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Relationship of *HLA-G* expression and its 14-bp insertion/deletion polymorphism with susceptibility to colorectal cancer

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ABSTRACT. Human leukocyte antigen (HLA) G, a non-classical HLA class I (MHC class I) molecule, is characterized by a low degree of polymorphism, unlike classical MHC-I. The 14-base pair insertion/deletion (indel) polymorphism (rs16375) in exon 8 of the 3′ untranslated region has been reported to control *HLA-G* expression transcriptionally and post-transcriptionally. We evaluated a possible association between the 14-bp indel polymorphism of *HLA-G* and the level of this protein in serum [*sHLA-G1*] and in primary-tumor tissue in colorectal cancer (CRC) of a Saudi population. A total of 105 patients with CRC and 105 healthy controls were analyzed for the 14-bp indel polymorphism. *sHLA-G1*; histological presence of *HLA-G* was also investigated for association with CRC. Lower prevalence of the heterozygous genotype of the 14-bp indel polymorphism was

observed among the patients with CRC, though the difference was not quite significant (P = 0.052). In addition, the *sHLA-G1* pattern did not show a significant difference between the patients with CRC and the controls. However, the expression pattern of *HLA-G* in colorectal tissue showed a heterogeneous profile, marked by a lack of expression in colorectal adenocarcinoma and normal cells and focal expression of the protein in the transition zone.

Key words: HLA-G; Colorectal cancer; Saudi Arabia; Immune tolerance; Genetic association

INTRODUCTION

The human leukocyte antigen (*HLA*) G is a non-classical major histocompatibility complex class I (MHC class I) protein and was first described by Geraghty et al. (1987). The *HLA-G* gene is located on the short arm of chromosome 6 within the *HLA* region (6p21.31), between genes *HLA-A* and *HLA-F* (Koller et al., 1989). The *HLA-G* gene consists of 7 introns and 8 exons coding for the heavy chain of the *HLA-G* molecule in addition to the 5′ promoter (5′ upstream regulatory region) and a 3′ untranslated region (3′-UTR). Exon 7 is missing in the mature mRNA owing to the presence of a stop codon in exon 6, and exon 8 is not translated (Donadi et al., 2011). *HLA-G* can be expressed as seven isoforms, including four membrane-bound (*HLA-G1*, *-G2*, *-G3*, and *-G4*) and three secreted isoforms (*HLA-G5*, *-G6*, and *-G7*) resulting from alternative splicing of its primary mRNA (Riteau et al., 2001). Furthermore, soluble *HLA-G1* (*sHLA-G1*) can be secreted into the medium via shedding of proteolytically cleaved surface *HLA-G1* (O'Brien et al., 2001).

HLA-G is primarily a regulatory molecule that enables the fetus to escape the reactions of the maternal immune system (Kovats et al., 1990). It was initially detected at high concentrations on trophoblasts at the foetal–maternal interface (Ferreira et al., 2017). It was later reported to be expressed in some specific regions such as the cornea, thymus, and pancreatic β-islets (Le Discorde et al., 2003). In the last decade, *HLA-G* was found to be associated with the regulation of both innate and adaptive immune responses by maintaining tolerance through its interaction with specific inhibitory receptors (Carosella et al., 2008).

Unlike the high degree of polymorphism in classical MHC class I genes, *HLA-G* manifests only limited polymorphism, distributed among the α1, α2, and α3 domains, and its expression can be altered by allelic variants (Koller et al., 1989; Fainardi et al., 2011). Multiple genetic polymorphisms in the 5′ upstream regulatory region and 3′-UTR have been reported, which may affect *HLA-G* expression via a change in the binding affinity of transcription factors, stability of mRNA, and microRNA targeting (Castelli et al., 2014).

