

# HLA-E\*0101 Associated with Recurrent Spontaneous Abortion

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## Abstract

**Objective:** Lack of HLA Ia antigens at fetomaternal interface has profound effect on histocompatibility between semiallogeneic fetus and mother but may pose jeopardy as natural killer (NK) cells of immune surveillance may attack cells lacking self HLA antigens. However, expression of non-classical antigens like HLA-G and HLA-E has been implicated in down regulation of immune response via interacting with KIR receptors of NK cells. In the present study, we have investigated HLA-E polymorphism in normal fertile women and recurrent spontaneous abortion, to demonstrate the role of HLA-E alleles in pregnancy.

**Materials and Methods:** In this study 200 recurrent spontaneous aborters (RSA) and 200 normal fertile controls were included. DNA was isolated by salting out procedure. Mutations in exon-2 and -3 were investigated by PCR-RFLP and ARMS-PCR.

**Results:** We have demonstrated E\*0101, E\*01031 and E\*01032 alleles among patients and controls. Allele E\*0101 was found to be associated with RSA patients ( $\chi^2=5.214$  and  $p=0.0224$ ). Further HLA-E\*0101 homozygotes were significantly higher among RSA patients ( $\chi^2=9.763$  and  $p=0.0018$ ) as compared to controls.

**Discussion:** Two major alleles E\*0101 and E\*0103 differ in their biological and biophysical properties. HLA-E\*0101 may be associated with recurrent spontaneous abortion because of decreased surface expression, lower peptide affinities and lower stability.

**Keywords:** amplification refractory mutation system, human leukocyte antigen-E, killer inhibitory receptor (KIR), recurrent spontaneous abortion

## Özet

### HLA-E\*0101'in Tekrarlayan Spontan Düşüklerle Bağlantısı

**Giriş:** Fetomaternal etkileşimde HLA Ia antijen eksikliğinin semialojenik fetüs ve anne arasındaki doku uyumuna önemli bir etkisi olmakla beraber tehlike de arz edebilir. İmmün sürveyansın NK hücreleri, HLA antijen eksikliği bulunan hücrelere saldırabilir. Ancak HLA-G ve HLA-E gibi klasik olmayan antijenlerin NK hücrelerindeki KIR reseptörleriyle etkileşime girmesi sonucunda, bağışıklık cevabı azalmaktadır. Bu çalışmada HLA-E allellerinin gebelikteki rolünü göstermek için normal doğurgan kadınlarda ve tekrarlayan spontan düşük hastalarında HLA-E polimorfizmi incelenmiştir.

**Materyal ve Metot:** Araştırmaya tekrarlayan spontan düşük yapan 200 kadın ve kontrol grubu olarak 200 normal doğurgan kadın alındı. DNA, "salting-out" yöntemi ile izole edildi. Exon-2 ve -3'teki mutasyonlar PCR-RFLP ve ARMS-PCR ile incelendi.

**Sonuçlar:** Hastalarda ve kontrol grubunda E\*0101, E\*01031, ve E\*01032 allelleri belirlenmiştir. Allel E\*0101'in tekrarlayan spontan düşüklerle bağlantısının olduğu saptandı ( $\chi^2=5.214$  ve  $p=0.0224$ ). Ayrıca HLA-E\*0101 homozigotları, kontrol grubuna göre tekrarlayan spontan düşük hastalarında daha yüksek ( $\chi^2=9.763$  ve  $p=0.0018$ ) çıkmıştır.

**Tartışma:** Başlıca iki allel; E\*0101 ve E\*0103, biyolojik ve biofiziksel özellikleri bakımından farklıdır. HLA-E\*0101 azalmış yüzey ekspresyonu, daha düşük peptit afinitileri, ve daha düşük stabilite nedeniyle tekrarlayan spontan düşüklerle bağlantılı olabilir.

**Anahtar sözcükler:** amplifikasyon refraktoryal mutasyon sistemi, insan lökosit antijeni-E, öldürücü inhibitör reseptör, tekrarlayan spontan düşük

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## Introduction

Immune system has evolved with various immunological armaments that not only distinguishes foreign antigens and pathogens but also removes them successfully. Human immune system recognizes non-self antigens and pathogens in an organized manner. Most crucial component of immune response is human leukocyte antigens (HLA) that contain over 100 genes (1). Human leukocyte antigens have been classified into classical and non classical antigens. HLA class I and II are classical antigens where as HLA-E, -F and -G are non classical antigens. HLA class I is expressed on all the living cells, where as class II antigens have restricted expression.

