International Journal of Reproduction, Contraception, Obstetrics and Gynecology
Matter TF et al. Int J Reprod Contracept Obstet Gynecol. 2013 Sep;2(3):277-283
www.ijrcog.org

pISSN 2320-1770 | eISSN 2320-1789

DOI: 10.5455/2320-1770.ijrcog20130904

Research Article

HLA-G and *HLA-E* Gene polymorphisms in idiopathic recurrent spontaneous abortion women in Gaza strip-Palestine

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Received: 2 July 2013
Accepted: 23 July 2013

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ABSTRACT

Human leukocyte antigens (HLA)-G and HLA-E are non-classical HLA class I antigens expressed on extravillous cytotrophoblast that invades decidua in the uterus during pregnancy and are postulated to help in maternal immunotolerance of the fetus. Published results on the relation between *HLA-G* and *HLA-E* polymorphisms and unexplained recurrent spontaneous abortion (RSA) are, however, controversial. This study was initiated in order to investigate whether *HLA-G* (*G*0103, G*0104* and *G0105N*) and *HLA-E* (*E*0101* and *E*0103*) polymorphisms are associated with risk of RSA in Palestinian women. Forty women with ≥2 first trimester unexplained RSAs and 40 age-matched fertile women were genotyped for *HLA-G* and *HLA-E* polymorphisms by PCR-RFLP technique.
The results revealed the absence of *HLA-G*0103* in the study population. No significant difference was observed between the RSA and the fertile groups in terms of the frequency of *HLA-G*0104*, *HLA-G0105N*, *HLA-E* 0101*, or *HLA-E*0103* alleles. Interestingly, *HLA-G*0105N/0104* genotype was present in the RSA group only and was encountered in 4 patients. The other genotypes showed comparable frequencies in both groups.
The presented data suggest that the investigated *HLA-G* and *HLA-E* alleles do not predispose to RSA in the study population. Meanwhile, the *HLA-G*0105N/0104* genotype may contribute to RSA through linkage disequilibrium with other genetic elements. Larger sample size and further studies are needed in order to confirm this observation.

Keywords: *HLA-G*, *HLA-E*, polymorphism, PCR-RFLP

INTRODUCTION

Currently, RSA is defined as the occurrence of ≥2 consecutive pregnancy losses before 20th week of gestation. It has been estimated that about 2% of women suffer from RSA. In around 50% of the cases the cause of miscarriage remains unidentified and thus termed "idiopathic". A large fraction of idiopathic RSA are thought to be caused by immunological rejection of the fetus by the mother (Kaariainen, 2006; Wang et al., 2010).

During pregnancy, the maternal immune system is in close contact with cells and tissues from the semi-allogenic fetus. Therefore, specific mechanisms must exist to ensure that maternal immune cells do not attack and harm the fetus (Haviid, 2006; Tien and Tan, 2007). Downregulation of the polymorphic classic major histocompatibility complex (MHC) antigen expression on fetal trophoblast cells is one documented mechanism (Devies, 2007).

The human major histocompatibility complex (MHC) locus at chromosome 6p21 encodes the classical human leukocyte antigen (HLA) class I proteins (A, B and Cw) and a range of non-classical HLA class Ib proteins such as HLA-E, F and G (Hunt, 2006).

Apps et al (2009) confirmed that in normal pregnancy primary villous trophoblast cells are *HLA* null whereas, extravillous trophoblast cells express *HLA-C, HLA-G* and *HLA-E*, but not the classical *HLA-A, HLA-B* or *HLA-DR* molecules.

Studies on the function of HLA-G molecules showed that HLA-G can inhibit natural killer (NK) and T cell-mediated fetal cell lysis, both through direct interaction with the receptors IL-T2 and IL-T4 and with the killer Ig-like receptor 2DL4 (KIR2DL4 receptor) (Devies, 2007). Other possible functions of HLA-G include suppression of T lymphocyte proliferation and influencing cytotoxic T-cells (Tc) lymphocytes and uterine NK cells by altering their secretion of cytokines, shifting the immune response from the pro-inflammatory type I to the anti-inflammatory type II (Haviid, 2006).