The sequence of *HLA-G* 3′-UTR contains approximately 1000 nucleotides and contains some polymorphic sites that may influence the expression profile of this protein (Geraghty et al., 1987). One common polymorphic site in the 3′-UTR is the 14–base pair (bp) insertion/deletion (indel) polymorphism (rs16375), which has been demonstrated to regulate alternative splicing of the mRNA isoforms, mRNA expression of HLA-G, and protein levels of *HLA-G* for the majority of soluble and membrane-bound isoforms (Amodio et al., 2014). After lung and breast malignant tumours, colorectal cancer (CRC) is the third most common cancer worldwide, and the fourth most common cause of

oncological deaths (Torre et al., 2015). Between 1994 and 2003, the age-adjusted incidence of CRC in Saudi Arabia increased almost two-fold, with a noticeable increase in the incidence between 2001 and 2006 (Ibrahim et al., 2008; Mosli and Al-Ahwal, 2012). According to the new report of the national Saudi Cancer Registry, a total of 1374 cases of CRC have been documented, and this number accounts for 11.5% of the total cancer incidence, thus ranking CRC first in the male population and third in the female population with an overall age-adjusted prevalence of 10.6 per 100,000.

In the last decade, *HLA-G* was implicated in the immune escape of cancer and infectious diseases. The *HLA-G* protein performs its function through its interaction with some inhibitory receptors like killer immunoglobulin-like receptor, KIR2DL4, on the surface of natural killer (NK) cells and immunoglobulin-like transcripts ILT2 and ILT4 in a wider range of immune cells including subsets of NK and T cells (Rajagopalan and Long, 2012; Naji et al., 2014). Furthermore, different expression profiles of the *HLA-G* gene under the influence of the 14-bp indel polymorphic site are associated with pathological conditions, including cancers (Ye et al., 2007; Silva et al., 2013; Teixeira et al., 2013; Jeong et al., 2014; Haghi et al., 2015; Coelho et al., 2016), viral infections (Zheng et al., 2009; Segat et al., 2014), parasitosis (Courtin et al., 2013), transplantation (Boukouaci et al., 2011; Chiusolo et al., 2012), and autoimmune and inflammatory diseases (Veit et al., 2009; Rizzo et al., 2013; Morandi et al., 2016). Therefore, the aim of this study was to evaluate the associations of the alleles and genotypes of this polymorphism (*HLA-G* 14-bp indel, rs16375) and *HLA-G* protein expression with CRC in Saudi Arabia population.

MATERIAL AND METHODS

Subjects

A total of 105 patients with CRC were recruited from two hospitals in the Kingdom of Saudi Arabia: 50 patients from King Fahad Specialist Hospital-Dammam-Eastern Province of Saudi Arabia and 55 cases from King Khaled University Hospital. Appropriate ethics approval was obtained from the local research ethics committee. The patients underwent staging and detailed treatment plan work-up through a consultant oncologist. For this study, 119 healthy individuals were enrolled as a control group. Fifty individuals of this control group were blood bank donors who visited the King Fahad Specialist Hospital-Dammam hospital, and the rest (59 individuals) visited King Khaled University Hospital. All the participants were asked for their consent according to the permit provided by the ethics committee of King Saud University for this study.

Genomic DNA was isolated from whole blood using a commercial kit: DNeasy Blood and Tissue Kit (Qiagen). Quantity and purity of each DNA sample was checked on a NanoDrop^{TM} 2000/2000c Spectrophotometer (Thermo Scientific, USA). Typing of the 14-bp *HLA-G* indel polymorphism in exon 8 was performed by polymerase chain reaction (PCR) with primers GE14HLAG (5′-GTGATGGGCTGTTTAAAGTGTCACC-3′) and RHG4 (5′-GGAAGGAATGCAGTTCAGCATGA-3′) (Hviid et al., 1999). The PCR protocol was as described by Al Omar et al. (2015).

