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HLA-E Polymorphism in Iraqi Women with Unexplained Recurrent Spontaneous Miscarriage (URSM)

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ABSTRACT

This study was conducted to determine the frequency of human leucocyte antigen (HLA)-E alleles in Iraqi women residing in Baghdad with and without recurrent spontaneous miscarriage (RSM) to estimate their function in the maintenance of gestation. A case-control study was approved. HLA-E genotyping was occurred by restriction fragment length polymorphism (RFLP) for 94 women with RSM and 87 fertile controls. The frequency of HLA-E *0101 allele was higher in patients with RSM while HLA-E*0103 allele was higher in controls, and the difference was statistically significant ($P = 3 \times 10^{-11}$). HLA- E*0101 / 0101 genotype was more frequent genotype in patients (61.7%), followed by HLA-E*0101 / 0103 (24.47%) and finally HLA-E*0103 / 0103 genotype (13.83%). The difference in the frequency of HLA-E*0101 / 0101 homozygous genotype in patients with RSM compared with that in the controls was statistically significant (Odd Ratio (OR) =6.63, 95% Confidence Intervolves (CI) = 3.23-13.87, Probability (P) = 5.6×10^{-9}). We found the higher frequency of homozygosity for HLA-E*0101 in Iraqi women with RSM. HLA-E*0101 homozygosity may thus be a dangerous factor for RSM.

Keywords: HLA-E, spontaneous miscarriage, polymorphism, gestation.

1. INTRODUCTION

The foetus is a semi-allograft and therefore the foetus is foreign to the mother genetically. Hence, the way of implantation may comprise mechanisms to prevent the rejection of allograft, however, the imbalance of immunological tolerance would laed to pathological pregnancy, like spontaneous miscarriage [1].

Unexplained recurrent spontaneous miscarriage (RSM) is traditionally defined as three or more sequential gestation losses before the 24th week of pregnancy after routine screening tests (normal uterine cavity, parental karyotypes, endocrine and infection parameters), is assumed to be caused by the immunological rejection of foetus by the mother [2].

The human major histocompatibility (HLA) complex locus at chromosome 6p21 encodes the classical human leucocyte antigen class I proteins (A, B and Cw) and a 'non-classical' HLA class Ib proteins such as HLA-E, F, and HLA-G [3, 4]. HLA-E has an extensive tissue distribution and plays a crucial role in the regulation of natural killer (NK) cell activity through its interaction with inhibitory (CD94 / NK G2A and CD94 / NKG2B) and activating (CD94 / NKG2C) NK receptors [5].

During gestation, the maternal immune system must tolerate the perseverance of allogeneic foetal cells in the maternal tissue. Foetal trophoblasts avoid a devastating maternal immune reaction by not expressing the classical HLA-A, HLA-B, HLA-DR, HLA-DQ and HLA-DP molecules that are the primary targets for allogeneic T cells. However, trophoblasts do express HLA-C and the non-classical HLA-E, HLA-F and HLA-G molecules [6]. NK cell-mediated cytotoxicity is thereby circumvented, but HLA- C is extremely polymorphic histocompatibility antigen that can also cause a cytotoxic T cell response [7, 8].

Suitable HLA-E expression in the trophoblast is essential to help trophoblasts attacking maternal decidua and vascular system; therefore there is an increase in uterine perfusion that is required during pregnancy. Nevertheless, if the HLA-E was decreased or not being expressed, the trophoblast cells ability will be decreased and inhibited from invading the uterus because it was perceived as non-self which has possessions as antigens that trigger the creation of antibodies in the mother. These antibodies associate to antigens, and immunological reaction happens that activate stimulation of pro-inflammatory cytokines, activated T-cells, and natural killer cells (NK) that will raid trophoblast cell itself, causing failure of the pregnancy product [9].

