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Laboratório de Imunogenética

TESE DE DOUTORADO

HLA-G EM DOENÇAS REUMATOLÓGICAS: ANÁLISE IMUNOGENÉTICA

Aluno de doutorado: Tiago Degani Veit

Orientador: Dr. José Artur Bogo Chies

Outubro de 2011

Porto Alegre – RS

Brasil

Universidade Federal do Rio Grande do Sul

TIAGO DEGANI VEIT

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Prof. Dr. José Artur Bogo Chies

Orientador

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“Senta na primeira classe e presta bem atenção no professor.”
Ao autor dessa frase, dirigida a mim em 1984, antes da primeira aula, dedico esta tese.

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SUMÁRIO

LISTA DE ABREVIATURAS 8

RESUMO 9

ABSTRACT 10

CAPÍTULO 1 - INTRODUÇÃO 11

CAPÍTULO 2 – Artigo 1 17

CAPÍTULO 3 – Artigo 2 33

CAPÍTULO 4 – Artigo 3 37

CAPÍTULO 5 – Artigo 4 44

CAPÍTULO 6 – Artigo 5 62

CAPÍTULO 7 – Discussão Geral 89

Influência da região 3'-não traduzida (3'UTR) e dos microRNAs na expressão do gene HLA-G 90

Influência de variantes polimórficas da região 3'-não traduzida (3'UTR) do gene HLA-G na suscetibilidade e curso de doenças auto-imunes. 92

Expressão da molécula HLA-G no plasma e líquido sinovial de pacientes com artrite 97

Perspectivas futuras 98

Considerações finais 101

REFERÊNCIAS BIBLIOGRÁFICAS 103

CAPÍTULO 8 - ANEXOS 106

ANEXO 1 - HLA-G +3142 polymorphism influences the susceptibility to infections in sickle cell anemia patients 107

ANEXO 2 - Association of the HLA-G 14 bp polymorphism with systemic lupus erythematosus. 114

ANEXO 3 - Association of the HLA-G 14-bp insertion/deletion polymorphism with juvenile idiopathic arthritis and rheumatoid arthritis 122

LISTA DE ABREVIATURAS

3'UTR – Região 3' não traduzida
AIJ – Artrite Idiopática Juvenil
AR – Artrite Reumatóide
CD – "Cluster of Differentiation"
Célula NK – Célula "Natural Killer"
DL – Desequilíbrio de Ligação
ELISA – "Enzyme-Linked Immunoabsorbent Assay"
FR – Fator Reumatóide
HCV – Vírus da Hepatite-C
HLA-A – Antígeno Leucocitário Humano A
HLA-DR – Antígeno Leucocitário Humano DR
HLA-G – Antígeno Leucocitário Humano G
hsa-miR – Homo sapiens microRNA
ILT2 – "Immunoglobulin-Like Transcript 2"
IL-10 – Interleucina 10
JEG-3 – Linhagem de coriocarcinoma humano HLA-G positiva
KIR2DL4 – "Killer cell immunoglobulin-like receptor 2DL4"
LES – Lúpus Eritematoso Sistêmico
M8 – linhagem tumoral humana
MHC – "Major Hystocompatibility Complex"
MTX – Metotrexato
pb – pares de base
rs1704 – SNP de referência 1704 ("reference SNP 1704")
rs1063320 – SNP de referência 1063320
sHLA-G – HLA-G solúvel
SNP – "Single Nucleotide Polymorphism"
TCR – "T-Cell Receptor"

RESUMO

Nas últimas décadas, a molécula HLA-G despontou como uma importante molécula imunossupressora. O fato de a molécula HLA-G estar envolvida em diversos mecanismos de imunorregulação e, considerando sua expressão em doenças inflamatórias, sugere a possibilidade de um papel dessa molécula na patogênese e no curso de doenças reumatológicas. Com base nisso, esta tese teve como objetivo avaliar a influência da molécula HLA-G, bem como das variantes genéticas do gene *HLA-G* na suscetibilidade e no curso de doenças reumatológicas, buscando correlacionar fatores genéticos, moleculares, clínicos e imunológicos. Neste trabalho, avaliamos a influência de variantes alélicas da região 3' não traduzida do gene HLA-G na suscetibilidade e curso do lúpus eritematoso sistêmico (LES), na suscetibilidade à artrite reumatóide (AR) e avaliamos a expressão de HLA-G solúvel (sHLA-G) em pacientes com AR e artrite idiopática juvenil (AIJ). Observamos que o mesmo haplótipo (D/G) parece estar associado tanto à suscetibilidade ao LES quanto à AR, apontando para o gene *HLA-G* como um potencial fator de suscetibilidade comum às duas doenças. Em AR, observamos maiores níveis de HLA-G solúvel no líquido sinovial de pacientes fator reumatóide-negativos (FR-) em comparação com pacientes FR+, e diferentes padrões de correlação entre os níveis plasmáticos de sHLA-G e parâmetros de atividade de doença após estratificarmos os grupos de pacientes para positividade para FR e gênero. Nossas observações, portanto, colocam a molécula e o gene HLA-G como elementos diretamente envolvidos na patogênese e curso dessas doenças e encorajam estudos futuros que procurem elucidar o papel da molécula HLA-G no curso de doenças reumatológicas.

ABSTRACT

In the last decades, HLA-G has emerged as a major immunosuppressive molecule. The fact that HLA-G is involved in various mechanisms of immunoregulation and given its expression in inflammatory diseases suggests the possibility of a role of this molecule in the pathogenesis and course of rheumatic diseases. This thesis aimed to evaluate the influence of HLA-G, as well as genetic variants of the *HLA-G* gene in the susceptibility and course of rheumatic diseases, seeking to correlate genetic, molecular, clinical and immunological factors. We evaluated the influence of allelic variants of the *HLA-G* gene 3 'untranslated region (3”UTR) in susceptibility and course of systemic lupus erythematosus, in the susceptibility to rheumatoid arthritis and we have also evaluated the expression of soluble HLA-G in patients with rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). We noted that the same haplotype (D/G) seems to be associated with susceptibility to RA and SLE, pointing to the *HLA-G* gene as a potential common susceptibility factor to both diseases. In RA, we observed higher levels of soluble HLA-G (sHLA-G) in synovial fluid (SF) from rheumatoid factor negative (RF-) patients as compared to RF+ patients, and different patterns of correlation between sHLA-G plasma levels and disease activity parameters after stratifying patient groups for RF positivity and gender. Our observations, therefore, put the gene and molecule HLA-G as directly involved elements in the pathogenesis and course of these diseases and encourage future studies that seek to elucidate the role of HLA-G in the course of rheumatic diseases.

CAPÍTULO 1 - INTRODUÇÃO

Capítulo 1 – INTRODUÇÃO

Reumatismo é um termo não-específico usado para descrever qualquer desordem dolorosa que afeta o sistema locomotor incluindo articulações, músculos, tecidos conjuntivos, tecidos suaves em volta das articulações e os ossos. As doenças reumáticas exercem um grande impacto nas sociedades e indivíduos, com custos econômicos para todos os países (Sangha, 2000).

A incapacidade funcional e laboral que as doenças reumatológicas geram tem um forte impacto sócio-econômico. Nos EUA, em 2001, a prevalência de artrites/sintomas articulares crônicos na população foi estimada em 33%, representando aproximadamente 69 milhões de pessoas (*Centers for Disease Control and Prevention (CDC*), 2002). Nesse mesmo país, estima-se que a artrite reumatóide (AR) afete cerca de 1,3 milhão de adultos, que o lúpus eritematoso sistêmico afete de 161 a 322 mil adultos e que 294 mil crianças sejam acometidas de artrite idiopática juvenil (AIJ) (Helmick *et al*., 2008). Grande esforço tem sido despendido nas últimas décadas na busca de novos tratamentos e da elucidação dos mecanismos de imunopatogênese e progressão desse grupo de doenças. No entanto, muitos desses mecanismos, incluindo aqueles associados à regulação da resposta imune, permanecem obscuros.

Nas últimas décadas, a molécula HLA-G despontou como uma importante molécula imunossupressora. O Antígeno Leucocitário Humano (HLA)-G é uma molécula de MHC (“Major Histocompatibility Complex” – Complexo Principal de Histocompatibilidade) de classe I (Figura 1A), dita “não clássica”, diferindo de outras moléculas de HLA de classe I “clássicas” - HLA-A, B, C - por apresentar expressão restrita a poucos tecidos em condições fisiológicas, baixo polimorfismo, diferentes isoformas geradas por “splicing” alternativo que podem ser tanto de membrana (G1-G4) ou secretadas (G5-G7 – Figura 2) e, interessantemente, por ser o único membro dessa família de moléculas capaz de formar dímeros, característica que parece ser decisiva para a sua atividade biológica (Clements *et al*., 2007) – Figura 1B.

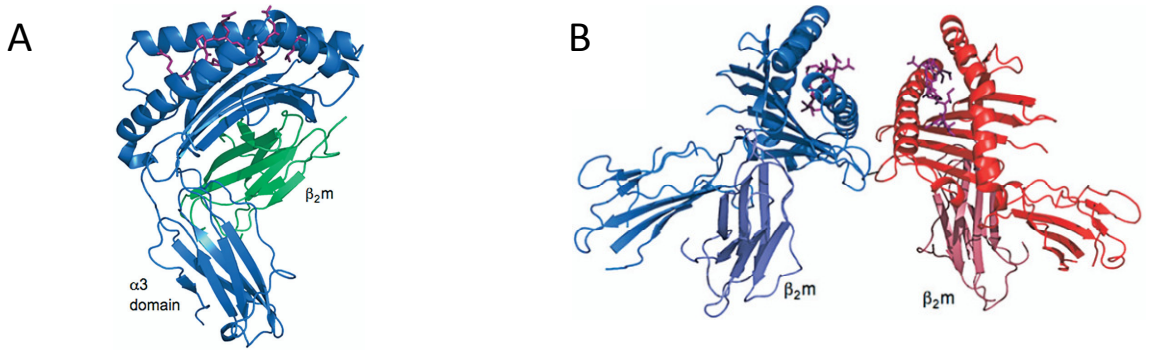

Figura 1. A) Estrutura da molécula HLA-G (isoforma G1), B) Dímero de HLA-G (Retirado de Clements *et al.*, 2007).

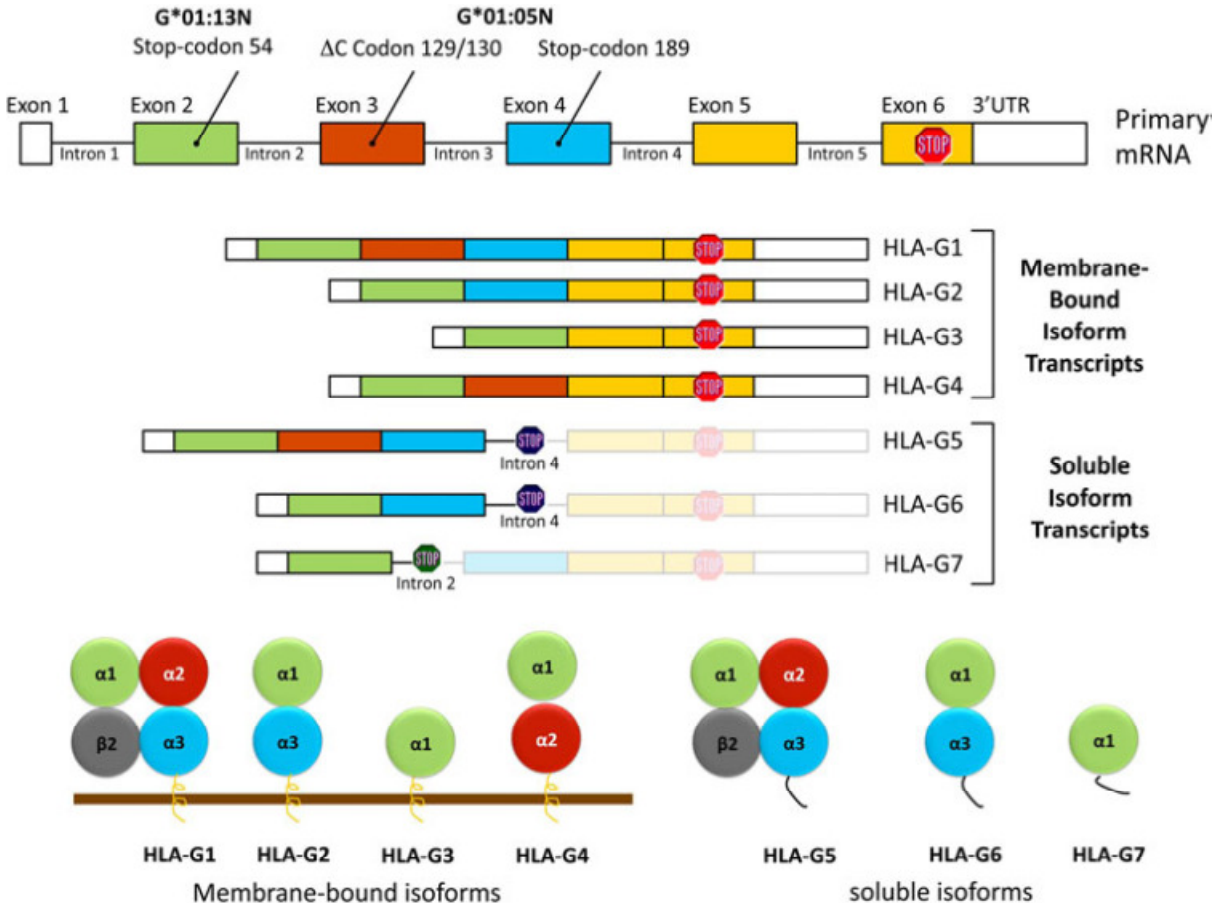

Figura 2. Isoformas de HLA-G produzidas por *splicing* alternativo do mRNA primário (Retirado de Donadi *et al.*, 2011).

HLA-G foi primeiramente descrita na interface materno-fetal, expressa pelo citotrofoblasto, atraindo a atenção da comunidade científica pelo seu possível papel na manutenção da gravidez, papel que vem ganhando crescente sustentação com o passar dos anos, através de diversos trabalhos já publicados na literatura. A molécula HLA-G é capaz de suprimir as respostas imunes, contribuindo para o escape e tolerância imunológicas (Carosella *et al.*, 1999). Diversos estudos apontam para um papel imunorregulador da molécula HLA-G através de diversos mecanismos, como inibição da proliferação e atividade citotóxica de células T e NK, indução de células T supressoras, inibição da apresentação de antígenos e da maturação de células dendríticas, indução de células apresentadoras de antígenos tolerogênicas, indução do aumento do número de receptores inibitórios, entre outros mecanismos que serão abordados com mais detalhe no capítulo 2. Por outro lado, a molécula HLA-G pode comportar-se também como uma molécula ativadora em células NK, efeito que parece ser importante no processo de remodelação do endométrio durante a gestação (Rajagopalan *et al.*, 2006).

O fato de a molécula HLA-G estar envolvida em diversos mecanismos de imunorregulação e, considerando sua expressão em doenças inflamatórias, sugere a possibilidade de um papel dessa molécula na patogênese e no curso de doenças reumatológicas. Além disso, os estudos oriundos da dissertação de mestrado (Anexos 1 e 2) claramente apontam para a influência de variantes genéticas do gene HLA-G na suscetibilidade e morbidade associada a esse grupo de doenças. Com base nisso, esta tese teve como objetivo avaliar a influência da molécula HLA-G, bem como das variantes genéticas do gene *HLA-G* na suscetibilidade e no curso de doenças reumatológicas, buscando correlacionar fatores genéticos, moleculares, clínicos e imunológicos.

O estudo está dividido em mais seis capítulos e três anexos. O capítulo 2 caracteriza-se como um artigo de revisão aceito na revista *Current Immunology Reviews* em 2009 e publicado em 2010 (Veit *et al.*, 2010), trazendo uma revisão abrangente da molécula HLA-G no que diz respeito a sua atividade biológica, os

mecanismos de regulação da expressão gênica, as regiões polimórficas com potencial papel nessa regulação e também uma revisão do estado da arte no que diz respeito à associação da molécula e de variantes genéticas do gene *HLA-G* em diversas situações fisiológicas e patológicas, com especial enfoque às doenças inflamatórias, incluindo as que serão abordadas no presente estudo.

O capítulo 3 caracteriza-se como um artigo de hipótese sobre o potencial papel de um SNP da região 3' não traduzida (3'UTR), localizado na posição +3142 (rs1063320) na regulação pós-transcricional da expressão de HLA-G, publicado na revista *Transplant Immunology* em 2009 (Veit & Chies, 2009), que acabou motivando outros trabalhos contidos nesse estudo, como os artigos dos capítulos 4, 5 e anexo 1. O leitor mais atento reparará que os capítulos 2 e 3 não obedecem a uma ordem cronológica, o que pode ser comprovado pelo fato de o artigo do capítulo 2 conter, entre suas referências, o artigo de hipótese do capítulo 3. Tal arranjo foi feito com vistas a favorecer a compreensão do leitor sobre o estudo.

O capítulo 4 é o primeiro artigo de resultados desta tese, descrevendo a associação de um haplótipo envolvendo os polimorfismos de inserção/deleção de 14 pb (rs1704) e +3142C>G, localizados na 3'UTR, composto pelos alelos de deleção de 14 pb e +3142G (D/G) com a suscetibilidade ao lúpus eritematoso sistêmico (LES) em mulheres, tendo observado também a associação de menores índices de atividade da doença em pacientes duplo-heterozigotos. O artigo foi publicado em 2011 na revista *Lupus* (Consiglio *et al.*, 2011).

No capítulo 5, são apresentados dados não publicados descrevendo a associação do mesmo haplótipo mencionado no capítulo 4 (D/G), desta vez em mulheres com AR. Além da coorte de Porto Alegre, contamos também com uma coorte confirmatória de pacientes e controles de Belém do Pará.

O capítulo 6 caracteriza-se por um estudo da expressão de HLA-G em plasma e líquido sinovial de pacientes com AR e AIJ. Nesse estudo, foram observados níveis aumentados de HLA-G solúvel (sHLA-G) no líquido sinovial de pacientes com AR, em comparação a pacientes com osteoartrite (OA) e pacientes

com trauma agudo ou crônico do joelho, particularmente em pacientes negativos para fator reumatóide (FR-). Também foram observados altos níveis de sHLA-G no líquido sinovial de pacientes com AIJ. Ainda, são mostrados padrões distintos de correlação entre os níveis de HLA-G no plasma de acordo com a positividade para fator reumatóide e sexo dos pacientes.

No capítulo 7 procuramos integrar os conhecimentos atingidos durante o período de trabalho, buscando correlacioná-los com o conhecimento já existente sobre HLA-G e doenças reumatológicas. Também são levantadas algumas questões pertinentes e futuras perspectivas de estudo por ocasião dos novos conhecimentos atingidos.

O anexo 1 caracteriza-se pelo primeiro estudo envolvendo o polimorfismo +3142C>G no nosso grupo de pesquisa, em pacientes com anemia falciforme, no qual observamos uma associação do alelo C com a proteção contra a infecção pelo vírus da hepatite C (HCV). Esse trabalho foi publicado na revista *Tissue Antigens* (Cordero *et al*., 2009) e apresentado sob a forma de tese de doutorado por Elvira Alicia Aparicio Cordero em 2009.

O anexo 2 fez parte do trabalho de mestrado e é o primeiro artigo a ser publicado pelo nosso grupo de pesquisa analisando o polimorfismo de 14 pares de base (14 pb) da 3'UTR do gene *HLA-G* em LES, publicado na revista *Lupus*, em 2009 (Veit *et al*., 2009).

O anexo 3 é um artigo que também fez parte do trabalho de mestrado e avaliou a influência do polimorfismo de 14 pb em AIJ e AR, encontrando uma associação do alelo de deleção com a suscetibilidade à AIJ em pacientes do sexo feminino. O artigo foi publicado na revista *Tissue Antigens* em 2008 (Veit *et al*., 2008).

CAPÍTULO 2 – Artigo 1

HLA-G - From Fetal Tolerance to a Regulatory Molecule in Inflammatory Diseases

Tiago Degani Veit, Priscila Vianna, José Artur Bogo Chies

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HLA-G - From Fetal Tolerance to a Regulatory Molecule in Inflammatory Diseases

Tiago Degani Veit, Priscila Vianna and José Artur B. Chies*

Immunogenetics Laboratory, Genetics Department, Post-Graduation Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

Abstract: The Human Leukocyte Antigen G (HLA-G) is a non-classical class I MHC which is characterized by low polymorphism at the DNA level, limited tissue distribution in non-pathological conditions and the expression of both membrane-bound and soluble isoforms by alternative splicing. This molecule has become the object of interest because of its possible role in pregnancy maintenance. HLA-G seems to be involved in the induction and maintenance of tolerance between the mother immune system and the semi-allogeneic fetus at the fetal-placentary interface, and also seems to play an important role in embryo implantation. Besides, several studies point out to a broader immunoregulatory role for this molecule. Here we review the potential roles of the HLA-G molecule on the immune system, the unique regulatory region of its gene, the influence of gene polymorphisms on HLA-G expression, as well as several situations in which this molecule has been involved, such as pregnancy, transplantation, cancer, viral infections and, more recently, inflammatory diseases.

Keywords: HLA-G, immune regulation, genetics, pregnancy, cancer, inflammatory diseases.

INTRODUCTION

> *"Although experimental evidence is needed before a final decision can be reached regarding functionality of nonclassical class I gene products, the data presented here favor the hypothesis that nonclassical genes are largely nonfunctional and are essentially pseudogenes. It is possible, however, that certain nonclassical genes have acquired new functions different from that of classical genes".*

This passage, extracted from an article written by Hughes and Nei in 1989 [1], reflects the relative skepticism of scientists about the role of nonclassical HLA on human biology twenty years ago. At that time, there had been 31 years since the discovery of the first HLA allele, 21 years since the adoption of the HLA nomenclature and six years since the mapping of the HLA gene region at 6p21.3. The HLA classic molecules were fairly well studied and their role in tissue rejection was already well established. Since nonclassical HLA molecules did not seem to present foreign antigens and were shown to be almost monomorphic, it was reasonable to think of them as essentially nonfunctional genes. Two years later, HLA-G, which had been named 6.0 because of the fragment length in which it was found [2], had its expression detected in trophoblast [3, 4] revealing the tip of the iceberg that represents the importance of this molecule on the immune system. Eight years later, HLA-G expression was described in tumor cells [5], making evident that this molecule was not exclusively expressed during pregnancy. Further descriptions of the expression of HLA-G in transplants and its induction in viral infections have widened even more the spectrum of situations in which this molecule has a potential role. More recently, HLA-G research is beginning to focus on another potential area of interest, which is inflammatory diseases. Here we discuss the intriguing features of the HLA-G molecule, including the unique regulatory regions of this gene and the consequent diverse roles this molecule has in the immune system.

*Address correspondence to this author at the Department of Genetics, UFRGS, Av. Bento Gonçalves, 9500, Caixa Postal 15053 Zip Code 91501-970, Porto Alegre, RS, Brazil; Tel: +55-51-3308-6740; Fax: +55-51-3308-7311; E-mail: jabchies@terra.com.br

THE HLA-G GENE AND MOLECULE

HLA-G is a class Ib molecule, whose structure resembles that of classic HLA class I molecules, constituted by three alpha domains non-covalently associated to a β2-microglobulin chain. Nevertheless, unlike its classic counterparts, HLA-G exhibits low polymorphism at its coding region, presents a restricted expression pattern in healthy conditions and possesses a unique characteristic among HLA molecules, which is to form HLA-G multimers. Also, by alternative splicing the HLA-G gene can give rise to seven different protein isoforms. All these characteristics contribute to the increasing interest of scientists in this molecule, and some of these features have proved to influence HLA-G biological functions.

As previously mentioned, HLA-G, the most studied non-classic HLA molecule, has limited polymorphism as compared to classic HLA molecules: it possesses 36 alleles described to date, which encode 14 different proteins, as compared to 673, 1077 and 360 alleles from HLAs A, B and C (Anthony Nolan Research Institute, September 2008, http://www.anthonynolan.org.uk/research/hlainformaticsgroup). This limited polymorphism is distributed along the three alpha domains, while in classic HLA molecules it is concentrated around the peptide binding groove. The HLA-G limited polymorphism clearly restricts the peptide repertoire capable of binding it and, indeed, it was shown that in placenta, a single peptide accounts for 15% of eluted peptides from HLA-G molecules [6]. The bound peptide sits deeper in the cleft than in classic HLA molecules, and its

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buried area seems to be even higher than other non-classic MHC molecules, such as HLA-E and Qa-2 (a mouse non-classic molecule) [7]. These special characteristics of the HLA-G make it unlikely that this molecule performs a role in antigen presentation. However, more studies are needed to address the real influence of the bound peptide on receptor binding.

HLA-G proteins can occur in different isoforms: four membrane-bound (G1-G4) and three secreted (G5-G7) forms have been described to date [8], and are generated by alternative splicing. Depending on the cell type and physiological condition, different HLA-G isoforms are produced [9-11]. All isoforms contain at least the alpha-1 domain, and HLA-G1 is the complete isoform. In G5-G7 isoforms, intron 4 is not spliced out, which introduces a stop codon and thus the transmembrane and cytoplasmic domains are not translated, resulting in secretable, soluble proteins [12-16]. HLA-G also possesses a cytoplasmic tail that is shorter than those from HLA-A, B and C, due to a frameshift mutation at exon 6 [2]. This feature has important implications for HLA-G expression as it unfolds an endoplasmic reticulum (ER) retrieval motif that results in a slower turnover and prolonged expression of HLA-G at the cell surface [17] as compared to classic HLA molecules.

HLA-G expression is highly tissue-restricted: besides being expressed in fetal tissues, such as trophoblast cells, HLA-G constitutive expression was found only in adult thymic medulla, cornea, pancreatic islets, erythroid and endothelial-cell precursors. However, HLA-G expression can be induced in situations such as transplantation, inflammatory diseases, in tumor cells, multiple sclerosis and in viral infections.

IMMUNOTOLEROGENIC FEATURES OF HLA-G

The Description of a Suppressive role

HLA-G expression was first described at the cytotrophoblast and therefore the first studies on this molecule concentrated on its role on pregnancy. During pregnancy, the maternal immune system is in close contact with cells and tissues from the semiallogeneic fetus. This suggests that specific mechanisms must be operating to modulate and moderate the maternal immune system in order to prevent the fetus rejection - i.e, promoting the acceptance of the semiallogeneic fetus. Indeed, certain complications during pregnancy, such as pre-eclampsia, have been associated with a Th1 immune response [18-20]. The etiology and pathogenesis of pre-eclampsia involve a combination of maternal-fetal genetic and immunological factors. To protect the fetus from the mother's immune system, preventing T-cell mediated cytolysis, the cytotrophoblasts are devoid of HLA-A or B and express very low HLA-C. In addition, expression of HLA-G by these cells inhibits activation of maternal T cells, natural killer (NK) and antigen-specific CTL (cytotoxic T cell) cytolysis *via* specific receptors [21,22], and IL-10 secreting cells may stimulate the HLA-G expression [23].

Immunoregulatory Functions of HLA-G

The role of HLA-G in the maintenance of pregnancy as an immunosuppressive molecule, the various reports of its expression in different types of cancer, as well as the emphasis in recent years in analyses of the mechanisms used by tumors to avoid the immune system recognition and destruction provided the impetus to investigate the mechanisms through which HLA-G exerts its regulatory functions. Several mechanisms have been identified (Fig. **1**). As was previously mentioned, HLA-G was shown to inhibit the cytotoxic activity of CTL and NK cells [24, 25]. Similarly, HLA-G is able to protect class I-negative cells or allogeneic tumors from NK-mediated anti-tumor immunity [26-28]. Also, it was shown to inhibit CD4+ T cell alloproliferative responses [29], the proliferation of T and NK cells [30-32], and also to act on APCs by inhibiting their maturation and function [33].

From now on, we will discuss some of the reasons why HLA-G seems to be capable of performing a major regulatory role in the immune system: (i) HLA-G was reported to bind many types of receptors, some of which are widely distributed among immune cells; (ii) HLA-G may exert long-term immunotolerogenic effects through the generation of suppressor cells; and (iii) even cells which do not transcribe HLA-G may temporarily become HLA-G$^+$ acquiring a suppressive profile through intercellular uptake of HLA-G containing membrane patches, a mechanism also known as *trogocytosis*.

Receptors for HLA-G

HLA-G exerts its effects through binding to specific receptors on various immune cell types (Fig. **1**). The leukocyte receptor complex of chromosome 19 includes two polymorphic gene families: leukocyte immunoglobulin-like receptors (LILR) and killer cell immunoglobulin-like receptors (KIR). Among LILR molecules, LILR1 (ILT-2, CD85j) and LILR2 (ILT4, CD85d) are inhibitory receptors that recognize all HLA class I molecules [34]. LILR1 is expressed by B cells, some T and NK cells, and all monocytes, whereas LILR2 is specific of myeloid lineages. LILRB1 and LILRB2 have been convincingly shown to bind HLA-G [35-41].

HLA-G has been found in different conformations at the cell surface. An interesting fact is that HLA-G can exist as dimers of two β2m-associated HLA-G complexes. The HLA-G homodimer is linked by disulfide bonds between cysteines located at position 42 of the HLA-G chain, at the α1 domain [42, 43]. This cysteine is unique among HLA-I molecules, implying that the formation of these structures in this group of molecules is restricted to HLA-G. It has been shown that this homodimeric complex dramatically increases LILRB1 binding, with a higher affinity and slower dissociation rates than monomers. Recent data show that the LILR1 and LILR2 binding sites of HLA-G dimers are more accessible than those of HLA-G monomers [44]. This increased avidity resulted in augmented signaling through LILRB1 [45] and ~100 -fold lower concentration of dimers needed to induce signaling [44].

KIR2DL4 (CD158d) is a member of the KIR gene family which is thought to bind HLA-G [46-49]. Structural analyses and signaling assays *in vitro* indicate that this molecule can function as an activator or inhibitory molecule [50, 51]. Although a number of different works have reported KIR2DL4 binding HLA-G [47,48, 52-54], either they lack replicability by other workers or lack data concerning

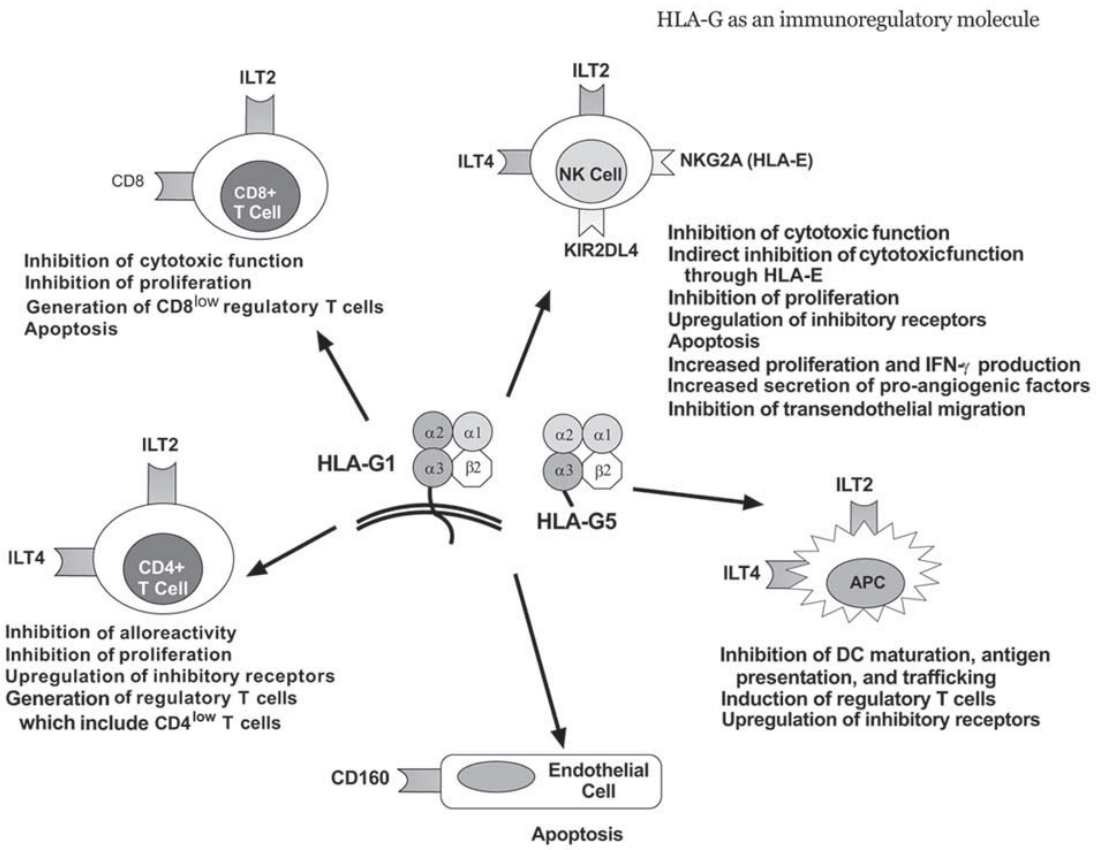

Fig. (1). Immunoregulatory activities mediated by HLA-G, Target cells and receptors. Modified from Ref. [61].

appropriate control molecules [55-56]. A possible explanation for these contradictory findings is that KIR2DL4 binds HLA-G through a low affinity interaction that only takes place when the ligand is concentrated in endosomal compartments [47, 55].

