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Association between sHLA-G and HLA-G 14-bp deletion/insertion polymorphism in Crohn's disease

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Abstract

The aim of this study was to evaluate the association between the HLA-G 14-bp deletion/insertion (Del/Ins) polymorphism and soluble (s) HLA-G production in patients with Crohn's disease (CD). We analyzed also the sHLA-G molecules by ELISA and western blot in plasma samples. Among unselected patients, the 14-bp Del/Ins polymorphism was not significantly associated with increased CD risk neither for alleles (*P* = 0.371) nor for genotypes (*P* = 0.625). However, a significant association was reported between the 14-bp Del/Ins polymorphism and CD, in particular in young-onset CD patients for alleles [*P* = 0.020, odds ratio (OR) = 2.438, 95% confidence interval (CI): 1.13–5.25] but not with adult-onset CD patients. A significant association was reported concerning the genotype Ins/Ins for young-onset CD patients (*P* = 0.029, OR = 3.257, 95% CI: 1.08–9.77). We observed also a significant increase in sHLA-G measured by ELISA in CD patients compared to controls (*P* = 0.002). The 14-bp Del/Del and 14-bp Del/Ins genotypes are the high HLA-G producers. Among sHLA-G^{positive} patients, 43% of subjects present dimers of HLA-G. The presence of dimers seems to be related to the advanced stages of the disease. The 14-bp Del/Ins polymorphism is associated with an increased risk of CD particularly in young-onset CD patients and controls sHLA-G plasma levels. Dimers of sHLA-G are frequent in advanced disease stages. The above findings indicate that the genetic 14-bp Del/Ins polymorphism in exon 8 of the HLA-G gene is associated with the risk of CD and suggest a role for sHLA-G as a prognostic marker for progressive disease.

Keywords: 14 bp, conformation, Crohn's disease, HLA-G, polymorphism

Introduction

Crohn's disease (CD), an inflammatory bowel disease, is characterized by a chronic inflammation commonly localized in the ileocecal area (1). This auto-immune disease is associated in part with genetic background and also with immunologic factors (2). In fact, CD is linked to increased levels of T_h1 cytokines (including IFN-γ, tumor necrosis factor-α and IL-12), as well as higher concentrations of T_h17 cytokines (including IL-17A, IL-17F, IL-22, IL-21 and IFN-γ) (2, 3).

HLA-G is an immune-modulatory molecule encoded in the short arm of chromosome 6. It is a non-classical HLA-I molecule characterized by low allelic polymorphism and a restrictive tissue expression in comparison with classical HLA-I antigens (4). After alternative splicing of the primary transcript, seven HLA-G isoforms can be obtained: four membranous isoforms (HLA-G1, G2, G3 and G4) and three soluble isoforms (HLA-G5, G6 and G7). HLA-G possesses an unpaired cysteine residue at position 42 on an external loop of the peptide-binding groove that enables the dimerization (5). Leukocyte immunoglobulin-like receptors have a greater affinity for the dimeric form that increases the signaling transduction (6).

HLA-G molecules mediate immunosuppressive functions through the inhibition of immune cells. Indeed, HLA-G inhibits the lysis of NK cells (7–9), the alloproliferation of $CD4^+$ T cells (10, 11) and the antigen-presentation of dendritic cells (12). It enhances, in the other side, the production of regulatory T

cells (13) and the apoptosis of $CD8^+$ cells (14). Importantly, the 14-bp Del/Ins polymorphism in the 3′-untranslated region of HLA-G (rs66554220) controls mRNA stability (15). In particular, the insertion (Ins) has been associated with lower levels of HLA-G expression (16–18). Taking into account that the 14-bp Del/Ins polymorphism could influence HLA-G protein levels, and the reported implication of HLA-G molecules in patients with CD (19), first, we studied the contribution of this polymorphism on the susceptibility to CD in Tunisian samples, stratifying by disease onset, behavior, location and surgical resection.

Secondly, we analyzed the plasma levels of soluble (s) HLA-G and tried to correlate it to HLA-G 14-bp Del/Ins genotype either for CD patients or for healthy controls.

