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ORGINAL ARTICLE

GENETIC POLYMORPHISMS OF HLA-G ANTIGEN IN IRAQI WOMEN WITH UNEXPLAINED RECURRENT MISCARRIAGE.

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Abstract

This study was designed to search for HLA-G polymorphisms and its relationships with unexplained recurrent spontaneous abortion (URSA) among Iraqi women. In this study 300 aborted women have been chosen and they had history of recurrent spontaneous abortion as case group; in all cases full history and complete examinations including body weight, height and body mass index were done. Furthermore, all of the patients were screened for various known causes of miscarriages, including Anticardiolipin antibodies (IgM) , antiphospholipid (IgM), Coagulation factors including protein C, protein S, Antithrombin III, activated protein C resistance(APCR), and investigation of toxoplasmosis antibodies (IgM) and cytomegalovirus antibody (IgM), rubella antibody (IgM), as well homocysteine level , TSH and progesterone hormones. Based on the results of the screening test 33.6 % of cases are of unknown cause, So out of 300, only 100 case were enrolled in this study to investigate the association between RSA and three HLA-G alleles (HLA-G*0103, HLA-G*0104 and HLA-G*0105N) and 14-bp insertion/deletion polymorphism in exon 8 of the 3untranslated region (3 UTR)frequency and genotypes in idiopathic RSA in Iraqi women compared with 100 control women without previous history of RSA, with at least two successful pregnancies In addition, the ELISA was conducted to investigate HLA-G serum levels in women with URSA and healthy women.

The frequency of the allele and genotypes in the patient group and control group was determined using PCR-restriction fragment length polymorphism (PCR-RFLEP). Exon 3 of *HLA-G* gene amplified using specific primers then digested by *PpuM-I* and *BseR-I* restriction enzymes, in order to detect *HLA-G*0105N* and *HLA-G*0104 alleles* respectively. While exon 2 of *HLA-G* gene amplified using specific primers then digested with *HinfI* restriction enzyme to detect *HLA-G*0103* allele. Genotypes for the mentioned alleles determined and tested for their association with URSA patients. Results of *HLA-G* allele frequency showed significant association with increased risk of URSA patients. *HLA-G*0105N* and *HLA-G*0104* allele was present in URSA patients and fertile controls. HLA-G*0105N allele was found in 48.7% of the patient group while, it was present in 51.3% of the control group, odds ratio was 11.5759. HLA-G*0104 allele was present with high significant frequency of 71.3% in the patient group and 28.7% in

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the controls, odds ratio was 4.8947. While HLA-G*0103 allele was not encountered neither in the patient group nor in the control group.
Aforementioned alleles genotyped, and two genotypes were encountered in both URSA patients and fertile control groups (*HLA-G*0105N/0105N* and *G*0104/0104*) whereas the third genotype (*HLA-G*0104/0105N*) was absent in the control group. *HLA-G*0104/0105N* genotype was interesting as it was evident in 10 patients (100%) whereas none of the fertile controls showed this genotype. Other genotypes presented in both URSA patients and fertile controls**.** *HLA-G*0105N/0105N* genotype showed the frequency (12%) in patient groups and (13%) in control groups with odds ratio (0.9126) in both groups, (30%) of the patients revealed in *HLA-G*0104/ 0104* genotype whereas (27%) of the control group harbored this genotype with odds ratio 1.1587.

