Abstract. Aberrant expression of human leukocyte antigen G (HLA-G), an immunosuppressive molecule, has been observed in various cancer types. The exact mechanism of HLA-G expression is unclear. HLA-G expression is associated with low expression levels of microRNA (miR)-148a and miR-152. To the best of our knowledge, the prognostic value of the expression levels of miR-148a, miR-152 and HLA-G has not been investigated in colon cancer. The aim of the present study was to investigate the relationship between the presence of HLA-G molecules and the expression levels of miR-148 and miR-152 in colon cancer. In addition, the association of both HLA-G and miR expression with survival results and clinicopathological data was determined. An immunohistochemical method was used for detection of HLA-G expression in the tumor tissues and adjacent healthy tissues of 108 patients with colon cancer. Reverse transcription-quantitative PCR was used for miR analysis. HLA-G was expressed in 82 (75.9%) of the cancer samples, and there was no staining in normal tissues. HLA-G expression >20%, which was found in 41.7% (45/108) of the patients with colon cancer, was significantly positively associated with patient survival (P=0.039). Additionally, miR-148a levels were lower in the tumor tissues compared with the adjacent healthy tissues. No differences were found for miR-152 expression. In addition, mir-148a levels were found to be significantly lower (P=0.001) in HLA-G-positive tumor tissues than HLA-G-negative tumor tissues, but no such relationship was found for miR-152 levels. The presence of HLA-G expression was associated with poor survival outcomes. HLA-G is one of the prognostic factors in colon cancer, and decreased miR-148a expression in colon cancer tissues may be associated with HLA-G-mediated carcinogenesis.

Introduction

Colon cancer is one of the most common cancers in the world. Many mechanisms responsible for colon cancer have been identified in detail, including activation of oncogenic signaling pathways resulting from genetic mutations, inactivation of tumor suppressor genes, and epigenetic changes (1). Important features of tumor cells during colon cancer development are their ability to suppress of immune system cells and their avoidance of immune destruction. In this process, tumor cells secrete cytokines such as transforming growth factor beta, which reorganize the microenvironment and inhibit the infiltration of T cells. As a result of major histocompatibility complex class 1 mutations, tumor neoantigens are lost, and immune system's ability to recognize cancer cells decreases. Cytotoxic activity is suppressed by the overexpression of HLA-G on the cell surface as a consequence of the production of miR (2).

HLA-G is a non-classical major histocompatibility class Ib molecule, the presence of which was first demonstrated in placental extravillous trophoblast cells (3). HLA-G creates an immunotolerant environment and prevents fetal rejection by maternal immune system cells (4,5). While its physiological presence is limited to pancreatic island cells, thymic epithelial cells, activated monocytes, corneal keratocytes, and erythroblasts, its pathological expression has been demonstrated on the surface of various cancer cells (6). HLA-G can inhibit the activation and proliferation of T and B cells, the cytotoxic function of T and NK cells, and neutrophil functions. HLA-G shows an immunosuppressive feature by binding to various receptors on the cell surface, including immunoglobulin-like transcript receptor 2 on lymphoid and myelomonocytic cells (ILT-2) on lymphoid and myelomonocytic cells, ILT-4 on dendritic cells (4,5). While its physiological presence is limited to pancreatic island cells, thymic epithelial cells, activated monocytes, corneal keratocytes and erythroblasts, its pathological expression has been demonstrated on the surface of various cancer cells (6). HLA-G can inhibit the activation and proliferation of T and B cells, the cytotoxic function of
KRAS, NRAS types of cancer, including colon cancer. It has been reported apoptosis, tumor development, and metastasis (10). Recently, mechanisms, such as metabolism, proliferation, differentiation, protein-coding genes. Thus, miRs control various cellular transcriptional regulator by binding to the 3'UTR region of noglobulin-like receptor on natural killer cells (7‑9).