The PCR products (210/224 bp) were analysed based on the presence or absence of a specific band on a 3% agarose gel stained with ethidium bromide and visualized on an

ultraviolet transilluminator with a gel documentation system (BioRad Gel225 Doc™XR+, Hercules, CA, USA) (Figure 1)

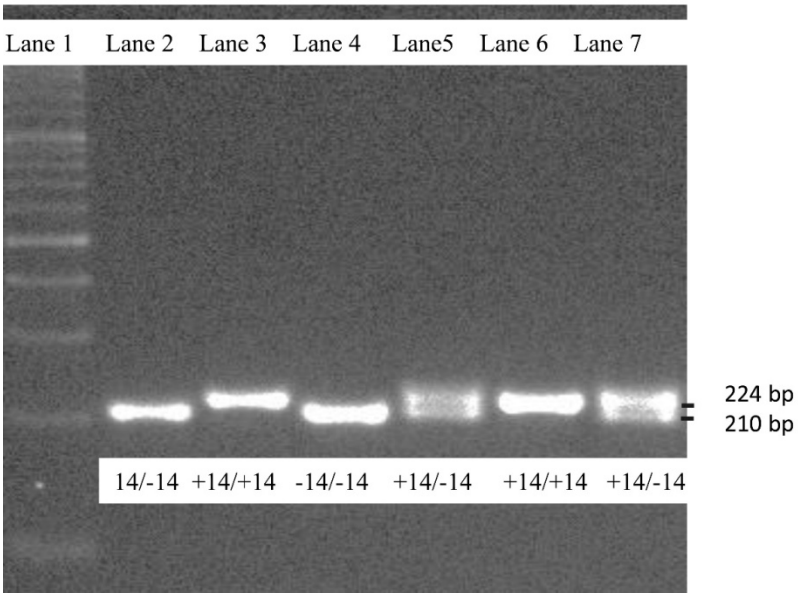

Figure 1. Genotyping of *HLA-G* 14-bp indel polymorphism in agarose gel. Lane 1 is the 100 bp DNA ladder. Lanes 2 and 4 are homozygous -14-bp/-14bp; lanes 3 and 6 are homozygous +14-bp/+14-bp and lanes 5 and 7 are heterozygous +14-bp/-14-bp

Quantitation of *sHLA-G1* by an enzyme-linked immunosorbent assay (ELISA)

The serum levels of *sHLA-G1* were determined by the double-sandwich ELISA technique using the Soluble Human leucocyte antigen G1 ELISA Kit (MyBioSource, California, USA, catalogue No. MBS2600014). All the samples were analyzed in duplicate. Optical density was measured at 450 nm on a microplate reader (Anthos 2020; Anthos Labtec Instruments, Eugendorf, Wals, Austria). The final concentration of *sHLA-G1* (in ng/mL) for each sample was calculated via a standard curve by means of standard solutions.

Immunohistochemical analysis of *HLA-G*

Standard immunohistochemical analysis was performed on formalin-fixed paraffin-embedded tumor tissue samples from patients with CRC by the protocols reported by Alomar et al., (2016). Briefly, tissue sections of 3-µm thickness prepared on a Leica RM2235 Rotary Microtome (Leica Biosystems, Wetzlar, Germany), were deparaffinized, and incubated for 30 mn with an anti-*HLA-G* monoclonal antibody (1:50; Abcam, ab52455, UK, Cambridge). The UltraView Universal HRP detection Kit was the source of secondary antibodies and was applied using a BenchMark XT automated staining system (Ventana, Arizona, USA). The immunostained tissue sections were visualized under an Olympus BX51 light microscope and DP72 equipped with an Olympus Digital Camera (magnification 100× and 200×; Olympus America Inc., Center Valley, PA, USA).

Statistical analysis

The allele and genotype frequencies of the 14-bp indel polymorphism of *HLA-G* were determined by direct counting. Binary logistic regression analysis was performed to calculate odds ratios (ORs) and 95% confidence intervals (CIs). Statistical significance was assessed by two-tailed Fisher's exact test at the level of 5%. These statistical analyses were performed in the SigmaPlot software, version 11.0, Build 11.0.0.77 (GmbH, Germany). For ELISA data analysis, the two groups were compared using the nonparametric Mann–Whitney test in GraphPad Prism, version 8 (GraphPad Software, San Diego, CA).

