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ORIGINAL ARTICLE

The balance of the immune system between HLA-G and NK cells in unexplained recurrent spontaneous abortion and polymorphisms analysis

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Abstract Human leukocyte antigen (HLA)-G is involved in immunoregulatory processes and particularly in pathogenesis of inflammatory disorders such as recurrent spontaneous abortions (RSA). The purpose of the current study was to examine whether two single nucleotide polymorphisms (SNPs) of *HLA-G* gene (rs1736936 and HLA-G*0105N) influence susceptibility to recurrent spontaneous abortion. Genomic DNA from 117 RSA patients and 117 normal fertile control individuals was isolated using the salted out method. The two single nucleotide polymorphisms in *HLA-G* gene were analyzed using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Differences between the two groups were analyzed by SPSS19 software using Chi-square test. The results revealed a significant increase in HLA-G*0105N allele in the proportion of whole group of RSA women compared with fertile controls (P value = 0.015), OR (95 % CI) = 2.054 (1.798–2.347), as well as an absence of homozygosity for HLA-G*0105N in the study population. No significant difference was observed between the RSA and the fertile groups in terms of alleles and genotypes frequency of rs1736936 (P value = 0.323), OR (95 CI %) = 1.056 (0.844–1.319). The presented data suggest that the investigated HLA-G*0105N allele is potentially associated with RSA through linkage disequilibrium with other genetic elements. Meanwhile, the rs1736936 SNP do not predispose to RSA in the study population.

Keywords Promoter polymorphism · rs1736936 · HLA-G · Recurrent miscarriage · Recurrent spontaneous abortion · HLA-G*0105N

Introduction

Recurrent spontaneous abortion (RSA), defined as the occurrence of two or more consecutive abortions prior to 20 weeks from the last menstrual period, occurs in ≈1 in 300 pregnancies [5, 25, 26, 41]. The etiology of RSA is often unclear, with considerable controversy regarding diagnosis and treatment. Some reasonably accepted etiologic causes include: chromosomal abnormalities, antiphospholipid antibody syndrome (APS), endocrine disorders, thrombophilias, immunologic abnormalities, infections, and environmental factors. Nevertheless, nearly 50 % of cases of RSA remain unexplained and are found to be associated with certain maternal immune responses against the fetus [8, 16, 32, 37, 38].

Pregnancy is an immunological paradox state where semi-allogeneic fetal tissues are in close contact with the maternal immune system but are not rejected [28, 35]. In particular, this protection has shown to be conferred by the expression of human leukocyte antigen (HLA)-G on the trophoblast cell surface. HLA-G, a non-classical HLA Class I molecule first described on the surface of invasive cytotrophoblasts at the fetal–maternal interface, is a crucial factor in the modulation of the maternal immune system during pregnancy [4, 9, 27, 39, 40]. The immunomodulatory function of HLA-G occurs through interaction with

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NK cell inhibitory receptors such as KIR2DL or ILT-2 (immunoglobulin-like transcript-2) [15].

Other possible functions for HLA-G include suppression of T-lymphocyte proliferation, altering cytokine secretion by cytotoxic T-lymphocytes and uterine natrual killer (UNK) cells, shifting the immune response from pro-inflammatory type I to anti-inflammatory type II and interferon (IFN)-γ secretion by UNK cells to support normal pregnancy and fetal tolerance [2, 25, 30]. Moreover, soluble HLA-G (sHLA-G) is able to induce apoptosis of both activated NK and CD8 T cells. Unlike other *HLA* genes, *HLA-G* has very unique characteristics including seven membrane-bound (G1, G2, G3, and G4) and soluble (G5, G6, and G7) isoforms that arise from alternative splicing and very few amino acid polymorphisms [10]; this means HLA-G protein is essentially invariant in the human population, and as a result, the maternal immune system is unlikely to recognize the trophoblast cells as foreign. Lack of expression of MHC Class 1 molecules by a trophoblast cell exposes it to attack by NK cells; according to some studies, there is a decreased amount of sHLA-G in the serum of women experiencing RSA [18]. These observations suggest that the HLA-G/NK interaction at the maternal–fetal interface could be critical in determining the outcome of pregnancy.