Cells, ILT‑4 expressed by dendritic cells, and killer cell immuno‑transcript (ILT) receptor 2 on lymphoid and myelomonocytic the cell surface some of which are are immunoglobulin‑like immunosuppressive feature by binding to various receptors on T and NK cells, and neutrophil functions. HLA‑G shows an 2

The mechanisms controlling HLA‑G expression are largely unknown. The 3'UTR region in the HLA‑G structure has an important role in regulating the expression of the molecule and functions as the target of miRs. Previous studies have shown that miR148‑a and miR‑152 bind to the 3'UTR terminal of the HLA‑G molecule, and downregulate it. HLA‑G overexpression is accompanied by low miR‑148a and/or low miR‑152 levels in these studies (12‑14). Based on previous studies, we hypothesized that there may be a similar relationship of HLA‑G expression with miR‑148a and miR‑152 levels in colon cancer.

Materials and methods

Patients and tissue samples. Pairs of colon tumor and adjacent nontumorous tissues of 108 patients with colon cancer were collected. All patients were treated with surgery between 2013 and 2016 at the GATA Haydarpasa Training Hospital (GHTH). We included patients who were operated from the beginning of January 2013 to the end of December 2016 in our study. The last patient we included in the study was followed up for 5 years. Previous patients had longer follow-up. Our study protocol complied with the principles of the Declaration of Helsinki. Informed consent forms were obtained from all patients. Patients who did not agree to give informed consent for participating in the study were excluded. Formalin‑fixed, paraffin‑embedded tissue blocks were retrieved from the pathology archives. Follow‑up was conducted from the first 2 years, then every 6 months for 3 years, and then yearly. During follow‑up visits, whole and routine blood examinations were performed, serum carcinoembryonic antigen levels were tested, and a complete physical examination was conducted. Patients also received abdominal ultrasound and chest X‑rays or a chest, abdominal, and pelvic computerized tomography scan once yearly. Colonoscopy was performed 1 year after surgical resection and repeated in Years 3 and 5. The patients were followed for at least 5 years post‑operation. Clinicopathological data were retrieved from the patients' medical files and pathology reports. In addition, patients' survival times were confirmed via telephone inquiry until September 2021. Time of recurrence or death was noted. Our study was approved by the ethics committee of GHTH with approval number 37/24.02.2016.

Immunohistochemical staining. We determined the expression level of HLA‑G using the immunohistochemistry method. Blocks greater than 0.5 cm in diameter and containing at least 50% tumor were chosen for evaluation. We cut 4‑μm‑thick sections from paraffin‑embedded tissue blocks and mounted them on poly‑lysine‑coated slides. The slides were deparaffinized in xylene and rehydrated in 70% ethanol. Endogenous peroxidase activity was blocked using a 0.3% hydrogen peroxide solution containing methanol at room temperature for 30 min. Antigen retrieval was performed at 120°C (autoclave) for 5 min in a 10‑nmol/l sodium citrate buffer (pH 6.0), and then anti‑HLA‑G mouse monoclonal antibody (4H84‑sc21799; Santa Cruz Biotechnology, Inc.; dilution range: 1/100) was incubated for 2 h. Afterwards, the samples were washed in a 0.01‑M phosphate‑buffered saline solution. Subsequently, the binding sites of the primary antibody were visualized using the ultraView Universal DAB Detection Kit (Ventana Company), and the Ventana BenchMark automated system; Ventana Company. Finally, sections were counterstained with hematoxylin and mounted with glycerol gelatin. The entire tumor area in the samples was examined by two experienced pathologists under a light microscope at 4x magnification. In cancer cells, HLA‑G stained cytoplasmically. The percentage of staining in the entire area was recorded. The presence of HLA‑G expression in colon cancer tissue was found in different proportions, from negative to 95% positivity. Overall, 75.9% (n=82) of colon cancer samples were HLA‑G positive. Kaplan‑Meier survival analysis was performed to determine the minimum proportion of HLA‑G expression which reaches statistic significance to patient survival. The data showed that the cut‑off value for HLA‑G was 20%, which was significantly associated with patient survival (P=0.039). Therefore, 41.7% (n=45) of the patients were considered HLA‑G positive.

