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Original Article

Genetic polymorphism association of *HLA-G* (*G+3142 C>G, G*01:03,* and *G*01:05N*) with acute lymphocytic leukemia in Saudi Arabia

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Abstract: Human leukocyte antigen-G (*HLA-G*) is linked to the development of human malignancies via immune escape mechanisms. The chief variations for *HLA-G* were found in three prime untranslated regions (3'UTR). The current study aims to evaluate the distribution of *HLA-G rs1063320 (G+3142 C>G)*, *HLA-G*01:03*, and *HLA-G*01:05N* polymorphisms with risk of acute lymphocytic leukemia (ALL) in Saudi Arabia. This case-control study analyzed 232 samples from 117 patients with ALL and 115 healthy controls (HCN) using the PCR-RFLP method. Associations between *HLA-G* and ALL risk were analyzed using allele contrasts. The *HLAG rs1063320 G+3142 C>G* polymorphism results showed a reduced risk of ALL in the dominant *G/C* model odds ratios (OR) = 0.34, 95% confidence interval (CI) = 0.12-0.98, *P* = 0.041), stratified by age. However, those stratified by gender, showed decreased risk of ALL in all genetic inheritance models tested: codominant model *C/G* versus *G/G* (OR = 0.24, 95% CI = 0.06-0.99), *C/C* versus *G/G* (OR = 0.01, 95% CI = 0.00-0.12), *P* = 0.0001), and dominant model *C/G-C/C* versus *G/G* (OR = 0.12, 95% CI = 0.03-0.47, *P* = 0.000004), and the recessive model *C/C* versus *G/G-C/G* (OR = 0.03, 95% CI = 0.00-0.24, *P* = 0.0001), log-additive (OR = 0.12, 95% CI = 0.04-0.35, *P* = 0.0001). Conversely, the *G*01:03* allele was not found in ALL or HCN, whereas the *G*01:05N* allele showed polymorphic frequencies that were not significant. In conclusion, the *HLA-G +3142 C>G* polymorphism significantly decreased the prevalence of ALL stratified by gender and age polymorphisms in the risk of ALL in the pediatric Saudi population.

Keywords: Acute lymphocytic leukemia, *HLA-G* polymorphisms, genotyping, non-classical *HLA*, RFLP, Saudi Arabia

Introduction

Cancer ranks as the second most prevalent cause of death on a global scale after heart disease, thus it imposes a notable economic burden on both developed and developing nations. In addition, approximately 1,918,030 novel cancer cases and 609,360 deaths due to malignant tumors have occurred in the United States alone [1, 2]. Cancer is becoming increasingly common in the Middle East at an astonishing rate. The top five cancers in children in the Arab World in 2020 were identical to those reported globally [3, 4]. Childhood cancer ranks as the second most prevalent cause of mortality, and leukemia accounts for 28% of all cases [1]. More than 18,000 children in Arab countries are annually diagnosed with cancer [4]. Leukemia is one of the five most common cancers in Saudi Arabia [5].

The *HLA-G* molecule differs from HLA class I genes by having few alternative molecular codes. Although the coding region is the most conserved [6, 7]. The *HLA-G* molecules function as immunological checkpoints [8], regulating immune responses by binding to receptors on immune cells, including the inhibitory receptors ILT2 and ILT4 [9]. HLA-G expression plays a vital role in preserving the tolerance between mother and fetus and may impact the development of many diseases, such as autoimmune disorders, malignancies, and viral infections [10, 11]. The *HLA-G* rs1063320 SNP *G +3142 C>G* is located in the 3'-UTR [12]. The presence of G at position +3142 enhances its affinity for

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Table 1. PCR-RFLP for *HLA-G* rs1063320 (G+3142 C>G), G*01:03 and G*01:05N

HLA-G	Primers	RE	Product size (bp)	Specificities
rs1063320 (G+3142 C>G)	*F: 5'-CATGCTGAACTGCATTCCTTCC-3'*	*BseSI*	460	C
	R: 5'-CTGGGCCGGAGTTACTCACT-3'		316+90	G
G*01:03 (Exon 2)	*F: 5'-TCCATGAGGTATTTCAGCGC/G-3'*	*HinfI*	79+125+27	G*01:03
	R: 5'-CTGGGCCGGAGTTACTCACT-3'		106+175	All but G*01:03
G*01:05N (Exon 3)	*F: 5'-CACACCCTCCAGTGGATGAT-3'*	*PpuMI*	276	G*01:05N
	R: 5'-GGTACCCGCGCGCTGCAGCA-3'		108+168	All but G*01:05N

F, forward; R, reverse; rs, reference SNP cluster ID; RE, restriction endonuclease; bp, base pair.

three microRNAs (miRNA-148a, miRNA-148b, and miRNA-152) and decreases *HLA-G* mRNA stability and production [13]. Several studies in different populations have investigated the association between corroborating the unfavorable role of *G+3142 C>G* and cancer risk, including cervico-vaginal cancer [14], papillomavirus [15], cervical squamous cell carcinoma [16], breast cancer [17, 18], thyroid tumors [19], and colorectal cancer [20].