Methods

Patients

Blood samples were obtained from subjects with CD recruited from the Department of Gastroenterology in Charles Nicole Hospital of Tunis. Patients had not been treated by immunosuppressive therapeutics. Altogether, 44 patients (20 males and 24 females) were recruited with a mean age 36.89 ± 12.29 (SD; age range: 20–69 years) (Table 1). The sex ratio was evaluated to 0.83. CD diagnosis was based on clinical, radiological, endoscopic and histopathologic findings. Patients with uncertain diagnosis or with other auto-immune disease were excluded from the study. Several parameters were collected including age, family history, disease localization, smoking habits, surgical therapy, chemical therapy and extra-intestinal manifestations.

The control population consisted of 71 healthy subjects including 30 males and 41 females with a mean age 34.04 ± 11.58 (age range: 19–64 years).

All patients and control subjects were Tunisian. The study was approved by the local ethics committee. Subjects were stratified based on their age-at-CD onset in two groups: young-onset CD (age ≤25 years) and adult-onset CD (age >25 years). Patients were also stratified based on the disease behavior in inflammatory, stenotic phenotypes according to the Montreal classification (20).

14-bp Del/Ins gene polymorphism

Genomic DNA was extracted from blood using the salting method. 14-bp Del/Ins genotyping was performed by PCR as previously described (21). Briefly, DNA was amplified with a set of primers: 5′-GTG ATG GGC TGT TTA AAG TGT CAC C-3′, 5′-GGA AGG AAT GCA GTT CAG CAT GA-3′. The 35 cycles of PCR were performed at 94°C for 30 s, 64°C for 60 s and 72°C for 60 s, and final cycle of 72°C for 10 min.

DNA fragments were electrophoresed on 3% agarose gels containing ethidium bromide. The insertion allele was visualized as a 224-bp band, while the deletion allele was seen as 210-bp bands. The genotyping call rate exceeded 98%—no significant differences between cases and healthy controls.

Soluble HLA-G measurement by ELISA

sHLA-G levels [shedding HLA-G1 (sHLA-G1) molecules generated by metalloproteinases proteolytic cleavage (22) and HLA-G5 molecules] were measured in plasma of CD patients (n = 30) and controls (n = 25) in duplicate as previously reported in the Essen Workshop (23). We used the monoclonal antibody (mAb) MEM-G9 (Exbio, Praha, Czech Republic) as a capture antibody and antibody to β2-microglobulin as the second specific antibody.

We measured also HLA-G5 levels according to the Essen Workshop (23), with the 5A6G7 (Exbio) as capture antibody and W6/32 (Exbio) as secondary antibody. The limit of sensitivity was 1.0 ng ml^{-1}.

sHLA-G immunoprecipitation and western blot analysis

221-G1 cell culture supernatants and samples were biotinylated with 0.2 mg ml^{-1} EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) in pH 8.0 PBS for 30 min at 4°C (17). Samples were then immunoprecipitated for 2 h at room temperature with anti-HLA-G MoAb (MEM-G9; Exbio), washed twice in PBS and incubated overnight with protein G-Sepharose beads (Santa Cruz, CA, USA) at 4°C. The samples were washed twice and suspended in 20 μl of Laemmli Buffer (Bio-Rad, Segrate, Milan, Italy). The protein concentration in immunoprecipitates was quantified by the Bradford assay (Bio-Rad Laboratories) using plasma bovine albumin (Sigma-Aldrich) as the standard. The purified sHLA-G1 molecules obtained from untreated 221-G1 culture supernatants were used as positive controls. Total protein was denatured at 100°C for 5 min. Proteins were loaded with or without reducing buffers in 10% TGX-Pre-cast gel (Bio-Rad), with subsequent electroblotting transfer onto a PVDF membrane (Millipore). The membrane was incubated with an HRP-conjugated streptavidin (Thermo Scientific, Rockford, IL, USA) and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by the Bio-Rad Gel Doc (Bio-Rad,