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Introduction:-

The human leukocyte antigen-G gene (*HLA-G*) is located on the short arm of chromosome 6 within the *HLA* region . It consists of 7 introns and 8 exons that code for the heavy chain of the HLA-G molecule. Exons 7 and 8 are always absent in the mature mRNA because of the presence of a stop codon in exon 6 **(1).** Seven expressed isoforms have been described, 4 of which (HLA-G1-G4) are membranous and 3 (HLA-G5-G7) of which are soluble molecules (**2**). The proteolytic cleavage of the HLA-G1 isoform generates the soluble HLA-G1 form (**3**). Compared to the classical HLA molecules, the *HLA-G* gene contains a modest 46 polymorphisms that map to either the coding or non-coding regions. At the protein expression level, only 15 variants have been reported (**4**). The polymorphisms mapped to the non-coding regions, particularly those in the 5' upstream regulator region (5' UTR) and 3' untranslated regions (3' UTR), reportedly influence the function of HLA-G molecules and have been implicated in some pathologies such as infertility, preeclampsia, failure in *in vitro* fertilization, and RSA **(1,5).** Several studies have demonstrated the importance of the 3' UTR in the *HLA-G* expression profile **(6,7)**. This region contains several regulatory elements, including a poly-A signal and AU-rich motifs involved in maintaining mRNA stability and isoform alternative splicing patterns, which may influence the function of HLA-G, particularly during pregnancy (**8**)gly, the 14-base pair (bp) (5'-ATTTGTTCATGCCT-3') insertion/deletion (indel) polymorphism mapped to position 3741 in the 3' UTR of exon 8 has gained interest (**6**). Thus, several reports have indicated that this indel polymorphism is related to *HLA-G* mRNA stability and splicing patterns involved in generating HLA-G isoforms (**6,9,10**). Moreover, the 14-bp insertion allele was reported to be associated with low levels of both *HLA-G* mRNA and circulating soluble HLA-G (sHLA-G) isoforms (**10,11**). It was also reported that plasma levels of sHLA-G were dramatically lower with the genotype +14-bp/+14-bp than with +14-bp/-14-bp and -14-bp/-14-bp genotypes (**7,10**). Thus, based on the results of several studies, the HLA-G molecule is considered to be a key player during early and mid-term pregnancy by contributing to the maintenance of gestation throughout pregnancy (**8,12,13**).

RSA was initially defined as death of the fetus before 24 weeks of gestation at least 2 consecutive times (**14**). It occurs in 2-4% of reproduce tive-aged women, of which 40-55% cases remain unexplained (**15,16**). RSA is one of the most common complications associated with early pregnancy and remains a challenge in gynecology (**17**). Different potential etiologic factors have been implicated in this condition, such as endocrine regulation, autoimmune reaction, thrombophilia, environmental, psychological, and genetic background, and viral infections (**15,18,19**).

The interface between the fetus and mother may contribute to the development and maintenance of the pregnant uterus as an immune-privileged site. In fact, the immunologic relationship between the mother and the fetus is determined by fetal antigens and the maternal immune system. Inadequate recognition of fetal antigens may result in failed pregnancy (**20,21**). Since its discovery, the crucial role of the non-classical HLA-G molecule in pregnancy success has been demonstrated (**22,23**). Various studies have reported that HLA-G molecules are responsible for maintaining the immune-regulated and tolerogenic environment during pregnancy (**8,24,25**). Indeed, these molecules are predominately expressed on extravillous cytotrophoblasts at the fetal-maternal interface during pregnancy (**26**). Currently, there is convincing experimental support for expression of HLA-G molecules conferring protection against cytolysis mediated by different maternal cytotoxic subpopulations, such as those of the natural

killer cells, T lymphocytes, and dendritic cells. Another study indicated that altered expression of HLA-G molecules is associated with RSA (**27**).

In this study, we examined the association between successful and unsuccessful pregnancies and the *HLA-G* 14-bp insertion/deletion polymorphism using 2 groups of women: an URSA group and a normal, fertile control group of unrelated women in a Iraqi population.

Material And Methods:-

Subjects:-

This study included 100 cases of Iraqi women (mean age 34.18 ± 6.22 years) who had URSA and had consulted the Al-yarmouk teaching Hospital, Baghdad, Iraq, between April 2014 and June 2015. The control group included 100(mean age 34.67 ± 7.75 years) unrelated, normal fertile Iraqi women with 2 or more uncomplicated pregnancies, without a history of RPL, and with at least 2 live births. Patients with anatomical, endocrine, or metabolic disorders or immunodeficiency and autoimmune diseases were excluded from the study. Ethical approval for the study was obtained from the medical ethics committee of Al-Mustansiryia medicine College /Al-Yarmouk Hospital. All patients and controls provided informed consent and agreed to give blood samples for this case-control study.