RNA extraction and polyadenylation. Total RNA was isolated using the RecoverAll Total Nucleic Acid Isolation kit (Life Technologies; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. The quantity of the isolated RNA was assessed using a NanoDrop 1000 spectrophotometer; Thermo Fisher Scientific, Inc. Polyadenylation of total RNA was performed with Escherichia coli poly (A) polymerase (Life Technologies; Thermo Fisher Scientific, Inc.) at 37°C for 30 min according to the manufacturer's instructions for the poly(A) tailing kit. RNAs were stored at 70°C.

Reverse transcriptase reaction. Single‑stranded cDNA was synthesized using the miScript II Reverse Transcription Kit (Qiagen Sample and Assay Technologies). A reverse transcriptase mixture was prepared by adding 4 μl of a HiSpec buffer, 2 μl of a miScript nucleic mix, 2 μl of Script RT, 2 μl of template RNA, and 10 μl RNase‑free water. This mixture was kept at 37°C for 60 min and then incubated at 95°C for 5 min to deactivate the reverse transcriptase. For reverse transcription‑quantitative PCR (RT‑qPCR) assays of miRNAs, cDNA was generated from equal amounts of total RNA per sample. The cDNAs were stored undiluted at 20°C.

qPCR. qPCR was done using a miScript SYBR‑Green Kit (Qiagen Sample and Assay Technologies). The 25‑μl PCR mixture contained 10 μl of Quntitect SYBR‑Green PCR
master mix, 1 μl of miScript universal forward and reverse primer, 5 μl of template cDNA, and 8 μl of RNase-free water. The nucleotide sequences for miR-148a were forward 5'-TCA GTGCACCTACGAGGGTGT-3' and reverse 5'-GCTGTC ACGATACGCTACGT-3' and for miR-152 were forward 5'-TCAGTGTCATGACGACAGTTGGAA-3' and reverse 5'-GCTGTCACGATCAGCTACGT-3'. All amplifications were conducted with a Rotor-Gene Q 5Plex real-time PCR system (Qiagen Sample and Assay Technologies). The cycles were completed with qPCR at 94˚C for 5 sec, 58˚C for 20 sec, and 72˚C for 30 sec, with a total of 45 amplification cycles. In order to evaluate the specificity of the PCR products formed, melting curve analyses were performed between 55 and 95˚C, with a sample temperature increase rate of 0.1˚C/sec. RNU6B small nuclear RNA with serial dilutions was used as a reference for normalizing the expression levels of miRNAs. The relative expression levels of miR-148a and miR-152 were calculated using the 2^ΔΔCT method (16). The ΔΔCT values were compared with the threshold values (CTcycle threshold) of the amplification curve of each miRNA, and the CT of the target miRNAs were determined. For each miRNA, ΔCT (Delta Cycle Threshold) values were calculated with the ΔCT=CT miRNA-CT U6RNA formula in both tumor tissue and non-tumor tissue. The Rotor-Gene Q Series software v. 1.7 (Qiagen Sample and Assay Technologies) was used for this calculation. The relative expression ratio of miR-148a and miR-152 was presented as the fold change. A relative expression ratio of <1.0 was considered low expression in cancer cells relative to the nontumorous control.

Statistical analysis. Statistical analyses were performed using SPSS v. 25.0 (IBM Corp.). Disease-free survival (DFS) was calculated from the date of surgery to the date of disease relapse or death from any cause. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Survival analyses were performed using the Kaplan-Meier analysis method. The effects of various prognostic factors related to tumor and patient characteristics on DFS and OS were investigated with the log-rank test. The effects of multiple prognostic factors on DFS and OS were investigated using the multivariate Cox regression test. Comparisons between groups for quantitative data were made with the Mann-Whitney U test and Kruskal-Wallis H test. Categorical comparisons between groups were calculated with Pearson's Chi-squared test, continuity correction, and Fisher's exact test. Spearman's correlation test was used to determine the level of correlation between miR-148a and miR-152 levels. Wilcoxon's signed-rank test was used to determine whether there was a difference between the levels. The results were evaluated at the 95% confidence interval and a significance level of P<0.05.