The coding region of *HLA-G*, like the classical HLA genes, consists of seven introns and eight exons. The *HLA-G* gene is of modest polymorphism with 44 described alleles, and due to presence of synonymous alleles, they encode 14 distinct functional proteins with all isoforms (HLA-G*0101, *0102, *0103, *0104, *0106, *0107, *0108, *0109, *0110, *0111, *0112, *0114, *0115 and *0116) and modified proteins encoded by the null *G*0105N allele; defined by a 1597C deletion in exon 3 (Donadi et al, 2011). The nucleotide polymorphisms in the 3 HLA-G alleles investigated in the present work as compared to the wild-type *G*0101* allele is illustrated in Table 1 below, and this explains how those alleles are discriminated by PCR-RFLP.

Table 1: *HLA-G* allele assignment based on nucleotide substitutions (Modified from HLA nomenclature. www.anthonynolan.org.uk).

Allele	Exon 2	Exon 3	
	Codon 31	Codon 110	Codon 130
G*0101	ACG	CTC	CTG
G*0103	**T**CG	CTC	CTG
G*0104	ACG	**A**TC	CTG
G*0105N	ACG	CTC	-TG

Positions of amino acid variability are indicated in bold letters. Codon 31 encodes threonine (ACG) or serine (TCG); codon 110 encodes either a leucine (CTC) or an isoleucine (ATC). The 1597 delC mutation of the codon 130 in G*0105N is also indicated.

The HLA-G system presents 7 protein isoforms, generated by alternative splicing of the primary transcript, 4 of them being membrane-bound and three soluble proteins. The HLA-G1 isoform is the only isoform expressed at the cell surface whereas, the HLA-G2, -G3 and -G4 are retained in the endoplasmic reticulum (Donadi et al., 2011; Ober and Aldrich, 1997).

Soluble HLA-Gs can also affect peripheral immune cells and modulate their function for the benefit of pregnancy. It has been shown that sHLA-G may play a key role in implantation of the embryo, as plasma levels were reduced in early miscarriage as compared with normal pregnancy. In addition, following an IVF procedure only those embryos which secreted sHLA-G gave rise to successful pregnancy (Kaariainen, 2006; Ober and Aldrich, 1997).

Polymorphic sites present in the coding and non-coding regions of the *HLA-G* gene may potentially affect the biological functions of HLA-G e.g., interaction with cell receptors, isoform production and modulation of the immune response (Donadi et al., 2011).

HLA-E, on the other hand, is minimally polymorphic with four non-synonymous alleles: *HLA-E*0101, HLA-E*0102, HLA-E*0103* and *HLA-E*0104* (Matte et al., 2000). HLA-E plays an important 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. Surface expression of HLA-E requires, and is therefore controlled by, the availability of the signal sequences of other HLA class I molecules including *HLA-A, -B, -C*, and -G (Antoun et al., 2009). The *HLA-E*0101* and *HLA-E*0103* alleles are distinguished by an A>G single nucleotide polymorphism (SNP) at codon 107 in exon 3 (Matte et al., 2000) that allows genotyping by PCR-RFLP.

Previous studies on the association between *HLA-G* and *HLA-E* polymorphisms and RSA in different populations showed discrepant results. For example, studies on Finnish, Japanese, Hungarian, Danish and Chinese Han populations (Karhukorpi et al., 1997; Yamashita et al., 1999; Penzes et al., 1999; Hviid et al., 2002; and Yan et al., 2006, respectively) suggest that there is no association of *HLA-G* genotypes with pregnancy outcome whereas, the studies on Americans, German and Indian women support the association of particular HLA-G genotypes with RSA (Aldrich et al., 2001; Pfeiffer et al., 2001; Abbas et al., 2004, respectively).

Similar trends have been reported for *HLA-E* alleles. For instance Mossad et al. (2011) in Egypt and Tripathi et al. (2006) in North India have found an increased frequency of homozygosity for HLA-E*0101 in RSA women. In the contrary, Steffensen et al. (1998) and Hirohiko et al. (2006) in Danish and Japanese RSA women, respectively, observed no such association.

The purpose of the present study was to investigate the distribution of three *HLA-G* (*HLA-G*0103*, *HLA-G*0104* and *HLA-G*0105N*) and two *HLA-E* (*HLA-E*0101* and *HLA-E*0103*) alleles and their genotypes in patients who

had experienced two or more RSA, compared with a group of normal fertile women.

METHODS

After having all the study participants' written informed consent and the approval of the local ethics committee, samples of peripheral blood (4 mL) were collected into EDTA tubes from 40 women who experienced at least two first trimester unexplained consecutive pregnancy losses. The RSA women were free from identifiable causes of miscarriage including chromosomal, anatomic, endocrine and autoimmune causes. They were also normal in terms of hereditary thrombophilia risk SNPs i.e., *Factor V* (G1691A), *Factor II* (G20210A) and *MTHFR* (C677T) mutations.

