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HLA-G and susceptibility to develop celiac disease

Eulalia Catamo [a,*], Luisa Zupin [b], Ludovica Segat [b], Fulvio Celsi [b], Sergio Crovella [a,b]

[a] University of Trieste, 34127 Trieste, Italy
[b] Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", 34137 Trieste, Italy

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ABSTRACT

The Human Leukocyte Antigen-G has immunomodulatory function and its expression has been associated with several diseases. In our study we analyzed HLA-G polymorphisms in order to evaluate their possible association with susceptibility to celiac disease development. A total of 420 celiac patients and 509 controls were genotyped for HLA-G polymorphisms. We sequenced 800 bp upstream the ATG codon (5′ upstream regulatory region) and the whole 3′ untranslated region of the HLA-G gene, whereas the ΔC deletion at exon 3 was detected by RFLP-PCR.

Five polymorphisms (namely −477 C>G, −369 C>A, 14 bp del/ins, 3187 A>G, 3196 C>G) and one haplotype (TCGGTACGAAITCCCGAG) were significantly more frequent in celiac patients than controls and associated with increased disease susceptibility. The 14 bp I/I, 3187 G/G, 3196 G/G genotypes and TCGGTACGAAITCCCGAG haplotype, were still significantly associated with increased disease susceptibility (and in addition also the 3003 C/C genotype) when the analysis was restricted to patients and controls presenting the DQ2.5 or DQ8 HLA-DQ celiac disease risk haplotypes.

Our findings indicate an association between HLA-G gene polymorphisms and susceptibility to celiac disease development, suggesting that HLA-G molecule is possibly involved in the pathogenesis of the disease.

1. Introduction

The human leukocyte antigen (HLA)-G is a protein belonging to the human major histocompatibility complex (MHC).

The *HLA-G* gene (6p21.3) is a non-classical class I HLA composed of eight exons and seven introns [1,2]; HLA-G mRNA produces seven isoforms (four membrane-bound, G1-G4, and three soluble, G5-G7) by alternative splicing [3].

HLA-G is predominantly expressed on the extravillous cytotrophoblast cells of the placenta where it is associated with maternal-fetal tolerance and, in non-pathological conditions, its expression is highly restricted to specific tissues such as thymus, cornea, pancreas and hematopoietic cells where HLA-G is present at low levels [4].

HLA-G molecules are observed in different pathological conditions such as tumors, viral infections, autoimmune diseases, inflammations, etc. [5–11]; moreover HLA-G expression and gene polymorphisms have been associated with several disorders [12–16].

HLA-G has an important role in regulating the immune system; indeed, the molecule is able to inhibit the cytotoxic activity of Natural Killer cells (NK) and T cell-mediated cytolysis (CTL) [17]. HLA-G can inhibit the response of alloproliferative CD4+ T cells, proliferation of T and NK cells, and the maturation and function of antigen presenting cells (APC) [18–20]. In addition, HLA-G has a tolerogenic effect due to its capacity of generating suppressor cells by binding to specific receptors and it can induce apoptosis in endothelial cells [21,22].

Considering HLA-G expression in pathological conditions and its immunomodulatory functions, a role for this molecule in celiac disease (CD) can be hypothesized.

CD is a chronic inflammatory disease with autoimmune features caused by immune response to ingested wheat gluten and related cereal proteins in genetically predisposed individuals

Abbreviations: APC, antigen-presenting cell; AU, Absorbance Units; CD, celiac disease; CD4+, CD4 positive; C.I., confidence interval; CTL, cytotoxic T lymphocyte; EMA, anti-endomysial antibodies; F, female; HC, healthy controls; HLA-G, Human Leukocyte Antigen-G; IFN-γ, interferon-gamma; LD, linkage disequilibrium; M, male; MAF, minor allele frequency; MHC, major histocompatibility complex; NK, Natural Killer; NR, not risk; O.R., odds ratio; PCR, polymerase chain reaction; RFLP-PCR, restriction fragment length polymorphism-polymerase chain reaction; sHLA-G, soluble human leukocyte antigen-g; SNP, single nucleotide polymorphism; tTG, tissue Transglutaminase; URR, upstream regulatory region; UTR, untranslated region.

