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Research Article

The Role of *CD28R* Gene Variants in Pregnancy Loss Among Females Infected with Human Herpes 2 Virus

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Abstract

Background: Abortion is the termination of a pregnancy by removal or expulsion of an embryo or fetus from the uterus before it is capable of survival. A co-stimulation molecule, CD28's aberrant expression can control T-cell activation and influence the strength of the immunological response. Particularly if obtained during pregnancy, the sexually transmitted infection known as herpes simplex virus type 2 (HSV-2) may be linked to spontaneous abortion. ***Objective***: To investigate the effect of CD28R gene polymorphism as well as HSV2 infection in females suffering from recurrent miscarriage (RM). ***Methods***: This case-control study involved a total of 200 placental tissue samples, with 100 obtained from female patients experiencing recurrent miscarriage (RM) and the remaining 100 from placentas exhibiting unremarkable pathological changes, serving as an apparently healthy control group with normal vaginal delivery. Polymerase chain reaction (PCR) was employed to detect HSV-2 DNA sequences, while Sanger sequencing was used to identify polymorphisms in the CD28R gene. ***Results***: Recurrent miscarriage patients were older on average than the control group, which appeared to be in good health. A substantial positive association was found between mother age, the number of abortions, the number of participants, and the week of the abortion. 27% of the cases tested positive for HSV-2 DNA, according to PCR results, while 73% tested negative. Statistically significant differences were found among groups based on CD28R gene genotyping. ***Conclusions***: Polymorphisms of the CD28R gene and presence of HSV-2 may be considered risk factors for recurrent abortion among Iraqi women.

Keywords: CD28R, HSV2, PCR, Recurrent miscarriage, Sequencing.

دور المتغيرات الجينية *CD28R* في فقدان الحمل بين الإناث المصابات بفيروس الهربس البشري 2

الخلاصة

الخلفية: الإجهاض هو إنهاء الحمل عن طريق إزالة أو لفظ جنين الجنين من الرحم قبل أن يصبح قادرا على البقاء على قيد الحياة. جزيء التحفيز المشترك، يمكن للتعبير الشاذ ل CD28 التحكم في تنشيط الخلايا التائية والتأثير على قوة الاستجابة المناعية، خاصة إذا تم الحصول على العدوى المنقولة جنسيا أثناء الحمل، فإن العدوى المنقولة جنسيا المعروفة باسم فيروس الهربس البسيط من النوع 2 (HSV-2) قد تكون مرتبطة بالإجهاض التلقائي. **الهدف**: التحقيق في تأثير تعدد أشكال الجين CD28R وكذلك عدوى فيروس الهربس البسيط 2 لدى الإناث اللواتي يعانين من الإجهاض المتكرر (RM). **الطرائق**: تضمنت دراسة الحالة والشواهد هذه ما مجموعه 200 عينة من أنسجة المشيمة، مع 100 تم الحصول عليها من مريضات يعانين من RM والباقي 100 من المشيمة تظهر تغيرات مرضية غير ملحوظة، حيث كانت بمثابة مجموعة ضابطة صحية على ما يبدو مع ولادة مهبلية طبيعية. تم استخدام تفاعل البوليميراز المتسلسل (PCR) للكشف عن تسلسل الحمض النووي لفيروس HSV-2، بينما تم استخدام تسلسل سانجر لتحديد تعدد الأشكال في جين CD28R. **النتائج**: كان مرضى الإجهاض المتكرر أكبر سنا في المتوسط من المجموعة الضابطة، والتي بدت بصحة جيدة. تم العثور على ارتباط إيجابي كبير بين عمر الأم وعدد حالات الإجهاض وعدد المشاركين وأسبوع الإجهاض. 27٪ من الحالات كانت إيجابية للحمض النووي لفيروس HSV-2، وفقا لنتائج تفاعل PCR، بينما كانت نتيجة اختبار 73٪ سلبية. تم العثور على اختلافات ذات دلالة إحصائية بين المجموعات بناء على التنميط الجيني للجين CD28R. **الاستنتاجات**: يمكن اعتبار تعدد أشكال الجين CD28R ووجود HSV-2 من عوامل الخطر للإجهاض المتكرر بين النساء العراقيات.

