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

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ABSTRACT

This particular exploration was done to determine allele's frequency of human leucocyte antigen (HLA) - E of Iraqi females residing in Baghdad with and without recurrent spontaneous miscarriage (RSM) to look at the functionality of theirs in the maintenance of gestation. A case control study was approved. Genotyping of HLA -E was carried out by restriction fragment length polymorphism (RFLP) for ninety four females with RSM and eighty seven fertile controls. HLA-E *0101 allele frequency was a lot more frequent in women with RSM while HLA- E*0103 allele was much higher in control groups, moreover, the real difference was statistically significant (P = 3 x 10^{-11}). HLA- E*0101/0101 genotype was much more frequent genotype in people (61.7 %), accompanied by HLA- E*0101/0103 (24.47 %) and lastly HLA E*0103/ 0103 genotype (13.83 %). The main difference in the frequency of HLA- E*0101/ 0101 homozygous genotype in females with RSM in comparison to fertile group was statistically significant, Odd Ratio (OR) = 6.63, 95 % Confidence Intervals (CI) = 3.23- 13.87, Probability (P) = 5.6 x 10^{-9}). This specific analysis was discovered to be additional frequency of homozygosity for HLA -E*0101 in Iraqi females with RSM. HLA- E*0101 homozygosity may, thus, be viewed as a risky factor for RSM.

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

1. INTRODUCTION

The foetus is often a semi allograft and therefore, the foetus varies to the mother genetically. Thus, the means of implantation may comprise mechanisms to avoid the refusal of the allograft, nonetheless, the imbalance of immunological tolerance can lead to pathological pregnancy, like impulsive miscarriage [1].

Unexplained recurrent impulsive miscarriage is frequently referred to as 3 or more sequential gestation losses prior to the 24th week of pregnancy after typical evaluation (parental karyotypes, frequent uterine cavity, endocrine and illness parameters), is assumed for a result of the immunological refusal of foetus by the mothers [2].

The human major histocompatibility (HLA) complex locus at giving chromosome 6p21 encodes the classical human leucocyte antigen class I protein (A, B and Cw) and additionally a' non-classical' HLA class Ib proteins as HLA- E, F, and HLA -G [3, 4]. HLA-E has a considerable tissue distribution and also plays an important action in the regulation of natural killer (NK) cell activity through the user interface of it is with inhibitory (CD94, NK G2A and CD94, NKG2B) and stimulating (CD94, NKG2C) NK cell receptors [5].

During gestation, the maternal body's immune system must endure the tenacity of allogeneic foetal cells inside the maternal cells. Foetal trophoblasts stay away from a terrible maternal immune response by not creating the classical HLA -A, HLA-DR, HLA-B, HLA-DQ and HLA-DP molecules which are truly the key objectives for allogeneic T cells. Nevertheless, trophoblasts display HLA- C and furthermore, the' non-

classical' HLA -E, HLA- G and, HLA- F molecules [6]. NK cell mediated cytotoxicity is therefore circumvented, but HLA -C is very polymorphic histocompatibility antigen that could, additionally, result in a cytotoxic T cell response [7, 8].

The appropriate appearance of HLA- E in the trophoblast is very essential to assist trophoblasts assaulting maternal decidua furthermore to vascular system; as an outcome, there is an increase in uterine perfusion that is vital during pregnancy.

Nevertheless, if the HLA- E was reduced but not being conveyed, the trophoblast cells strength is reduced and inhibited from invading the uterus since it had been considered non self as antigens which trigger the generation of antibodies in the mother. These antibodies are regarding antigens, and immunological outcome happens which trigger stimulation of pro inflammatory cytokines, triggered T cells, and natural killer cells (NK) which will attack trophoblast cell itself, leading to failure of the pregnancy produce [9].

HLA-E is a fairly polymorphic with two non-identical alleles termed up to right now (HLA-E*0103 and HLA-E*0101). HLA-E*0103 allele differs from HLA- E*0101 by an amino acid replacing (glycine to arginine at position 107 of the 2-α heavy chain domain) and additionally by a cell surface expression [10, 11]. In the brightness of the potential effect of HLA- E in the maintenance of pregnancy, it's essential to learn the biology of the different alleles of HLA- E [12].

Thus, the target of the evaluation was investigative the HLA -E gene polymorphism of Iraqi females with RSM and normal fertile females to look at the impact of HLA-E alleles on upkeep of pregnancy.