* Corresponding author at: IRCCS Burlo Garofolo, Via dell'Istria 65/1, 34137 Trieste, Italy. Fax: +39 040 3785540.
E-mail address: eulalia.catamo@gmail.com (E. Catamo).

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[23]. The majority of celiac patients (around 90%) carry the HLA-DQ2.5 haplotype (encoded by DQA1∗05, DQB1∗02 and DRB1∗03 alleles), whereas a smaller percent of subjects carry the HLA-DQ8 haplotype (encoded by DQA1∗03, DQB1∗0302 and DRB1∗04 alleles) [24].

In celiac patients, the ingestion of gluten triggers a mucosal Th1 type inflammatory response with production of large amounts of interferon-γ (IFN-γ) [25,26]. HLA-G molecule may counteract the development of celiac disease or may balance its inflammation as a consequence of its immunomodulatory properties.

Torres et al. [27] demonstrated an association between soluble HLA-G (sHLA-G) expression and CD as well as a correlation between increased levels of sHLA-G expression and susceptibility to develop CD. The authors hypothesized that the enhanced expression of sHLA-G found in CD patients could be part of a mechanism to restore gluten tolerance [27]. Moreover, an association between HLA-G polymorphism and CD has already been described by Fabris et al. [28]: the authors reported that the 14 bp insertion in the 3′UTR of *HLA-G* gene, conferred an increased risk of CD in addition to the risk conferred by HLA-DQ2.5 in Italian CD patients.

In order to replicate Fabris et al. [28] findings, reporting the association of *HLA-G* gene with celiac disease, we sequenced the 800 bp upstream to the ATG codon (HLA-G 5′ upstream regulatory region, URR) as well as the whole 3′ untranslated region (UTR), and investigated the presence of a cytosine deletion at exon 3 (ΔC), in a group of Italian CD patients and healthy controls.

2. Material and methods

2.1. Patients and controls

We retrospectively selected for this study genomic DNAs from 420 CD Italian patients (European Caucasian, mean age 21.05 ± 15.5 years) (262 female, mean age 23.8 ± 17.31 years, and 158 male mean age 16.72 ± 10.84 years, female (F)/male (M) ratio 1.6). The 420 CD Italian patients analyzed in this study represent a selected subset of a bigger historical cohort (around 2500 samples) of celiac individuals that were recruited at the Gastroenterology Service of IRCCS Burlo Garofolo (Trieste, Italy) from July 2000 to December 2009. Then, we selected 294 DQ2.5 subjects (181 females and 113 males, F/M ratio 1.6) and 126 DQ8 subjects (81 females and 45 males, F/M ratio 1.8) to be further analyzed for HLA-G polymorphisms. The few individuals carrying the minor/null risk haplotype DQ2.2 have been excluded from the study, as well as DQ2/DQ8 heterozygotes individuals.

The DQ2.5 and DQ8 frequencies in our study (70% and 30% respectively) do not reflect the HLA Class II distribution in CD Italian patients from North East Italy but reflect the choice to select a sufficient number of individuals in order to allow an adequate sample size in both high risk haplotypes groups; moreover all patients were not previously analyzed by Fabris et al. [28].

CD diagnosis was made according to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition guidelines [29]: after the evaluation of clinical symptoms, anti-tissue Transglutaminase (anti-tTG) serological test was performed (with the ELISA Eu-tTG kit; Eurospital, Trieste, Italy), and EMA antibodies were subsequently screened (with the Antiendomysium Kit; Eurospital) for results confirmation when ambiguous serological results were achieved. Molecular HLA typing was used to exclude/include CD in doubtful cases. In symptomatic CD patients with normal intestinal mucosa and positive serology, after HLA typing, immunohistochemical analysis was performed to determine the number of CD3-positive intraepithelial lymphocytes, using CD4, CD8, and CD25 as population markers [30,31].