HLA-E is slightly polymorphic with two non-synonymous alleles termed so far (HLA-E*0101 and HLA-E*0103). HLA-E*0103 allele differs from HLA-E*0101 by an amino acid substitution (glycine to arginine at position 107 of the *a*2 heavy-chain domain) and by a cell surface expression [10, 11]. In the light of the possible role of HLA-E in the maintenance of pregnancy, it is necessary to understand the biology of the various alleles of HLA-E [12].

Therefore, the objective of this study was to investigate the HLA-E gene polymorphism in Iraqi women with RSM and normal fertile women to estimate the effect of HLA-E alleles on the maintenance of pregnancy.

2. MATERIALS AND METHODS

Patients and healthy fertile controls*:* A case-control study was conducted and included 300 female patients were recruited from outpatient's clinics of Obstetrics and Gynecology Department, Al-Yarmouk and Al-Elwiya teaching Hospital. The median age of the patients was 29.23 years (range 19–39), and the average number of RSM was 4 (range 3–6). All women selected were not having any prior live births (primary miscarriage). Eighty-seven healthy Iraqi women with no history of previous miscarriage, living in the same geographic place as patients and has the same ethnic origin, were taken as controls. The mean age of healthy controls was 29.89 years (range 20–38). Five milliliters of blood was taken from each control and women with RSM. The Written informed agreement was obtained from the patients and controls after approving the study protocol by local ethical committee.

The patients had regular menstruation and underwent examinations into different known causes of RSM including Anticardiolipin antibodies (IgM), antiphospholipid (IgM), Coagulation factors including protein C, protein S, Antithrombin III, activated protein C resistance, and investigation of toxoplasmosis antibodies (IgM) and cytomegalovirus antibody (IgM), rubella antibody (IgM), activated partial thromboplastin time (APTT) as well as homocysteine level. Only 94 patients from 300 were chosen for our study because they gave normal value for previous tests. Therefore, they considered as cases with unexplained recurrent spontaneous miscarriage (RSM).

2.1 DNA extraction

Total genomic DNA from both groups were collected in tubes containing EDTA was extracted and purified by using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), and adhering to the manufacturer protocol.

2.2 Amplification of DNA fragments using RFLP - PCR technique

Analysis of the single nucleotide polymorphism (SNP) of the HLA-E gene was done by using restriction fragment length polymorphism (RFLP) analysis. The polymorphism in HLA-E locus is practically restricted to HLA- E*0101 and HLA- E*0103 alleles, and they differ by amino acid substitution at codon 107 in exon3. The codon 107 in E*0101 encodes arginine (called ER107) and in E*0103 encodes glycine (called EG107). The genomic differences between the 2 HLA-E alleles were created by using PCR-RFLP analysis. The DNA-amplified product is cut into fragments of different lengths by restriction enzymes, resulting in pieces are separated according to their lengths by gel electrophoresis. The appearance of the HLA-E*0103 allele was distinguished by the presence of a restriction site for *Hpa*II enzyme, which cuts the HLA-E*0103 allele into two fragments, (260 and 20) bp, and leaves the HLA-E*0101 allele without cut giving a band at 280 bp.

Genomic DNA was amplified using the PCR with various primers (forward and reverse) as described previously [13]. The Sequence of primers (alpha DNA) was used as follows: forward primer HLA-E: 5 GGC TGC GAG CTG GGG CCC GCC 3, reverse primer E- EG2.2: 5 AGC CCT GTG GAC CCT CTT 3. PCR was carried out in 25 µl reaction volumes: 2 µl (1µM) of each primer, 12.5 µl Green Go Taq® master, 5 µl gDNA (100ng/ µl) and 5.5 µl of H2O. The PCR conditions consisted of 35 cycles: 94°C for 45 s, 61 °C for 45 s and 72 °C for 45s with a final extension at 72 °C for 7 min. The PCR products were digested with the Restriction endonuclease *Hpa*II enzyme (10.000 U / ml; New England Biolabs Inc., Ipswich, MA, USA) can cleave amplified DNA at specific sites in each exon. Master mix (total volume 10 *µl*) was prepared as follows: 1 *µl* of *Hpa*II enzyme, 5 *µl* PCR product 1 *µl* enzyme buffer and 3 *µl* H2O.The enzyme was incubated with the PCR products at 37 C° for at least 4 h. Five microliters of PCR products were mixed with 2 *µl* loading buffer and fragment separation was carried out by 3% agarose gel and visualized by staining with ethidium bromide and exposed to ultraviolet light. The PCR product was detected in the appearance of DNA molecular weight marker (3000-100 bp). The PCR product was distinguished at 280 bp for E*0101 allele and at 260 + 20 bp for the E*0103 allele (Fig. 1).

