Association of CD46 IVS1-1724 C>G Single Nucleotide Polymorphism in Iranian Women with Unexplained Recurrent Spontaneous Abortion (URSA)

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ABSTRACT

There are several known and unknown factors for unexplained recurrent spontaneous abortion (URSA). Among them, complement regulatory protein CD46 plays a pivotal role in preventing uncontrolled activation of complement and successful continuation of pregnancy. We aimed in this study to investigate the possible association of CD46 IVS1-1724 C>G polymorphism with RSA in Iranian women.

141 women with RSA and 153 women with normal pregnancy were enrolled in this study. RSA was confirmed as the history of having at least three consecutive abortions without any known immunologic, pathologic and genetic reason. Genomic DNA was extracted and RFLP-PCR was done using a specific primer pair and HindIII restriction enzyme. Statistical analysis was done for determining the genotype and allele frequency, and also for odds ratio (OR).

Statistical analysis showed no significant difference in genotype frequency between two RSA and normal groups. However G allele was significantly more frequent in fertile women and represented as a protective allele (p=0.04, OR=0.8, CI 95%).

In contrary to similar studies in other two ethnic populations, our study showed no genotype differences in CD46 IVS1-1724 C>G Single nucleotide polymorphis (SNP) between RSA and fertile women. On the other hand, G allele was revealed as a protective allele for RSA. CD46 polymorphisms may predict the outcome of pregnancy; however, more studies in different ethnic groups are required.

Keywords: Abortion; CD46; PCR-RFLP; Recurrent spontaneous abortion; Single nucleotide polymorphis

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INTRODUCTION

Recurrent spontaneous abortion (RSA) is defined as the loss of three or more consecutive pregnancies before

20 weeks of gestation that afflicts 1-3% of pregnant women. There are several factors which have been involved in occurring RSA including genetic, anatomic, immunologic and endocrine factors, and infections as well as unexplained factors.[1,2] Uncontrolled activation of complement is one of the immunologic factors that has been proved to play a role in pre-term termination of pregnancy and RSA. Therefore, regulation of complement activation by several regulatory proteins including CD46, CD55, C4b-binding protein and CD59, is a key event in maintaining pregnancy.[3]

Membrane cofactor protein (MCP, CD46) is a transmembrane glycoprotein with broad expression in all nucleated cells and multiple isoforms resulting from tissue-specific alternative splicing. The gene that codes for human CD46 consists of 14 exons, and as a member of regulators of complement activation (RCA) gene family is located on chromosome 1q32. By binding to C3b and C4b, CD46 acts as a cofactor for complement factor I and inhibits formation of C3 convertase complex and sustaining activation of complement pathways.[4-7] Several mutations and single nucleotide polymorphisms (SNPs) have been identified in CD46 gene which have been associated to preeclampsia,[8,9] hemolytic uremic syndrome,[10,11] and also to recurrent pregnancy loss.[12,13] However, the exact role of such SNPs in diseases remains unclear.

IVS1-1724 C>G SNP has been first identified by Bora et al.[14] It is a biallelic polymorphism in the first intron flanked between exons 1 and 2 and could be determined by HindIII restriction endonuclease. All individuals express CD46 as two isoforms of 56 KDa and 66 KDa, and substitution of C nucleotide in HindIII restriction site (AAGCTT) by G nucleotide leads to the predominant expression of lower molecular weight isoform (so-called L phenotype, CD46H*2 allele). Therefore, individuals homozygote for C allele predominantly express upper molecular weight isoform and are named U phenotype (CD46H*1 allele). Heterozygosity for C and G leads to an equal expression of both isoforms and called E phenotype (CD46H*1/*2). Risk et al. reported higher frequency of CD46H*2 allele in RSA women.[12] Findings from two other studies by Wang et al. and Zutshi et al. also showed significant association between CD46H*2 allele and RSA.[13,15]

In this study, we aimed to study the possible association between CD46 IVS1-1724 C>G SNP with predisposition to unexplained recurrent spontaneous abortion (URSA) in Iranian women.