The *HLA-G*01:05N* allele is a well-known part of the human leukocyte antigen (HLA) system [21]. It is known for its part in controlling the immune system and its effects on many diseases. This allele is classified as a null variant due to a specific mutation that leads to a truncated protein product, significantly affecting its functionality [22]. *HLA-G*01:05N* is distinguished by the deletion of a single base, spliced *HLAG4* isoforms, and full-length soluble isoforms *HLA-G5* [21]. The *HLA-G*01:05N* null allele is distinguished by a single base-pair deletion in exon 3 [23], it blocks *HLA-G1* and *-G5* translation, in exon 5 in the codon 297 (TAG) [24]. The reduced expression of HLA-G associated with the *01:05N variant may cause altered immunological responses, possibly increasing the susceptibility to certain diseases or influencing transplant results [9, 21].

Several investigations have examined the impact of *HLA-G* polymorphisms on the chance of developing leukemia; however, the results have been inconsistent [15, 25]. In CLL, *HLA-G* expression is linked to impaired immunodeficiency in tumor cells [26]. In acute leukemia, soluble *HLA-G* levels are increased [27]. *HLA-G* expression has been identified as a prognostic marker in B-CLL [28-30], and ALL [31]. This study aimed to evaluate the genetic polymorphisms of *HLA-G rs1063320 (G+3142 C>G), G*01:03*, and *G*01:05N* alleles in patients of Saudi Arabia with ALL.

Materials and methods

Ethics statement

The ethics committee of King Khaled Hospital University (KKHU) in Riyadh, Saudi Arabia, gave its approval to this research project prior to its implementation. The ethical norms of King Saud University's Institutional Review Board (IRB) were adhered to throughout the course of this investigation (IRB code: E-20-5346).

Participants

The participants in this study were 117 pediatric patients with ALL (male = 73 and female = 44), identified between April 2017 and March 2020. The healthy control (HCN) group consisted of 115 ethnically matched individuals (male = 77 and female = 38). As a retrospective study, control patients had no diseases linked to any form of cancer and no history of immune disorders.

Genomic DNA extraction

Placed in EDTA vials, three ml of blood was taken from individuals who were diagnosed with ALL and from HCN. Following the directions provided by the manufacturer, genomic DNA was extracted with the use of the DNeasy Blood & Tissue Kit which was manufactured by QIAGENE GmbH, Germany. In this investigation, both forward and reverse primers sequences were used, and they are included in **Table 1**.

Genotyping for HLA-G

Genotyping of HLA-G involves the analysis of genetic variations within the HLA-G gene to

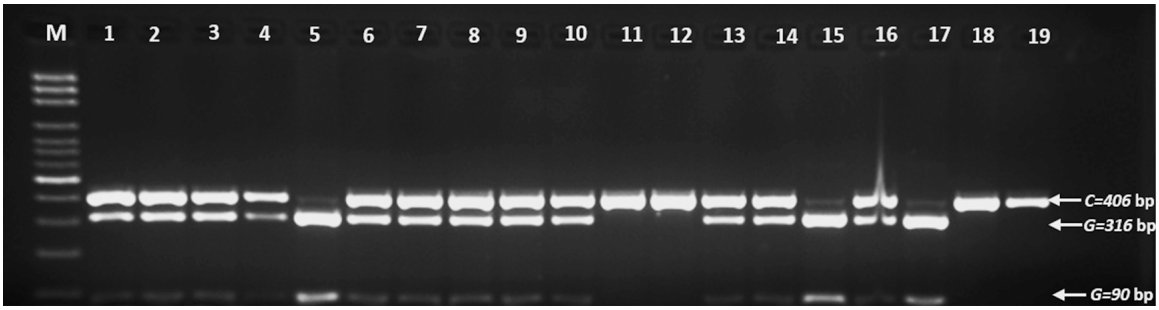

Figure 1. A photograph of the PCR-RFLP products of *HLA-G* rs1063320 (G+3142 C>G); *G* allele was digested with *BseS-I*, three bands were produced: 406 bp C allele, 316 and 90 bp G allele; lanes 1-4 and 6-10, as well as 13, 14, and 16: CG; 5, 15, and 17: GG; 11, 12, 18, and 19: CC; Lane M: 100 bp DNA Marker.