Table 1. The characteristics of CD patients and controls

Characteristics	Patients	Controls	*P* value
Female *n* (%)/male *n* (%)	24 (54.5)/20 (57.7)	41 (45.5)/30 (42.3)	0.740[a]
Age [M ± SEM (25% P–75% P)]	36.89 ± 1.85 (27.00–47.00)	34.04 ± 1.37 (24.00–43.00)	0.118[b]
Disease onset: young (%)/adult (%)	18 (40.9)/26 (50.1)	—	
Disease duration [M ± SEM (25% P–75% P)]	6.57 ± 0.81 (2.00–8.00)	—	

M, mean; P, percentile; SEM, standard error of the mean.
[a]2 × 2 contingency table: χ^2 = 011.
[b]Mann–Whitney test: U = 1301.

Milano, Italy). Monomers were detected at 39 kDa, dimers at 78 kDa.

Statistical analysis

Statistical analysis was performed with SPSS (16.0) and by Graphpad prism 5. Comparison between baseline characteristics and levels of sHLA-G among patients and controls were estimated by the Mann–Whitney *U*-test.

The differences in genotypic/allelic frequencies between patients and controls were evaluated by the chi-square (χ^2) test (2×2 contingency table for alleles and 2×3 contingency table for genotypes). Pearson χ^2 or Fisher's exact test was used to assess inter-group significance. Spearman test was used to test for correlations between quantitative variables.

14-bp polymorphism was tested for Hardy–Weinberg equilibrium using http://oege.org/software/hwe-mr-calc.shtml. Two-tailed P values <0.05 were considered statistically significant.

Results

Study population

A total of 44 patients with CD were included in this study. Table 1 summarizes their main demographic and clinical characteristics. No significant differences were noticed either for subgroups stratified by genders ($P = 0.113$) or for the participants' age ($P = 0.118$). Among patients, the mean of CD duration was 6.57 ± 0.81 years.

HLA-G genetic polymorphism typing

Allelic and genotypic frequencies of 14-bp HLA-G polymorphism among CD cases and control subjects are presented in Tables 2 and 3. No Hardy–Weinberg equilibrium deviation was observed in the control and CD samples for the 14-bp HLA-G polymorphism ($\chi^2 = 0.041$; $P = 0.839$).

No different allele and genotype frequencies were observed between CD patients and control subjects ($\chi^2 = 0.801$, $P = 0.371$) (Table 2). When we stratified according to CD young-onset ($n = 18$; mean age of CD onset: 25.56 ± 1.20 years, range: 20–38 years old) and adult-onset ($n = 26$; mean age of CD onset: 43.36 ± 1.97 years, range: 29–69 years old), we found an increased frequency of the Ins allele in young-onset CD patients ($\chi^2 = 5.358$, $P = 0.020$) (Table 3) in comparison with controls. Similarly, there was an over-representation of the Ins/Ins genotype ($\chi^2 = 4.718$, $P = 0.029$) (Table 3). In young-onset CD patients, the Ins allele was associated with a 2.438-fold [95% confidence interval (CI): 1.131–5.254] higher risk of CD susceptibility compared with the Del allele. Moreover, when we considered the three genotypes separately, we found evidence of a tendency to a statistical significance for the association between the three Del/Ins 14-bp genotypes and CD susceptibility in young-onset patients ($\chi^2 = 5.560$, $P = 0.062$). In particular, the Ins/Ins genotype was associated with a 3.257-fold (95% CI: 1.086–9.770) increased risk for CD susceptibility compared with the Del/Del and Del/Ins genotypes (Table 3). No differences were observed in adult-onset patients (data not shown).

Allelic and genotypic frequencies of the 14-bp polymorphism in CD patients after stratification for clinical phenotypes

Patients with CD were stratified for disease behavior (inflammatory, stenotic or inflammatory and stenotic phenotype), disease location (ileum and colon/ileum only or colon only) and surgical resection (resection or not) (Table 4). The Ins allele was frequent in the stenotic phenotype (62.5%) and in patients with CD located in both colon and ileum (56.2%) in comparison with total CD patients (51.1%). However, no statistically significant association was found after the three cited stratifications. No correlation was found between 14-bp polymorphism and extra-intestinal manifestations (data not shown).

