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Genetic polymorphism of HLA-G gene (*G*01:03*, *G*01:04*, and *G*01:05N*) in Iraqi patients with inflammatory bowel disease (ulcerative colitis and Crohn's disease)

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Abstract

Background: Human leukocyte antigen-G (HLA-G) has been proposed to influence susceptibility to inflammatory bowel disease (IBD). Therefore, the genetic association between HLA-G alleles and two clinical phenotypes of IBD (ulcerative colitis [UC] and Crohn's disease [CD]) was evaluated in Iraqi patients. A case-control study was performed on 50 UC and 50 CD patients and 100 healthy controls (HC). Three HLA-G alleles (*G*01:03*, *G*01:04*, and *G*01:05N*) were determined using sequence-specific polymerase chain reaction assay followed by product digestion with restriction endonucleases (*Hinf-I*, *BseR-I*, and *PpuM-I*, respectively).

Results: The *G*01:03* allele was not detected in IBD patients (UC and CD) or HC, while *G*01:04* and *G*01:05N* alleles showed polymorphic frequencies. The allele *G*01:04* was significantly associated with susceptibility to UC (odds ratio [OR] = 2.55; 95% confidence interval [CI] = 1.27–5.13; corrected probability [*pc*] = 0.018) and CD (OR = 4.45; 95% CI = 2.11–9.41; *pc* < 0.001). The allele *G*01:05N* was also associated with increased risk of UC (OR = 4.17; 95% CI = 1.32–13.21; *pc* = 0.032) and CD (OR = 4.75; 95% CI = 1.53–14.78; *pc* = 0.014). These associations were more pronounced in IBD (UC + CD), and a significantly increased risk for IBD was found with the alleles *G*01:04* (OR = 3.32; 95% CI = 1.86–5.95; *pc* < 0.001) and *G*01:05N* (OR = 4.46; 95% CI = 1.59–12.47; *pc* = 0.008). A stratification of IBD patients according to some demographic and clinical characteristics revealed that frequencies of both alleles showed no significant differences between the subgroups of patients in each stratum. Soluble HLA-G was not influenced by HLA-G alleles in patients or HC. UC was an exception, and the presence of *G*01:04* allele was associated with a significantly higher mean of soluble HLA-G compared to patients without the allele (189.6 ± 24.0 *vs*. 168.6 ± 27.2 ng/mL; *p* = 0.033).

Conclusion: This study indicated that *HLA-G*01:04* and *HLA-G*01:05N* alleles may influence susceptibility to UC and CD in Iraqi patients.

Keywords: Inflammatory bowel disease, Ulcerative colitis, Crohn's disease, HLA-G, Null allele

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Background

Inflammatory bowel disease (IBD) has recently been viewed as a gastrointestinal disorder with an increasing burden worldwide [1]. The disease is characterized by chronic inflammation of the gastrointestinal tract due to exaggerated inflammatory responses against intestinal microbiota in genetically susceptible individuals [2]. In most cases, two clinical phenotypes of IBD are recognized: ulcerative colitis (UC) and Crohn's disease (CD) [3]. In UC, the inflammation occurs in the colonic mucosa starting from the rectum and extending proximally in a continuous manner to involve part of, or the entire, colon. CD is characterized by transmural inflammation involving any part of the gastrointestinal tract [4]. The exact etiology of IBD is still not precisely defined and its pathogenesis not well elucidated. Nevertheless, it is suggested that interactions between intestinal microbes, environmental factors and host genetic predisposition are required to trigger irregular immune responses that are participated in provoking chronic relapsing and remitting inflammation [2].

There is overwhelming evidence drawn from population-based studies that genetic factors play an important role in pathogenesis of IBD [5]. The risk of contracting the disease increases in blood-relatives of patients, and the disease concordance rate is higher among monozygotic twins than among dizygotic twins [6]. Further, genome-wide association studies have described the importance of multiple genetic loci in increasing the risk of IBD, particularly those involved in modulating the inflammatory response [7]. Studies in patients of European and non-European ancestry have identified more than 200 IBD susceptibility loci that are proposed to have a functional role in the intestinal inflammatory immune response [8, 9]. Among these loci are genes located in a region of human chromosome 6 (6p21) termed inflammatory bowel disease 3 (IBD3). The region is of interest at the genome level because it encompasses genes of the classical and non-classical human leukocyte antigen (HLA) system, as well as HLA-class III genes, such as tumor necrosis factor (TNF)-α gene (*TNFA*) [10]. HLA gene products play a major functional role in immune responses. Further, HLA alleles have shown associations with susceptibility to different diseases; especially those related to immune dysregulation [11]. Besides, patients with IBD are at a greater risk of developing autoimmune and inflammatory diseases that have established HLA-associations; for instance, psoriasis, ankylosing spondylitis, type 1diabetes, and multiple sclerosis [12]. HLA-G is one such locus, the products of which play a major role in modulating immunity due to their tolerogenic properties [13].