Forty healthy women with at least two live births and no history of pregnancy loss served as the study control. Both groups were recruited from Gaza strip private Ob/Gyn clinics, IVF centers and hospitals. All subjects were in the age 18-35 years and their husbands were not their family relatives.

Genomic DNA (gDNA) was extracted from peripheral blood using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instructions.

HLA-G and *HLA-E* genotyping was carried out on gDNA by PCR-RFLP method.

HLA-G Genotyping

Genotyping of *HLA-G*0103*, *HLA-G*0104* and *HLA-G*0105N* alleles was done as described by Gazit et al., (2004). Briefly, two sets of exon specific primers were used:

Exon 2: Forward: 5`-TCCATGAGGTATTTCAGCGC-3`, Reverse: 5`-CTGGGCCGGAGTTACTCACT-3`
Exon 3: Forward: 5`-CACACCCTCCAGTGGATGAT-3`, Reverse: 5`-GGTACCCGCGCGCTGCAGCA-3`

PCR reactions were carried out in 20 µl volumes: 2 µl (1µM) of each primer, 10 µl of PCR Green Go Taq® master mix (Promega, USA), 2 µl of gDNA (100ng/ µl) and 4 µl of H2O. The PCR conditions for *HLA-G* exon 2 consisted of 35 cycles: 94°C for 1min, 57°C for 1 min and 72°C for 1 min with a final extension at 72°C for 7 min. Conditions for *HLA-G* exon 3 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.

Following exon 2 and exon 3 amplifications, aliquots (10 µL) of the PCR products were digested with the following restriction endonucleases: Exon 2 with Hinf-I and Exon 3 with PpuM-I, and BseR-I. The digestion products were resolved on 2% agarose gel and detected by staining with ethidium bromide. Allele assignments was based on the patterns of the DNA fragments as shown in Table 2.

Table 2: Allele assignment scheme for *HLA-G* gene and *HLA-E* alleles.

Gene	Exon	Restriction Enzymes	Product size (bp)	Specificity
HLA-G	2	Hinf-I	79+175+27	G*0103
			106+175	All but G*0103
	3	PpuM-I	276	G*0105N
			108+168	All but G*0105N
		BseR-I	276	G*0104
			40+236	All but G*0104
HLA-E	3	HpaII	280	E*0101
			260+20	E*0103

HLA-E Genotyping

Genotyping of the two *HLA-E* alleles (*HLA-E*0101* and *HLA-E*0103*) was performed following Mosaad et al. (2011). In brief, *HLA-E* exon 3 was amplified using the following primers: Forward 5`-GGC TGC GAG CTG GGG CCC GCC-3`, reverse primer 5`-AGC CCT GTG GAC CCT CTT-3`. PCR was carried out in 20 µl reaction volumes: 2 µl (1µM) of each primer, 10 µl Green Go Taq® master, 2 µl gDNA (100ng/ µl) and 4 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 enzyme HpaII and the digestion products were resolved on 3% agarose gel and detected by staining with ethidium bromide. Interpretation of the patterns of the DNA fragments was done as indicated in Table 2.

Statistical analysis

Statistical significance of the differences between the patient and control groups were estimated by independent samples t-test. Odds Ratio (OR) and 95% confidence intervals (CI) were analyzed by Fisher's exact test. Statistical significance was set at *P-value* <0.05.

RESULTS

None of the investigated *HLA-G* alleles showed significant difference between RSA cases and controls. *HLA-G*0105N* and *HLA-G*0104* were present in comparable frequencies in both groups. Moreover, the *HLA-G*0103* was not detected in the study population (Table 3).

In terms of genotypes, the two *HLA-G*0105N/0105N* and *G*0104/0104* were evident in both groups whereas the third genotype (*HLA-G*0105N/0104*) was absent in the control group.

*HLA-G*0105N/0104* genotype was interesting as it was evident in 4 patient whereas none of the fertile controls showed this genotype. The *HLA-G*0105N/0105N* genotype showed similar frequency (12.5%) in both groups, 30% of the patients harbored *HLA-G*0104/0104* genotype whereas 27.5% of the control group possessed this genotype (Table 4).