PATIENTS AND METHODS

Samples

In this study, 141 women diagnosed with RSA and 153 women with the experience of normal pregnancy and full-term delivery were enrolled. This study was approved by Medical Ethics Committee (code NO. TBZMED.REC.1394.338), and informed consent was signed by all participants. All women with RSA did not have any defined etiology for recurrent abortion and had experienced three or more abortions .They were tested for any genetic and anatomical abnormalities, and were negative for infectious diseases and anti-phospholipid antibody. Women with normal delivery without any history of undefined abortion, autoimmunity and infectious disease were considered as the control group.

Genotyping of CD46 IVS1-1724 C>G SNP

One milli liter peripheral whole blood was collected in tubes containing ethylene diamine tetraacetic acid (EDTA) anti-coagulant. Standard salting out method[16] was used to extract genomic DNA, and all DNAs were stored at -20°C until genotyping. Quality and quantity of all DNAs were evaluated and determined by ultraviolet (UV) spectrophotomety and agarose gel electrophoresis. Flanking region of IVS1-1724 C>G was amplified by conventional polymerase chain reaction (PCR) using specific forward and reverse primers (Table 1). Twenty-five μl PCR reaction mixture was prepared from 2.5 μl 10× PCR buffer, 1 μl dNTPs (10 mM) (Thermo Scientific Inc., USA), 1.5 μl $MgCl_2$ (25 mM), 1 μl each forward and reverse primers (10 pM) (Bioneer Inc., South Korea), 0.5 μl Taq DNA polymerase (5u/ μl) (Thermo Scientific Inc., USA) and 50 ng template DNA. All reactions were run in 37 cycles and included annealing temperature at 54°C for 30 seconds. Specific bands were visualized by agarose gel (1.5%) electrophoresis of small fraction of PCR products and ethidium bromide staining, and confirmed in comparison with GeneRuler 100 bp DNA Ladder (Thermo Scientific Inc., USA). PCR products were then subjected to digestion with 10 u/μl HindIII restriction enzyme (Thermo Scientific Inc., USA). Digestion patterns were also documented by agarose gel (3%) electrophoresis and ethidium bromide staining. Genotype of each sample was determined

using specific digestion pattern according to Table 1. Random samples from each possible genotype were confirmed by direct sequencing of PCR products (Bioneer Inc.).

Statistical Analysis

Statistical analysis was performed by SPSS software version 16 (SPSS Inc., Chicago, IL, USA) and frequency of genotypes and alleles in two groups were compared by Chi-squared and logistic regression tests. A *p*-value less than 0.05 was considered as statistically significant.

RESULTS

DNA extraction and PCR reactions using specific primers resulted in 243 bp specific band verified and visualized by agarose gel electrophoresis. This step was followed by digestion using HindIII restriction enzyme that led to three different digestion patterns, each of which corresponding to defined genotype of CD46 IVS1-1724 C>G SNP (Table 1, Figure 1). Genotypings by HindIII digestion were confirmed by direct sequencing (Figure 2). Accordingly, the most frequent genotype in RSA group was C/C (CD46H*1/*H1), seen in 79 out of 141 RSA women (56%). On the other hand, C/G (CD46H*1/*H2) genotype was the most frequent genotype in fertile women (70 out of 153, 46%). There was no significant statistical difference in the frequency of genotypes between the two groups. More frequent allele were C (CD46H*1) in both RSA (n=206) and fertile women (n=202). However, G (CD46H*2) allele was significantly more frequent in fertile women (n=104) in comparison with RSA women (n=76) (*p*=0.04, OR=0.8, CI 95%). Detailed results are presented in Table 2.

