The relationship between cytokine gene polymorphism and unexplained recurrent spontaneous abortion in Saudi females

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ABSTRACT

الأهداف: التحقق في العلاقة بين الأشكال المتعددة للتركيبات النووية للاجهاضات المتكررة غير المبررة لدى السيدات والأشكال المتعددة للنيوكليوتايد (TNF–α 238– G / A، 308– G / A، IL–6 634– C G / وIL–10 592– C / A) في منطقة المروج من 3 انترلوكين مختلفة (TNF–α، IL–6، والجينات IL–10).

الطريقة: تتكون مجموعة الدراسة من 65 امرأة (34.1 6.2) متوسط العمر، تتراوح أعمارهم بين 45–15 عام) تم تحويلهم إلى عيادة الإجهاض المتكرر بمستشفى الملك خالد الجامعي بالرياض، المملكة العربية السعودية خلال الفترة من 6 يناير2010م إلى6 يناير2011م. تتألف مجموعة التحكم من 65 سيدة لديهن على الأقل اثنين من الحمل الناجح وليس لهم تاريخ في الإجهاض. تم سحب عينات الدم وتم استخراج الحمض النووي باستخدام عدة Puregene تنقية DNA. باستخدام تفاعل البلمرة سلسلة تضخيم والمنطقة المروج والتسلسل على المنظم ABI لدراسة مواقع متعددة الأشكال من الاهتمام. تم تحديد جميع الأشكال في حالات وعينات السيطرة.

النتائج: كانت هنالك علاقة وثيقة بين G فقط 308– A / تعدد الأشكال في الجين المروج TNF–α وحدوث الاجهاضات المتكررة غير المبررة، وعدم وجود ارتباط مهم مع وظائف أخرى.

خاتمة: يمكن أن تعدد الأشكال الجينية لجينTNF –α في موقف 308– كما أنه ليس هنالك أي تبرير للعامل الوراثي المهيئ للاجهاضات المتكررة.

Objectives: To investigate the relationships between unexplained recurrent spontaneous abortion (RSA) and single nucleotide polymorphisms tumor necrosis factor-alpha (TNF-α) (-238 G/A, -308 G/A), interleukin (IL)-6 (-634 G/C) and IL-10 (-592 C/A) in the promoter region of 3 different interleukin (TNF-α, IL-6, and IL-10) genes.

Methods: The study group comprised 65 women (mean age: 34.1±6.2; range: 15-45 years) with unexplained RSA, consecutively referred to the Recurrent Abortion Clinic, King Khaled University Hospital, Riyadh, Kingdom of Saudi Arabia from January 2010 to January 2011. The control group consisted of 65 females with at least 2 successful pregnancies and no history of abortion. Blood samples were drawn and deoxyribonucleic acid (DNA) was extracted using Puregene DNA purification kit. Utilizing polymerase chain reaction, the promoter region was amplified and sequenced on an Applied Biosystems Integrated sequencer to study the polymorphic sites of interest. All polymorphisms were identified in the case and control samples.

Results: A significant association was identified only between the -308 G/A polymorphism in the TNF-α gene promoter and the occurrence of unexplained RSA, and there was no significant association with other positions.

Conclusion: The TNF-α gene polymorphism at position -308 could be a genetic predisposing factor for unexplained RSA.