The investigated *HLA-E* alleles also did not show significant association with RSA (Table 3). The *HLA-E*0101* allele was observed in comparable frequencies in RSA patients (77.5 %) and fertile controls (73.7%). Similarly, *HLA-E*0103* allele was encountered in equivalent frequencies in patients (22.5 %) and controls (26.3%).

Table 3: *HLA-G* and *HLA-E* alleles frequencies in RSA patients and fertile controls.

Gene	Allele	Group		OR	95% CI	Independent Samples t-Test	
		Pt N= 40 N(%)	Cont N= 40 N (%)			t–test	P value
HLA-G alleles	*G*0105N*	14 (17.5%)	10 (12.5%)	1.48	0.68- 3.26	0.89	0.38
	*G*0104*	28 (35.0%)	22 (27.5%)	1.42	0.78- 2.59	1.02	0.31
	*G*0103*	___	___	___	___	___	___
HLA-E alleles	*E*0103*	18 (22.5%)	21 (26.3%)	0.82	0.43- 1.55	0.56	0.58
	*E*0101*	62 (77.5%)	59 (73.7%)	1.23	0.64- 2.35	0.56	0.58

Table 4: *HLA-G* and *HLA-E* genotypes observed in RSA patients and fertile controls.

Gene	Genotype	Group		OR	95% CI	t-test	P value
		Pt N= 40 N(%)	Cont N= 40 N (%)				
HLA-G	*HLA-G*0104:0104*	12 (30.0%)	11 (27.5%)	1.13	0.61-2.08	0.25	0.81
	*HLA-G*0104:0105N*	4 (10%)	0 (0%)	-	-	2.05	0.04
	*HLA-G*0105N:0105N*	5 (12.5%)	5 (12.5%)	1.00	0.43-2.31	0.00	1.00
HLA-E	*HLA-E*0101:0101*	24 (60.0%)	22 (55%)	1.23	0.70-2.15	0.45	0.65
	*HLA-E*0101:0103*	14 (35.0%)	15 (37.5%)	0.90	0.50-1.60	0.23	0.82
	*HLA-E*0103:0103*	2 (5.0%)	3 (7.5%)	0.65	0.20-2.08	0.46	0.65

The distribution of HLA-E genotypes was also similar in both groups and no significant differences were observed (Table 4).

DISCUSSION

HLA-G is the predominant HLA expressed by fetal tissues at the feto-maternal interface. The HLA-G antigen is postulated to play an essential role during pregnancy by protecting the semi-allogeneic fetus from recognition and destruction by maternal immune cells (Yan et al., 2006) and, altered expression of *HLA-G* on the fetal extravillous cytotrophoblast has been implicated in the etiology of recurrent miscarriages (Akhter et al., 2012). Along with HLA-G, trophoblasts also express HLA-E antigen (Dahl and Hviid, 2012).

Some investigators speculated that pregnancy outcome is influenced by *HLA-G* and/or *HLA-E* level of expression and resulting isoforms; both aspects have been shown to rely on the allelic combination present in the individual (Pfeiffer et al., 2001). Studies on association between adverse pregnancy outcome and polymorphism in *HLA-E* and *HLA-G* genes in various populations, however, have yielded contradictory results.

Our results showed that *HLA-G*0103* allele was not evident in the RSA patients or in the control subjects. This result indicates that *HLA-G*0103* allele is rare in our population and the small sample size investigated here precluded detection of this allele. Low frequency of *HLA-G*0103* has been reported in several other populations including Spanish (0%), Chinese Han (0.3%), Koreans (0.5%), Portuguese and Danish (2%). However, *HLA-G*0103* with a frequency of (24.2%) constitutes the major HLA-G allele in North Indians (Park et al., 2011) thus, making this allele an example of inter-population genetic variation.

*HLA-G*0105N and HLA-G*0104* alleles were present in individuals from both groups. Though the two alleles were of slightly higher frequency in the patient group their difference did not reach statistical significance. These two alleles may still be important in RSA in our population and further work employing a larger sample size is needed to resolve this point.

On the other hand, lack of association between RSA and *HLA-G*0105N* and *HLA-G*0104* alleles found in this study is in consistence with studies performed on Finnish, Japanese, Chinese, and Danish populations (Yan et al, 2006). In contrast, Aldrich et al, (2001) 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. Moreover, Pfeiffer et al. (2001) found significant increase of *HLA-G*0105N* allele in patients with RSA.

