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A tug-of-war between tolerance and rejection – New evidence for 3′UTR HLA-G haplotypes influence in recurrent pregnancy loss

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ABSTRACT

HLA-G is a molecule essential to the maintenance of the maternal-fetal interface tolerance, thus contributing to a healthy pregnancy. Here we investigate the role of HLA-G single nucleotide polymorphisms (SNPs) and whether a specific HLA-G haplotype influence or not recurrent pregnancy loss (RPL) risk. A total of 296 DNA samples from RPL (N = 140) and controls (N = 156) were evaluated. The HLA-G 3′UTR region was sequenced and eight major SNPs were evaluated (14pb insertion/deletion, +3003T/C, +3010C/G, +3027C/A, +3035C/T, +3142G/C, +3187A/G, +3196C/G). A high linkage disequilibrium (LD) among all pairs and a perfect LD between +3010C/G and +3142G/A (D′ = 1.0, r^2 = 1.0) were observed. Our data showed an increased risk to +3010CC genotype carriers in comparison with control [odds ratio (OR) 2.05 95% confidence interval (CI) 1.05–4.00, p = 0.035] and to a decreased risk of RPL in +3142CC genotype carriers (OR = 0.49 95%CI 0.25–0.95, p = 0.035) and +3187AG genotype carriers (OR = 0.58 95%CI 0.35–0.94, p = 0.029). A total of eight haplotypes were observed in the sample, being UTR-1 and UTR-2 the most represented. An association between UTR-1 haplotype carriers with a reduced risk of both RPL and secondary RPL was observed. Our results indicate that the HLA-G 3′UTR plays important roles in RPL and might be an important marker of susceptibility to this, and possible to other, pregnancy disorders.

1. Introduction

Recurrent pregnancy loss (RPL) is historically defined as three consecutive pregnancy losses prior to 20 weeks. Nevertheless, due to a high risk of miscarriage in subsequent pregnancies after two consecutive pregnancy losses, some guidelines consider two consecutive pregnancy losses as a relevant point, that deserves evaluation in patients with no prior live birth (reviewed in Christiansen [1]). When defined by the presence of at least two consecutive miscarriages, RPL affects 2–5% of couples [1,2]. This disorder is classified in different clinical subgroups according to the woman reproductive history. Primary RPL is characterized by consecutive losses and no prior successfully pregnancy, while at least one successful pregnancy, regardless of the number of miscarriages, characterizes the secondary RPL [3]. Notably, after the identification of possible etiologic factors, a specific cause of RPL is clearly identified in only ~50% of all cases [3].

As the fetus is not genetically identical to its mother, it is reasonable to think that mechanisms must exist to allow the mother to carry the fetus throughout gestation without a rejection. Identified in the maternal-placental interface mainly on extravillous trophoblast cells (EVT), Human leukocyte antigen (HLA)-G has a relevant role in reproduction, it contributes to trophoblast invasiveness, vascular remodeling, decidual cell differentiation and establishment of a immuno tolerogenic environment [4–7].

Among the non-classical class I molecules, *HLA-G* is the best characterized and when compared to its classical class I counterparts, HLA-G is a low polymorphic molecule. At present, 51 alleles

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have been recognized by the international ImMunoGeneTics project (IMGT) [8]. Seven different isoforms of HLA-G are reported as the result of the alternative splicing of messenger RNA three of which are soluble (sHLAG5-G7) and four are membrane bound (G1-G4) [9]. In addition, a soluble G1 (sHLA-G1) form can be generated by cell surface proteolytic shedding [10].

In pregnancy, sHLA-G5, HLA-G1 and its shed form seem to be molecules of great importance [11]. Although functionality of the different isoforms is poorly understood, they might be involved in the modulation of immune activity. Through interaction with inhibitory receptors immunoglobulin-like transcript (ILT)2, ILT-4 and the killer immunoglobulin-like (KIR)2DL4, HLA-G promotes tolerance by regulating innate and adaptive responses (reviewed in Rebmann et al. [12]). Therefore, specific interactions contribute to: (a) the immune suppression of the cytotoxic activity of the decidual NK cells (dNK); (b) the inhibition of proliferation of CD4 + T lymphocytes, CD8+ T and B lymphocytes and induction of apoptosis of CD8+ T cells via FAS-FAS-L pathway and; (c) the placental stimulation and development through the secretion of angiogenic factors by dNK cells and macrophages [4,13,14].