There is evidence that, like classical sHLA class I antigens [57, 58], sHLA-G induces apoptosis of activated CD8+ T cells and NK cells through binding to CD8, leading to ligand (L) upregulation, soluble FasL secretion and activated CD8+ cell apoptosis by Fas/sFasL interaction [59]. However, it is likely that sHLA-G molecules do not play a major role in physiological conditions, since their level in serum is about one order of magnitude below that required to induce CD8+ T and NK cell apoptosis *in vitro* [59, 60]. Thus, the potential role of HLA-G in inducing apoptosis through CD8 would be restricted to pathological conditions associated with a marked increase in the level of sHLA-G in serum or in a given anatomic site. Also, whether sHLA-G cooperates with classical sHLA class I antigens in inducing apoptosis and whether this effect is additive or synergistic remains to be determined [61].

Other receptors have been implicated in HLA-G binding, although further studies are needed to confirm these findings. NK receptors KIR2DL1, KIR2DL2/3 and KIR3DL1 were reported as capable of binding HLA-G but these results have never been replicated [62, 63]. Also, sHLA-G1 has been shown to inhibit *in vitro* and *in vivo* angiogenesis by inducing endothelial cell apoptosis upon binding to the CD160/BY55 receptor [63, 64]. CD160 is expressed by endothelial cells and also T and NK cells. However, blocking with specific monoclonal antibodies to CD160 or HLA-G was not shown, and other HLA-I molecules might bind CD160 [55, 63, 65]. HLA-G also seems to have an influence on CD94/NKG2 binding to HLA-E: the affinity of both CD94/NKG2A and CD94/NKG2C for HLA-E is highest when a leader peptide derived from HLA-G is bound, as compared with a leader peptide from a classical HLA-I molecule [66]. This could be a potential mechanism of discrimination between fetal and maternal cells by NK cells; however, the binding of HLA-E presenting the HLA-G leader peptide to decidual leukocytes expressing CD94/NKG2 receptors has not been functionally investigated [55].

HLA-G Suppressor Cells

There is increasing evidence that, besides its direct inhibitory effects, HLA-G may exert long-term immunotolerogenic effects through the generation of suppressor cells. Several HLA-G-related suppressor cells have been identified [reviewed in 67]. **Naturally occurring HLA-G$^+$ regulatory T cells** are present in the peripheral blood under physiological conditions. These cells can be CD4$^+$ or CD8$^+$ and constitutively express HLA-G1 at their

surfaces. HLA-G1$^+$ T cells are hyporesponsive and mediate their suppressive functions through soluble factors that include HLA-G, but not IL-10 or TGF-B. Their occurrence has been identified also in sites of inflammation [68]. **HLA-G$^+$ T cells can also be induced** through allostimulation and produce soluble HLA-G5 and, at rare occasions, HLA-G1 [69, 70]. Although their origin is unclear, these cells are suppressive and limit the alloproliferation of autologous CD4 T cells. **HLA-G-induced regulatory T cells** were first described *in vitro* after allogeneic stimulation by HLA-G1$^+$ APCs. These cells were hyporesponsive and inhibit the proliferation of autologous T cells. They are not characterized by a particular phenotype and their mechanisms of action remain largely unknown but, although HLA-G is directly responsible for their induction, they do not exert their regulatory functions through HLA-G [71-73]. **HLA-G-induced tolerogenic dendritic cells** (DC) are matured in the presence of HLA-G tetramers, in which the stimulation ability is markedly reduced. Moreover, these cells are capable of inducing the generation of CD4$^+$C25$^+$CTLA4$^+$ and IL-10-producing regulatory T cells [74, 75]. APCs can also express HLA-G under pathological conditions. These cells were found in transplanted tissues, in tumors, during inflammatory diseases and viral infections [76-79]. They have been shown to block the reactivity of T cells and to induce suppressor T cells [71], and seem to have an important physiologic role in acute B-chronic lymphocytic leukemia [80]. **Adult bone marrow mesenchimal stem cells** (MSC) are multipotent cells capable of differentiating into several lineages and which possess strong immunomodulatory properties. Recently, it was shown that HLA-G was a key contributor to MSC immunosuppressive functions [81, 82].

In conclusion, HLA-G-dependent regulatory cells are diverse, and given such different origins, modes of induction, phenotypes and mechanisms of action. It is very unlikely that all these cells might play the same role in the same situations.

Trogocytosis and HLA-G

Trogocytosis is a cell-to-cell contact-dependent uptake of membranes and associated molecules. Nevertheless, during trogocytosis, all molecules contained within a certain membrane area are transferred, including some that do not participate in the cell-to-cell crosstalk, and are therefore transferred nonspecifically. Most of the studies on trogocytosis were carried out on murine T cells and show that CD4$^+$ and CD8$^+$ T cells can respectively acquire MHC Class II and MHC Class I molecules from antigen presenting cells (APC) in an antigen-specific manner [83-85]. Recently, trogocytosis of HLA-DR, CD80, and HLA-G1 from APC by T cells was demonstrated in humans, and was shown to follow the same rules as in the murine system [31, 86, 87]. Functionally, CD8$^+$ T cells that acquired their MHC Class I ligands became susceptible to antigen-specific cytolysis [84, 85]. Then, after HLA-DR and CD80 acquisition, T cells stimulated resting T cells in an antigen-specific manner, behaving as APCs themselves [86-88] whereas acquisition of HLA-G1 rendered T cells immunosuppressive [31]. This might constitute a cheap and efficient way of modulating presentation/stimulation capabilities of the immune system. It was shown that NK cells can acquire MHC Class I proteins [89-93] and viral receptors [90] from their targets.

Indeed, it was demonstrated that HLA-G1 can be acquired from tumor cells by activated but not resting NK cells by trogocytosis, and this can be a mechanism of immune escape for HLA-G-negative tumor cells [31]. Almost all activated NK cells can acquire detectable levels of HLA-G1 in a few minutes by a cell-to-cell contact-dependent mechanism. Differently from HLAG1-expressing cells, NK cell-surface expression of acquired HLA-G1 is temporary, as these cells do not transcribe HLA-G. Functionally, NK cells that acquire HLA-G1 stop proliferating, are no longer cytotoxic, and behave as suppressor cells capable of inhibiting cytotoxic functions of other NK cells. All these functional changes are due to acquired HLA-G1, and could be abrogated by blocking HLA-G1 or its receptor LILR1 at the NK cell surface.

HLA-G REGULATION AND POLYMORPHISMS AT REGULATORY REGIONS

Despite the efforts to clarify the mechanisms of regulation of HLA-G expression, the mechanisms underlying HLA-G expression remain largely unknown. Since the description of the differential expression of HLA-G and classic HLA molecules in trophoblast cells, a different pattern of expression control was suggested for these genes. A clue to the differences in protein expression, as compared to another HLA genes, is given by the HLA-G unique promoter region, which exhibits several differences in relation to the promoters of classic HLA genes (and also to other non-classic HLAs). The HLA-G promoter region presents many typical elements deleted or modified, rendering HLA-G expression unresponsive to classical HLA stimulator factors such as nf-kB, IRF1 and CIITA [94]. In addition, while in classic HLA genes the promoter elements are located within 220 bp upstream the ATG start codon, the regulatory elements of HLA-G are located on a region that spans ~1.5 kb upstream from the start codon (Reviewed in [95]) (Fig. **2**). Several factors have been shown to influence HLA-G gene expression: stress [96] and treatment with leukemia inhibitory factor is able to activate the HLA-G gene [97], and IL-10 [23], IFNs [98, 99], GM-CSF [100], glucocorticoids [101] and progesterone [102,103] are able to stimulate HLA-G expression.

Another striking characteristic of the HLA-G promoter is its high polymorphism. To date, more than 30 SNPs (Single Nucleotide Polymorphisms) have been identified in this region (National Center for Biotechnology Information, October 2008 http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?chooseRs=all&go=Go&locusId=3135), many of which within or very close to known transcription factor binding sites or regulatory elements [104]. Thirteen haplotypes have been identified based on 27 polymorphisms in three different ethnic groups, and evidence for selection for maintaining two different promoter haplotype lineages, consistent with a history of balancing selection at this region, was reported. These different haplotype lineages probably present different promoter activity patterns [105]. The research group of Carole Ober showed that a subset of promoter haplotypes associated with the allele G*010101 (G*010101b and G*010101c) presented higher promoter activity than another

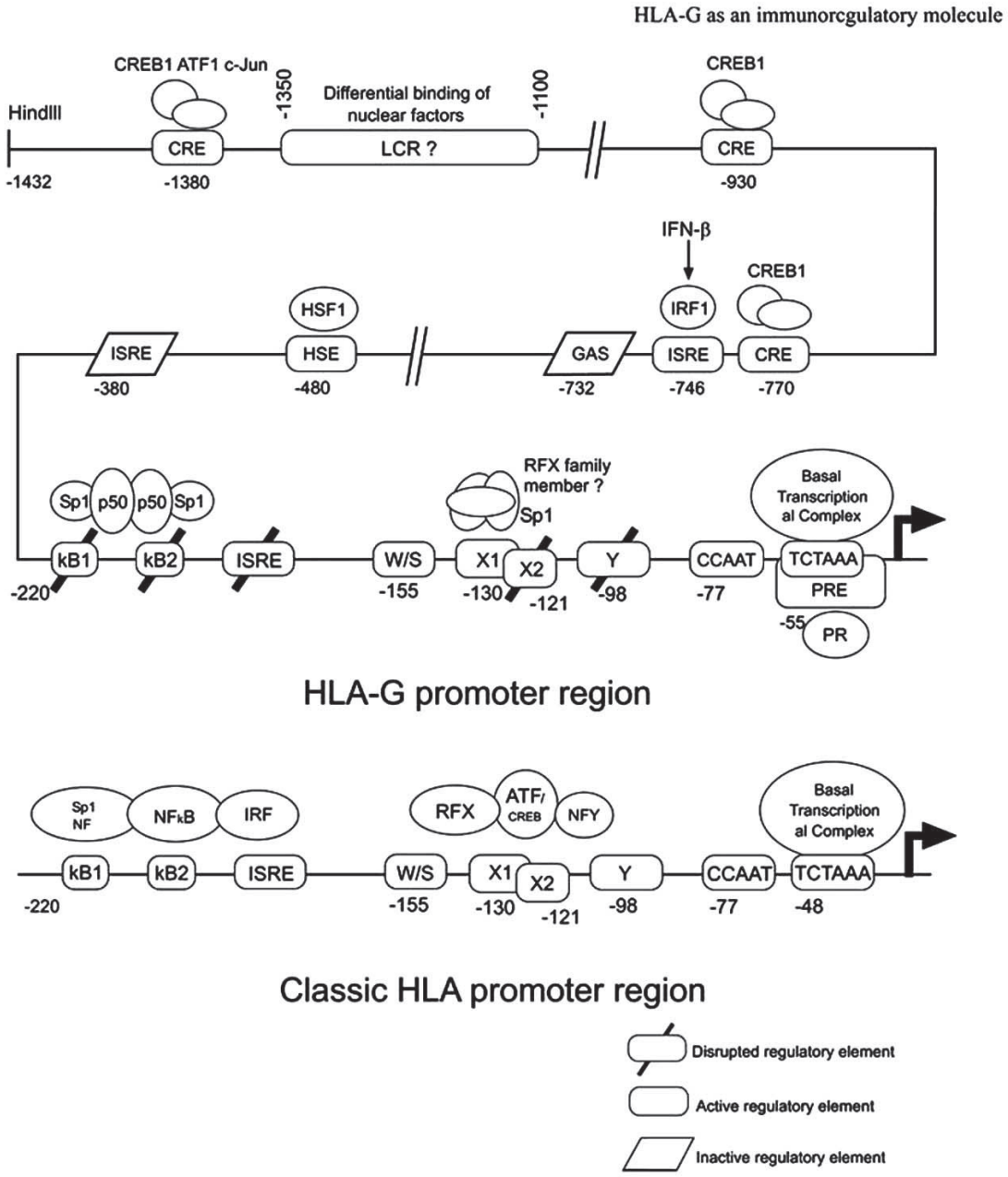

Fig. (2). Schematic representation of the human leukocyte antigen-G (HLA-G) gene promoter [updated from Ref. 190]. Numbers indicate location of regulatory boxes relative to ATG (bp). LCR ?, putative locus control region; CRE, three functional CRE/TRE sites bound by CREB1, ATF1, and c-Jun; ISRE, interferon (IFN) sequence responsive element. HSE, heat shock element that binds heat shock factor-1; GAS, non-functional IFN-gactivated site. kB2 and kB1, referred to as enhancer A within classical HLA class I promoter, are disrupted within the HLA-G promoter and display affinity for P50, a subunit of nuclear factor-kB (NF-kB). The conserved X1 half of X box associates with RFX and Sp1 *in vitro*;_?_indicates that RFX member factor is not yet identified. X2 and Y boxes are mutated, thus avoiding CIITA-induced trans-activation of HLA-G gene. PRE, a novel progesterone response element which is juxtaposed to the TCTAAA box and, through bindong of PR, is thought to enhance HLA-G transcription.

subset (G*010101a, G*010301 and G*010102). The enhanced expression of these alleles was mainly due to a polymorphism at position -725 (rs1233334) [106]. Interestingly, the -725G allele was previously associated to recurrent miscarriage [104], contradicting results which had associated a lower HLA-G expression with miscarriage rates [107,108].

In addition to promoter polymorphisms, epigenetic factors might contribute to HLA-G transcriptional regulation. Methylation status of the HLA-G promoter has been inversely correlated with HLA-G expression [109]. Still, ovarian carcinomas presented high levels of methylation at the surrounding region of a hypoxia response element (HRE), the binding site of HIF-1, which has recently been reported as a negative regulator of HLA-G expression in human tumor cell lines [110]. These data suggest that methylation may be a mechanism used by tumors to avoid HLA-G inactivation.

The 3' untranslated region (3'UTR) also seems to play an important role on HLA-G expression, mainly through post-transcriptional regulatory mechanisms. A 14bp insertion/deletion polymorphism located at position +2960 at exon 8 (rs1704) has attracted the attention of several scientific groups due to its potential role both on HLA-G alternative splicing and on RNA stability. It was previously shown that transcripts with the 14pb sequence (*ins*) could undergo an additional splicing step which removes 92 bases from the region in which this sequence is located. This deletion is thought to influence mRNA stability as the HLA-G transcripts with the 92 bases spliced out were shown to be more stable than the "complete" mRNAs in placental cells after actinomycin treatment [111]. However, the *ins* allele was shown to be less expressed than the 14bp deletion allele (*del*) in several situations [112-116]. The 14 bp polymorphism is so far the most studied HLA-G polymorphism, and its implications on pregnancy and disease will be discussed later. Another interesting element at the 3'UTR is a putative microRNA binding site, which is a potential target for miR-148a, miR-148b and miR-152 [117]. Inside this 20 nucleotide region lies a C/G polymorphism, at position +3142 (rs1063320). Tan and cols. performed *in silico* and *in vitro* tests which showed that the G allele favors microRNA targeting therefore favoring the repression of HLA-G expression. We hypothesized that the +3142 polymorphism is directly responsible for the regulation of the HLA-G expression at the translation level, and a co-responsible for HLA-G protein levels, together with the HLA-G promoter region [118]. A third polymorphism which seems to influence post-transcriptional control of HLA-G expression is a SNP (A/G) at position +3187 (rs9380142), located near an adenylate/uridylate (AU)-rich motif, a known cis-acting degradation signal of the mRNAs of certain lymphokines, cytokines and proto-oncogenes. According to Yie and cols, the HLA-G mRNAs with the G allele were shown to be less rapidly degraded than the mRNA containing the A allele after actinomycin treatment. The frequency of the A allele in this study was shown to be significantly higher in preeclamptic women [119].

HLA-G AND PREGNANCY

Human embryo implantation is a complex process that requires both the ability of the embryo to implant into the uterus and adequate endometrial receptivity. All evidence indicates that during this process, several embryo- and endometrium-led adaptations in the maternal immune system are needed to allow establishment of a viable pregnancy. The relationship between HLA-G and human embryo implantation acts as a driver in the immune tolerance of the semiallogeneic fetus by the mother. As previously discussed, the 14 bp deletion/insertion polymorphism influence both HLA-G isoform splicing patterns and mRNA stability and was already implicated in pregnancy complications such as pre-eclampsia and recurrent abortions [120-124]. Pre-eclampsia (PE) is a systemic disorder of unknown origin that is characterized by abnormal vascular response to placentation, increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation cascade, and endothelial cell dysfunction [125]. In pre-eclamptic placentas, the HLA-G extravillous cytotrophoblast invasion is reduced and this defect was associated with a lack of HLA-G expression [126-128]. Such defective HLA-G expression may contribute to the immune and vascular abnormalities associated with this pathology [126,129]. The strong HLA-G expression by invasive trophoblasts may, in part, explain maintenance of the semi-allogeneic fetus during pregnancy. It is thought that HLA-G inhibits the activation of maternal T cells and natural killer cells resident in deciduas favoring a Th2 type cytokine response [22, 129, 25]. Several studies have suggested the importance of the maternal HLA-G expression during cleavage-stage embryo development and during the course of pregnancy [130]. Some researchers reported that the levels of secreted soluble HLA-G protein are higher than the capacity proposed for soluble HLA-G release by the embryo, so the signals secreted by the embryos are not in the order of magnitude of estimated HLA-G protein concentrations [131]. These findings shed some light onto the contribution of the HLA-G protein for a successful pregnancy.

HLA-G, TRANSPLANTATION AND ONCOLOGY

Transplantation and oncology are two particularly clear situations in which HLA-G is involved. Since the first description of HLA-G expression by tumor cells in 1998 [5], numerous studies have been performed on more than one thousand malignant lesions, showing that although turned off in surrounding normal areas, HLA-G gene transcription and protein expression is switched on in various tumor lesions [132]. In the context of transplantation, HLA-G expression might be beneficial and promote tolerance to grafts. The expression of HLA-G was studied in more than 1000 patients after heart, [133, 9, 134] kidney, [135] liver, [73] and liver-kidney [73, 72, 9] transplantation, with those expressing HLA-G in the graft and/or at the plasma exhibiting significantly better graft acceptance. Thus, in transplanted patients, titration of HLA-G might be used as a monitoring tool to determine and follow tolerance status, which could then be used to adjust immunosuppressive therapies. In this context, patients with high HLA-G titers could be candidates for a reduction in immunosuppressive treatment, whereas HLA-G-negative (or with low levels) patients would have a comparatively higher risk of rejection. Furthermore, HLA-G itself might be used as a therapeutic tolerogenic agent, exogenously provided to HLA-G-negative patients as complementary and/or alternative therapy [136].

In the context of oncology, studies on several malignant lesions showed that HLA-G transcription and protein expression may be switched on in tumor lesions and protect them from NK cytolysis. It was also shown that HLA-G expression by tumor lesions protected against cytolysis [137,138] correlated with malignancy in ovarian and breast carcinomas [139], as well as in melanocytic lesions [140],

and with unfavorable outcome in chronic lymphocytic leukemia [80], and gastric and colorectal cancers [141]. High HLA-G plasma levels were also recently observed in patients with neuroblastoma and correlated with relapse [142]. Expression of HLA-G has been evidenced in different malignant hematopoietic diseases, but most particularly in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and B chronic lymphocytic leukemia (B-CLL). Thus, HLA-G expression would favor tumor development by impairing antitumor immunity. In this way, HLA-G titration in peripheral blood might be used for diagnosis and/or monitoring, but in this context, high titers of HLA-G would represent a negative factor [139, 80]. In HLA-G positive patients, HLA-G itself might finally constitute a therapeutic target: if expressed as a membrane-bound protein, as observed in some hematological malignancies [80], HLA-G could be used as a tumor marker to deliver therapy. Alternatively, HLA-G could be blocked or deleted as a contributor to tumor immunosuppression and/or tumoral escape [143].

As previously discussed, new aspects of HLA-G biology, namely the highly inhibitory function by HLA-G multimers; its inhibitory function through regulatory cells and trogocytosis, have been reported that are critical to HLA-G pathologic relevance and should help design HLA-G based diagnosis and therapeutic strategies.

HLA-G AND VIRAL INFECTIONS

One of the several mechanisms adopted by viruses to evade the human immune system is by interference with the expression of HLA antigens. Normally, alterations in the self HLA cell profile are easily detected by immune surveillance and these cells are depleted. In viral infections, such as HIV, the virus down regulates cell surface class I classical antigens (HLA-A and HLA-B) to avoid HIV-specific CTL responses, but the expression of HLA-G remains unaffected or at least not decreased. The HIV infected cells are resistant to lysis by NK cells, interaction of HLA-G1 with KIR of NK cells can inhibit the antigen-specific HLA-restricted CTL response [60]. Various NK cell receptors that recognize MHC-independent ligands can regulate important cytolytic NK functions. This immunoregulation could be achieved by increased expression of HLA-G during viral infections. When HIV Nef down regulates the surface class I antigens by interacting with their cytoplasmic domain [144], it may not be able to interact with non-classical HLA-I antigens such as HLA-G, which has a truncated domain [2]. In spite of the inability of viral Nef to downregulate HLA-G, some changes could indirectly influence the expression of HLA-G, particularly increased interleukin 10 [145]. In the immunoregulation of viral infections, the stage of infection has a profound effect on the microenvironment, which, in turn, could alter HLA-G expression.

Other acute viral infections such as human cytomegalo-virus (CMV) and herpes simplex both decrease cell surface expression of HLA-G1, but can increase HLA-G1 expression upon reactivation [146, 147]. Further, HLA-G polymorph-isms are also associated with the risk of HIV, HCV and CMV infections [41, 148-153]. Besides their immunoregul-atory properties, the impact of this non-classical class I antigen HLA-G in the susceptibility to viral infections needs further investigation.

A NEW FIELD OF STUDY - INFLAMMATORY DISEASES

HLA-G expression in inflammatory diseases is a relatively recent area of research. The first studies described HLA-G expression in muscle fibers in various inflammatory myopathies [154], in atopic dermatitis [78] and psoriatic skin [155]. It was promptly proposed, based on the findings that HLA-G seems to shift T-helper responses towards a Th2-type response, [156-159] that HLA-G would act as a tissue-protective molecule in inflammatory responses. Numerous studies have been conducted since then, and are displayed on Table **1**.

An interesting finding concerning inflammatory bowel disease is that HLA-G expression was observed in ulcerative colitis (UC) intestinal biopsies, but not in Crohn's disease (CD), which constitutes a potential distinction between the two diseases [160]. Quite surprisingly, Rizzo et al. have reported that concerning peripheral blood mononuclear cells (PBMC), the CD patients were those who expressed the higher levels of HLA-G, whereas UC patients presented no detectable levels, even after LPS stimulation, which led the authors to suggest sHLA-G produced by PBMC as a less invasive diagnostic tool in the early phases of the diseases [161]. A genetic association between the 14bp *del* allele and UC had been described, indicating that this allele might constitute a risk factor for UC. The same study also reported an increased frequency of the allele in CD patients who underwent ileocecal resection, suggesting that HLA-G may also have a role on CD course [162].

In celiac disease, an immune-mediated disorder characterized by an immune response to ingested gluten, HLA-G expression was higher in the co-occurrence of autoimmune or genetic diseases, and also depended on gluten ingestion, possibly indicating that the enhanced expression of sHLA-G in this disease could be related to tolerance towards oral antigens [163]. Allergic Rhinitis is a good example of the coincidence of HLA-G expression with a Th2 phenomenon. Higher levels of sHLA-G were detected in both seasonal and perennial allergic rhinitis groups and a strong correlation between clinical severity and sHLA-G levels was observed [164, 165].

HLA-G has also been studied in Multiple Sclerosis (MS). MS is considered an autoimmune inflammatory demyelinating disease of the central nervous system. HLA-G was shown to be strongly expressed in MS lesions and in areas with lymphocytic and monocytic inflammation [166]. Cerebrospinal fluid (CSF) and intrathecal synthesis of sHLA-G was higher in MS than in controls. In this context, HLA-G levels directly correlated with IL-10 levels and to better prognosis markers [167, 168]. Moreover, HLA-G expression was shown to be increased in patients after treatment with Interferon-β (IFN-β), a major immunomodu-latory agent used in the treatment of MS. HLA-G derived from monocytes, the primary source of HLA-G in MS, was shown to inhibit both Th1 (IFN-g, IL-2) and Th2 (IL-10) cytokine production by antigen-stimulated autologous CD4

Table 1. HLA-G in Inflammatory Diseases

Disease	Type of Study	N	Organ/Fluid/ Cell/Site	HLA-G Expression	Outcome	Polymorphism	Associated Allele/Genotype	Refs.
Allergic rhinitis - perennial	Expression	25	serum	Higher - correlation with severity	-	-	-	[165]
Allergic rhinitis - seasonal	Expression	60	serum	Higher - correlation with severity	-	-	-	[164]
Asthma	Genetic	867[a]	-	-	susceptibility (GWS)	HLA-G locus	Positive association	[176]
	Genetic	936[b]	-	-	susceptibility	2 SNPs for HLA-G	No association	[177]
	Genetic	180	-	-	susceptibility if	-1306 (rs1736936)	AA - protective	[117]
						+1489 (rs1130356)	No association	
					mother is affected	14 pb (rs1704)	Ins/ins -protective	
						+3142 (rs1063320)	GG - protective	
	Expression	53	Plasma	Higher in atopic asthma patients	-	-	-	[178]
	Expression	20	PBMC	Lower upon LPS stimulation	-	-	-	[179]
Asthma (isocyanate induced)	Expression	20	PBMC	Higher (without LPS stimulation)	-	-	-	[180]
Atopic dermatitis	Expression	9	Skin	Present	-	-	-	
Behcet's disease	Genetic	312	-	-	susceptibility	multiple	G*010101 - protection	[186]
Celiac disease	Expression	24	small intestine	Present - sHLA-G	-	-	-	[163]
Idiopathic dilated cardiomiopathy	Genetic	117	-	-		14 pb (rs1704)	Del	[187]
Inflammatory bowel disease	Expression	43	Colon	CD - absent UC - present	-	-	-	[160]
	Expression	28	PBMC	CD - present UC - absent	-	-	-	[161]
	Genetic	628	-	-	CD - Ileocecal resection	14 pb (rs1704)	Ins	[162]
Inflammatory myopathies	Expression	20	Muscle	Present	-	-	-	[154]
Juvenile idiopathic arthritis	Genetic	106	-	-	susceptibility	14 pb (rs1704)	Del (Females)	[172]
Kawasaki disease	Genetic	92	-	-	susceptibility	GWS	HLA-G locus	[184]
Multiple sclerosis	Expression	50	CSF	Present - 42% of patients	-	-	-	[167]
	Expression	17	PB monocytes	Lower Up-regulated upon INF-β treatment	-	-	-	[166]
	Expression	69	Intrathecal	More frequent	-	-	-	[168]
			CSF	More frequent in MRI stable MS	-	-	-	

(Table 1) contd…..

Disease	Type of Study	N	Organ/Fluid/ Cell/Site	HLA-G Expression	Outcome	Polymorphism	Associated Allele/Genotype	Refs.
			Serum	Decreased in clinically stable MS	-	-	-	
	Genetic	698	-	-	susceptibility	-725 (rs1233334)	No association	[170]
						*105N (rs41557518)	No association	
						14 pb (rs1704)	No association	
Pemphigus vulgaris	Genetic	24	-	-		14 pb (rs1704)	Del	[188]
	Expression	ND	Skin	Present	-	-	-	[189]
Rheumatoid arthritis	Expression	106	Serum	Decreased Positively correlated to DAE	-	-	-	[171]
	Expression	30	PBMC	Increased upon MTX treatment	-	-	-	[115]
	Genetic	156	-	-	susceptibility	14 pb (rs1704)	No association	[115]
	Genetic				response to MTX	14 pb (rs1704)	Del/Del	
	Genetic	130	-	-	response to MTX	14 pb (rs1704)	No association	[173]
	Genetic	265	-	-	susceptibility	14 pb (rs1704)	No association	[172]
Sarcoidosis	Genetic	47	-	-	susceptibility	multiple	14bp-containing alleles (NS)	[185]
	Expression	ND	Granulomas	Rare and weak	-	-	-	
Septic shock	Expression	64	Plasma	Increased Higher increase in survivors	-	-	-	[175]
Systemic Lupus Erythematosus	Expression	50	Serum	Higher	-	-	-	[181]
			Lymphocytes	Higher	-	-	-	
	Expression	130	Plasma	Lower	-	-	-	[116]
	Genetic	200	-	-	susceptibility	14 pb (rs1704)	Ins	
	Genetic	293	-	-	susceptibility	14 pb (rs1704)	Ins/Del	[182]
Systemic sclerosis	Expression	21	Skin	Present - 57% of patients Associated with better prognosis	-	-	-	[174]
Type 1 Diabetes	Genetic	2321[c]	-	-	susceptibility	GWS	HLA-G locus	[183]

[a] Families/Trios from 4 different samples [b] children from 2 different samples [c] families

CD - Chron's disease; CSF - Cerebrospinal fluid; DAE - Disease associated epitopes; GWS - Genome-wide scan; LPS - Lipopolysaccharide; MRI - Magnetic resonance imaging; MS - Multiple sclerosis; MTX - Methotrexate; ND - not determined; NS - not significant; PB - Peripheral blood; PBMC - Peripheral blood mononuclear cells; SLEDAI - Systemic lupus erythematosus disease activity index; UC - Ulcerative colitis.

T cells [166]. Recently, it was shown a trend towards an inverse correlation between CSF concentrations of sHLA-G and sHLA-I and between CSF levels of sHLA-G and sFas in relapsing-remitting MS, suggesting that sHLA-G could play an immunomodulatory role in MS through Fas/FasL-mediated mechanisms [169]. Another study addressed the possible genetic influence of HLA-G polymorphisms on multiple sclerosis, but no significant differences were observed for the analyzed polymorphisms [170].

In Rheumatoid Arthritis (RA), lower plasma levels of HLA-G were reported as compared to healthy individuals. However, HLA-G levels in these patients correlated with the presence of disease-associated epitopes, which could represent a link to genetic factors or may merely be an indirect consequence of disease activity or a combination of

both [171]. To date, attempts to find a genetic association between HLA-G and susceptibility to RA have failed [115, 172]. However, an interesting finding concerning RA therapy is that methotrexate (MTX) was shown to induce HLA-G expression *in vitro* and that the response to MTX was reported as being influenced by the HLA-G 14-bp genotype (with the del/del genotype being the most favorable), although these results were not reproduced on a second study [173]. Interestingly, in Juvenile Idiopathic Arthritis, a significant association between the deletion allele and disease susceptibility in girls was reported by our group [172].

In Systemic Sclerosis, an autoimmune disease characterized by widespread cutaneous and visceral fibrosis and obliterative small vessel vasculopathy, HLA-G expression was reported in skin biopsies, and this expression was associated with a lower frequency of vascular cutaneous ulcers, telangiectasias and inflammatory polyarthralgia and with a higher survival rate [174]. An interesting work revealed evidences of the potential importance of HLA-G in controlling situations of acute inflammation: it was reported that HLA-G5 had a marked and persisting elevation in septic shock and a significantly higher concentration of this molecule was present in survivors in comparison to nonsurvivors, therefore being identified as a potential predictor of survival in this situation [175].

Other disease associative studies concerning HLA-G presented controversial results. In Genome Wide Scan studies in Asthma, the HLA-G gene has been marked as a susceptibility gene in four independent samples [176], but these results were not replicated by a second study [177]. Polymorphisms at the promoter region and also at the 3' UTR were implicated in asthma susceptibility [117], however, further studies trying to characterize HLA-G expression produced conflicting results: although HLA-G plasma levels were elevated in atopic asthma patients [178], another study reported a decreased production of HLA-G (and IL-10) by peripheral blood mononuclear cells stimulated with lipopolysaccharide, which was reversed by exogenous IL-10, indicating that a specific deficit of IL-10 secretion in patients with asthma could prevent the normal production of sHLA-G1/HLA-G5 molecules [179]. Also, in isocyanate-induced asthma, spontaneous production of sHLA-G by PBMC was significantly higher in patients [180]. In Systemic Lupus Erythematosus (SLE), a study reported higher levels of HLA-G in the patients' plasma [181] while another study reported lower levels [116]. Also, this last study reported a genetic association with the 14-bp polymorphism, with the ins/ins genotype as a risk factor for SLE development. However, this result was not replicated by our research group, which instead observed an increased frequency of the heterozygous genotype, with genotypic frequencies departing from the expected Hardy-Weinberg equilibrium [182]. It is possible that environmental and/or specific ethnic factors are contributing to such conflicting results.

