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Susceptibility role of soluble HLA-G and HLA-G 14-bp insertion/deletion polymorphism in inflammatory bowel disease

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Abstract

Background: Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders of the gastrointestinal tract. It is fundamentally related to a dysregulated immune response in the intestinal mucosa against microbiota in genetically predisposed individuals. Among the genetic and immunological factors that are suggested to have role in etiology and pathogenesis of IBD are human leukocyte antigen (HLA)-G molecules. Therefore, soluble HLA-G (sHLA-G) serum level and genetic association with HLA-G 14-bp insertion (Ins)/deletion (Del) polymorphism was analyzed in 100 IBD patients; 50 ulcerative colitis (UC) and 50 Crohn's disease (CD), and 100 controls.

Results: sHLA-G level was significantly elevated in IBD patients compared to controls (174.7 ± 27.1 vs. 126.8 ± 15.1; corrected probability [pc] < 0.001). The level was also elevated in UC patients compared to CD patients but the difference was not significant (180.5 ± 27.1 vs. 168.9 ± 26.3; $p = 0.059$). Receiver operating characteristic analysis confirmed the significance of sHLA-G in total IBD, UC, and CD patients (area under curve = 0.944, 0.961, and 0.927, respectively). The genetic association was analyzed under five genetic models (allele, recessive, dominant, overdominant, and codominant). At the allele level, *Del* allele frequency was significantly increased in total IBD patients (Odds ratio [OR] = 1.93; 95% confidence interval [CI] = 1.27–2.94; pc = 0.018) and CD patients (OR = 2.08; 95% CI = 1.23–3.54; pc = 0.042) compared to controls. Among UC patients, a similar increased frequency was observed, but the pc value was not significance (OR = 1.79; 95% CI = 1.07–3.00; $p = 0.031$). At the genotypic level, Del/Del genotype was associated with a significantly increased IBD-risk in total patients under codominant model (OR = 4.06; 95% CI = 1.56–10.56; pc = 0.024). sHLA-G level was not influenced by the Ins/Del polymorphism.

Conclusions: This study demonstrated a significant increase in serum level of sHLA-G in UC and CD patients. Further, HLA-G 14-bp Ins/Del polymorphism may be associated with susceptibility to IBD, particularly CD.

Keywords: Inflammatory bowel disease, Ulcerative colitis, Crohn's disease, Soluble HLA-G, 14-bp Ins/Del polymorphism

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Background

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders of the gastrointestinal tract. It is fundamentally related to a dysregulated immune response in the intestinal mucosa against microbiota in genetically predisposed individuals [1]. Two major phenotypes of IBD are clinically recognized: ulcerative colitis (UC) and Crohn's disease (CD). The inflammation in UC is restricted to the colon and rectum, while in CD, it involves any part of the gastrointestinal tract in a non-continuous fashion [2]. Their clinical presentations are similar, but diarrhea and bleeding are most likely presented in UC, whereas watery diarrhea and vague symptoms are encountered in CD [3]. Etiology of both phenotypes is incompletely understood, but complex interactions between genetic, environmental, immunological, and gut microbiomial factors are suggested to be involved. Their interactions orchestrate a cascade of inflammatory responses in the intestinal mucosa [4]. Among the genetic and immunological factors that are proposed to have a role in etiology and pathogenesis of IBD are human leukocyte antigen (HLA)-G molecules, which are encoded by the non-classical major histocompatibility (MHC) class I genes that are mapped to the short arm of chromosome 6 (6p21.3) [5–7].

HLA-G molecules are characterized by restricted tissue expression. Under non-pathological conditions, they are principally expressed on the extra-villous cytotrophoblast cells and tissues at the feto-maternal interface [8]. Functionally, HLA-G molecules create a state of tolerance to protect the fetus from the maternal immune response against the fetal semi-allograft [9]. The tolerogenic properties of HLA-G molecules are augmented through interactions with cell-surface receptors present on natural killer (NK) cells, T and B cells and antigen-presenting cells [10]. However, upregulation of HLA-G has also been demonstrated under pathological conditions, especially in diseases characterized by inflammation. Therefore, it has been suggested that these molecules enhance immune surveillance as a possible mechanism of tissue protection against inflammatory responses [11–13]. Seven isoforms of HLA-G molecules are recognized (HLA-G1–G7). The first four isoforms (G1–G4) are cell-surface bound proteins, while G5–G7 are soluble proteins (sHLA-G) [8].

The HLA-G gene is presented with low allelic polymorphism compared other classical HLA-class I molecules. Up to April 2020, 78 alleles have been recognized at the DNA level and 21 alleles at the protein level, as well as, four null alleles have also been registered [14]. In addition, HLA-G gene exhibits a further polymorphism, which is 14 base-pairs (14-bp) insertion (Ins)/deletion (Del) at the 3′ untranslated region (3′ UTR) of exon 8. Investigations have linked this polymorphism to post-transcriptional regulation of HLA-G molecules, and thus its effect on the stability of mRNA has been proposed [15, 16].