2.3 Statistical analysis: Statistical significance of the difference between the patient and control groups was

assessed by independent samples t-test. Odds Ratio (OR) and 95% confidence intervals (CI) were investigated by Fisher's exact test. The probability was corrected for the multiple comparisons by Bonferroni. Statistical significance was set at *P-value* ≤ 0.05

3. RESULTS AND DISCUSSION

The frequency of HLA-E *0101 and *0103 alleles were contrasted with those in patients with RSM and fertile women as controls (Table 1). The HLA-E*0101 allele was higher in patients with RSM (73.94%) than in fertile controls (39.66.2%), and HLA-E*0103 allele was higher in fertile controls (60.34%) than in patients with RSM (26.06%). The difference in the frequency of HLA-E alleles between the two groups was statistically significant ($P = 3x10^{-11}$).

When examining the distribution of HLA-E genotypes in patients and controls group, HLA-E*0101 / 0101 genotype was the most frequent genotype in patients with RSM (61.7%), followed by HLA-E*0101 / 0103 genotypes (24.47%) and at last HLA-E*0103 / 0103 genotypes (13.83%). Relating to controls, HLA-E*0101 / 0103 and 0103 / 0103 genotypes were had the same frequency (40.23%). While HLA-E*0101 / 0101 genotype have a lower frequency (19.54%). The difference in the frequency of HLA-E*0101 / 0101 homozygous genotype in patients with RSM when compared with that in the fertile controls was statistically significant after correction for multiple comparisons (OR = 6.63, 95% CI =3.23-13.87, Table 2).

Table 1: Frequency of HLA-E alleles in patients with recurrent spontaneous miscarriage and fertile controls.

HLA-E Alleles	Patients (N=94)		Control (N = 87)		OR	95% CI	*p*-value
	N	%	N	%			
0101	139	73.94	69	39.66	4.32	2.70-6.91	$3x10^{-11}$
0103	49	26.06	105	60.34	0.23	0.14-0.37	$3x10^{-11}$

HLA, human leucocyte antigen; OR, odds ratio; 95% CI, 95% confidence interval.
*Significant *P*-value if ≤0.05. Bonferroni-corrected *P*-value for multiple comparisons

Table 2: Frequency of HLA-E genotypes in patients with recurrent spontaneous miscarriage and fertile controls.

HLA-E genotypes	Patients (N=94)		Control (N = 87)		OR	95% CI	*p*-value
	N	%	N	%			
0101: 0101	58	61.70	17	19.54	6.63	3.23-13.87	$5.6x10^{-9}$
0101: 0103	23	24.47	35	40.23	0.48	0.24-0.95	0.017
0103: 0103	13	13.83	35	40.23	0.24	0.11-0.52	$5x10^{-5}$

HLA, human leucocyte antigen; OR, odds ratio; 95% CI, 95% confidence interval.
*Significant *P*-value if ≤0.05. Bonferroni-corrected *P*-value for multiple comparisons