Nowadays, genome wide scans are playing a major role in unfolding disease susceptibility gene alleles and regions. A thorough genetic analysis of the MHC region, using a dataset generated by the Type-1 Diabetes (T1D) genetics consortium with 2965 genotype markers across the MHC in 2321 T1D families has put HLA-G at the main list of candidate genes for susceptibility to type-1 diabetes. Four SNPs near the HLA-G locus have been identified as genomic risk markers for T1D suggesting that a fine mapping of the *HLA-G* gene, with the surrounding region to include putative regulatory sites, is warranted in T1D [183]. A similar study, analyzing 2360 SNPs within the MHC region in 92 Kawasaki disease patients, reported HLA-G as the only candidate locus to have a significant association with this acute, self-limited vasculitis of infants and children, manifested as fever and signs of mucocutaneous inflammation [184].

The 14-bp polymorphism has been studied in other situations and has been associated to several diseases: the insertion allele had been observed more frequently in patients with sarcoidosis [185] and in Behcet's disease [186], while the deletion allele was reported as a risk factor for idiopathic dilated cardiomyopathy [187] and for Pemphigus vulgaris (PV) [188]. HLA-G expression at the skin of PV patients has been recently reported [189] However, in order to establish these associations, these results must be replicated in future work.

Although the number of studies in autoimmune and/or pro-inflammatory diseases is still limited, these studies suggest a broad spectrum of pathologic situations in which HLA-G may be involved. There is promising evidence that therapeutic strategies which include control (stimulation or inhibition) of HLA-G expression in such situations may influence disease activity and prognosis.

CONCLUSIONS AND FUTURE PERSPECTIVES

After two decades of studies, research on HLA-G has achieved maturity. From the status of a monomorphic molecule, whose utility in the biology of humans was neglected and even put into doubt, HLA-G has turned into an intriguing molecule with unique structural features, and there is a consistent amount of information indicating that it is capable of exerting direct and long-lasting effects on the immune system. As we have shown throughout this review, HLA-G expression is a two-edged sword. While beneficial in pregnancy and in transplantation, its expression in tumors and viral infections would be detrimental by impairing antiviral and antitumor immunity respectively. Given the broad immunoregulatory functions of this molecule and the wide spectrum of situations in which it might be involved, it is tempting to think of it as a potential tool for clinical purposes. This would include not only its use as a diagnostic and prognostic tool, but also as a molecular target to deliver therapy, as a therapeutic agent administered exogenously or by controlling its endogenous expression. However, some steps still must be undertaken in order that the accumulated knowledge on HLA-G reverts into clinical benefits for patients.

One important issue yet to be clarified is the role of the multiple isoforms and structures that HLA-G can form. When approaching HLA-G isoforms, the majority of the papers analyzed HLA-G1 and G5 whereas the other isoforms have been poorly assessed. Still, the most important fact that is yet to be taken into account by future studies is that multimers may be responsible for the majority of HLA-G inhibitory function. It is now clear that the relationship

between HLA-G structure and its functional relevance needs to be established *in vivo* beyond doubt. The development of new tools for analyzing HLA-G structures will allow a reanalysis of data, which might greatly strengthen the relevance of HLA-G in disease. Also, future studies should concentrate on the investigation of different types of HLA-G-dependent suppressor cells, probably the main elements responsible for the long lasting effects of HLA-G on the immune system. Is seems unlikely that all HLA-G-dependent suppressor cells play the same roles and are present at the same contexts; therefore, the identification of those cells and their roles in a given (pathological) context will greatly help in elucidating mechanisms of disease onset and progression, as well as the development of strategies for counteracting those mechanisms.

Another important step to accomplish is a better understanding of the HLA-G gene regulation. Although many regulatory elements have been identified at the HLA-G promoter and 3' UTR regions, the relevance of these elements and possible interaction that may exist between them remains elusive. Assessing this issue will be crucial to understanding how HLA-G expression is up-regulated in certain tumors and which could be the better approaches to influence HLA-G expression according to what would be required for each situation.

One might interfere on HLA-G transcription, act on HLA-G alternative splicing, or block the function of HLA-G or of HLA-G-driven suppressor cells [138]. In this regard, the development of an animal model would allow the *in vivo* validation of the concept that the immunogenicity of HLA-G^+ tumor cells might be enhanced by blocking the expression and function of HLA-G. This approach might define whether strategies aimed at blocking HLA-G by using a specific antibody affect the clinical course of HLA-G^+ malignant diseases.

Finally, there are many situations where the possible influence of HLA-G has been reported by association studies, or even by measuring its levels in blood or *in situ*, but there is still poor evidence of the direct influence of HLA-G. The majority of inflammatory diseases previously cited can be included as examples. For them, further studies will be needed to evaluate the real influence of HLA-G. Although the various accumulated evidence of HLA-G broad regulatory functions may have caused much enthusiasm among scientists, it is worth to keep in mind that "HLA-G is no panacea and does not have to be correlated with everything to be useful and important in some given situations" [136].

> *"Increasingly complex, HLA-G? Yes, but not increasingly chaotic because the field of HLA-G research benefits from a true asset: focus. Indeed, there might be disagreements over such technical subtlety, or such particular result, but there are few arguments over what HLA-G is, can do, and should be used for: HLA-G is at minima a tolerogenic molecule that should be used to monitor and cure. This is not bad for a molecule that barely existed 10 years ago".*
>
> *Carosella et al. 2008*

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CAPÍTULO 3 – Artigo 2

Tolerance versus immune response — MicroRNAs as important elements in the regulation of the HLA-G gene expression

Tiago Degani Veit, José Artur Bogo Chies

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Brief communication

Tolerance versus immune response — MicroRNAs as important elements in the regulation of the HLA-G gene expression ☆

T.D. Veit, J.A.B. Chies *

Genetics Department, Post-Graduation Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

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ABSTRACT

HLA-G is a class Ib HLA which has gained much attention due to its multiple functions on the immune system. HLA-G exerts several immunomodulatory effects, being beneficially implicated in embryo implantation and fetal survival but, conversely, being potentially detrimental in tumors and viral infections. Such a two-edged sword behavior suggest that HLA-G expression is under tight regulation. However, to date, little is known about the regulation of this gene and previous works have been unable to well correlate HLA-G regulation at the mRNA level with the polymorphic variants at the genomic level. Here we present the hypothesis that an element, which was until now neglected, might play a role in HLA-G expression regulation: MicroRNAs might participate in the regulation of the HLA-G gene expression through a putative microRNA binding site at its 3′ UTR region. Inside the 20 nt region of this microRNA binding site lies a C/G polymorphism, which was shown to be responsible for differential microRNA binding affinity and translation suppression. The role of microRNA binding on the regulation of HLA-G gene expression (and therefore on tolerance versus immune response) can be easily tested through relatively simple steps: Confirming the expression of those three complementary microRNAs in human cells which express HLA-G, followed by examination of the correlation between HLA-G mRNA and protein production controlling for HLA-G genotypes and microRNA levels; finally, selective inhibition of microRNA activity with anti-sense oligos restoring HLA-G production would access microRNA influence on HLA-G expression which, if confirmed, might help in the development of strategies to the management of several conditions in which HLA-G is involved, including pregnancy complications, transplantation, and cancer.

1. Introduction

HLA-G is a class Ib HLA molecule which was first characterized by its expression at the maternal–fetal interface and which has gained much attention due to its multiple functions on the immune system. As an HLA molecule, it shares structural properties of its classic counterparts HLA-A, B and C. However, unlike its counterparts, it is characterized by limited tissue distribution in healthy conditions and by the expression of seven different isoforms that can be either membrane-bound (G1–G4) or secreted (G5–G7). Since it was first described, in cytotrophoblasts, this molecule has attracted much attention due to its immunotolerogenic properties. HLA-G is capable of interacting with several receptors (ILT-2, ILT-4, KIR2DL4, CD8, CD160) present in various cells of the immune system, such as NK cells, T and B lymphocytes and antigen-presenting cells (APC). It can elicitate immune suppression by several mechanisms, such as inhibition of cytotoxicity, proliferation and/or differentiation, induction of tolerogenic APC or suppressive T and NK cells, induction of apoptosis, as well as up regulation of inhibitory receptors, among other features [reviewed in 1–2]. All this features have made HLA-G an attractive target in different situations in which immune tolerance is involved, such as pregnancy and its complications, transplantation, cancer and viral infections, as well as in inflammatory and autoimmune diseases [see, for example, 3]. The research in HLA-G is increasing dramatically, with over 300 references listed in the last year [4].

The fact that HLA-G exerts several immunomodulatory effects, being beneficially implicated in embryo implantation and fetal survival but, conversely, being potentially detrimental in tumors and viral infections suggests that its expression is under tight regulation. The singular organization of its promoter region, with several typical elements deleted or modified [5], reflects the HLA-G unique expression pattern among HLA molecules. This region is highly polymorphic and there are strong evidences that balancing selection might be occurring [6].

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* Corresponding author. Department of Genetics, UFRGS, Av. Bento Gonçalves, 9500, Caixa Postal 15053 Zip Code 91501-970, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 6740; fax: +55 51 3308 7311.
E-mail address: jabchies@terra.com.br (J.A.B. Chies).

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The 3′ untranslated region (UTR) seems also to have an important role on the regulation of gene expression. A 14 bp insertion/deletion polymorphism at this region has attracted the attention of many scientific groups due to its role on HLA-G alternative splicing and also on RNA stability. It was previously shown that transcripts with the 14pb sequence could undergo an additional splicing step which removes 92 bases encompassing the region in which this sequence is located. This deletion is thought to influence mRNA stability as the HLA-G transcripts with the 92 bases spliced out were shown to be more stable than the "complete" mRNAs in placental cells after actinomycin treatment [7]. Although the number of transcripts that undergo 92 bases deletion seems to vary among +14 bp alleles and cell lineages and, in most cases, they do not represent a majority of the transcripts [7,8], it would be expected that, overall, homozygous individuals for the insertion allele presented a higher HLA-G expression, due to the presence of these more stable transcripts. However, the proclaimed mRNA stability conferred by the 14 bp insertion seems not to have a positive effect in enhancing HLA-G expression. Instead, in heterozygote trophoblasts the measure of mRNA originated from each allele revealed that the +14 bp allele was less expressed than the −14 bp allele [8]. Moreover, several studies have repeatedly reported the association of the 14 bp insertion and lower soluble HLA-G levels and even the lack of detectable HLA-G expression in the plasma of homozygotes for the insertion allele [9–12].

It has been shown that HLA-G expression is a fundamental prerequisite for embryo implantation and to the maintenance of pregnancy [reviewed in 13] and the +14/+14 genotype has been associated to recurrent spontaneous abortions and to the failure of *in vitro* fertilization [14], suggesting that this genotype could be associated to lower HLA-G levels which could, in turn, predispose to these complications. In women with pre-eclampsia, a pregnancy condition that seems to be associated to lower HLA-G production, it was observed that the placentas with the lowest HLA-G transcription levels were homozygous to the +14 bp allele and that none of the controls of the study presented this genotype [15]. It was also reported an association between the HLA-G 14 bp insertion, as well as a particular allele (G*0106) containing the 14 bp insertion and preeclampsia [16–18] although controversial data come from other studies [19,20].

So, there is evidence that, although the 14pb insertion is associated to the generation of a more stable mRNA population, these transcripts seem to have, if so, a minimal effect on overall HLA-G expression. Moreover, instead of an enhancement on HLA-G expression, a decreased protein expression is associated to the insertion allele. The existing contradiction between the stability of the transcripts originated by the +14 alleles and the observed low HLA-G levels associated to this variant *in vivo* constitutes a paradox, which we name "The 14 bp polymorphism paradox". Although this polymorphism is considered to be responsible by the alternative splicing event occurring at the 3′ in the HLA-G transcript, the lower *in vivo* expression associated to the +14 bp allele is difficult to explain, even considering that transcripts lacking the 92 bases do not represent a majority of the transcripts. The answer to this paradox may involve other unknown characteristics related to this polymorphism or even other nearby polymorphisms in linkage disequilibrium (LD) with the 14 bp polymorphism.

We believe that an interesting approach to determine how the HLA-G molecule expression is regulated should include the analysis of cellular entities which are becoming very popular among research groups: MicroRNAs. MicroRNAs are small RNAs (20–22 nt) which have important and diversified functions in human biology, being involved in cell differentiation and developing, apoptosis, hematopoiesis and tumorigenesis, among other phenomena. These RNAs are able to supress gene expression when binding to specific sites at mRNA 3′ regions, through translation repression and/or by inducing RNA degradation [reviewed in 21]. At present, it is estimated that up to 30% of human genes might be regulated by microRNAs [22].

2. Are microRNAs the hidden element underlying the HLA-G 14 bp paradox?

The *HLA-G* gene has a microRNA binding site at its 3′ UTR region, less than 200 bp away from the 14pb polymorphic site. This site is a potential target for three microRNAs — miR-148a, miR-148b e miR-152 [23]. Inside this 20 nt region lies a C/G polymorphism, at position +3142 (rs1063320). To test the influence of this po0lymorphism in microRNA binding, Tan and cols. performed *in silico* and *in vitro* tests which showed that the G allele favor the targeting of the three microRNAs to the binding site and therefore direct the repression of HLA-G expression. It is important to note that both alleles present a frequency of around 50% in several different populations (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1063320) and it was previously reported that the +3142 and the 14 bp polymorphisms are in LD (r^2=0.63 for the Chicago population) [23].

We therefore hypothesize that the +3142 polymorphism is more directly responsible for the regulation of the HLA-G expression at the translation level, than the 14 bp polymorphism. It could explain, for example, the reason why the transcripts originated from the +14 bp allele, although more stable, do not reflect in higher HLA-G protein levels: Its no use having more stable transcripts if they are not efficiently translated!

The hypothesis concerning the microRNA influence on HLA-G expression can be easily tested. The first step would consist in detecting the expression of those three complementary microRNAs in human cells which express HLA-G. These cells can either be lineages cultivated ex-vivo, such as the JEG-3 choriocarcinoma cell line, or freshly collected cells known to express the HLA-G molecule (trophoblasts, thymocytes, monocytes etc.). Second step would be to examine the correlation between HLA-G mRNA and protein production by these cells, controlling for 14 bp and +3142 genotypes and by the microRNA levels. It would be particularly interesting to investigate these correlations in both healthy and pathological samples. For example, several papers report HLA-G expression in a variety of tumors [24]. In breast cancer, a malignancy in which HLA-G is expressed in 50% of the cases, miR-148 and miR-152 were shown to be down-regulated [25]. Normal and preeclamptic placentas would also be good candidates to test this hypothesis. The final step would consist of the selective inhibition of microRNA activity with anti-sense oligos, which should determine an increase on HLA-G protein levels, especially on those cells homozygous for the G allele.

3. Evolutive considerations

When observing the homologous region of the HLA-G 3′ UTR region presented in the *Pan troglodytes* and *Macaca mulata* genome projects we observed that both species present the G allele at the position homologous for the *Homo sapiens* +3142 position and the +14 bp allele. In primates, until now, only the 14 bp insertion was observed, suggesting that it might be the ancestor allele, shared between humans and higher primates [26]. So, it is possible that the haplotype composed by the G at the +3142 position and the 14pb insertion is in fact the ancestor haplotype between humans and primates. It is important to note that the 14 bp deletion, in spite of occurring only in humans, is a high prevalent allele in the majority of the populations analyzed to date. This suggests that, in some point of the human evolutionary history, the appearance of this mutation at the HLA-G gene might have provided an adaptive advantage [27]. Nevertheless, we can postulate that it was not the −14 bp allele *per se* that provided such adaptive advantage but instead another variant in LD with this allele. From that, it is possible to conjecture that the C allele at the +3142 position, which is present on the HLA-G most abundant haplotype (G*010101), is the main responsible for this advantage, by lessening the affinity between microRNAs and their binding site at the 3′ region of the HLA-G gene and thus boosting HLA-G production by the fetus and rendering it less prone to aggression by the mother's immune system.

4. Conclusions and perspectives

Concluding, we hypothesize that the 14pb polymorphism is not the main element on the regulation of gene expression at the RNA level, although its role on alternative splicing cannot be neglected. We have exposed consistent arguments about the strong possibility of the +3142 polymorphism as an important factor concerning HLA-G expression regulation. The rs1063320 polymorphism might influence microRNA binding to the 3′ region of the HLA-G mRNA and thus influence translation rates or even RNA degradation. We are aware that concentrating on only one polymorphism does not solve the whole problem concerning HLA-G expression. Nevertheless, we put in consideration the influence of a variable which has been neglected until now. The study of the influence of microRNAs on HLA-G expression may shed light on many issues concerning the role of this molecule in several pathological conditions, including pregnancy miscarriages, pre-eclampsia, transplantation and cancer. Since HLA-G expression is shown to be present in several types of cancer, therapeutic strategies involving control of HLA-G expression through microRNA could be viewed as interesting approaches, making the tumor more prone to be targeted by the immune system. Conversely, in situations where HLA-G expression is associated to a better outcome, like transplantation and pregnancy, HLA-G expression could be improved through the neutralization of its targeting microRNAs. Therefore, microRNA control could be viewed as interesting tools in order to control the immune system in a variety of conditions. We strongly suggest that future studies concerning HLA-G should consider this polymorphism and the microRNAs that target this gene.

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CAPÍTULO 4 – Artigo 3

Association of the HLA-G gene +3142C>G polymorphism with systemic lupus erythematosus

Camila Rosat Consiglio, **Tiago Degani Veit**, Odirlei André Monticielo, Tamara Mucenic, João Carlos Tavares Brenol, Ricardo Machado Xavier, José Artur Bogo Chies

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Association of the *HLA-G* gene +3142C>G polymorphism with systemic lupus erythematosus

C. R. Consiglio[1]*, T. D. Veit[1,2]*, O. A. Monticielo[3], T. Mucenic[3], R. M. Xavier[3], J. C. T. Brenol[3] & J. A. B. Chies[1,2]

1 Genetics Department, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
2 Post-Graduation Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
3 Rheumatology Division, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

Key words
human leukocyte antigen G; immunogenetics; polymorphism; systemic lupus erythematosus

Correspondence
José Artur Bogo Chies
Departament of Genetics
Universidade Federal do Rio Grande do Sul
Av. Bento Gonçalves, 9500
Caixa Postal 15053
91501-970 Porto Alegre
Rio Grande do Sul
Brazil
Tel: +55 51 3308 6740
Fax: +55 51 3308 7311
e-mail: jabchies@terra.com.br

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Abstract

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease that affects several organs and systems. Its etiology remains unknown, although it is probably multifactorial. The human leukocyte antigen G (HLA-G) is a nonclassic major histocompatibility complex I molecule characterized by restricted expression and low DNA polymorphism. HLA-G plays a role in immunosuppression through different mechanisms. In inflammatory diseases, it has been postulated that HLA-G expression may be a possible mechanism of tissue protection against exacerbated inflammatory response. On the 3′ untranslated region (3′ UTR) of the *HLA-G* gene, there is an insertion/deletion polymorphism of 14 bp (rs1704) that was shown to influence the mRNA stability. The influence of this polymorphism in disease susceptibility is controversial. Also in the 3′ UTR there is a single nucleotide polymorphism C/G (rs1063320) on the position +3142, at a possible binding site for microRNAs (miRNAs) and having an influence on miRNA affinity. In this study, we analyzed the +3142C>G and the 14 bp polymorphisms in 195 SLE European-derived female patients. Our findings show a significant increase of the +3142G allele frequency among patients as compared with controls (0.58 *vs* 0.47, $P = 0.011$). Also, patients presented a higher frequency of the GG genotype (OR = 1.90, 95% CI: 1.08–3.42). Double heterozygotes for the two polymorphisms presented a milder mean systemic lupus erythematosus disease activity index (SLEDAI) than heterozygotes for only one of the variants or non-heterozygous individuals (1.56 *vs* 3.15 and 3.26, respectively, corrected $P = 0.044$). These results suggest the involvement of the HLA-G molecule on SLE susceptibility and outcome.

Introduction

Systemic lupus erithematosus (SLE) is a systemic autoimmune and inflammatory disease that mainly affects women in reproductive age. Its pathogenesis is characterized by autoantibody production, mainly antinuclear antibodies, and the deposition of immunocomplexes, promoting intense inflammation of several organs and systems. SLE is a disease with clinical heterogeneity, resulting in a spectrum of different symptoms and disease activity. These variable clinical outcomes are a result of its probably multifactorial etiopathogenesis, where the interaction of genetic and environmental factors contributes to disease susceptibility. There is an evident genetic component, as has been shown in studies that observed heritability higher than 66%, a siblings risk ratio between 8 and 29 and concordance of monozygotic twins (24%–69%) and dizygotic twins (2%–9%) (1, 2). Genome-wide linkage studies show a strong linkage with the region 6p21.1-q15, which encompasses the human leukocyte antigen (HLA) region (3).

The HLA-G is a class I nonclassical major histocompatibility complex (MHC) molecule characterized by restricted expression and low DNA polymorphism. Seven different isoforms of HLA-G can be produced by alternative splicing, where HLA-G1 to -G4 are membrane-bound and HLA-G5 to -G7 are soluble molecules. It has been shown that the

*Both authors equally contributed to this work.

HLA-G plays a major role in immunosuppression, interacting with cells of the immune system and suppressing the immune response by different mechanisms (4). HLA-G was shown to inhibit the cytotoxic activity of natural killer and cytotoxic T cells, CD4+ T cell alloproliferative response and also to act on antigen-presenting cells by inhibiting their maturation and function. Additionally, HLA-G is capable of exerting long lasting effects on the immune system through the interaction with inhibitory receptors, by modulating cytokine production, by the induction of HLA-E expression, by the up-regulation of inhibitory receptors and through the generation of suppressor cells, among other mechanisms (5). The expression of HLA-G was first described in fetal cytotrophoblast, where it is involved in the induction and maintenance of tolerance between the mother and the fetus (6, 7). In inflammatory diseases, it has been postulated that the HLA-G expression may be a possible mechanism of tissue protection against inflammatory responses (8). For instance, Rizzo et al. have shown that SLE Italian and Danish patients have a lower expression of HLA-G when compared with controls (9).

The *HLA-G* gene is located on chromosome 6 (6p21.31) and several polymorphisms have been studied in inflammatory disease susceptibility. On the 3′ untranslated region (3′ UTR), an insertion/deletion polymorphism of 14 bp (rs1704) was shown to influence mRNA stability (10). Transcripts with the 14 bp insertion are able to undergo an additional splicing step that removes 92 nucleotides (nt) and this was shown to have an effect on the stability of the mRNA, as mRNAs lacking the 92 nt appear to be more stable *in vitro* (10). In spite of this, several studies have repeatedly reported the association of the 14 bp insertion and lower soluble HLA-G levels and even the lack of detectable HLA-G expression in the plasma of homozygotes for the insertion allele (9, 11–13). This polymorphism has been investigated in SLE by previous studies, which observed conflicting results (9, 14). Also on the 3′ UTR of the HLA-G gene, there is a single nucleotide polymorphism C>G on the position +3142 (rs1063320) which is known to be within a putative binding site for microRNAs (miRNAs), which is thought to be important for the regulation of the *HLA-G* gene expression (5). The G allele was shown to favor the targeting of three miRNAs (miR-148a, miR-148b and miR-152), possibly resulting in a higher repression of translation and mRNA degradation and, consequently, less expression of HLA-G, whereas the C allele would yield a binding site with lower affinity for these miRNA (15). In this study, we analyzed the influence of the +3142C>G and the 14 bp ins/del polymorphisms of the HLA-G gene on SLE susceptibility and outcome.

Material and methods

Patients and controls

The SLE patients were recruited from the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre, RS, Brazil. All the patients included in the study satisfied the American College of Rheumatology (ACR) revised criteria for SLE (16). The group totalized 195 European-derived women diagnosed after 20 years of age. The ethnic classification was based on the physical appearance, which was judged by the researcher at the time of blood collection and on data about the ethnicity of parents/grandparents reported by the participants. The SLE disease activity index (SLEDAI) (17) was applied to each patient as a measure of disease activity. A total of 122 controls with the same ethnic, sex and age profile were recruited. This group was from the urban population of Porto Alegre and other cities from the same geographic area of the patients.

The study's protocol was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre and informed consent was obtained from all patients and controls.

Polymerase chain reaction amplification of the +3142C>G and 14 bp polymorphism in exon 8 (3′ UTR) of the HLA-G gene and genotyping

Patients' and controls' DNA was isolated from peripheral blood cells using a salting out method (18). The genotyping of the 14 bp polymorphism of the HLA-G gene was performed as previously described (19). Briefly, 100 ηg of genomic DNA was amplified in a 25 ml reaction, with final concentrations as follows: polymerase chain reaction (PCR buffer) 1×, dNTP 0.2 mM, $MgCl_2$ 1.5 mM, Taq DNA polymerase 0.75 U and 10 ρmol of each primer (GE14HLAG 5′-GTGATGGGCTGTTTAAAGTGTCACC-3′, RGH4 5′-GG AAGGAATGCAGTTCAGCATGA-3′. The thermocycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s, 64°C for 60 s and 72°C for 60 s; final extension of 72°C for 10 min. The amplified PCR products were visualized in 6% polyacrylamide gel stained with ethidium bromide, with the amplicon sizes being 224 bp for the 114 bp allele and 210 bp for the 214 bp allele.

The PCR of the +3142C>G polymorphism was performed as described (20): 200 ηg of genomic DNA were added to a final volume of 25 μl, with final concentrations as follows: PCR buffer 1×, 2.0 mM $MgCl_2$; 0.2 mM of each dNTP; 1.0 U of Taq-polymerase and 10 ρmol of each primer (GMIRNAF 5′-CATGCTGAACTGCATTCCTTCC-3′, GMIRNAR 5′-CTGGTGGGACAAGGTTCTACTG-3′). Thermocycling conditions were as follows: 94°C for 5 min; 32 cycles of 94°C for 30 s, 65.5°C for 30 s and 72°C for 60 s followed by a final extension step at 72°C for 5 min.

The amplified PCR products were cleaved with 3 U of the restriction enzyme BaeGI (New England Biolabs Inc., Ipswich, MA) according to manufacturer's instructions. Restriction fragment length polymorphism products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide, with amplicon sizes of 406 bp for the C allele and 316 and 90 bp for the G allele.

Statistical analysis

The +3142C>G and 14 bp polymorphisms genotype were compared with Hardy–Weinberg (HW) expectations using chi-squared tests. The allelic and genotypic frequencies of the HLA-G polymorphisms of controls and SLE patients were compared using the chi-squared test (with Yates correction when necessary) or Fisher exact test. Haplotype frequencies were estimated with the Mlocus software (21), which uses an expectation maximization algorithm (22). Bonferroni correction for multiple comparisons was applied when the *P* value was significant. Relative risks were estimated by the odds ratio. Means for SLEDAI were analyzed by Kruskall–Wallis tests. The significance level was set at $\alpha = 0.05$ (two-tailed) and all statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL) and winpepi (23).

Results

A total of 195 SLE female patients and 122 healthy female controls were genotyped for the two *HLA-G* 3′ UTR polymorphisms. Mean age of controls was 48.3 ± 15.4 years, while the mean age of patients was 50.2 ± 13.1 years; the mean age at diagnosis was 35.8 ± 11.5 years. Further patient details are displayed in Table 1. As previously published, when comparing genotype frequencies of patients and controls with expected HW frequencies, we observed a heterozygote excess for the 14 bp polymorphism (59.3, $P = 0.014$) among patients (24). However, no departures from HW equilibrium were observed among patients or controls concerning the +3142C>G polymorphism (data not shown).

In this study, a significant increase of the +3142G allele frequency was observed among patients as compared with controls (0.58 *vs* 0.47, $P = 0.011$). Also, patients presented a higher frequency of the GG genotype (OR = 1.90, 95% CI: 1.08–3.42, Table 2). Further analysis of estimated haplotype frequencies showed an increased frequency of the del/G haplotype in patients (0.16 *vs* 0.07), whereas the frequency of the del/C haplotype was decreased as compared with controls (0.40 *vs* 0.51, $P = 0.0019$, Table 3).

The influence of *HLA-G* genotype in the patients' disease activity was undertaken by assessing the patients' first SLEDAI. Because SLEDAI values did not follow a normal distribution, a nonparametric test was applied to evaluate differences among genotypes. Among the 195 patients, 164 had their first SLEDAI score (meaning the SLEDAI at diagnosis) registered on their records. As it is shown in Table 4, patients homozygous for the insertion allele or the G allele presented the highest mean SLEDAI among genotypes (3.44 *vs* 2.06), although not significant after correction for multiple comparisons ($P_{corr} = 0.096$). Conversely, both the heterozygotes for the 14 bp or the +3142C>G polymorphisms presented the lowest mean SLEDAI among their respective genotypes, and double heterozygotes presented a milder mean disease activity score than heterozygotes for only one of the polymorphisms or non-heterozygous individuals (1.56 *vs* 3.15 and 3.26 respectively, corrected $P = 0.044$). We also assessed the influence of the *HLA-G* genotype in clinical and immunological features individually, but no statistically significant differences were observed.

Table 1 Patients' clinical features

Patients' features	*N*[a] (%)	Mean	SD
Patients' age (years)	195	50.2	13.1
Age at diagnosis (years)	195	35.8	11.5
Disease time (years)	195	14.4	8.1
Controls' age (years)	122	48.3	15.1
Malar rash	102/195 (52.3)	—	—
Discoid rash	31/195 (15.9)	—	—
Photosensitivity	162/195 (83.1)	—	—
Oral ulcers	68/195 (34.9)	—	—
Arthritis	166/195 (85.1)	—	—
Serositis	57/195 (29.2)	—	—
Nephritis	72/195 (36.9)	—	—
Neurologic disorders	22/195 (11.3)	—	—
Hematologic disorders	147/195 (75.4)	—	—
Hemolytic anemia	60/195 (30.8)	—	—
Leukopenia/Lymphopenia	111/195 (56.9)	—	—
Thrombocytopenia	35/195 (17.9)	—	—
Immunologic disorders	131/194 (67.5)	—	—
Anti-DNA	85/194 (43.8)	—	—
Anti-Sm	38/194 (19.6)	—	—
Anticardiolipin	55/194 (28.4)	—	—
Lupic anticoagulant	14/194 (7.2)	—	—
False-positive VDRL	11/194 (5.7)	—	—
Anti-Ro	73/178 (41.0)	—	—
Anti-La	23/178 (12.9)	—	—
Anti-RNP	60/178 (33.7)	—	—
Anti-Scl70	5/178 (2.8)	—	—
ANA	193/194 (99.5)	—	—
Sjögren	(11.1)	—	—
SLEDAI	168	1.0[b]	0, 4.0[c]
SLICC	187	1.0[b]	0, 2.0[c]

ANA, antinuclear antibody; RNP, ribonucleoprotein; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, Systemic Lupus International Collaborating Clinics; VDRL, venereal disease research laboratory test.

[a]Number of patients with the feature/total analyzed.

[b]Median.

[c]Percentiles 25, 75.

Discussion

In this study, we analyzed the role of two 3′ UTR polymorphisms of the *HLA-G* gene in the susceptibility and clinical outcome of SLE patients, with special emphasis to the +3142C>G polymorphism, which has not yet been studied in this disease. Considering the inflammatory character of SLE, a role for the HLA-G molecule seems suitable in the etiopathology of this disease.

Table 2 HLA-G genotype frequencies in patients and comparison with controls

	Patients *N* (frequency)	Controls *N* (frequency)	Odds ratio (95% CI)	*P* (allele)
14 bp ($n = 193$)				
Del/del	51 (0.26)	40 (0.33)	0.71 (0.42–1.21)[a]	–
Ins/del	114 (0.59)	60 (0.50)	—	—
Ins/ins	28 (0.15)	21 (0.17)	—	—
Del	216 (0.56)	140 (0.58)	—	NS
Ins	170 (0.44)	102 (0.42)	—	—
+3142 ($n = 192$)				
GG	61 (0.32)	24 (0.20)	**1.90 (1.08–3.42)**[b]	—
CG	100 (0.52)	67 (0.55)	—	—
CC	31 (0.16)	31 (0.25)	—	—
G	222 (0.58)	115 (0.47)	—	**0.011**
C	162 (0.42)	129 (0.53)	—	—

HLA-G, human leukocyte antigen G; NS, not significant. The odds ratio for genotypes and the *P* for alleles are in boldface.
[a]Del/Del as risk genotype.
[b]GG as risk genotype.

Table 3 HLA-G haplotype-estimated frequencies

Haplotype	Patients	Controls	Residuals (*P*)[a]	*P*
Del/C	157 (0.40)	127 (0.51)	−2.67 (0.007)	0.0019
Del/G	64 (0.16)	18 (0.07)	3.38 (<0.001)	—
Ins/C	8 (0.02)	5 (0.02)	0.04 (0.97)	—
Ins/G	163 (0.42)	100 (0.40)	0.40 (0.61)	—

[a]Of patients in relation to controls.