HLA-G belongs to the non-classical class I HLA gene family, and is located within the major histocompatibility complex (MHC) in the chromosomal region 6p21.3 [13]. HLA-G was first described to play a major role in maintaining tolerance in the maternal-fetal interface [14]. Subsequently, HLA-G was shown to be involved in modulating innate and adaptive immune responses in a variety of pathological conditions; for instance, viral infections, malignancies, autoimmune diseases, transplant outcomes, and inflammatory diseases. These results highlighted that HLA-G encodes for molecules important for immune system functions [15]. Seven isoforms of HLA-G have been identified; four of them are membrane-bound (G1–G4), and three are soluble (G5–G7) [16].

The magnitude of HLA-G gene expression is controlled by variants in the promoter (5′-upstream regulatory region; 5′ URR) and in the 3′ untranslated region 3 (3′UTR) [13]. However, the HLA-G gene is described as a polymorphic locus with low number of alleles compared to other HLA loci. To date (14 October 2020), 82 alleles have been recognized at the DNA level (https://hla.alleles.org). A further polymorphism is exhibited by the HLA-G gene; it is 14 base-pairs (14-bp) insertion (Ins)/deletion (Del) at the 3′ UTR of exon 8 [17]. Numerous studies have examined the association between HLA-G variants and various diseases, especially inflammatory and immune-mediated diseases. Despite inconsistent observations made, it is suggested that HLA-G alleles are important biomarkers for monitoring the disease predisposition and progression [15].

With respect to the role of HLA-G molecules in etiology of IBD or their influence on pathogenicity of disease, few studies have been conducted with inconsistent findings. HLA-G expression in intestinal biopsies of UC and CD patients was first analyzed in 2004. The results depicted that expression of these molecules only occurred in the biopsies of UC patients, while CD samples showed no expression [18]. In a subsequent in vitro study, spontaneous secretion of sHLA-G was found in supernatant of cultured peripheral blood mononuclear cells (PBMCs) obtained from CD patients, while PBMCs obtained from UC patients or healthy control showed no secretion of sHLA-G [19]. A more recent immunohistochemical study demonstrated increased HLA-G expression in plasma cells/lymphocytes infiltrating the lamina propria in biopsies of UC and CD patients compared to controls, but greater cell staining was found in UC cells compared to CD cells [20]. In a study by our group, serum level of sHLA-G was significantly elevated in UC and CD patients compared to controls [21].

HLA-G polymorphisms have not been well investigated in IBD. Two previous studies suggested the importance of HLA-G 14-bp Ins/Del polymorphism in susceptibility to IBD [22, 23]. A third study by our group also favored a similar suggestion [21]. To the best knowledge of investigators, other HLA-G variants have not been investigated in IBD. In this study, the genetic

association between three alleles of HLA-G gene (G*01:03, G*01:04 and G*01:05N) and susceptibility to IBD (UC and CD) was analyzed in Iraqi patients.

Methods

Populations investigated

A case-control study was conducted during January–June 2019 to investigate the genetic association of HLA-G alleles (*G*01:03*, *G*01:04 G*01:05N*) with two phenotypes of IBD; UC and CD. One hundred patients diagnosed with IBD (50 UC and 50 CD) were recruited from outpatient clinics in three hospitals in Baghdad (Al-Kindy Teaching Hospital, Baghdad Teaching Hospital and Gastroenterology and Hepatology Teaching Hospital). Standard clinical, radiological, endoscopic and histopathological criteria were followed in the diagnosis of UC and CD [24]. Patients presented with indeterminate colitis or other gastrointestinal autoimmune diseases were excluded. Data related to age, gender, cigarette-smoking, disease duration, family history of IBD, laboratory findings (hemoglobin; Hb, white blood cell count; WBC, erythrocyte sedimentation rate; ESR and sHLA-G), disease extension (UC: ulcerative proctitis, left-sided colitis and extensive colitis; CD: ileocecal colitis and ileocecal + colon), symptoms (abdominal/colon pain, diarrhea, and fever), and extra-intestinal manifestations (aphthous ulcer, arthralgia, skin rash, appendectomy, bowel stricture, colostomy, fistula, and hemorrhoid) were recorded for each patient (Table 1). A control sample of 100 healthy individuals (HC) matched to patients for age and gender was also included. Written consent to participate in the study was obtained from all participants. The approval of Ethics Committee in the target hospitals to conduct the study was obtained (Approval number: N264 on 13 January 2019).