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INTRODUCTION

Recurrent miscarriages (RM) are defined clinically as three or more consecutive miscarriages before the twentieth week of pregnancy and are regarded as post-implantation failures in spontaneous conception. Numerous maternal and fetal variables are linked to the diverse condition known as RM [1]. Furthermore, a primary spontaneous abortion was defined as one that did not result in any previous births, whereas a secondary spontaneous abortion was defined as one that happened following at least one successful

pregnancy. Epidemiological studies revealed that the likelihood of losing a pregnancy rose in tandem with the number of abortions. For example, the likelihood of an abortion may rise to 24% after two miscarriages and to 40% after four losses [2]. The immune system is essential for detecting external invaders and preserving the body's immunological balance. Consequently, RSA may be linked to immune system anomalies. There are two types of immune cells: adaptive immune cells (T and B cells) and innate immune cells (NK cells, neutrophils, macrophages, and myeloid-derived suppressor cells, or MDSCs). Pregnancy success may be impacted by an imbalance in these immune cells. When the immune system is dysregulated, immune cells may target the embryo or the placenta [3]. The fact that CD28 was constitutively expressed on the surface of NK cells, plasmocytes, CD4+, CD8+ T cells, and CD3+ peripheral blood T cells was discovered by Hansen in 1980 [4]. B7 molecules, which were mostly formed on the surface of antigen-presenting cells (APCs), would provide the co-stimulatory signal when T cells were activated, facilitating the production of cytokines, cytotoxic impact, and T cell proliferation [5]. Comparing B7-1-deficient and CD28-deficient rats to wild-type animals, for instance, the proportion of regulatory T cells in their spleen dropped to 10% and 25%, respectively [6]. In contrast to paternal antigens among embryo cells, the mother's own Treg would limit maternal alloimmunity reactions to protect the child during pregnancy [7]. Furthermore, CD28 was the primary driver of Treg proliferation [8,9]. Herpes simplex virus type 2 (HSV-2) is a virus with an enclosed double-strand DNA genome that is disseminated throughout the world. It is a member of the Herpesviridae family [10]. HSV-2 caused skin disruptions and propagated throughout the epithelial mucosa. It then spreads to the lumbosacral ganglia, which are nerve tissues, to start a dormant infection. The vaginal tract can be infected by this virus. Generally speaking, this virus is one of the primary causes of viral sexually transmitted infections (STDs) worldwide [11,12]. HSV-2 is responsible for a high proportion of genital herpes infections. During their reproductive years, females are particularly vulnerable to HSV-2 infection, which can potentially spread to the developing embryo during pregnancy [13]. Intrauterine HSV-2 transmission causes abortion, stillbirth, and congenital defects in live fetuses before the 20th week of pregnancy [14]. This study aims to investigate the effect of CD28R gene polymorphism as well as HSV2 infection in female patients suffering from recurrent miscarriage (RM).

METHODS

Study design and setting

This case-control study involved 200 placental tissue samples and blood collected from female patients experiencing recurrent pregnancy loss, along with samples from apparently healthy individuals with normal vaginal delivery serving as the control group. The participants were from Babylon Teaching Hospital for Women and Children in Hilla City, Iraq. The study population ranged in age from 17 to 49. Between July 2024 and February 2025, specimens were aseptically collected, moved into sterile test tubes with a sterile cap, and transported to the microbiology lab for examination.

Viral genome extraction

The PathoGene Spin™ DNA/RNA Extraction Kit (iNtRON Biotechnology Co., Korea), which is intended to separate high-quality nucleic acids from a range of infections and specimens, was used to extract the viral genome in order to identify HSV-2 DNA. Low elution volumes are used in the kit to allow for sensitive downstream analysis.

Total DNA extraction

To examine the polymorphism in the CD28R gene, all of the DNA must be extracted. It was accomplished by using the G-Spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology Co., Korea), which is designed to extract DNA fragments up to 50 kb in size and 20–30 kb in size. Two milliliters of venous blood and about 25 milligrams of placental tissues were put into an EDTA tube that was obtained from RM patients and apparently healthy controls (AHC). The DNA was then promptly kept at -20°C until it was needed.