Results

Patient characteristics. Analyses of HLA-G and miRNA expression were performed on 108 patients with colon cancer stages I-III. The mean age at the time of diagnosis was 69.31±10.35 years. Of the 108 samples analyzed, 60 were from male and 48 from female subjects. Patients' clinicopathological features are shown in Table I.

HLA-G expression in colon cancer. In the immunohistochemical staining, HLA-G was detected as a brown stained product in tumor tissue but not stained in normal colon tissue (Fig. 1). Comparing the HLA-G-positive and negative patient groups, the former group showed significantly higher rates of lymph node positivity (53 vs. 29%, P=0.003) and presence of perineural invasion (49 vs. 21%, P=0.004) and significantly lower pathologically early-stage disease rates (7 vs. 22%, P=0.038). In addition, miR-148a levels were significantly lower (P=0.001) in the HLA-G-positive tumor tissues than in negative ones (Table I). For miR-148a expression, levels were downregulated 2.5 fold or more in tumor tissue compared with normal colon tissue, but no such relationship was found for miR-152 expression (Table II).

Survival analyses. The mean follow-up period was 87.46±26.29 months, and the median follow-up period was 90 (14-127) months. A total of 32 (29.6%) patients received adjuvant fluoropyrimidine-based chemotherapy. The rate of adjuvant therapy was significantly higher (46.6 vs. 17.4%, P=0.041) in the HLA-G positive group. The mean disease-free survival (DFS) time was 80.41±34.13 months, and the median DFS time was 90 (7-127) months. During the follow-up period, 26 patients (24.1%) relapsed, 23 patients (21.3%) died due to disease, and 3 patients (2.8%) died due to other causes. According to the Kaplan-Meier survival analysis results, the 10-year DFS, and OS (overall survival) probabilities of the patients were 75.9, 72.8 and 65.2%, respectively. Patients with positive HLA-G expression had a lower 10-year DFS rate (92.1 vs. 53.3%, P<0.001). According to the results of the univariate Cox regression analysis, miR-148a levels were found to be associated with DFS (P=0.014) (Table III). According to the multivariate Cox regression analysis, the presence of HLA-G expression, grade 3 tumor, and N2 status were shown to be independent factors that negatively affected survival (Table IV). Survival curves according to HLA-G status are shown in Figs. 2 and 3.

Discussion

In our study, we found that the level of miR-148a in cancer tissue was significantly lower than in healthy adjacent tissue, but the difference between miR-152 levels was not significant. Specifically, among the 108 patients with colon cancer, miR-148a was weakly expressed in 82 cases (76%; P<0.001), and miR-152 was weakly expressed in 48 cases (44%; P=0.123). However, no significant differences in miR-152 expression was found between the tumor tissue and non-tumor tissue. The mean fold changes for miR-148a and miR-152 were 0.68 and 0.88, respectively. In a study conducted with 101 gastric cancer patients, it was shown that 70% of cases had low expression of miR-148a and 58% of cases had low expression of miRNA-152 when compared to nontumor tissue. The median fold change was 0.28 and 0.50, respectively (17). In a similar study, although miR-152 levels were found to be low in ovarian cancer tissue, no differences were found for miR-148a levels (18). Among
Table I. HLA-G expression and clinicopathological characteristics of patients.