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

Characteristics		UC (*N* = 50)	CD (*N* = 50)	HC (*N* = 100)	*p*
Mean age ± SD; year		31.5 ± 10.1	30.5 ± 9.7	31.2 ± 9.8	0.864
Gender; *N* (%)	Male	28 (56.0)	28 (56.0)	57 (57.0)	0.990
	Female	22 (44.0)	22 (44.0)	43 (43.0)	
Current cigarette-smokers; *N* (%)		40 (80.0)	35 (70.0)	39 (39.0%)	**< 0.001**
Disease duration; *N* (%); year	≤ 3	20 (40.0)	25 (50.0)	NA	0.314
	> 3	30 (60.0)	25 (50.0)	NA	
Positive family history; *N* (%)		7 (14.0)	8 (16.0)	NA	0.779
Disease extension; *N* (%)	Ulcerative proctitis	20 (40)	NA	NA	
	Left-sided colitis	15 (30)	NA	NA	
	Extensive colitis	15 (30)	NA	NA	
	Ileocecal colitis	NA	43 (86.0)	NA	
	Ileocecal + colon	NA	7 (14.0)	NA	
Symptoms; *N* (%)	Abdominal/colon pain	35 (70.0)	31 (62.0)	NA	0.527
	Diarrhea	30 (60.0)	26 (52.0)	NA	0.546
	Fever	25 (50.0)	24 (48.0)	NA	1.000
Extra-intestinal manifestations; *N* (%)	Aphthous ulcer	11 (22.0)	13 (26.0)	NA	0.308
	Arthralgia	32 (62.0)	40 (80.0)	NA	0.118
	Skin rash	7 (14.0)	3 (6.0)	NA	0.318
	Appendectomy	7 (14.0)	6 (12.0)	NA	1.000
	Bowel stricture	2 (4.0)	5 (10.0)	NA	0.436
	Colostomy	8 (16.0)	3 (6.0)	NA	0.200
	Fistula	10 (20.0)	6 (12.0)	NA	0.414
	Hemorrhoid	4 (8.0)	5 (10.0)	NA	1.000
Laboratory findings; mean ± SD	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/h)	54.3 ± 22.5	58.8 ± 42.4	NA	0.876
	sHLA-G (ng/mL)[a]	180.5 ± 27.1	168.9 ± 26.3	126.8 ± 15.1	**< 0.001**

UC ulcerative colitis, *CD* Crohn's disease, *HC* healthy controls, *Hb* hemoglobin, *WBC* white blood cell, *ESR* erythrocyte sedimentation rate, *SD* standard deviation, *p* LSD (least significant difference) or two-tailed Fisher's exact probability (significant *p* value is indicated in bold), *NA* not applicable

[a]The mean was based on 30 samples of each group (UC, CD, or HC)

Determination of HLA-G alleles

From each participating subject, 3 mL of peripheral blood was drawn in ethylene-diamine-tetra-acetic-acid (EDTA) tube. The blood was processed to isolate genomic DNA using DNA purification kit (Geneaid, Taiwan), and instructions of manufacturer were followed. Three alleles, *HLA-G*01:03*, *HLA-G*01:04*, and *HLA-G*01:05N*, were determined using sequence-specific polymerase chain reaction (PCR) assay followed by product digestion with restriction endonucleases (*Hinf-I*, *BseR-I*, and *PpuM-I*, respectively), as presented in Table 2 and previously described [25]. 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 of each forward and reverse primer (10 pmol/μL) and 5.5 μL nuclease-free water. The optimized thermocycling conditions were initial denaturation at 94 °C (5 min), followed by 35 cycles of denaturation at 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 (5 min). The amplified PCR products were digested with the restriction endonucleases *Hinf-I* (*G*01:03*), *BseR-I* (*G*01:04*), and *PpuM-I* (*G*01:05N*). The digestion products were electrophoresed in 3% agarose gels, and migrating bands were visualized with UV trans-illuminator after staining with ethidium bromide. Assignments of HLA-G alleles were based on migrating DNA fragments. For *G*01:03*, the two bands defining the allele were not observed (79 and 125 bp), and instead bands of 106 and 175 bp were found after digestion with *Hinf-I*, indicating absence of *G*01:03*. *G*01:04* was defined by a band of 276 bp after digestion with *BseR-I*. Finally, *G*01:05N* was also defined by a band of 276bp but after digestion with *PpuM-I* (Table 2 and Fig. 1).