The role of HLA-G molecules in pathogenesis of IBD or their effect on susceptibility to disease has not been well-elaborated. In 2004, the expression of HLA-G in intestinal biopsies was analyzed in UC and CD patients. It was found that HLA-G was only expressed in biopsies of UC patients, whereas CD samples showed no expression [17]. In a further study, the level of sHLA-G was determined in supernatants of cultured peripheral blood mononuclear cells (PBMCs) obtained from 18 UC and 10 CD patients. The results showed different expression of sHLA-G between the two phenotypes of IBD, and a spontaneous secretion of sHLA-G was depicted in CD patients but not in UC or healthy subjects [18]. In a more recent immunohistochemical study, expression of HLA-G was noticed in UC and CD biopsies; however, the expression of HLA-G5 isoform was differentially correlated with levels of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) [7]. With regard to HLA-G 14-bp Ins/Del polymorphism, two previous studies suggested the significance of this polymorphism in IBD risk [19, 20].

Due to the growing importance of HLA-G molecules in etiology and pathogenesis of IBD (UC and CD), this study analyzed serum level of sHLA-G in UC and CD of Iraqi Arab patients categorized according to gender, cigarette-smoking status, disease duration, family history, disease extension and medication. The genetic association of HLA-G 14-bp Ins/Del polymorphism with both phenotypes of IBD was also analyzed.

Methods

Populations studied

During January–June 2019, a case-control study was conducted on 100 Iraqi Arab IBD patients (50 UC and 50 CD). Ethnicity matched 100 healthy subjects were taken as controls. The patients attended the outpatient gastrointestinal clinics at Al-Kindy Teaching Hospital, Baghdad Teaching Hospital and Gastroenterology and Hepatology Teaching Hospital in Baghdad for diagnosis and treatment. The diagnosis was based on standard clinical, radiological, endoscopic, and histopathological criteria [3]. Patients with indeterminate colitis or other related autoimmune diseases were excluded. The patients were stratified according the following parameters: age, gender, current cigarette-smoking status, disease duration, family history, laboratory findings (hemoglobin; Hb, white blood cell count; WBC and erythrocyte sedimentation rate; ESR), disease extension, symptoms, extra-intestinal manifestations, and medication (Table 1). The control sample included blood donors who were healthy and there serum profile for anti-pathogen antibodies was negative (Central Blood Bank, Baghdad). All participants gave

Table 1 Baseline characteristics of inflammatory bowel disease (ulcerative colitis and Crohn's disease) patients and controls

Characteristics[a]	UC (*N* = 50)	CD (*N* = 50)	Controls (*N* = 100)	*p*
Age	31.5 ± 10.1	30.5 ± 9.7	31.2 ± 9.8	0.864
Gender				0.990
Male	28 (56.0)	28 (56.0)	57 (57.0)	
Female	22 (44.0)	22 (44.0)	43 (43.0)	
Cigarette-smokers	40 (80.0)	35 (70.0)	39 (39.0%)	**< 0.001**
Disease duration (years)				0.314
≤ 3	20 (40.0)	25 (50.0)	NA	
> 3	30 (60.0)	25 (50.0)	NA	
Positive family history	7 (14.0)	8 (16.0)	NA	0.779
Hb (mg/dL)	11.0 ± 3.0	10.5 ± 3.5	NA	0.682
WBC ($\times 10^9$/L)	8.1 ± 3.6	7.7 ± 3.1	NA	0.783
ESR (mm/hour)	54.3 ± 22.5	58.8 ± 42.4	NA	0.876
Disease extension				
Ulcerative proctitis	20 (40.0)	NA	NA	
Left-sided colitis	15 (30.0)	NA	NA	
Extensive colitis	15 (30.0)	NA	NA	
Ileocecal	NA	43 (86.0)	NA	
Ileo-colonic	NA	7 (14.0)	NA	
Symptoms				
Abdominal/or colon pain	33 (66.0)	33 (66.0)	NA	1.000
Diarrhea	29 (58.0)	27 (54.0)	NA	0.840
Fever	22 (44.0)	27 (54.0)	NA	0.424
Extra-intestinal manifestations				
Aphthous ulcer	9 (18.0)	15 (30.0)	NA	0.241
Arthralgia	34 (68.0)	38 (72.0)	NA	0.504
Skin ulcer	7 (14.0)	3 (6.0)	NA	0.318
Appendectomy	6 (12.0)	7 (14.0)	NA	1.000
Bowel stricture	4 (8.0)	3 (6.0)	NA	1.000
Colostomy	5 (10.0)	6 (12.0)	NA	1.000
Fistula	10 (20.0)	6 (12.0)	NA	0.414
Hemorrhoids	6 (12.0)	3 (6.0)	NA	0.487
Medication				0.556
Protocol A	14 (28.0)	12 (24.0)		
Protocol B	12 (24.0)	8 (16.0)		
Protocol C	11 (22.0)	11 (22.0)		
Protocol D	13 (26.0)	19 (38.0)		
Adalimumab or infliximab doses				0.614
0	12 (24.0)	8 (16.0)		
1–10	20 (40.0)	18 (36.0)		
11–20	13 (26.0)	18 (36.0)		
21–30	5 (10.0)	6 (12.0)		

[a]Data are either mean ± standard deviation or absolute number followed by percentage in parentheses: *UC* ulcerative colitis, *CD* Crohn's disease, *Hb* hemoglobin, *WBC* white blood cell, *ESR* erythrocyte sedimentation rate, *protocol A* anti-inflammatory and immunosuppressive, *protocol B* anti-inflammatory and antibiotics, *protocol C* anti-inflammatory, immunosuppressive and antibiotics, *protocol D* immunosuppressive only. *p* LSD (least significant), Pearson's chi-squared or Fisher's exact test probability, *NA* not applicable. Significant *p* is bold-marked

written consent to participate in the study. The approval of Ethics Committees at the target hospitals was obtained to conduct the study (N264 on 13/01/2019). All participants (50 UC and 50 CD and 100 controls) were genotyped for HLA-G 14-bp Ins/Del polymorphism, while serum level of sHLA-G was determined in 90 randomly selected participants (30 UC, 30 CD, and 30 controls).