After confirming the diagnosis of celiac disease, all patients started a gluten-free diet, whose effects were measured using tTG serology (Eu-tTg kit; Eurospital). Values below 7 AU (Absorbance Units) were considered as negative; values comprised between 7 and 10 AU were considered borderline and required EMA analysis; values above 10 AU were considered positive. The mean serum anti-tTG value was 39.5 ± 5.3 AU (range 13–174) in CD patients; normally, the introduction of a gluten-free diet leads to a decline in the number of tTG autoantibodies under the cutoff value of 7 AU.

As healthy controls (HC) we enrolled 509 European–Caucasian individuals (mean age 40.64 ± 10.29 years) (277 females, mean age 37.9 ± 8.68 years, 232 males, mean age 43.9 ± 11.12 years, F/M ratio 1.2), of whom 148 HC (85 female and 63 male, F/M ratio 1.3) with DQ2.5, 67 HC (32 females and 35 males, F/M ratio 0.9) with DQ8 haplotypes and 294 not carrying DQ2.5 or DQ8 (named as NR) (160 females and 134 males, F/M ratio 1.19).

All subjects had no clinical signs related to the disease, no familiar history of CD and were not on gluten-free diet. CD was excluded by testing the subjects for the presence of anti-tTG antibodies (mean serum anti-tTG value 1.8 ± 0.3 AU range 0.1–5.3).

The study was approved by the local ethical committee (Burlo Garofolo Protocol No. CE/V-71).

2.2. DNA extraction and HLA genotyping

Genomic DNAs were extracted from peripheral whole blood for all patients and controls using the EZ1 DNA extraction kit and automatic DNA extraction system (Qiagen, Milan), following the manufacturer's indications.

Two diagnostic kits from Eurospital (Trieste, Italy) were used for HLA Class II risk typing in CD patients and controls: the Eu-Gen kit used from 2000 to 2005 was not able to determine heterozygous individuals, while the Eu-Gen Risk used after 2005 was able to do it.

2.3. HLA-G genotyping

The 800 bp upstream the ATG codon in the *HLA-G* 5′URR, and the entire 3′UTR (about 450 bp) were amplified by PCR using the following primers: 5′URR forward 5′-CACGGAAACTTAGGGCTA CGG-3′, 5′URR reverse 5′-GCGTCTGGGGAGAATGAGTCC-3′; 3′UTR forward 5′-GCTGTGCTATGAGGTTTCTTG-3, 3′UTR reverse 5′-CGTGT ACTGTGGAAAGTTCTCA-3′, and sequenced with the Sanger method on the ABI PRISM 3130XL automated DNA sequencer (Applied Biosystems, USA). Sequences were handled using the 4Peaks (http://mekentosj.com/4peaks/) and Codon-Code Aligner (http://www.codoncode.com/aligner/) software.

HLA-G ΔC deletion at exon 3 (typical of the G∗0105N allele) was detected by RFLP-PCR [32]. PCR products were digested over night at 37 °C with the PpuMI endonuclease (New England Biolabs, Beverly, MA) and subsequently separated on a 3% agarose gel. The ΔC allele showed one fragment of 276 bp, while C allele showed two fragments of 108 and 168 bp. Direct sequencing of 100 randomly chosen amplicons was used as RFLP-PCR genotyping quality control.

2.4. Statistical analyses

Allele and genotype frequencies were calculated by direct gene counting. Statistical significance of difference in allele and genotype frequencies was calculated by Fisher's exact test. The odds ratio (O.R.) and 95% confidence interval (C.I.) were also computed.

Haplotypes and the eventual presence of linkage disequilibrium (LD) between *HLA-G* polymorphisms were evaluated by using the free available online software Arlequin 3.11 (http://cmpg.unibe.ch/software/arlequin3) [33]. Statistical significance of