Statistical analysis

Baseline data were given as mean ± standard deviation (continuous variables) or number and percentage (categorical variables). Least significant difference (LSD) was used to assess significant difference between continuous variables, while two-tailed Fisher's exact or Pearson's chi-square test was used for categorical variables. HLA-G alleles (*G*01:03*, *G*01:04* and *G*01:05N*) were given as number and percentage. Logistic regression analysis was used to determine genetic association of HLA-G allele with UC or CD. The association was expressed as odds ratio (OR) and 95% confidence interval (CI). Two-tailed Fisher's exact probability (p) was used to assess statistical significance of association. Bonferroni correction was applied to adjust the p value due to multiple comparisons. A corrected p (pc) ≤ 0.05 was considered significant. The statistical package IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) was employed to carry out these analyses. Power of sample size was estimated using G*Power software (version 3.1.9.4).

Results

Power of sample size (PSS)

At 0.05 α error of probability and 0.3 effect size, the PSS (1−β error probability) of 50 cases was 0.71, which was below the marginal limit (0.80). When UC and CD cases were combined into a single group (i.e. IBD), the PSS was raised to 0.93. Thus, statistical verification of sample size was proposed.

Baseline data of patients and controls

UC and CD patients matched HC for age and gender distributions, and no significant differences were recorded. In addition, no significant variations between UC and CD patients were observed regarding family history of IBD, disease duration, symptoms or extra-intestinal manifestations. For cigarette-smoking, 80 and 70% of UC and CD patients were smokers, respectively compared to 39% among HC (p < 0.001). In the case of laboratory findings, Hb, WBC, ESR, and sHLA-G means showed no significant variations between UC and CD patients; however, sHLA-G mean was significantly elevated in both IBD phenotypes compared to HC (Table 1).

HLA-G alleles

The *G*01:03* allele was not detected in IBD patients (UC and CD) or HC, while *G*01:04* and *G*01:05N* alleles showed polymorphic frequencies, and their distribution in IBD patients (total, UC, or CD) showed significant variations compared to HC. Whereas, the comparison between UC and CD patients revealed no significant differences in the distribution of *G*01:04* and *G*01:05N* allele frequencies. The allele *G*01:04* was significantly

Table 2 Assignment of *HLA-G*01:03*, *HLA-G*01:04*, and *HLA-G*01:05N* alleles [25]

Exon	Primer	Restriction endonuclease	Product size (bp)	HLA-G allele
2	F: 5′-TCCATGAGGTATTTCAGCGC-3′	*Hinf-I*	79 + 125	*G*01:03*
	R: 5′-CTGGGCCGGAGTTACTCACT-3′		106 + 175	All but *G*01:03*
3	F: 5′-CACACCCTCCAGTGGATGAT-3′	*BseR-I*	276	*G*01:04*
	R: 5′-GGTACCCGCGCGCTGCAGCA-3′		40 + 236	All but *G*01:04*
		PpuM-I	276	*G*01:05N*
			108 + 168	All but *G*01:05N*

F forward, *R* reverse, *bp* base pair

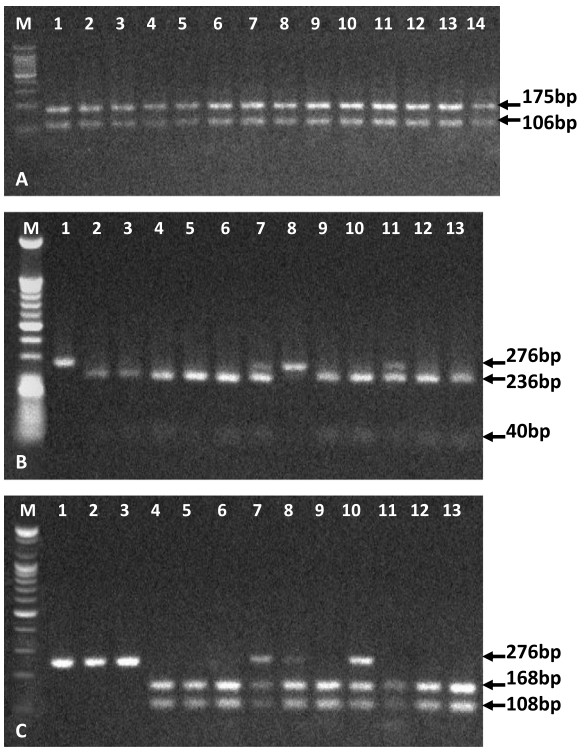

Fig. 1 Agarose gel electrophoresis of HLA-G gene PCR products in 3% agarose at 5 V/cm^2. **a** PCR products digested with *Hinf-I* showing two bands (106 and 175 bp); Lanes 1–14: absence of *HLA-G*01:03* allele. **b** PCR products digested with *BseR-I* showing three bands (40, 236, and 276 bp bands); lanes 1 and 8: homozygous *HLA-G*01:04* allele; lanes 7 and 11: heterozygous *HLA-G*01:04* allele; lanes 2–6, 9–10, and 12–13: absence of *HLA-G*01:04* allele. **c** PCR products digested with *PpuM-I* showing three bands (108, 168, and 276 bp bands); lanes 1-3: homozygous *HLA-G*01:05N* allele; lanes 7–8 and 10: Heterozygous *HLA-G*01:05N* allele; lanes 4–6, 9, and 11–13: absence of *HLA-G*01:05N* allele. Lane M: 100 bp DNA ladder. The bands were visualized using UV trans-illuminator after staining with ethidium bromide