Determination sHLA-G

Serum level of sHLA-G was determined using human HLA-G enzyme-linked immunosorbent assay kit (catalog no.: E-EL-H1663) according to instructions of manufacturer (Elabscience, China).

Genotyping of HLA-G 14-bp Ins/Del polymorphism

Genomic DNA was isolated from EDTA blood using DNA purification kit (Geneaid, Taiwan) following instructions of manufacturer. Sequence-specific polymerase chain reaction (PCR) assay was accomplished to genotype 14-bp Ins/Del polymorphism in exon 8 of *HLAG* gene (3′-untranslated region) using a thermocycler (Eppendorf, Germany) followed by agarose gel electrophoresis as previously depicted [21]. Briefly, the reaction mix (25 μL) consisted of 5 μL DNA (60 ng/mL), 12.5 μL 1× Green Master mix (Bioneer, Korea), 1 μL forward primer (5′-GTGATGGGCTGTTTAAAGTGTCACC-3′; 10 pmol/μL), 1 μL reverse primer (5′-GGAAGGAATGCAGT TCAGCATGA -3′; 10 pmol/μL) and 5 μL nuclease-free water. The optimized thermocycling conditions were initial denaturation at 94 °C min (5 min), followed by 35 cycles of denaturation and 94 °C (30 s), annealing at 60 °C (30 s), and extension at 72 °C (30 s), and a final extension cycle at 72 °C for 5 min. The amplified PCR products were electrophoresed in 3% agarose gels. The bands were visualized with UV trans-illuminator after staining with ethidium bromide. Product sizes were 210 bp and 224 bp for *Del* and *Ins* alleles, respectively.

Statistical analysis

Data were statistically analyzed using the statistical package IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.). Categorical variables were given as numbers and percentages. Continuous variables were tested for normality distribution (Kolmogorov-Smirnov and Shapiro-Wilk tests). All continuous variables were normally distributed; therefore, they were presented as mean ± standard deviation (SD). Pearson's chi-squared and Fisher's exact tests were used to compare categorical variables. One-way analysis of variance (ANOVA) was used to compare continuous variables followed by the post hoc test LSD (least significant difference). Receiver operating characteristic (ROC) was used to determine area under curve (AUC), sensitivity, specificity, and cut-off value. Direct gene counting method was used to estimate allele frequencies of Del/Ins 14-bp polymorphism. Assumption of Hardy-Weinberg equilibrium (HWE) was tested using Pearson's chi-squared goodness-of-fit test. Logistic regression analysis was employed to calculate odds ratio (OR) and 95% confidence interval (CI) under five genetic models (allele, recessive, dominant, overdominant and codominant). A probability (p) value ≤ 0.05 was considered significant after applying Bonferroni correction (corrected p; pc). G*Power software (version 3.1.9.4) was used to estimate power of sample size.

Results

Power of sample size

Power of sample size (1−β error probability) was estimated for UC and CD. At an α error of probability of 0.05 and an effect size of 0.3, the power for a sample size of 50 cases was 0.71, which was below the marginal power of 0.8. However, merging UC and CD cases in one group (IBD) elevated the power to 0.93.

Baseline characteristics of populations studied

Mean age and gender distribution showed no significant variation between IBD patients (UC and CD) and control. However, most of UC and CD patients were cigarette-smokers (80.0 and 70.0%, respectively), while among control, smokers accounted for 39.0%. Such difference was statistically significant ($p < 0.001$). There were no significant differences between UC and CD patients regarding disease duration (≤ 3 and > 3 years), positive family history, laboratory findings (Hb, WBC, and ESR), symptoms (abdominal pain, diarrhea and fever), and extra-intestinal manifestations (aphthous ulcer, arthralgia, skin ulcer, appendectomy, bowel stricture, colostomy, fistula, and hemorrhoids). With regard to disease extension, UC patients were classified as ulcerative proctitis (40%), left-sided colitis (30%), and extensive colitis (30%), while most of CD patients were classified under ileocecal colitis (86%). For medication, four protocols were used: anti-inflammatory and immunosuppressive, anti-inflammatory and antibiotics, anti-inflammatory, antibiotics and immunosuppressive, and immunosuppressive only. Patients who received the immunosuppressive medicines adalimumab or infliximab were divided into four groups according to the number of doses received: 0, 1–10, 11–20, and 21–30 doses (Table 1).