| Features                        | HLA-G expression |  \( \chi^2/Z \) | P-value |
|---------------------------------|------------------|----------------|---------|
| Mean age (SD)                   |                  |                |         |
| Negative (n=63)                 | 68.98 (10.59)    | -0.069\(^a\)  | 0.945   |
| Positive (n=45)                 | 69.76 (10.10)    |                |         |
| Age group, n (%)                |                  |                |         |
| <70 years                       | 26 (41.3)        | 1.090\(^b\)   | 0.297   |
| ≥70 years                       | 37 (58.7)        |                |         |
| Sex, n (%)                      |                  |                |         |
| Male                            | 37 (58.7)        | 0.347\(^b\)   | 0.556   |
| Female                          | 26 (41.3)        |                |         |
| Tumor localization, n (%)       |                  |                |         |
| Cecum                           | 9 (14.3)         | 6.043\(^b\)   | 0.418   |
| Ascending colon                 | 8 (12.7)         |                |         |
| Hepatic flexura                 | 5 (7.9)          |                |         |
| Transvers colon                 | 4 (6.3)          |                |         |
| Splenic flexura                 | 4 (6.3)          |                |         |
| Descending colon                | 12 (19.0)        |                |         |
| Sigmoid colon                   | 21 (33.3)        |                |         |
| Tumor side, n (%)               |                  |                |         |
| Right colon                     | 26 (41.3)        | 0.000\(^b\)   | >0.999  |
| Left colon                      | 37 (58.7)        |                |         |
| Histopathology, n (%)           |                  |                |         |
| Adenocarcinoma                  | 55 (87.3)        | 0.210\(^b\)   | 0.647   |
| Mucinous carcinoma              | 8 (12.7)         |                |         |
| Tumor invasion, n (%)           |                  |                |         |
| pT1/2                           | 15 (23.8)        |                |         |
| pT3/4                           | 48 (76.2)        |                |         |
| Nodal status, n (%)             |                  |                |         |
| pN0                             | 45 (71.4)        | 11.788\(^b\)  | 0.003\(^d\) |
| pN1                             | 15 (23.8)        |                |         |
| pN2                             | 3 (4.8)          |                |         |
| Stage, n (%)                    |                  |                |         |
| I                               | 14 (22.2)        | 6.518\(^b\)   | 0.038\(^d\) |
| II                              | 30 (47.6)        |                |         |
| III                             | 19 (30.2)        |                |         |
| Grade, n (%)                    |                  |                |         |
| I/II                            | 58 (92.1)        |                |         |
| III                             | 5 (7.9)          |                |         |
| Lymphovascular invasion, n (%)  |                  |                |         |
| Negative                        | 42 (66.7)        | 2.739\(^b\)   | 0.098   |
| Positive                        | 21 (33.3)        |                |         |
| Perineural invasion, n (%)      |                  |                |         |
| Negative                        | 50 (79.4)        | 8.320\(^b\)   | 0.004\(^d\) |
| Positive                        | 13 (20.6)        |                |         |
| Adjuvant chemotherapy, n (%)    |                  |                |         |
| Yes                             | 11 (17.4)        |                |         |
| No                              | 52 (82.6)        |                |         |
| Mean miR-148a expression in tumor tissues (SD) | 0.83 (0.44) | -5.038\(^b\) | <0.001\(^d\) |
| Mean miR-152 expression in tumor tissues (SD) | 0.86 (0.40) | -0.545\(^a\) | 0.586   |

\(^a\)Z, Mann-Whitney U test; \(^b\)\(\chi^2\), \(\chi^2\) test; \(^c\)\(\chi^2\), Fisher's exact test. \(^d\)P<0.05. HLA-G, human leukocyte antigen G; miR, microRNA.
Table II. miRNA levels in non-tumor and tumor tissues.

| Variable | ΔCT mean (SD)       | ΔCT median (minimum-maximum) | Z*   | P-value |
|----------|---------------------|------------------------------|------|---------|
|          |                     |                              |      |         |
| miR-148a |                     |                              |      |         |
| Non-tumor| 1.33 (0.57)         | 0.92 (0.02-3.02)             | -5.238 | <0.001b |
| Tumor    | 0.66 (0.44)         | 0.68 (0.01-2.87)             |      |         |
| miR-152  |                     |                              |      |         |
| Non-tumor| 0.84 (0.41)         | 0.85 (0.01-2.62)             | -1.279 | 0.123   |
| Tumor    | 0.93 (0.41)         | 0.88 (0.01-2.74)             |      |         |

Z=Wilcoxon Signed Ranks test  ΔCT=CT miRNA-CT U6RNA. *P<0.05. ΔCT, delta cycle threshold; miRNA/miR, microRNA.