associated with susceptibility to UC (OR = 2.55; 95% CI = 1.27–5.13; *pc* = 0.018) and CD (OR = 4.45; 95% CI = 2.11–9.41; *pc* < 0.001), but the risk was higher in CD than in UC. Frequency of *G*01:05N* allele also showed a significantly elevated frequency in UC (18 *vs.* 5%; OR = 4.17; 95% CI = 1.32–13.21; *pc* = 0.032) and CD (20 *vs.* 5%; OR = 4.75; 95% CI = 1.53–14.78; *pc* = 0.014) patients compared to HC. These associations were more pronounced in IBD group (UC + CD), and a significantly increased risk for IBD was found with the alleles *G*01:04* (OR = 3.32; 95% CI = 1.86–5.95; *pc* < 0.001) and *G*01:05N* (OR = 4.46; 95% CI = 1.59–12.47; *pc* = 0.008) (Tables 3 and 4).

Stratification of HLA-G alleles according to characteristics of patients

*G*01:04* and *G*01:05N* alleles were stratified in total IBD patients according to the demographic and clinical characteristics given in Table 1. The frequency distribution of the two alleles showed no significant differences between the subgroups of patients in each stratum (Tables 5 and 6).

Impact of HLA-G alleles on sHLA-G

The data on sHLA-G serum level were obtained from an article previously published by our group in this journal [21]. The level did not show significant differences between participants (patients or controls) who had the *G*01:04* or *G*01:05N* allele and those who did not have the allele. UC was an exception, and the presence of *G*01:04* allele was associated with a significantly higher mean of sHLA-G compared to patients without the allele (189.6 ± 24.0 *vs.* 168.6 ± 27.2 ng/mL; *p* = 0.033) (Fig. 2).

Discussion

In a previous study, we showed that serum level of sHLA-G was significantly elevated in current samples of UC and CD patients compared to HC. Further, the genetic association with an exon 8 polymorphism of HLA-G gene (14-bp Ins/Del polymorphism) was also analyzed,

Table 3 Frequency of HLA-G alleles in inflammatory bowel disease patients (ulcerative colitis and Crohn's disease) and controls

HLA-G allele	Model	HC (*N* = 100)		UC (*N* = 50)		*p*	CD (*N* = 50)		*p*	IBD (*N* = 100)		*p*
		N	%	*N*	%		*N*	%		*N*	%	
*G*01:03*	Absent	100	100.0	50	100.0	1.000	50	100.0	1.000	100	100.0	1.000
	Present	0	0.0	0	0.0		0	0.0		0	0.0	
*G*01:04*	Absent	61	61.0	19	38.0	**0.009**	13	26.0	**< 0.001**	32	32.0	**< 0.001**
	Present	39	39.0	31	62.0		37	74.0		68	68.0	
*G*01:05N*	Absent	95	95.0	41	82.0	**0.016**	40	80.0	**0.007**	81	81.0	**0.004**
	Present	5	5.0	9	18.0		10	20.0		19	19.0	

HC healthy controls, *UC* ulcerative colitis, *CD* Crohn's disease, *IBD* inflammatory bowel disease, *p* two-tailed Fisher exact probability compared to controls (significant *p* value is indicated in bold)

Table 4 Logistic regression analysis of *HLA-G*01:04* and *HLA-G*01:05N* alleles in inflammatory bowel disease patients (ulcerative colitis and Crohn's disease)

Comparison	HLA-G allele	Model	OR (95% CI)	*p*	*pc*
UC *vs.* HC	*G*01:04*	Absent	Reference		
		Present	2.55 (1.27–5.13)	**0.009**	**0.018**
	*G*01:05N*	Absent	Reference		
		Present	4.17 (1.32–13.21)	**0.016**	**0.032**
CD *vs.* HC	*G*01:04*	Absent	Reference		
		Present	4.45 (2.11–9.41)	**< 0.001**	**< 0.001**
	*G*01:05N*	Absent	Reference		
		Present	4.75 (1.53–14.78)	**0.007**	**0.014**
IBD *vs.* HC	*G*01:04*	Absent	Reference		
		Present	3.32 (1.86–5.95)	**< 0.001**	**< 0.001**
	*G*01:05N*	Absent	Reference		
		Present	4.46 (1.59–12.47)	**0.004**	**0.008**
UC *vs.* CD	*G*01:04*	Absent	Reference		
		Present	0.57 (0.25–1.33)	0.284	0.496
	*G*01:05N*	Absent	Reference		
		Present	0.88 (0.33–2.36)	1.000	1.000