Primer selection

The primers were designed as the following: HSV2 primer sequence *F'5-*TACGCTCTCGTAAATGCTTC-3 *R'5-*GCCCACCTCTACCCACAA-3. CD28R primer sequence F 5-AAG GAT GCA GTT TAG GGT CTA GAT T-3, R 5-GAT CAA GCC AAC ATT GTC CAT TGG-3 *PCR product = 886 bp*

PCR technique

The PCR Reaction Mixture, which had a total volume of about 25 microliters and included the following components: master mix (12.5 µl), forward and reverse primers (1 µl each), nuclease-free water (5.5 µl), and extracted DNA (5 µl), was used to perform the polymerase chain reaction using a conventional heat cycler (Biometra, Germany) (Table 1).

DNA genotyping and sequencing

The reactions were finally placed in the Biometra, Germany thermal cycler, which had been preheated to 94°C and configured with the appropriate cycle parameters listed in Table 1. Using certain primers, the target region of HSV2 and CD28R polymorphisms were amplified. The PCR products were subsequently electrophoresed using 1.5% agarose gel, and the gel documentation system was used to view the results. The PCR data were then automatically sequenced using the PCR forward (F) primer as the sequencing primer for both TIM3 and CD28R. The sequences were analyzed using the NCBI reference database's DNA sequences and Geneious

Bioinformatics software version 2 for sequence data processing and alignment. The sequencing was done at Macrogen Company in Geumcheon, Seoul, South Korea.

Table 1: Steps of PCR technique (thermal conditions) used for genome amplification

Genes	No. of cycles	Initial denaturation (min)	Denaturation (min)	Annealing (sec)	Extension (min)	Final extension (min)
CD28R	40	95˚C/5	95˚C/1	62.5˚C/45	72˚C/2	72˚C/5
HSV2	35	95˚C/5	95˚C/1	58˚C/45	72˚C/2	72˚C/5

Ethical consideration

The Declaration of Helsinki's guiding principles were adhered to in this investigation. Patients gave their written and verbal consent prior to sample collection. On May 18, 2024, the study protocol, permission form, and subject information were examined and authorized by a local ethics commission under project number M239543.

Statistical analysis

For statistical analysis, SPSS version 24 software was used to analyze HSV2 between Recurrent Miscarriage patients and control groups. The significant differences between the viral infection patient and the control group were calculated, with a *p*-value < 0.05. Chi-square test was utilized for data analysis at a significance level of $p < 0.05$.

RESULTS

Table 2 shows that the mean age of patients with Recurrent Miscarriage (RM) was 37.22±11.53 years, which is higher than the mean age of the apparently healthy controls (35.47±12.32 years). However, the difference between the two groups was not statistically significant ($p = 0.06$).

Table 2: Distribution of women with recurrent miscarriage and apparently heathy controls according to their age

Study groups	No.	Age (year)	Range (year)
AHC	100	35.47±12.32	18-48
Recurrent miscarriage	100	37.22±11.53	17-46
Total	200	*p*-value = 0.3	

Values were expressed as mean±SD.

Maternal age, the number of abortions, the number of participants, and the week of abortion all showed a strong, extremely significant positive connection ($p < 0.001$). Table 3 showed a strong significant positive connection ($p < 0.001$) in the number of abortions.in all cases and groups.

Table 3: Comparison of miscarriage patterns between women with and without clinical spontaneous abortion (n=100 in each group)

Variables	Patients			Controls		
Maternal age	17-28	29-39	39-49	17-27	28-38	39-49
Number of participants	48	46	6	55	31	14
Age of participants	23±5.7	33±6.3	42±9.6	25±9.7	32±8.5	42±9.8
Number of abortions	1.6±6.4***	2.5±5.1**	4.5±10.9**	----	----	----
Week of abortion	12±7.9	13±8.6	14±11.7	----	---	---

--- : no discernible relationship; *** $p < 0.001$; ** $p < 0.01$.

However, there was no discernible relationship in the control groups. The PCR results indicated that 27% were positive for HSV-2 DNA, while 73% were negative, as demonstrated in Table 4 and Figure 1.