As observed in our study, patients of SLE had a significantly higher frequency of the +3142G allele ($P = 0.011$) and the GG genotype (OR = 1.90, 95% CI: 1.08–3.42) when compared with healthy controls, suggesting this genotype as a disease susceptibility factor. The +3142C>G polymorphism might affect disease susceptibility by its influence on post-transcriptional regulation of HLA-G expression, as suggested by Tan et al. in asthma patients (15). To date, conflicting results have been reported concerning the analysis of another *HLA-G* 3′ UTR polymorphism, the 14 bp ins/del. Rizzo et al. (9) observed an increased frequency of the 14 bp insertion among Italian SLE patients, while our group observed an excess of heterozygotes among Brazilian patients (24) and Wu et al. described a lack of association between this variant and SLE among Chinese SLE patients (25). It is interesting to point out that the 14 bp polymorphism is in partial linkage disequilibrium (LD) with the +3142C>G polymorphism. It is possible that these conflicting data are because of the LD with the +3142C>G polymorphism or with other variants differently present in these ethnically diverse populations. Further analysis of the +3142C>G polymorphism on those, and other, populations would help to clarify the influence of 3′ UTR polymorphisms in the pathology of SLE.

Table 4 Genotype and first SLEDAI index in SLE patients

	Mean (SD)	Mean rank	*P*	P_{corr}
14 bp				
Ins/ins	3.39 ± 4.18	94.57	0.053[a]	0.212
Ins/del	2.11 ± 3.48	74.05	—	—
Del/del	2.96 ± 3.66	88.11	—	—
+3142				
C/C	3.00 ± 3.83	90.46	0.026[a]	0.104
C/G	1.85 ± 3.01	73.41	—	—
G/G	3.44 ± 4.20	92.89	—	—
Ins/ins or G/G	3.44 ± 4.09	90.71	0.024[b]	0.096
Other	2.06 ± 3.31	74.49	—	—
Double hetero	1.56 ± 2.91	67.42	0.011[a]	0.044
Single hetero	3.15 ± 3.90	84.88	—	—
Other	3.26 ± 4.00	90.67	—	—

SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index.
[a]Kruskal–Wallis test.
[b]Mann–Whitney test.

The haplotype analysis conducted in this study showed that while the frequencies of the ins/C and ins/G haplotypes were similar between patients and controls, a significant increase of the del/G allele frequency among patients was observed, while the del/C haplotype was shown to be overrepresented in controls (Table 3). This difference suggests the involvement of the del/G haplotype in lupus susceptibility and clarifies previous results obtained when analyzing the 14 bp polymorphism alone, where a heterozygote excess was observed among patients. In brief, now it is clear that this previously described heterozygote excess is because of an increased frequency of the del/G haplotype.

A recent study investigated the haplotype structure of the 3′ UTR of the *HLA-G* gene (26). According to this study, the del/G haplotype fits in the so-called UTR-3′-extended haplotype, which is associated with three alleles G*010401, G*010403 and G*010404, which are closely related in terms of evolution (27). Intriguingly, G*010401, the most prevalent allele associated to this haplotype, was previously shown to be a high producer allele (28). However, it has been shown that the majority of the miRNAs which were described as potentially targeting the 14 bp region have higher affinity for the deletion allele. Also, the deletion of the 92 nt region, which occurs only with the 14 bp insertion, could favor the stability of these shorter transcripts, as this region is a target for several miRNAs (29). Nevertheless, it should be pointed out that the regulation of HLA-G expression at the 3′ UTR remains elusive and further studies are needed in order to clarify these mechanisms.

The SLEDAI ranking analysis among genotypes of the two 3′ UTR polymorphisms showed no significant association between disease severity and homozygosis to a particular allele. Interestingly, both the heterozygotes for the 14 bp or the +3142C>G polymorphism presented a lower mean

SLEDAI. Taking the two polymorphisms together, a milder mean activity score was significantly observed on double heterozygotes when compared with the other genotypes. A similar trend was observed in our previous study, where we analyzed the 14 bp polymorphism alone (24). It was already shown that the *HLA-G* seems to be under balancing selection (30). In this way, heterozygosis to *HLA-G* alleles would be expected to confer some kind of advantage to their carriers. It can be suggested, concerning the complexity and multiplicity of clinical manifestations in SLE, that HLA-G expression would be triggered in a wider range of situations in heterozygous patients, as compared with homozygous patients, because of the presence of different regulatory regions in each given allele. Further analysis of HLA-G levels and also microRNA expression in SLE may clarify the importance of the HLA-G molecule in disease susceptibility and outcome.

In conclusion, we have observed differences in the genotypic, allelic and haplotypic frequencies of the two 3′ UTR polymorphisms analyzed, with emphasis at an increased frequency of the G allele and the del/G haplotype among SLE patients, pointing out to a role of the *HLA-G* gene in disease susceptibility. Also, double heterozygous patients presented lower disease activity scores, reinforcing previous data pointing to a role of this molecule in disease outcome. Although we cannot rule out the existence of other polymorphic loci in LD with the analyzed HLA-G polymorphisms as involved in the SLE etiopathogenesis, our results support a role of the HLA-G molecule in the pathology of SLE.

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CAPÍTULO 5 – Artigo 4

Association of an *HLA-G* 3'UTR haplotype with the susceptibility to Rheumatoid Arthritis.

Tiago Degani Veit; Clayton Pereira Silva de Lima; Laura Corso Cavalheiro; Claiton Viegas Brenol; João Carlos Tavares Brenol; Ricardo Machado Xavier; Eduardo José Melo dos Santos; José Artur Bogo Chies

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Association of an *HLA-G* 3'UTR haplotype with susceptibility to Rheumatoid Arthritis.

Tiago Degani Veit [1]; Clayton Pereira Silva de Lima [2]; Laura Corso Cavalheiro [3]; Claiton Viegas Brenol [3]; João Carlos Tavares Brenol [3]; Ricardo Machado Xavier [3]; Eduardo José Melo dos Santos [2]; José Artur Bogo Chies [1,*]

[1] Universidade Federal do Rio Grande do Sul - Laboratório de Imunogenética;

[2] Universidade Federal do Pará - Laboratório de Genética Humana e Médica

[3] Hospital de Clínicas de Porto Alegre - Serviço de Reumatologia

*Corresponding author: José Artur Bogo Chies

Departament of Genetics

Universidade Federal do Rio Grande do Sul

Av. Bento Gonçalves, 9500

Caixa Postal 15053

91501-970 Porto Alegre, RS

Brazil

Tel: +55 51 3308 6740

Fax: +55 51 3308 7311

e-mail: jabchies@terra.com.br

Short title: HLA-G 3'UTR polymorphisms and Rheumatoid Arthritis

ABSTRACT

HLA-G is a non classical HLA molecule characterized by limited tissue distribution under normal physiological conditions and low polymorphism at DNA and protein level. It has been shown that the HLA-G plays a major role in immunosuppression, interacting with cells of the immune system and suppressing the immune response by different mechanisms. In the present study, we sought to investigate the genetic influence of the two *HLA-G* 3'UTR polymorphisms – the 14 bp insertion/deletion (rs1704) and the +3142C>G (rs1063320) – in the susceptibility to rheumatoid arthritis in a Southern-Brazilian RA cohort and in an independent confirmatory group from the North of Brazil. A total number of 466 RA patients and 502 controls was PCR genotyped for these polymorphisms. After adjusting for city of origin and gender, we observed that female patients presented a higher frequency of the deletion-G (D/G) haplotype (OR=1.754, 95% CI=1.187–2.592, P=0.005). Although only the female RF+ group of patients presented statistical significance (1.784, 95%C.I = 1.193–2.667, P=0.005), the OR of the RF- female group was quite similar (1.689), suggesting that the susceptibility to RA conferred by the D/G haplotype is not affected by the positivity to RF. Our results suggest that the D/G and the corresponding HG0104 might be a risk factor for RA in females.

Introduction

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory disease that can lead to joint deformities and permanent physical disability. Onset is likely triggered by environmental factors in susceptible individuals. Several genes have been indicated so far in the pathogenesis of RA. The most important genetic region is the Human Leukocyte Antigen (HLA) region, which contributes to approximately half of the genetic susceptibility for RA. Alleles from the HLA-DRB1 locus, notably those carrying the “shared epitope” sequence, are associated with an increased risk and a more severe phenotype in RA (1). The most recent evidences suggest such that such HLA associations are stronger or specific for anti-citrullinated protein antibodies (ACPA) positive disease. However, growing evidence has indicated that this locus does not entirely explain the HLA contribution to disease risk. [reviewed in (2)].

HLA-G is a non-classical HLA molecule characterized by limited tissue distribution under normal physiological conditions and low polymorphism at DNA and protein level. HLA-G presents unique characteristics as compared to its classical counterparts such as the expression of multiple isoforms generated by alternative splicing, which can be membrane-bound (G1-G4) or secreted (G5-G7). Moreover, it is the only HLA molecule capable of forming dimers, which seem to be the main contributors in its biologic activity. It has been shown that the HLA-G plays a major role in immunosuppression, interacting with cells of the immune system and suppressing the immune response by different mechanisms. These mechanisms include the inhibition of cytotoxic activity of cytotoxic T lymphocytes (CTL) and NK cells, protection of class I-negative or allogeneic tumors from NK-mediated anti-tumor immunity (3). Also, it was shown that the HLA-G molecule can inhibit CD4+ T cell alloproliferative responses(4), the proliferation of T and peripheral blood NK cells (5-7), and also can act on APCs by inhibiting their maturation and function (8). Additionally, HLA-G may exert long-term immunotolerogenic effects through the generation of suppressor cells [reviewed in (9)]. Such features render HLA-G as an attractive candidate gene for susceptibility to immune mediated diseases. The 3’ untranslated region (3’UTR) seems to play an important role on HLA-G

expression, mainly through post-transcriptional regulatory mechanisms. A recent work from Castelli et al. has described eight different haplotypes in a Brazilian population (10). *In silico* analysis of this region has identified numerous putative sites for microRNA binding, which may influence HLA-G expression depending on the allele and/or biological context. According to the latest information, eleven polymorphic positions have been identified (11), many of which overlapping putative microRNA binding sites. Among the 3'UTR polymorphisms, a 14 bp insertion/deletion (INDEL) (rs1704), which may be, among other things, involved in alternative splicing processes, seems to be important for post-transcriptional regulation. Two previous studies have assessed the influence of the 14 pb INDEL (rs1704) in the susceptibility to RA, but no association was observed (12, 13). However, our group has observed a positive association of a 3'UTR haplotype encompassing the 14bp locus and another 3'UTR polymorphism, which seems to be involved in microRNA binding - +3142C/G (rs1063320) – and disease susceptibility in patients with systemic lupus erythematosus (14). In the present study, we sought to investigate the genetic influence of the two *HLA-G* 3'UTR polymorphisms in the susceptibility to rheumatoid arthritis in a Southern-Brazilian RA cohort and in an independent confirmatory group from the North of Brazil.

Methods

The study encompassed samples from two distinct medical centers in Brazil: the Southern sample comprised 337 RA European-derived patients and 283 healthy European-derived controls from the urban region of Porto Alegre, State of Rio Grande do Sul, Southern Brazil, situated at 30.02°S and 51.13°W. The Northern sample comprised 129 RA patients and 219 healthy controls from the admixed population of Belém, Northern Brazil, situated at 1.27°S and 48.30°W, totalizing 466 RA patients and 502 controls. Patients from Porto Alegre were under the care of the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre (HCPA), while the patients from Belém were collected from several medical centers. All patients were diagnosed according to the American College of Rheumatology's criteria for the classification of Rheumatoid Arthritis.

Patients having another connective tissue disease, other than secondary Sjögren syndrome, were excluded. Patients were followed at the Rheumatology Outpatient Clinic of the Hospital de Clínicas de Porto Alegre.

Polymerase chain reaction amplification of the +3142C>G and 14bp polymorphism in exon 8 (3'UTR) of the HLA-G gene and genotyping

Patient and control DNA was isolated from peripheral blood using a salting out method (15). The genotyping of the 14bp polymorphism of the HLA-G gene was performed as previously described (16). Briefly, 100 ηg of genomic DNA was amplified in a 25 mL reaction, with final concentrations as follows: polymerase chain reaction (PCR buffer) 1x, dNTP 0.2 mM, MgCl2 1.5mM, Taq DNA polymerase 0.75 U and 10 ρmol of each primer (GE14HLAG 5'GTGATGGGCTGTTTAAAGTGTCACC-3', RGH4 - 5'-GGAAGGAATGCAGTTCAGCATGA-3'. The thermocycling conditions were as follows: 94ºC for 2 min; 35 cycles of 94ºC for 30 s, 64ºC for 60 s and 72ºC for 60 s; final extension of 72ºC for 10 min. The amplified PCR products were visualized in 6% polyacrylamide gel stained with ethidium bromide, with the amplicon sizes being 224 bp for the 114 bp allele and 210 bp for the 214 bp allele.

The PCR of the +3142C>G polymorphism was performed as described (17): 200 ηg of genomic DNA were added to a final volume of 25 µL, with final concentrations as follows: PCR buffer 1×, 2.0 mM MgCl2; 0.2 mM of each dNTP; 1.0 U of Taq-polymerase and 10 ρmol of each primer (GMIRNAF-5'-CATGCTGAACTGC ATTCCTTCC-3', GMIRNAR-5' CTGGTGGGACAAGGTTCTACTG-3'). Thermocycling conditions were: 94ºC for 5 min; 32 cycles of 94ºC for 30 s, 65.5ºC for 30s and 72ºC for 60s followed by a final extension step at 72ºC for 5 min.

The amplified PCR products were cleaved with 3U of the restriction enzyme BaeGI (New England Biolabs Inc., Ipswich, MA) according to manufacturer's instructions. RFLP products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide, with amplicon sizes of 406bp for the C allele and 316 and 90bp for the G allele.

Statistical analysis

The +3142C>G and 14bp genotypic frequencies were compared with Hardy–Weinberg (HW) expectations using chi-squared tests. The allelic and genotypic frequencies of the HLA-G polymorphisms of controls and SLE patients were compared using the chi-squared test (with Yates correction when necessary) or Fisher's exact test. Haplotype frequencies were estimated with the Mlocus software (18), which uses an expectation maximization algorithm (19). Bonferroni correction for multiple comparisons was applied when the P value was significant. Relative risks were estimated by the odds ratio. Means for SLEDAI were analyzed by Kruskall–Wallis tests. The significance level was set at $\alpha = 0.05$ (two-tailed), and all statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) and winPEPI (20).

Results

Mean patients age and the mean age at diagnosis were, respectively, 60.3±12.2 and 45.8±13.7 years in Porto Alegre and 54.7±13.7 and 43.5±15.6 years in Belém, The frequency of RA patients that were positive for rheumatoid factor (RF+) was 0.834 in Porto Alegre and 0.853 in Belém. Further patient details are displayed on Table 1.

The polymorphisms in the *3'UTR* showed linkage disequilibrium ($P < 0\ 001$). The values of D' between the 14bp polymorphism and the +3142G>C in controls and RA patients were 0.948 and 0.924 and the values for r_2 were 0.511 and 0.479, respectively. The 14 bp insertion allele (I) segregated almost exclusively with the +3142G (G) allele (0.992 in controls and 0.987 in patients) and the deletion (D) allele had a higher probability to segregate with the +3142C (C) allele (0.749 in controls and 0.730 in patients).

The observed genotype distribution was in agreement with Hardy-Weinberg equilibrium (HWE) expectations for all polymorphisms. A significant difference in the allelic and estimated haplotypic frequencies was observed between healthy

controls of Porto Alegre and Belém, with an increased frequency of the +3142G allele and deletion-G (D/G) haplotype in the control population from Belém (0.585 vs. 0.511 and 0.199 vs. 0.112), whereas the frequency of the deletion-C (D/C) haplotype was increased in the control population from Porto Alegre (0.474 vs 0.409 – see Table 2). Therefore, all further analyses were adjusted for city of origin.

We analyzed the haplotype distribution between RA patients and healthy controls stratified by gender and city of origin. We observed an increased frequency of the D/G haplotype estimated frequency in female patients as compared to female controls (Porto Alegre: 0.131 vs. 0.074, P=0.019; Belém: 0.247 vs. 0.179, P=0.060). To further investigate the influence of the D/G haplotype in the susceptibility to RA, we compared the frequencies of patients and controls carrying the D/G allele in homozygosis or heterozygosis. Double heterozygotes were assumed to be non-carriers of the D/G allele, as the number of double heterozygotes expected of being I/C-D/G was shown to be lower than one in all patient/control groups, exception made to the women patients from Belém, which was 1.78 – see Table 4. We performed multivariate logistic regression analyses in the unified sample to examine whether gender is a relevant factor to the association between RA and the D/G variant in *HLA-G*. The odds ratio (OR) in the unified sample, adjusted for city of origin, is 1.196 [95% CI 0.896–1.600, P=0.229, Table 4]. Next, we examined whether gender is an effect modifier of the association between the D/G variable and RA. When computing the ORs for men and women separately, a striking difference becomes apparent: the OR for females is 1.754 (95% CI=1.187–2.592, P=0.005) while for males it is 0.660 (95% CI=0.348–1.251, P=0.203). We also evaluated whether this effect was restricted to a subgroup of patients after stratifying the analysis for positivity to RF. Although only the female RF+ group of patients presented statistical significance (1.784, 95%C.I = 1.193–2.667, P=0.005), the OR of the RF- female group was pretty similar (1.689), suggesting that the susceptibility to RA conferred by the D/G haplotype is not affected by the positivity to RF. Although pointing to the opposite direction, the OR for male patients yielded no significant results (Table 5). We also investigated whether the HLA-G genotype influenced the age of diagnosis, but no

significant differences were observed (data not shown). In males, a higher frequency of the I/D haplotype was observed (Porto Alegre: 0.476 vs. 0.401, P=0.166; Belém: 0.550 vs. 0.409, P=0.242), although not yielding a significant OR (1.672, 95% CI=0.924–3.026, P=0.089).

Discussion

In this work, we assessed the influence of two polymorphisms located at the 3'UTR of the *HLA-G* gene in the susceptibility to rheumatoid arthritis. Due to the inflammatory nature of this disease and the immunoregulatory features attributed to the HLA-G molecule, the *HLA-G* gene seemed to be a good candidate for association with RA. Although two previous works have assessed the influence of a 3'UTR polymorphism in the susceptibility to RA (14 bp), this is the first work to analyze the influence of 3'UTR haplotypes in this disease.

Here, a gender-specific association between the *HLA-G* 3'UTR D/G haplotype has been reported. We have observed that women carrying the *HLA-G* D/G haplotype were overrepresented in the patient group as compared to female controls. Such type of association had already been reported by our group in systemic lupus erythematosus (SLE), another autoimmune disease with an even higher prevalence in females as compared to RA (14). In that study, a higher frequency of the D/G haplotype had been reported in female patients. It is clear that sex hormones have a profound effect on immune system development and function and it has been previously shown that they are capable of regulating miRNA expression. It is therefore possible that the miRNA profile in women differentially interferes with HLA-G expression. In SLE, an increased expression of miR-148a, which binds the region encompassing the+3142C>G SNP, was described in PBMCs (21), and this increased expression of miR-148a was also demonstrated in splenocytes from estrogen-treated orchiectomized male C57BL/6 mice (22).

It has been previously hypothesized that many autoimmune diseases (ADs) might share a common genetic origin, with some polymorphisms being common risk factors for the development of ADs while others being disease-specific (23). Our

findings therefore suggest a broader involvement of the HLA-G molecule in the pathogenesis of autoimmune diseases.

One may argue that the observed association would likely be a tag marker to a functional SNP or haplotype localized on the 5' upstream regulatory region (5'URR). Indeed, a very recently published work by Castelli et al. has performed a comprehensive study of polymorphic sites along the *HLA-G* gene (24), revealing a very high linkage disequilibrium between the 3'UTR and the 5'URR. However, this same study reveals that the D/G haplotype G haplotype fits in the so called UTR-3 extended haplotype, which is associated exclusively with the HG0104 extended haplotype lineage, encompassing only three HLA-G alleles, namely G*010401, G*010404 and G*010405. The 5'URR of this lineage differs from the HG010102 lineage (which is associated to the majority of the I/G alleles) by only 2 or 3 out of 25 variation sites. Thus, unless one of these 3 SNPs was the functional responsible for the different regulation of this haplotype, we would also expect a higher prevalence of the I/G haplotype in females, which was not the case. Therefore, our chance is high that the two above-mentioned polymorphisms are the true responsibles for an increased susceptibility to RA which was described in this study. Nonetheless, we cannot rule out the possibility of LD of this allele with a functional allele in other another locus at the MHC since this region characterized by high LD in all its extension and LD between the HLA-G locus and class I and II genes had been previously described (25-27).

Interestingly, the directly detectable frequency of D/G-positive controls differed significantly between cities and genders, with a higher frequency in the population from Belém and, controlling for city, in males as compared to females (Porto Alegre: 0.259 vs. 0.137; Belém: 0.394 vs. 0.304). This differences are significant as determined by a logistic regression analysis with D/G as dependent variable and gender and city as predictors ($OR_{Belém}$=2.188, P<0.001; OR_{males}=1.776, P=0.007), i.e. the odds for controls from Belém to be carriers of the D/G are 2.19 times higher than controls from Porto Alegre, and the odds for men to be carriers of the D/G haplotype is 1.77 times higher than that for women. Our data therefore suggest a gender-specific transmission ratio distortion (TRD) of the D/G haplotype

among healthy controls. This hypothesis, however, still remains to be confirmed by the genotyping of further independent samples.

Concluding, we have described an association between a HLA-G 3'UTR haplotype with the susceptibility to rheumatoid arthritis. We hypothesize that in a specific milieu (considering hormonal levels as an important feature) the interaction of certain microRNAs with this particular HLA-G haplotype (D/G) could result in decreased HLA-G expression in situations of antigenic challenge, in genetically predisposed individuals. This context could favor the triggering of autoimmune diseases, mainly in females.

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Table 1: Rheumatoid Arthritis patient and control features.

	Porto Alegre (South)			*Belém (North)*		
	Freq.(N)	*Mean±SD*	*N*	*Freq.(N)*	*Mean±SD*	*N*
RA						
Females	0.813 (274)		337	0.922 (119)		129
Age		60.3±12.2	334		54.7±13.7	129
Age at diagnosis		45.8±13.7	309		43.5±15.6	129
Disease time (years)		14.3±8.2	336		13.1±8.6	129
RF positivity	0.834 (281)		337	0.853 (110)		129
Controls						
Females	0.413 (117)		283	0.571 (125)		219
Age		47.5±11.6	283		39.2±15.0	219

Table 2: HLA-G +3142 genotype, allele and haplotype frequencies in patients and controls

	Controls POA N (frequency)	*RA POA N (frequency)*	*Controls Belém N (frequency)*	*RA Belém N (frequency)*
14 bp				
DD	100 (0.353)	110 (0.326)	77 (0.352)	46 (0.357)
ID	132 (0.466)	168 (0.499)	113 (0.516)	63 (0.488)
II	51 (0.180)	59 (0.175)	29 (0.132)	20 (0.155)
P-value	0.717		0.802	
D	332 (0.587)	389 (0.576)	267 (0.610)	155 (0.601)
I	234 (0.413)	287 (0.424)	171 (0.390)	103 (0.399)
P-value	0.729		0.872	
+3142				
CC	67 (0.230)[b]	76 (0.226)	36 (0.168)[b]	21 (0.154)
CG	145 (0.512)[b]	157 (0.468)	110 (0.496)[b]	58 (0.482)
GG	73 (0.258)[b]	104 (0.306)	73 (0.336)[b]	50 (0.364)
P-value	0.342		0.817	
C	279 (0.489)[c]	309 (0.458)	182 (0.415)[c]	100 (0.388)
G	291 (0.511)[c]	365 (0.542)	256 (0.585)[c]	158 (0.612)
P-value	0.279		0.473	
Haplotype[a]				
D/C	268 (0.474)[d,e]	301 (0.448)	178 (0.409)[d,e]	144 (0.361)
D/G	64 (0.112)[d,f]	85 (0.126)	86 (0.199)[d,f]	91 (0.239)
I/C	7 (0.012)[d]	7 (0.010)	2 (0.006)[d]	11 (0.026)
I/G	227 (0.402)[d]	279 (0.415)	168 (0.386)[d]	144 (0.373)
P-value	0.760		**0.022**	

[a] Estimated frequencies
[b] P=0.062
[c] P = 0.022
[d] P=0.0011
[e] residual P = 0.046
[f] residual P < 0.001

Table 3: *HLA-G* D/G haplotype carrier frequencies in patients and comparison with controls.

	Controls POA N (freq)	*RF- patients POA N (freq)*	*RF+ patients POA N (freq)*	*Controls Belém N (freq)*	*RF- patients Belém N (freq)*	*RF+ patients Belém N (freq)*
All						
D/G carrier	59 (0.208)	13 (0.232)	67 (0.238)	75 (0.342)	6 (0.316)	44 (0.400)
non-carrier	224 (0.792)	43 (0.768)	214 (0.762)	144 (0.658)	13 (0.684)	66 (0.600)
Females						
D/G carrier	16 (0.137)	11 (0.234)	56 (0.247)	38 (0.304)	6 (0.375)	42 (0.408)
non-carrier	101 (0.863)	36 (0.766)	171 (0.753)	87 (0.696)	10 (0.625)	61 (0.592)
Males						
D/G carrier	43 (0.259)	2 (0.222)	11 (0.204)	37 (0.394)	0 (0.0)	2 (0.286)
non-carrier	123 (0.741)	7 (0.778)	43 (0.796)	57 (0.606)	3 (1.000)	5 (0.714)

Table 4. Number of expected D/G-I/C subjects in patient and control samples according to estimated haplotype frequencies and the number of double heterozygotes.

	Gender	*D/G*I/C (A)*	*D/C*I/G (B)*	*A/(A+B)*	*Double Heterozygotes*	*Expected number*[a]
Controls						
Porto Alegre	Female	0.001381	0.203338	0.006748	53	0.357618
	Male	0.000964	0.181440	0.005287	56	0.296084
Belém	Female	0.001782	0.163018	0.010813	43	0.464963
	Male	0.000000	0.149381	0.000000	36	0.000000
Patients						
Porto Alegre	Female	0.001089	0.184557	0.005865	97	0.568905
	Male	0.001954	0.189986	0.010178	26	0.264640
Belém	Female	0.007038	0.131216	0.050903	35	1.781600
	Male	0.000000	0.165000	0.000000	4	0.000000

[a] A/(A+B) x Double Heterozygotes

Table 5: Association of the D/G haplotype with RA in the unified cohort.

	OR [a]	*95%C.I.*	*P value*
OR_{All}	1.196	0.894-1.600	0.229
$OR_{females}$	**1.754**	**1.187-2.592**	**0.005**
OR_{males}	0. 660	0.348-1.251	0.203
$OR_{RF+\ females}$	**1.784**	**1.193-2.667**	**0.005**
$OR_{RF+males}$	0.711	0.359-1.409	0.328
$OR_{RF-\ females}$	1.689	0.864-3.303	0.126
$OR_{RF-\ males}$	0.476	0.101-2.242	0.348

[a] Odds ratios (OR) were determined by a logistic regression model with the RA status as outcome variable and the D/G haplotype as predictor. OR_{all} was computed for the unified sample of Porto Alegre and Belém, for computation of OR_{males}, $OR_{females}$ and the following OR, the unified sample was separated into male and female individuals. All ORs are controlled for the city of origin.

CAPÍTULO 6 – Artigo 5

Soluble HLA-G in plasma and synovial fluid from patients with Rheumatoid Arthritis and Juvenile Idiopathic Arthritis

Tiago Degani Veit; Vera Rebmann; Peter Horn; Mauricio Busatto; Claiton Viegas Brenol; João Carlos Tavares Brenol; Ilóite Maria Scheibel; Sandra Helena Machado; Luiz Roberto Stigler Marczyk, João Luiz Ellera Gomes, Ricardo Machado Xavier; José Artur Bogo Chies.

Em preparação para submissão à revista Tissue Antigens

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Tiago Degani Veit [1]; Vera Rebmann [2]; Peter Horn [2]; Mauricio Busatto [1]; Claiton Viegas Brenol [3]; João Carlos Tavares Brenol [3]; Ilóite Maria Scheibel [4]; Sandra Helena Machado [3]; Luiz Roberto Stigler Marczyk [5] João Luiz Ellera Gomes [5;] Ricardo Machado Xavier [3]; José Artur Bogo Chies [1,*]

[1] Laboratório de Imunogenética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

[2] Institut für Tranzfusionsmedizin, Universitätsklinikum Essen, Essen, Germany

[3] Serviço de Reumatologia, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

[4] Hospital da Criança Conceição, Grupo Hospitalar Conceição, Porto Alegre, Brazil

Serviço de Ortopedia, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

*Corresponding author: José Artur Bogo Chies

Departament of Genetics

Universidade Federal do Rio Grande do Sul

Av. Bento Gonçalves, 9500

Caixa Postal 15053

91501-970 Porto Alegre, RS

Brazil

Tel: +55 51 3308 6740

Fax: +55 51 3308 7311

e-mail: jabchies@terra.com.br

Short title: sHLA-G in RA and JIA

Abstract

The purpose of this study was to investigate the HLA-G expression in plasma and synovial fluid (SF) from rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) patients. Samples were obtained from 70 RA patients, 19 osteoarthritis (OA) patients, 19 knee trauma patients and 24 JIA patients and 36 healthy children(only plasma). Two ELISA methods were used to quantify sHLA-G in plasma and SF, while a Luminex-based receptor binding assay (ILT2) was also used to quantify plasma samples. In the G233 ELISA the RA group presented significantly increased SF levels as compared to adult control groups (medians: 13.06, 5.61 and 4.37 ng/mL; P=0.004). Stratifying by RF positivity, a significantly higher median level of sHLA-G in SF was observed in RF negative (RF-) patients as compared to RF positive (RF+) patients [273,99 vs. 69,84, P=0.018 (MEM-G/9); 52.87 vs. 9.27, P=0.031 (G233)]. The JIA group presented an even higher median value than the RA group for both ELISA measures. SF HLA-G correlated positively with plasma HLA-G by the two measures (MEM-G/9: r_s=0.591, P=0.026; G233: r_s=0.508, P=0.064). Different patterns of correlation between soluble HLA-G and disease parameters were observed among groups stratified by gender and RF positivity. In RF+ females, positive correlations between sHLA-G capable of binding ILT-2 and DAS28 CRP, CDAI, number of swollen joints and patient VAS were observed. In RF+ males, intriguingly, we observed a negative correlation between plasma HLA-G (G233) and DAS28 CRP (r_s=0.943, P=0.005). In RF- females, conversely, various disease parameters were positively associated to sHLA-G (MEM-G/9). The sHLA-G levels in JIA patients presented distinct patterns of correlation with disease activity markers according to gender. Such findings encourage further studies with the aim to investigate the role of sHLA-G in the course of these two rheumatic diseases.

Introduction

HLA-G is a nonclassic HLA class I molecule which was first characterized by its expression at the maternal-fetal interface. It is characterized by limited tissue distribution in healthy conditions and by the expression of seven different isoforms that can be either membrane-bound (G1-G4) or secreted (G5-G7). Since it was first described, in cytotrophoblasts, this molecule has attracted much attention due to its immunotolerogenic properties. HLA-G is capable of interacting with several receptors (ILT-2, ILT-4, KIR2DL4, CD8, CD160) present in various cells of the immune system, such as NK cells, T and B lymphocytes and antigen-presenting cells (APC). Several mechanisms of immunosuppression were described to date, such as inhibition of cytotoxicity, proliferation and/or differentiation, induction of tolerogenic APC or suppressive T and NK cells, induction of apoptosis, as well as up regulation of inhibitory receptors, among other features (Reviewed in 1). All this features have made HLA-G an attractive target in different situations in which immune tolerance is involved, such as pregnancy and its complications, transplantation, cancer and viral infections. In recent years, a convincing body of scientific evidence has indicated that the expression of HLA-G, plays a role in regulation of inflammation in autoimmune diseases (1, 2). The first studies on this area described the HLA-G expression in muscle fibers in various inflammatory myopathies, in atopic dermatitis and psoriatic skin (3-5). It was promptly proposed, based on the findings that HLA-G seems to shift T-helper responses towards a Th2-type response, that it would act as a tissue-protective molecule in inflammatory responses (6-8), and numerous studies have been conducted since then. HLA-G expression was described in ulcerative colitis (UC) patients. Together with IL-10, HLA-G expression on the surface of intestinal epithelial cells in this disease may participate in the suppression of the activity of pro-inflammatory cytokines and in the return of the immune system to a basal state. This phenomenon was not observed in Crohn's disease (CD), which constitutes a potential differentiation factor for the two diseases (9). In multiple Sclerosis (MS), HLA-G was shown to be strongly expressed at lesions and in areas with lymphocytic and monocytic inflammation (10). Moreover, HLA-G expression was shown to be increased in patients after treatment with Interferon-beta (IFN-β) the

major immunomodulatory agent used in the treatment of MS. HLA-G derived from monocytes, the primary source of HLA-G in MS, was shown to inhibit both Th1 (IFN-g, IL-2) and Th2 (IL-10) cytokine production by antigen-stimulated autologous CD4 T cells (10).