Serum level of sHLA-G

Serum level of sHLA-G was significantly elevated in IBD patients compared to controls (174.7 ± 27.1 vs. 126.8 ± 15.1; pc < 0.001). The level was also elevated in UC patients compared to CD patients but the difference was not significant (180.5 ± 27.1 vs. 168.9 ± 26.3; $p = 0.059$) (Table 2). ROC analysis confirmed the significance of sHLA-G antigens in total IBD patients, as well as UC

Table 2 Serum level of soluble HLA-G antigens in inflammatory bowel disease (ulcerative colitis and Crohn's disease) patients and controls

Group	sHLA-G (mean ± SD); ng/ml	p_1	p_2
Controls (*N* = 30)	126.8 ± 15.1		
IBD (*N* = 60)	174.7 ± 27.1	**< 0.001**	
UC (*N* = 30)	180.5 ± 27.1	**< 0.001**	0.059
CD (*N* = 30)	168.9 ± 26.3	**< 0.001**	

Significant *p* is bold-marked
IBD inflammatory bowel disease, *UC* ulcerative colitis, *CD* Crohn's disease, p_1 LSD (least significant difference) probability compared to control, p_2 UC vs. CD

and CD patients (AUC = 0.944, 0.961, and 0.927, respectively) (Fig. 1).

sHLA-G and characteristics of patients

UC male patients showed a significantly elevated mean of sHLA-G antigens compared to female patients (192.0 ± 24.1 vs. 170.5 ± 26.2; pc = 0.03) or control males (192.0 ± 24.1 vs. 173.5 ± 25.1; pc = 0.05). Cigarette-smoker UC patients also showed a significant increase in mean of sHLA-G compared to smoker CD patients (187.9 ± 24.2 vs. 162.8 ± 25.9; pc = 0.04). However, distributing UC and CD patients according to the other characteristics revealed no significant variations in sHLA-G levels (Table 3).

Frequency of HLA-G 14-bp Ins/Del polymorphism in controls

Genotype frequencies of HLA-G 14-bp Ins/Del polymorphism in controls of this study were in a good agreement with HWE equilibrium. The allele *Del* was more frequently observed than *Ins* allele (59 vs. 41%). However, allele and genotype frequencies of the HLA-G 14-bp Ins/Del polymorphism showed significant variation compared with other world populations (Table 4).

HLA-G 14-bp Ins/Del polymorphism-disease association

Five genetic models (allele, recessive, dominant, overdominant, and codominant) were adopted to assess the genetic association of HLA-G 14-bp Ins/Del polymorphism with IBD, UC and CD. At the allele level (*Del* vs. *Ins*), frequency of *Del* allele frequency was significantly increased in total IBD patients (OR = 1.93; 95% CI = 1.27–2.94; pc = 0.018) and CD patients (OR = 2.08; 95% CI = 1.23–3.54; pc = 0.042) compared to controls. In UC, a similar increased frequency was observed, but the significance was lost when the *p*-value was corrected (OR = 1.79; 95% CI = 1.07–3.00; *p* = 0.031). At the genotypic level, none of the adopted genetic models revealed variation with a corrected significant *p* value between patients and controls. The codominant model was an exception in total IBD patients. The Del/Del genotype vs. Ins/Ins maintained a significantly corrected *p* value (OR = 4.06; 95% CI = 1.56–10.56; pc = 0.024) (Table 5).

Impact of HLA-G 14-bp Ins/Del polymorphism on sHLA-G level

Serum level of sHLA-G antigens was inspected in the three genotypes of HLA-G 14-bp Ins/Del polymorphism of IBD patients (UC and CD) and controls, and no significant impact of genotypes was recorded (Table 6).

Discussion

Among the characteristics that showed significant variation in UC and CD compared to controls was cigarette-smoking. Most of the patients were smokers (80 and 70%, respectively). This may underline a casual relation between IBD and cigarette-smoking; however, the literature disclosed that smoking has a dichotomous effect in UC and CD [38]. In CD, it has been established that smoking is a prominent environmental risk factor, and has a significant impact on disease development among smokers. Moreover, increased risk of relapse, steroid requirements, and hospitalization were associated with tobacco exposure. Conversely, cigarette-smoking is considered an environmental protective factor in UC, and a reduced risk of disease has been reported in active smokers. However, a reduced risk of developing UC has also been identified in patients who have never smoked. Therefore, the association between cigarette-smoking and UC is not yet fully understood [39].

The principal aim of present study was to understand the role HLA-G molecules in etiology and pathogenesis of UC and CD. Two approaches were adopted. In the first, serum level of sHLA-G was determined. In both IBD phenotypes, the level was significantly elevated in patients compared to controls. Further, ROC analysis depicted that sHLA-G occupied a significant AUC in IBD, but was higher in UC than in CD (0.961 vs. 0.927). Accordingly, it is suggested that sHLA-G molecules are risk factors with detrimental effect on the pathological course of UC and CD. There is no direct evidence to support these findings and previous studies investigating sHLA-G role in pathogenesis of UC or CD have not been well-elaborated. Consistent with this study, a significantly elevated level of sHLA-G was reported in CD patients compared to controls [20]. However, in an earlier study, the mucosal expression of HLA-G was detected in UC samples, while biopsies of CD patients did not show the expression of HLA-G molecules [17], whereas, a recent study demonstrated their gene and protein expression in biopsies of UC and CD patients [7]. In an in vitro study, the supernatant of cultured PBMCs obtained from UC and CD patients was examined for sHLA-G molecules using ELISA. This time, UC patients showed no detectable level of sHLA-G, while