Table 4: Percentage of HSV2 positive signals in women patients with recurrent pregnancy loss by using PCR technique

HSV-2	n (%)	*p*-value*
Positive	27(27)	0.03
Negative	73(73)	
Total	100(100)	

Values were expressed as frequency and percentage. * Chi-Square test.

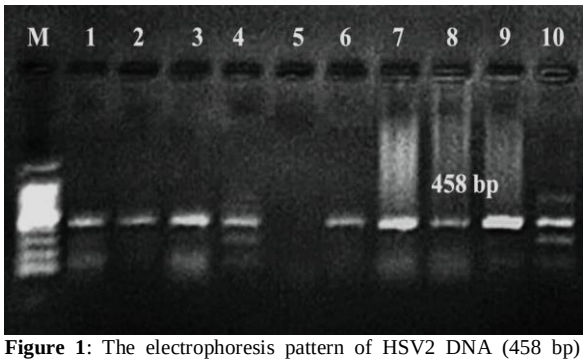

Figure 1: The electrophoresis pattern of HSV2 DNA (458 bp) detection in Placental tissues of recurrent pregnancy loss patients and healthy control groups. Lane 1 - 4 lane 6--- 10 refers to HSV-2 DNA samples; Electrophoresis conditions, 1% agarose, 75 V, 20 mA for 1h (5 µl in each well), stained with red safe solution.

There was a statistically significant difference among the patient group ($p = 0.03$). In women with recurrent miscarriage (RM), the age group most commonly affected by human herpes 2 virus DNA infection was 28-38 years, constituting 42% (15 out of 38 cases). This was followed by the age ranges 17-27 years and 39-49 years, which accounted for 10 (39%) and 2 (19%), respectively, as shown in Table 5.

Table 5 : Frequency of HSV2-PCR among the patients with RM according to the age stratum

Age Stratum	Years	HSV2-DNA		*p*-value*
		Positive	Negative	
17-27	59(59)	10(10)	49(49)	
28-38	36(36)	15 (15)	21 (21)	0.04
39-49	5(5)	2(2)	3(3)	
Total	100(100)	27(27)	73(73)	

Values were expressed as frequency and percentage. * chi-square test.

The statistical analysis indicated significant differences among these age groups ($p < 0.05$). The current results indicated that the distribution of DNA polymorphism for the CD28R gene was as follows: AA genotype in 20%, AC in 30%, and CC in 50% among patients with recurrent miscarriage (RM). In the control group, these frequencies were 70% for AA, 10% for AC, and 20% for CC. There were significant differences between the groups based on genotyping ($p < 0.05$). The AC genotype was more common in

patients (30%) compared to controls (10%), and the CC genotype was notably higher in patients (50%) than controls (20%), both showing significant associations. Regarding allele frequencies, the C allele was more prevalent in patients (65%) than controls (25%), with statistical analysis indicating a significant difference (p = 0.002). The 95% confidence interval for the C allele frequency difference was 0.17 (0.05–0.58), as shown in Table 6.

Table 6: Genotype distribution and odd ratio of CD28R gene polymorphisms between the RMI patients and AHC

Genotype I-CD28	Patients	Control	*p*-value	OR 95%
AA	20(20)	70(70)		
AC	30(30)	10(10)	0.05	0.09(0.008-1.10)
CC	50(50)	20(20)		0.11(0.01-0.8)
Total number	100(100)	100(100)		
A allele	35(35)	75(75)	0.002	0.17(0.05-0.58)
C allele	65(65)	25(75)		

Values were expressed as frequency and percentage. Samples were submitted in NCBI, and the accession number of nucleotide sequences of CD28R as new recording: LC872329 ; LC872330; LC872331.