UC ulcerative colitis, *CD* Crohn's disease, *IBD* inflammatory bowel disease, *HC* healthy controls, *vs.* versus, *OR* odds ratio, *CI* confidence interval, *p* two-tailed Fisher's exact probability, *pc* Bonferroni-corrected probability (significant *p* value is indicated in bold)

and the results indicated the susceptibility potential of this polymorphism to IBD [21]. These findings motivated us to extend the analysis of HLA-G gene in IBD to include three alleles (*G*01:03*, *G*01:04* and *G*01:05N*). The *G*01:03* allele was not evident in IBD patients or controls, while *G*01:04* or *G*01:05N* were determined with polymorphic frequencies. With respect to *G*01:03*, the global estimated allele frequency was 6.3% (range 0.0% in Iberian populations from Spain and Han Chinese from South China to 11.9% in people of African ancestry from the southwestern USA) [17]. Prakash and colleagues also reported that *G*01:03* was not recorded in populations from India, South Korea, Poland, Spain, Ghana, and Zambia, but in Denmark, a remarkably high frequency was found (43%) [26]. For *G*01:04* and *HLA-G*01:05N*, their allele frequencies were higher than the estimated global frequencies (39 and 5% *vs.* 17.3 and 3.3%, respectively) [17]. In Iraq, frequency of the three alleles was only available for females; it was 5.2, 17.0, and 9.4%, respectively [27]. Inbreeding could theoretically lead to elevated frequencies of *G*01:04* and *G*01:05N* alleles, because Iraqis generally obey some traditional practices (i.e., high prevalence of consanguineous marriage), which may influence HLA allele frequencies [28].

The present study sought to investigate the role of *G*01:04* and *G*01:05N* alleles in genetic susceptibility to IBD in UC and CD patients. Suggestive evidence for the association of both alleles with susceptibility to UC and CD was presented by the study. It was observed that *G*01:04* and *G*01:05N* alleles were associated respectively with 2.55- and 4.17-fold higher risk of susceptibility to UC. Similar observations were made in CD patients, and the corresponding ORs were 4.45 and 4.75, respectively. Further, no significant differences between UC and CD patients were found regarding frequencies of the two alleles. Therefore, it is possible to suggest that *G*01:04* and *G*01:05N* alleles are involved in a common pathogenic mechanism between UC and CD. To highlight this suggestion, data were reanalyzed considering UC and CD in one group (i.e. IBD). This time, the association between the two alleles and susceptibility to IBD was sustained, and the disease risk increased by 3.32 and 4.46 times, respectively. Accordingly, *G*01:04* and *G*01:05N* alleles may represent novel biomarkers for both IBD phenotypes (UC and CD).

*G*01:04* has not been well screened in inflammatory diseases, but it has been reported to be coincided with *Del* allele of the 14-bp Ins/Del polymorphism at the 3′ UTR of exon 8 in HLA-G gene, and is associated with high serum level of sHLA-G [29]. The present study demonstrated that serum level of sHLA was significantly increased in UC patients carrier for *G*01:04* allele compared to patients without the allele. The *Del* allele has also been associated with susceptibility to IBD, particularly CD, in Iraqi patients [21]. Thus, both alleles (*G*01:04* and *Del*) could act synergistically in the context of susceptibility to inflammatory diseases due to their association with immunosuppressive functions of natural killer (NK) cells. Recently, *G*01:04* has been shown to be a powerful catalyst in decidual NK cell activation. It also

Table 5 *HLA-G*01:04* and *HLA-G*01:05N* frequencies in inflammatory bowel disease patients classified according to demographic and clinical characteristics