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory disease that can lead to joint deformities and permanent physical disability. Onset is likely triggered by environmental factors in susceptible individuals. Synovial fluid of RA patients differs from that of healthy people by presenting a large cellular infiltrate consisting mainly of macrophages and T cells, plasma cells, dendritic cells, neutrophils and activated fibroblasts. Extensive work has shown that the interactions of these cell populations within the rheumatoid synovium contribute to disease progression. A number of therapeutic interventions aimed at specific cell lines, such as the lineages of T and B cells, has shown effectiveness in treating the disease (11). The production of cytokines, predominantly pro-inflammatory cytokines, plays a fundamental role in the initiation and perpetuation of chronic inflammation at the synovial membrane. In particular, TNF-alpha plays an important role for both chronic inflammation and to the progressive destruction of bone that characterizes this disease. Moreover, direct, cell-cell, interactions between macrophages, T cells, B cells, fibroblasts and other cell types present in the synovium may contribute to perpetuate the state of chronic inflammation in RA through induction of cytokines, chemokines and perpetuation of the activated state of cells within the joint (12).

In RA, lower plasma levels of HLA-G were reported. However, HLA-G levels in these patients correlated positively with disease severity parameters and the presence of disease-associated epitopes. Our group has previously assessed the influence of two HLA-G 3' untranslated region (3'UTR) polymorphisms in RA susceptibility – the 14 bp polymorphism (rs1704) and the +3142C>G (rs1063320) – observing that a haplotype composed by the 14 pb deletion (D) and the +3142G allele (G) may be involved in the susceptibility to RA in females (Veit, T.D. *et al.*, unpublished data). Concerning RA therapy, methotrexate (MTX) was shown to induce HLA-G expression in vitro and the response to MTX seemed to be

influenced by the HLA-G 14-bp genotype, with the D/D genotype being the most favorable (13). In juvenile idiopathic arthritis (JIA), another form of arthritis that involves children and adolescents under 16 years of age, HLA-G expression has been previously detected in synovial fluid from patients (14). Also, we have observed an increased frequency of the 14 bp D/D genotype in females, suggesting that this genotype might be involved in disease susceptibility (15).

The purposes of this study were to investigate the HLA-G expression in plasma and synovial fluid from RA and JIA patients, focusing the attention to potential correlations between sHLA-G levels and disease activity parameters, as well as the influence of *HLA-G* genetic variants.

Methods

Subjects and sampling

Plasma and/or SF samples were obtained from 70 RA patients (60 women and 10 men) diagnosed according to the American College of Rheumatology's criteria for the classification of Rheumatoid Arthritis (RA) [23]. Patients having another connective tissue disease, other than secondary Sjögren syndrome, unresolved malignancies or acute infections were excluded from the study. Patients were followed at the Rheumatology Outpatient Clinic of the Hospital de Clínicas de Porto Alegre. The disease activity scores involving 28 joint count (DAS28) were assessed during study period. At each visit, clinical assessment consisted of swollen joint counts (SJC) and tender joint counts (TJC) at 28 joints, pain visual analogue scale (VAS), evaluator global assessment (EGA) and patient global assessment (PGA) of disease activity by VAS, health assessment questionnaire (HAQ) (16), C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR).

The patient's disease activity was measured by DAS28 ESR (using erythrocyte sedimentation rate as acute phase marker) (17), DAS28 CRP (using C-reactive protein as acute phase marker) (18) and CDAI (clinical disease activity index) (19),

using the following formulas: DAS28 ESR= (0.56 × TJC1/2) + (0.28 × SJC1/2) + (0.7 × ln(ESR)) + (0.014 × PGA [in mm]), DAS28 CRP= (0.56 × TJC1/2) + (0.28 × SJC1/2) + (0.36 × ln(CRP+1)) + (0.014 × PGA [in mm]) + 0.96 , and CDAI = TJC + SJC + EGA [cm] + PGA [cm]. Patients had their medical records reviewed for further clinical and radiographic data. Clinical data included atlantoaxial subluxation and extra-articular (EA) manifestations (rheumatoid nodules, amyloidosis, vasculitis, pneumonitis and episcleritis). Erosive disease was characterized by the presence of erosions in any of the hands and feet x-rays. From these 70 patients, 15 had collected also synovial fluid. Also, 19 OA patients (14 women and 5 men) and 19 patients undergoing knee surgery due to acute or chronic knee injury (6 women and 13 men) had their SF and plasma collected and were used as control groups.

Twenty four JIA patients (15 girls and 9 boys) diagnosed according to the American College of Rheumatology and redefined according to the International League of Associations for Rheumatology classification (20) were analyzed. The patients were recruited in three Medical Centers from Porto Alegre, and their relatives signed an informed consent. All patients were diagnosed before 16 years of age and presented inflammatory arthritis in, at least, one joint persistent for more than one year at the time of the research. This criterion was used to the exclusion of persistent reactive arthritis. As controls, we collected plasma from 36 children (10 girls and 21 boys) undergoing elective surgery.

Collection of plasma and synovial fluid

Plasma samples were obtained from peripheral blood. Within 2 hours after venipuncture, total blood was centrifuged at 1900g for 7 minutes. Plasma was aseptically collected, aliquoted and stored at -80℃ until analysis.

The synovial fluid samples were collected by arthrocentesis of the knee at the time of medication administration or intra-articular knee surgery. Patients whose synovial fluid had become contaminated with blood were excluded from the study.

Synovial Fluid was transferred to an EDTA vacutainer (BD), spun down at 10000g for 5 min to remove infiltrated cells and stored at -80℃ until analysis.

sHLA-G determinations

The determination of sHLA-G was performed as described previously with minor modifications (21, 22). Three different methods were used to measure sHLA-G in plasma and synovial fluid: two different ELISA assays using and a ILT-2 receptor binding assay using the Luminex® platform. For sHLA-G ELISA the specific capture reagents were the monoclonal antibodies MEM-G/9 and G233 (Exbio, Czech Republic), respectively. Bound molecules were detected by a polyclonal antiserum rabbit anti-human β_2-microglobulin (Dako, Hamburg, Germany) followed by Envision goat antirabbit horseradish peroxidase (Dako, Germany). Plasma and SF samples were diluted 1:2 in PBS. In both assays, purified sHLA-G5 protein served as standard reagent and 3,3′,5,5′-tetramethybenzidine as substrate solution. After stopping the enzyme reaction with 1 M H_2SO_4, the optical density was measured at 450 nm (Biotek Instruments, Winooski, VT). Determination of plasma and synovial fluid sHLA-G levels was performed by three-parameter curve fitting. For the calculation of the ELISA detection limits, a standard curve starting from a concentration from 8 ng/ml was performed in equimolar dilution steps of 5 and 1 ng/ml, respectively. The results obtained were subjected to the software DINTEST (Institut für Rechts-und Verkehrsmedizin, Universitätsklinikum Heidelberg, Germany). According to this procedure, the detection limits of sHLA-G MEM-G/9 and G233 ELISA were 0.390 and 0.937 ng/ml, respectively.

For measurement of ILT2 receptor binding to solubilized HLA-G in plasma, Luminex-x-MAP technology and instruments were used (Luminex). Microspheres (Luminex) with color code 36 were covalently coupled with the G233 mAb (45). Binding of the Abs was performed as recently described (46). G233 coupled microspheres were incubated with plasma diluted 1:4 in Luminex buffer. The bound HLA-G molecules were exposed to recombinant human ILT2 receptor protein fused to the Fc region of human IgG1 (R&D Systems). Thereafter, the bound ILT2 receptor was detected by an anti-human ILT2 mAb (BD Biosciences),

PE conjugated. Measurement of the microspheres was carried out by the Luminex 100 IS System (Luminex). In total, 100 microspheres of each color were analyzed in each sample. The results were presented in geometrical fluorescence intensity. For the determination of HLA-G molecules recognized by the ILT2 receptor, HLA-G5 was used in a concentration ranging from 10–0.625 ng/ml. Luminex buffer was used as a negative control.

Polymerase chain reaction amplification of the +3142C>G and 14bp polymorphism in exon 8 (3'UTR) of the HLA-G gene and genotyping

Patient and control DNA was isolated from peripheral blood using a salting out method (23). The genotyping of the 14bp polymorphism of the HLA-G gene was performed as previously described (24). Briefly, 100 ηg of genomic DNA was amplified in a 25 mL reaction, with final concentrations as follows: polymerase chain reaction (PCR buffer) 1x, dNTP 0.2 mM, MgCl2 1.5mM, Taq DNA polymerase 0.75 U and 10 ρmol of each primer (GE14HLAG - 5' – GTGATGGGCTGTTTAAAGTGTCACC - 3', RGH4 - 5'- GGAAGGAATGCAGTTCAGCATGA - 3'. Thermocycling conditions were as follows: 94ºC for 2 min; 35 cycles of 94ºC for 30 s, 64ºC for 60 s and 72ºC for 60 s; final extension of 72ºC for 10 min. The amplified PCR products were visualized in 6% polyacrylamide gel stained with ethidium bromide, with the amplicon sizes being 224 bp for the 114 bp allele and 210 bp for the 214 bp allele.

The PCR of the +3142C>G polymorphism was performed as described (25): 200 ηg of genomic DNA were added to a final volume of 25 μL, with final concentrations as follows: PCR buffer 1×, 2.0 mM MgCl2; 0.2 mM of each dNTP; 1.0 U of Taq-polymerase and 10 ρmol of each primer (GMIRNAF - 5' – CATGCTGAACTGCATTCCTTCC - 3', GMIRNAR - 5' - CTGGTGGGACAAGGTTCTACTG-3'). Thermocycling conditions were: 94ºC for 5 min; 32 cycles of 94ºC for 30 s, 65.5ºC for 30s and 72ºC for 60s followed by a final extension step at 72ºC for 5 min.

The amplified PCR products were cleaved with 3U of the restriction enzyme BaeGI (New England Biolabs Inc., Ipswich, MA) according to manufacturer's instructions. RFLP products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide, with amplicon sizes of 406bp for the C allele and 316 and 90bp for the G allele.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL) or GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). According to nonparametric distribution of sHLA-G plasma and SF levels, levels of respective groups are presented as medians and differences between groups were assessed using the Mann–Whitney U or Kruskall-Wallis H tests. Speaman's nonparametric correlation coefficients and their p values have been calculated for sHLA-G and parameters of RA and JIA activity.

Results

Patient and control clinical features

The mean RA patient age was 57.4±10.6 years. Mean disease duration time was 12.0±9.4 years. At the time of sampling, 59 patients were taking methotrexate, 39 were under treatment with prednisone, 29 with NSAID (non-steroidal anti-inflammatory drugs), 15 with leflunomide, 6 with anti-malaric drugs (chloroquine diphosphate or hydroxyhloroquine), 4 with sulphasalazine and 3 patients were on biologic therapy with infliximab. Other RA patient and adult control details can be viewed on table 1. The mean age at the JIA group was 8.6±4.2 years, with a mean disease time of 5.3±4.6. Further details are displayed on Table 2.

sHLA-G in clinical patient groups

The median values for sHLA-G in plasma the RA, OA and trauma groups for the MEM-G/9 ELISA were 30.29, 23.10 and 50.05 ng/mL, respectively. In the G233

ELISA those values were lower (9.29, 7.47 and 16.59 ng/mL respectively), but correlated well to the MEM-G/9 assay (r_s=0.946, P<0.001). The ILT-2 assay yielded results comparable to the two ELISA tests (13.32, 0.0 and 15.05 FI units, respectively). In all three assays, no significant differences were observed between RA, OA and trauma groups (Figure 1). The sHLA-G levels in plasma were higher in male RA patients as compared to females according to the MEM-G/9 and ILT2 assays (MEM-G/9: 155.68 vs. 29.27 ng/mL– P=0.039; ILT2: 72.71 vs. 12.39 FI units – P=0.019). The JIA group presented higher medians as compared to the children control group, although not significant (MEM-G/9: 95.16 vs. 41.64; G233: 22.21 vs. 9.31; ILT2: 0.0 vs. 0.0 – Figure 1).

The two ELISA methods were also used to measure HLA-G levels in SF. In the G233 ELISA the RA group presented significantly increased levels as compared to the OA and trauma groups (medians: 13.06, 5.61 and 4.37 ng/mL; P=0.004 – Figure 1). This trend was also seen in the MEM-G/9 ELISA, although not achieving statistical significance (93.44, 56.98 and 36.66 ng/mL respectively; P=0.056). The JIA group presented an even higher median value than the RA group for both ELISA measures (MEM-G/9: 203.77 ng/mL; G233: 14.13ng/mL). SF HLA-G correlated positively with plasma HLA-G by the two measures (MEM-G/9: r_s=0.591, P=0.026; G233: r_s=0.508, P=0.064). No significant correlations were observed between SF and plasma for the two adult control groups (data not shown). In the JIA group, only one of the ELISA methods yielded a positive correlation (G233: r_s=0.673, P=0.023; MEM-G/9: r_s= -0.045, P=0.894).

In order to see whether the higher sHLA-G levels observed in RA were restricted to a subgroup of patients, we stratified the analysis by sex and RF positivity. Interestingly, a significantly higher level of sHLA-G in SF was observed in RF negative (RF-) patients as compared to RF positive (RF+) patients [medians: 273,99 vs. 69,84, P=0.018 (MEM-G/9); 52.87 vs. 9.27, P=0.031 (G233)]. Since no significant differences in plasma HLA-G levels had been observed between RF+ and RF- patients, in order to see if the observed higher levels in SF were a mere reflex of the sampling of RF- patients with higher plasma HLA-G levels, we performed a linear regression model considering the log of synovial HLA-G as a

dependent variable and RF positivity, gender and the log of sHLA-G in plasma as independent variables. According to these models, after adjusting for plasma HLA-G and gender, RF positivity was still significant as a predictor of sHLA-G levels in SF concerning G233 and also presented a trend towards significance concerning MEM-G/9 (P=0.033 and 0.107 – Table 2). Figure 1 shows the plasma and SF levels of HLA-G and HLA-I with the RA group already split into RF+ and RF- patients. Further analyses were performed considering RF+ and RF- as two distinct groups.

sHLA-G levels and RA disease activity parameters

Correlation coefficients and parameters of RA disease activity were calculated (Tables 4a and 4b). Different patterns of correlation were observed among groups stratified by gender and RF positivity. In RF+ females, while no significant association was observed between any of the evaluated and sHLA-G plasma levels measured by ELISA (MEM-G/9 and G233), however, positive correlations between sHLA-G capable of binding ILT-2 and DAS28 CRP, CDAI, number of swollen joints and patient VAS were observed (Table 4a). In RF- females, conversely, DAS28 ESR, DAS28 CPR, CDAI, number of tender joints and physician VAS were positively associated to sHLA-G measured by MEM-G/9, but the same trend was observed for G233. However, no significant correlations were observed between disease parameters and sHLA-G capable of binding ILT-2. Finally, no significant correlations were observed between sHLA-G levels in SF and disease parameters (Table 4a).

In RF+ males, intriguingly, we observed a negative correlation between plasma HLA-G (G233) and DAS28 CRP (r_s=0.943, P=0.005), a trend that was also observed with the other two sHLA-G plasma tests (Table 4b). Other disease activity markers like CDAI, HAQ, number of tender joints and patient VAS also yielded negative correlation values, although not significant. Again, no significant correlations were observed between sHLA-G levels in SF and disease parameters in males, possibly because of the restricted number of male patients.

The sHLA-G levels in JIA patients presented distinct patterns of correlation with disease activity markers according to gender (Table 5). Female JIA patients exhibited a positive correlation of sHLA-G in plasma and ESR (MEM-G/9: r_s=0.648, P=0.043; G233: r_s=0.552, P=0.098), while in male patients presented a (non-significative) negative correlation with ESR. Moreover, male JIA patients presented a negative correlation between sHLA-G plasma levels and plasma CRP (MEM-G/9 and G233: r_s=0.808, P=0.028). Strikingly, this same parameter correlated positively with SF HLA-G (MEM-G/9: r_s=0.894, P=0.041).

HLA-G levels and HLA-G 3'UTR genotype

The median values of HLA-G levels in plasma and synovial fluid according to genotype are given in Table 6. No significant differences were observed among 14bp and +3142 genotypes when stratifying by clinical group.

Discussion

In this work, we assessed sHLA-G levels in plasma and synovial fluid from RA and JIA by different measurement protocols. HLA-G expression has been described as a mechanism of tissue protection against inflammatory aggression and its expression has been described in various inflammatory conditions, including multiple sclerosis, inflammatory bowel disease and JIA (9, 14, 26). Also, polymorphisms at the *HLA-G* gene were implicated in the susceptibility to JIA in females (15) and, recently, we have observed an association between an HLA-G haplotype and susceptibility to RA. Thus, we found it interesting to analyze the expression of this molecule in SF and plasma from RA and JIA patients and its correlation with clinical parameters. To our knowledge, this is the first report of a quantification of sHLA-G in synovial fluid from RA patients.

Here, a significantly increased level of sHLA-G was reported in SF of RA patients. More specifically, RF- patients showed a significantly higher level of sHLA-G in SF. In the RF- patient group, all of the three patients whose SF fluid was collected presented very high sHLA-G levels and a moderate disease activity score (CDAI= 8, 15 and 16). Conversely, the RF+ patients with the three highest sHLA-G levels

in SF presented CDAI scores between 23.2 and 37.3, suggesting that, in this group, other mechanisms may be disrupting the sHLA-G protective effect. Another possibility would be that HLA-G may be acting as an immunoactivator. This is the case in pregnancy, where HLA-G binding KIR2DL4 is thought to induce the release of proinflammatory cytokines and pro-angiogenic factors un uterine NK (uNK) cells (27). Interestingly, it was previously shown that a subpopulation of NK cells, characterized as $CD56_{bright}$, is enriched at the synovial membrane of RA patients (28, 29). This population of NK cells could modulate disease progression in RA by producing cytokines and chemokines, as well as through cell-cell interactions with other immune cells within the joint (11).

Verbruggen and cols have described lower plasma HLA-G levels as compared to healthy controls. In our study, plasma sHLA-G levels in RA patients were comparable to those in OA and adult trauma groups. It is important to point out, however, that in our study neither of the "control" groups (OA and trauma patients) fit properly to the concept of "healthy". The same work reported that sHLA-G in plasma correlated positively with disease severity markers. In our study, this was partially confirmed, mainly in female patients, but the pattern of correlation was different between RF+ and RF- subgroups. In RF+ patients, only the HLA-G measured by ILT-2 assay correlated positively to RA disease parameters, i.e. the amount of sHLA-G which is capable of binding the ILT-2 receptor. It has been previously postulated that HLA-G dimers would be the main responsibles for HLA-G biologic activity, binding ILT-2 and ILT-4 receptors with a ~100-fold affinity as compared to monomers (30). Therefore, one could imply that RA disease parameters are correlating positively with the amount of HLA-G dimers in RF+ patients. On the other hand, this was not observed in RF- female patients. Instead, disease activity parameters correlated positively with the two ELISA measures, mainly MEM-G/9. These tests, however, have the limitations of being capable of detecting only β_2-microglobulin isoforms (G1 and G5) and not distinguishing sHLA-G monomers from dimers. Thus, we cannot rule out that, in spite of a positive correlation observed between sHLA-G and disease parameters, this has little to do with the sHLA-G that is biologically relevant. In RF+ males, we found a negative and significant correlation between plasma sHLA-G (G233) and DAS28 CRP

(r_s=0.943, P=0.005). This trend was also observed in the other plasma measurements and even in the two SF measurements (Table 4.2). This is consistent with a putative protective effect of sHLA-G against inflammatory aggression in these patients, as described in the literature. The correlation coefficients observed between DAS28 CRP, patient VAS and sHLA-G in the ILT-2 assay in males (-0.696 and -0.667), although not significant, are particularly contrasting with those observed in RF+ females (0.334 and 0.340), which showed significant P-values. Again, however, it is necessary, due to the limited number of patients, to interpret these findings with caution.

In JIA, significant differences were observed in the correlations of sHLA-G levels and disease activity parameters between males and females. While in females plasma sHLA-G showed a positive correlation pattern with disease parameters, males showed a negative correlation pattern in plasma, whereas, in synovial fluid, a positive correlation pattern was observed (Table 5) in males, but not in females. Another study has reported lower sHLA-G levels in sera from JIA patients as compared to the sera from healthy controls. Those results are divergent from ours, where plasma sHLA-G levels were slightly higher in JIA patients as compared to control children. There is a chance that such discrepancies may be due to sample handling, as it has been previously shown that sHLA-G measuring in sera samples has proven problematic (31-33).

Due to the exploratory nature of this study, no adjustments for multiple testing using the Bonferroni method were made. In this case, the chance of our results having false-positives (type 1 error) are not negligible and the observed associations between sHLA-G levels and disease activity markers in RA and JIA should remain a hypothesis requiring validation in larger cohorts. Still, the results taken as a whole point to different trends concerning the expression of sHLA-G and its correlation with disease-associated markers which, we think, should be taken into account in future studies.

Concluding, we confirm the expression of sHLA-G in SF from RA patients. Moreover, we observed high levels of sHLA-G in SF of both RA and JIA patients. Distinct patterns of correlation between sHLA-G levels and disease activity

parameters were observed according to body fluid, gender and RF positivity. Such findings encourage further studies with the aim to investigate the role of sHLA-G in the course of these two rheumatic diseases.

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Table 1. RA Patients and adult controls clinical features.

	RA (n=70)	**OA (n=19)**	**Trauma (n=19)**
Males:Females	10:60	5:14	13:6
European-derived (%)	64(91.4)	17 (89.4)	15 (78.9)
Plasma	55	-	-
SF	1	2	3
Plasma and SF	14	17	16
Age± (years)	57.4±10.6	61.8±12.3	35.0±12.4
Disease duration (years)	12.0±9.4		
DAS28 ESR(n)	4.05±1.55 (59)		
DAS28 CRP(n)	3.64±1.28 (57)		
CDAI (n)	15.21±10.54 (58)		
HAQ (n)	1.18±0.76 (56)		
Rheumatoid factor positivity (%)	56 (78.9)		
Bone erosions (%)	61 (87.1)		
Rheumatoid nodules (%)	14 (19.7)		
Amiloidosis (%)	2 (2.8)		
Episcleritis (%)	3 (4.2)		
Sub-luxation (%)	11(15.5)		
Sjogren's syndrome (%)	2 (2.8)		

DAS = Disease Activity Score, CDAI = Clinical Disease Activity Index, HAQ = Health Assessment Questionaire.

Table 2. JIA patients and control children clinical features.

	JIA	**Control Children**
Males:Females	9:15	21:15
Plasma	9	36
SF	4	-
Plasma and SF	11	-
Age (years)	8.6±4.2	6.5±4.4
Disease duration (years)	5.3±4.6	-
JIA subtype		
Oligoarticular	15	-
Polyarticular RF-	7	-
Systemic-onset	2	-

SF = Synovial Fluid; RF = Rheumatoid Factor

Table 3: Linear regression model of sHLA-G levels in SF from RA patients.

SF sHLA-G[a]	**ELISA G233** (P=0.003)		**ELISA MEM-G/9** (P=0.017)	
	Beta	P	Beta	P
RF positivity	-0.443	0.033	-0.363	0.107
sHLA-G plasma[a]	0.520	0.016	0.596	0.017
Sex (male)	-0.331	0.072	-0.216	0.287

SF = Synovial Fluid; RF = Rheumatoid Factor. [a] Log_{10}.

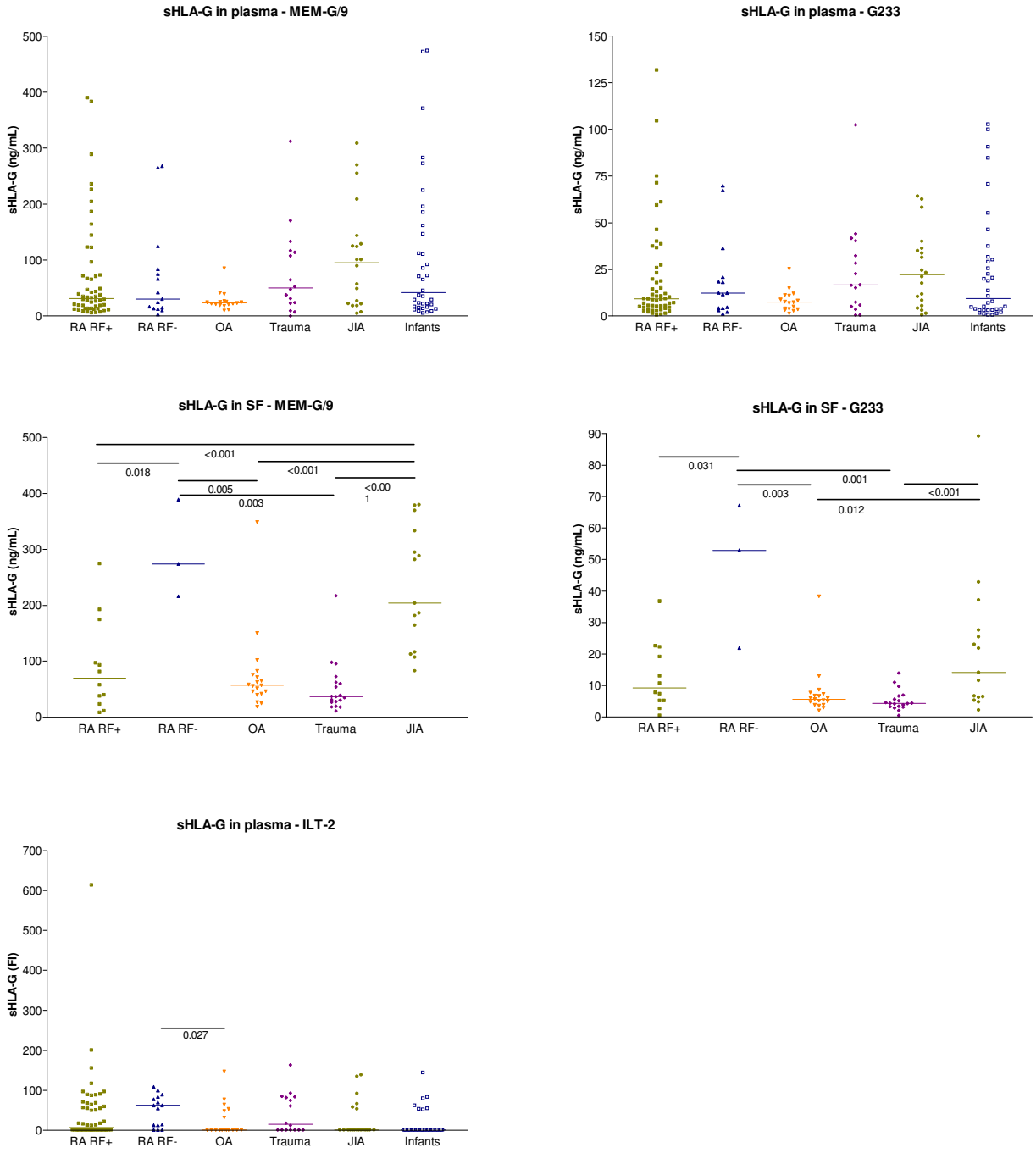

Figure 1: Soluble HLA-G levels in plasma and synovial fluid. RA = Rheumatoid arthritis; RF+ = rheumatoid factor positive; RF- = rheumatoid factor negative; OA = Osteoarthritis; Trauma = adults with knee injury; JIA = Juvenile Idiopathic Arthritis; Infants = control children; FI = fluorescence intensity; SF = synovial fluid.

Table 4.1 – sHLA-G and RA disease activity parameters stratified by RF positivity - females

	sHLA-G in plasma (MEM-G/9)			sHLA-G in plasma (G233)			sHLA-G in plasma (ILT2)[a]			SF sHLA-G (MEM/G-9)			SF sHLA-G (G233)		
Disease parameter	r_s	P	n	r_s	P	n	r_s	P	n	r_s	P	n	r_s	P	n
RF+															
DAS28 ESR	0.037	>0.10	37	*-0.028*	>0.10	37	0.251	>0.10	37	-0.143	>0.10	6	-0.238	>0.10	6
DAS28 CRP	0.029	>0.10	37	*-0.028*	>0.10	37	***0.334***	**0.044**	37	0.190	>0.10	6	0.119	>0.10	6
CDAI	0.072	>0.10	39	*0.013*	>0.10	39	***0.415***	**0.009**	39	0.143	>0.10	6	0.000	>0.10	6
HAQ	0.052	>0.10	39	0.046	>0.10	39	0.293	0.070	39	-0.048	>0.10	7	-0.333	>0.10	7
Tender joints	-0.148	>0.10	41	-0.165	>0.10	41	0.263	0.096	41	-0.643	>0.10	6	-0.500	>0.10	6
Swollen joints	0.128	>0.10	43	0.055	>0.10	43	**0.423**	**0.005**	43	0.378	>0.10	6	0.234	>0.10	6
ESR	0.107	>0.10	40	0.063	>0.10	40	0.050	>0.10	40	0.033	>0.10	7	-0.117	>0.10	7
CRP	0.107	>0.10	41	0.097	>0.10	41	0.209	>0.10	41	0.500	>0.10	7	0.633	0.067	7
Patient VAS	-0.008	>0.10	39	-0.062	>0.10	39	**0.340**	**0.034**	39	0.357	>0.10	6	0.464	>0.10	6
Phisician VAS	0.028	>0.10	39	*-0.037*	>0.10	39	0.151	>0.10	39	0.162	>0.10	6	-0.180	>0.10	6
RF-															
DAS28 ESR	**0.734**	**0.007**	12	**0.664**	**0.018**	12	0.243	>0.10	12	-	-	1	-	-	1
DAS28 CRP	**0.709**	**0.015**	11	0.600	0.051	11	*-0.041*	>0.10	11	-	-	1	-	-	1
CDAI	**0.673**	**0.023**	11	0.573	0.066	11	*0.023*	>0.10	11	1.000	-	2	1.000	-	2
HAQ	0.073	>0.10	11	0.078	>0.10	11	-0.481	>0.10	11	-1.000	-	2	-1.000	-	2
Tender joints	**0.598**	**0.040**	12	0.489	>0.10	12	0.117	>0.10	12	1.000	-	2	1.000	-	2
Swollen joints	0.400	>0.10	12	0.341	>0.10	12	0.081	>0.10	12	-1.000	-	2	-1.000	-	2
ESR	0.502	>0.10	11	0.479	>0.10	11	0.282	>0.10	11	-	-	1	-	-	1
CRP	0.114	>0.10	11	0.040	>0.10	11	-0.148	>0.10	11	-	-	1	-	-	1
Patient VAS	0.141	>0.10	11	0.150	>0.10	11	-0.452	>0.10	11	-1.000	-	2	-1.000	-	2
Phisician VAS	**0.601**	**0.050**	11	0.483	>0.10	11	-0.194	>0.10	11	1.000	-	2	1.000	-	2

DAS = Disease Activity Score, CDAI = Clinical Disease Activity Index, HAQ = Health Assessment Questionaire, ESR = Erythrocyte Sedimentation Rate, CRP = C-Reactive Protein, VAS = Visual Assessment of Pain. r_s= Spearman's correlation; n = number of measurements; P = probability of significant correlation between the indicated parameters.

Table 4.2 – sHLA-G and RA disease activity parameters stratified by RF positivity - males

	sHLA-G in plasma (MEM-G/9)			sHLA-G in plasma (G233)			sHLA-G in plasma (ILT-2)			sHLA-G in SF (MEM/G-9)			sHLA-G in SF (G233)		
Disease parameter	r_s	P	n	r_s	P	n	r_s	P	n	r	P	n	r	P	n
RF+															
DAS28 ESR	0.371	>0.10	6	0.029	>0.10	6	0.029	>0.10	6	1.000	-	2	1.000	-	2
DAS28 CRP	-0.771	0.072	6	**-0.943**	**0.005**	6	-0.696	>0.10	6	-1.000	-	2	-1.000	-	2
CDAI	-0.500	>0.10	5	-0.500	>0.10	5	0.154	>0.10	5	-0.500	>0.10	3	0.500	-	3
HAQ	-0.400	>0.10	4	-0.400	>0.10	4	-0.400	>0.10	4	-0.500	>0.10	3	-1.000	-	3
Tender joints	-0.564	>0.10	7	-0.636	>0.10	7	0.018	>0.10	7	-0.500	>0.10	3	0.500	>0.10	3
Swollen joints	0.000	>0.10	7	-0.185	>0.10	7	0.224	>0.10	7	-0.500	>0.10	3	0.500	>0.10	3
ESR	0.739	0.058	7	0.631	>0.10	7	0.209	>0.10	7	1.000	-	2	1.000	-	2
CRP	0.334	>0.10	7	0.000	>0.10	7	-0.206	>0.10	7	1.000	-	2	1.000	-	2
Patient VAS	-0.600	>0.10	5	-0.600	>0.10	5	-0.667	>0.10	5	-0.500	>0.10	3	-1.000	-	3
Phisician VAS	-0.200	>0.10	5	-0.200	>0.10	5	0.051	>0.10	5	0.500	>0.10	3	1.000	-	3
RF-															
DAS28 ESR	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1
DAS28 CRP	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1
CDAI	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1
HAQ	-	-	1	-	-	1	-	-	1	-	-	0	-	-	0
Tender joints	-	-	2	-	-	2	-	-	2	-	-	1	-	-	1
Swollen joints	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1
ESR	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1
CRP	-1.000	-	2	-1.000	-	2	-1.000	-	2	-	-	1	-	-	1
Patient VAS	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1
Phisician VAS	1.000	-	2	1.000	-	2	1.000	-	2	-	-	1	-	-	1

DAS = Disease Activity Score, CDAI = Clinical Disease Activity Index, HAQ = Health Assessment Questionaire, ESR = Erythrocyte Sedimentation Rate, CRP = C-Reactive Protein, VAS = Visual Assessment of Pain. r_s= Spearman's correlation; n = number of measurements; P = probability of significant correlation between the indicated parameters.