DISCUSSION

Although miscarriages are a common pregnancy outcome, it can be difficult to assess their occurrence due to uneven documentation and registration. Over the last ten years, there have been changes in the way abortion services are provided. In most nations, at least half of all abortions are medication abortions. Over 90% of all abortions were carried out for 13 weeks in most nations, and over two-thirds of all abortions occurred before the first nine weeks of pregnancy. Both the percentage of pharmaceutical abortions and the percentage of abortions performed before 9 weeks of pregnancy have increased throughout the last ten years [15]. Since the majority of spontaneous abortions take place in the first few weeks of pregnancy, they are sometimes mistaken for menstrual flow. Determining the rate of unplanned and spontaneous abortions is typically highly challenging because there is a chance of fraudulent reports in nations where abortion is illegal. Furthermore, the majority of spontaneous abortions in low- and middle-income nations have not been reported to or documented in their official health system, making research on this topic extremely difficult [16]. Herpes simplex type 2 infection during pregnancy can result in congenital and neonatal herpes, miscarriage, and premature labor. Gathering trustworthy epidemiologic data through seroprevalence studies is one of the main responsibilities of the disease control program [17;18]. HSV2-DNA was found in 27 (27%) of the RM females in the current investigation. Our findings are consistent with research conducted overseas by Robb et al. [19], who discovered a substantial correlation between placental HSV positivity and a poor pregnancy outcome (39% positive out of 200 cases).Prior research revealed either no association or a weak association between HSV infection and unfavorable pregnancy outcomes. In order to investigate the prevalence of HSV, Chow *et al*. [20] used multiplex PCR to test 105 pregnant women; they were unable to find any HSV infection; this could be because there were not many abortion cases in their sample. There is conjecture regarding the mechanisms through which HSV infection contributes to pregnancy loss. The Th1 to Th2 cytokine shift pathway may be dysregulated as a result [21]. Natural killer cell activity may rise as a result of reactivated endometrial HSV infection, and this has been linked to a significant increase in pregnancy loss [22]. Our findings are consistent with those of Smith and Robinson [24] and Bujko *et al*. [23]. The natural ligands for B7-1, CD28, and B7-2 (also known as CD86) are primarily expressed on activated dendritic cells and macrophages. When B7 molecules interact with CD28, they promote the production of cytokines such as IL-2, IL-5, and IL-4, and this enhancing effect is amplified in the presence of second messengers. Additionally, the co-stimulatory signal through CD28 influences various intracellular signaling pathways, including the PI3K pathway, the GRB-2/SOS-mediated p21Ras pathway, and the ITK/EMT pathway, all of which contribute to modulating immune responses [25,26]. The maladapted CD28/B7 pathway was thought to be the main cause of RSA development since immunological failure was thought to be one of the contributing elements. The idea was that blocking the expression of B7-1 and B7-2 could postpone the mother's rejection of an allogenic embryo in matings that were likely to result in miscarriages. Furthermore, methods for altering the CD28-mediated co-stimulatory signal to avoid the abortion linked to impaired immunity have been studied [27]. The latest CD28R results revealed The AC genotype seems to be more prevalent in patients (30%) than in controls (10%); however, the statistical analysis shows a strong association between genotype and disease. Likewise, the CC genotype showed a strong connection and was more prevalent in patients than in controls. In terms of allele frequency, the C allele was more common in female patients than in controls. In cases where the allele of rs3181097 was G but not A, the *p*-value was statistically substantially different. Chen *et al.* [28], who identified the CD28 single nucleotide polymorphism (SNP) in this haplotype, are in agreement with our findings. Thus, it was proposed that rs3181097 of the CD28 gene was also a significant SNP in transfusion reaction (TR). Variations in the gene's promoter region would affect the expression level because it contains many SNPs (rs5742909, rs4553808, rs16840252, rs62182595, and rs3181097) [29]. Consequently, the degree of CD28 and CTLA-4 expression may be connected to the

mechanism of TR caused by these SNPs. However, research on the functions of the rs62182595 and rs3181097 SNPs is still lacking. As a result, the mechanism of TR caused by rs3181097 was not verified. TR was influenced by a number of factors, including the patient's physical condition and the blood product's numerous potential causes. Consequently, it was determined that the immunity-related SNPs identified in CD28 and B7 were also RSA risk factors. Particularly, B7-2 rs1129055 was substantially associated with the altered risk of pneumonia-induced sepsis [30].

Conclusion

The CD28R SNP G significantly reduces the risk of abortion and directly decreases the level of CD28 gene expression. This suggests that SNP may play a key role in immunological regulation, which may lead to the development of RM. The primary HSV-2 infection in pregnant women may be linked to reactivation, which results in recurrent abortions and HSV2 infections in the vaginal tract.

Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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