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ORIGINAL ARTICLE

Human leukocyte antigen-G polymorphisms in periodontitis

Letícia Grando Mattuella[a], Lisiane Bernardi[b], Francis Maria Báo Zambra[c], Milene Borges Campagnaro[d], Rui Vicente Oppermann[e], Léder Leal Xavier[d], José Artur Bogo Chies[c] and Letícia Algarves Miranda[f]

[a]Dental School, Universidade Caxias Do Sul (UCS), Caxias Do Sul, Brazil; [b]Morphological Sciences, Universidade Federal Do Rio Grande Do Sul (UFRGS), Porto Alegre, Brazil; [c]Immunogenetics Lab, Universidade Federal Do Rio Grande Do Sul (UFRGS), Porto Alegre, Brazil; [d]Tissue Biology Lab, Pontifícia Universidade Católica Do Rio Grande Do Sul (PUCRS), Porto Alegre, Brazil; [e]Dental School, Universidade Federal Do Rio Grande Do Sul (UFRGS), Porto Alegre, Brazil; [f]Dental School, University of Western Australia (UWA), Perth, WA, Australia

ABSTRACT

Objective: This study evaluated human leucocyte antigen-G gene polymorphisms in patients with periodontitis and healthy controls.

Material and methods: The insertion/deletion polymorphism of 14 bp and a single nucleotide polymorphism (SNP) C > G on the position +3142 of the 3′ untranslated region of the gene were analyzed in chronic periodontitis ($n = 62$), aggressive periodontitis ($n = 24$) patients and healthy individuals ($n = 47$).

Results: Considering the 14 bp insertion/deletion, a significant deviation from Hardy-Weinberg expectations in the chronic periodontitis group was observed, but not in the other groups. No significant deviations were observed in patients and control groups considering the +3142 C > G SNP. A significant increased frequency of homozygotes for the 14 bp deletion allele was observed in the chronic periodontitis group as compared to controls. This group also presented a higher frequency of the deletion allele, which was marginally not significant. Concerning this polymorphism, no significant differences were observed between the aggressive periodontitis and healthy control groups. In addition, no significant differences were seen amongst patients and controls when considering the +3142 C > G frequencies.

Conclusion: No differences were found amongst patients and controls when considering the +3142 C > G SNP haplotypes frequencies, but a significant increased frequency of homozygotes for the 14 bp deletion allele was observed in chronic periodontitis patients compared to healthy controls, suggesting a susceptibility role of this polymorphism in the pathogenesis of this condition.

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Introduction

Periodontitis is a multifactorial inflammatory disease in which the tooth supporting collagen fibres of the periodontal ligament and bone are mainly broken down due to the development of an exacerbated immune-inflammatory response to the dental biofilm [1]. According to the American Academy of Periodontology [2], chronic periodontitis (CP) is classified as a slow progression disease related to an accumulation of bacteria biofilm. Aggressive periodontitis (AP) is characterized by a rapid periodontal destruction, generally occurring in young patients, and usually the severity of the disease is not compatible with the amount of biofilm [3]. The inter-individual differences in the outcome and course of periodontitis are not explained only by microbial factors. It is known that only a limited group of individuals develop AP whilst others have no or only slow disease progression [4].

Human leukocyte antigen (HLA) gene polymorphisms can provide important susceptibility or resistance factors to periodontitis [4]. The HLA system, if implicated in periodontitis, may be associated with low or non-responsiveness to bacterial antigen, which could result in disease progression [5]. HLA-G is a class I non-classical major histocompatibility complex (MHC) molecule characterized by restricted expression and low DNA polymorphism. It has limited tissue distribution under normal physiological conditions and by alternative splicing can produce seven different isoforms of HLA-G, four membrane-bound (HLA-G1 to HLA-G4) and three soluble ones (HLA-G5 to -G7) [6,7].

As an HLA molecule, the HLA-G shares structural properties of its classical counter-parts HLA-A, B and C [6]. On the 3′ untranslated region (UTR) of the HLA-G gene an insertion/deletion (ins/del) polymorphism of 14 base pair (bp) (rs1704) influences the mRNA stability [8], play a role in alternative splicing and is associated with different levels of HLA-G in plasma [9]. Also, in this region, there is a single nucleotide polymorphism (SNP) C > G on the position +3142 (rs1063320) which is known to be within a putative binding site for microRNAs, which is thought to be relevant for the regulation of the HLA-G expression [6].

The HLA-G molecule has important immunomodulatory properties in both physiological and pathological conditions.