The 3′ untranslated region (UTR) of *HLA-G* is of particular interest as it impacts the expression patterns of the gene due to the presence of regulatory elements such as AU-rich motifs, polyadenylation signals and miRNA binding sites [15]. Several *HLA-G* SNPs have been associated with an increased maternal risk to pregnancy disorders such as preeclampsia, infertility and recurrent miscarriage [5,16–18]. Three polymorphic sites have been reported to modify HLA-G expression: (1) the 14-bases pair (bp) insertion/deletion (ins/del) which is associated to mRNA stability [19]; (2) the +3142G which has been described as influencing micro RNA (miRNA) binding thus interfering on mRNA availability [20,21] and; (3) the +3187A described to affect mRNA stability due to a proximal AU-rich motif [22]. In despite of being a quite short segment, the 3′UTR presents at least eight polymorphic sites that are more frequently evaluated in worldwide populations. Recently, several other single nucleotide polymorphisms and 3′UTR haplotypes have been characterized [15,23].

A study evaluating the influence of 3′ UTR haplotypes and the expression patterns of sHLA-G in healthy non-pregnant individuals of a Brazilian cohort and French showed that some haplotypes were associated with high (UTR-1) and low (UTR-5, UTR-7) HLA-G levels in blood plasma [24]. Although conflicting results exist due to methodological issues, HLA-G UTR-1 haplotype seems to be one of the most important determinants on sHLA-G expression in pregnant and non-pregnant individuals [11,25]. Genetic variability in this region has been approached in a wide range of association studies, but in most cases, a limited set of putative functional variants was evaluated, whereas only few studies have evaluated the full sequence variation in unrelated healthy, non-healthy individuals as well as in the pregnancy outcome [16,17,26–28]. Therefore, and since the individual genetic variability in the 3′UTR might impact the pattern of HLA-G expression, we performed a comprehensive analysis of the *HLA-G* 3′ UTR in recurrent pregnancy loss and healthy multiparous women, in order to evaluate the maternal influence of this genetic *locus* in pregnancy outcome.

2. Materials and methods

2.1. Subjects

The present study enrolled a total of 296 women (156 multiparous control and 140 RPL idiopathic women). The subjects were recruited between 2000 and 2011 from the Prenatal Diagnosis Clinic of the Medical Genetics Service of the *Hospital de Clínicas de Porto Alegre* (HCPA), located in Southern Brazil. All women reporting at least two pregnancy losses before 24 weeks of gestation with the same partner and with no report of full-term pregnancy were invited to participate in the study [29,30]. Clinical and demographic data was obtained through a structured interview (obstetric history, family history of malformations, weight, height, occupation, use of tobacco, alcohol consumption, drug use). All women were subjected to a preliminary standard diagnostic protocol evaluating known causes of pregnancy losses [hysteroscopy, laparoscopy, ultrasound, karyotypic examination, immunological risk factors (anticardiolipin, lupus anticoagulant, antinuclear antibodies) and hormonal status (gonadotrophins, FSH, LH, prolactin, thyroid hormones, thyroperoxidase] before inclusion in the study. Exclusion criterion was considered by the presence of any clinical condition that could prevent full-term pregnancies.

The control group consisted of 156 healthy multiparous women with at least two successful pregnancies reported and no history of pregnancy loss and/or infertility, randomly selected to participate in the study during blood collection for routine laboratory analyses at the HCPA. This study was approved by the Research Ethics Committee of the Research and Postgraduate Studies Group of the HCPA, under the protocol number #11-242. Written informed consent in accordance with the Declaration of Helsinki was obtained from every participant before their inclusion in the study.

2.2. DNA extraction

Genomic DNA was obtained from the blood samples and saliva. The DNA extraction from blood was performed according to Lahiri and Nurnberger [31] and the DNA from saliva samples was extracted using the Oragene® DNA collection kit (DNA Genotek Inc., Canada), in accordance with the manufacturer's protocol.

2.3. HLA-G 3′ UTR analysis

The 3′ UTR of *HLA-G* gene (hg38 assembly chr6:29830768-29831270) was amplified by polymerase chain reaction (PCR) as previously described [32]. The PCR products were directly sequenced using the reverse primer GmiRNA in an ABI 3730 XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Finally, *HLA-G* polymorphic variants were assessed by interpretation of chromatogram peaks using the FinchTV software version 1.4.0 (available on http://www.geospiza.com/Products/finchtv.shtml). In the total, 133 RPL and 152 control samples were efficiently sequenced while seven and four samples, respectively, were excluded from genetic analysis due to low reactions quality.

2.4. Haplotype analysis

Linkage disequilibrium (LD) between pairs of the genetic variants evaluated in the study was assessed by r2 and D′ coefficient using the Haploview 4.1 software [33]. Haplotype inference was performed using a Bayesian method implemented in the PHASE software version 2.1 using default parameters [34]. Ten independent runs with different seed values for the random number generator were used to check the consistency across the results. The haplotype overall frequencies generated from PHASE showed highly consistent results across different runs. One sample of each group was excluded from the haplotype analysis due to a low confidence in the estimate. The haplotype Hardy-Weinberg equilibrium expectation was tested by exact test implemented in the Arlequin software version 3.5.1.3 [35]. All haplotype were named accordingly with previous studies [15,23].