of previous pregnancy	4.18 ± 1.5		3.17 ± 1.13		0.031

Figure (2) showed the distribution of TNF- α genotype in RPL cases and normal women which revealed the presence of the TNF- α fragment (430 pb) in all cases of the RPL women while it was disappeared in all DNA samples of normal pregnant women .

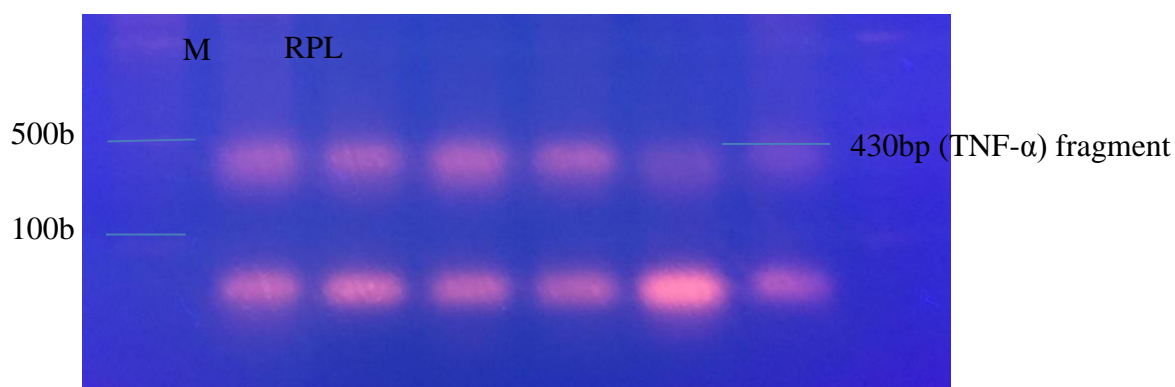


Figure (2): 2% agarose gel electrophoresis of TNF- α fragment in RPL cases. M: DNA marker

IL-6 gene (400 Pb.) was present in all RPL DNA samples while it was disappear from all DNA samples of normal pregnant women. The IL-6 gene (400Pb) can be seen in figure (3)

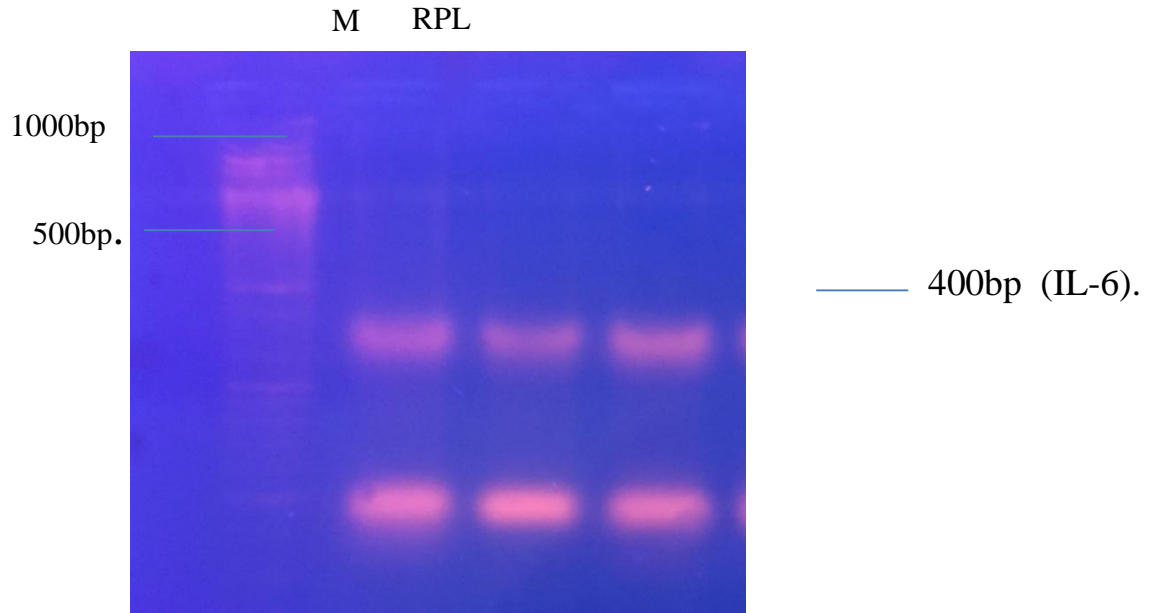


Figure (3): 2% agarose gel electrophoresis of IL-6 fragment in RPL cases.