Table 1. Sequences of primers and digestion pattern for CD46 IVS1-1724 genotypes

CD46 SNP	Primer sequence	Restrction enzyme	genotype/ digestion pattern
IVS1-1724	**F:** 5'- AGAGACCCTGTCTCAAAACAAACAAAACAC-3' **R:** 5'- CACTTTCTCTAGGTTTCATACCTG-3'	HindIII	G/G: 243 bp G/C: 243/185/55 bp C/C: 185/55 bp

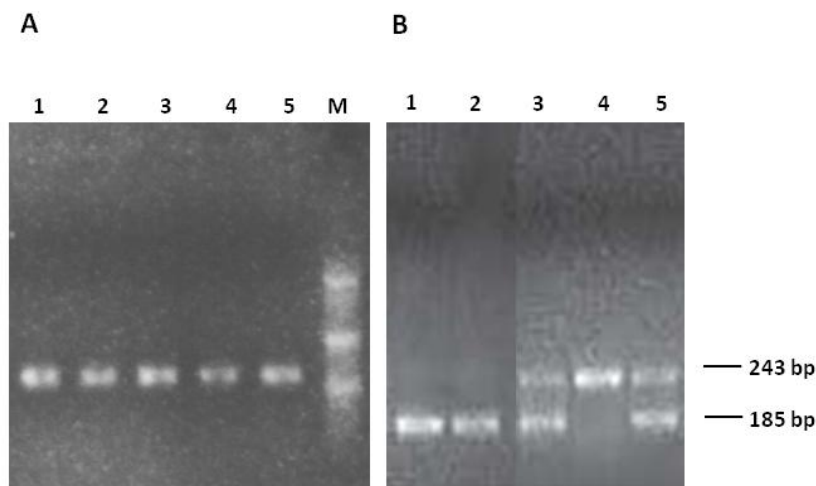F, forward primer; R, reveres primer; bp, base pair

Figure 1. Representative results for digestion pattern of CD46 IVS1-1724 C>G SNP. M: DNA size marker. (A) Lanes 1 to 5 show specific 243 bp specific PCR product. (B) Digestion of PCR products by HindIII restriction endonuclease revealed three digestion patterns corresponding to the three different genotypes. Lanes 1 and 2: CC, lanes 3 and 5: CG, and lane 4: GG.

Table 2. Genotype and allele frequencies of CD46 IVS1-1724 in RSA and normal groups

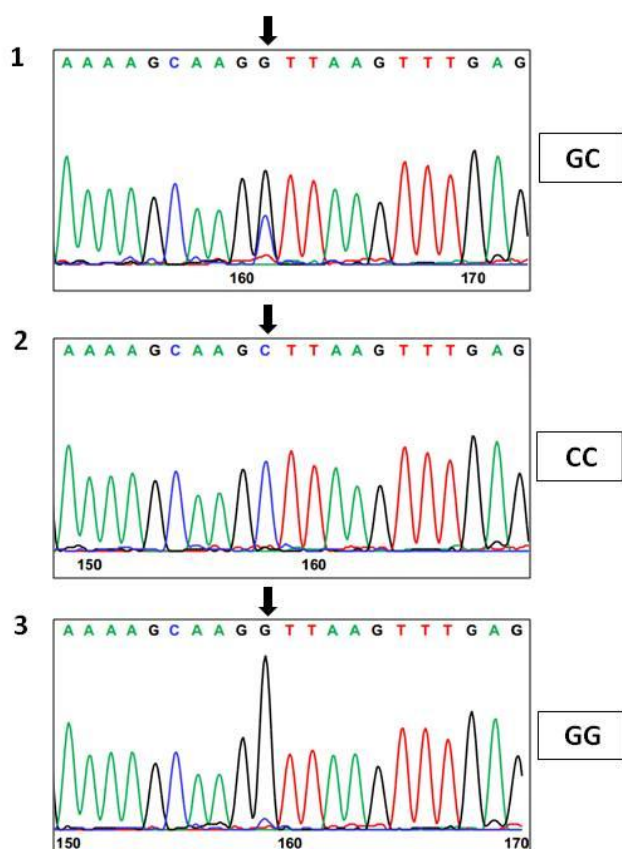| CD46 IVS1-1724	Frequency (percent)		p-value	Odds ratio, CI 95%.
	RSA group n=141	normal group n=153		
genotype				
C/C (CD46H*1/*H1)	79(56%)	66(43%)		
C/G (CD46H*1/*H2)	48(34%)	70(46%)		
G/G (CD46H*2/*H2)	14(10%)	17(11%)	0.1	
allele				
C (CD46H*1)	206	202		
G (CD46H*2)	76	104	0.04	0.8