Increase of sHLA-G in CD patients

CD patients and controls analyzed for sHLA-G plasma levels presented similar age (mean ± SEM, patients: 38.6 ± 2.38 versus controls: 34.22 ± 1.58, Mann–Whitney: $P = 0.156$) and similar sex ratio [males/females, patients: 14/16 (ratio = 0.88) versus controls: 26/32 (ratio = 0.81)].

The means of sHLA-G were 5.20 (SEM = 1.06) ng ml^{-1} and 2.14 (SEM = 0.45) ng ml^{-1} in CD patients and controls, respectively (Fig. 1A) and were statistically different ($P = 0.002$). In addition, the number of sHLA-G^{positive} CD patients was enhanced ($n = 25/30$, 76.7%) compared with controls ($n = 25/58$, 43.1%) ($P = 3 \times 10^{-4}$).

Table 2. Distribution of allelic and genotypic frequencies of Del/Ins 14-bp polymorphism in CD patients (without stratification) and controls

14-bp Del/Ins (rs66554220)	Patients ($n = 44$), n (%)	Controls ($n = 71$), n (%)	χ^2	P value	OR (95% CI)
Alleles					
Del	43 (48.9)	78 (54.9)	0.80	0.37	1.27 (0.75–2.17)
Ins	45 (51.1)	64 (45.1)			
Genotypes[a,b]					
Del/Del	11 (25)	21 (29.6)	0.28	0.59	0.79 (0.34–1.86)
Ins/Ins	12 (27.3)	14 (19.7)	0.89	0.35	1.53 (0.63–3.70)
Del/Ins	21 (47.7)	36 (50.7)	0.09	0.76	0.89 (0.42–1.89)

14 bp, 14 base pairs; Del, deletion; Ins, insertion; OR, odds ratio.
[a]2×3 contingency table: $\chi^2 = 0.94$, $P = 0.63$.
[b]Hardy–Weinberg equilibrium: $\chi^2 = 0.04$, $P = 0.98$.

Table 3. Distribution of allelic and genotypic frequencies of Del/Ins 14-bp polymorphism in young-onset CD Patients (stratification by disease onset) and Controls

14-bp Del/Ins (rs66554220)	Patients (n = 18), n (%)	Controls (n = 71), n (%)	χ^2	P value	OR (95% CI)
Alleles					
Del	12 (33.3)	78 (54.9)	5.36	**0.02**	2.44 (1.13–5.25)
Ins	24 (66.7)	64 (45.1)			
Genotypes[a]					
Del/Del	2 (11.1)	21 (29.6)	—	0.14[b]	0.30 (0.06–1.44)
Ins/Ins	8 (44.4)	14 (19.7)	4.72	**0.03**	3.26 (1.08–9.77)
Del/Ins	8 (44.4)	36 (50.7)	0.23	0.64	0.78 (0.28–2.20)

14 bp, 14 base pairs; Del, deletion; Ins, insertion; OR, odds ratio. Significant P values are highlighted in bold.
[a]2 × 3 contingency table: $\chi^2 = 5.56$, $P = 0.06$.
[b]Fisher's exact test.

Table 4. Distribution of allelic and genotypic frequencies of Del/Ins 14-bp polymorphism in CD patients after stratification for clinical phenotypes

Genotype/allele	Disease behavior			Disease location		Resection or not	
	Inflammatory phenotype, n = 20/43 (%)	Stenotic phenotype, n = 12/43 (%)	Inflammatory and stenotic phenotype, n = 11/43 (%)	Location in both ileum and colon, n = 19/43 (%)	Location in ileum only or colon only, n = 24/43 (%)	No resection	Resection
Del	23 (57.5)	9 (37.5)	10 (45.5)	21 (43.8)	21 (55.3)	28 (50)	14 (50)
Ins	17 (42.5)	15 (62.5)	12 (54.4)	27 (56.2)	17 (44.7)	28 (50)	14 (50)
Del/Del	8 (40)	2 (16.7)	1 (9.1)	4 (16.7)	7 (36.8)	8 (28.6)	3 (21.4)
Ins/Ins	5 (25)	5 (41.7)	2 (18.2)	7 (29.2)	5 (26.3)	8 (28.6)	3 (21.4)
Del/Ins	7 (35)	5 (41.7)	8 (72.7)	13 (54.2)	7 (36.8)	12 (42.9)	8 (57.1)