PCR programs optimization:-

Total genomic deoxyribonucleic acid DNA was extracted from peripheral blood using the blood DNA isolation kit of Promega Company Several PCR programs were adopted during this study. First, optimization program shown in table (2-3) was applied to all primers with gradient temperature in the range of (53-65 ºC). After determination of the most perfect annealing temperature for each primer, a new program was set for each of the primers. A successful PCR is achieved when a single sharp band with a specific molecular size appears on the gel following electrophoresis. Analysis of the single nucleotide polymorphism of the HLA-G gene were done by using restriction fragment length polymorphism (RFLP) analysis. PCR reactions were performed using the followings Specific primers were provided by (Promega) for each exons (2,3,8) designed for HLA-G . All primers were supplied as a lyophilized product of different picomols concentrations. Lyophilized primers must be dissolved in a free nuclease water to give a final concentration of 100 Pmole/µl and kept as a stock in -20ºC as recommended by the provider. To prepare 10µM concentration as work primer re-suspend 10 µl in 90 µl of free nuclease water. Exons, restriction enzymes, the size of digested fragments that are used for screening by PCR-RFLP and alleles for HLA-G gene polymorphisms was performed according to Tamam and Fadel(**28).**The Primer sequence of HLA-G exon 2 was Forward: 5'- TCC ATG AGG TAT TTC AGC GC -3'.Reverse : 5'- CTG GGC CGG AGT TAC TCA CT -3'.For exon 3 , Forward: 5′- CAC ACC CTC CAG TGG ATG AT -3′ ,Reverse: 5′- GGT ACC CGC GCG CTG CAG CA -3′.While for exon8 :Forward : 5'-GTG ATG GGC TGT TTA AAG TGT CAC C -3' and Reverse : 5'-GGA AGG AAT GCA GTT CAG CAT GA -3'.Ampilification was done using Go Taq®Green Master Mix which contains pure deoxynucleotiedes (dNTPs) , bacterially derived Taq DNA polymerase , $MgCl_2$, reaction buffer and two dyes (blue and yellow) that allow the monitoring of progress during electrophoresis. In order to reduce the risk of contamination and achieve homogeneity of reagents all Amplification must be performed on ice in aseptic conditions in laminar air flow by using 200 µl tight cap Eppendorf tubes.

Determination exon 2 of HLA –G:-

The DNA samples were amplified for exon 2 by using a pair of specific primers as reported by (28) . Using polymerase chain reaction (PCR), PCR was performed in a total volume of (25 µl),and the PCR cycling conditions were as follows: Initial denaturation temp = 95C min at 5 min,Denaturation 95C for 30 sec and annealing temperature 58C for 30 sec and extension temperature at 72C for 30 sec and the final extension step was 72C for 5 min (35 cycle).The PCR products and the ladder marker were resolved by electrophoresis on 1.5% agarose gel. The electrophoresis was undertaken at 70 V for 2 h and gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain. DNA ladder (100bp DNA ladder) was used to estimate the molecular size of the bands**.**PCR products digestion reactions were performed on ice by using restriction endonuclease enzymes ***Hinf-I*** , and the mixture was incubated at (37 ^{0}C) for 1 hour in water bath. Digestion products were run on 1.5% agarose gel after staining with ethidium bromide, 5 µl of the mixture was taken and loading it in the wells. Run at (70V/cm^2) for 2hrs , 1X TBE buffer was used, then it was visualized under UV light using ultraviolet transilluminator.The size of the digestion products was then determined by electrophoresis on agarose gel. The wild-type of exon 2 gene product was detected as a 281-bp band. For HLA-G*0103 allele detected by presence of 175 bp band **.**

Determination the exon 3 of HLA-G:-

The DNA samples were amplified for exon 3 by using a pair of specific primers which were demonstrated by using polymerase chain reaction (PCR), PCR was performed in a total volume of (25 µl)**,** and the PCR cycling conditions were as follows: Initial denaturation temp = 95C min at 5 min,Denaturation 95C for 30 second, annealing temperature 61C for 30 sec and extension temperature at 72C for 30 sec and the final extension step was 72C for 5 min (35 cycle**).**

The PCR cycling conditions were as optimized and the PCR products and the ladder marker (100bp DNA ladder) were electrophoresed on 1.5% agarose gel after staining with ethidium bromide. The electrophoresis was undertaken at (70 V/cm^2)for 2 h and gel viewed under UV trans-illuminator .PCR products of promoter polymorphisms for exon3 (276 bp) was digested with ***Ppum-I*** *and* ***BseR-I*** restriction endonuclease enzymes and were performed on ice. The wild-type of exon 3 gene product was detected as a 276-bp band. For HLA-G*0105N allele detected by presence of 276 bp (not digested) band, the same for presence of HLA-G*0104 allele produce 276 bp band

Determination the exon 8 of HLA-G:-

The extracted DNA samples were amplified for exon 8 by using a pair of specific primers, as demonstrated, by using polymerase chain reaction (PCR). The reaction volume was (25 µl) .The PCR cycling conditions were as optimized and the PCR products and the ladder marker (25/100bp mixed ladder) were electrophoresed on 3% agarose gel. The electrophoresis was undertaken at 70 V for 2 h and the gel was viewed under UV trans-illuminator after staining it with ethidium bromide stain.