Matching of these antigens is a prerequisite for organ transplantation. Mammalian pregnancy is like an allogeneic transplantation, as this makes mother to bear a semiallogeneic fetus that also possesses paternal antigens and are always exposed to maternal immune response (2). Maternal T cells can attack, perceiving paternal HLA molecules as foreign. To cope with this situation HLA class I and II antigens are absent from normal syncytiotrophoblast cells, which constitute the outermost layer of fetus and is exposed to maternal immune response. Further absence of HLA class I antigens are reported in blastocyst and preimplantation embryos (3). This provision should suffice to protect the fetus from immune attack of maternal T cells, but not from the NK (Natural Killer) cells. In absence of HLA class I antigens, NK cells are released from inhibition, to kill the target cells. Though various immune mechanisms work in the favor of pregnancy but still there inter-relation and self sufficiency is not confirmed. It is proposed that nature also expresses certain immunoregulatory components as HLA-G and HLA-E to protect fetus from NK cell cytotoxicity (4-6).

HLA-E has wide tissue distribution expressed at most of the cells in the body (7). HLA-E is also expressed at certain immune privileged site i.e. fetomaternal interface that with the exception of HLA-G is devoid of various HLA class I antigens (8). HLA-E is oligomorphic and broadly consists of only two variants having different amino acid sequences (9,10). One more peculiar property of HLA-E is requirement of HLA class I leader peptide for efficient surface expression (11). Though HLA-E is expressed at various other cells in the body its expression at placenta anticipates some important immuno down regulating functions. It is more conspicuous with the evidence that it has the highest affinity for HLA-G derived peptides (12), which is already present at fetomaternal interface and thus may make more efficient surface expression and immuno regulating properties of HLA-E at placenta. HLA-E however, is known for its capacity to inhibit NK cell response (13), and thus may be helpful in preventing NK cell response against fetus during normal pregnancy.

Further HLA-E exists in nearly equal proportions in different populations that symbolize its maintenance due to selection pressure for some of its particular function (14). To fill the

lacuna in immuno regulatory circuits evident at fetomaternal interface at the time of normal pregnancy, it would be interesting to evaluate its role, by investigating the frequency distribution of both the alleles of HLA-E in normal as well as in spontaneous aborters.

## Materials and Methods

### Samples and genomic DNA extraction

All the patients were North Indian women attending outpatient Department of Medical Genetics of Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow and other hospitals of the city, for the evaluation of recurrent spontaneous abortions (RSA). Each woman had 3 or more RSA. They underwent investigations for various causes of RSA viz.; day 21 progesterone, thyroid stimulating hormone (TSH), anti-cardiolipin antibodies (ACLA), antinuclear antibodies (ANA), activated partial thromboplastin time, kaolin coagulation time, platelet count, hysterosalpingography, pelvic ultrasonography (USG) and karyotyping of both husband and wife. After informed consent 200 women were selected to participate in the present study.

Total 200 healthy North Indian women with at least three live births and no history of previous miscarriage were taken as controls. Two ml blood was taken from both controls and RSA women. Ethical clearance was taken to conduct this study by the ethical committee of SGPGIMS and those giving informed consent were enrolled in the study. Genomic DNA was extracted from peripheral blood by salting out procedure (15).

### PCR-RFLP Analysis

Polymorphism at codon-82/83 and at codon-157 was investigated through PCR-RFLP analysis. Primer pairs AE1 (5'-TGAAGTATTTCCACACTTCCGTGTCCCGGCC-3')/AE2 (5'-AATCTGGGACCCGAAGATTCGAGGGGACCC-3') and AE7 (5'-CAAAATGCCACAGGGTGGTGGCGACGGG-3')/AE8 (5'-GGAGATGGGAGAGTAGCCCTGTGGACCTC-3') were used to amplify HLA-E exon-2 and -3 respectively. PCR amplification was done in 100 µl of reaction volume having 200 ng of DNA, 0.25 mM of each dNTP, 15 pmol of each primer, 10 mM Tris-HCL (pH-9), 1.5 mM MgCl<sub>2</sub>, 50 mM KCL and 0.01% Gelatin and 2.5 U of Taq polymerase. Amplification was done with initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 58°C for exon-2 or 64°C for exon-3 for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

Further amplified PCR products were digested with Bsh1236I and BspLI to detect the polymorphism at codon 82/83 (exon-2) and 157 (exon-3) respectively, according to manufacturer's instructions (MBI Fermentas). The digested PCR products were run on 9% nondenaturing polyacrylamide gel, and stained with ethidium bromide.