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Recurrent spontaneous abortion (RSA) is defined as "3 or more consecutive pregnancy losses before the 20th week of gestation".[1] Several factors have been implicated as causative factors in the etiology of abortion, including genetic and environmental factors. However, in almost 50% of the cases the etiology remains unexplained. The known causes cover uterine abnormalities, genetic factors, thromobophilic, endocrine, metabolic, and immunologic factors, infections and other environmental and unexplained factors.[1] In the face of unknown etiological factors, dysregulated immunity has been proposed as a potential mechanism underlying RSA.[2,3] Some studies have led to the awareness that immunological factors play an important role in establishing a successful pregnancy. Considerable evidence has accumulated indicating that cytokines play an important role in the maintenance of pregnancy by modulating the immune system.[2] Several studies have shown that cytokines play a major role in reproductive phenomena, where T-helper type 2 Th2- dominant response has been associated with normal pregnancy, and Th1 response has been related to pregnancy failure.[4] Enhanced uterine expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interferon-gamma (IFN-γ), interleukin (IL)-1β, and IL-6 has been associated with embryo loss.[5] Anti-inflammatory cytokines such as IL-10 appear to protect against inflammation-induced miscarriage.[6] Research efforts have focused on single nucleotide polymorphisms (SNP) in cytokine genes,[3] and various SNPs have been reported to be associated with infectious and inflammatory conditions, including the risk of pre-labour rupture of the amniotic membranes and preterm labor.[7] Polymorphisms in the promoter regions, exons or introns of certain cytokine genes, influence the level of cytokine production and result in high, intermediate or low levels of cytokines.[8] The SNPs that generally occur in the promoter region, putatively act as transcriptional regulators, hence, many studies have been directed towards the relationships between SNPs in the promoter region of TNF-α at -1031T/C, -863C/A, -857C/T, -376G/A, -308G/A, -238G/A, and +488G/A, IL-6 at - 634C/G and IL-10 at -1082G/A, -819C/T, -592C/A, and RSA.[9-11] Studies in some populations show association while in others no association has been observed. Interestingly, variation in the prevalence of different polymorphic forms is a frequent finding and this makes it necessary to conduct association studies in each individual population.[9,12,13] The aim of this study was to investigate the association between unexplained RSA and genetic polymorphisms of TNF-α, IL-10, and IL-6 genes. In this study, we report our results of 4 SNPs in 3 cytokine genes (-308G/A, -238G/A in TNF-α, - 634G/C in IL-6, and -592 C/A in IL-10).

Methods. The patients participating in this case control study were referred to the Recurrent Abortion Clinic, King Khaled University Hospital, Riyadh, Kingdom of Saudi Arabia from January 2010 to January 2011. This study was approved by the Institutional Review Board (IRB), College of Medicine, King Saud University. The study group comprised of 65 women (mean age: 34.1 ± 6.2 years, range: 15-45 years) with unexplained RSA, consecutively referred. The reference population (controls) consisted of 65 women who had at least 2 children, and were without known pregnancy losses or any known medical illnesses. Routine analysis at the hospital laboratory were performed to exclude known causes of abortion: parental karyotypes; hormone levels; toxoplasmosis; cytomegalovirus; rubella; anti-phospholipid antibodies; protein C; protein S; glucose level; hysteroscopy; hysterosalpingography; and serial ultrasound when needed. The criteria for inclusion were: females presenting with unexplained RSA after all the tests mentioned above were normal. The protocol of this investigation was approved by the Medical Ethics Committee of King Khalid University Hospital and the Ethical Committee of King Saud University, Riyadh, Saudi Arabia. All women were required to sign the informed consent form to participate. Ten ml blood samples were extracted by venipuncture in ethylenediaminetetraacetic acid (EDTA) tubes from each case and control and genomic deoxyribonucleic acid (DNA) was extracted using the DNA Puregene purification kit. The purified DNA was amplified by polymerase chain reaction (PCR) using sequence-specific primers (**Table 1**) designed using the Primer 3 program for amplification of selected regions in the promoter region of TNF-α, IL-6, and IL-10 genes. Each PCR reaction required 2 µL (50 ng/µl) of DNA, 1 µL of forward primer (10 µM/µl), 1 µL of reverse primer (10 µM/µl), 2 µl (10x PCR buffer), 1 µL dNTPs (2.5 mM), 17.5 µl distilled water, and 0.25 µ1 hot star Taq-polymerase (5 U/ul) to perform the reaction. The cycling conditions were as follows: an initial denaturation at 95^{0}C for 15 minutes, followed by 36 cycles at 95^{0}C for 30 seconds and 60^{0}C for 30 seconds. The final extension step was at 72^{0}C for 10 minutes. After PCR, each fragment generated was subjected to sequencing using Applied Biosystems Integrated (ABI) 3130xl Genetic Analyzer following the kit manufacture instructions.

All statistical analyses were carried out using the Statistical Package for Social Sciences version 12 for Microsoft Window (SPSS Inc, Chicago, IL, USA) and

the genotype and allele frequencies of the patient were compared with control using X^2 and Fisher exact test analysis and the difference was considered statistically significant if $p<0.05$.