Since HLA-G has immunomodulatory properties, understanding the role of polymorphic sites on its gene function may allow an individualized approach for future use of HLA-G for therapeutic purposes. Building on the above-noted observations, it was critical to probe for functional polymorphisms in this gene when studying pregnancy disorders with unknown etiology. Based on nucleotide sequence variations in the coding region of the *HLA-G* locus, 44 different alleles were observed in previous studies in which frequencies seem to vary from population to population [7, 13, 36]. Using quantitative analysis, it has recently been reported that the allelic variants of *HLA-G* such as 0105 N, 0106, 010401, 010108, etc. are related to lower level of sHLA-G known as 'low-secretor' alleles and thus may contribute to RSA susceptibility (Table 1) [17].

All previous studies describing the role of *HLA-G* polymorphisms in women with RSA reported that the null allele G*0105N was more frequent in RSA patients as compared to normal fertile controls. HLA-G*0105N (defined by a C deletion at exon 3) was the first *HLA-G* null allele found to occur independently in different ethnic groups. This deletion disrupted the reading frame, resulting in incomplete formation of HLA-G1, HLA-G4, and HLA-G5 isoforms; thus, it was postulated that reduced expression of HLA-G in individuals carrying an HLA-G*01:05 N allele would be associated with an increased risk for miscarriage [22]. On the other hand, HLA-G*0105N allele has been postulated to be protective against intrauterine infections in pregnancy. So, both their possible roles may coexist [6].

Table 1 HLA-G allele assignment based on nucleotide substitutions with clinical relevance

Risk factor for RSA	Yes (low-secretor)	No
HLA-G allele	0105N	010101
	0106	010106
	0103	010110
	010102	010403
	010103	
	010401	
	010108	
	010105	

Results are adapted from Aruna et al. [7], Sipak-Szmigiel et al. [36], Moreau et al. [27], and Abbas et al. [1]

Variation within the *HLA-G* promoter site must be essential for the function of this non-classical HLA-I protein, and so this could influence expression of the HLA-G locus [34]. The aim of present study was to examine whether two single nucleotide polymorphisms in the *HLA-G* gene (rs1736936 at promoter site and HLA-G*0105N located at exon 3) were associated with susceptibility to RSA.

Materials and methods

Study populations

In this case–control study, peripheral blood samples were obtained from women who had undergone evaluations of recurrent spontaneous abortions (RSA) at the Yazd Infertility Center over the 2013–2014 time frame. A total of 117 patients with two or more recurrent spontaneous miscarriages (as a case group) and 117 healthy women without any history of miscarriage and at least one normal birth (as a control group) were recruited to the study. The following additional data were obtained from all the RSA patients: age/medical history. At the time of this study, all the women were confirmed to have normal menstrual cycles and were healthy. In addition, all of the study subjects were without anatomical, microbial, viral, or genetic disease (evaluated through vaginal sonography or HSC,[1] pap-smear and karyotyping or cytogenetic tests, respectively), and hormone profile tests and ovulation/tubal patency tests were normal. They also underwent investigations for known causes of RSA, including evaluation of thyroid

[1] Diagnostic hysteroscopy.

stimulating hormone (TSH), anti-cardiolipinantibodies (ACLA), antinuclear antibodies (ANA), activated partial thromboplastin time, kaolin coagulation time, platelet count, and hysterosalpingography. The Ethics Committee of Yazd Hospital as well as the University of Medical Sciences (Yazd, Iran) gave formal approval for the studies prior to the subject recruitment.