Results:-

In this case-control study, we examined the distribution of alleles and genotypes of the most commonly studied *HLA-G* 14-bp indel polymorphism among women with at least 3 unexplained RSA and healthy women with at least 2 live births and without a history of RSA. Clinical and demographic characteristics of the URSA and control subjects are reported in Table 1. We observed that among RSA subjects, the number of spontaneous abortions varied from 3 to 6. The percentage of women with 3, 4, 5, 6, 7, and 8 RSAs were 37.5, 26.56, 17.2, 4.7, 6.25, and 8%, respectively. Within the RSA group, 36% of women had no children (100% abortions), while for the remaining women, the loss rate was greater than 50%.

Table 1: Clinical and demographic characteristics of the cases and control subjects.

Characteristic	Cases	Controls	P value
Mean age (years)	29.23±4.76	29.89±5.22	p>0.05
body mass index BMI(Kg/m^2)	22.37±4.24	22.78±3.88	p>0.05
Menarche (years)	13.46±1.62	13.35±1.57	p>0.05
Irregular menstrual history %	65	15	p<0.01
Number of pregnancies	4.37±1.22	2.6±1.38	p<0.01
Abortion	3.5±0.72	0	p<0.001

Serum HLA-G levels in patient with URSA

The estimated levels of serum HLA-G for unexplained recurrent spontaneous abortion (URSA) women were 19.5±2.34 pg/ml (mean ± S.D) while in healthy pregnant women were 35.6±3.12 pg/ml within first trimester. The difference was statistically highly significant ($p < 0.001$). In this study, we found that the median maternal serum sHLA-G levels in the women with missed abortions were lower compared with the healthy controls matched by maternal age, BMI, and gestational age.

Isolation of Genomic DNA:-

A single sample of whole blood with anticoagulant was collected from a group of 100 URSA cases with unexplained cause in addition from 100 healthy women**.** The tubes of blood were stored at -20C, prior to DNA isolation and the blood samples were thawed and mixed well and an aliquot was used for DNA isolation. The blood samples were then placed back into the freezer and stored again at -20C for further isolation when required. The isolated DNA was loaded onto agarose gel together with a DNA concentration series of 60-500 ng/µl. Following electrophoresis and Ethidium bromide staining, the isolated DNA had migrated to the same position as the DNA of the standard series. It was a single well defined band without streaking, indicating the absence of DNA fragmentation. From the concentration series the DNA concentration was determined with a final concentration of

60ng/µl. The purity of the DNA was determined by measuring the absorbance at 260 and 280 nm and calculating the ratio of 260/280 nm. The ratio was 1.8 which was within the range 1.7-2.0 required for DNA that was used for further molecular diagnostics.

Amplification of exons 2, 3 and 8 of HLA-G gene:-

Polymerase chain reactions was performed for the amplification of the exons 2,3 and 8 in HLA-G gene in all URSA patient and control groups. Agarose gel electrophoresis was used to confirm this amplification for each exon following optimization experiment when sharp, single bands with the accurate molecular sizes were obtained for each exon. The following figures demonstrate the amplified exons of HLA-G gene which are: exon 2 (figure 1), exon 3 (figure 1) and exon 8 (figure 3). Amplification was achieved for all patients and controls..

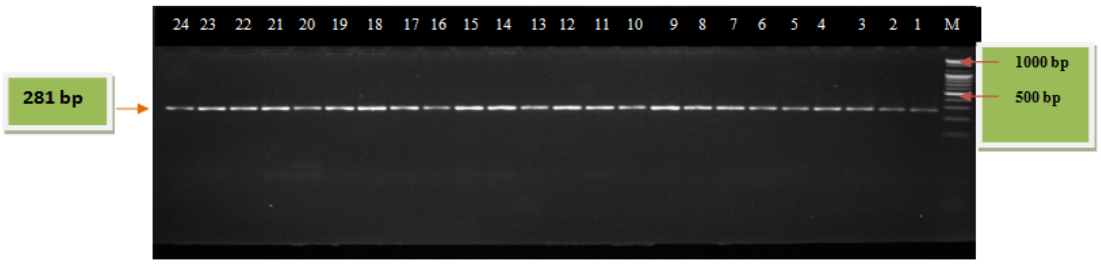

Figure 1: The PCR products and the ladder marker were resolved by electrophoresis on 1.5% agarose gel. The electrophoresis was undertaken at 70 V for 2 h and gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain. DNA ladder (100bp ladder) was used to estimate the molecular size of the bands. PCR products of exon 2 of HLA-G gene of the molecular size 281 bp. M: Marker, lanes 1-5: control samples, lanes 6-24: patients sample.