CONTACT Leticia Algarves Miranda leticia.algarvesmiranda@uwa.edu.au Dental School, University of Western Australia (UWA), M512, 17 Monash Avenue, Nedlands, 6009 Perth, WA, Australia

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HLA-G gene has been shown to be expressed in sites of inflammation and in several inflammatory diseases, such as juvenile idiopathic arthritis (JIA) [10], rheumatoid arthritis (RA) [10], systemic lupus erythematous (SLE) [11,12], multiple sclerosis [13], angiographic coronary artery disease [14]. The possible roles of HLA-G in inflammation include present peptides similarly to classical HLA-class I molecules and interact with most immune cells such as T and B cells, NK cells, monocytes, macrophages, neutrophils and dendritic cells, impairing their function. Recently, an upregulation of HLA-G expression and/or release has been described as an immune scape mechanism performed by virus and bacteria to avoid recognition by immune effector cells and reduce the inflammatory response [15].

Until now, several HLA gene polymorphisms have been investigated as possible markers of susceptibility to periodontitis and studies vary in inclusion criteria, methodological procedures and results [16–20]. Concerning HLA-G, its relation to CP and AP has not been described so far. Since HLA-G molecule seems to be involved in several other inflammatory disorders as mentioned above, the aim of the present study was to determine the influence of two polymorphisms located at the 3′UTR of the HLA-G gene in patients with CP and AP.

Material and methods

Patients and controls

One hundred and thirty three patients were recruited from the Department of Periodontology at the Federal University of Rio Grande do Sul (UFRGS), Brazil. Their age ranged from 12 to 64 years. After clinical examination, the patients who fulfilled the clinical inclusion criteria were invited to participate in the present study, which was submitted to and approved by the UFRGS (Protocol No. 47/05) and PUCRS (Protocol No. 272/08) Ethics Committees. All patients and individuals from the control group were thoroughly informed about the purpose and methods of the study and a written consent was obtained from each of them. All periodontal examinations were performed by post-graduate periodontologists. Subjects were classified into the following groups: healthy control ($n = 47$), CP ($n = 62$) and AP ($n = 24$). Control healthy subjects had at least 20 teeth, gingival bleeding index less than 20%, attachment loss in proximal sites $\leq$2 mm [21]. CP subjects were aged between 35 and 60 years of age and had at least 20 teeth, attachment loss $\geq$4 mm in at least 10 teeth, probing depth $\geq$6 mm in at least 5 teeth and periodontal bleeding in at least 10 teeth [21]. AP subjects were those who had four or more teeth with attachment loss $\geq$4 mm in individuals between 14 and 19 years of age or $\geq$5 mm in individuals between 20 and 29 years of age [3]. A questionnaire was applied to all participants regarding their educational level, smoking habits and general health. For the present research, individuals diagnosed with HIV and/or diabetes, pregnant or lactiferous women and anyone who took immunomodulatory/anti-inflammatory/antibiotics drugs in the 6 months prior to the study were excluded.

Genotyping of the HLA-G gene polymorphism

Blood samples (15 ml) were obtained by venipuncture from each patient using BD™ Vacutainer tubes (BD Diagnostics, NJ, USA) and DNA was extracted with a salting-out method [22].

Genotyping was performed by PCR amplification using specific primers and the manufacturers' protocols were followed. The +3142 C > G polymorphism genotyping was performed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay as described by Cordero et al. [23]. Briefly, the PCR samples were prepared with 200 ng genomic DNA to a final volume of 25 μl, with final concentration of 10 pmol of each specific primer (GMIRNAF 5′-CATGCTGAACTGCATTCCTTCC-3′ and GMIRNAR 5′-CTGGTGGGACAAGGTTCTACTG-3′, Invitrogen Corporation, CA, USA), 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, PCR buffer 1× and 1.0 U of Taq DNA polymerase (Invitrogen Corporation, CA, USA). Samples were submitted to 94 °C for 5 min, 32 cycles of 30 s at 94 °C, 30 s at 65.5 °C and 60 s at 72 °C followed by a final extension step of 5 min at 72 °C, in a thermocycler. The PCR products were digested with 3 U of the restriction enzyme BaeGI (New England Biolabs Inc., MA, USA), according to the manufacturer's instructions, producing 316 and 90 bp fragments for the G allele or a single undigested 406 bp fragment for C allele, which were visualized under UV irradiation in a 2% agarose gel stained with ethidium bromide.