The PCR product for exon-2 was of 346 bp having five restriction sites for BSH1236I. Out of five restriction sites only one would have been polymorphic. In allele E\*0101, 01031

and 01032 RFLP there were six fragments. In allele E\*0102 there is conversion of CG to GC which abolishes restriction site. Hence two fragments of 104 bp and 83 bp may remain intact making band of 187 bp and thus only five fragments may be produced. The PCR product for exon-3 was of 381 bp having four restriction sites for BspLI restriction endonuclease, hence allele E\*0101, 0102, 01031 and 01032 PCR RFLP produces five fragments. However, in allele E\*0104 there is A to G substitution which creates one more restriction site and may split the band of 190 bp into two bands of 159 bp and 31 bp, over all producing six fragments.

**ARMS-PCR Analysis**

Tetra primer Amplification Refractory Mutation System (ARMS) PCR was used to detect polymorphism at codon 77 and 107 by using four primers in a single reaction to amplify specific alleles as well as a control fragment. Primer set- 5'-TGAAGTATTTCCACACTTCCGTGTCCCGGCC-3', 5'-CAGGGACACCGCACAGATTTTCCGAGTCGAC-3', 5'-ATTGTAGTAGCCGCGCAGCGTCCGCGGA-3' and 5'-AATCTGGGACCCGAAGATTCGAGGGGACCC-3' were used to examine polymorphism at codon 77 of exon-2, where as polymorphism at codon 107 of exon-3 was detected by primer set- 5'-CAAAATGCCACAGGGTGGTGGC-GAC GGG-3', 5'-ATGCATGGCTGCGAGCTGGGGCCC-GAAA-3', 5'-GAACTGTTCATACCCGCGGAGGAAGC-GACC-3' and 5'-GGAGATGGGAGAGTAGCCCTGTG-GACCCTC-3'. The composition and condition of ARMS amplification are described elsewhere (16). The amplified PCR products were run on 9% nondenaturing polyacrylamide gel, and stained with ethidium bromide. ARMS PCR for codon-77 produced a band of 346 bp as a control where as at polymorphic site, 'C' and 'T' corresponded to bands of 159 bp and 245 bp respectively. Where as for codon 107 the control was a band of 381 bp and at polymorphic site 'A' and 'G' produced band of 290 bp and 158 bp respectively.

Allele frequencies were compared by  $\chi^2$  test using SPSS (version 9.0.0).

**Results**

Mutation detection at codon-77, 82/83, 107 and 157 can assign all known alleles of HLA-E known so far. The absence of allele E\*0102 and E\*0104 in both RSA patients and normal fertile controls was confirmed as no 'C' at position 246 (exon-2) and 'G' at position 198 (exon-3) could be detected by PCR-RFLP. Further analysis of codon 77 and 107 by ARMS-PCR have revealed the presence of cytosine and/or thymine at nucleotide 230 (exon-2) and adenine and/or guanine at position 48 (exon-3). Using this information allele E\*0101, E\*01031 and E\*01032 was assigned in both the groups. Allele E\*0101 was the most predominant allele in both the groups. It was present in 72.25% RSA patients and 64.75% controls, followed by E\*01032 and E\*01031 (Table 1). Further allele E\*0101 was found to be significantly higher in RSA patients ( $\chi^2=5.214$ ,  $p=0.0224$ ). On comparing the genotypic distribution of HLA-E alleles it was

found that E\*0101 homozygotes were found to be significantly higher in RSA patients ( $\chi^2=9.763$  and  $p=0.0018$ ) than in the normal fertile control, whereas E\*0101/E\*01031 heterozygotes were significantly higher among controls ( $\chi^2=10.256$  and  $p=0.0014$ ) (Table 2).

**Discussion**

HLA-E is a heterodimeric HLA class I molecule consisting of a heavy chain and a light chain ( $\beta$ -2 microglobulin). The heavy chain is of ~45 kDa consisting of 8 exons. Similar to typical HLA class I organization, exon one encodes the leader peptide, exons 2 and 3 encode the  $\alpha$ -1 and  $\alpha$ -2 domains, constituting peptide binding domain, exon 4 encodes the  $\alpha$ -3 domain, exon 5 encodes the transmembrane region, and exons 6 and 7 encode the cytoplasmic tail. The heavy chain is anchored in the membrane through transmembrane domain (17).