RESULTS

In this study, we analyzed 210 Saudi subjects (105 CRC cases and 105 healthy controls) for the association between the *HLA-G* 14-bp indel polymorphism and CRC. Clinical and demographic characteristics of the studied groups are given in Table 1. No significant difference in the sex distribution was noted (P = 0.4). The mean age, however, was significantly older among the patients with CRC (57.9 ± 13 years) than among the controls (47.4 ± 11.6 years; P < 0.001). We found that 83% of the cancers were localized in the colon, and 59% of them were at stage II.

Table 1. Demographic and main clinical data of the colorectal cancer patients and controls.

Characteristic			Cancer (105)	Control (105)
Gender	Male		55.2%	48.6%
	Female		44.8%	51.4%
Age			57.9±13.1	47.4 ± 11.6
Localization	Colon		82.9%	-
	Rectum		17.1%	-
Stage		0	2	-
		II	59	-
		III	33	-
		IV	11	-

The allele frequencies and the genotype distributions were compared between the case (CRC) and control group for risk assessment. The results are summarized in Table 2.

Table 2. Distribution of the 14‑bp insertion/deletion allele and genotype in patients with colorectal cancer and controls.

Genotype	Control (n=119)	CRC % (n= 105)	OR	CI	P value
14-bp insertion	117 (0.49)	107 (0.51)	Ref		
14-bp deletion	121 (0.51)	103 (0.49)	0.93	0.64-1.34	0.77
ins/ins	27 (0.23)	33 (0.31)	Ref		
del/del	29(0.24)	31 (0.30)	1.3	0.71-2.35	0.45
Heterozygous allele	63 (0.53)	41 (0.39)	0.56	0.33-0.97	0.052

CRC = colorectal cancer; OR = odds ratio; CI = confidence interval.

The three *HLA-G* 14-bp indel genotypes in the control group were in Hardy–Weinberg equilibrium (χ^2 = 0.42, P = 0.51). No significant differences in the *HLA-G* 14-bp indel alleles were observed between the patients and controls. Genotype comparisons showed a greater proportion of the heterozygous genotype of the 14-bp indel polymorphism among the controls than among the patients with CRC, but this difference did not reach significance (P = 0.052). Associations according to sex and age were also assessed (Tables 3–6). The heterozygous genotype was more frequent among female controls than among female patients with CRC, but once again, the difference was not significant (P = 0.134; Table 3). A similar result was obtained in the analysis of the male groups (P = 0.69; Table 4).

Table 3. Distribution of the 14‑bp insertion/deletion allele among females.

Genotype	Control % (n= 54)	CRC % (n= 47)	OR	CI	P value
14-bp insertion	54	52	1.23	0.71-2.16	0.48
14-bp deletion	54	42	0.80	0.46-1.40	0.48
del/del	10	11	1.34	0.51-3.52	0.62
ins/ins	10	16	2.27	0.91-5.66	0.1
Heterozygous allele	34	20	0.43	0.19-0.96	0.064

CRC = colorectal cancer; OR = odds ratio; CI = confidence interval.

Table 4. Distribution of the 14‑bp insertion/deletion allele among males.

Genotype	Control % (n= 51)	CRC % (n= 58)	OR	CI	P value
14-bp insertion	50	39	0.66	0.38-1.1	0.15
14-bp deletion	52	61	1.50	0.86-2.6	0.15
del/del	16	20	1.45	0.64-3.30	0.48
ins/ins	15	9	0.52	0.20-1.34	0.24
Heterozygous allele	20	21	0.83	0.38-1.82	0.69

CRC = colorectal cancer; OR = odds ratio; CI = confidence interval.

To evaluate the CRC association of the 14-bp indel polymorphism adjusted for age, we stratified the patients and controls into groups <50 and >50 years of age (Tables 5 and 6). No significant differences in the frequencies of the allele or in the genotype distribution were observed between the patients and controls of the two subgroups.

Table 5. Distribution of the 14‑bp insertion/deletion allele among patients >50 years old.