Characteristic		N	*HLA-G*01:04* allele model				p	*HLA-G*01:05N* allele model				p
			Absent		Present			Absent		Present		
			N	%	N	%		N	%	N	%	
Gender	Male	56	19	33.9	37	66.1	0.672	49	87.5	7	12.5	0.075
	Female	44	13	29.5	31	70.5		32	72.7	12	27.3	
Cigarette-smoking	Smoker	75	25	33.3	50	66.7	0.805	64	85.3	11	14.7	0.077
	Non-smoker	25	7	28.0	18	72.0		17	68.0	8	32.0	
Family history	Yes	15	2	13.3	13	86.7	0.134	13	86.7	2	13.3	0.729
	No	85	30	35.3	55	64.7		68	80.0	17	20.0	
Abdominal/colon pain	Present	66	22	33.3	44	66.7	0.822	56	84.8	10	15.2	0.188
	Absent	34	10	29.4	24	70.6		25	73.5	9	26.5	
Diarrhea	Present	56	19	33.9	37	66.1	0.672	49	87.5	7	12.5	0.075
	Absent	44	13	29.5	31	70.5		32	72.7	12	27.3	
Fever	Present	49	14	28.6	35	71.4	0.524	42	85.7	7	14.3	0.310
	Absent	51	18	35.3	33	64.7		39	76.5	12	23.5	
Aphthous ulcer	Present	24	7	29.2	17	70.8	0.806	16	66.7	8	33.3	0.069
	Absent	76	25	32.9	51	67.1		65	85.5	11	14.5	
Arthralgia	Present	72	20	27.8	52	72.8	0.160	56	77.8	16	22.2	0.260
	Absent	28	12	42.9	16	57.1		25	89.3	3	10.7	
Skin rash	Present	10	4	40.0	6	60.0	0.722	7	70.0	3	30.0	0.396
	Absent	90	28	31.1	62	68.9		74	82.2	16	17.8	
Appendectomy	Yes	13	3	23.1	10	76.9	0.541	11	84.6	2	15.4	1.000
	No	87	29	33.3	58	66.7		70	80.5	17	19.5	
Bowel stricture	Present	7	2	28.6	5	71.4	1.000	6	85.7	1	14.3	1.000
	Absent	93	30	32.32	63	67.7		75	80.6	18	19.4	
Colostomy	Present	11	4	36.4	7	63.6	0.741	9	81.8	2	18.2	1.000
	Absent	89	28	31.5	61	68.5		72	80.9	17	19.1	
Fistula	Present	16	7	43.7	9	56.3	0.380	12	75.0	4	25.0	0.498
	Absent	84	25	29.8	59	70.2		69	82.1	15	17.9	
Hemorrhoid	Present	9	3	33.3	6	66.7	1.000	6	66.7	3	33.3	0.366
	Absent	91	29	31.9	62	68.1		75	82.4	16	17.6	

p Two-tailed Fisher's exact probability

Table 6 *HLA-G*01:04* and *HLA-G*01:05N* frequencies in inflammatory bowel disease patients (ulcerative colitis and Crohn's disease) classified according to disease extension

Disease extension		N	*HLA-G*01:04* allele				p	*HLA-G*01:05N* allele				p
			Absent		Present			Absent		Present		
			N	%	N	%		N	%	N	%	
UC	Ulcerative proctitis	20	4	20.0	16	80.0	0.094	15	75.0	5	25.0	0.366
	Left-sided colitis	15	8	53.3	7	46.7		12	80.0	3	20.0	
	Extensive colitis	15	7	46.7	8	53.3		14	93.3	1	6.7	
CD	Ileocecal colitis	43	11	25.6	32	74.4	0.867	33	76.7	10	23.3	0.319
	Ileocecal + colon	7	2	28.6	5	71.4		7	100.0%	0	0.0	

UC ulcerative colitis, *CD* Crohn's disease, *p* Pearson's chi-square probability

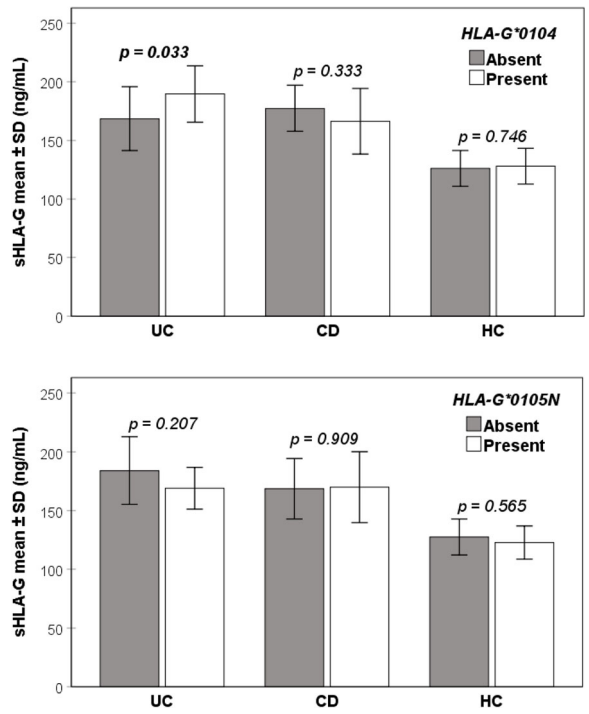

Fig. 2 Means of soluble HLA-G level distributed according to absence and presence of *HLA-G*01:04* and *HLA-G*01:05N* allele in ulcerative colitis (UC) and Crohn's disease (CD) patients and healthy controls (HC). *p*: Least significant difference probability (significant *p* value is indicated in bold)

