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# Association of HLA-G*01:01:02:01/G*01:04:01 polymorphism with gastric adenocarcinoma

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ABSTRACT

Human leukocyte antigen-G (HLA-G) plays an important role in tumor cell escape from immune surveillance and HLA-G polymorphisms might service as a potential risk factor for clinical outcomes in GAC (gastric adenocarcinoma). We investigated the association between HLA-G polymorphisms as well as soluble HLA-G level and accordance of GAC. This case-control study included 100 GAC patients and 102 unrelated Iranian individual's samples as control. The clinical stages ranged from I to IV. PCR-RFLP method was carried out in order to specify the genotypes of the HLA-G gene. Concentrations of sHLA-G in serum were determined with the sHLA-G-specific enzyme linked immunosorbent assay (ELISA) kit. The G*01:04:01 and G*01:01:02:01 alleles were the predominant alleles in GAC patients and healthy controls. The G*01:01:03:01 and G*01:01:08 allele distributions are significantly higher among controls comparing to cases and seem to have protective effect (*P* value = 0.026 and 0.007 respectively). There is a substantial differences in G*01:01:02:01/G*01:04:01 genotype frequencies between cases and controls (OR = 2.8, *P* value < 0.001). The G*01:01:03:01/G*01:04:01 and G*01:01:02:01/G*01:01:08 genotypes frequency are higher among controls in comparison to patients (*P* value = 0.028 and 0.007 respectively). The polymorphisms in HLA-G could affect GAC induction and its outcome. Also, increased sHLA-G levels in serum might be a useful biomarker for diagnosis.


## 1. Introduction

Gastric adenocarcinoma (GAC) is the most common gastrointestinal malignancy in Iran [1] and the second leading cause of cancer death worldwide [2]. Some epidemiological studies have suggested that several environmental factors may be involved in the development of GAC, including smoking [3], alcohol consumption [4], pathogenic infections [5]and nutritional deficiency [6]. Nevertheless, only some of the exposed individuals actually developed GAC during their life, suggesting that genetic makeup may confer susceptibility to GAC. Despite advances made in cancer diagnosis and treatment over the last decades, the prognosis of these cancers still remains poor. A number of tumor markers such as plasma CEA and SCC-Ag have extensively been investigated for diagnosis. These markers have shown different variety of sensitivity and specificity [7].

In recent years, strong evidence has emerged that tumor progression could be actively controlled by a host's immune system. However, the effectiveness of the antitumor immune response is relatively poor, indicating that the ability of the immune system to recognize and eliminate tumor cells might be impaired. Human leukocyte antigen-G (HLA-G) molecules are nonclassical HLA-class I molecules that have the same HLA-class I structure. The mRNA splicing provides seven isoforms: four membrane-bound (HLA-G1 to -G4) and three soluble HLA-G (sHLA-G) (HLA-G5 to -G7) [8]. A great number of studies have investigated the involvement of HLA-G in tumor development and progression [9,10]. Like HLA-class I genes, the HLA-G gene is composed of eight exons and seven introns [11]. Most single nucleotide polymorphisms (SNPs) in the HLA-G coding sequence, mainly in exons 2, 3, and 4, which code the α1, α2, and α3 domains respectively, are conserved substitutions. Nonsynonymous SNPs generate 17 different HLA-G peptide

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sequences (G*01:01–G*01:017) and reported SNPs generate 46 HLA-G allelic variants [12]. In contrast to coding regions, three regions of the gene including the 5′-upstream regulatory (5′URR), the promoter and the 3′-untranslated (3′ UTR) are polymorphic [13]. These genetic variations were previously found to influence the HLA-G mRNA and protein expression levels [14] and has been associated with several disorders, including recurrent spontaneous abortion [15], preeclampsia [16], asthma [17] and pemphigus vulgaris [18]. Furthemore, soluble HLA-G has been shown to be involved in producing pro-angiogenic cytokines in NK cells [19] and induce apoptosis in CD8+ T and NK cells by binding to CD8+ through Fas/FasL-dependent mechanism [20]. Increased sHLA-G levels have been reported in patients with melanoma, neuroblastoma, lymphoproliferative disorders, breast, ovarian and colorectal carcinoma in compare to healthy controls or benign neoplasms [21]

Although the role of HLA-G was extensively evaluated, only a few studies have focused on relationship between HLA-G polymorphisms and clinic-pathological parameters. The aim of the present study is to evaluate the relation and likely involvement of the HLA-G gene polymorphisms in GAC risk in Iranian GAC patients.