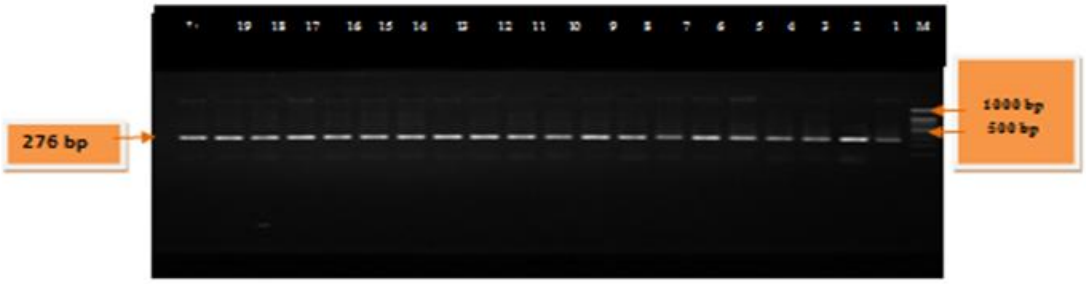

Figure 2: The PCR products and the ladder marker (100bp ladder) were electrophoresed on 1.5% agarose gel. The electrophoresis was undertaken at 70 V for 2 h and gel viewed under UV trans-illuminator after staining it with ethidium bromide stain. PCR products of exon 3 of HLA-G gene of the molecular size 276 bp. M: Marker, lanes 1-5: control samples, lanes 6-20: patient samples.

Figure 3: The PCR products and the ladder marker (25/100bp mixed ladder) were electrophoresed on 3% agarose gel. The electrophoresis was undertaken at 70 V for 2 h and the gel was viewed under UV trans-illuminator after staining it with ethidium bromide stain. The wild-type exon 8 gene product was detected as a 224-bp band. M: Marker, lanes 1-5: control samples, lanes 6-19: patient samples.

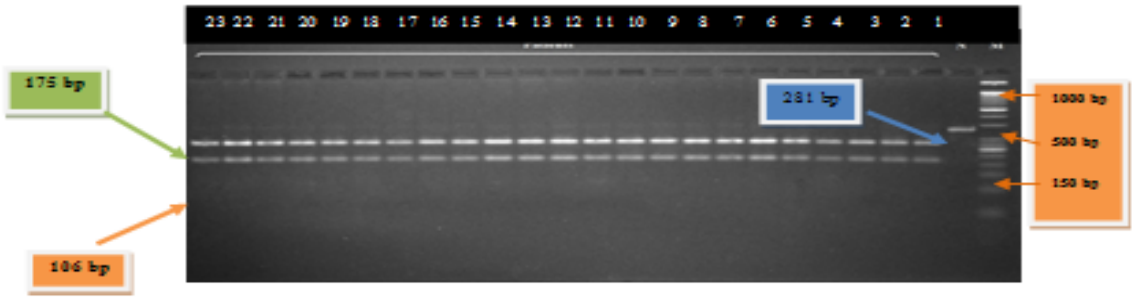

Figure 4: A photograph of 1.5% agarose gel showing the PCR-RFLP product for exon 2 of *HLA-G* gene after digestion with *Hif-I* restriction enzyme. Gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain . Lane M: 25/100 bp mixed DNA Marker . Lane N: DNA before cutting. Lane 1-23: Two fragments (175+ 106) bp band indicate absence of *HLA-G*0103* allele in patient groups.