2. MATERIALS AND METHODS

2.1 Patients and controls

A case control evaluation was conducted which included 300 female people are recruited from outpatient's clinics of Obstetrics and Gynecology Department, Al-Elwiya and Al-Yarmouk teaching Hospital. The median age of those was 29.23 years (range 19 to 39), and the quantity of RSM was four (range 3- 6). All selected women weren't have any prior live births (primary miscarriage). Eighty-seven healthy Iraqi women with no history of last miscarriage, residing in comparable geographic place as individuals and in addition has exactly the same ethic origin, had been considered as controls. The mean age of control was 29.89 years (range 20- 38). Five millilitres of blood was used out of both females and those influence with RSM. The Written educated contract was from the patients and fertile controls after approving the analysis process by local ethical committee.

Regular menstruation was had by the individuals and underwent examinations into various known causes of RSM like Anticardiolipin antibodies (IgM), antiphospholipid (IgM), Coagulation factors impacting protein C, protein S, Antithrombin III, activated protein C resistance, and exploration of toxoplasmosis antbodies (IgM) additionally cytomegalovirus antibody (IgM), rubella antibody (IgM), activated partial thromboplastin time (APTT) in addition to homocysteine level. Only 94 unique from 300 were selected because of this research since they provided normal values for previous assessments. So, they considered as situations of unexplained recurrent impulsive miscarriage.

2.2 DNA extraction

Total genomic DNA from both groups organizations had been collected in tubes with EDTA was extracted as well as pure using Wizard Genomic DNA Purification Kit (Promega, USA), WI, Madison, and adhering together with the producer protocol.

2.3 Amplification of DNA fragments and RFLP - PCR technique:

Analysis of the single nucleotide polymorphism (SNP) of the HLA- E gene was carried out by utilizing restriction fragment length polymorphism (RFLP) analysis. The polymorphism in HLA- E locus is basically restricted to HLA E*0101 and HLA- E*0103 alleles, therefore they differ by amino acid replacing for codon hundred seven in exon3. The codon hundred seven in arginine is encoded by E*0101 (called ER107) and also in E*0103 encodes glycine (called EG107). The genomic disparities between the two HLA -E alleles are already produced by using PCR - RFLP analysis. The DNA amplified item is cut into fragments of various measurements by restriction enzymes, leading to pieces are separating based on the measures of theirs by gel electrophoresis. The appearance of the HLA-E*0103 allele was distinguished by the presence of a restriction site for *HpaII* enzyme, which often cuts the HLA- E*0103 allele into 2 fragments, (260 + 20) bp, and leaves the HLA- E*0101 allele without cutting giving a band 280 bp.

Genomic DNA was amplified by PCR technique utilizing the following primers (forward and also reverse) as mentioned before [13]. The Sequence of primers (alpha DNA) was utilized as follows: advanced 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 25µl reaction volumes: 2µl (1M) of each primer, 12.5 µl Green Go Taq master mix, 5µl gDNA (100ng/µl) as well as 5.5 µl of H2O. The PCR conditions were made up of 35 cycles: 94C for 45 s, 61 C for 45 s and 72 C for 45s with a very last extension at 72 C for 7 min. The PCR products are broken down together with the Restriction endonuclease *HpaII* enzyme (10.000 U? ml; New England Biolabs Inc., MA, Ipswich, USA) is able to cleave amplified DNA at some sites in every exon. Master mix (total amount ten µl) was prepared as follows 1 µl of *HpaII* enzyme, 5µl PCR product, 1µl enzyme buffer and 3µl H2O.The enzyme was incubated with the PCR products at 37 C for at least 4 hrs. 5 µl of PCR products are combined with 2 µl loading buffer in addition to fragment separation was performed by3 % agarose gel and visualized by staining with ethidium

bromide and also then put through ultraviolet light. The PCR product was recognised in the look 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 any E*0103 allele (Fig. 1).

2.3 Statistical analysis

Statistical significance of the big difference between the individuals and healthy control group was analysed by independent samples *t*- test. Odds Ratio (OR), in addition to 95 % confidence intervals (CI), have been analysed by Fisher's exact test. The probability was corrected for numerous comparisons by Bonferroni. Statistical significance was established for P- value≤ 0.05

3. RESULTS AND DISCUSSION

The frequency of HLA- E *0101 as well as *0103 alleles are in contrast to individuals with fertile group and RSM (Table 1). The HLA- E*0101 allele was much more frequent in females with RSM (73.94 %) than in women that are fertile (39.66 %), and HLA- E*0103 allele was much higher in fertile females (60.34 %) than in females with RSM (26.06 %). The actual effect on the frequency of HLA- E alleles between the two groups had been statistically significant ($P = 3x10^{-11}$).