Similarly, the mean of sHLA-G1 was increased in CD patients compared with controls (3.87 ± 0.92 versus 1.10 ± 0.32 ng ml^{-1}) ($P < 0.0001$, Fig. 1B). The number of positive plasma samples for sHLA-G1 was enhanced for CD patients (n = 21/30, 70% versus 12/58, 20.7%) ($P = 1.6 \times 10^{-5}$).

On the contrary, the levels of HLA-G5 were lower in CD patients compared with controls without reaching statistical significance (1.33 ± 0.67 versus 1.05 ± 0.31 ng ml^{-1}) (P = 0.37, Fig. 1C). The number of positive plasma samples for HLA-G5 was enhanced for controls (n = 16/58, 27.6% versus 5/30, 16.7%) (P = 0.3).

There was no significant association between age, sex or duration of illness with sHLA-G, sHLA-G1 or HLA-G5 levels (data not shown).

Association of sHLA-G to 14-bp Del/Ins polymorphism

According to previous studies (17, 24), the presence of one or two 14-bp Del alleles (14-bp Del/Del and 14-bp Del/Ins genotypes) is associated with higher HLA-G production. Similarly, we observed that CD patients with high-producer genotypes (14-bp Del/Del, Del/Ins) expressed increased sHLA-G mean plasma levels [7.36 ± 1.58 (SEM) ng ml^{-1}] in comparison with the low-producer genotype (14-bp Ins/Ins) (5.14 ± 1.00 ng ml^{-1}) even without reaching a statistical significance (P = 0.869) (Fig. 2A). A similar profile was found for sHLA-G1 concentrations, where high-producer genotypes presented a mean of 6.17 ± 1.42 versus 4.32 ± 1.30 ng ml^{-1} for low producers, even without reaching a statistical significance (P = 0.282) (Fig. 2B). On the contrary, the 14-bp Del/Del and 14-bp Del/Ins genotypes presented lower concentrations of HLA-G5 (1.20 ± 0.81 ng ml^{-1}) compared with the 14-bp Ins/Ins genotype (1.69 ± 1.12 ng ml^{-1}), even without reaching a statistical significance (P = 0.54) (Fig. 2C). The majority of high sHLA-G producers belong to the adult-onset CD patients subgroup (71.9%) compared with low sHLA-G producers that mainly belong to the young-onset CD patients subgroup (58.3%).

Association of sHLA-G dimers with advanced CD stages

Since HLA-G molecules have both monomeric and dimeric conformations, we performed western blot analysis on plasma samples positive for sHLA-G in ELISA. We easily distinguished the monomeric and dimeric conformations of sHLA-G (Fig. 3). The western blot analysis indicated 43% of CD patients with sHLA-G dimers versus 78% for controls.

We evaluated the presence of sHLA-G dimers in association with clinical characteristics (Table 5). We observed a significant difference between genders in CD subgroups based on the presence/absence of sHLA-G dimers (P = 0.04). Indeed, males present increased sHLA-G dimers compared with females (70 versus 30%) (P = 0.04). The absence of HLA-G dimers in patients with CD seems to be related to progressed