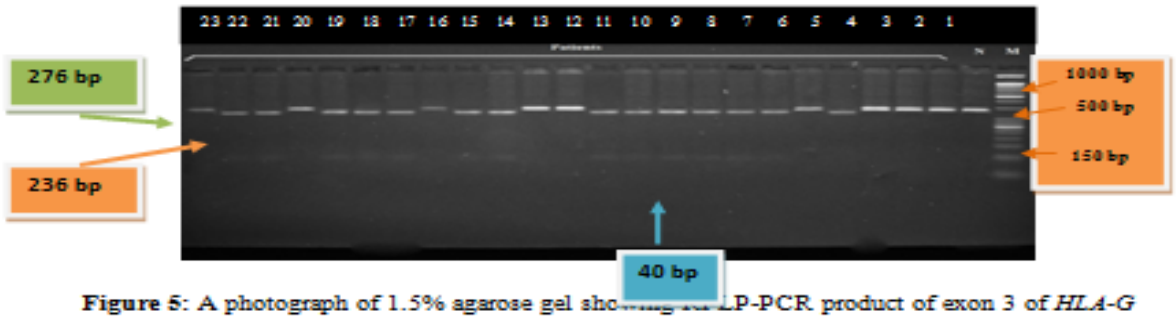

Figure 5: A photograph of 1.5% agarose gel showing the PCR-RFLP-PCR product of exon 3 of *HLA-G* gene after digestion with *Bsgr-I* restriction enzyme. Gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain Lane M: 25/100 bp mixed DNA ladder,Lane N :DNA before cutting , lanes 4,6-11,14,15,17-19,21,22 : 236 bp band indicate absence of *HLA-G*0104* allele, lanes 1-3,5,12,13,16,20,23: 276 bp band indicate presence of *HLA-G*0104* allele.

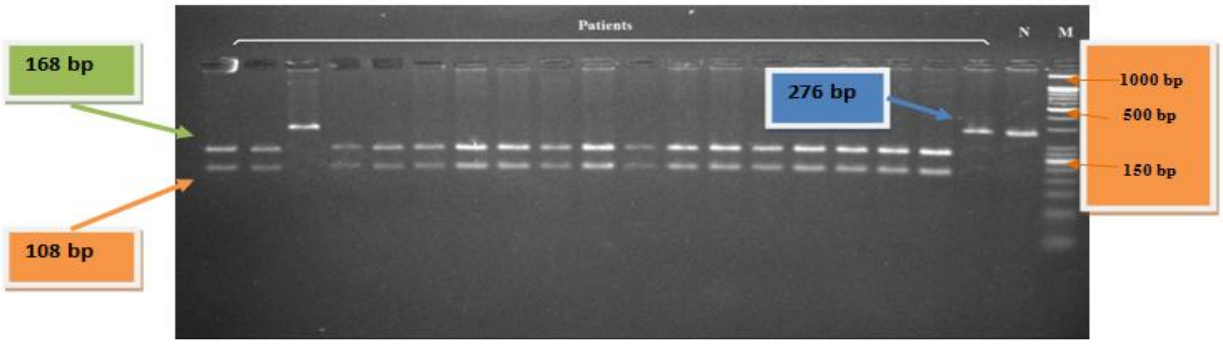

Figure 6: A photograph of 1.5% agarose gel showing the PCR-RFLP product for exon 3 of HLA-G gene after digestion with *PpuMI* restriction enzyme. Gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain Lane M: 25/100 bp mixed DNA marker ,N: DNA before cutting, lanes 1,17 : 276 bp band indicate presence of HLA-G*0105N null allele, lanes 2-16,18,19 : 168+108 bp bands indicate absence of HLA-G*0105N null allele.

PCR- RFLP genotyping of exons 2 and 3 of HLA-G gene:-

Exon 2 was amplified using the specific primers and the PCR product was then digested by *HinfI* restriction enzyme to produce fragments with 79, 175 and 27 bp bands in the presence of HLA-G*0103 allele (Table 2), while production of 106 and 175 bp bands indicate absence of HLA-G*0103 allele. Exon 3 of HLA-G gene was amplified

under the conditions previously described using primers. The PCR product was then subjected to RFLP analysis using *PpuM-I* and *BseR-I* restriction enzymes to detect HLA-G*0105N and *HLA-G*0104* alleles, respectively.

Table 2: HLA- G polymorphic motifs : presence of HLA-G*0105N is indicated by the presence of 276 bp band after digestion with *PpuMI*, HLA-G*0104 is evident by the presence of 276 bp band after digestion with *BseRI*, and HLA-G *0103 is revealed by presence of 79+175+ 27 bp bands after digestion with *HinfI*.

Restriction enzymes	*HLA-G*0105N*	*HLA-G*0104*	*HLA-G*0103*
PpuMI	+ **(276)**	- **(108+168)**	- **(108+168)**
BseRI	- **(40+ 236)**	+ **(276)**	- **(40+ 236)**
HinfI	- **(106+ 175)**	- **(106 + 175)**	+ **(79+ 175 + 27)**