For the genotyping of the 14 bp polymorphism of the HLA-G gene, samples were amplified by conventional PCR using specific primers, according to Hviid et al. [24]. The amplification was performed with 100 ng of genomic DNA in a 25 μl reaction, with final concentrations as follows: 10 pmol of each primer (GE14HLAG 5′-GTGATGGGCTGTTTAAAGTG TCACC-3′, RGH4 5′-GGAAGGAATGCAGTTCAGCATGA-3′, Invitrogen Corporation, CA, USA), dNTP 0.2 mM, $MgCl_2$ 1.5 mM, PCR buffer 1× and Taq DNA polymerase 0.75 U (Invitrogen Corporation, CA, USA). Samples were submitted to 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 60 s at 64 °C and 60 s at 72 °C followed by a final extension of 10 min at 72 °C. PCR products were plotted on 8% polyacrylamide gel stained with ethydium bromide and visualized under UV irradiation. The 14 bp ins allele amplification yielded a 224 bp fragment whereas the del allele amplification yielded a fragment of 210 bp.

Statistical analysis

The 14 bp and 3142 C > G genotypic frequencies were compared to HW expectations using Chi-squared tests. The allelic and genotypic frequencies of the HLA-G gene polymorphisms of periodontitis patients and controls were compared using the Fisher's exact test or Chi-squared test (with Yates correction when necessary). Haplotype frequencies were estimated with the MLocus software [25], which uses an expectation maximization algorithm [26]. Relative risks were estimated by the odds ratio. The significance level was set at $\alpha = 0.05$ (two-tailed), and all statistical analyses were

Table 1. Main characteristics of the studied subjects.

	Healthy group	Periodontitis group	
		Chronic	Aggressive
Male/Female gender (%)	14/33 (29.8/70.2)	23/39 (37.1/62.9)	04/20 (16.7/83.3)
Age (mean and SD)	29.7 (±0.7)	48.1 (±7.8)	34.9 (±9.8)
Ethnic group (%)			
Caucasian	47 (100)	52 (83.9)	20 (83.3)
Non Caucasian	0 (0)	10 (16.1)	04 (16.7)
Smoking status (%)			
Non smoker	34 (72.3)	25 (40.3)	15 (62.5)
Former smoker	06 (12.8)	22 (35.5)	03 (12.5)
Smoker	07 (14.9)	15 (24.2)	06 (25)

performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and winPEPI [27].

Results

General characteristics of the subjects included in the study are presented in Table 1.

HLA-G genotypic, allelic and haplotype frequencies in patients and controls are displayed on Table 2. A significantly increased genotypic frequency of the 14 bp del allele was observed in the CP group as compared to controls (0.484 against 0.234, OR = 3.07, 95% CI: 1.24–7.87). The CP group presented a higher frequency of the del allele, which was marginally not significant (0.653 against 0.533, $p = .053$). No significant differences were observed between the AP and healthy control groups concerning the genotypic and allelic frequencies of this polymorphism. Also, no significant differences were observed amongst patients and controls when considering the +3142 C > G SNP frequencies.

Considering the estimated haplotype frequencies from both polymorphisms, we observed an increased frequency of the del/G haplotype in CP patients (0.177 against 0.085) and, conversely, a decreased frequency of the ins/G haplotype (0.346 against 0.478). However, these differences did not reach statistical significance ($p = .057$).

With respect to the 14 bp ins/del polymorphism, we observed a significant deviation from HW expectations in the CP group ($p = .047$), but not in the healthy control and AP groups, due to a deficit in heterozygosis. No significant deviations were observed in patients and control groups considering the +3142 C > G SNP.

Discussion

This study assessed the potential association of two polymorphisms of the HLA-G gene in patients with CP and AP. These polymorphisms have been studied in several other tissue-destructive inflammatory diseases [10–14] but not in periodontitis. Considering the immune-inflammatory tissue-destructive nature of periodontitis, a role for a molecule involved in several immunoregulatory processes, such as the HLA-G molecule, seems suitable to be investigated in the etiopathogenesis of these diseases.

We observed a significant increased genotypic frequency for the HLA-G 14 bp del allele for the CP patients compared to controls, showing an OR of 3.07 (95% CI: 1.24–7.87). Although no statistical significance was observed in the HLA-G −14 bp allele, this polymorphism was more frequent in the patients groups (CP and AP). Interestingly, Veit et al. [10] also found that the HLA-G −14 bp allele was a risk factor for another chronic tissue-destructive condition, juvenile idiopathic arthritis.