exerts protective effects against NK cell-mediated lysis. These findings underlie the exceptional role of *G*01:04* as a mediator of immune tolerance [30]. In an earlier study, it was found that women with HIV-1 infection and bacterial vaginosis and carrying *G*01:04:04* allele expressed the highest levels of genital HLA-G molecules [31]. In lung inflammatory diseases, such as cystic fibrosis, *G*01:04* was associated with lower survival rates and higher frequency of chronic rejection after lung transplantation [32]. It was also reviewed that *G*01:04* showed elevated frequency in couples with unexplained recurrent miscarriages, while it exhibited a protective role in one of the chronic inflammatory diseases (acute renal rejection and end-stage kidney disease) [33]. It has also been concluded that HLA-G haplotypes that include *G*01:04* allele may be a good candidate marker for inflammation in asthma patients [34]. Together, these findings enforce the susceptibility role of *G*01:04* in inflammatory diseases including IBD.

The null allele *G*01:05N* was also significantly associated with risk of UC and CD in current samples of Iraqi patients. The allele has not been investigated in IBD, but its susceptibility role in etiology of other human ailments has been suggested; for instance, Behçet's disease, autistic spectrum disorders, recurrent pregnancy loss, and preeclampsia [35–37]. The *G*01:05N* allele represents a single base deletion causing a frame-shift reading that leads to a premature stop codon at the beginning of exon 4. As a consequence, this allele is associated with incomplete formation of the HLA-G isoforms G1, G4, and G5 that possess the α3 domain, while G2, G3, and G7 isoforms (lack the α3 domain) show normal expression and sustain the immune tolerogenic function of HLA-G [15, 38]. The allele is also associated with low serum level of sHLA-G, and unlike *G*01:04*, it coincided with *Ins* allele of the 14-bp Ins/Del polymorphism [39]. The *Ins* allele has been considered a risk factor for celiac disease (IBD-related disease) [40]. Both conditions are characterized by chronic intestinal inflammation, and may share similar etiology and immunopathogenesis [41]. Another study reported a positive association between *G*01:05N* and an infectious disease (HIV infection) in adult Caucasian females, and the authors suggested that this allele could be considered a marker of susceptibility or it might have a direct functional effect on the development of HIV infection [42].

Collectively, these data indicate that HLA-G plays an important role in regulating the inflammatory response in UC and CD. It has been indicated that HLA molecules exert immune-suppressive effects on different immune cells, including CD4+ and CD8+ T cells, NK cells, antigen-presenting cells, and B cells. In addition, they participate in inducing T regulatory cells and IL-10 producing dendritic cells [43]. In this context, it has been demonstrated that CD4+CD25+ T cells were elevated in UC patients with primary sclerosing cholangitis [44]. Two SNPs of HLA-G (rs66554220 and rs1063320) were also associated with low frequency of regulatory CD8+ CD28- T cells [45]. Further studies indicated that HLA-G locus have been associated with higher susceptibility to UC and CD or higher severity of the two diseases [21, 23, 46]. HLA-G has also been indicated as predictive marker of response to therapy in inflammatory and infectious diseases, as well as cancer [47]. Thus, it is possible that these immunological and genetic signatures of HLA-G contribute to the susceptibility and persistence of UC and CD. However, this study might have provided preliminary evidence to suggest that HLA-G polymorphisms play a role in susceptibility to both phenotypes of IBD. Further studies are warranted to confirm or refute these results and to evaluate the role of *G*01:04* and *G*01:05N* alleles in etiology and pathogenesis of IBD. It should be noted that although statistical validation of sample size was established, the study was still limited due to lower sample size and better evaluation of HLA-G alleles in IBD should be based on a larger sample size.

Conclusions

The results of this study indicated that *HLA-G*01:04* and *HLA-G*01:05N* alleles are associated with susceptibility to UC and CD in Iraqi patients.

Abbreviations

bp: Base-pairs; CD: Crohn's disease; CI: Confidence interval; Del: Deletion; ESR: Erythrocyte sedimentation rate; Hb: Hemoglobin; HC: Healthy controls; HLA: Human leukocyte antigen; IBD: Inflammatory bowel disease; Ins: Insertion; LSD: Least significant difference; MHC: Major histocompatibility complex; ND: Not detected; NK: Natural killer; OR: Odds ratio; *p*: Probability; PBMC: Peripheral blood mononuclear cell; *pc*: Corrected *p*; PSS: Power of sample size; SD: Standard deviation; sHLA-G: Soluble HLA-G; UC: Ulcerative colitis; URR: Upstream regulatory region; UTR: Untranslated region; 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. AHA managed data, carried out statistical analyses, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

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 (Approval number: N264 on 13/01/2019).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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