When examining the distribution of HLA- E genotypes in females with RSA and control groups, HLA E*0101/ 0101 genotype was received a greater recurring genotype in females with RSM (61.7 %), accompanied by HLA E*0101/ 0103 genotype (24.47 %) and at last HLA E*0103/ 0103 genotype (13.83 %). Relating to control groups, HLA E*0101/ 0103 and 0103/ 0103 genotypes have been an equivalent frequency (40.23 %). While HLA E*0101/ 0101 genotype has a reduced frequency (19.54 %). The variance in the frequency of HLA- E*0101/0101 homozygous genotype in females with RSM when as contrasting with which in the fertile class was statistically significant after correction for many 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

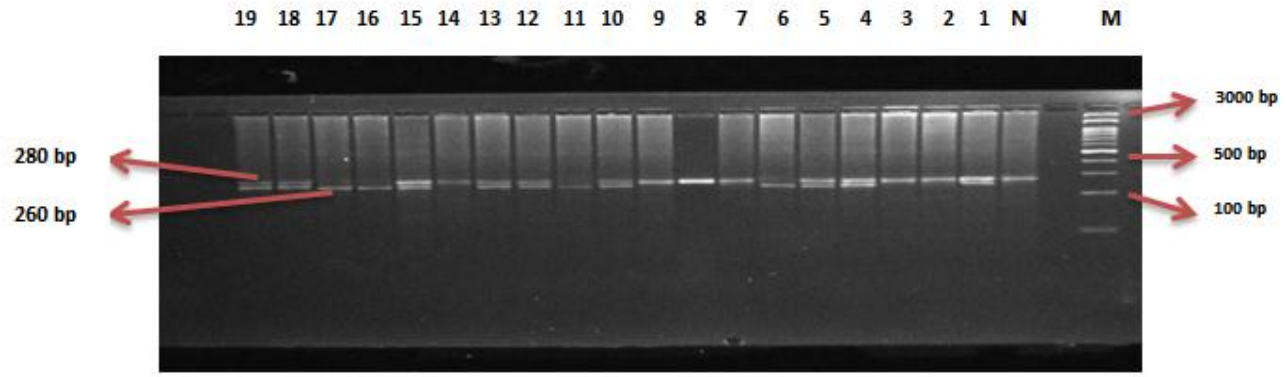

Figure 1: A photograph of 3 % agarose gel demonstrating the PCR -RFLP product for exon 3 of HLA-E gene after digestion with *HpaII* restriction enzyme. Lane M: 100/3000 bp mixed DNA marker, N: negative control, lanes (1,4,5,10,12,13,15,18,19) indicate the appearance of heterozygous HLA E*0101 allele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

HLA-E is a non classical HLA type I gene with two adversative products HLA-E*0101 and HLA-E*0103. They're discovered in numerous tissues [14], HLA-E*0103 being conveyed at drastically higher amounts compared to HLA-E*0101. These variations depend on the affinity for accessible peptides also as on the balance of refolded complexes [11].

The HLA- E expression is recognized in B cells, T cells, triggered T lymphocytes and also other cells like placenta and trophoblast cells [15, 16 and 17]. HLA-E expression at the maternofoetal interface is revitalizing due to trophoblast absences all many other classical class I antigens and expresses the HLA -E accompanied by HLA- G and also almost as certain extent HLA- C. The HLA -E expression here might be involved in immunomodulation of maternal immune reaction to the foetus [12].

Surface expression of HLA- E needs incredibly preserved peptide made out of the signal sequence of various other class I molecules including HLA-C, HLA-G, HLA-B, and HLA-A but not HLA- F [18]. The precision of any peptide is very essential, as the only proper peptide is loaded on HLA- E allowing expression and then a defence of target cells by an interaction of HLA- E/ peptide complex as well as CD94/ NKG2 receptor of NK cells [11, 19].

The consequences of the present work discovered that HLA- E*0101 allele is regarded as the regular allele present in our learned populations that the HLA- E allele frequencies were much like those earlier talked about in numerous populations as Africans [13], Caucasian, African American in addition to Hispanic populations [t20], Indians [12] Euro-Caucasoid and Afro Caribbean, and Indo Asian [5]. Conversely, the gene frequency of HLA- E*0103 is drastically greater compared to HLA- E*0101 allele in the Japanese [20] and Chinese populations [21].