Genotype	Control % (n= 38)	CRC % (n=81)	OR	CI	P value
14-bp insertion	33	82	1.31	0.76-2.28	0.33
14-bp deletion	43	81	0.75	0.43-1.31	0.33
del/del	7	25	1.65	0.63-4.32	0.36
ins/ins	12	24	0.91	0.39-2.1	0.83
Heterozygous allele	19	32	0.65	0.3-1.41	0.32

CRC = colorectal cancer; OR = odds ratio; CI = confidence interval.

Table 6. Distribution of the 14‑bp insertion/deletion allele among patients <50 years old.

Genotype	Control % (n= 67)	CRC % (n=24)	OR	CI	P value
14-bp insertion	32	21	1.5	0.7-3.2	0.33
14-bp deletion	48	21	0.66	0.31-1.41	0.38
del/del	19	6	0.84	0.29-2.44	0.79
ins/ins	35	9	0.54	0.21-1.42	0.24
Heterozygous allele	13	9	2.49	0.89-6.94	0.134

CRC = colorectal cancer; OR = odds ratio; CI = confidence interval.

Serum *sHLA-G1* levels of 33 randomly selected patients with CRC and of 30 controls were determined. A box plot of the results is presented in Figure 2. The *sHLA-G1* concentration was higher among the patients with CRC than in the controls, but the difference did not reach significance (P = 0.057). The median value was 0.75 ng/mL (range 0.69 - 3.47) among the patients and 0.43 ng/mL (range 0.03 - 3.27) among the controls. No significant differences were observed when the 14-bp indel polymorphism genotype, sex, and CRC grade of the patients were considered.

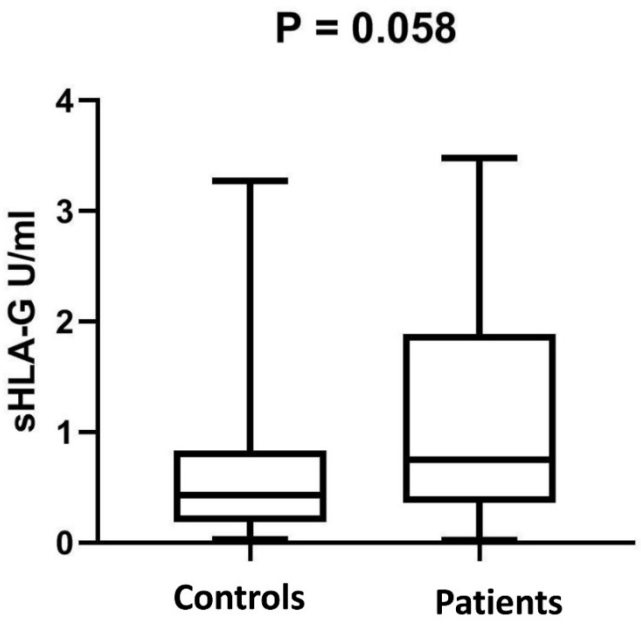

Figure 2. Comparison of *HLA-G* serum level in CRC patients and healthy controls by ELISA assay using T-test analysis.

Immunohistochemical analysis was performed to determine the expression of the *HLA-G* protein in six adenocarcinoma tissue samples (Figure 3). Two sectioned samples did not show any signal, i.e. tested negative for HLA-G. The four other tissue sections showed no *HLA-G* signal in malignant and normal cells but yielded a positive signal in the transition zone between the normal tissue and adenocarcinoma.