Discrepancy between results could be due to many reasons including population genetic variation unrelated to investigated *HLA* alleles, presence of nucleotide polymorphism somewhere else in the *HLA* allele e.g., in the 5`-, 3`-untranslated or intronic regions, epigenetic alterations and linkage disequilibrium to other sequence variants in the vicinity of the studied *HLA* locus. In support of this point Ober et al. (2003) work showed a promoter-region -725 C/G single nucleotide polymorphism (SNP) that is associated with risk of fetal loss. Moreover, Manaster et al. (2012) have demonstrated that micro-RNAs (miR-148a and miR-152) down-regulate *HLA-G* expression by binding its 3'UTR and that this down-regulation of HLA-G affects NK-cell receptor (LILRB1) recognition and consequently, abolishes the LILRB1-mediated inhibition of NK cell killing.

Studies that support association of *HLA-G*0105N* allele with RSA hypothesized that *HLA-G*0105N* is associated with significantly lower serum concentrations of soluble HLA-G (sHLA-G) and decreased amount of HLA-G1 isoform (Pfeiffer et al., 2001; Discorde et al., 2005; Moreau et al., 2003). This finding, however, was not replicated by other researchers and in the contrary was refuted by documenting *HLA-G*0105N* heterozygous and homozygous women whose gestations and deliveries were normal (Hviid et al, 2002; Discorde et al., 2005). Moreover, *HLA-G*105N* is present in a relatively high frequency in the fertile controls (12.5%, this study) and as reported by Park et al. (2011) in other populations e.g., Iranians (18.%) and North Indians (15.4%).

In the present study, the presence of *HLA-G*0104:0105N* genotype in patients and its absence in the fertile group may be a mere coincidence (due to the small sample size) or may identify a subset of patients with a specific genetic predisposition to pregnancy loss.

Our results showed that *HLA-G*0104:0104* and *HLA-G*0105N:0105N* genotypes are present in similar frequencies in both RSA patients and fertile controls, which confirms that homozygosity for *HLA-G*0105N* is not associated with recurrent pregnancy loss as reported in previous studies (Discorde et al., 2005; Abbas et al., 2004).

HLA-E antigens are believed to help the fetus avoid maternal immune surveillance, possibly by playing a role both as a modulator of NK cell activity (by interacting with the CD94-NKG2A receptor) and as an antigen presenting molecule (Audus et al., 2002). The immunomodulating activity of HLA-E may be helpful in the success of pregnancy, as >90% of decidua lymphocytes are CD94/NKG2+ NK cells (Mallia et al., 2012).

The two investigated *HLA-E* alleles differ by a single amino acid (R107G) and this substitution may alter the structure of the molecule and consequent biological functions (Veiga-Castelli et al., 2012).

Our results showed that the two investigated *HLA-E* alleles are present in comparable frequencies in the two study groups. This result is in agreement with some previous studies which found no difference in allelic distribution of *HLA-E* between RSA and fertile controls (Kanai et al., 2006; Steffensen et al., 1998). Moreover, *HLA-E*0101* proved to be the prevalent allele in our population (~ 75%) and this is in agreement to those previously reported in various populations such as Africans, Caucasian, African-American and Hispanic populations, Indians and also Euro-Caucasoid, Afro-Caribbean, and Indo-Asian. On the other hand, the frequency of **0103* is significantly higher than that of **0101* in the Japanese and Chinese populations (Mosaad et al., 2011).

As with HLA-G, results on association between *HLA-E* alleles and RSA are also controversial. For example,

Mossad et al. (2011) and Tripathi et al. (2006) have found an increased frequency of homozygosity for HLA-E*0101 in RSA women whereas, Steffensen et al. (1998) and Hirohiko et al. (2006) observed no such association. Discrepancies between findings on different population could be due to the same reasons put forward for *HLA-G* above.

Finally, the presented data suggest that the investigated *HLA-G* and *HLA-E* alleles have no clear-cut association with increased risk of RSA. Meanwhile, the combination of particular *HLA-G* and *HLA-E* alleles may have some effect on patients suffering from RSA. Larger sample size and more studies are needed in order to confirm this observation.

Funding: None
Conflict of interest: None declared
Ethical approval: The study was approved by local ethics committee

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DOI: 10.5455/2320-1770.ijrcog20130904
Cite this article as: Matter TF, Sharif FA. *HLA-G* and *HLA-E* Gene polymorphisms in idiopathic recurrent spontaneous abortion women in Gaza strip-Palestine. Int J Reprod Contracept Obstet Gynecol 2013;2:277-83.