Results. The demographic data of the unexplained RSA patient and controls are presented in **Table 2**. Age and body mass index (BMI) were matched in both groups ($p>0.05$), while, there were significant difference in the number of pregnancies and number of children and BMI in the 2 groups ($p<0.05$).

Genotyping results. TNF-α -238 G/A polymorphism. The -238 G/A polymorphism in the promoter region of TNF-α was studied in a 430bp fragment generated after PCR. The mutation was present in the Saudi population and most patients and controls were homozygous to the mutation. **Table 3** presents the genotype and allele frequency of the G and A alleles in patients and controls. No significant differences ($p=0.255$) were identified in the genotype of allele frequencies of G and A alleles in patients and controls.

TNF-α -308 G/A polymorphism. The TNF-α -308 mutation was present in 430bp fragment produced following PCR. This was a G/A transition and the wild type homozygous (GG), heterozygous (GA) and homozygous for the variant (AA) were all identified. Most of the patients and controls were homozygous to the mutated allele. The genotype and allele frequencies of the wild type and mutant allele showed a statistically significant difference when the RSA patients were compared to the controls (**Table 4**).

IL6-634 G/C polymorphism. The -634 G/C polymorphism in the promoter region of the IL6 gene was located in a 400 bp fragment produced on PCR amplification. Wild type homozygous (GG), heterozygous (GC) and mutated homozygous (CC) forms were identified. **Table 5** presents the genotype and allele frequency of IL6 -634 G/C polymorphism in patients and controls. Most patient and control samples were homozygous for the wild type G allele. However, there were no significant differences in the genotype and allele frequencies between patients and controls.

IL10-592 C/A polymorphism. The -592 C/A polymorphism in the promoter region of IL-10 gene, was located in a 420bp PCR product obtained on PCR amplification. **Table 6** presents the genotype and allele frequencies for C and A, where most of the patients and controls were homozygous to the mutation. No significant differences ($p=0.66$) were identified in the frequencies of C and A allele patients with RSA and controls.

Table 1 - Primer sequences used during the study to amplify the sequence of interest in the promoter region of the 3 genes.

Genes	5'-3' primer sequence	Product size
TNF-α	Forward: CAGGCCTCAGGACTCAACAC Reverse: AAGGAAGTTTTCCGCTGGTT	430
IL-6	Forward: AGGCAAACCTCTGGCACA Reverse: TTCTAGCCTGTTAATCTGGTCAC	400
IL-10	Forward: CTGTGCCTCAGTTTGCTCAC Reverse: GTCTTGGGTATTCATCCCAGG	420

Table 2 - Demographic and clinical characteristics of unexplained RSA patients in comparison with the control group.

Parameter	Patient	Control	*P*-value
	Mean ± standard error of mean		
Age, years	34.1 ± 0.77	34.6 ± 0.97	0.6
Height, m	156.97 ± 0.75	158.56 ± 0.68	0.414
Weight, kg	75.15 ± 2.45	71.34 ± 1.64	0.126
Body mass index, kg/m^2	30.66 ± 1. 01	28.28 ± 0.62	0.047*
No. of pregnancies	6.5 ± 0.38	3.9 ± 0.22	0.0001*
No. of children	2.1 ± 0.27	3.8 ± 0.22	0.04*

RSA - recurrent spontaneous abortion, *statistically significant

Table 3 - Genotype and allele frequencies of -238 G/A polymorphism in tumor necrosis factor-α gene promoter region in unexplained recurrent spontaneous abortion patients compared with the control group.

Genotype	Control		Patients		Control versus patients		*P*-value
	n (%)				OR	CI	
GG	57	(87.7)	55	(84.6)	0.77	0.28-2.10	0.612
GA	8	(12.3)	7	(10.7)	0.86	0.29-2.53	0.784
AA*	0		3	(4.6)	0	0	0.24
Total	**65**		**65**				
Allele	**Control**	**(Frequency)**	**Patients**	**(Frequency)**			
G	122	(0.9)	117	(0.9		0.24-1.48	0.255
A	8	(0.1)	13	(0.1)		0.68-4.24	

OR - odds ratio, CI - confidence interval, *Fisher exact test

Table 4 - Genotype and allele frequencies of -308 G/A polymorphism in tumor necrosis factor-α gene promoter region in unexplained recurrent spontaneous abortion patients compared with the control group.

