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Association of genetic variants in the 3′UTR of HLA-G with Recurrent Pregnancy Loss

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ABSTRACT

Human Leukocyte Antigen (HLA)-G is involved in reprogramming immune responses at fetal-maternal interface during pregnancy. We evaluated the genetic diversity of the 3′ Un-Translated Region (UTR) of *HLA-G*, previously associated with *HLA-G* mRNA post-transcriptional regulation, in women with unexplained Recurrent Pregnancy Loss (RPL), with 2 pregnancy losses (RPL-2, n = 28), or 3 or more pregnancy losses (RPL-3, n = 24), and in 30 women with a history of successful pregnancy. Results showed in RPL-2, but not in RPL-3, women compared to controls: i) higher frequency of the 14 bp Ins allele, in single and in double copy; ii) significantly lower frequency of DelG/X genotype, iii) reduced frequency of the UTR-2, and UTR-3 haplotypes; iv) higher frequencies of the UTR-5, UTR-7, and UTR-8 haplotypes. This pilot study supports the relevance of performing 3′UTR *HLA-G* genetic screening, not limited to a specific polymorphism, but considering the extended haplotypes, as a possible predictor of pregnancy outcome.

1. Introduction

Sporadic miscarriage is the most frequent complication of early pregnancy. Approximately 70% of conceptions fail prior to live birth with most losses occurring prior to implantation or before the missed menstrual period [1]. Recurrent Pregnancy Loss (RPL) was initially defined as three or more consecutive pregnancy losses before 20–22 weeks of gestation [2]. Later, a large retrospective study indicated no differences in the frequency of diagnostic factors between women with two or more pregnancy losses [3]. Based on these results the Practice Committee of the American Society for Reproductive Medicine defined RPL as two or more failed pregnancies [4]. The prevalence of RPL is considerably lower than the prevalence of sporadic miscarriage, ranging from 0.8–1.4% to 2–3%, if biochemical losses (i.e. pregnancies that fail before ultrasound or histologic confirmation) are included [5]. Causes of RPL include chromosomal abnormalities, autoimmunity, such as untreated hypothyroidism and diabetes mellitus, uterine anatomic abnormalities, heritable thrombophilia, infections, and environmental cues [6]. The incidence of fetal chromosomal abnormalities, which is identified in 29–60% of women with RPL, decreases as the number of miscarriages increases, suggesting that other mechanisms may play a role in pregnancy loss [7]. The correction of endocrine disorders, and the treatment of the anti-phospholipid syndrome (APS), the most common autoimmune cause of pregnancy complications, have been shown to be effective in controlling miscarriage [6]. However, prevention of RPL remains an unmet medical need.

The uterus and the placenta constitute a unique site of immune modulation where the semi-allogeneic fetus is tolerated by the maternal immune system. Several years of research have identified the non-classical Human Leukocyte Antigen (HLA)-G, physiologically expressed on extravillous trophoblasts [8], as a molecule operatives in promoting tolerance during pregnancy [9]. HLA-G inhibits cytotoxic $CD8^+$ T cells and natural killer (NK) cells, and allo-specific $CD4^+$ T cell proliferation, modulates the activity of antigen presenting cells (APCs), and induces the differentiation of myeloid and T regulatory cells (reviewed in [10]). At the fetal maternal inferface during pregnancy the expression of HLA-G on trophoblasts prevents their NK-mediated lysis [11], and HLA-G-

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expressing regulatory T cells and APCs are highly represented [12,13], supporting the critical role of HLA-G in promoting tolerance during pregnancy. In pregnancy complications, such as preeclampsia, it has been repetitively demonstrated a reduced levels of HLA-G expression in placental tissues [14,15] and in the maternal circulation [15,16], and defects in HLA-G-expressing regulatory cells [13]. Moreover, in RPL reduced levels of HLA-G have been reported [17].

The HLA-G *locus* contains several polymorphisms in the non-coding regions, including those present at the 3′Un-Translated Region (UTR), which influence HLA-G expression [18]. Among the 3′UTR of *HLA-G* polymorphisms, the most studied are an insertion/deletion (Ins/Del) of 14 base pairs (bp) that has been associated with *HLA-G* mRNA stability [19,20], the G/C in position +3142 that can be involved in micro(mi)RNA-mediated post-transcriptional regulation [21], and the G/A at position +3187 that lands in an AU-rich region [22]. Other and less studied Single Nucleotide Polymorphisms (SNPs) are +3001 C/T, +3003 T/C, +3010 G/C, +3027 C/A, +3035 C/T, and +3196 C/G, which have been proposed as potential miRNA binding sites [23]. These polymorphisms arrange in haplotypes, named UTRs [18], and the most frequent are from UTR-1 to UTR-8 [24]. The association between 3′UTR haplotypes of *HLA-G* and protein expression has been recently studied: haplotypes containing 14 bp Del (i.e. UTR-1) are associated with high levels of soluble HLA-G, whereas those with 14 bp Ins (i.e. UTR-2, and UTR-5) with low HLA-G levels [25].