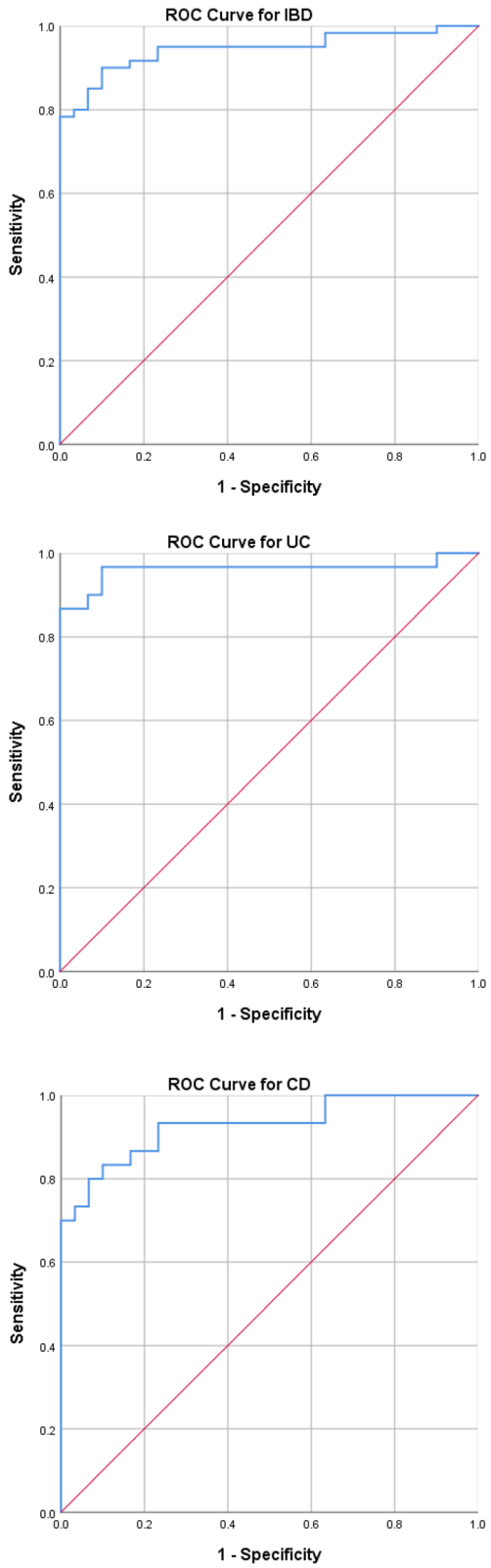

Fig. 1 Receiver operating characteristic (ROC) analysis for sHLA-G antigens showing area under curve (AUC) in IBD (AUC = 0.944; 95% CI = 0.898–0.990; $p < 0.001$; sensitivity = 90.0%; specificity = 90.0%; cut-off value = 145.2 ng/ml), UC (AUC = 0.961; 95% CI = 0.902–1.000; $p < 0.001$; sensitivity = 96.7%; specificity = 90.0%; cut-off value = 145.2 ng/ml) and CD (AUC = 0.928; 95% CI = 0.860–0.993; $p < 0.001$; sensitivity = 83.3%; specificity = 83.3%; cut-off value = 145.2 ng/ml)

Table 3 Serum level of soluble HLA-G antigens in ulcerative colitis and Crohn's disease distributed according to some characteristics of patients

Characteristic	sHLA-G (mean ± SD); ng/ml		*p* (pc)
	UC (*N* = 30)	CD (*N* = 30)	
Gender			
Male	192.0 ± 24.1	173.5 ± 25.1	**0.005 (0.050)**
Female	170.5 ± 26.2	164.9 ± 27.5	0.542 (NS)
p (pc)	**0.003 (0.030)**	0.292 (NS)	
Cigarette smoking status			
Smoker	187.9 ± 24.2	162.8 ± 25.9	**0.004 (0.040)**
Non-smoker	165.9 ± 27.9	175.0 ± 26.2	0.419 (NS)
p (pc)	**0.033** (NS)	0.181(NS)	
Disease duration (years)			
≤ 3	175.1 ± 32.2	164.9 ± 24.0	0.362 (NS)
> 3	184.1 ± 23.4	172.9 ± 28.7	0.233 (NS)
	0.376 (NS)	0.417 (NS)	
Family history			
Yes	193.3 ± 31.7	174.7 ± 23.4	0.332 (NS)
No	178.6 ± 26.5	167.8 ± 27.2	0.152 (NS)
p (pc)	0.352 (NS)	0.647 (NS)	
Disease extension			
Ulcerative proctitis	185.4 ± 32.3		
Left-sided colitis	170.2 ± 19.3		
Extensive colitis	182.6 ± 25.0		
Ileocecal		171.6 ± 25.2	
Ileo-colonic		155.3 ± 30.5	
p (pc)	0.457 (NS)	0.212 (NS)	
Abdominal/or colon pain			
Present	184.7 ± 29.9	165.1 ± 23.6	**0.030** (NS)
Absent	169.1 ± 12.5	174.0 ± 29.7	0.662 (NS)
p (pc)	0.558 (NS)	0.841 (NS)	
Diarrhea			
Present	184.6 ± 23.1	165.0 ± 27.6	**0.032** (NS)
Absent	172.3 ± 33.6	171.9 ± 25.8	1.000 (NS)
p (pc)	0.224 (NS)	0.485 (NS)	
Fever			
Present	192.3 ± 27.0	169.4 ± 28.7	0.051 (NS)
Absent	171.6 ± 24.2	168.6 ± 25.5	0.725 (NS)
p (pc)	**0.040** (NS)	1.000 (NS)	
Medication			
Protocol A	191.9 ± 23.6	167.6 ± 32.7	0.085 (NS)
Protocol B	189.7 ± 26.3	159.8 ± 10.3	0.079 (NS)
Protocol C	167.5 ± 15.3	181.8 ± 19.2	0.199 (NS)
Protocol D	170.8 ± 32.0	165.2 ± 29.4	0.741 (NS)
p (pc)	0.119 (NS)	0.184 (NS)	