In the existing investigation, the frequency of HLA-E*0101 allele was drastically greater in females with RSM and definitely the frequency of HLA- E*0103 was substantially greater in a fertile group (P= 3x10 $^{-11)}$. A few prior scientific tests was conducted to calculate the significance of HLA- E alleles in the RSM and discovered non-significant associations in contrary to the outcomes of the existing study [22 ,24]. Nevertheless, the work of Tripathi *et al.* [12] on Indians found a greater frequency of HLA- E*0101 in comparison to HLA- E*0103, therefore the variance was considerable *(*P = 0.043). The differences in the distribution of HLA-E alleles between various studies could be due to ethnic variation.

In the present analysis, a major correlation was discovered between homozygosity and RSM of HLA-E*0101 allele (odd ratio (OR) = 6.63, confidence interval 95 % CI = 3.23 -13.87, P = 5.6 x 10 $^{-9}$). Exactly precisely the same outcome was discussed by Tripathi *et al.* [12], and they claim which this specific allele may be having a number of significant impact that's loaded when it seems like in a double dose.

A conspicuous theory in the region of immune related RSM could be the point that certain cases of RSM are due to a maternal immune response to the foetus as well as placenta. This particular theory concludes the placenta is outcome foreign tissue (transplant) which, ordinarily, mechanisms happen to be in a place to avoid from a maternal immune response to this particular foreign tissue. It's theorized that in a number of situations of RSM these managing systems are unsuccessful, permitting the maternal immune system to react to foetal antigens. A mechanism that could limit maternal immune responses is immunosuppression of the populations of leucocytes extant at the maternofoetal interface [25].

Expression of HLA -E at the fetomaternal interface plays an important impact in the effective pregnancy resulting from the capability of it's to downregulate maternal immune response [12]. In the decidua (within the epithelial tissue), the component of the interface where foetal maternal interaction develops, CD56 positive NK cells have become the main kind of lymphocytes and also convey CD94: NKG2A complicated, suggesting that HLA- E protein on the trophoblasts might be distinguished by the maternal immune cells. Therefore, the expression of HLA E might play an important component in staying away from the foetus from being assaulted by maternal body's immune system [26]. Research looking at lowering the expression of HLA- E*0101 together with the lower stability of it is could weaken the ability of it to downregulate NK cells and so, is regarding RSM [12].

Kanai *et al.* [23] theorized the difference in HLA E types between the mother and foetus might produce an abnormal immune response and thereby trigger expectant complications like RSM. They evaluated the idea by looking at the polymorphism of HLA -E protein in normal couples and couples with RSM in the Japanese publication, and also found no substantial difference in the spread of HLA- E alleles or even in the variety of shared HLA -E alleles between couples with individuals and RSM with confirmed fertility. Nevertheless, far more experiments have to offer whether foetal HLA- E*0101 homozygosity with the reduced HLA- E expression on the trophoblast leading to unsuccessful suppression of decidual NK cells with anti- trophoblast activity.

It was learned that HLA- E*0101 allele is imperceptible on the HLA -E*0101 cell surface [23, 24] and unfinished expression of this specific allele at the fetomaternal screen particularly when contained in a homozygous status as found in this specific study may result in maternal NK cell stimulation, resulting in RSM. The presence of greater frequency of HLA -E*0103 allele and also HLA- E*0103 homozygous genotypes in command than in females with RSM may help support the suggestion that increases in the amount of

expression of HLA- E*0103 provide far better suppression of NK cells as well as permit pregnancy preservation in fertile settings.

The idea of HLA- E allele homozygosity connection with diseases was talked about earlier 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 explain the relation between HLA- E*0101 homozygosity and acute bacterial infection to follow HLA matched not related donor hematopoietic stem cell transplantation and then realized the look of HLA -E*0101 allele may result in a bad demonstration of bacterial peptides by the donor derived antigen presenting cells (APC). Furthermore, the higher frequency of HLA -E*0103 homozygosity discovered in people with nasopharyngeal cancer might supply enhanced inhibitory signals to NK cells which allow tumor salvation [28].

4. CONCLUSION

This particular study detected the greater frequency of homozygosity for HLA -E*0101 in Iraqi females with RSM. HLA- E*0101 homozygosity may, thus, be regarded as a harmful element for RSM. More scientific research have to explain whether foetal HLA- E*0101 homozygosity is associated to lower HLA- E expression on the trophoblast triggering insufficient suppression of decidual NK cells with anti -trophoblast activity.

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