Immuno regulatory function of HLA-E is evident with their property to inhibit NK cell cytolysis by interacting with killer inhibitory receptors. NK cells are important components of immune surveillance that can eliminate cells appearing as non-self due to reduced HLA class I expression. These NK cells can attack invasive trophoblast cells of placenta as these cells are also devoid of HLA class I antigens with few exceptions. One group of receptors of NK cells are of C type lectine like receptor, which may mediate various effector functions, includes NKG2x that heterodimerises with CD94. These NKG2x may be of various types depending on the

**Table 1.** HLA-E allele frequency among RSA patients and normal fertile control

E*Allele	RSA patients (n=120)	%	Controls (n=120)	%
E*0101	289*	72.25	259*	64.75
E*0102	0	0	0	0
E*01031	42	10.5	60	15
E*01032	69	17.25	81	20.25
E*0104	0	0	0	0
Total	400	100	400	100

\* $\chi^2=5.214$  and  $p=0.0224$

**Table 2.** Genotypic distribution of HLA-E alleles

HLA-E genotype	RSA patients		Controls	
0101/0101	103 <sup>A</sup>	51.5%	72	36%
0101/01031	25 <sup>B</sup>	12.5%	50	25%
0101/01032	58	29%	65	32.5%
01031/01031	03	1.5%	02	1%
01031/01032	11	5.5%	06	3%
01032/01032	0	0%	05	2.5%
Total	200	100%	200	100%

A\* $\chi^2=9.763$  and  $p=0.0018$   
 B\* $\chi^2=10.256$  and  $p=0.0014$

chain they possess i.e. A, B, C, E and H, which finally concludes their behavior as activating or inhibitory effector receptors. HLA-E imparts most of their immunological properties by interacting through these NKG2/CD94 heterodimeric receptors (18), which may be activating (NKG2C/E/H) or inhibitory (NKG2A/B). As NK cells bear a complex mixture of activating and inhibitory receptors, the final outcome of effect is product of various opposing activating and inhibitory signals generated during target cell interaction, where the nature of receptor as well as other microscopic biophysical properties may play a conclusive role. It is further seen that NKG2A, a NK cell inhibitory receptor has comparatively higher affinity for ligand (19) as well as a higher level of expression in general compared to activating receptors of NK cells (20). All these evidences strongly suggest that in the presence of any inhibitory ligand, outcome of NK cell effector response would be inhibitory, even if they possess activating receptors too. Thus evidences seen so far unanimously indicate towards the ability of HLA-E to down regulate NK cells and hence their importance in pregnancy.

Though initial studies about the role of HLA in pregnancy has indicated the increased sharing of HLA classical class I and II antigens to be associated with loss of fetus, various studies resulted in inconsistent data and recent meta analysis in 2005 by Baydoun & Saftlas has concluded on no association of HLA class I with recurrent spontaneous abortion (21). Further HLA class I classical antigens are found to be absent on fetomaternal interface suggesting no functional implication of these antigens in pregnancy. Another potential HLA antigen present on fetomaternal interface is HLA-G. HLA-G is known to have some immunodownregulatory properties. Studies have supported association of various HLA-G polymorphisms with loss of pregnancy (22-24). But finding of HLA-G null individual, homozygous for HLA-G 0105N allele (25,26) has indicated the presence of alternative immunoregulatory mechanism during pregnancy. The ability of HLA-G 0105N null allele to provide peptide for HLA-E expression, co-expression of HLA-E/HLA-G derived peptide and NK cells suggest crucial role of HLA-E in the immunobiology of pregnancy even in the absence of functional HLA-G (27). Though expression analysis of HLA-E by Bhalla et al. 2006 revealed a similar pattern of expression among RSA patients and normal fertile women (28), the allele dependent differential expression of HLA-E has also been reported (29). This reflects that the expression may differ depending upon the allelic composition of RSA patients and controls tested.

The importance of these molecules at fetomaternal interface is suggested by comparative significance and efficient effector mechanism of HLA-E on placenta. In contrast to HLA class I, in normal condition HLA-E only binds to the restricted set of nonamer peptides derived from leader sequence of HLA class I antigens at the time of processing (11).