Table 3 Serum level of soluble HLA-G antigens in ulcerative colitis and Crohn's disease distributed according to some characteristics of patients *(Continued)*

Characteristic	sHLA-G (mean ± SD); ng/ml		*p* (pc)
	UC (*N* = 30)	CD (*N* = 30)	
Adalimumab or infliximab doses			
0	189.7 ± 26.3	159.8 ± 10.3	0.079 (NS)
1–10	185.6 ± 23.7	172.8 ± 28.1	0.229 (NS)
11–20	176.6 ± 24.6	161.2 ± 26.8	0.193 (NS)
21–30	156.5 ± 47.5	197.8 ± 11.5	0.217 (NS)
p (pc)	0.334 (NS)	0.144 (NS)	

Significant *p* is bold-marked
SD standard deviation, *UC* ulcerative colitis, *CD* Crohn's disease, *protocol A* anti-inflammatory and immunosuppressive, *protocol B* anti-inflammatory and antibiotics, *protocol C* anti-inflammatory, immunosuppressive, and antibiotics, *protocol D* immunosuppressive only, *p* LSD (least significant) or one-way ANOVA (analysis of variance) probability, *pc* Bonferroni-corrected probability, *NS* not significant (*pc* > 0.05)

the supernatant of CD patients showed spontaneous production of sHLA-G [18]. Further accomplishing data suggested that immunosuppressant therapies are able to normalize the production of sHLA-G in CD, while in UC patients such therapy initiates their release. This effect on sHLA-G release was not influenced by age and gender of patients, as well as, disease activity, localization, or duration [40]. In this study, medication showed no significant effect on sHLA-G level. However, there was a tendency for sHLA-G to have a decreased level in UC patients who received immunosuppressive therapy, and the decrease paralleled the number of therapy doses. In CD patients, the response to medication was different. Patients treated with protocol C (anti-inflammatory, antibiotics and immunosuppressive) showed the highest level of sHLA-G among the other medication groups. Further, the increased level of sHLA-G paralleled the number of immunotherapy doses (the level was

Table 4 Allele and genotype frequencies of HLA-G 14-bp insertion/deletion polymorphism in healthy populations from Iraq and other countries

Country	*N*	HLA-G 14-bp allele		HLA-G 14-bp genotype			HWE *p*	Reference
		Ins	*Del*	Ins/Ins	Ins/Del	Del/Del		
Iraq	100	82 (41.0)	118 (59.0)	20 (20.0)	42 (42.0)	38 (38.0)	0.187	Present study
Algeria	100	77 (38.5)	123 (61.5)	15 (15.0)	47 (47.0)	38 (38.0)	0.940	[22]
Brazil	120	107 (44.6)	133 (55.4)	26 (21.7)	55 (45.8)	39 (32.5)	0.427	[23]
China	292	193 (33.1)	391(66.9)	35 (12.0)	123 (42.1)	134 (45.9)	0.411	[24]
Egypt	100	120 (60.0)	80 (40.0)	38 (38.0)	44 (44.0)	18 (18.0)	0.401	[25]
Greece	192	176 (45.8)	208 (54.2)	39 (20.3)	98 (51.0)	55 (28.7)	0.698	[26]
India	383	347 (45.3)	419 (54.7)	86 (22.4)	175 (45.7)	122 (31.9)	0.127	[27]
Iran	210	180 (42.9)	240 (57.1)	52 (24.8)	76 (36.2)	82 (39.0)	< 0.001	[28]
Italy	451	351 (38.9)	551 (61.1)	65 (14.4)	221 (49.0)	165 (36.6)	0.514	[29]
Japan	777	389 (25.0)	1165 (75.0)	58 (7.5)	273 (35.1)	446 (57.4)	0.075	[30]
Korea	491	232 (23.6)	750 (76.4)	17 (3.5)	198 (40.3)	276 (56.2)	0.009	[31]
Mexico	214	195 (45.6)	233 (54.4)	41 (19.2)	113 (52.8)	60 (28.0)	0.346	[32]
Poland	465	385 (41.4)	545 (58.6)	77 (16.5)	231(49.7)	157 (33.8)	0.607	[33]
Saudi Arabia	119	117 (49.2)	121 (50.8)	27 (22.7)	63 (52.9)	29 (24.4)	0.519	[34]
Spain	33	17 (25.8)	49 (74.2)	2 (6.1)	13 (39.4)	18 (54.5)	0.863	[35]
Tunisia	170	174 (51.2)	166 (48.8)	42 (24.7)	90 (52.9)	38 (22.4)	0.439	[36]
Turkey	191	188 (49.2)	194 (50.8)	48 (25.1)	92 (48.2)	51 (26.7)	0.615	[37]
p		**< 0.001**		**< 0.001**				