Genotype	Control		Patients		Control versus patients		*P*-value
	n (%)				OR	CI	
GG	47	(72.3)	33	(50.8)	0.39	0.19-0.82	0.011*
GA	14	(21.5)	24	(37.0)	2.13	0.98-4.64	0.054
AA	4	(6.2)	8	(12.3)	2.14	0.61-7.50	0.223
Total	**65**		**65**				
Allele	(Frequency)						
G	108	(0.831)	90	(0.692)		0.25-0.83	0.008*
A	22	(0.169)	40	(0.308)		1.21-3.94	

OR - odds ratio, CI - confidence interval, *statistically significant

Table 5 - Genotype and allele frequencies of -634 G/C polymorphism in IL6 gene promoter region in unexplained recurrent spontaneous abortion patients compared with the control group.

Genotype	Control		Patients		Control versus patients		*P*-value
	n (%)				OR	CI	
GG	54	(83.1)	53	(81.5)	0.90	0.37-2.22	0.82
GA	8	(12.3)	9	(13.9)	1.15	0.41-3.18	0.80
AA	3	(4.6)	3	(4.6)	1.00	0.19-5.15	1.00
Total	**65**		**65**				
Allele	(Frequency)						
G	116	(0.831)	115	(0.889)		0.43-2.00	0.84
A	14	(0.169)	15	(0.111)		0.50-2.34	

OR - odds ratio, CI - confidence interval

Table 6 - Genotype and allele frequencies of -592 C/A polymorphism in IL10 gene promoter region in unexplained recurrent spontaneous abortion patients compared with the control group.

Genotype	Control		Patients		Control versus patients		*P*-value
	n (%)				OR	CI	
GG	32	(49.2)	28	(43.1)	0.78	0.39-1.56	0.482
GA	23	(35.4)	28	(43.1)	1.38	0.68-2.80	0.369
AA	10	(15.4)	9	(13.8)	0.88	0.33-2.34	0.88
Total	**65**		**65**				
Allele	(Frequency)						
G	87	(0.67)	84	(0.65)		0.54-1.51	0.694
A	43	(0.33)	46	(0.35)		0.66-1.85	

OR - odds ratio, CI - confidence interval

Discussion. Evidence exists that inter-individual variation in the inflammatory cytokine response may affect the risk of unexplained RSA.[12] Etiologies are usually manifold, and RSA is considered as a syndrome rather than a disease, where up to 40% of cases have unknown etiology.[14] During early pregnancy, plasma levels of pro-inflammatory cytokines are higher, while levels of anti-inflammatory cytokines are lower in women who miscarry than in those who maintain their pregnancy.[8] Trophoblast activated peripheral blood mononuclear leukocytes from women with a history of RSA produce more pro-inflammatory cytokines but less anti-inflammatory cytokines than women without a history of RSA.[7] In investigating the contribution of these complex cytokine cascades to the pathogenesis of RSA, it is difficult to distinguish molecular mechanisms that are causal from those that are epiphenomena of the disease process. Animal models may not reflect the

same pathogenic processes that occur in women who experience RSA.[15] In human studies, dominant Th1 immune responses in peripheral blood lymphocytes have been documented, which reflect the systemic contribution of Th1 cytokines to RSA or multiple implantation failures in IVF cycles.[16]