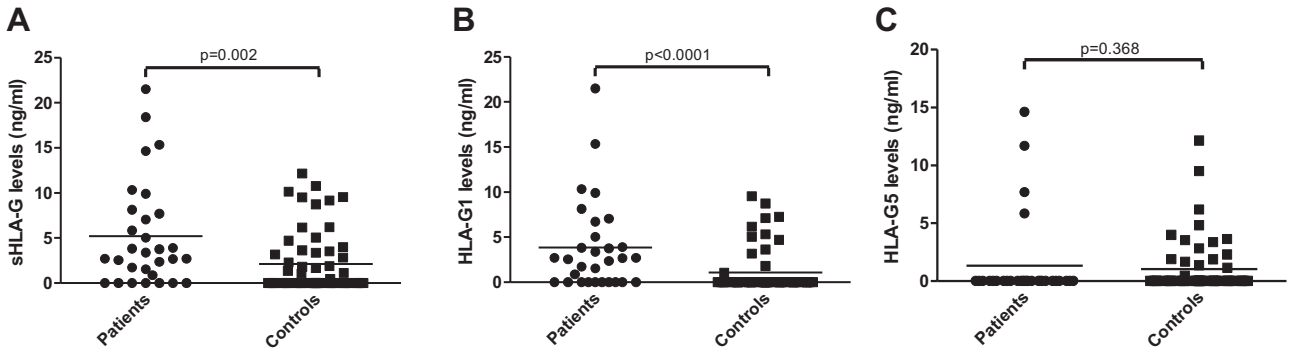

Fig. 1. Box-plot of serum sHLA-G (sHLA-G1 and HLA-G5) (A), sHLA-G1 (B) and HLA-G5 (C) concentrations in patients with CD and in healthy controls. Mean levels are indicated by horizontal lines. *P* values were obtained by Mann–Whitney test.

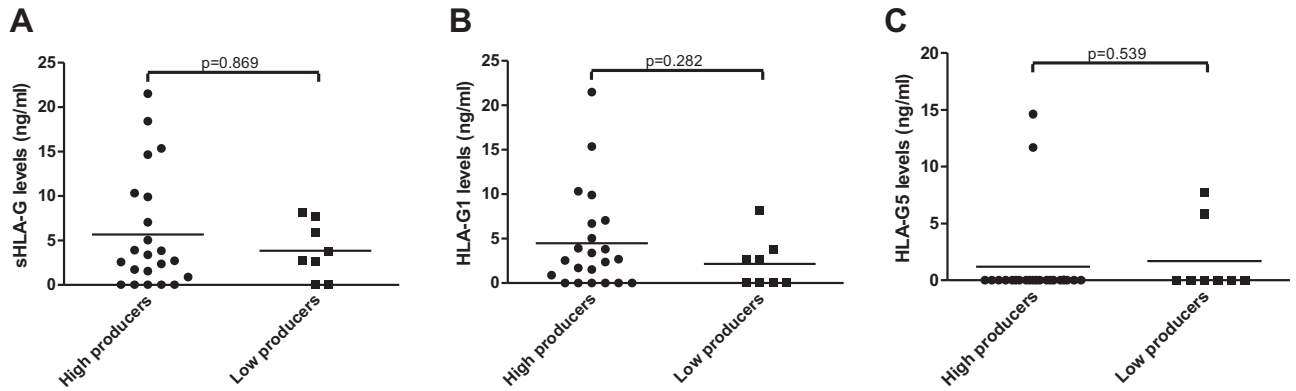

Fig. 2. Box-plot of serum sHLA-G (sHLA-G1 and HLA-G5) (A), sHLA-G1 (B) and HLA-G5 (C) concentrations in patients with CD subdivided according with HLA-G 14-bp Ins/Del genotypes. Mean levels are indicated by horizontal lines. *P* values were obtained by Mann–Whitney test. High producers: 14-bp Del/Del, Del/Ins; low producers: 14-bp Ins/Ins.

stages of CD characterized by an inflammatory phenotype (69.2% of CD patients with dimers) and an extended location covering the ileum or the colon (46.2% of CD patients with dimers) even without reaching a statistical significance (Table 5).

Discussion

Previous work has evaluated the role of HLA-G molecules in CD. In particular, Torres *et al*. (25) studied intestinal samples of CD patients and, by an immunohistochemistry technique, demonstrated that CD intestinal biopsies did not present HLA-G expression. The distribution of the 14-bp Del/Ins polymorphism in CD patients was investigated by Glas *et al*. (26). They observed a significant increase of the Ins allele and the Ins/Ins genotype in those CD cases positive for ileocecal resection. Also Rizzo *et al*. (19) evaluated HLA-G expression in CD patients. Non-activated peripheral blood mononuclear cells from CD patients spontaneously secrete sHLA-G.