Significant *p* is bold-marked
Ins insertion, *Del* deletion, *HWE* Hardy-Weinberg equilibrium, *p* Pearson's chi-squared test probability

Table 5 Allele and genotype frequencies of HLA-G 14 bp insertion/deletion polymorphism in inflammatory bowel disease (ulcerative colitis and Crohn's disease) patients and controls

Genetic model	Allele/Genotype	HC (*N* = 100)	IBD (*N* = 100)			UC (*N* = 50)			CD (*N* = 50)		
		N (%)	*N* (%)	OR (95% CI)	*p* (pc)	*N* (%)	OR (95% CI)	*p* (pc)	*N* (%)	OR (95% CI)	*p* (pc)
Allele	*Ins*	82 (41.0)	53 (26.5)	Reference		28 (28.0)	Reference		25 (25.0)	Reference	
	Del	118 (59.0)	147 (73.5)	1.93 (1.27–2.94)	**0.003 (0.018)**	72 (72.0)	1.79 (1.07–3.00)	**0.031** (NS)	75 (75.0)	2.08 (1.23–3.54)	**0.007 (0.042)**
Recessive	Del/Del	38 (38.0)	54 (54.0)	Reference		27 (54.0)	Reference		27 (54.0)	Reference	
	Ins/Del + Ins/Ins	62 (62.0)	46 (46.0)	1.92 (1.09–3.37)	**0.024** (NS)	23 (46.0)	1.92 (0.96–3.81)	0.064 (NS)	23 (46.0)	1.92 (0.96–3.81)	0.064 (NS)
Dominant	Ins/Ins	20 (20.0)	7 (7.0)	Reference		5 (10.0)	Reference		2 (4.0)	Reference	
	Ins/Del + Del/Del	80 (80.0)	93 (93.0)	3.32 (1.34–8.26)	**0.010** (NS)	45 (90.0)	2.25 (0.79–6.40)	0.129 (NS)	48 (96.0)	6.00 (1.34–26.81)	**0.019** (NS)
Overdominant	Ins/Del	42 (42.0)	39 (39.0)	Reference		18 (36.0)	Reference		21 (42.0)	Reference	
	Ins/Ins + Del/Del	58 (58.0)	61 (61.0)	1.13 (0.64–1.99)	0.666 (NS)	32 (64.0)	1.29 (0.64–2.60)	0.408 (NS)	29 (58.0)	1.00 (0.50–1.99)	1.000 (NS)
Codominant	Ins/Ins	20 (20.0)	7 (7.0)	Reference		5 (10.0)	Reference		2 (4.0)	Reference	
	Ins/Del	42 (42.0)	39 (39.0)	2.65 (1.01–6.96)	**0.047** (NS)	18 (36.0)	7.11 (1.53–32.98)	**0.012** (NS)	21 (42.0)	5.00 (1.07–23.44)	**0.041** (NS)
	Del/Del	38 (38.0)	54 (54.0)	4.06 (1.56–10.56)	**0.004 (0.024)**	27 (54.0)	2.84 (0.95–8.51)	0.062 (NS)	27 (54.0)	1.71 (0.56–5.28)	0.384 (NS)
HWE-*p*		0.187		0.991		0.449			0.396		

Significant *p* is bold-marked

Ins insertion, *Del* deletion, *HC* healthy control, *IBD* inflammatory bowel disease, *UC* ulcerative colitis, *CD* Crohn's disease, *OR* odds ratio, *CI* confidence interval, *p* probability, *pc* Bonferroni-corrected *p*, *NS* not significant ($p > 0.05$), *HWE* Hardy-Weinberg equilibrium

Table 6 Serum level of sHLA-G antigens in inflammatory bowel disease (ulcerative colitis and Crohn's disease) patients and controls distributed according to HLA-G 14-bp insertion/deletion genotypes

Group[a]	sHLA-G (mean ± SD); ng/ml			*p*
	Ins/Ins	Ins/Del	Del/Del	
Control (*N* = 19:5:6)	122.2 ± 16.6	135.5 ± 15.2	126.0 ± 14.5	0.314
UC (*N* = 19:6:5)	183.7 ± 17.9	180.0 ± 46.8	179.8 ± 22.4	0.958
CD (*N* = 21:7:2)	172.5 ± 34.9	165.0 ± 26.2	169.9 ± 27.0	0.894

UC ulcerative colitis, *CD* Crohn's disease, *SD* standard deviation, *Ins* insertion, *Del* deletion, *p* one-way ANOVA (analysis of variance) probability
[a] Numbers of Ins/Ins, Ins/Del, and Del/Del are given in parentheses, respectively

increased in patients who received 21–30 doses). These findings indicted that UC and CD patients responded differently to the administrated therapy. Previous in vitro study also suggested that immunosuppressant therapies may act differently in UC and CD patients [40].