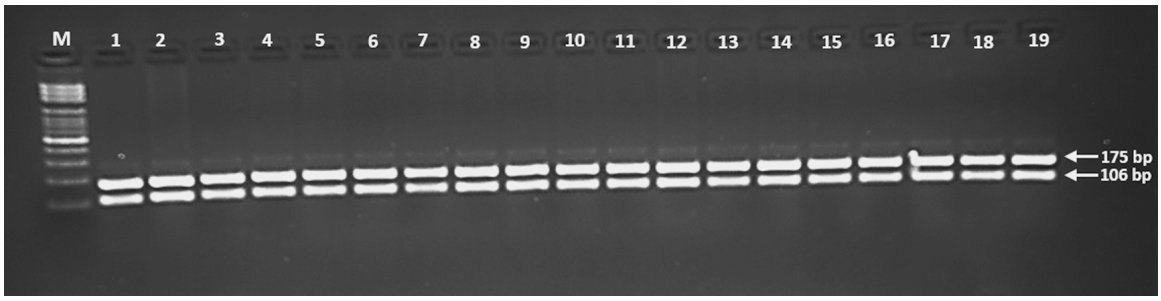

Figure 2. A photograph of the PCR-RFLP products of *HLA-G**01:03 allele, digested with *Hinf-I*, shows two bands (106 and 175 bp); lanes 1-19 show the absence of *HLA-G*01:03* allele; lane M: 100 bp DNA marker.

understand its polymorphisms and their associations with ALL. Using the PCR-RFLP technique, the genotyping of HLA-G rs1063320 (G+3142 C>G) as well as two alleles (HLA-G*01:03 and HLA-G*01:05N) was carried out. In brief, the reaction mix for HLA-G +3142 C>G comprised of the following components: DNA (1 µL), 7.5 µL 2X (GoTaq® Master Mixes, Promega, USA), forward and reverse primers (0.5 µL), and nuclease-free water. The total volume of the reaction mix was 15 µL. The thermocycling settings were as follows: a three-minute initial denaturation at 95°C was followed by thirty seconds of denaturation at 94°C, thirty seconds of annealing at 57-65°C, forty-five seconds of elongation at 72°C, and finally five minutes of final elongation at 72°C.

The digestion products were separated on 2.5% agarose by gel electrophoresis (Bio-Rad Laboratories, GmbH, Munchen). The bands were stained with ethidium bromide (Sigma-Aldrich, US) to ensure that the primers were working and that alleles were present or absent, and they were visualized by a UV trans-illuminator (BioDocAnalyze, Biometra GmbH, Germany).

The specific enzymes used for digestion were *BseSI (rs1063320)*, *Hinf-I (G*01:03)*, and *PpuM-I (G*01:05N)*. The procedures were conducted according to the instructions provided by the manufacturer (Thermo Fisher Scientific).

The digested products were separated by electrophoresis using 2.5% agarose gels. *HLA-G rs1063320 (G+3142 C>G)* was defined as a band of 406 bp. After digestion with *BseS-I*, three bands were observed at 406, 316, and 90 bp (**Figure 1**). In addition, for *HLA-G*01:03*, two bands of 106 and 175 bp were found after digestion with *Hinf-I* (**Figure 2**). While *HLA-G*01:05N was defined by a 276 bp band after PpuMI digestion*, three bands of 108, 168, and 276 bp were observed (**Figure 3**).