Genotyping

Peripheral blood samples from the controls and patients were collected into EDTA-coated tubes, and molecular analyses were performed using DNA extracted from peripheral blood leukocytes after a standard salting out procedure. The polymorphisms of the *HLA-G* gene were genotyped using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Two sets of exon-specific primers were used as shown in Table 2. The cycling condition for promoter region consisted of 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 58 °C for 40 s, 72 °C for 30 s; and a final cycle extension at 72 °C for 5 min [20]. The cycling condition for exon 3 consisted of 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 61 °C for 45 s, 72 °C for 45 s; and a final cycle extension at 72 °C for 7 min [25]. Following the amplifications, PCR products were digested with restriction endonucleases *MluCI* (#R0538 s for promoter) and *PpuM-I* (#R0506 s for exon-3) and followed manufacturer protocols (NEB Inc., Beverly, MA, USA). The digestion products were then resolved over 2 % agarose gels and detected by staining with green viewer.

Statistics

Statistical analysis was conducted using SPSS19 Version 19 software (SPSS, Inc., Chicago, IL, USA). The goodness of fit between observed and estimated genotype and allele frequencies was determined by a Chi-squared test. Odds ratios (OR) were calculated with a confidence interval of 95 %. A difference was considered significant at P value <0.05.

Results

HLA-G genotype frequencies were in agreement with Hardy–Weinberg equilibrium, and the frequencies were consistent with those previously reported by other investigators. All the RSA and fertile control women had the mean age of (31.62 ± SD 3.372 years) and (32.12 ± SD 2.487 years) with respect (range 19–37 years), and number of miscarriages ranged between 2 and 5. Need to choose matched cases and controls on the basis of health status and age made the sample size limited. Distribution of the genotype frequencies in the RSA patient and control groups is shown in Table 3. The genotype and allele frequencies of rs1736936 between RSA patients and healthy controls were found not to be significantly different (P value = 0.323), OR (95 CI %) = 1.056 (0.844–1.319); although, HLA-G*0105N allele demonstrated a significant increase in the proportion of whole group of RSA women compared with fertile controls (P value = 0.015), OR (95 % CI) = 2.054 (1.798–2.347), which confirms association of HLA-G*0105N allele with recurrent pregnancy loss as reported in previous studies [3, 27, 31].

Discussion

Studies of couples with several cases of RSA have provided support to the theory that genes in the HLA region are associated with RSA susceptibility [21]. HLA-G is a non-classical human leukocyte antigen expressed primarily at the maternal–fetal interface. The expression of HLA-G in these areas further illustrates that HLA-G not only seems to play a role in trophoblast invasion, but also might activate maternal immuno-competent cells without compromising pregnancy [12]. HLA-G has a very unique structure that permits a restricted peptide presentation and, as a result the maternal immune system, is unlikely to recognize the trophoblast cells as foreign [29]. Otherwise, lack of expression of MHC class 1 molecules by a trophoblast cell exposes it to attack by NK cells and this can result in a loss of pregnancy maintenance, leading to embryo loss as unexplained recurrent miscarriage [42].

The interaction of HLA-G expressed at the surface of trophoblast cells with ILT2 from NK cells has been shown to confer protection against NK-cell-mediated cytolysis [33]. According to some studies there is a decreased amount of soluble HLA-G in the serum of women experiencing RSA [3, 13, 14]. The tight transcriptional control of *HLA-G* may be attributed to its unique promoter region in which rs1736936 SNP is close to known regulatory elements and C-containing sequences can act with Sp1 transcription factor (SP1); however, SP1 disappears in T-containing sequences [11, 19]. In this regard, this allele may influence HLA-G expression levels. A role for rs1736936 in RSA pathogenesis was first proposed in this study. The cited polymorphism was previously seen as associated with susceptibility to non-segmental vitiligo (NSV) in the Korean population [20]; however, no significant differences in allele and genotype frequencies existed between patients with rheumatoid arthritis (RA) and healthy control subjects [19]. Our data indicate that the difference in rs1736936 frequencies is not significant in this study. Thus, this change-in-function promoter

Table 2 Primers used in determination of HLA-G exon 3 (codons 130) and promoter polymorphisms in PCR–RFLP analysis