While IL-10 gene fragment (420 pb.) was recorded in both of RPL and normal DNA samples and it can be seen in figure (4)

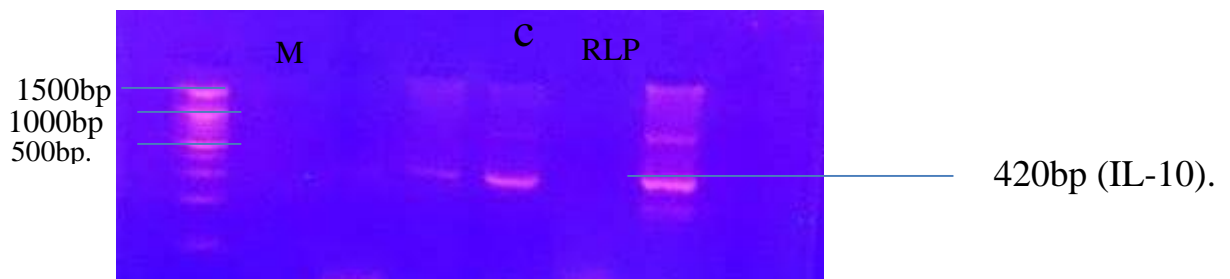


Figure (4): Agarose gel electrophoresis of IL-10 gene in both control and RPL cases.

DISCUSSION

This study was designed to determine the association of the polymorphisms in an inflammatory cytokines (IL-6, TNF- α) and anti-inflammatory (IL-10) and recurrent pregnancy loss in women of Basra /Iraq, which is represented the first study dealing with these cytokine genes polymorphism and RPL cases of Basra's women in compared with other

normal, so the recurrent pregnancy loss requires careful consideration of genetic, anatomic, endocrine, infectious and immunological factors.

The results showed the presence of the TNF- α gene (-238 G/A, -308 G/A promoter region) in 430bp fragment produced by PCR, in RPL women while it was disappear in all women with normal pregnancy and this agreed with Alkhuriji *et al* (2013) and Liu *et al* (2015) who found that TNF- α -308 promoter polymorphism could be a genetic predisposing factor for unexplained RPL .As well as it was agreed with sharief *et al* (2014) in Basra who they found an increased TNF- α cytokine in serum of women who are suffering from RPL. Recently, Li *et al.* in (2016) reported the association of TNF- α genetic polymorphisms with recurrent pregnancy loss risk systematically and meta-analysis.

Certain cytokine gene polymorphisms influence the level of cytokine production and associated with susceptibility to diseases and/or different clinical features/outcomes of diseases (Bidwell *et al.*, 2001), moreover the TNF- α is a potent cytokine with a wide range of pro-inflammatory activities and the circulation levels of TNF- α are higher in patients with a subsequent miscarriage compared to those with a successful pregnancy, suggesting that this cytokine may be act as an etiologic factor in recurrent miscarriage (Jenkins *et al.*, 2000 and Raghupathy 1997 and Liu *et al.*, 2015); while on the other hand Kaur and Kaur (2011) founded that there was no association of between the polymorphism in the promoter regions of tumor necrosis factor (TNF- α) cytokine and miscarriage .

In comparison between RPL women and normal pregnant women, the results revealed that the frequencies of IL-6(634G/C) promoter region, (400bp) were more abundant in RPL women than normal pregnant women and that proved a possible association of polymorphism IL-6(634G/C) and the increased frequency of recurrent abortion status in the studies of Ma *et al.*, (2012) and Lee *et al.*,(2015) were they confirmed by meta-analysis that IL-6 -634 G/C polymorphisms is associated with susceptibility to RPL and the IL-6-634C/G polymorphism might be a possible genetic protective factor for RPL.

The frequency of anti-inflammatory cytokine IL-10 gene polymorphism (-592 C/A) in the promoter region (420bp) was detected in both RPL women and normal pregnant women which is suggested that IL-10 gene polymorphism has no association with RPL and that agreed with Alkhuriji *et al* (2013) who found that there was no significant association between this gene position and RPL, while Parveen *et al.*, (2013) demonstrated that IL-10 plays an important role in the maintenance of normal pregnancy as it act as an immunosuppressive by keeping a balance of pro- and anti-inflammatory signals that coordinate the satisfactory development of pregnancy, placental growth, and remodeling for favorable pregnancy outcome.

Suggested the IL-10 gene polymorphism screening might have some relevance in patients with RPL (Motahareh *et al.* 2014),

CONCLUSION

In conclusion, recorded results suggest that the TNF- α (-238 G/A, -308 G/A promoter region and IL-6 -634 G/C polymorphism) may be considered as a risk factors for RPL in Basra women.

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