We have used these strategies to examine associations between maternal carriage of cytokine polymorphisms and RSA by investigating certain genetic polymorphism in the promoter region of TNF-α, IL-10 and IL-6 gene in Saudi females. The TNF-α is a potent cytokine with a wide range of pro-inflammatory activities. Circulating 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 an etiologic factor in recurrent miscarriage.[2,3] Additionally, certain genetic polymorphisms in the TNF-α promoter region are associated with higher levels of TNF-α in the blood. Functional polymorphisms at position -308 and -863 in the promoter region of the human TNF-α gene have been reported to be associated with altered TNF-α promoter activity and with different plasma levels of TNF-α in humans.[17] We investigated both these polymorphisms in the promoter region of TNF-α. The G/A substitution at position -238 was polymorphic although there were no association with the occurrence of RSA, and no significant differences (p=0.212) were identified in the frequencies of G and A allele in females with unexplained RSA and the controls. The G/A substitution at position -308 in the promoter of the TNF-α gene showed significant differences (p=0.04) in frequencies of the genotype and G/A allele in women with RSA and controls. Previous studies, which investigated the association of RSA and TNF-α polymorphisms reported contradictory results. Babbage et al,[18] showed that the -308A TNF-α polymorphism was not associated with RSA in a Caucasian population of 43 women with RSA, while Reid et al,[19] presented evidence for an increased risk of RSA for carriers of the TNF-α -308A allele, although these differences did not show statistical significance. In this regard, our results are in line with the results of Reid et al,[19] and show a significant association between the -308A TNF-α polymorphism and RSA susceptibility.

The IL-6 levels in maternal serum, amniotic fluid, vaginal fluid, and placenta have been found to increase during the process of normal labor compared with the non-labor state.[20] Relationships between RSA and IL-6 promoter gene polymorphisms were reported previously.[9] Saijo et al[9] investigated the relationships between RSA and polymorphism at -634C-G in the promoter region of the IL-6 gene in the Japanese population, and reported significantly lower risk of RSA in the carriers of G allele compared to the women with the wild type C allele. Ma et al,[13] investigated the relationship between RSA and polymorphisms of IL-6 (-634C/G) in Chinese population and reported significantly lower frequencies of the GG genotype and the G allele in the RSA group versus the control group, and suggested the IL-6 (-634C/G) polymorphism might be a possible genetic protective factor against RSA. In the present study, our results contradict the results found in the Japanese and Chinese populations, and show that most patients (81.5%) and controls (83.07%) were homozygous for the wild type allele (GG), and 13.85% of the patients and 12.31% of the controls existed in the heterozygous (GC) state. No difference was observed in the frequencies of the wild type and mutant allele in patients with RSA and the controls. In our study population, the IL-6 (-634C/G) polymorphism was not associated with RSA. The Th1 and Th2 cells reciprocally regulate each other's function through their respective cytokines.[21] Among the Th2 cytokines, IL-10 plays a key role in Th2 immunity.

Levels of IL10 vary as an anti-inflammatory response. It is encoded by the IL10 gene and many SNPs have been reported in the proximal (at position -1082A/G, -819T/C and -592A/C) and distal regions of the gene. Several polymorphisms are reportedly involved in IL-10 transcription rate, thereby directly affecting IL-10 production levels.[22] The role of IL-10 levels in unexplained RSA pathogenesis remains controversial. It was suggested that increased IL-10 expression was associated with successful pregnancy, whereas low levels were linked to recurrent fetal loss.[2] Serum IL-10 concentrations are low in pre-eclampsia, another common disorder of pregnancy; thus, IL-10 could be an important anti-inflammatory cytokine contributing to the outcome of pregnancy.[23] During this study, our results showed that the -592 C/A polymorphism in the IL10 gene promoter is present in unexplained RSA patients and controls, where most of the patients (43.1%) and controls (49.2%) are homozygous to the wild type allele (CC). The heterozygous (CA) state also occurs in 43.1% of patients and 35.4% of the controls, while the mutant genotype (AA) occurs in 13.8% in patients and 15.4% in controls. The frequencies of the genotypes and alleles do not show any significant difference between the unexplained RSA patients and controls, and hence cannot be considered as a clinically important polymorphism linked to unexplained RSA. In this regard, our results agree with several studies which showed that multiple combinations of genetic polymorphism of IL-10 were not associated with RSA,[11,18,24] but disagree with the findings of Zammiti et al,[25] who demonstrated an association between IL-10

-592C/A and -819C/T promoter polymorphisms among Tunisian RSA patients.

The limitation of the study is that IL-18 was not studied as there is new evidence of its association with recurrent spontaneous abortion.[26,27] Future studies in this area is recommended.

In conclusion, the findings of this study show that only the -308G/A polymorphism in TNF-α is associated with unexplained RSA, while -238G/A polymorphism in TNF-α gene promoter -634G/C in IL-6 gene promoter and -592 C/A polymorphisms in IL10 gene promoter do not show any association with unexplained RSA.

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