Table 5: HLA-G, HLA-I and JIA disease severity parameters.

	Plasma sHLA-G (MEM-G/9)			Plasma sHLA-G (G233)			Plasma sHLA-G (ILT2)[a]			SF sHLA-G (MEM/G-9)			SF sHLA-G (G233)		
Disease parameter	r_s	P	n	r_s	P	n	r_s	P	n	r_s	P	n	r_s	P	n
Females															
Tender joints	0.775	>0.10	4	*0.775*	>0.10	4	0.816	>0.10	4	-	-	1	-	-	1
Swollen joints	0.276	>0.10	9	*0.276*	>0.10	9	0.134	>0.10	9	-0.247	>0.10	8	*-0.247*	>0.10	8
ESR	**0.648**	**0.043**	10	0.552	0.098	10	0.425	>0.10	10	0.132	>0.10	8	0.431	>0.10	8
CRP	0.248	>0.10	9	0.009	>0.10	9	0.302	>0.10	9	0.062	>0.10	8	0.062	>0.10	8
Males															
Tender joints	-1,000	-	2	-1,000	-	2	-1,000	-	2	-	-	1	-	-	1
Swollen joints	0.000	>0.10	7	0.000	>0.10	7	-0.509	>0.10	7	0.775	>0.10	4	-0.775	>0.10	4
ESR	-0.429	>0.10	7	-0.429	>0.10	7	-0.579	>0.10	7	0.600	>0.10	5	-0.300	>0.10	5
CRP	**-0.808**	**0.028**	7	**-0.808**	**0.028**	7	-0.147	>0.10	7	**0.894**	**0.041**	5	0.224	>0.10	5

ESR = Erythrocyte Sedimentation Rate, CRP = C-Reactive Protein; r_s= Spearman's correlation; n = number of measurements; P = probability os significant correlation between the indicated parameters.

Table 6: Median (n) HLA-G levels according to genotype.

		Plasma			Synovial fluid	
14bp (rs1704)		ELISA MEM-G/9	ELISA G233	Luminex ILT2	ELISA MEM-G/9	ELISA G233
DD	RA RF+	32.98(18)	10.67(18)	0.0 (18)	58.05 (1)	19.21(1)
	RA RF-	12.79(4)	4.13(4)	6.69(4)	-	-
	OA	21.60(5)	9.87(5)	0.0 (5)	40.45(5)	5.39(5)
	Trauma	35.24(4)	10.12(4)	0.0(4)	28.03(4)	4.13(4)
	JIA	24.46(6)	9.52(6)	0.0(6)	237.62(6)	6.50(6)
	C. Children	45.73(17)	10.76(17)	0.0(17)	-	-
DI	RA RF+	32.35(30)	9.19(30)	12.54(30)	40.03(9)	7.78(9)
	RA RF-	83.73(7)	20.77(7)	62.07 (7)	273.99 (3)	52.87(3)
	OA	23.50(8)	3.92(8)	0.0 (8)	65.26(8)	7.29(8)
	Trauma	85.91(8)	25.50(8)	36.45(8)	37.04(10)	4.46(10)
	JIA	124.93(11)	24.57(11)	0.0(11)	192.90(8)	18.59(8)
	C. Children	33.27(18)	6.00(18)	0.0(18)	-	-
II	RA RF+	16.12(6)	5.40(6)	34.10(6)	143.07(2)	14.78(2)
	RA RF-	30.30(3)	11.61(3)	62.07(3)	-	-
	OA	26.35(3)	6.95(3)	48.30(3)	51.51(3)	4.97(3)
	Trauma	30.83(4)	10.12(4)	83.97(4)	54.06(5)	4.24(5)
	JIA	22.43(1)	4.15(1)	0.0(1)	-	-
	C. Children	110.43(1)	20.53(1)	0.0(1)	-	-
+3142 (rs1063320)						
CC	RA RF+	32.98(14)	10.67(14)	0.0(14)	58.05(1)	19.21(1)
	RA RF-	13.28(3)	4.15(3)	0.0(3)	-	-
	OA	21.60(5)	9.87(5)	0.0(5)	40.46(5)	5.39(5)
	Trauma	22.67(3)	5.06(3)	0.0(3)	36.67(3)	4.44(3)
	JIA	48.81(3)	11.69(3)	0.0(3)	248.81(4)	16.08(4)
	C. Children	102.01(10)	27.27(10)	0.0(10)		
CG	RA RF+	32.35(24)	9.20(24)	13.17(24)	97.31(5)	13.06(5)
	RA RF-	99.53(6)	27.23(6)	50.52(6)	244.98(2)	37.40(2)
	OA	21.83(7)	3.84(7)	0.0(7)	62.99(7)	6.19(7)
	Trauma	112.05(6)	37.03(6)	36.44(6)	37.04(8)	4.46(8)
	JIA	89.68(13)	21.17(13)	0.0(13)	288.84(7)	23.06(4)
	C. Children	24.94(20)	5.04(20)	0.0(20)	-	-
GG	RA RF+	24.18(16)	11.61(16)	54.56(16)	93.44(6)	7.79(6)
	RA RF-	41.74(5)	12.37(5)	62.07(5)	388.72(1)	67.10(1)
	OA	25.80(4)	7.86(4)	50.80(4)	65.26(5)	6.78(5)
	Trauma	52.29(7)	16.71(7)	83.35(7)	42.62(8)	4.20(8)
	JIA	63.91(2)	16.92(2)	0.0(2)	182.03(3)	6.29(3)
	C. Children	90.77(6)	20.24(6)	0.0(6)	-	-

CAPÍTULO 7 – Discussão Geral

Discussão Geral

Nos capítulos que se sucederam, foram apresentados os resultados da análise da influência do gene e molécula HLA-G na suscetibilidade e curso de doenças reumatológicas, duas delas comprovadamente de caráter auto-imune – artrite reumatóide e lúpus eritematoso sistêmico, e uma doença reumatológica pediátrica, a artrite idiopática juvenil. A seguir, serão retomados alguns pontos-chave dos resultados obtidos durante a pesquisa a fim de integrar o conhecimento obtido durante o período de estudo.

Influência da região 3'-não traduzida (3'UTR) e dos microRNAs na expressão do gene HLA-G

Desde o começo do trabalho, em 2007, até o final, muitos avanços no conhecimento sobre a molécula HLA-G foram feitos, principalmente no que diz respeito ao estudo de variantes polimórficas na região reguladora do gene. Até 2007, o polimorfismo de 14 pb (rs1704) era quase que exclusivamente o único polimorfismo da 3'UTR a ser abordado em estudos de associação, graças, principalmente, ao trabalho de Rousseau *et al.* (Rousseau, 2003), que demonstraram que os transcritos apresentando a seqüência de 14 pb (inserção) poderiam passar por uma etapa adicional de splicing que retira 92 bases da região em que a seqüência está contida. Essa deleção influenciaria a estabilidade do mRNA, já que os transcritos de HLA-G sem as 92 bases mostravam-se mais estáveis do que os transcritos "completos" em células HLA-G positivas JEG-3 e células M8 transfectadas com o alelo G*010102, após tratamento actinomicina. No entanto, vários estudos subseqüentes haviam repetidamente relatado a associação do alelo ins e baixos níveis de sHLA-G e até mesmo a ausência de quantidades detectáveis sHLA-G no plasma de homozigotos para o alelo ins (Hviid *et al.*, 2003; Hviid *et al.*, 2004; Rizzo *et al.*, 2008; Rizzo *et al.*, 2005; Rizzo *et al.*, 2006). Essas observações paradoxais inquietavam-nos, sendo que várias

vezes tivemos problemas para explicar a alunos de iniciação científica, e mesmo a colegas de doutorado e professores, sobre o que na época chamamos de “o paradoxo do (polimorfismo de) 14 pb”. Em junho de 2008, durante o IV curso da RELAGH (Rede Latino-Americana de Genética Humana), fui introduzido ao conceito de microRNAs na palestra da professora Dra. Iscia Lopes-Cendes, o que teve um papel decisivo no direcionamento desse trabalho de doutorado. No mesmo dia, procurei trabalhos na sobre microRNAs e HLA-G no sítio *Pubmed*, encontrando o trabalho de Tan *et al.* (Tan *et al*., 2007), que descrevia a ligação alelo-específica de microRNAs à região contendo o polimorfismo +3142C>G (rs1063320) e a sua ligação com a suscetibilidade à asma. A partir disso, foi produzida a primeira publicação da tese, em que hipotetizamos que a resposta para o paradoxo poderia estar em outro polimorfismo em desequilíbrio de ligação (DL) com a inserção/deleção de 14 bp. O polimorfismo +3142C>G pareceu ser um bom candidato para explicar o paradoxo do 14 bp, pois situa-se a cerca de 200 pb a jusante da região de 14 pb e apresenta DL parcial com o polimorfismo 14bp (Veit & Chies 2009 – Capítulo 3). Uma hipótese que nos passou despercebida na época, provavelmente pelo direcionamento induzido pelo trabalho de Tan *et al.,* era de que a própria região contendo a inserção/deleção de 14 pb pudesse explicar o paradoxo do 14 pb, sendo ela própria uma região de ligação de microRNAs. Alguns meses mais tarde, Castelli *et al.* publicaram uma análise *in silico* da região 3’ do gene que identificou vários sítios potenciais de ligação de microRNAs (Figura 3) e, não surpreendentemente, descreveu a região de inserção/deleção de 14 pb como uma dessas potenciais regiões, o que nos deixou um pouco frustrados por não termos pensado nessa possibilidade na época. Ainda assim, o artigo de hipótese teve relativamente boa aceitação e, mais importante, direcionou o trabalho de doutorado no sentido de investigarmos a existência de associação do polimorfismo +3142C>G em diferentes grupos de pacientes. O primeiro trabalho publicado por nosso grupo a analisar o papel desse polimorfismo foi em pacientes com anemia falciforme (Cordero *et al*., 2009), em que o alelo C mostrou-se um fator de proteção dose-dependente contra a infecção pelo vírus da hepatite-C (HCV). Esse alelo confere uma menor estabilidade na ligação dos microRNAs hsa-miR-148a, 148b e 152 em

comparação ao alelo G (em DL parcial com o alelo de inserção de 14 pb), resultando em um escape do controle pós-transcricional promovido por esses microRNAs e, consequentemente, em uma maior expressão de HLA-G. Como já foi previamente discutido no capítulo 6, a molécula HLA-G está envolvida não apenas na inibição da resposta imune, mas também na ativação de certas células do sistema imune em determinadas situações. Em certas sub-populações de células NK, a interação de HLA-G com o receptor KIR2DL4 é capaz de promover a liberação de citocinas pró-inflamatórias e fatores pró-angiogênicos em células NK uterinas (uNK) (Rajagopalan *et al.* 2006). A partir disso, não seria de se estranhar que a expressão de HLA-G pudesse vir a desempenhar um papel importante no controle da infecção por HCV nesses pacientes através da ativação de células NK. Interessantemente, um sítio de ligação semelhante foi descrito na 3'UTR do gene *HLA-C*, um gene cuja molécula é conhecida por interagir mais extensamente com receptores de células NK. Hipotetizou-se que um polimorfismo de deleção de um nucleotídeo dentro dessa região resultaria em uma maior expressão de HLA-C e que isso estaria ligado a um melhor controle da infecção por HIV em pacientes soropositivos (Kulkarni *et al.*, 2011). Sem dúvida, esse estudo dá uma sustentação ainda maior aos nossos resultados em anemia falciforme e encoraja a pesquisa de novas associações entre polimorfismos no gene *HLA-G* e infecções virais.

Influência de variantes polimórficas da região 3'-não traduzida (3'UTR) do gene HLA-G na suscetibilidade e curso de doenças auto-imunes.

Apesar do interessante resultado observado em anemia falciforme, o foco principal do trabalho sempre foi as doenças reumatológicas. Em lúpus eritematoso sistêmico (LES), havíamos observado um excesso de heterozigotos para o polimorfismo de 14 pb em mulheres caucasóides (Veit *et al.*, 2009 - Anexo 2). Já na época da produção do artigo de hipótese, antevíamos a possibilidade de que a análise do SNP em +3142 pudesse clarificar nossas observações prévias com o 14 pb, indicando-nos um alelo de risco. Essa possibilidade foi confirmada

através da associação do haplótipo D/G com a suscetibilidade ao lúpus em mulheres. Também pudemos observar menores níveis médios de atividade da doença em pacientes duplo-heterozigotos. Interessantemente, essa observação é coerente com os dados da literatura que sugerem uma seleção balanceadora atuando no gene HLA-G (Castelli *et al.*, 2011; Tan *et al.*, 2005). Estudos com o objetivo de investigar a influência do HLA-G na susceptibilidade e fisiopatologia do LES estão ainda longe de chegar a um consenso. Um estudo recente relatou que os monócitos e células dendríticas a derivadas de pacientes com LES apresentaram uma produção reduzida de HLA-G e uma indução reduzida de HLA-G sob estimulação com IL-10 (Monsivais-Urenda *et al.*, 2011), sugerindo que a expressão (reduzida) de HLA-G pode desempenhar um papel no curso da doença. Dois estudos com amostras da Espanha e China têm relatado níveis

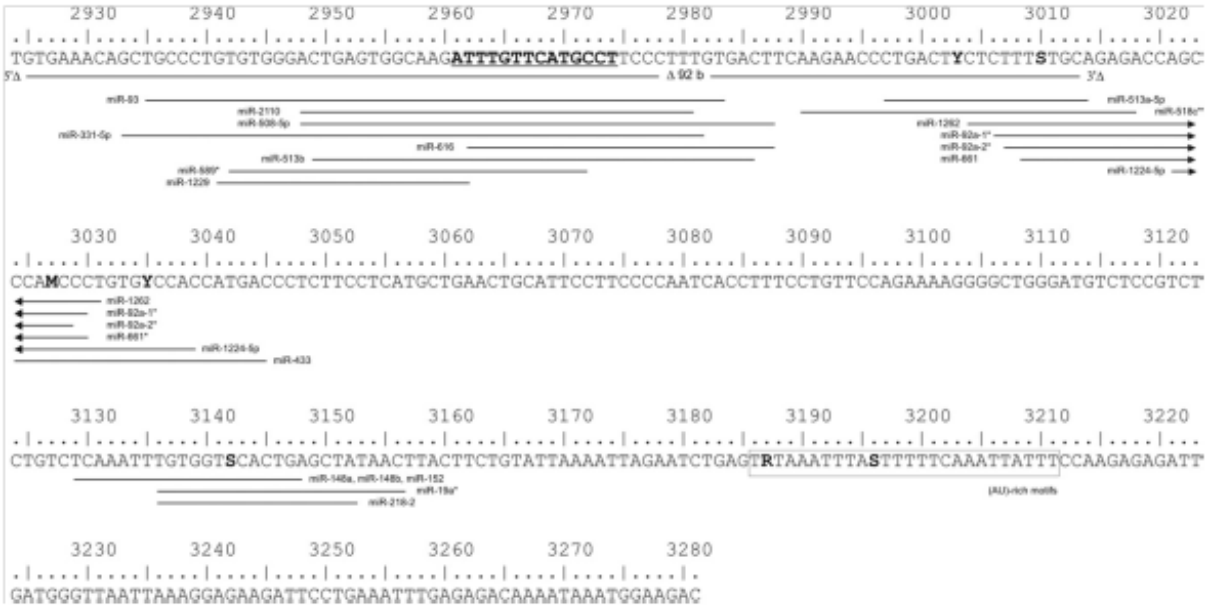

Figura 3. Mapa da região 3' não traduzida e regiões potenciais de ligação de microRNAs. (Retirado de Castelli *et al.*, 2009)

plasmáticos mais elevados em HLA-G em pacientes com LES (Rosado *et al.*, 2008; Wu *et al.*, 2009), enquanto outro estudo com pacientes italianos e dinamarqueses relatou níveis circulantes mais baixos da molécula (Rizzo *et al.*,

2008). Neste último estudo, esses níveis mais baixos foram associados a uma maior freqüência do alelo de inserção de 14pb. Esses resultados, como comentado no artigo que compõe o capítulo 4, não foram replicados em nossos pacientes.

Interessantemente, o mesmo haplótipo D/G encontrou-se associado à suscetibilidade à AR, outra doença de caráter auto-imune (Capítulo 5). Nesse estudo, além de uma amostra do nosso laboratório, contamos com uma segunda amostra de pacientes e controles da cidade de Belém do Pará. O fato de a maior frequência de portadores do alelo D/G em pacientes ter sido replicada nas duas amostras adiciona uma credibilidade adicional ao achado científico e encoraja pesquisas futuras no sentido de esclarecer o papel da molécula HLA-G no processo patogênico dessas doenças auto-imunes. Os dois polimorfismos que compõem o que chamamos de "haplótipo D/G" nesse trabalho são marcadores de um haplótipo estendido descrito muito recentemente por Castelli *et al.*, denominado por esse grupo de HG0104, que engloba um grupo muito específico de alelos com praticamente a mesma região promotora – G*010401, G*010403, G*010404 e G*010405 – e cuja 3'UTR é também encontrada em outros primatas (Castelli *et al.*, 2011). O alelo mais prevalente associado a esse haplótipo foi previamente descrito como de alta expressão em plasma (Rebmann *et al.*, 2001). No entanto, não se pode fazer uma generalização de tal observação e inferir-se que um excesso na expressão de HLA-G em determinadas condições possa contribuir para a manifestação do processo auto-imune pelo fato do perfil de expressão de microRNAs poder variar enormemente de tecido para tecido e entre situações fisiológicas. Isso permite que mesmo o oposto seja verdadeiro, isto é, um déficit na expressão de HLA-G devido à presença desse alelo sob determinado perfil de expressão de microRNAs e outras moléculas reguladoras. Finalmente, embora tenhamos nos concentrado em analisar os polimorfismos da região 3', não se pode, de forma alguma, desprezar o papel da região promotora do gene na regulação da expressão do HLA-G. É muito possível que polimorfismos do promotor que compõem o haplótipo HG0104 estejam diretamente envolvidos na expressão diferencial da molécula HLA-G de forma a

tornar esse haplótipo um fator de suscetibilidade às doenças auto-imunes aqui estudadas.

Desde o primeiro artigo, publicado em 2008, em pacientes com AIJ (Veit *et al.*, 2008 - Anexo 3), até o presente, um padrão curioso tem se apresentado com respeito à influência do gene *HLA-G* em doenças reumatológicas: as associações observadas foram apenas em pacientes do sexo feminino. Pode-se argumentar que, pelo fato de os pacientes desse grupo de doenças apresentarem mais representantes do sexo feminino, obter-se-ia um maior poder de análise para esse grupo de pacientes. De fato, todas as nossas coortes de pacientes apresentavam mais mulheres do que homens, em proporções variando de 2:1 (AIJ) até 8:1 (LES). O que chama a atenção em todos os trabalhos é que os pacientes homens simplesmente não apresentavam uma tendência a acompanhar os resultados observados em mulheres. Ao contrário, quase sempre representaram um fator de confusão nas análises, quando não estratificadas por sexo. Todas essas observações apontam para uma interação sexo-específica entre as variantes polimórficas do gene HLA-G e a suscetibilidade a esse grupo de doenças, o que nos leva a acreditar fortemente na influência tanto direta quanto indireta de hormônios na expressão de HLA-G. Diretamente, através da ligação de elementos de resposta a hormônios na região promotora do gene, como o sítio de ligação a progesterona já descrito na literatura (Yie *et al.*, 2006), ou através da indução de microRNAs mediada por hormônios, que tenham como alvo a 3'UTR do gene *HLA-G*.

Para concluir, os resultados observados nesse trabalho apontam fortemente para a influência genética de variantes alélicas do gene HLA-G na suscetibilidade a doenças inflamatórias e auto-imunes. Não se pode, no entanto, esquecer que, por tratar-se de um gene localizado no complexo principal de histocompatibilidade (MHC), existe a possibilidade de desequilíbrio de ligação com alelos já consagrados como fatores de suscetibilidade a essas doenças. Faz-se premente, portanto, para um maior respaldo de nossos resultados, que seja levada a cabo a genotipagem dos alelos HLA-DRB1 que possuem o epitopo compartilhado (DR1, DR4 e D10), no caso da AR, e, no caso do LES, os alelos

DR2, DR3 e os genes do complemento, localizados na região de classe III do MHC, entre outros, a fim de controlar nossos resultados para a presença desses alelos de risco. Não obstante, acreditamos que a interação de fatores hormonais inerentes ao sexo, associados a situações de estresse que funcionem como gatilho para processos auto-imunes (como infecções agudas, transfusões de sangue, estresse psicológico, etc.) possam, em algum momento, interagir com as variantes polimórficas do gene *HLA-G* implicadas na suscetibilidade a essas doenças, levando a um desequilíbrio da homeostase imunológica e ao início do processo auto-imune. Esse desequilíbrio pode ser devido tanto a um déficit quanto a um excesso da produção de HLA-G em determinado contexto imunológico. Futuros trabalhos terão a missão de elucidar a ligação entre o haplótipo implicado na suscetibilidade à AR e ao LES e os mecanismos de patogênese dessas doenças.

Expressão da molécula HLA-G no plasma e líquido sinovial de pacientes com artrite

No capítulo 6 deste trabalho, descrevemos pela primeira vez a presença de HLA-G solúvel no líquido sinovial de pacientes com AR e, pela segunda vez, sua presença em pacientes com AIJ. Nesse estudo, usamos três métodos diferentes para dosar HLA-G: os dois protocolos de ELISA usando os anticorpos de captura MEM-G/9 e G233 e o método de ligação ao receptor ILT2 na plataforma Luminex, sendo todos os experimentos realizados na Universitätsklinikum Essen, Institut für Tranzfusionsmedizin, em Essen, na Alemanha. Esse último método, desenvolvido pelo laboratório da Prof[a]. Vera Rebmann, distingue-se dos métodos de ELISA por fornecer uma estimativa da proporção de HLA-G capaz de ligar-se ao receptor endógeno, ILT2, o que é muito vantajoso para a análise dada a diversidade de isoformas da molécula e a grande diferença na afinidade de monômeros e dímeros de HLA-G, estes últimos tidos como os principais responsáveis por sua atividade biológica. Por esse método, observamos maiores níveis de HLA-G no plasma de pacientes com AR FR- em comparação a pacientes com OA, e uma maior expressão de HLA-G em pacientes com AR FR- do sexo masculino em relação a pacientes com lesões no joelho do mesmo sexo. Como o foco original do estudo estava voltado para a expressão de HLA-G no líquido sinovial, não foi coletado plasma de indivíduos saudáveis. Assim, não pudemos confirmar as observações de Verbruggen *et al.*, que observaram menores níveis de sHLA-G no plasma de pacientes com AR em comparação com controles saudáveis. Podemos, contudo, afirmar que os pacientes FR+ apresentaram níveis comparáveis aos outros grupos de pacientes e ao grupo de crianças controles.

Diferentes padrões de correlação entre os níveis de HLA-G no plasma e parâmetros de atividade de doença em pacientes com AR e AIJ foram observados em nosso estudo. Em pacientes com AR, a diferença mais evidente se dá entre pacientes FR+ e FR- do sexo feminino, os primeiros apresentando correlações positivas com os níveis de HLA-G medidos por ILT2, e o segundo grupo, com os níveis medidos por ELISA, o que levanta a possibilidade de que haja uma produção diferencial de HLA-G entre os dois subgrupos, tanto no que diz respeito

à produção de formas diméricas quanto à expressão de isoformas incompletas da molécula (G2, G3, G4, G6, G7). Entre homens e mulheres FR+, algumas discrepâncias podem ser notadas, como a correlação negativa entre parâmetros de atividade e os níveis de sHLA-G medidos por ELISA e o não acompanhamento, por parte dos homens, das correlações positivas observadas nas mulheres quando medidas pelo ensaio de Luminex com ILT2. Tais observações, infelizmente, carecem de um maior número de amostras a fim de nos fornecerem correlações mais confiáveis e necessitarão de outros estudos que validem esses resultados.

Perspectivas futuras

Os resultados apresentados nesta tese levantam muitas questões a serem investigadas. A confirmação da presença de HLA-G solúvel no líquido sinovial de pacientes com AR levanta as seguintes perguntas a serem respondidas: “qual o papel desempenhado pela molécula HLA-G nesse contexto?”, “o papel que desempenha é o mesmo nos diversos subtipos, ou difere, de acordo com o subtipo, ou mesmo com outros fatores, como sexo?”. Se observamos a associação de um haplótipo D/G (ou, extrapolando, HG0401) com duas doenças auto-imunes diferentes (AR e LES), podemos agora perguntar-nos “de que maneira o haplótipo associado influencia a suscetibilidade a essas doenças?”, ou “Esse haplótipo estaria envolvido em outras doenças auto-imunes?”, ou mesmo “A associação encontrada não seria fruto de um desequilíbrio de ligação entre esse haplótipo e outros genes envolvidos na suscetibilidade a essas doenças?”, entre várias perguntas pertinentes que poderiam ser formuladas a partir de nossas observações. Muitas delas não possuirão resposta imediata devido à grande complexidade associada às suas respostas.

Talvez um dos melhores exemplos dessa complexidade seja dada pela diversidade de microRNAs capazes de se ligarem à 3’UTR do gene *HLA-G*. Após a primeira análise em profundidade da região 3', tornou-se evidente que o efeito dos microRNAs na expressão HLA-G será muito difícil de analisar, já que o perfil

de microRNA pode variar substancialmente entre tecidos e situações fisiológicas, diferentemente de outros marcadores fisiológicos, como os auto-anticorpos, que podem estar presentes vários anos antes dos primeiros sintomas dessas doenças (Aho *et al.*, 1991; Arbuckle *et al.*, 2003).

Uma das perguntas que pode e deve ser elucidada refere-se à existência de desequilíbrio de ligação entre o haplótipo de risco descrito nesse trabalho D/G e seu haplótipo estendido associado (HG0401) com alelos de risco já estabelecidos na literatura presentes na região do MHC. Referimo-nos aqui aos alelos DRB1 do epitopo compartilhado (DR1, DR4, DR10) no caso da AR , e de DR2, DR3 e alelos do sistema complemento associados ao LES. Existem trabalhos mostrando que existe um desequilíbrio não desprezível entre alelos de HLA-G e de HLA-A e entre os primeiros e alelos de HLA de classe II, como os DRB1 (Hviid & Christiansen, 2005; Morales *et al.*, 1993; Ober *et al.*, 1996).

Agora que confirmamos a existência de HLA-G na sinóvia de artrite reumatóide, é possível perguntarmos: qual o papel desse HLA-G solúvel no curso da AR (e da AIJ)? Se, hipoteticamente, depletássemos o HLA-G desse compartimento, o que observaríamos: uma melhora ou uma piora do processo inflamatório? A corrente dominante na literatura tem descrito o HLA-G como uma molécula com um papel imunorregulador da molécula HLA-G através de diversos mecanismos já descritos, como a inibição da proliferação e atividade citotóxica de células T e NK, indução de células T supressoras, inibição da apresentação de antígenos e da maturação de células dendríticas, indução de células apresentadoras de antígenos tolerogênicas, indução do aumento do número de receptores inibitórios, entre outros mecanismos (revisado em Veit *et al.* 2010, Capítulo 2). Grande parte desses efeitos é mediada pela interação entre HLA-G e receptores inibitórios como ILT-4 (CD85d) e ILT-2 (CD85j). Por outro lado, a molécula HLA-G pode se comportar também como uma molécula ativadora, através da interação com o receptor KIR2DL4, expresso em células NK. Especificamente, foi mostrado que dímeros de HLA-G são capazes de induzir a produção de citocinas pró-inflamatórias como IL-6, IL-8 e TNF-α em células NK uterinas (Li *et al.*, 2009). Em outro estudo, Rajagopalan *et al.* observaram que a

sinalização mediada por HLA-G via KIR2DL4 parece ocorrer via internalização do complexo ligante/receptor em endossomos, resultando na secreção de fatores pró-inflamatórios e pró-angiogênicos. A sinalização, nesse caso, seria mediada por HLA-G solúvel, que pode ser secretado ou clivado da membrana de células HLA-G positivas (Rajagopalan *et al.*, 2006).

Uma quantidade significativa de células NK foi previamente descrita na articulação de pacientes com AR (Dalbeth & Callan, 2002; Pridgeon *et al.*, 2003). As células *Natural Killer* (NK) são linfócitos do sistema imune inato com um papel importante na eliminação de tumores e células infectadas por vírus. Além do seu papel na imunidade inata, células NK também podem modular a resposta imune adaptativa por meio de diversos mecanismos, incluindo a produção de citocinas, sinalização célula-célula e eliminação direta de outras células do sistema imune. Células NK são células grandes, granulares e de curta duração que representam de 10-15% dos linfócitos circulantes e 5% dos linfócitos no tecido linfóide (Orange & Ballas, 2006). As células NK humanas são fenotipicamente caracterizadas pela expressão de CD56 e pela ausência de CD3, o antígeno associado ao receptor de células T (TCR) em linfócitos T. Funcionalmente, o próprio CD56 permite reconhecer dois subconjuntos diferentes de células NK, $CD56^{bright}$ (alta densidade) e $CD56^{dim}$ (baixa densidade): em torno de 90% das células NK do sangue periférico (PB) e baço são $CD56^{dim}CD16^{+}$ e mostram forte atividade citotóxica. Em contrapartida, a maioria das células NK nos gânglios linfáticos e as tonsilas são $CD56^{bright}CD16^{-}$ e possuem baixa atividade citotóxica, porém são capazes de expressar diversas citocinas como TNF-α e IFN-γ em resposta a diferentes estímulos (Orange & Ballas, 2006).

As células NK constituem o grupo de células do sistema imune menos estudado em artrite reumatóide, uma doença primariamente caracterizada como uma doença do sistema imune adaptativo. Entretanto, diferentes estudos apontam para um potencial papel destas células em doenças auto-imunes. Interessantemente, foi previamente mostrado que uma sub-população de células NK, caracterizada como $CD56^{bright}$, encontra-se enriquecida na membrana sinovial de pacientes com AR (Dalbeth & Callan, 2002; Pridgeon *et al.*, 2003). Esta

população de células NK pode modular a progressão da doença na AR pela produção de citocinas e quimiocinas, bem como através de interações célula-célula com outras células do sistema imune dentro da articulação. Foi previamente observado que as células NK sinoviais são postas em co-cultura com monócitos *in vitro*, elas induzem a sua diferenciação em osteoclastos. A depleção de células NK de camundongos antes da indução da artrite induzida por colágeno reduz a gravidade da artrite subseqüentes e quase completamente evita a erosão óssea (Soderstrom *et al.*, 2010).

Hipotetizamos que o HLA-G solúvel produzido por diferentes tipos celulares presentes na sinóvia inflamada, ao interagir com receptores em células NK, particularmente com o receptor KIR2DL4, pode levar essas células a secretar fatores pró-inflamatórios e pró-angiogênicos que, no contexto da AR, podem influenciar no remodelamento indesejável do tecido inflamado, contribuindo para a progressão da destruição articular que caracteriza a doença. Esta hipótese baseia-se também na observação de que, estes mesmos fatores, durante a gravidez, têm grande importância na remodelação do endométrio e formação da placenta, a fim de garantir o adequado aporte de nutrientes para o feto. Acreditamos que futuros ensaios funcionais com o intuito de analisar o efeito resultante da expressão de HLA-G dentro do compartimento sinovial e em outros sítios de inflamação em outras doenças inflamatórias terão muito a acrescentar na compreensão do papel regulador dessa molécula no contexto desse grupo de doenças.

Considerações finais

Neste trabalho, avaliamos a influência de variantes alélicas da região 3' não traduzida do gene HLA-G na suscetibilidade e curso do lúpus eritematoso sistêmico, na suscetibilidade à artrite reumatóide e avaliamos a expressão de HLA-G solúvel em pacientes com artrite reumatóide e artrite idiopática juvenil. Observamos que o mesmo haplótipo (D/G) parece estar associado tanto à suscetibilidade ao LES quanto à AR, apontando para o gene *HLA-G* como um

potencial fator de suscetibilidade comum às duas doenças. Em AR, observamos maiores níveis de HLA-G solúvel no líquido sinovial de pacientes FR- em comparação com pacientes FR+, e diferentes padrões de correlação entre os níveis plasmáticos de sHLA-G e parâmetros de atividade de doença após estratificarmos os grupos de pacientes para positividade para FR e gênero. Nossas observações, portanto, colocam a molécula e o gene HLA-G como elementos diretamente envolvidos na patogênese e curso dessas doenças e encorajam estudos futuros que procurem elucidar o papel da molécula HLA-G no curso de doenças reumatológicas.