The present study was aimed at gaining further insight into the role of the genetic polymorphism HLA-G 14-bp Del/Ins in CD and the possible effect on HLA-G expression. Our results provide suggestive evidence for an association of this HLA-G polymorphism with the susceptibility to CD in young-onset CD patients. In fact, we observed an increased Ins allele and Ins/Ins genotype frequency in young-onset CD patients, where the Ins allele was associated with a 2.438-fold higher risk of susceptibility to CD compared with the Del allele. Moreover, the Ins/Ins genotype was associated with a 3.257-fold increased risk of CD susceptibility compared with the Del/Del and Del/Ins genotypes. These data sustain the proposed role of the HLA-G 14-bp Del/Ins polymorphism in CD. Similarly to Glas *et al*. (26), we found a tendency for an increase in Ins allele frequency in those CD patients with the stenotic phenotype and location in both ileum and colon. These results could explain the maintenance of local inflammatory conditions in CD patients, where an increase in the Ins allele frequency could subtend to a lower HLA-G expression.

To confirm this point, we considered sHLA-G plasma levels and we observed significantly higher levels of sHLA-G and in particular of the sHLA-G1 isoform and an increased number of positive sHLA-G samples in CD patients in comparison with controls. As membranous HLA-G has not been checked in intestinal samples from CD patients (25), we hypothesized that sHLA-G1 could not be issued from the local site of inflammation but from circulating immune cells including regulatory T cells (27, 28), and dendritic cells (29), that subtend a dysregulated immune response.

sHLA-G1 could derive from membrane-bound HLA-G1 cleavage due to type 2 metalloproteinases, which are highly expressed in CD patients (30–32). This hypothesis is reinforced by the recent finding by Rizzo *et al*. that showed three possible highly specific cleavage sites for matrix metalloproteinase-2 (33). The systemic production of sHLA-G molecules

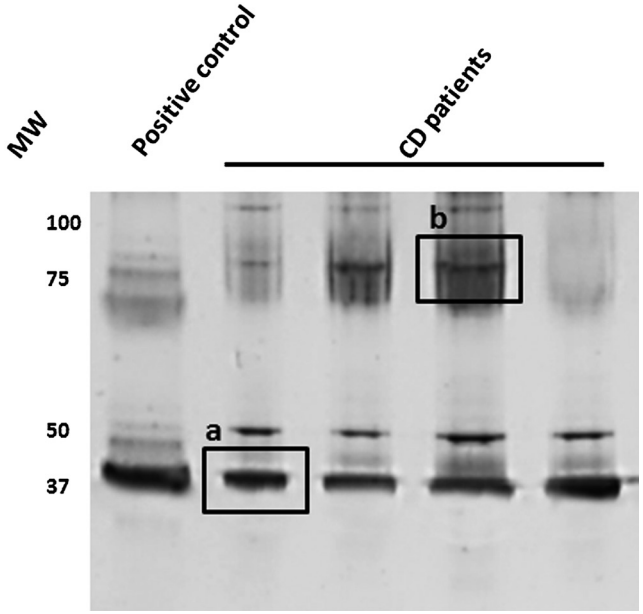

Fig. 3. sHLA-G expression and dimerization in patients with CD. Positive control corresponds to 221-G1 culture supernatant; (a) monomers of sHLA-G (sHLA-G1 and HLA-G5) (39 kDa); (b) dimers of sHLA-G (78 kDa). Samples were immunoprecipitated with MEM-G9 monoclonal antibody.