## 2. Materials and methods

### 2.1. Subjects

This case-control study included 100 GAC patients and 102 healthy individuals as control. GAC patients were recruited from an ongoing population-based cohort study in Golestan North-East of Iran. The clinical factors were assessed by the general rules for gastric adenocarcinoma [22]; the clinical stages ranged from I to IV(TNM classification, 1997). The female: male gender ratio was 4 (20 female and 80 male patients), and the mean age was 64.47 ± 9.42 years (range, 43–81 years). The control subjects had a mean age of 60.6 ± 9.53 years (range, 40–82 years) and a female: male gender ratio of 2.2 (33 female and 69 male subjects). The control subjects were randomly recruited from a group of healthy blood donor Iranian individuals, visiting Iranian Blood Transfusion Organization (Tehran, Iran) with no evidence of any personal or family history of cancer or other major illnesses. Written informed consent was obtained from all subjects. The study was approved by the Medical Ethics Committee of the Tehran University of Medical Sciences (TUMS).

### 2.2. JEG-3 cell line culture

JEG-3 cell line was obtained from the National Cell Bank of the Pasteur Institute of Iran. The cell line was used as a control for a specific genotype of HLA-G. The JEG-3 cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

### 2.3. DNA extraction

Genomic DNA was isolated and purified from the JEG-3 cells and the whole blood samples collected in 5% EDTA using PureGene DNA Extraction Kit (Gentra Puregene Blood Kit, USA). The concentration, purity and integrity of DNA were assessed by NanoDrop (Thermo Scientific 2000c) and electrophoresis was done on 2% agarose gel.

### 2.4. PCR-RFLP analysis

HLA-G genotyping was carried out with polymerase chain reaction followed by restriction fragment length polymorphism. PCR was performed with 300–500 ng of genomic DNA in a total volume of 25 μL of the reaction mixture containing 0.4 μM of the primers (Exon 2: forward: 5′-TCCATGAGGTATTTCAGCGC -3′, reverse: 5′-CTGGGCCGGAGTTACTCACT -3′) and (Exon 3: forward: 5′-CAC ACC CTC CAG TGG ATG AT-3′, reverse: 5′-GGT ACC CGC GCG CTG CAG CA-3′).

Other ingredients included 1.1x reaction buffer (Roche), 4.5 mM MgCL2, 250 μM dNTP and 2.5U of Taq DNA polymerase (Genet Bio). The PCR cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 10 cycles of 94° C for 15 s, 63 °C for 30 s, and 68 °C for 1 min and another 29 cycles of 94 °C for 15 s, 54.7 °C for 30 s and 68 °C for 1 min. The program was followed by a final extension step at 72 °C for 8 min.

Following the amplification of exons 2 and 3, aliquots (10 μL) of the PCR products were digested with the restriction endonucleases Msp-I, Hinf-I and Apa-I for the PCR products of exon 2 and Acy-I and BseR-I for the PCR products of exon 3. Digested fragments of the PCR products were separated by a 2.5% agarose gel and detected by staining with Ethidium bromide. Obtained results were interpreted based on the previous information [18].

### 2.5. sHLA-G enzymed-linked immunosorbent assay

sHLA-G levels in serum of 50 GAC patients and 50 age and sex matched unrelated healthy individuals were determined. Concentrations of sHLA-G in serum were determined with the sHLA-G specific enzyme linked immunosorbent assay (ELISA) kit (sHLA-G kit; Glory science, USA0 according to the manufacturer's instruction. the final concentration was determined by optical density based on the standard curves. The detection limits were 1 U/ml.

### 2.6. Statistical analysis

The genotype frequencies were tested for the Hardy–Weinberg equilibrium for both patients and controls using the X2 analysis. Both allelic and genotypic correlation between patients and controls were analyzed using chi-square test with Pearson and Yates correction and cross tabulation, where appropriate. The odds ratio (OR) and the 95% confidence intervals (CI) were calculated for all data. Differences in levels of sHLA-G between healthy controls and malignant patients were compared using the Student's *t*-test after calculation tests for normality by Kolmogorov–Smirnov normality test using Graph pad software (P value was greater than 0.1). All analyses were performed using the SPSS Statistical Analysis software (version 16) and VassarStats: Website for Statistical Computation. *P* value less than 0.05 were considered statistically significant.

## 3. Results

HLA-G allele frequencies in GAC patients and healthy controls showed in Table 1. The genotype distributions of HLA-G alleles were in Hardy–Weinberg equilibrium for both patients and controls. The G*01:04:01 and G*01:01:02:01 alleles were the predominant alleles in GAC patients and healthy controls. The G*01:01:05, G*01:01:07, G*01:01:08 and G*01:05 N alleles were absent in GAC group but exists in the control group. G*01:01:03:01 allele distribution is significantly higher among controls comparing to cases and seems to have protective effect (OR = 0.44, *P* value = 0.026). The G*01:01:08 allele found among controls ($n = 8$) but not patients and seems to have protective effect too (*P* value = 0.007).

In order to evaluate the possible differences regarding gender of patients, frequencies were statistically analyzed (Table 2). There were no significant differences between male and female patients.