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CAPÍTULO 8 - ANEXOS

ANEXO 1

HLA-G +3142 polymorphism influences the susceptibility to infections in sickle cell anemia patients

Elvira Alicia Aparicio Cordero, Tiago Degani Veit, Maria Aparecida Lima da Silva, Sidia Maria Callegari-Jacques, Lúcia Mariano da Rocha Silla, José Artur Bogo Chies

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HLA-G polymorphism influences the susceptibility to HCV infection in sickle cell disease patients

E. A. A. Cordero[1,*], T. D. Veit[2,*], M. A. L. da Silva[3], S. M. C. Jacques[2,4], L. M. D. R. Silla[3] & J. A. B. Chies[2]

1 Post-Graduation Program in Medical Sciences, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil
2 Genetics Department, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil
3 Hematology Division, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil
4 Departamento de Estatística, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Key words
hepatitis C; human leukocyte antigen-G; polymorphism; sickle cell disease

Correspondence
José Artur Bogo Chies
Department of Genetics, UFRGS
Av. Bento Gonçalves, 9500,
Caixa Postal 15053, 91501–970
Porto Alegre, RS
Brazil
Tel: +55 51 3308 6740
Fax: +55 51 3308 7311
e-mail: jabchies@terra.com.br

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Abstract

Despite its well known monogenic etiopathogenesis, sickle cell disease (SCD) is characterized by a striking variability of clinical presentation. There is growing evidence that genetic factors may be involved in this variability. Human leukocyte antigen (HLA)-G is a non-classical HLA molecule which was shown to be expressed at sites of inflammation and in inflammatory diseases. Besides its large and highly polymorphic promoter region, the 3' UTR region seems also to play an important role on regulating HLA-G expression. We investigated the influence of the 14 pb (rs1704) and the +3142 (rs1063320) HLA-G polymorphisms in 93 SCD patients in order to evaluate its potential role on clinical parameters. Twenty-one patients presented an HCV infection. Among all SCD patients 16 (22.2%) were homozygous for the +3142C genotype, none of them hepatitis C (HCV) positive. Controlling for blood transfusions in the last year, the C allele represented a dose dependent protection effect for HCV infection (PR = 0.41; 95% CI: 0.24–0.71). The +3142C allele was also underrepresented among patients with history of respiratory-tract infections. Our results support a role of the +3142 polymorphism in the susceptibility to infections, in particular to HCV infection, and suggest a possible interference of the HLA-G molecule in the response to infections, among SCD patients.

Introduction

Sickle cell disease (SCD) is the most common hereditary illness in the world, affecting mainly people of African ancestry (1). The illness is characterized by a mutation in the sixth codon of the beta hemoglobin gene (2) which results in the substitution of valine for glutamic acid in position six of beta chain of the hemoglobin. The resulting abnormal hemoglobin (Hb S) is responsible for alterations in erythrocytes that culminate in the obstruction of the microcirculation, ischemia, tecidual necrosis and organic dysfunction (3, 4). In Brazil, over 8000 people present SCD and about 2 million present the sickle trait (heterozygotes). Since the S allele was originated in Africa, its prevalence presents regional variations due to the processes of miscegenation and migration of the Brazilian population, ranging from 1%–2% in Southern Brazil to 6%–10% on the Northeast (5, 6). Despite its well known monogenic etiopathogenesis, SCD is characterized by a striking variability of the clinical presentation ranging from an early-onset life-threatening disease to a milder condition compatible with an almost normal life course. Unpredictability of the major complications is also a characteristic feature of this disease. Although environmental factors undoubtedly play a role in this variability, there is growing evidence that other genetic factors may be involved as well. Recently, SCD has been proposed to be viewed as a chronic inflammatory disease, where the primary vascular effects end up driving the patient's immune system to a state of constant activation (7). Following this perspective, recent studies have focused on the immunogenetic modulation of both innate and adaptive immune responses (8–12). Particularly, it has been reported that both classical and non-classical human leukocyte antigen (HLA) alleles might play a role in the susceptibility to infections in SCD patients (11, 12).

HLA-G is a non-classical HLA molecule, characterized by limited polymorphism, by the expression of both membrane-bound and soluble isoforms and by its limited

* Both authors contributed equally to this work

tissue distribution under normal physiological conditions. However, this molecule was shown to be largely expressed at sites of inflammation and in inflammatory diseases (13–16), being able to mediate the inhibition of the cytotoxic activity of Natural Killer (NK)- and CD8 T-cells and affect CD4 T-cell functions and dendritic cell maturation (17). Therefore, it was suggested that HLA-G plays an important role in regulating the immune response in these conditions. The regulation of HLA-G gene expression seems to be complex and is yet poorly understood. Besides its large and highly polymorphic promoter region (18), the 3' UTR seems to play an important role in regulating HLA-G expression. A 14 bp insertion/deletion polymorphism (19) (rs1704) located at this region was shown to play a role in alternative splicing and was also associated with different levels of HLA-G in plasma (20, 21). This polymorphism has been shown to be associated to susceptibility to some inflammatory diseases (13, 15, 22–27). Another polymorphism located at this region, at the position +3142 (rs1063320), is thought to influence microRNA (miRNA) binding, thus influencing RNA turnover and miRNA-mediated repression of translation (28). An association between this polymorphism and asthma susceptibility has also been reported. It was previously suggested by our group that this polymorphism, which is in linkage disequilibrium with the 14 bp polymorphism (28), might better explain the differences observed in HLA-G protein expression in plasma than the 14 bp polymorphism (29). In the present study, we investigated the influence of these two polymorphisms on SCD disease course.

Materials and methods

Study population

The patients with sickle cell disease (SCD) were recruited at the Hemoglobinopathy Unit at Hospital de Clınicas de Porto Alegre. Ninety-three agreed to participate in the study (45 men and 48 women). All patients were African-derived and were from the urban population of Porto Alegre, the capital of the southernmost state of Brazil. The arisen issue on the skin color-based classification criteria that is used in Brazil is well documented and has been already assessed by our group in previous works (9, 10). Patient data (gender, age, ethnic origin, clinical manifestations, hematological parameters, etc) were obtained from their medical records.

The control group comprised 264 healthy individuals of African decent from the urban population of Porto Alegre and Rio de Janeiro (30). The study protocol was approved by the Medical Ethics Committee of the Hospital de Clınicas de Porto Alegre, Brazil and informed consent was obtained from all individuals.

Genotyping of the HLA-G gene polymorphisms

DNA was isolated from peripheral blood cells using a salting-out procedure (31). For the polymerase chain reaction (PCR) analysis of the +3142 polymorphism, 200 ng of genomic DNA were added to a final volume of 25 μL, with final concentrations as follows: PCR buffer 1X, 2.0 mM $MgCl_2$; 0.2 mM of each dNTP; 1.0 unit of *Taq*-polymerase and 10 pmol of each primer (GMIRNAF-5'-CATGCTGAACT GCATTCCTTCC-3', GMIRNAR-5' CTGGTGGGACAAGG TTCTACTG-3'). Thermocycling conditions were: 94°C for 5 min; 32 cycles of 94°C for 30 s, 65.5°C for 30 s and 72°C for 60 s followed by a final extension step at 72°C for 5 min. The amplified PCR products were cleaved with 3U of *BaeG* I (New England Biolabs Inc., Ipswich, MA) at 37°C for three hours, according to manufacturer's instructions. RFLP products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethydium bromide and visualized under ultraviolet light. The C allele yielded an intact fragment of 406 bp while the G allele yielded fragments of 316 and 90 bp. The 14 bp polymorphism at exon 8 of HLA-G gene was detected through PCR analysis as previously described (32). Briefly, 200 ng of genomic DNA were added to a final volume of 25 μL, with final concentrations as follows: PCR buffer 1X, 1.5 mM $MgCl_2$; 0.4 mM of each dNTP; 1 unit of *Taq*-polymerase and 10 pmol of each primer (GE14HLAG-5'-GTGATGGGCTGTTTAAAGTGTCACC-3', HRG4-5'-GGA AGGAATGCAGTTCAGCATGA-3'). Thermocycling conditions were adapted to 94°C for 2 min; 35 cycles of 94°C for 30 s, 64°C for 60 s and 72°C for 60 s followed by a final extension step at 72°C for 10 min. The amplified PCR products were analyzed by electrophoresis in a 6% polyacrylamide gel stained with ethydium bromide and visualized under ultraviolet light. The 14 bp insertion allele (ins) amplification yielded a 224 bp fragment whereas the deletion allele (del) amplification yielded a fragment of 210 bp.

Statistical analysis

HLA-G genotypic frequencies were compared to Hardy–Weinberg expectations using chi-square tests. Haplotype frequencies were estimated with the MLOCUS software (33), which uses an expectation maximization algorithm (34). HLA-G allelic frequencies and HLA-G genotypes of controls and SCD patients were compared using the χ^2 test (with Yates correction when $df = 1$). Bonferroni correction for multiple comparisons was applied when the *P* value was significant. Prevalence rations were obtained using Poisson Regression with robust variance. The significance level was set at $\alpha = 0.05$ (two-tailed) and all statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL) and winPEPI (35).

Results

Ninety-three SCD patients (45 women and 48 men) were genotyped for the HLA-G polymorphisms. The mean (± SD) patient's age was 27.3 ± 12.2 years. Eighty-eight patients were under treatment with Hydroxyurea (HU) by the time of analysis. Further details about the patients' clinic status are displayed on Table 1. The allelic, genotypic and the estimated haplotype frequencies observed in the patients group were similar to those obtained in a control group composed by African-descendent individuals (Table 2) and both groups

Table 1 Patient and control features and patient clinical manifestations

	n (%)	Mean	SD
Controls (total $n = 264$)			
Females	99 (37.5)		
Males	165 (62.5)		
Age (years)		37.3	12.1
SCD (total $n = 93$)			
Females	45 (48.4)		
Males	48 (51.6)		
Age (years)		27.3	12.2
Age at HU treatment (years)		19.4	11.8
Clinical features or manifestations			
Pain crisis	84 (90.3)		
≥Blood transfusions[a]	36 (37.5)		
Priapism	10 (20.8)[b]		
HCV infection	21 (22.6)		
Foot ulcers	13 (14.0)		
Bone[c]	21 (22.6)		
Neurological[d]	15 (16.1)		
Respiratory[e]	48 (51.6)		
Gastroenterological[f]	57 (61.3)		
Circulatory[g]	21 (22.6)		
Endocrinal	2 (2.2)		
Sensorial[h]	6 (6.5)		
Genital-urinary	11 (11.8)		
Lymphatic	6 (6.5)		

HU, Hydroxyurea; SCD, sickle cell disease.
[a]At least one, during one year follow-up.
[b]Only male patients (total = 48).
[c]Osteonecrosis/osteomyelitis.
[d]Cerebrovascular accident /mental retardation/epilepsy.
[e]Upper respiratory tract infections/bronchopneumonia/asthma/rhinitis/sinusitis.
[f]Gastric ulcer/cholelithiasis/hepatomegaly/splenomegaly.
[g]Deep vein thrombosis/systemic artherial hypertension/pulmonary hypertension/cardiomegaly.
[h]Otitis and retinopathies.

Table 2 HLA-G allelic, genotypic and haplotypic frequencies in patients and comparison with controls

	Patients *N* (frequency)	Controls *N* (frequency)	*P*-value[a]
14 bp			
Ins/ins	15 (0.16)	34 (0.13)	0.378
Ins/del	47 (0.52)	122 (0.47)	
Del/del	29 (0.32)	103 (0.40)	
Del allele	105 (0.58)	328 (0.63)	
+3142			
CC	16 (0.17)	47 (0.18)	0.977
CG	47 (0.51)	130 (0.49)	
GG	30 (0.32)	87 (0.33)	
G allele	107 (0.58)	304 (0.58)	
Haplotype[b]			
Del/C	71 (0.38)	212 (0.40)	0.334
Del/G	38 (0.20)	123 (0.23)	
Ins/C	9 (0.05)	13 (0.03)	
Ins/G	68 (0.37)	180 (0.34)	

HLA-G, human leukocyte antigen-G.
[a]Pearson χ^2.
[b]Estimated frequencies as described in the text.

did not deviate from Hardy-Weinberg equilibrium (data not shown).

Twenty-one SCD individuals (23%) were infected with HCV, a frequency similar to that of other studies with SCD patients (36–42), but much higher than the expected prevalence on the general Southern Brazilian population (1%–2%) (43). None of these patients was homozygous for the +3142C allele (CC) as compared to 16 individuals (22%) from the group without HCV. The frequency of the C allele was lower in the infected group as compared to the HCV-negative group (respectively 21% and 47%, $P = 0.003$) (Table 3). Interestingly enough, while none of the CC individuals was in the HCV infected group, 19% of the heterozygous and 40% of the patients homozygous for the G allele (GG) were in the infected group (linear trend $\chi^2 = 7.467$ $P = 0.006$), suggesting a dose effect associated to this polymorphism on the susceptibility to HCV infection. Since blood transfusion is a known factor for HCV infection, a Poisson regression model was performed to estimate the risk controlling for this variable (blood transfusions in the last year-yes or not). The adjusted relative risk of acquiring an HCV infection is presented in Table 4: an increased risk of 2.4 times (0.95 CI: 1.4–4.2) is expected for each additional G allele in the patient genotype. No differences between infected and non-infected subjects were observed concerning the 14 bp polymorphism frequencies (Table 3). However, at the haplotype level, a higher frequency of the del/C genotype was observed among patients without HCV while the ins/G haplotype was overrepresented among patients with HCV infection (Table 3).

Forty-eight patients presented a history of respiratory tract affections (upper respiratory tract infections, bronchopneumonia, asthma and/or rhinitis/sinusitis–Table 1). Moreover, among them 42 (87.5%) had presented at least one episode of bronchopneumonia and/or other upper respiratory tract infection. When considering bronchopneumonia and upper respiratory tract infections separately, we observed that in both groups there was a decreased frequency of the CC genotype (although not statistically significant) when compared to individuals without these characteristics. It is also interesting to point out that none of the 15 patients with upper respiratory tract infections presented the CC genotype (Table 5). This association yielded a significant *P*-value when considering both bronchopneumonia and upper respiratory tract infections as a single group ($P = 0.040$), although not significant after correcting for multiple comparisons ($P = 0.12$–Table 5). No differences were observed when considering the 14 bp polymorphism alone, or for haplotypes involving both polymorphisms (*P* interval: 0.18–1).

We also evaluated a potential influence of the HLA-G genotype on hematological parameters, such as adult and fetal Hb levels, leukocyte and platelet counts, and bilirubin and LDH (lactate dehydrogenase) levels. No differences were observed between these parameters and the analyzed polymorphisms

Table 3 Comparison of HLA-G allelic, genotypic and haplotypic frequencies in SCD patients with and without HCV infections

	HCV-positive *N* (frequency)	HCV-negative *N* (frequency)	*P*-value	Prevalence ratio (95% CI)
14 bp				
Ins/ins	4 (0.19)	11 (0.15)	0.132	0.35
Ins/del	14 (0.67)	34 (0.47)		(0.11–1.10)[a]
Del/del	3 (0.14)	27 (0.38)		
Del allele	0.476	0.611		
+3142				
CC	0 (0.00)	16 (0.22)	0.006	0(0)[b]
CG	9 (0.43)	38 (0.53)		
GG	12 (0.57)	18 (0.25)		
G allele	0.214	0.486		
Haplotype[c]				Residuals (P)[d]
Del/C	9 (0.22)	61 (0.42)	0.013	−2,46 (0.01)
Del/G	11 (0.26)	27 (0.19)		1,05 (0.29)
Ins/C	0 (0)	9 (0.06)		−1,66 (0.10)
Ins/G	22 (0.52)	47 (0.33)		2,33 (0.02)

CI, confidence interval; HLA, human leukocyte antigen; SCD, sickle cell disease.
[a]Taking del/del as reference genotype.
[b]Taking C/C as reference genotype.
[c]Estimated frequencies.
[d]For HCV-positive patients.

Table 4 Adjusted prevalence ratios of development of HCV infection for the number of HLA-G +3412 G alleles in the genotype, using Poisson regression model with robust variance

Variable	PR	95% CI	*P*
HLA-G +3142G	2.44	1.41–4.17	0.001
Blood transfusion	2.6	1.2–6.0	0.015

HLA, human leukocyte antigen; CI, confidence interval; PR, prevalence ratio.

(*P* interval: 0.16–0.87). To evaluate a potential interference of the HLA-G genotype on the effect of HU, we compared the above-mentioned parameters before and after HU treatment. Again, no significant differences were observed among genotypes and haplotypes considering the two HLA-G polymorphisms (*P* interval: 0.38–0.98).

Discussion

In this work, we assessed the influence of two polymorphisms located at the 3'UTR of the HLA-G gene on clinical features of SCD patients. Although being a disease of monogenic inheritance, the marked variability of symptoms in SCD has led to the search for genetic features that could explain, at least partially, such clinical differences. A marked feature in SCD patients is the high prevalence of recurrent infections. It has been proposed that SCD is a chronic inflammatory condition and that the high occurrence of infections among SCD patients is a consequence of this status of chronic inflammation rather than any immunodeficiency (7, 44). Since associations between both classical and non-classical HLA molecules and disease outcome in SCD patients have been reported by previous studies and since HLA-G is a molecule that interferes in both innate and adaptive immunity, it constitutes an interesting target to be evaluated on SCD course.

In our study group, 23% of the patients were positive for HCV and, of those patients, 86% had previous history of at least one blood transfusion. Because SCD patients are at a higher risk of blood transfusions due to their frequent episodes of hemolytic anemia, they constitute a risk group for HCV and other diseases transmitted by blood derivates. However, the human body resists to the HCV virus through the host's immune system; this resistance, according to our results, seems to be partially dependent on the HLA-G genotype. As previously shown, no patients homozygous for the C allele were found in the HCV infected group and, conversely, the frequency of homozygotes for the G allele was more than doubled in the infected group as compared to the non-infected group. The Poisson regression model was consistent with a dose effect, suggesting that CC patients are more protected than heterozygous patients and that these are more protected than GG patients against HCV infection (the adjusted prevalence ratio for the number of C alleles was 0.41, 95% CI: 0.24–0.71). Haplotype analysis pointed to a similar situation, where the Del/C haplotype was significantly more represented among HCV-negative patients (Table 3). It should be noticed that the most significant association observed in our study was not with an infection caused by a pathogen which would explore directly the intrinsic dysregulation of the SCD patient, but rather with an infection that is an indirect consequence of the patients' special needs.

Table 5 +3142 Genotype and respiratory tract infections in SCD patients

	HCV	GG	CG	CC	*P* value	PR (95% CI)[a]	$\%_{G\ allele}$
Upper respiratory	Yes	6 (40.0)	9 (60.0)	0 (0.0)	0.120	0 (0)	30.0
Tract infections	No	24 (30.8)	38 (48.7)	16 (20.5)			44.9
Bronchopneumonia	Yes	13 (36.1)	20 (55.6)	3 (8.3)	0.129	0.44 (0.15–1.25)	36.1
	No	17 (29.8)	27 (47.4)	13 (22.8)			43.5
Respiratory tract	Yes	16 (38.1)	23 (54.8)	3 (7.1)	0.040[b]	0.37 (0.13–1.05)	34.5
Infections	No	14 (27.5)	24 (45.1)	13 (25.5)			49.2

CI, confidence interval; SCD, sickle cell disease.
[a]PR, Prevalence Ratio, considering CC as a protection factor.
[b]Corrected $P = 0.12$.

This finding is somewhat surprising considering the immunosuppressive role of the HLA-G molecule and the context in which it is being analyzed. Considering HCV infection alone, it could be expected that the C allele, which is associated to a lesser miRNA binding to the HLA-G mRNA molecule hence to a higher HLA-G expression, would be associated to an increased risk of infection by HCV. However, our results revealed that instead of being a risk factor for HCV infection, the C allele seems to confer protection against HCV, by a mechanism associated to an increase on HLA-G expression. At first glance, this idea seems paradoxal, since the immunosuppression mediated by molecules such as HLA-G could, *a priori*, predispose to infections. However, considering SCD as a chronic inflammatory disease, where the frequent vaso-occlusive events and a constant state of hemolytic anemia may easily lead to an altered state of immune homeostasis, the presence of immune regulatory molecules such as HLA-G could favor a better immune behavior against foreign pathogens. Another finding that supports this view is the fact that homozygosity for the C allele was underrepresented, although not statistically significant, in the group with history of respiratory infections. It is important to point out that the loss of statistical significance of the association of the +3142 polymorphism with respiratory tract infections after correction for multiple comparisons could be due to the small sample size. Therefore, if a resistance to HCV or other infections conferred by the C allele is restricted to the very specific immune context of SDC patients or if it can be extrapolated to other risk groups remains to be elucidated.

An association between HLA-G polymorphisms and HCV infections has been previously reported (45). Martinetti et al. have observed that the homozygosity for the 14 bp deletion (which is associated to higher expression of HLA-G) as well as the presence of an allele which contains this deletion (*010401) seem to be risk factors for vertical transmission of HCV whereas the *0105N allele, which contains the 14 bp insertion, confers protection. However, in our study, the 14 bp genotype was not related to susceptibility to HCV infection. A possible explanation for these conflicting results is the distinct contexts of exposure to the HCV virus focused by each study: vertical transmission of HCV implicates an overcoming of the placental barrier before entering the child's organism, while there is no such barrier when acquiring the virus directly through blood transfusions, which was probably the main case in our study. If HLA-G plays a role on the permissiveness of the placenta to the passage of the virus, and we should point out that HLA-G molecule is a major component of the immune system in this environment, this could perfectly explain the observed discrepancies between our data and the above-mentioned study. Also, different ethnic backgrounds could account for the discrepancy between our results and those from Martinetti et al.

Taken together, our results support the role of HLA-G in the susceptibility to infections in SCD patients, especially HCV. Moreover, they constitute an indirect evidence of the role of microRNAs in the regulation of HLA-G expression through their interaction with the region which encompasses the +3142 polymorphism.

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ANEXO 2

Association of the HLA-G 14 bp polymorphism with systemic lupus erythematosus.

Tiago Degani Veit, Elvira Alicia Aparicio Cordero, Tamara Mucenic, Odirlei André Monticielo, João Carlos Tavares Brenol, Ricardo Machado Xavier, Andrés Delgado-Cañedo, José Artur Bogo Chies

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PAPER

Association of the HLA-G 14 bp polymorphism with systemic lupus erythematosus

TD Veit[1,2], EAA Cordero[1], T Mucenic[3], OA Monticielo[3], JCT Brenol[3], RM Xavier[3], A Delgado-Cañedo[4] and JAB Chies[1,2]

[1]Genetics Department, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil; ; [2]Post-Graduation Program in Genetics and Molecular Biology, UFRGS, Porto Alegre, Brazil; ; [3]Rheumatology Division, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil; and ; and [4]Laboratório de Cardiologia Molecular e Celular – Instituto de Cardiologia/Fundação Universitária de Cardiologia, Porto Alegre, RS, Brazil

Human leukocyte antigen-G (HLA-G) is a nonclassical class I major histocompatibility complex molecule which is induced at the course of inflammatory pathologies, and its expression has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses, therefore acting as a mechanism of immune surveillance. We investigated the influence of the 14 bp polymorphism of the *HLA-G* gene on systemic lupus erythematosus (SLE) by analyzing 293 patients with SLE and 460 healthy controls. The patient's group was not in Hardy–Weinberg equilibrium, presenting an excess of heterozygotes ($P = 0.014$). The heterozygote group exhibited lower systemic lupus erythematosus disease activity indexes than the homozygous deletion group and the homozygous insertion group (mean value = 2.29 against 2.97 and 3.4, respectively, $P = 0.035$). Photosensitive patients showed a higher frequency of heterozygotes and an equivalent lower frequency of homozygotes for deletion; on the other hand, patients without arthritis presented a higher frequency of heterozygotes than the arthritis group and also a lower frequency of the del/del genotype. Overall, our results support the idea of a role of the HLA-G insertion/deletion polymorphism and therefore a role for the HLA-G molecule, on the pathology of SLE. *Lupus* (2008) **00,** 1–7.

Key words: HLA-G; polymorphism; systemic lupus erythematosus

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune inflammatory disease that involves many organs and systems. It is characterised by autoantibody production mainly directed against nuclear antigens as well as by immune complex formation and deposition, which lead to intense inflammatory response and tissue damage.

The etiopathogenesis of SLE remains unclear, but it is probably multifactorial. It is thought that interactions among genetic, hormonal, immunological and environmental factors contribute to the development of SLE. Therefore, a combination of genes, instead of a single gene, predisposes to immunological disorder that leads to defective mechanisms of immunological tolerance, allowing antibody production against autoantigens, immune complex formation and deposition.

The human leukocyte antigen-G (HLA-G) is a nonclassical class I major histocompatibility complex molecule which is characterised by low polymorphism at DNA level, limited tissue distribution in nonpathological conditions and expression of both membrane-bound and soluble isoforms by alternative splicing.[1] Recent studies show that HLA-G is induced at the course of inflammatory pathologies such as myositic lesions, psoriatic lesions on skin and multiple sclerosis.[2–4] Furthermore, HLA-G expression on the surface of epithelial intestinal cells seems to play a role on the suppression of proinflammatory cytokines in ulcerative colitis.[5] HLA-G expression has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses, therefore acting as a mechanism of immune surveillance.[6,7] Recently, Verbruggen, *et al.*[8] observed

Correspondence to: José Artur Bogo Chies, Department of Genetics, UFRGS, Av. Bento Gonçalves 9500, Caixa Postal 15053, Zip Code 91501-970 Porto Alegre, RS, Brazil. Email: jabchies@terra.com.br
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that soluble HLA-G levels were lower in patients with rheumatoid arthritis (RA) compared with controls, but they were positively correlated with parameters of RA disease activity. HLA-G levels were shown to be altered in patients with SLE,[9,10] suggesting that differential expression of the HLA-G molecule might play a role on the immunopathology of rheumatic autoimmune diseases.

Expression of HLA-G may be influenced by genetic variants on the *HLA-G* gene. Particularly, it was shown that a *HLA-G* gene polymorphism, a 14 bp insertion/deletion in exon 8 of the gene, might play a role in mRNA stability[11] and is, to some extent, associated with alteration in the plasmatic levels of soluble forms of HLA-G.[12,13] The involvement of this polymorphism in situations where immune regulation is required, such as in the maintenance of pregnancy,[14,15] success of in-vitro fertilization,[16] development of preeclampsia[17,18] and other pathological conditions such as sarcoidosis,[19] has been also described. In our laboratory, we have found a positive association between the HLA-G −14 bp/−14 bp (del/del) genotype and susceptibility to juvenile idiopathic arthritis (JIA).[20] Considering that SLE is a chronic inflammatory disease, and considering the described presence of high serum HLA-G levels in patients with SLE, it would be interesting to investigate the influence of this polymorphism in SLE, assessing the frequency of the 14 bp polymorphic variants.

Materials and methods

Patients and controls

All patients were under the care of the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre, the capital of the southernmost state of Brazil. The study protocol was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre, and informed consent was obtained from all patients.

The group of patients with SLE was comprised of Q1 293 individuals, 224 (76.4%) of which were identified as European-derived and 67 (23.6%) were identified as African-derived. This classification was based on physical appearance as judged by the researcher at the time of blood collection and on data about the ethnicity of parents/grandparents reported by the participants. The issue arisen on the skin colour-based classification criteria that is used in Brazil is well documented[21] and has been already assessed by our group in previous works.[22]

All patients satisfied the American College of Rheumatology criteria[23] and had their records reviewed or underwent a medical interview for documentation of clinical and laboratory data. The systemic lupus erythematosus disease activity index (SLEDAI)[24] and the systemic lupus international collaborating clinics (SLICC)/ACR damage index[25] were Q2 applied to each patient as a measure of disease activity and cumulative damage. The evaluated clinical manifestations are presented in Table 1. The control group was composed of 460 healthy individuals, with proportions of skin colour similar to the patient's group: 356 (77.4%) were identified as European-derived and 104 (22.6%) were identified as African-derived. The controls were from the urban population of Porto Alegre or other cities from the same geographic area of the patients. An informed consent was obtained from all subjects.

Polymerase chain reaction amplification of exon 8 of the HLA-G *gene and genotyping*

DNA was isolated from peripheral blood cells using a salting out method.[26] Polymerase chain reaction (PCR) amplification of exon 8 of the *HLA-G* gene was performed as previously described.[15] The amplified PCR products were visualised in 6% polyacrylamide gel stained with ethydium bromide. Amplicon sizes for the 14 bp polymorphism were 224 bp for the +14 bp allele and 210 bp for the −14 bp allele. All experiments included a control for both homozygous genotypes.

Statistical analysis

HLA-G genotypic frequencies were compared with Hardy–Weinberg (HW) expectations using chi-square tests. HLA-G allelic frequencies and HLA-G genotypes were compared among patient and control groups using the chi-square test (with Yates correction when necessary) or Fisher exact test. Bonferroni correction for multiple comparisons was applied when the P value was significant. Relative risks were estimated by the odds ratio. Means for SLICC and SLEDAI were analysed by Kruskall–Wallis tests. The significance level was set at $\alpha = 0.05$ (two-tailed), and all statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, Illinois, USA) and winPEPI.[27]

Results

We analysed 293 patients with SLE (267 women and 26 men) for the 14 bp insertion/deletion polymorphism. The mean patient's age was 44.5 ± 14.2 years, and the mean disease diagnostic

Table 1 Disease clinic features

Patient's features	*European-derived* (n = *224*)	*African-derived* (n = *67*)	*Whole* (n = *293*)[a]
Females	89.7% (224)	95.5% (67)	91.1% (293)
Age (years)	45.1 ± 14.5 (223)	42.8 ± 13.2 (67)	44.5 ± 14.2 (292)
Age at diagnosis (years)	31.3 ± 13.8 (211)	32.5 ± 12.6 (62)	31.6 ± 13.5 (274)
Malar rash	53.4% (221)	43.1% (65)	50.9% (287)
Discoid rash	14.9% (221)	14.1% (64)	14.7% (286)
Photosensitivity	76.5% (221)*	57.8% (64)*	72.4% (286)
Oral ulcers	35.3% (221)	21.9% (64)	32.2% (286)
Arthritis	81.4% (221)	84.6% (65)	77.3% (287)
Serositis	30.0% (220)	36.5% (63)	31.7% (284)
Nephritis	42.1% (221)	43.1% (65)	42.7% (288)
Neurologic disorders	12.2% (221)	12.2% (64)	11.2% (286)
Haematologic disorders	73.8 %(221)	82.8 %(64)	75.5 %(286)
Haemolytic anaemia	32.6% (221)**	21.9% (64)**	30.1% (286)
Leukopenia/lymphopenia	55.9% (220)***	73.4% (64)***	59.6% (285)
Thrombocytopenia	16.8% (220)	18.8% (64)	17.2% (285)
Immunologic disorders	63.1% (214)	70.3% (64)	64.9% (279)
Anti-DNA	44.9% (214)	46.9% (64)	45.5% (279)
Anti-Sm	15.0% (214)	25.0% (64)	17.2% (279)
Anticardiolipin	23.5% (213)	25.0% (64)	24.1% (278)
Lupic anticoagulant	4.7% (213)	1.6% (64)	4.0% (278)
False positive VDRL	2.3% (213)	2.3% (64)	2.2% (278)
ANA	97.6% (218)	100% (65)	98.6% (285)
Sjogren	13.1% (191)	13.8% (58)	13.1% (251)
SLEDAI	2.58 ± 3.46 (159)	2.90 ± 4.51 (50)	2.66 ± 3.73 (159)
SLICC	1.13 ± 1.49 (190)	1.02 ± 1.33 (58)	1.11 ± 1.46 (190)

Abbreviations: VDRL, venereal disease research laboratory test; ANA, antinuclear antibody; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus international collaborating clinics.
[a]Included two patients whose ethnicity was not determined.
*$P = 0.005$.
**$P = 0.018$
***$P = 0.018$.

age was 31.6 ± 13.5 years (see Table 1). When subdividing the patient's group according to ethnicity, few differences on disease features were found between the two groups. The European-derived group presented a higher proportion of individuals with photosensitivity (76.5% vs 57.8%) (which might result from the difficulty to detect this disease feature in patients with dark skin colour), less leukopenia/lymphopenia (55.9% vs 73.4%) and more haemolytic anaemia than the African-derived group. All other disease features presented in Table 1 showed no statistically significant differences among the groups, including the SLEDAI and SLICC indexes (data not shown). When subgrouping the patients with SLE by gender, it was observed that female patients presented a higher frequency of arthritis (83.5% against 65.4%, $P = 0.031$). No male patients presented neurological disorders, against 12.3% of the females. However, this difference was not statistically significant, probably due to the low number of male patients.