Table 5. The characteristics of CD patients sHLA-G^{positive} based on presence/absence of HLA-G dimers

	CD patients		*P* value[a]
	HLA-G dimers	No HLA-G dimers	
Female *n* (%)/male *n* (%)	3 (30)/7 (70)	10 (76.9)/3 (23.1)	**0.04**
Age [M ± SEM (25% P–75% P)]	40.80 ± 3.83 (32.75–49.25)	39.38 ± 4.13 (24.50–50.50)	0.80[b]
Disease duration [M ± SEM (25% P–75% P)]	9.20 ± 2.35 (1.00–16.75)	4.15 ± 0.66 (1.50–6.00)	0.15[b]
Disease behavior			
Inflammatory phenotype, *n* (%)	3 (30)	9 (69.2)	0.09
Stenotic phenotype, *n* (%)	2 (20)	1 (7.7)	0.56
Inflammatory and stenotic phenotype, *n* (%)	5 (50)	3 (23.1)	0.22
Disease location			
Location in both ileum and colon, *n* (%)	8 (80)	7 (53.8)	0.37
Location in ileum only or colon only, *n* (%)	2 (20)	6 (46.2)	0.38
Anal-perianal fistulas			
No, *n* (%)/yes, *n* (%)	6 (60)/4 (40)	12 (92.3)/1 (7.7)	0.12
Abscess			
No, *n* (%)/yes, *n* (%)	9 (90)/1 (10)	12 (92.3)/1 (7.7)	1
Resection or not			
No resection, *n* (%)	5 (50)	10 (76.9)	0.22
Resection, *n* (%)	5 (50)	3 (23.1)	

M, mean; P, percentile; SEM, standard error of the mean. Significant *P* values are highlighted in bold.
[a]2 × 2 contingency table: Fisher's exact text.
[b]Mann–Whitney test.

in CD patients could, tentatively, represent a way to counteract the inflammatory condition.

As a confirmation, of the previous data on the effect of the HLA-G 14-bp Del/Ins polymorphism on HLA-G expression (16–18), we observed that patients with high-producer genotypes (Del/Del, Del/Ins) expressed an increased sHLA-G mean level in comparison with the sHLA-G low-producer genotype (14-bp Ins/Ins) in CD patients. A similar profile was found for sHLA-G1 concentrations. We report here clear evidence of the correlation of the Del allele to enhanced production of sHLA-G, while low sHLA-G producers (Ins/Ins genotype) were included essentially in young-onset CD patients and were mainly characterized by HLA-G5 secretion. We could hypothesize a different cytokine environment, in young-onset CD patients, that could sustain HLA-G5 production also in the low-producer genotype. These results confirm the association

of the Ins allele with low sHLA-G concentrations and sustain the proposed role of this HLA-G polymorphism in controlling HLA-G expression also in a pathological condition as CD. However, the presence of higher levels of systemic sHLA-G in CD patients in comparison with controls could, tentatively, represent a way to counteract an inflammatory condition.

Since HLA-G can present monomeric and dimeric conformation, we evaluated the plasma composition in both controls and CD patients. We observed 43% of CD patients with sHLA-G dimers versus 78% for controls. These data are of extreme interest, since they could explain the contrasting results obtained with the levels of sHLA-G in plasma samples. In fact, even if we found higher levels of sHLA-G in CD plasma samples, they are mainly characterized by a monomeric conformation, that is known to have a lower affinity for inhibitory receptors (6). These results sustain the proposed importance of evaluating not only HLA-G expression but also the monomer/dimer conformation. Additionally, the immunoprecipitation and western blot revealed some extra bands at 53 KDa that could be HLA-G-like molecules (34). When we correlated sHLA-G conformations with clinical characteristics, we observed the presence of sHLA-G dimers in CD patients with advanced stages of CD characterized by an inflammatory and stenotic phenotype and an extended location covering the ileum and the colon. We could hypothesize that sHLA-G dimers are increased in advanced stages of CD as an attempt to counteract inflammation.

To our knowledge, hitherto, this is the first study to describe the genotype of the 14-bp Del/Ins polymorphism, the association with HLA-G expression and the monomer/dimer conformation in CD patients. Our data strengthen the proposed functional role of HLA-G molecules in CD and suggest a potential use for clinical purposes as a prognostic marker for progressive disease.

Conflict of interest statement: The authors declared no conflicts of interest.

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