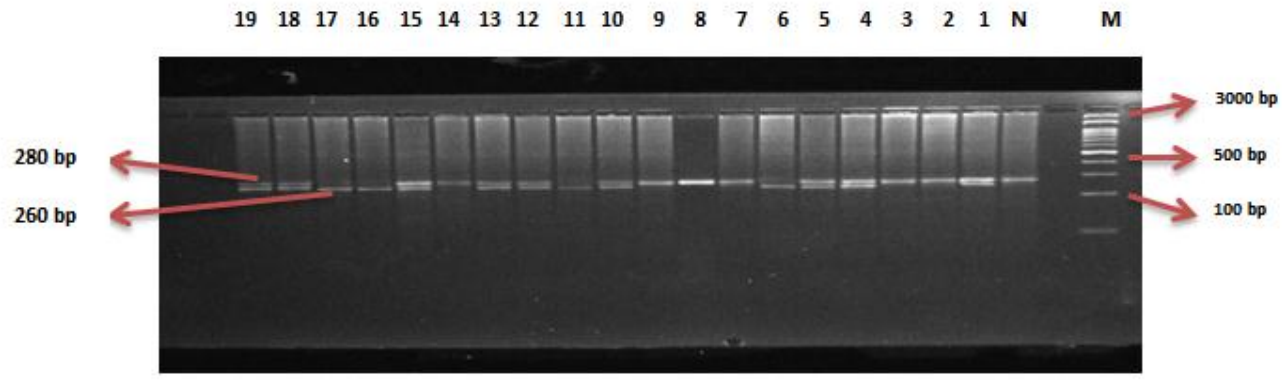

Figure 1: A photograph of 3% agarose gel showing the PCR-RFLP product for exon 3 of HLA-E gene after digestion with *HpaII* restriction enzyme. Lane M: 100/3000 bp mixed DNA marker, N: negative control, lanes (1,4,5,10,12,13,15,18,19) indicate the appearance of heterozygous HLA-E*0101 allele bands (280+260) bp. Lanes (2, 3, 7, 8, 9, 14) indicate the presence of homozygous HLA-E*0101 allele bands (280bp). Lanes (6, 11, 16, 17) show the presence of homozygous HLA-E*0103 allele bands (260bp+20bp not visible).

HLA-E is a non-classical HLA class I gene with two adversative products HLA-E*0101 and HLA-E*0103. They are discovered on most tissues [14], HLA-E*0103 being expressed at significantly higher levels than HLA-E*0101. These variations depend on the affinity for accessible peptides and on the stability of refolded complexes [11].
The HLA-E expression is observed in T cells, B cells, activated T lymphocytes and different other cells including placenta and trophoblast cells [15, 16, and 17]. HLA-E expression at the maternofetal interface is exciting because of trophoblast absences all other classical class I antigens and expresses the HLA-E accompanied by HLA-G and up to certain extent HLA-C. The HLA-E expression here may be involved in immunomodulation of maternal immune response to the fetus [12].

Surface expression of HLA-E needs highly preserved peptide derived from the signal sequence of other class I molecules such as HLA-A, HLA-B, HLA- C and HLA-G but not HLA-F [18]. The precision of peptide is very important, as the only suitable peptide can be loaded on HLA-E allowing expression and then a defense of target cells by an interaction of HLA-E / peptide complex and CD94 / NKG2 receptor of NK cells [12, 19].

The consequences of the present work shown that HLA- E*0101 allele is the most frequent allele appear in our studied population and that the HLA-E allele frequencies were similar to those previously described in different populations such as Africans [13], Caucasian, African- American and Hispanic populations [20], Indians [12] and also Euro-Caucasoid, Afro-Caribbean, and Indo-Asian [5]. Conversely, the gene frequency of HLA-E*0103 is significantly higher than that of HLA-E*0101 in the Japanese [20] and Chinese populations [21].

In the current study, the frequency of HLA-E*0101 allele was significantly higher in patients with RSM and the frequency of HLA-E*0103 was significantly higher in fertile controls (P = $3x10^{-11}$). Some prior studies were conducted to estimate the significance of HLA-E alleles in the RSM and reported non-significant relations in contrary to the results of the current study [22–24]. Though, the work of Tripathi *et al.* [12] on Indians shown a higher frequency of HLA- E*0101 compared with HLA-E*0103, and the variance was significant (P = 0.043). The alteration in the distribution of HLA-E alleles between various studies may be due to ethnic variation.
In the present study, a significant correlation was found between RSM and homozygosity of HLA-E*0101 allele (odd ratio (OR) = 6.63, confidence interval 95% CI = 3.23–13.87, P = 5.6×10^{-9}).