Figure 2. Representative results for direct sequencing of CD46 IVS1-1724 C>G SNP. Selected samples from any genotype were sequenced and confirmed as GC (1), CC (2) and (GG) in HindIII restriction site (AAGCTT).

DISCUSSION

Several undetermined and also defined factors including anatomical, genetic and immunologic factors have been proposed for the predisposition of pregnant women to recurrent spontaneous abortion (RSA). Unwanted activation of complement is an immunologic event and could result in abortion. Therefore, the action of complement regulatory proteins might be a barrier against complement-mediated rejection of fetus. Of several soluble and membrane bound regulatory proteins, MCP (CD46) functions as a cofactor for factor I in degradation of C3 convertase complex in all three pathways of complement activation. Mutations and SNPs in CD46 gene have been widely investigated in clinical conditions in which activation of complement plays a major role in immunopathogenesis of diseases e.g. RSA. Multiple mutations and SNPs in CD46 gene have been identified and showed to be correlated with some defined clinical settings in which complement activation plays major role.

Bora et al. showed that C/G substitution in HindIII restriction site in both normal individuals and five cell lines is correlated to different CD46 expression phenotypes called as U, L and E phenotypes.[14] Higher frequency of L phenotype (G nucleotide, CD46H*2 allele) in women with recurrent spontaneous abortion was showed by Risk et al.[12] However, this finding was not significant due to very low number of individuals (17 normal and 18 RSA) and heterogeneity of study populations (males and females). Wang et al. studied maternal CD46 polymorphism and polymorphism in the promoter region of the IL-1β gene in 131 caucasian recurrent pregnancy loss (RPL) women and 72 fertile controls.[13] They found statistically significant higher frequency of G allele (CD46H*2) in PRL group and postulated that this allele could lead to more active Th1 response and pregnancy loss, although the exact relation between inherited allele and lower or higher expression or function of CD46 in unclear. This argument was based on the finding that cross-linking of

cell surface CD46 on monocytes with antibody or complement fragments leads to lower production of IL-12, the main Th1-promoting cytokine.[17] CD46 is also a receptor for Measles virus[18] and suppression of Th1 response by the virus could be resulted via this mechanism. Accordingly, Zutshi et al. investigated the same polymorphism (so-called CD46 IVS1-1724 C>G) in 44 Indian women with RSA and 44 normal controls.[15] They also found significant higher frequency of CD46H*2 allele in women with RSA.

In this study, we also investigated possible association of CD46 IVS1-1724 C>G with RSA in 141 Iranian women in comparison with 153 fertile women. Frequency of CD46H*2 allele was found as dominant in fertile but not in RSA women (Table 2). Advantage of our study over the previous investigations is higher number of both study populations specially RSA group. However, our finding is in contrary to those studies. The possible explanation for such controversial results could be attributable to ethnic differences among study populations i.e. caucasian population in USA, Indian and Iranian populations. According to our results and in contrary to other studies, CD46H*2 is not a predisposing allele for URSA.

In conclusion, CD46 genotyping could be a predictive marker for predisposition of women to URSA. However, more studies in several ethnicities and in concomitant with other candidate genes are needed for such a claim.

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