A lot of efforts have been made to define whether the genetic variations influencing the HLA-G expression can be associated with tolerance and/or pregnancy complications. Several studies have been conducted to define the influence of the 14 bp Ins/Del in recurrent miscarriage (RSA) with contrasting results [26–28]. Recent meta-analysis studies attributed the variability in the reported results to the heterogeneity of the cohorts analyzed and to the criteria used for patient selection [29,30]. Nevertheless, Wang et al. [29] reported an association between the 14 bp Ins with recurrent miscarriage and an overall risk of RSA independently from the number of pregnancy losses, while Fan et al. [30] reported the same association only in women with three or more miscarriages. Thus far, the association between 3′UTR polymorphisms, not limited to the 14 bp Ins/Del, or the extended 3′UTR *HLA-G* haplotypes and RPL has been not investigated.

2. Subjects and methods

2.1. Study subjects

Subjects recruited in the study were admitted to the High-risk Pregnancy Outpatients Clinics of the San Raffaele University Hospital, Milan, Italy for investigation and treatment. The study sample included 52 patients diagnosed with RPL as two or more consecutive pregnancy losses confirmed by the Hospital records, according with the definition of the American Society for Reproductive Medicine [4]. Patients were subsequently divided in two different groups: women with 2 pregnancy losses (RPL-2, n = 28), and women with 3 or more pregnancy losses (RPL-3, n = 24). The control group comprised 30 age-matched women from couples with no history of miscarriages and a previous history of successful pregnancy (HP) (Table 1). Among the RPL group 28.8% had signs of autoimmunity, 15.4% had dysthyroidism, 1.9% had hypertension, and 17.3% had congenital thrombophilia, and none of them had uterine abnormalities as assessed by hysteroscopy or uterine ultrasonography. Conversely, 26.7% of HP had uterine anatomic abnormalities and after correction delivered successfully, and none of them was affected by other diseases. All RPL patients and their male partners had normal karyotypes. No others investigations on the male partners were performed. All patients were regularly menstruating and all had normal thyroxin levels in the plasma. Human peripheral blood was obtained upon informed consent in accordance with local ethical committee approval and with the Declaration of Helsinki.

Table 1
Patients characteristics and pregnancy history.

	RPL-2 women	RPL-3 women	HP women
Patients (N)	28/52	24/52	30
Age (mean ± SD)	36.3 ± 4.3	36.5 ± 4.3	36.1 ± 4.0
Caucasian (N, %)	26/28 (92.8)	22/24 (91.7)	27 (90)
Miscarriages (N, %)	57/69 (82.6)	92/112 (82.1)	0
Intrauterine fetal death (N, %)	1/69 (1.4)	1/112 (0.9)	0
Live birth (N, %)	11/69 (16)	19/112 (17)	38 (100)

2.2. DNA isolation

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs), separated by density gradient centrifugation over Lymphoprep (Nycomed Amersham), using a commercial kit (QIAamp, QIAGEN, Italy) according to the manufacturer' s instructions. The DNA samples were stored at −20 °C in a freezer compartment.

2.3. Amplification and sequencing of 3′UTR of the HLA-G gene (or genotyping)

100 ng of genomic DNA were amplified in a 25 μl reaction containing 1× polymerase chain reaction (PCR) buffer (Roche, USA), 0.2 mM dNTP mix (Roche, USA), 1.5 mM $MgCl_2$ (Roche, USA), 0.8 U *Taq* Polymerase (Roche, USA), and 1 μM of each primer (For: 5′ TCACCCCTCACTGTGACTGA 3′; Rev: 5′ TTCTCATGTCTTCCATTTATTTTGTC 3′). The initial denaturation step was carried out at 95 °C for 3 min, followed by 30 cycles at 93 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s, and by a final extension step at 72 °C for 10 min. The amplification product was evaluated using a 2.5% agarose gel, purified using a commercial kit (Wizard SV Gel and PCR Clean-Up System, Promega, WI, USA) according to the manufacturer' s instructions. The Rev primer (5′ TTCTCATGTCTTCCATTTATTTTGTC 3′) by PrimmBiotech (Milan, Italy) was used to perform direct sequencing on both strands of purified amplification products. Sequencing pherograms were analyzed with the CodonCode Aligner software (Centerville, MA), and the 14 bp Ins/Del (rs1704), +3003 C/T (rs1707), +3010 C/G (rs1710), +3027 A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142) and +3196 C/G (rs1610696) polymorphic sites were individually annotated. Haplotype and genotype construction were assigned and named according to previous report [24] (Supplementary Table 1).

2.4. Statistical analysis

HLA-G 3′UTR allele and genotype frequencies were obtained by direct count. Single comparisons of allele and genotype frequencies between populations were performed using the Fisher test and, when necessary, Bonferroni correction for multiple comparisons was applied. Differences were regarded as significant at $^{*}P < 0.05$. The results were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA).

3. Results and discussion

The main objective of this pilot study was to investigate the possible association of 3′UTR polymorphisms and haplotypes of