following figures are representative examples of the *HLA-G* gene alleles (*HLA-G*0103*, *HLA-G*0105N* null allele and *HLA-G*0104*) investigated in this study. HLA-G, a gene with few alleles, rather than the highly polymorphic *HLA-A* and *-B* genes, are the predominant HLA expressed by fetal tissues at the feto-maternal interface. The HLA-G antigen is thought to play an essential role during pregnancy by protecting the semi-allogeneic fetus from recognition and destruction by maternal immune cells (29) and altered expression of *HLA-G* on the fetal extravillous cytotrophoblast has been implicated in the etiology of recurrent miscarriages (30).The frequencies of HLA-G alleles illustrated in table 3-4 (HLA-G*0104, HLA-G*0105N and HLA-G*0103). HLA-G*0104 alleles were found in both groups with higher frequency reach statistical significance (p= 1.7×10^{-12}), it was 60.0% of the patient groups while, the frequency was 25.0 % in the control groups, odds ratio was 4.50 with EF = 0.47 and (95% C.I.: 2.94 - 6.89). HLA-G*0105N allele was present only in patient groups with a frequency of 3.0%, odds ratio was 13.40. This high odd ratio indicates 13 times more frequently an abortion develops in individuals carrying the allele as compared to the frequency of the allele in individuals lacking it. The confidence interval of HLA-G*0105N was very wide (95% CI: 0.76-237.87) therefore a larger sample for the 2 selected groups is needed to confirm this satiation. *HLA-G*0103* allele was not detected neither in the patient group nor in the control group. It seems that *HLA-G*0103* allele is rare in our population and the small sample size investigated here could not detect that allele. Other alleles (not studied) were demonstrated high PF= 0.60 may contribute to confer protection from abortion.

Table 3: Table: Observed numbers and percentage frequencies of HLA-G alleles in unexplained recurrent spontaneous abortion patients and controls**.**

HLA-G Genotypes	Patients (No.=100)		Controls (No.=100)		Odd Ratio	EF or PF	*p*	95% C.I.
	No.	%	No.	%				
0104	120	60.0	50	25.0	4.50	0.47	1.7×10^{-12}	2.94 - 6.89
0105N	6	3.0	0	0.0	13.40	0.03	0.03	0.76 - 237.78
0103	0	0.0	0	0.0	-	-	-	-
Others	74	37.0	150	75.0	0.20	0.60	2.0×10^{-14}	0.13 - 0.30
Total	200	100.0	200	100.0				

EF: Etiological Fraction; PF: Preventive Fraction; *p*: Fisher's exact probability; C.I.: Confidence Interval

The possible importance of HLA-G in RSA is more likely to be based on HLA-G polymorphisms or mutations which could result in altered functional capacity in the interaction with other proteins or in the concentrations of membrane bound and soluble HLA-G isoforms, or may be alterations in mutual levels of these different isoforms (4).The lack of association between RSA and HLA-G*0105N, HLA-G*0103N alleles observed in this study is in consistence with studies performed on Finnish, Japanese, Hungarian, Chinese, and Danish populations (27). In contrast, Aldrich (31) reported that the presence of HLA-G*0104 and HLA-G*0105N alleles in either partner was found to be significantly associated with increased risk for subsequent miscarriage. Further Pfeiffer (6) results reported significant increase of HLA- G*0105N allele in patients with RSA (4,16). Discrepancy between results could be due to nucleotide polymorphisms in the 5'- or 3'-untranslated regions or due linkage disequilibrium to other alleles in the vicinity of the HLA-G locus. For instance, Ober (29) have provides evidence for an association between a promoter region (- 725 C/G) single nucleotide polymorphism and fetal loss rates. HLA-G*0105N null

allele is characterized by a frameshift mutation in exon 3 and the presence of a stop codon in exon 4, thus leading to incomplete HLA-G1, -G5 and, -G4 isoforms and normal HLA-G2, -G3, and -G7 isoforms (32).

Studies that support association of HLA-G*0104N allele with RSA women, hypothesized that HLA-G*0104N is associated with significantly lower serum concentrations of soluble HLA-G. Lower soluble HLA-G concentrations have been demonstrated to be correlated also with adverse pregnancy outcome after IVF (6). Other studies have confirmed that HLA-G genotype influences circulating sHLA-G levels during pregnancy but that does not necessarily increase the risk of RSA (33,34).

Another explanation is that nonsense-mediated decay; a eukaryotic regulatory process that degrades mRNA with premature termination codons, could be responsible for the higher degradation of HLA-G1 transcripts in cells expressing the null allele (HLA- G*0104N) compared with other alleles, where in HLA-G2 isoforms, the HLA-G*0104N allele does not lead to a distorted protein, because exon 3, which contains the frame shift mutation, has been spliced out. The HLA-G*0104N allele can therefore, be only responsible for a decreased amount of HLA-G1 isoforms. So, other HLA-G isoforms exist and may compensate for the loss of HLA-G function (4). This is further supported by finding the HLA-G*0104N allele (in both heterozygous and homozygous forms) in healthy adults whose gestations and deliveries were normal, suggesting that the HLA-G isoforms encoded by this allele possess functions able to compensate for the low level or absence of both the HLA- G1 and -G5 proteins and to maintain the immune privileged status of the fetal- maternal interface (35).