As interaction of these nonamer peptides are decisive for the surface expression of HLA-E, their comparative affinity with

the peptide could be conclusive for the efficiency of HLA-E induced immunoregulation of NK cells. Further when peptides of various origins were investigated, it was seen that interaction of HLA-E has highest affinity with HLA-G derived peptide (12), which is the most prominent HLA antigen expressed at fetomaternal interface. This is suggestive of influential role played by HLA-E at fetomaternal interface in spite of its wide tissue distribution throughout the body.

Two protein variants of HLA-E are present in the human population (16,30), distinguished by a single sequence dimorphism at position 107, arginine (E\*0101; HLA-E<sup>R</sup>) or glycine (E\*0103; HLA-E<sup>G</sup>). As this dimorphism exists in equal frequencies and hence is expected to be maintained through strong balancing selection (14), some studies have shown the association of this locus with susceptibility to nasopharyngeal carcinoma (31), type I diabetes mellitus (32) and susceptibility to HIV infection (33). Some previous studies have also suggested the association of HLA-E polymorphism with recurrent spontaneous abortion (34).

In the present study E\*0101 was significantly higher among RSA patients compared to controls ( $\chi^2=5.214$ ,  $p=0.0224$ ). Also this was predominant among both groups. We could not demonstrate the presence of E\*0102 and E\*0104 among our samples, which is in line with the recent evidences that these alleles have sequences similar to other known alleles and may not exist as was initially demonstrated. Further E\*0101 homozygotes were significantly higher among RSA patients ( $\chi^2=9.763$  and  $p=0.0018$ ) than in the normal fertile controls. That shows the critical role of E\*0101 allele among RSA patients. E\*0101/E\*01031 heterozygotes were significantly higher among controls ( $\chi^2 =10.256$  and  $p=0.0014$ ) (Table 2), which further indicates that the disease causation by E\*0101 homozygotes are disrupted by being present in heterozygous configuration.

A very high level of polymorphism is maintained in HLA class I classical antigens under selective pressure of presenting mutating and diverse pathogenic antigens. Contrary to this HLA-E exhibits very low level of allelic polymorphism, as is involved in presenting a limited set of peptides of HLA-I origin in normal conditions (11) suggesting their role in well regulated and predicted immunosurveillance and immunoregulation. Initially their low polymorphism questioned their role in CTL recognition but later it was unequivocally seen that human T cells may recognize HLA-E antigens (35).

These two major alleles of HLA-E exist nearly in equal frequencies in different populations. Kanai et al. in 2001 have demonstrated HLA-E polymorphism among RSA patients and normal fertile controls, and demonstrated HLA-E<sup>G</sup> was higher than HLA-E<sup>R</sup> but could not see any difference of HLA-E polymorphism between patients and controls (9). Though, these two protein variants differ at codon 107, which is definitely not involved in peptide presentation, but both variants have substantial differences in surface expression. These biophysical differences between these two major alleles of HLA-E were shown by Strong et al. in 2003 (29).



Among interaction of HLA-E variants and peptides of different origin, peptide/HLA-E<sup>R</sup> complex always has a lower surface expression compared to peptide/HLA-E<sup>G</sup> complex (29). This in turn may be regulated by the difference in their thermal stability. HLA-E protein variant alone and their peptide complex revealed, HLA-E<sup>G</sup> more thermally stable than HLA-E<sup>R</sup> (29). It has therefore confirmed that subtle but significant difference in thermal stability may influence their surface expression.

The crucial role of HLA-E in the pregnancy resides in its ability to down regulate NK cell cytotoxicity by interacting with their inhibitory receptors, hence lower expression of HLA-E<sup>R</sup> (E\*0101) and their lower stability may impair their efficiency to down regulate NK cells. This study has revealed an association of E<sup>R</sup> with the RSA probably due to the decreased surface expression and less stability and thus less immunoregulation, which may be required for the progression of pregnancy. It is speculated that the function of NK cell is regulated by a balance between activating and inhibitory signals provided by their heterocladic receptors upon recognition of specific ligands i.e. HLA-C, HLA-G, HLA-E expressed on invading trophoblast (36). It is also demonstrated that aborting women usually have a limited repertoire of inhibitory receptors of the KIR family (inhKIR), and most of them are for the fetal HLA-Cw antigens (36). Similarly studies about the HLA-E specific KIR inhibitory receptor of NK cells and HLA-E polymorphism could reveal some interesting results, further demonstrating immunoregulating properties of HLA-E in pregnancy.

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