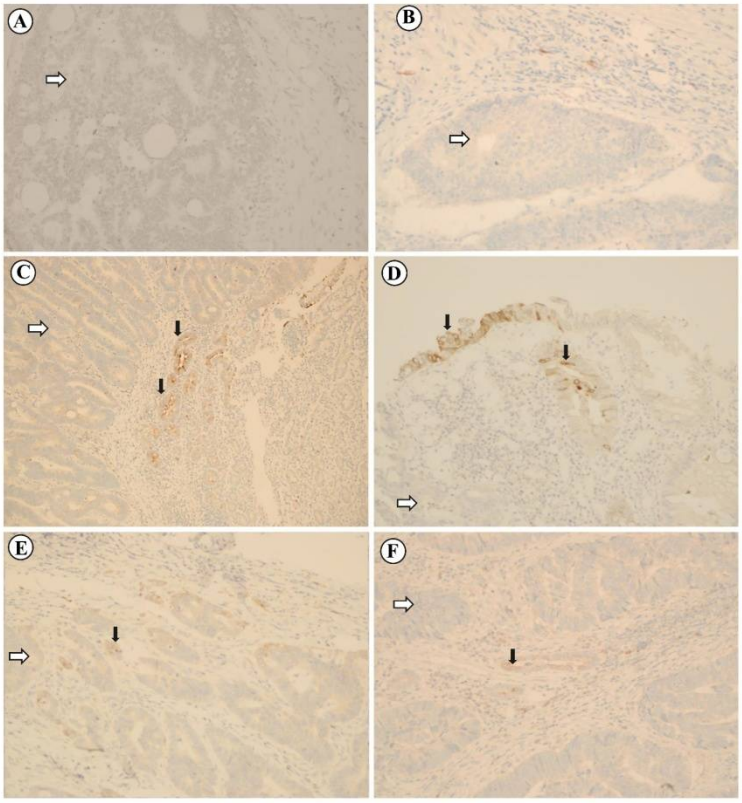

Figure 3. Immunohistochemical staining of *HLA-G* in colon tissues containing cancer tissue (white vertical arrows), with dysplastic lesions and normal colon tissue, in the same slide. A: negative control; B: negative *HLA-G* staining; C and D: focal positivity at the luminal surface of the dysplastic cells at the transition between benign/dysplastic cells and malignant cells (black vertical arrows. E and F: a focal weak positivity at luminal surface of the dysplastic cells (vertical black arrows). The malignant cells are negative (horizontal white arrows). A, B, E and F = 20x; C and D = 10x.

DISCUSSION

The biological function and clinical importance of *HLA-G* have been extensively studied in many human diseases including cancer (Ge et al., 2014; Reimers et al., 2014; Morandi et al., 2016; de Almeida et al., 2018). Even though its expression is restricted to some healthy tissues including trophoblasts in pregnant women, the cornea, thymus, and pancreas, *HLA-G* proteins have been reported to be expressed in some diseases affected tissues like tumors, transplanted organs, and inflamed tissues (Le Discorde et al., 2002; Le Discorde et al., 2003; Lin and Yan, 2018). The inter-individual variation in the level of *HLA-G* expression in pathological and normal physiological conditions has been attributed to some genetic variability within the regulatory regions of the gene. Some polymorphic sites in the 5′-UTR and 3′-UTR were reported to influence the expression of *HLA-G* and the type of isomer being expressed (Dias et al., 2015). The 14-bp indel polymorphism in the 3′-UTR region of *HLA-G* has been widely studied and was analysed along with other polymorphism sites (single-nucleotide polymorphisms) influencing the stability of