Statistical analysis

In control subjects, the Hardy-Weinberg equilibrium (HWE) was tested for genotype frequencies. We used the two-tailed Fisher's exact test; *HLA-G* (*G+3142 C>G, G*01:03,* and *G*01:05N*) was tested in terms of numerical values and percentages and was used to establish the cor-

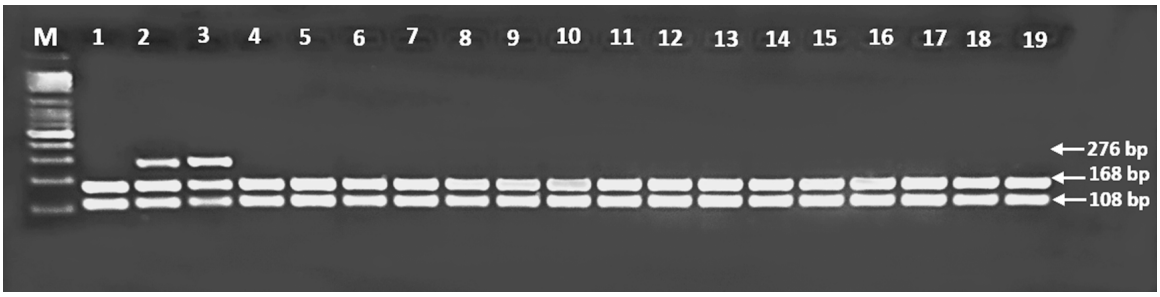

Figure 3. A photograph of *HLA-G**01:05N, PCR-RFLP products digested with *PpuM-I*; Lanes 2 and 3 show heterozygous *HLA-G**01:05N allele; lanes 1 and 4-19 show homozygote, absence of *HLA-G**01:05N allele; Lane M: 100 bp DNA marker.

Table 2. Frequency of *HLA-G +3142 C>G SNP*, *G*01:03* and *G*01:05N* alleles in patients with ALL and HCN

HLA-G alleles		HCN	ALL	X^2	OR	95% CI	*P-value*
		N = 115	N = 117				
	Alleles (%)						
*G*01:03*	***A**CG*	230 (100.0%)	234 (100.0%)	NS	NS	NS	NS
	***T**CG*	0.0 (100.0%)	0.0 (100.0%)				
*G*01:05N*	***C**TG*	223 (97.0%)	230 (98.3%)	0.89	1.80	(0.52-6.25)	0.38
	-TG	7 (3.0%)	4 (1.7%)		0.55	(0.16-1.92)	

ALL, acute lymphoblastic leukemia; HCN, healthy control; N, number of individuals; Allele, allele frequency; OR, odds ratio; CI, confidence interval; -TG, deletion C from codon 130 position 1597 in exon 3 in *G*01:05N*; TCG, Sequences at codon 31 encode a serine in *G*01:03* and ACG encode a threonine in all but *G*01:03*. Bold letters indicate the positions of amino acid variability.

relation between alleles. To evaluate the risk of ALL, OR and 95% CI, and *p-value* ≤ 0.05 were determined.

The analysis methods utilized in this study effectively elucidated the relationship between HLA-G genetic variants and ALL susceptibility, by employing SNP genotyping, using SNPStats software [32], statistical modeling, and stratification by age and sex.

Results

In total, 232 patients were enrolled in this study, including 117 patients with ALL and 115 unrelated HCN. We investigated the *HLA-G rs1063320 (G+3142 C>G)* SNP and the variants of two alleles, *G*01:03* and *G*01:05N*, in the 3'-UTR *HLA-G* polymorphic sites. The genotyping results for the *G+3142 C>G* polymorphism are shown in **Figure 1**. The *G*01:03* allele was not present with ALL or HCN. Contrarily, the *G*01:05N* allele exhibited variable frequencies and distribution with ALL compared to HCN.

The allele frequencies of *G+3142 C>G*, *G*01:03*, and *G*01:05N* with ALL and in HCN are shown in **Table 2**. The G+3142 G SNP was less common with ALL (55.0%) than in those in the HCN group (63.3%). The *G+3142* SNP was more prevalent with ALL (45.0%) than in those in the HCN group (36.7%). The results of *G+3142 C>G* are presented in **Table 3**. In addition, we conducted tests categorized by age, and the findings are shown in **Table 4**. In the dominant model, the SNP *G+3142 C>G* showed a substantial age-dependent difference. The SNP G+3142 *C>G* was found to reduce the risk of ALL in patients older than 18 years in the dominant group.