Actually, one study confirmed the association of HLA-G*0104 allele with URSA. That study noted that the association of HLA-G*0104 allele was unexpected as the allele defined conservative amino acid change of a leucine to an isoleucine at amino acid 110 in exon3, which encodes the α2 domain of the full length G1 isoforms. This suggests that there may, in fact, be a functional difference between these alleles and that the HLA-G1 isoforms play a critical role in fetal survival (31).

HLA-G*0103 [encoding a Thr to Ser substitution at codon 55 of the open reading frame (ORF) in the α-1 domain] (36). The frequency of the HLA-G*0103 allele (which was not detected in any of the investigated subjects) in this study was different from that reported in other ethnic groups. This allele frequency is 22.7% in India, 2.4% in American Caucasians and 3.0% in Danish people (27).

Initially, HLA-G was shown to protect HLA class I-deficient targets from NK cells- mediated lysis, through interaction with killer-cell inhibitory receptors (KIRs). This was demonstrated for both full-length membrane-bound and soluble isoforms (36). With so little information about the specific amino acid residues that are critical for inhibition for either NK cells or T cells, it is difficult to assess the impact of the different HLA-G alleles on these interactions (31). Most studies have focused on exons 2 and 3 (encoding the α1- and α2-domains, respectively) of the HLA-G protein. However, if HLA-G performs novel functions, polymorphisms in other coding regions of the gene may be functionally relevant. Because soluble HLA-G includes 21 amino acids encoded by sequences in intron 4, polymorphisms in this intron could also result in variability at the protein level and provide a source of allogeneic recognition in pregnancy (37) Limited polymorphism of HLA-G and its restricted expression to the cytotrophoblast prompted the hypothesis that it provides the barrier that shields the fetus from the innate and/or acquired immunity of the mother. However, subsequent studies indicated that this cannot be the exclusive mechanism of maternofetal tolerance because the same degree of HLA-G incompatibility exists between fertile and habitually aborting couples (38).

Frequency of *HLA-G* genotypes:-

The frequencies of the three HLA-G genotypes observed in this study are shown in table (3-5). Two genotypes were encountered in patients group (*HLA-G*0104/0105N and HLA-G*0105N/0105N*) whereas only the genotype *(HLA-G*0104/0104)* was present in both groups. *HLA-G*0104/0104* genotype showed high frequency reach statistical significance ($p = 3.5 \times 10^{-6}$), the frequency of patients group was 58% while, it was 25 % for controls group with odds ratio (4.14) in both groups, and (95% C.I. = 2.28- 7.54). The patients groups which revealed *HLA-G*0104/0105N* genotype had the frequency 4.0% whereas it absent from the controls group. *HLA-G*0105N/0105N* genotype was present in one patient (1.0%) whereas none of the fertile controls showed this genotype. Other genotypes (not studied) were showed high frequency in both groups and reach statistical significance ($p= 9.5\times10^{-8}$) and PF =0.60 which may contribute in protection from abortion.

Table 4: *HLA-G*0104* allele frequency in patients and controls

			Group		T0tal	Odds ratio (95%CI)
HLA-G*0104	**No**	**Count**	**38**	**75**	**113**	4.8947 (2.6687-8.9777) p< 0.0001*
		%	**33.6%**	**66.4%**	**100.0%**	
	yes	**Count**	**62**	**25**	**87**	
		%	**71.3%**	**28.7%**	**100.0%**	
Total		**Count**	**100**	**100**	**200**	
		%	50.0%	50.0%	100.0%	

Interestingly, the results showed that HLA-G*0103 allele was not evident neither in the URSA patients nor in the control subjects. It seems that HLA-G*0103 allele is rare in our population under study and the small sample size investigated here could not detect that allele. HLA-G*0105N allele was presented in URSA groups while absent in fertile control groups and the confidence interval was very wide (95% CI : 0.6315-212.2057) therefore a larger sample for the 2 selected groups is needed to confirm this satiation. On other hand HLA-G*0104 alleles were found in both URSA and fertile control groups with higher frequency reach statistical significance p<0.0001,and with odd ratio 4.8947.

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