The same consequence was reported by Tripathi *et al.* [12], and they propose that this allele may be having some significant influence that is added up when it appears in a double dose.

A conspicuous hypothesis in the area of immune-related RSM is that some cases of RSM are due to a maternal immune response to the fetus and placenta. This hypothesis concludes that the placenta is in effect a foreign tissue (transplant) and that, ordinarily, mechanisms are in place to avoid a maternal immune response to this foreign tissue. It is theorized that in some cases of RSM these controlling mechanisms fail, permitting the maternal immune system to react to fetal antigens. One mechanism that might limit maternal immune reactions is immunosuppression of the populations of leucocytes extant at the maternofetal interface [25].

Expression of HLA-E at the feto-maternal interface plays an important role in the successful pregnancy due to its ability to downregulate maternal immune response [12]. In the decidua (within the epithelial tissue), the area of the interface where fetal-maternal interaction arises, CD56-positive NK cells are the main type of lymphocytes and express CD94: NKG2A complex, suggesting that HLA-E protein on the trophoblasts can be distinguished by the maternal immune cells. Therefore, the expression of HLA-E might play an important role in avoiding the fetus from being attacked by maternal immune system [26]. Evidence viewing the lower expression of HLA-E*0101 and its lower stability may weaken its ability to downregulate NK cells and therefore, may be associated with RSM [12].

Kanai *et al.* [23] theorized that the difference in HLA-E type between the mother and fetus might cause an unusual immune reaction and thereby stimulate pregnant complications including RSM. They tested this idea by examining the polymorphism of HLA-E protein in normal couples and couples with RSM in the Japanese population, and they discovered no significant difference in the spread of HLA-E alleles or in the number of shared HLA-E alleles between couples with RSM and those with confirmed fertility. However, more studies are needed to illuminate whether fetal HLA-E*0101 homozygosity is related to the decreased HLA-E expression on the trophoblast leading to unsuccessful suppression of decidual NK cells with anti-trophoblast activity.

It was revealed that HLA-E*0101 allele is imperceptible on the HLA-E*0101 cell surface [23, 24] and incomplete expression of this allele at the fetomaternal interface particularly when existing in a homozygous state as shown in this study may result in maternal NK cell stimulation, causing RSM. The existence of higher frequency of HLA-E*0103 allele and HLA-E*0103 homozygous genotypes in control than in patients with RSM may support the postulate that increases in the level of expression of HLA-E*0103 provide better suppression of NK cells and permit pregnancy maintenance in fertile controls.

The theory of HLA-E allele homozygosity association with diseases was described before in type1 diabetes

mellitus (DM) [27], nasopharyngeal carcinoma (cavity cancer) [28], HIV [29] and post-transplant complications [30, 31]. Tamouza *et al.* [30] was the first to report the relation between HLA-E*0101 homozygosity and serious bacterial infections following HLA-matched unrelated donor hematopoietic stem cell transplantation and concluded that the appearance of HLA-E*0101 allele could cause inefficient demonstration of bacterial peptides by the donor-derived antigen-presenting cells (APC). Furthermore, the higher frequency of HLA-E*0103 homozygosity detected in patients with nasopharyngeal cancer may supply enhanced inhibitory signals to NK cells that allow tumor salvation [28].

4. CONCLUSION

This study detected the higher frequency of homozygosity for HLA-E*0101 in Iraqi women with RSM. HLA- E*0101 homozygosity may thus be a dangerous factor for RSM. More studies are needed to explain whether fetal HLA- E*0101 homozygosity is associated with lower HLA- E expression on the trophoblast leading to ineffective suppression of decidual NK cells with anti-trophoblast activity.

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