A biological explanation for this finding could involve the anti-inflammatory cytokine IL-10. Rizzo et al. [28] performed an in vitro study using peripheral blood mononuclear cells activated with lipopolysaccharide and observed a relationship between +14 bp/+14 bp cells and higher IL-10 levels. Considering IL-10 as an anti-inflammatory cytokine, the −14 bp allele could be associated to lower IL-10 levels in vivo and in inflammation situations, such as periodontitis. Indeed, in periodontitis patients, an absence of IL-5 and IL-10 expression was observed in gingival biopsies [29]. Górska et al. [30] compared severe CP patients with healthy controls and analyzed cytokine concentration in inflamed gingival tissue and serum samples. They found that although IL-10 levels were generally low or even undetectable in serum, the frequency of individuals expressing IL-10 positive cells was much higher in healthy gingival tissue as compared to CP patients. These findings are corroborated by Garlet et al. [31] who observed lower IL-10 expression in gingival biopsies from patients with AP. We also observed a trend towards lower serum levels of Th2 cytokines, including IL-5 and IL-10, in AP patients [32]. However, additional studies should specifically correlate IL-10 levels with HLA-G −14 bp polymorphism in periodontitis patients to clarify this issue. We have not observed any of the polymorphisms analyzed to be susceptibility indicators for the aggressive form of periodontitis. This may be due to the sample size of this group of patients presenting this more rare form of the disease. The present study could, thus, be considered an initial report and additional studies with a larger sample could render more reliable results.

When analyzing the +3142 C > G gene polymorphism, we had no haplotype ins/C in our study. However, del/C was more frequent, but not statistically significant, in the CP group. In the control and AP group, very similar results were found to ins/G and del/C. Different findings from this were demonstrated in lupus patients [33] and C hepatitis patients [23], where the haplotype del/C was suggested to be a susceptibility factor for these diseases.

An interesting meta-analysis has not observed a positive nor negative significant association between HLA gene polymorphisms and patients with chronic periodontitis. However, in aggressive patients, a positive significant association was described to HLA-A9 and HLA-B15 polymorphisms, whilst, HLA-A2 and HLA-B5 showed a negative association. It is important to mention that all studies included had Caucasian patients in their sample [4]. These same authors believe that differences between the prevalence of certain bacteria in the subgingival biofilm in different countries and in different ethnic groups may influence the results.

Racial genetic variations could also influence HLA-G gene polymorphisms. In the study of Lucena-Silva et al. [34], the majority of polymorphic sites showed a similar distribution in

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

	Controls frequency (N)	Chronic periodontitis frequency (N)	Aggressive periodontitis frequency (N)
14 bp			
Del/del	0.234 (11)	0.484 (30)	0.375 (9)
Ins/del	0.574 (27)	0.339 (21)	0.375 (9)
Ins/ins	0.191 (9)	0.177 (11)	0.250 (6)
OR (95%CI)[a]	–	**3.07 (1.24–7.87)**[b]	1.96 (0.58–6.46)
Del	0.533 (49)	0.653 (81)	0.562 (27)
Ins	0.467 (45)	0.347 (43)	0.438 (21)
p value	–	0.053	0.723
+3142			
CC	0.128 (6)	0.274 (17)	0.250 (6)
CG	0.617 (29)	0.403 (25)	0.375 (9)
GG	0.255 (12)	0.323 (20)	0.375 (9)
OR (95%CI)[c]	–	2.58 (0.86–8.72)	2.28 (0.52–9.73)
C	0.436 (41)	0.476 (59)	0.438 (21)
G	0.564 (53)	0.524 (65)	0.562 (27)
p value	–	0.585	1.000
Haplotype[d]			
del/C	0.436 (41)	0.475 (59)	0.437 (21)
del/G	0.085 (8)	0.177 (22)	0.125 (6)
ins/G	0.478 (45)	0.346 (43)	0.437 (21)
p value	–	0.057	0.732

[a]Taking del/del as reference genotype.
[b]$p_{corr} = .020$.
[c]Taking CC as reference genotype.
[d]Estimated frequencies.
Bold values indicate $p < 0.05$.

healthy individuals and the genetic background of Northeastern and Southeastern Brazilian populations could influence the HLA-G gene variability. Even though these findings are not related to the presence of a chronic inflammation as in the present study, it was observed a similarity in the allele and haplotypes frequencies for the 14 bp del/ins and +3142 C > G polymorphisms of the HLA-G gene when their results were compared to ours control group. This could be justified by the fact that the population of the present study is also originated from the South of Brazil, as the aforementioned study.

Concluding, no differences were found amongst patients (CP and AG) and controls when considering the +3142 C > G SNP haplotypes frequencies, but a significant increased frequency of homozygotes for the 14 bp del allele was observed in CP patients compared to healthy controls, suggesting a susceptibility role of this polymorphism in the pathogenesis of this condition.

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