Table 5 shows our findings from testing the inheritance models for the genotype frequencies of the G+3142 *C>G* SNP and susceptibility in patients with ALL stratified by sex. The G+3142 *C>G* variant decreased the risk for ALL in males in the codominant model *C/G VS G/G* (OR = 0.24, 95% CI = 0.06-0.99) and *C/C* versus *G/G* (OR = 0.01, 95% CI = 0.00-0.12), *P* = 0.0001. In addition, the dominant model was

Table 3. Genotypic association of the *HLA-G* +3142 C>G polymorphism in patients with ALL and HCN, displaying genetic models: codominant, dominant, recessive, over dominant, and log-additive

Model	Allele/Genotype	ALL	HCN	OR (95% CI)	*P-value*	AIC
G+3142	*G*	77 (55.0%)	105 (63.3%)	0.71 (0.44-1.12)	0.16	
	C	63 (45.0%)	61 (36.7)	1.41 (0.89-2.23)		
Codominant	*G/G*	22 (31.4%)	33 (39.8%)	1.00	0.33	214.8
	C/G	33 (47.1%)	39 (47%)	0.79 (0.39-1.60)		
	C/C	15 (21.4%)	11 (13.2%)	0.49 (0.19-1.26)		
Dominant	*G/G*	22 (31.4%)	33 (39.8%)	1.00	0.28	213.8
	C/G-C/C	48 (68.6%)	50 (60.2%)	0.69 (0.36-1.36)		
Recessive	*G/G-C/G*	55 (78.6%)	72 (86.8%)	1.00	0.18	213.2
	C/C	15 (21.4%)	11 (13.2%)	0.56 (0.24-1.32)		
Over dominant	*G/G-C/C*	37 (52.9%)	44 (53%)	1.00	0.98	215
	C/G	33 (47.1%)	39 (47%)	0.99 (0.53-1.88)		
Log-additive	---	---	---	0.71 (0.45-1.13)	0.15	212.9

AIC, Akaike information criterion.

Table 4. Correlations between *HLA-G* +3142 *C>G* polymorphism and susceptibility in ALL patients stratified by age

Model	Genotype	ALL (Age ≤ 18)	ALL (Age >18)	OR (95% CI)	*P-value*	AIC	BIC
Codominant	*G/G*	8 (21.1%)	14 (43.8%)	1.00	0.11	98.2	104.9
	C/G	20 (52.6%)	13 (40.6%)	0.37 (0.12-1.13)			
	C/C	10 (26.3%)	5 (15.6%)	0.29 (0.07-1.14)			
Dominant	*G/G*	8 (21.1%)	14 (43.8%)	1.00	**0.041**	96.4	100.8
	C/G-C/C	30 (79%)	18 (56.2%)	0.34 (0.12-0.98)			
Recessive	*G/G-C/G*	28 (73.7%)	27 (84.4%)	1.00	0.27	99.3	103.8
	C/C	10 (26.3%)	5 (15.6%)	0.52 (0.16-1.72)			
Over dominant	*G/G-C/C*	18 (47.4%)	19 (59.4%)	1.00	0.32	99.5	104
	C/G	20 (52.6%)	13 (40.6%)	0.62 (0.24-1.59)			
Log-additive	---	---	---	0.51 (0.26-1.02)	0.051	96.7	101.2

BIC, Bayesian information criterion. The significant *p-values* are indicated in bold.

Table 5. Correlations between *HLA-G +3142 C>G* polymorphism and susceptibility in patients with ALL stratified by sex

Model	Genotype	ALL Female	ALL Male	OR (95% CI)	*P-value*	AIC	BIC
Codominant	*G/G*	3 (10%)	19 (47.5%)	1.00	**<0.0001**	75.1	81.9
	C/G	13 (43.3%)	20 (50%)	0.24 (0.06-0.99)			
	C/C	14 (46.7%)	1 (2.5%)	0.01 (0.00-0.12)			
Dominant	*G/G*	3 (10%)	19 (47.5%)	1.00	**5e-04**	87.3	91.8
	C/G-C/C	27 (90%)	21 (52.5%)	0.12 (0.03-0.47)			
Recessive	*G/G-C/G*	16 (53.3%)	39 (97.5%)	1.00	**<0.0001**	77.7	82.2
	C/C	14 (46.7%)	1 (2.5%)	0.03 (0.00-0.24)			
Over dominant	*G/G-C/C*	17 (56.7%)	20 (50%)	1.00	0.58	99.3	103.8
	C/G	13 (43.3%)	20 (50%)	1.31 (0.50-3.39)			
Log-additive	---	---	---	0.12 (0.04-0.35)	**<0.0001**	74.7	79.2

The significant *p-values* are indicated in bold.

C/G-C/C VS G/G (OR = 0.12, 95% CI = 0.03-0.47, *P* = 0.000004). The recessive model was *C/C VS G/G-C/G* (OR = 0.03, 95% CI = 0.00-0.24, *P* = 0.0001), and log-additive (OR = 0.12, 95% CI = 0.04-0.35, *P* = 0.0001).