When comparing genotype frequencies of patients and controls to expected HW frequencies, we observed that the patient's group was not in HW equilibrium, presenting an excess of heterozygotes ($P = 0.014$), differently from the control group, which was in HW equilibrium. When subgrouping the patients according to ethnicity, we were able to observe that the excess of heterozygotes was concentrated on the European-derived group ($P = 0.005$), whereas the African-derived group presented no significant deviations from HW frequencies. However, no statistical differences on genotypic or allelic frequencies were observed between the two groups ($P = 0.261$ and 0.658, respectively, Table 2). Moreover, when comparing patients with controls, no significant differences on genotypic or allelic frequencies were observed neither by considering all patients as only one group nor by subgrouping patients and controls according to ethnicity (Table 2). Therefore, for the other patient features, the subsequent analyses were performed including all patients in one group.

The heterozygote group showed lower SLEDAI indexes than the homozygous −14 bp group and the +14 bp group (mean value = 2.29 against 2.97 and 3.4, respectively). Because SLEDAI and SLICC values did not follow a normal distribution, a non-parametric test was applied to test for differences among genotypes. A significant difference was

Table 2 Genotypic and allelic frequencies on patients with SLE and controls

	European-derived		*African-derived*		*Whole*	
	*Patients (%)**	*Controls (%)*†	*Patients (%)**	*Controls (%)*†	*Patients (%)*	*Controls (%)*
Genotype	*n* = 224	*n* = 356	*n* = 67	*n* = 104	*n* = 293	*n* = 460
ins/ins	30 (13.4)	59 (16.5)	11 (16.4)	11 (10.6)	41 (14.0)	70 (15.2)
ins/del	129 (57.6)	175 (49.1)	31 (46.3)	48 (46.1)	161 (54.9)	223 (48.5)
del/del	65 (29.0)	122 (34.4)	25 (37.3)	45 (43.3)	91 (31.1)	167 (36.3)
P value[a]	0.138		0.488		0.213	
Allele						
ins	189 (42.2)‡	293 (41.2)§	53 (39.5)‡	70 (33.7)§	242 (41.3)	363 (39.5)
del	259 (57.8)‡	419 (58.8)§	81 (60.5)‡	138 (66.3)§	340 (58.7)	557 (60.5)
P value[b]	0.76		0.299		0.418	

Abbreviations: ins/ins, homozygous +14 bp; ins/del, heterozygous; del/del, homozygous −14 bp; ins, 14 bp insertion; del, 14 bp deletion.
[a]Chi-square.
[b]Chi-square with Yates correction.
**P* = 0.261.
†*P* = 0.146.
‡*P* = 0.658.
§*P* = 0.062.

observed on SLEDAI indexes among genotypes, with the heterozygote group exhibiting the lowest mean rank ($P = 0.035$, Kruskal–Wallis test, see Table 3). When comparing SLICC indexes, the heterozygote group also presented the lowest mean values (1.087 against 1.133 and 1.162). However, those differences were not significant ($P = 0.966$); this might be because the SLICC index correlates positively with disease time ($r_s = 0.336$, $P < 0.001$, Spearman's correlation test), which could influence the analysis, whereas the SLEDAI index does not ($P = 0.181$). Indeed, the heterozygous group had the higher mean disease time among all genotypes (13.35 against 12.56 and 11.66 years). In the group of patients treated with cyclophosphamide, which is used to treat the patients with severe disease, a lower proportion of heterozygotes was observed compared with the other patients (50.5% against 55.6%), although not significant. No differences were observed in disease onset or age at diagnosis (data not shown).

Among all clinical features analysed, two presented a noteworthy difference in genotypic frequencies among positive and negative subjects, although not statistically significant after correction for multiple comparisons. Photosensitive patients showed a higher frequency of heterozygotes and an equivalent lower frequency of homozygotes for deletion; on the other hand, patients without arthritis presented a higher frequency of heterozygotes than the arthritis group and also a lower frequency of the del/del genotype (Table 3).

Discussion

In the present study, we assessed the frequency of the 14 bp insertion/deletion polymorphism in the *HLA-G* gene in SLE. Because the HLA-G molecule is involved in several immunoregulatory processes and

Table 3 Genotype and disease features in patients with SLE

		ins/ins	*ins/del*	*del/del*	
Disease index					*P* value
SLEDAI	Mean (SD)	2.66 (4.42)	2.29 (3.76)	2.97 (3.28)	
(*n* = 209)	Mean rank	118.30	95.94	115.34	0.035
SLICC	Mean (SD)	1.162 (1.55)	1.087 (1.42)	1.133 (1.48)	
(*n* = 249)	Mean rank	126.96	130.33	129.29	0.966
Patients' features		*n* (%)	*n* (%)	*n* (%)	Uncorrected *P*
Photosensitivity	Yes	30 (14.5)	123 (59.4)	54 (26.1)	0.030
	No	11 (13.9)	35 (44.3)	33 (41.8)	
Arthritis	Yes	30 (12.8)	125 (53.2)	80 (34.0)	0.021
	No	11 (21.2)	33 (63.5)	8 (15.4)	

Abbreviations: ins/ins, homozygous +14 bp; ins/del, heterozygous; del/del, homozygous −14 bp; SLEDAI, systemic lupus erythematosus disease activity index; SD, standard deviation; SLICC, systemic lupus international collaborating clinics.

in inflammatory disorders, HLA-G represents an excellent candidate gene for association to this disease.

The first intriguing finding in our analysis was the observation that this polymorphism was not on HW equilibrium in patients with lupus, which present an excess of heterozygotes. A possible explanation to this phenomenon could be based on a work of Mendes-Junior, *et al.*[28] who analysed this polymorphism in eight Amerindian populations from the Brazilian Amazon and suggested the occurrence of balancing selection at this locus. It is important to point out that although the insertion allele is associated with preeclampsia and miscarriages, the deletion allele seems to be associated with JIA, a childhood disease, [20] and the HLA-G −14 bp/−14 bp genotype was already associated to pemphigus vulgaris in Jewish patients[29] and with a higher risk of developing acute graft-versus-host disease in unrelated bone marrow transplantation for thalassaemia.[30] Besides, differential survival ability of the foetus in a pathogen-rich environment has been already proposed to explain the high frequencies of an *HLA-G* null allele in African populations.[31] In the perspective that individuals with SLE are prone to develop common, chronic and opportunistic infections (see review Zandman-Goddard and Shoenfeld, 2005[32]), heterozygosis for the 14 bp insertion/deletion polymorphism could confer advantage to individuals who otherwise would be prone to develop other diseases. In patients with lupus, the HLA-G genotype seems to influence disease severity, as the heterozygous individuals seemed to present a milder disease than their homozygote counterparts, which was verified by the SLEDAI indexes.

It is also possible that the heterozygote excess observed on our work is due to a hitch-hiking effect, i.e., to an excess of heterozygosis occurring at other sites within or near the *HLA-G* gene. Tan, *et al.*[33] have shown a strong evidence for balancing selection at the *HLA-G* promoter region, which is characterised by two different lineages of human haplotypes, which may have different promoter activity, resulting in differences that, as suggested by the authors, could result in different spaciotemporal expression patterns that meet different immunologic needs and be tissue-specific and/or development-stage specific.[33] The most common haplotypes of first and second lineages seem to be associated with alleles that are associated, respectively, with the deletion and insertion polymorphisms.[28] However, more work is needed to elucidate the mechanisms underlying the balancing selection acting in this locus because the regulation of the *HLA-G* gene, *per se*, is not yet completely understood. Also, it was previously shown that the HLA-G*010102, which is the most common allele with the 14 bp insertion, seems to be in linkage disequilibrium with HLA-DR1 and DR3, DQ2 and HLA-A1 alleles,[34–37] the latter three being part of the 8.1 ancestral haplotype, which is associated to several immunopathological diseases, including SLE.[38] Thus, it could be expected an increased frequency of the insertion allele among patients with SLE. This was not observed in our sample; however, it should be pointed out that the patients homozygous to the insertion allele presented the higher SLEDAI mean.

Among the several clinical features analysed in the present work, photosensitivity and arthritis occurred differentially in the patients subgrouped according to the *HLA-G* genotype. An influence of the HLA-G genotype on skin manifestations of lupus is not unlikely because the expression of this molecule in skin infiltrates has already been documented.[9] When considering arthritis, a higher frequency of heterozygotes was found in the patients without the development of arthritis, which corroborates the previously suggested protective role for this genotype on SLE. Also, a higher frequency of the del/del genotype was observed among individuals that developed arthritis (Table 3). The allelic frequency for the insertion allele was lower in the patients with arthritis than in the nonarthritis group (0.394 and 0.529, respectively). This result was similar to our previous finding on JIA, where women showed a higher frequency of the del/del genotype and a lower frequency of the insertion allele.[20] Thus, it is possible that in SLE, the insertion allele exerts a similar protective influence against development of arthritis, as suggested to exerts in JIA. This result, however, must be considered with caution, due to the relatively low number of patients who were negative for arthritis.

Two previous studies concerning HLA-G in SLE have presented conflicting results. Rosado, *et al.*,[9] who analysed genotype and expression patterns of HLA-G in SLE, have encountered a higher frequency of the +14 bp allele and also lower plasma levels of soluble HLA-G in patients with SLE. They also observed a significant increased frequency of the HLA-G +14 bp/+14 bp and a decreased frequency of the HLA-G −14 bp/−14 bp genotype in patients with SLE. The data about plasma levels of soluble HLA-G disagrees with the work done by Rosado, *et al.*,[9] in which soluble HLA-G levels were higher in patients with SLE. Our results also disagree with those of Rizzo, *et al.*[10] in the sense that the patient group deviates from the HW equilibrium and the insertion allele is not a risk factor for SLE. It is possible that environmental and/or ethnicity-specific factors are contributing for such conflicting results.

Concluding, we observed differences in the genotypic and allelic frequencies of the 14 bp polymorphism between patients with SLE and healthy controls represented mainly by a departure from HW equilibrium (excess of heterozygotes) in the patient's group. Also, heterozygous individuals presented lower SLEDAI values, suggesting that this polymorphism might play a role in disease outcome. Although we cannot exclude the existence of other polymorphic loci in linkage-disequilibrium with the analysed HLA-G variant, as involved on the etiopathogenesis of SLE, overall, our results support the idea of a role of the HLA-G insertion/deletion polymorphism and therefore a role for the HLA-G molecule, on the pathology of SLE. Q4 However, the conflicting results on the involvement of HLA-G in SLE suggest that more work is needed to elucidate the real role of this molecule in this disease.

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ANEXO 3

Association of the HLA-G 14-bp insertion/deletion polymorphism with juvenile idiopathic arthritis and rheumatoid arthritis.

Tiago Degani Veit, Priscilla Vianna, Ilóite Scheibel, Claiton Brenol, João Carlos Tavares Brenol, Ricardo Machado Xavier, Andres Delgado-Cañedo, Jorge Eduardo Gutierrez, Ana Paula Carneiro Brandalize, Lavinia Schuler-Faccini, José Artur Bogo Chies.

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Association of the HLA-G 14-bp insertion/deletion polymorphism with juvenile idiopathic arthritis and rheumatoid arthritis

Tiago Degani Veit[1,2], Priscilla Vianna[1,2], Ilóite Scheibel[3,4], Claiton Brenol[3], João Carlos Tavares Brenol[3], Ricardo Machado Xavier[3], Andres Delgado-Cañedo[5], Jorge Eduardo Gutierrez[1,5], Ana Paula Carneiro Brandalize[2], Lavinia Schuler-Faccini[1,2] & José Artur Bogo Chies[1,2]

1 Genetics Department, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
2 Post-Graduation Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
3 Rheumatology Division, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil
4 Rheumatology Division, Fundação Faculdade Federal de Ciência Médicas de Porto Alegre, Porto Alegre, Brazil
5 Laboratory of Molecular and Cellular Cardiology, Instituto de Cardiologia de Porto Alegre, Porto Alegre, Brazil

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Correspondence
José Artur Bogo Chies
Department of Genetics
Universidade Federal do Rio Grande do Sul
Av. Bento Gonçalves, 9500
Caixa Postal 15053
91501-970 Porto Alegre
Brazil
Tel: +55 51 3308 6740
Fax: +55 51 3308 7311
e-mail: jabchies@terra.com.br

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Abstract

We tested the possible association of the 14-bp polymorphism of the HLA-G gene in the course of two inflammatory diseases, rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). Patients and controls were genotyped for the 14-bp polymorphism by polymerase chain reaction with specific primers for the exon 8 of the human leukocyte antigen (HLA)-G gene and the amplified fragment was visualized in a 6% polyacrylamide gel. A total of 106 JIA patients, 265 RA patients, 356 healthy adults and 85 healthy children were genotyped for the 14-bp polymorphism. Female JIA patients presented a higher frequency of the −14 bp allele when compared with female healthy children (0.743 and 0.500, corrected $P = 0.003$), which reflected in the JIA group as a whole. This increased frequency of the −14-bp allele was observed in all JIA subtypes. In RA patients, no differences in allelic and genotypic frequencies were observed between patients and controls. No correlations were observed among genotype and disease severity or clinical manifestations. Our data suggest that the HLA-G −14 bp allele is probably a risk factor for JIA, mainly in females. Considering the differences observed in relation to gender, we suggest that hormonal differences can interfere with the development of JIA. Considering the RA patients, our data agree with results from the literature and highlight the differences in the etiology of RA and JIA.

Introduction

The human leukocyte antigen G (HLA-G) is a nonclassical class I major histocompatibility complex (MHC) which is characterized by low polymorphism at DNA level, limited tissue distribution in nonpathological conditions and the expression of both membrane-bound and soluble isoforms by alternative splicing (1). This molecule has become the object of interest for its possible role in pregnancy maintenance. HLA-G seems to be involved on the induction and maintenance of tolerance between the mother immune system and the semi-allogeneic fetus at the fetal–placentary interface and also seems to play an important role in embryo implantation (2–5).

Besides, several studies point out to a broader immunoregulatory role for this molecule. HLA-G seems to reduce cell sensitivity of several types of solid tumors, cells from lymphoproliferative disorders and allogeneic grafts to cytolysis mediated by natural killer (NK) cells and cytotoxic T cells, therefore acting as a mechanism of immune escape (1, 6–12). The expression of soluble HLA-G (sHLA-G) in blood and by heart and liver/kidney grafts has also been associated with significant better prognosis and fewer rejection episodes (1, 13–14).

Recent studies show that HLA-G is induced at the course of inflammatory pathologies such as myositic lesions, psoriatic lesions on skin, atopic dermatitis and multiple

sclerosis (15–18). Furthermore, HLA-G expression on the surface of epithelial intestinal cells seems to play a role on suppression of pro-inflammatory cytokines in ulcerative colitis (19). HLA-G expression has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses, therefore acting as a mechanism of immune surveillance (20–21). Recently, Verbruggen et al. observed that sHLA-G levels were lower in rheumatoid arthritis (RA) patients when compared with controls (22).

It was previously shown that a HLA-G gene polymorphism, a 14-bp insertion/deletion in exon 8 of the gene, might play a role in mRNA stability (23). The +14 bp allele is associated with lower levels of HLA-G mRNA and, to some extent, with lower levels of sHLA-G (24–26). The involvement of this polymorphism in the maintenance of pregnancy, in success of *in vitro* fertilization and in preeclampsia has been previously described (27–31).

It was previously described that interleukin (IL)-10, a cytokine known for its anti-inflammatory and immunosuppressive roles, induces HLA-G expression in human trophoblasts and monocytes (32), and that HLA-G seems also to induce IL-10 expression in peripheral blood mononuclear cells (PBMCs) (33). Rizzo et al. (34) investigated HLA-G and IL-10 levels in PBMCs activated with lipopolyssacharide (LPS) in relation to the 14-bp polymorphism. They observed a significant increase of IL-10 levels in PBMCs homozygous for the +14-bp allele when compared with the other genotypes. This data, allied to the recent description of a subset of T cells that express and secrete HLA-G and that are present in normal physiological and inflammatory conditions (35) are elements that incite the study of the influence of polymorphic variants of HLA-G in inflammatory diseases.

RA and juvenile idiopathic arthritis (JIA) are two inflammatory disorders that share some common features, such as a strong pro-inflammatory response directed to joints and tissue destruction. However, certain discrepancies concerning clinical [e.g. JIA presents a broad clinical heterogeneity, being classified in several subtypes and in RA, the vast majority of the patients are rheumatoid factor (RF) positive, while in JIA, there are few RF+ patients], molecular and genetic features suggest that they represent two quite different pathologies.

In the present work, we assessed the frequency of the 14-bp polymorphic variants in RA and JIA. Differences were observed in genotypic and allelic frequencies between these two disorders and a significant difference was observed in JIA patients subgrouped according to gender.

Materials and methods

Patients and controls

The RA patient group was comprised of 265 European-derived individuals with diagnosis of RA, satisfying the American College of Rheumatology criteria (36), that were under the care of the Division of Rheumatology of the Hospital de Clínicas de Porto Alegre (HCPA), the capital of the southernmost state of Brazil. Among them, 23 lost to follow up and 242 patients had their medical records reviewed for documentation of clinical, laboratory and radiographic data. Clinical data included pattern of joint involvement, atlantoaxial subluxation and extraarticular manifestations (rheumatoid nodules, amyloidosis, vasculitis, pneumonitis and episcleritis). Erosive disease was characterized by the presence of erosions in any of the X-rays of the hands and feet since the beginning of follow up in HCPA, otherwise patients were classified as having nonerosive disease. The disease activity score (DAS28) (37) and the health assessment questionnaire (HAQ) (38) were applied to each patient as a measure of disease activity and physical ability.

A total of 106 patients who fulfilled the criteria for juvenile RA as proposed by the American College of Rheumatology and redefined according to the International League of Associations for Rheumatology classification (39) were analyzed. The patients were recruited in three Medical Centers from Porto Alegre, and their relatives signed an informed consent. All patients were diagnosed before 16 years of age and presented inflammatory arthritis in, at least, one joint persistent for more than 1 year at the time of the research. This criterion was used for the exclusion of persistent reactive arthritis.

The JIA control group comprised 85 normal children with mean age of 5.2 ± 3.4 years. The RA control group was composed of 356 European-derived healthy individuals with a mean age of 53.5 ± 17.7 years. Both control groups were from the urban population of Porto Alegre, the same geographic area of the patients, and share the same ethnic origins of the patients. The study protocol was approved by the Ethics Committee of the HPCA and informed consent was obtained from all patients.

Polymerase chain reaction amplification of exon 8 of the HLA-G gene and genotyping

DNA was isolated from peripheral blood cells using a salting out method (40). HLA-G genotyping was performed as previously described (28). Briefly, 100 ηg of genomic DNA was amplified in a 25 μl reaction, with final concentrations as follows: polymerase chain reaction (PCR buffer) 1×, dNTP 0.2 mM, $MgCl_2$ 1.5 mM, Taq DNA polymerase 0.75 U and 10 ρmol of each primer (GE14HLAG – 5′-GTGATGGGCTGTTTAAAGTGTCAC C, RHG4 – 5′-GGAAGGAATGCAGTTCAGCATGA). Thermocycling conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 64°C for 60 s and 72°C for 60 s; final extension of 72°C for 10 min. The amplified PCR products were visualized in 6% polyacrylamide gel stained with ethidium bromide. The

amplicon sizes for the 14-bp polymorphism were: 224 bp for the +14 bp allele and 210 bp for the −14 bp allele.

Statistical analysis

HLA-G genotypic frequencies were compared with Hardy–Weinberg expectations using chi-squared tests. HLA-G allelic frequencies and HLA-G genotypes based upon the 14-bp deletion polymorphism in exon 8 of controls, JIA and RA patients were compared using the chi-squared test (with Yates correction when necessary) or Fisher's exact test. Bonferroni correction for multiple comparisons was applied when the P value was significant. Relative risks were estimated by the odds ratio. Means for DAS28 and HAQ were analyzed by one-way ANOVA and Kruskal–Wallis test, respectively. The significance level was set at $\alpha = 0.05$ (two-tailed) and all statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL) and WINPEPI (41).

Results

Juvenile idiopathic arthritis

We analyzed 106 JIA patients (70 females and 36 males) for the 14-bp insertion/deletion polymorphism. The mean disease age of onset was 4.8 ± 3.1 years (see further details in Table 1). Both control children and JIA patient groups were in Hardy–Weinberg equilibrium (data not shown). We observed a significant difference in the genotypic frequencies between the two groups, even after Bonferroni correction. We also observed an increased frequency of the −14 bp allele in the JIA group when compared with controls (Table 2). The results are similar if JIA patients are compared with the adult control group (data not shown). The genotypic frequencies differed significantly between gender inside the patients group: the −14 bp/−14 bp genotype was more frequent among JIA females (55.7% against 19.4%), while the +14/−14 and +14/+14 genotypes were more frequent among JIA males (66.7% against 37.1% and 13.9% against 7.1% respectively; see Table 3).

Hence, considering that our JIA sample was composed of 2/3 of female individuals and that the −14 bp allele seems to be more frequent in this group, we compared patients against controls subgrouping by gender (Table 3). Female patients presented a higher frequency of the −14 bp allele when compared with female controls (0.743 and 0.500, respectively, corrected $P = 0.003$). We observed an increased frequency of the −14 bp/−14 bp genotype among female patients when compared with female controls (corrected $P = 0.006$), while in males this genotype was observed in a low frequency, although not significant (OR = 0.46, $P = 0.190$). Females with the −14 bp/−14 bp genotype presented a higher chance of having JIA (OR = 4.67, 95% CI = 1.67–14.29). All these results are equivalent if the female JIA group is compared with the female healthy adult control group (data not shown).

Table 1 JIA and RA patients' characteristics and disease subtypes

	n (%)	Mean	Range
JIA (total, $n = 106$)			
Age (years)		9.3	4–18
Age at diagnosis		4.8	1–13
Disease time (years)		4.6	1–16
Oligoarticular	50 (47.2)		
Polyarticular			
RF−	38 (35.8)		
RF+	3 (2.8)		
Systemic	13 (12.3)		
Unknown subtype	2 (1.9)		
ANA positivity	15 (14.1)		
	n (%)	Mean	SD
RA (total, $n = 265$)			
Age (years)		58.1	12.67
Symptoms onset age		41.6	14.17
Age at diagnosis		46.6	13.64
Disease time (years)		11.7	6.74
DAS		4.21	1.33
HAQ		1.41	0.78
Erosions	205 (79.2)[a]		
EA manifestations	55 (22.1)[b]		
Rheumatoid nodules	42 (16.9)[b]		
Subluxations	10 (4.2)[c]		
RF positivity	220 (89.1)[d]		

ANA, antinuclear antibodies; DAS, disease activity score; EA, extraarticular; HAQ, Health Assessment Questionnaire; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; RF, rheumatoid factor.
[a] Out of 259.
[b] Out of 249.
[c] Out of 239.
[d] Out of 247.

We then observed the genotypic frequency distribution when subgrouping the patients according to JIA subtype. No significant differences were observed among subtypes when analyzing males and females together (data not shown). The genotypic frequency distributions were similar among the oligoarticular, polyarticular and systemic subtypes of JIA in females ($P = 0.986$), which were similar to the female JIA group as a whole. Similarly, the three male subtypes presented no differences in their genotypic frequency distributions ($P = 0.908$), which were similar to the male group as a whole. No influence of the HLA-G genotype on disease age onset was observed (data not shown).

Rheumatoid arthritis

We analyzed 265 RA patients (218 women and 47 men) for the 14 bp insertion/deletion polymorphism. The mean

Table 2 Genotypic and allelic frequencies on JIA and RA patients

	JIA, *n* (%) ($n = 106$)	JIA controls, *n* (%) ($n = 85$)	RA, *n* (%) ($n = 265$)	RA controls, *n* (%) ($n = 356$)
Genotype				
+14 bp/+14 bp	10 (9.4)	22 (25.9)	49 (18.2)	59 (16.6)
+14 bp/−14 bp	50 (47.2)	38 (44.7)	132 (49.1)	175 (49.2)
−14 bp/−14 bp	46 (43.4)	25 (29.4)	84 (31.2)	122 (34.3)
P value[a]	0.006		0.727	NA
Correction[d]	0.012			
OR (95% CI)[b]	1.81 (0.97–3.54)		0.89 (0.62–1.27)	NA
Allele				
+14 bp	70 (0.330)	82 (48.2)	230 (0.434)	293 (0.412)
−14 bp	142 (0.670)	88 (51.8)	300 (0.566)	419 (0.588)
P value[c]	0.0036		0.463	NA
Correction	0.0072			

JIA, juvenile idiopathic arthritis; NA, not applicable; RA, rheumatoid arthritis.
[a] Frequencies of each group of patients are compared to those of the control group using chi-squared test.
[b] Each patient group is compared to its respective control group, taking as a risk factor the -14bp/-14bp genotype.
[c] Same as footnote 'a' but using chi-squared with Yates correction.
[d] Bonferroni correction was applied by multiplying *P* values by the number of tests ($n = 2$).

patient age was 58.1 ± 12.7 years and the mean symptoms onset age was 41.6 ± 14.2 years (see further details on Table 1). Both control and RA patient groups were in Hardy–Weinberg equilibrium (data not shown). No significant differences were observed in the allelic and genotypic frequencies between RA patients and controls ($P = 0.727$ and 0.463 respectively; see Table 2). Unlike JIA, no differences on genotypic or allelic frequencies were observed between genders in RA patients (Table 3).

Next, we investigated whether the 14-bp polymorphism had any relation with disease severity or with different RA-associated clinical features. No patient presented pneumonitis, only one patient presented episcleritis (being heterozygous to the analyzed polymorphism), two patients had amyloidosis (both heterozygous) and four patients presented vasculitis (two heterozygous and two homozygous −14 bp). No differences were observed among the mean DAS28 or HAQ scores for each genotype ($P = 0.575$ and 0.700 respectively).

Table 3 Genotypic and allelic frequencies in JIA. Patients subgrouped by gender

	Genotype				Allele		
Groups (*n*)	−14 bp/−14 bp (%)	+14 bp/−14 bp (%)	+14 bp/+14 bp (%)	OR (95% CI)[a]	−14 bp (%)	+14 bp (%)	*P* value[f]
Females							
JIA patients (70)	39 (55.7)	26 (37.1)	5 (7.1)	**4.67 (1.67–14.29)**[b]	104 (74.3)	36 (25.7)	**0.003**[g]
JIA controls (33)	7 (21.2)	19 (57.6)	7 (21.2)		33 (50.0)	33 (50.0)	
RA patients (218)	74 (33.9)	105 (48.2)	39 (17.9)	1.15 (0.73–1.83)[c]	253 (58.0)	183 (42.0)	0.954
RA controls (159)	49 (30.8)	85 (53.5)	25 (15.7)		183 (57.5)	135 (42.5)	
Males							
JIA patients (36)	7 (19.4)	24 (66.7)	5 (13.9)	0.46 (0.14–1.36)[d]	38 (52.8)	34 (47.2)	NA[h]
JIA controls (52)	18 (34.6)	19 (36.5)	15 (28.8)		55 (52.9)	49 (47.1)	
RA patients (47)	10 (21.3)	27 (57.4)	10 (21.3)	0.46 (0.19–1.01)[e]	47 (50.0)	47 (50.0)	0.101
RA controls (196)	73 (37.2)	89 (45.4)	34 (17.3)		235 (59.9)	157 (40.1)	

JIA, juvenile idiopathic arthritis; NA, not applicable; RA, rheumatoid arthritis.
[a] Each patient subgroup is compared with its respective control group, according to gender, taking as a risk factor the −14 bp/−14 bp genotype.
[b] $P = 0.006$ after Bonferroni correction for multiple comparisons ($n = 3$).
[c] $P = 0.597$
[d] $P = 0.190$
[e] $P = 0.057$
[f] Each patient subgroup was compared to the respective control group, according to gender, using chi-squared with Yates correction.
[g] After Bonferroni correction ($n = 3$).
[h] The genotypic distribution on JIA males group was not in Hardy–Weinberg equilibrium.

No differences on allelic frequencies were observed concerning the clinical features evaluated in the presented study (Table 4). No correlation between HLA-G genotype and disease onset was observed (data not shown).

Discussion

In the present study, we assessed the frequency of the 14-bp insertion/deletion polymorphism in the HLA-G gene in RA and JIA. Although this polymorphism has been previously assessed in RA (42), this is the first case/control study in JIA. Because the HLA-G molecule seems to be involved in several immunoregulatory processes and, particularly in inflammatory disorders, HLA-G represents an excellent candidate gene for association to these two rheumatic diseases. The influence of the HLA-G molecule in RA was analyzed by two recent studies (22, 42), but there are no data concerning this molecule and JIA.

Our data suggest a gender-specific difference for the 14-bp insertion/deletion polymorphism in JIA, with a significantly higher frequency of the −14 bp allele among female patients, which seems to confer a higher disease susceptibility to the females that present this allele, particularly in homozygosis. In our sample, approximately 56% of the JIA females were homozygous for this allele as compared with approximately 19% of the males. Another interesting fact is that this increased frequency was not limited to a given subtype of the disease; all the subtypes represented in our sample presented an increased frequency of the −14 bp allele in the female subgroup. However, because of the reduced sample size in some subtypes, this fact must be interpreted with caution. We can speculate that there might be an immunoregulatory mechanism, common to the different subtypes of the disease that can be altered in the presence of the −14 bp allele.

Our data suggest that the HLA-G −14 bp allele is a risk factor for JIA. Rizzo et al. performed an *in vitro* study using PBMCs activated with LPS, observing that the IL-10 produced by those cells correlated directly with the HLA-G genotype, with +14 bp/+14 bp cells presenting the higher IL-10 levels (34). Considering IL-10 as an anti-inflammatory cytokine, if the −14 bp allele is associated to lower levels of IL-10 *in vivo*, in situations of inflammation, such as in JIA, the presence of this allele would be, *per se*, harmful. Further studies that evaluate the correlation between serum and synovial levels of IL-10 and HLA-G allelic variants in JIA will be necessary to clarify this issue.

In disagreement with what was observed in JIA females, the homozygous −14 genotype seems not to be a risk factor for JIA in males. The fact is intriguing because this is a childhood disease, in which physiological differences between genders, particularly hormonal levels, are not so pronounced. Still, a hypothesis for the differential involvement of the HLA-G molecule in JIA, according to gender, would be the possible regulation of the HLA-G gene by hormones. Indeed, Yie et al. (43) showed that HLA-G expression is regulated by progesterone through the presence of a progesterone response element at the 5′ upstream region of the gene. However, no significant differences were observed on the progesterone levels between JIA patients and controls (44) or between healthy children subgrouped by gender (45) that could point to this possibility.

Interestingly, Khalkhali-Ellis et al. (44) observed an association between low androgen levels and disease in 20 JIA female patients. Testosterone serum levels are higher in boys, when compared with girls, from uterine life to the first year of life (46). In recent years, studies both in animal models as well as in humans have highlighted an immunosuppressor role of testosterone (47–49). Therefore, it is possible that in situations where testosterone levels are present below a certain threshold (that would be attained/exceeded more frequently by boys), the HLA-G molecule would pass from a secondary role to an important factor in the regulation of inflammatory responses.

Concerning RA, our data are in accordance with the results obtained by Rizzo et al. (42), where no differences between genotypic and allelic frequencies for this polymorphism were observed when RA patients were compared with control individuals. When DAS28 and HAQ scores, which were used to evaluate disease severity, were analyzed according to HLA-G genotype, no correlation was observed. This data suggest that, differently from JIA, in RA the 14-bp polymorphism is not related to the disease physiopathology. However, we cannot rule out the possibility of involvement of other polymorphisms within the HLA-G gene with RA. The fact that RA patients present lower sHLA-G levels in serum (22) is indicative that HLA-G molecule might play a role in the physiopathology of RA.

Concluding, in this work we observed differences in the genotypic and allelic frequencies of the 14-bp

Table 4 Rheumatoid arthritis – clinical manifestations in individual carrying the −14 bp variant

Feature		% $_{\text{Allele } -14 \text{ bp}}$ (*n*)	*n*	*P* value
EA manifestations	Yes	57.3 (55)	96	0.709
	No	54.5 (194)	356	
Rheumatoid nodules	Yes	60.0 (42)	70	0.443
	No	54.2 (207)	382	
Erosions	Yes	53.7 (205)	382	0.333
	No	60.0 (54)	90	
Subluxations	Yes	45.5 (10)	22	0.540
	No	54.5 (229)	430	
RF positivity	Yes	54.5 (220)	404	0.462
	No	48.2 (27)	48	

EA, extraarticular; RF, rheumatoid factor.

polymorphism between RA and JIA and between JIA patients subgrouped according to gender. Our results strengthen the argument that RA and JIA are not etiopathologically related. We cannot exclude another polymorphic locus, even outside the HLA-G gene, in linkage disequilibrium with the analyzed HLA-G variants, as a true responsible for the obtained results. However, the increasing evidences linking HLA-G and autoimmune diseases and inflammatory conditions, together with the recent description of a new regulatory HLA-G+ T cell subset (35), strongly suggest that this molecule plays essential roles in the regulation of immune responses.

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