mRNA and alternative splicing (Castelli et al., 2010; Martelli-Palomino et al., 2013; Poras et al., 2017). The 14-bp insertion allele is associated with *HLA-G* mRNA instability resulting in weaker expression of the protein (Hviid et al., 2003). Although the role of the *HLA-G* 14-bp indel polymorphism has been examined in several diseases including different types of cancer, its association with CRC has not been reported frequently (Garziera et al., 2015; Garziera et al., 2016; de Almeida et al., 2018). In the present study, we explored the associations of the *HLA-G* 14-bp indel polymorphism and *HLA-G* expression with CRC in a Saudi population. We did not find a significant allelic association between the *HLA-G* 14-bp indel polymorphism and CRC among the examined blood samples. Nevertheless, with marginal significance, lower prevalence of the heterozygous genotype was observed among the patients with CRC (P = 0. 052). Stratification analyses according to sex and age did not reveal significant associations of the alleles or genotypes of rs16375 with CRC. Randomly selected patients with CRC and healthy individuals were tested here for the blood concentration of *sHLA-G1* by the double-sandwich ELISA method. This assay did not detect significant differences between the patients and healthy individuals. Regarding these results, very wide variation was noted within each group regardless of the 14-bp indel genotype, sex, and CRC grade of the patients. This result could be explained by a multitude of factors that could influence the levels of sHLA-G, as reported in other studies, including genetic polymorphisms of the 3′-UTR and 5′-UTR regulatory regions in addition to the 14-bp indel polymorphism and epigenetic mechanisms such as DNA methylation, histone acetylation, and transcriptional repressor RREB-1 (Larsen and Hviid, 2009; Castelli et al., 2011; van den Elsen, 2011; Martelli-Palomino et al., 2013; Svendsen et al., 2017). In other studies, high levels of s*HLA-G* in blood have been reported to be associated with cancers such as breast, ovarian, lung cancers, leukemia, gastric, oesophageal and colorectal carcinomas (Yie et al., 2007a; Yie et al., 2007b; He et al., 2010; Cao et al., 2011; Provatopoulou et al., 2012). The s*HLA-G* level was then associated in many cases with an unfavorable outcome of a disease and was suggested as a prognostic factor (Nuckel et al., 2005). The expression profile of *HLA-G* was assessed here in primary-cancer tissue by immunohistochemical analysis. *HLA-G* was detected in four out of six examined tissue samples, but only in dysplastic cells at the transition of a normal to malignant tissue. The colonic adenocarcinoma cells and the normal tissue yielded negative results. The two HLA-G–negative tissue samples belong to patients at the T2 N0 Mx stage. The dysplastic tissue samples expressing *HLA-G* belong to patients having nodal expansion or metastasis. This finding is in agreement with other studies showing aberrant expression of *HLA-G* in tissues of many cancers including CRC tumors (Paul et al., 1998; de Kruijf et al., 2010; Lin and Yan, 2015; Zhang et al., 2017; Babay et al., 2018). The correlation between the expression of *HLA-G* in tumour tissue and the clinical outcome of a disease (e.g. reduced overall survival) has also been reported in many studies (Zeestraten et al., 2014; Guo et al., 2015; Zhang et al., 2017; Babay et al., 2018). The exclusive neoexpression of *HLA-G* in the transition dysplasia between cancer and healthy tissue in the present study may be a contributing factor that favors tumor progression and carcinogenesis. In this context, studies have been focused on the role of *HLA-G* as an immunosuppressive molecule allowing cancer cells and viruses to evade cell-mediated immunity, and thus *HLA-G* may be considered a marker of disease (including cancer) progression (Reimers et al., 2014; Lin and Yan, 2018). This function is mainly mediated by the interaction of *HLA-G* with specific suppressive receptors expressed on the surface of

immune cells, e.g. immunoglobulin-like transcript (ILT2 and ILT4) and killer cell immunoglobulin-like receptor 2DL4 (Morandi et al., 2014; Zhang et al., 2015). The absence of *HLA-G* molecules should make tumor cells susceptible to removal by cytotoxic NK and $CD8^+$ T cells. Moreover, it was suggested that down-regulation of *HLA-G* via RNA interference or antibody blockade can prevent tumor recurrence and recover the immune function (Carosella et al., 2015; Porto et al., 2015). In conclusion, our study did not find statistically significant differences in the frequencies of the 14-bp insertion/deletion *HLA-G* alleles or genotypes between controls and patients with colorectal cancer in Saudi Arabia. Soluble *HLA-G* was marginally increased in patients with CRC. However, the protein expression in the primary tumor tissue showed a specific pattern characterized by focal expression at the level of the dysplastic cells, while the malignant and normal tissue did not express the protein. Although we did not find an association between the 14-bp polymorphism of the HLA-G, a role of this molecule in the progression of CRC particularly at the level of the primary tissue, could not excluded. *HLA-G* may intervene in the suppression of the cytotoxic immune response against tumor cells and in the creation of a microenvironment favorable to the progression of the tumor.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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