Polymorphism	Primer sequence	Product size (bp)
rs1736936	5′-CTCTGCTCCTTTTCCTCACCTC-3′	T allele: 105 and 154 bp;
	5′-CAAGTGCCTGACATTCTAGAAGC-3′	C allele: 259 bp
HLA-G*0105N	5′-CACACCCTCCAGTGGATGAT-3′	HLA-G*0105N: 276 bp;
	5′-GGTACCCGCGCGCTGCAGCA-3′	All but HLA-G*0105N allele: 108 and 168 bp

Results adapted from Matter and Sharif [25] and Le Discorde et al. [22]

Table 3 Genotypes and alleles frequencies of promoter single nucleotide polymorphism (rs1736936) and frequencies of HLA-G*0105N null allele in fertile and RSA women

				Case	Control	*p* value
rs1736936	Genotype	T/T	Count	31	37	0.323
			% within group	26.5 %	31.6 %	
		T/C	Count	57	60	
			% within group	48.7 %	51.3 %	
		C/C	Count	29	20	
			% within group	24.8 %	17.1 %	
	Allele	T	Count	88	97	0.198
			% within group	75.2 %	82.9 %	
		C	Count	86	80	0.472
			% within group	73.5 %	68.4 %	
	Functional		Count	111	117	0.015
HLA-G*0105N null allele			% within group	94.9 %	100 %	
	Heterozygot		Count	6	0	0.015
			% within group	5.1 %	0.0 %	
	Null homozygot		Count	0	0	–
			% within group	0.0 %	0.0 %	

polymorphism does not appear to be associated with RSA susceptibility.

In addition, the present study compared genotype frequencies of rs1736936 in other populations (Table 4). As seen, allele frequencies of rs1736936 in Iranian population (T, 0.508; C, 0.492) have a similar pattern to those in Asia as anticipated. The second polymorphism, HLA-G*0105N allele, was present in comparable frequencies in both groups. The mentioned allele, which is defined by a cytosine deletion at the first base of codon 130, changes the reading frame resulting in appearance of a stop codon near the beginning of exon 4, and therefore blocks translation of HLA-G1, HLA-G4, and HLA-G5. Therefore, the HLA-G*0105N allele might be associated with decreased amount of serum concentrations of sHLA-G and an increase in the incidence of trophoblast attack by the maternal NK cells [41].

Despite the fact that the null genotype is a contributing factor in spontaneous abortion, HLA-G*0105N is able to eradicate intrauterine pathogen contamination [22]. The frequency of this null allele has been found to be 13.8 % in Indian residents [1], 8 % in African-Americans, 4.8 % in Ghanaians, 3 % in Spaniards, 2.3 % in mixed German and Croatian, and 0.6 % in Danish subjects. In the present study, the HLA-G*0105N alleles were found with 5.1 % of frequency among the Iranians evaluated, a level similar to

Table 4 The human SNP database (dbSNP BUILD131) presents frequencies of genotype for rs1736936

Population	TT	TC	CC
Iranian	0.265	0.487	0.248
European	0.232	0.482	0.286
Sub-Saharan African	0.317	0.550	0.133
Japanese	0.477	0.432	0.091
Chinese	0.378	0.356	0.267
Korean	0.360	0.474	0.166
African American	0.333	0.333	0.333

Results shown are adapted in part from Kim et al. [19]

those in Ghanaian populations. Although worldwide population studies showed the highest allele frequency in the Middle East, this allele was not found in Japanese, American-Caucasian, Chinese [23], and Amerindian populations [6]. This variation in HLA-G*0105N polymorphism presence among different ethnic populations is possibly a result of evolution and suggests the allele may confer some advantage at the HLA-G immuno-functional level. On the other hand, this allele was consistently observed in the heterozygous state except for a few cases of homozygosity reported in African-Americans [24]. No homozygous individuals for this allele were found in the sample population assessed in the present study.

The data here suggest that *HLA-G* promoter polymorphisms might not have clear-cut associations with increased risk of RSA. Particular HLA-G*0105N alleles were associated with significantly lower serum concentrations of sHLA-G proteins and thus possible increases in RSA susceptibility. Clearly, a larger sample size and further studies are necessary to confirm this observation and better clarify the role of HLA-G at risk for RSA.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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