Irrespective of these inconsistencies, this study points to the role for sHLA-G molecules in pathogenesis of UC and CD. Both phenotypes presented with upregulated inflammatory responses, and sHLA-G molecules may encounter the exaggerated inflammatory response due to their immunomodulating effects [41]. The HLA-G molecules may protect local tissue by inhibiting the activity of NK cells and may also control the balance between T helper 1 and 2 cells in mucosa tissues [5].

In the second approach, allele and genotype frequencies of HLA-G 14-bp Ins/Del polymorphism were analyzed in IBD patients (UC and CD) and controls. As in other world populations, both alleles (*Ins* and *Del*) showed polymorphic frequencies in the Iraqi sample of controls (41 and 59%, respectively). In most of the populations investigated (Table 4), *Ins* was the allele of minor frequency and had a range of 23.6% in Koreans [31] to 49.2% in Saudi Arabians [34] and Turkish [37]; however, Egyptians [25] and Tunisians [36] showed the opposite profile, and the *Ins* allele frequency exceeded this range (60 and 51.2%, respectively). Therefore, the Iraqi *Ins* allele frequency fits well the presented range. However, the comparison between patients and controls depicted variations in allele and genotype frequencies of HLA-G 14-bp Ins/Del polymorphism. In total IBD patients, as well as CD patients, logistic regression analysis suggested a risk potential of the allele *Del*. In UC patients, a similar conclusion was reached but the difference was significant before correction of *p* value. In Caucasian German patients, allele and genotype frequencies of HLA-G 14-bp Ins/Del polymorphism showed no significant variations between UC or CD patients and controls, but there were significant differences between UC and CD patients. The heterozygous genotype and the Del+ phenotype were significantly elevated in UC patients compared to CD patients [19]. In a Tunisian study, neither allele nor genotype frequencies of HLA-G 14-bp Ins/Del polymorphism showed significant differences between CD patients and controls. However, the distribution of patients as young- and adult-onset showed significantly increased frequencies of *Ins* allele and Ins/Ins genotype in young-onset patients but not adult-onset patients [20]. To the best knowledge of investigators, no further study was performed to explore the association of HLA-G 14-bp Ins/Del polymorphism with UC or CD. Although conflicting results were obtained, a possible risk effect of the HLA-G gene in both phenotypes of IBD is suggested.

This study also addressed that the upregulated serum expression of sHLA-G in UC and CD patients might be influenced by HLA-G 14-bp Ins/Del variant. The analysis revealed that sHLA-G level was similarly distributed in the three genotypes of patients or controls, and no significant influence was observed. However, a previous study suggested inconsistent functional relevance, and the exon 8 polymorphism (14-bp Ins/Del) may influence the transcription activity of HLA-G gene [15]. The authors observed that HLA-G mRNAs harboring the 92-base deletion showed more stability than complete forms of mRNA. Accordingly, it was suggested that regulation of HLA-G post-transcription is controlled by this region. However, it has been reviewed that presence of *Del* allele in homozygous and heterozygous genotypes is correlated with higher production of sHLA-G (high-producer genotypes). A similar observation was made in CD patients, and Del/Del and Del/Ins genotypes were associated with an increased plasma level of sHLA-G, but no statistical significance was attended [20].

Conclusions

This study demonstrated a significant increase in serum level of sHLA-G in UC and CD patients. Further, HLA-G 14-bp Ins/Del polymorphism may be associated with susceptibility to IBD, particularly CD. However, the study was limited by low number of UC and CD patients, and increasing the sample size will certainly contribute to a further understanding of HLA-G role in immunopathogenesis of both IBD phenotypes.

Abbreviations

ANOVA: Analysis of variance; AUC: Area under curve; bp: Base-pairs; CD: Crohn's disease; CI: Confidence interval; Del: Deletion; ESR: Erythrocyte sedimentation rate; Hb: Hemoglobin; HLA: Human leukocyte antigen; HWE: Hardy-Weinberg equilibrium; IBD: Inflammatory bowel disease; Ins: Insertion; LSD: Least significant difference; MHC: Major histocompatibility complex; NK: Natural killer; OR: Odds ratio; *p*: Probability; PBMC: Peripheral blood mononuclear cell; pc: Corrected *p*; ROC: Receiver operating characteristics; SD: Standard deviation; sHLA-G: Soluble HLA-G; Th: T helper; UC: Ulcerative colitis; WBC: White blood cell

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Authors' contributions
SSA handled laboratory assessments, managed data and statistical analyses and contributed to writing and revising the manuscript. ENA and NHZ contributed to data handling, writing and revising the manuscript. NMA diagnosed the disease, managed data and revised the manuscript. AHA managed data, carried out statistical analyses, and wrote the manuscript. The authors read and approved the final manuscript.

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The participants provided their written informed consent to be included in the study. The College of Science (Al-Mustansiriya University) obtained the approval of the Ethics Committees at the target hospitals to carry out the study (N264 on 13 January 2019).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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