Discussion

HLA-G has been implicated in various pathological conditions, including cancer and autoimmune diseases. Its expression can be upregulated in tumors, contributing to immune evasion and tumor progression. Studies have shown that HLA-G can facilitate a tumor-driven immune escape mechanism, making it a potential target for therapeutic interventions in oncology.

This study looked at how variations in the HLA-G gene, specifically the HLA-G +3142 C>G polymorphism and two alleles, G01:03 and G01:05N, are linked to the risk of getting ALL in healthy, unrelated people. These findings provide notable and significant insights into the genetic determinants that may potentially impact ALL's vulnerability.

This is the first study to investigate the effects of the *G+3142 C>G SNP, G*01:03,* and *G*0105N* allele polymorphisms on the risk of ALL in the pediatric Saudi population. The genotyping findings for the *HLA-G +3142 C>G* indicated that the *G+3142 G* allele was less frequent patients with ALL (55.0%) than that in HCN (63.3%), whereas the *G+3142 C* allele was more common with ALL (45.0%) than in HCN (36.7%). The *p-values* and ORs in **Table 2** indicate that these differences were not statistically significant. This finding implies that the previously aforementioned polymorphism may not have a significant influence on the predisposition to ALL within the Saudi population. Results of this study were consistent with that of our previous studies where [33]. This frequency matches with the findings in other reports [17] and [34].

Our results showed a high risk between the *G+3142 C>G* and susceptibility in patients with ALL by age in the genetic dominant inheritance model only, which showed a decrease in the risk of ALL in the Saudi population. This was maintained only in the dominant model (*C/G-C/C*) after stratification by age ≤ 18 years (OR = 0.34, 95% CI = 0.12-0.98, *P* = 0.041). Conversely, our study results showed a significant association stratified by sex, and showed a protective effect against ALL in all inheritance models. The calculated OR suggests that the homozygous *G/G* and heterozygous C/G genotypes have a protective effect against ALL. Our findings suggest that the *G+3142 C>G* mutation may have a protective effect in patients with ALL. Therefore, the results of our study are consistent with another study [35]. This is also consistent with the findings of other researchers [36], who found that *G+3142 G>C* decreased the risk of rheumatoid arthritis in an Iranian patients. Another study [37] showed that none of the *HLA-G* variant locations studied were linked to susceptibility to AML. Contrarily, additional research [21] found that 3'-UTR *HLA-G* alleles are a risk for the development of CML. Another study [31] also mentioned that *HLA-G* expression in patients with ALL. Similarly, [38] it was observed that acute leukemia increases soluble *HLA-G* levels.

Saudi Arabia's population exhibits substantial genetic diversity, influenced by its complex demographic structure, which includes a mix of native and immigrant populations. This study examined two *HLA-G* alleles, *G*01:03 and G*01:05N*. The lack of the G*01:03 allele in persons with acute lymphoblastic leukemia (ALL) or healthy controls (HCN) indicates that it is uncommon among the Saudi population. This discovery is consistent with broader genetic studies that emphasize the substantial variation and distinct allele frequencies observed in various populations, especially in locations characterized by high levels of consanguinity, like Saudi Arabia [39]. Consanguineous marriages are quite common in South Africa (about 57.7%), contributing to a unique genetic environment. This genetic background is critical for understanding the distribution of many alleles, including those of the HLA-G gene, which contains the G*01:03 variant [40-42].

Recent study data has shown that the G01:03 allele is not found in patients with inflammatory bowel disease (IBD) [43], suggesting a low prevalence in both populations. Other alleles, such as G01:04 and G01:05N, had polymorphism frequencies and were linked to a higher risk of disorders such as ulcerative colitis (UC) and Crohn's disease (CD) [44]. The lack of G01:03 in both ALL and HCN populations shows that it

may not have a substantial role in the genetic susceptibility to both diseases in the Saudi population.

In conclusion, this study provides evidence suggesting a potential correlation between the *G+3142 C>G* SNP and ALL susceptibility in the Saudi population. This association was further influenced by the stratification according to age and sex. The observed protective effect of this genetic variation seems to be more prominent among younger individuals and females. Nevertheless, further investigations using more extensive sample sizes and a broader range of populations are necessary to validate these discoveries and provide a comprehensive understanding of the genetic predisposition to ALL. Understanding the genetic elements that contribute to the risk of acute ALL may have implications for disease prevention and personalized treatment strategies in the future.

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Disclosure of conflict of interest

None.

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