Figure 1. Representative image of immunohistochemical HLA-G expression. HLA-G showed cytoplasmic staining in tumor tissues. Scale bar, 500 µm (A, C and E) or 100 µm (B, D and F). (A) Negative HLA-G staining in cancer tissue (magnification, x4). (B) Negative HLA-G staining in cancer tissue (magnification, x20). (C) HLA-G staining <20% in cancer tissue (magnification, x4). (D) HLA-G staining <20% in cancer tissue (magnification, x20). (E) Positive HLA-G staining in cancer tissue (magnification, x4). (F) Positive HLA-G staining in cancer tissue (magnification, x20). HLA-G, human leukocyte antigen G.
| Category                     | No. | 5-year DFS, % | P-value | 5-year DSS, % | P-value | 5-year OS, % | P-value |
|------------------------------|-----|---------------|---------|---------------|---------|--------------|---------|
| **Age group**                |     |               |         |               |         |              |         |
| <70 years                    | 50  | 72.8          | 0.292   | 72.8          | 0.445   | 70.1         | 0.558   |
| ≥70 years                    | 58  | 79.3          |         | 78.1          |         | 60.2         |         |
| **Sex**                      |     |               |         |               |         |              |         |
| Male                         | 60  | 71.7          | 0.266   | 70.1          | 0.174   | 67.8         | 0.163   |
| Female                       | 48  | 81.2          |         | 84.5          |         | 73.9         |         |
| **Tumor localization**       |     |               |         |               |         |              |         |
| Cecum                        | 12  | 66.7          | 0.625   | 64.3          | 0.353   | 64.3         | 0.143   |
| Ascending colon              | 17  | 88.2          |         | 80.2          |         | 80.1         |         |
| Hepatic flexura              | 10  | 70.2          |         | 70.5          |         | 70.4         |         |
| Transvers colon              | 6   | 83.3          |         | 83.3          |         | 83.3         |         |
| Splenic flexura              | 11  | 63.6          |         | 63.6          |         | 63.6         |         |
| Descending colon             | 21  | 81.3          |         | 86.6          |         | 86.6         |         |
| Sigmoid colon                | 31  | 74.2          |         | 80.1          |         | 70.4         |         |
| **Tumor side**               |     |               |         |               |         |              |         |
| Right colon                  | 45  | 77.8          | 0.673   | 81.7          | 0.702   | 62.3         | 0.889   |
| Left colon                   | 63  | 74.6          |         | 75.9          |         | 68.2         |         |
| **Histopathology**           |     |               |         |               |         |              |         |
| Adenocarcinoma               | 92  | 75.8          | 0.634   | 78.1          | 0.835   | 66.4         | 0.851   |
| Mucinous carcinoma           | 16  | 81.3          |         | 81.3          |         | 81.3         |         |
| **Invasion**                 |     |               |         |               |         |              |         |
| pT1/2                        | 19  | 100           | 0.011a  | 100           | 0.021a  | 100          | 0.014a  |
| pT3/4                        | 89  | 70.8          |         | 71.4          |         | 59.1         |         |
| **Nodal status**             |     |               |         |               |         |              |         |
| pN0                          | 66  | 84.8          | <0.001a | 86.8          | <0.001a | 82.7         | <0.001a |
| pN1                          | 27  | 81.5          |         | 81.5          |         | 76.4         |         |
| pN2                          | 15  | 26.7          |         | 37.3          |         | 37.3         |         |
| **Stage**                    |     |               |         |               |         |              |         |
| I                            | 17  | 100           | 0.009a  | 100           | 0.023a  | 100          | 0.003a  |
| II                           | 50  | 78.2          |         | 80.5          |         | 75.5         |         |
| III                          | 41  | 63.4          |         | 67.2          |         | 55.1         |         |
| **Grade**                    |     |               |         |               |         |              |         |
| I/II                         | 94  | 79.8          | 0.002a  | 82.4          | 0.006a  | 71.4         | 0.001a  |
| III                          | 14  | 50.3          |         | 57.1          |         | 57.1         |         |
| **LVI**                      |     |               |         |               |         |              |         |
| Negative                     | 64  | 84.4          | 0.007a  | 84.4          | 0.001a  | 82.6         | <0.001a |
| Positive                     | 44  | 63.6          |         | 64.2          |         | 53.5         |         |
| **PNI**                      |     |               |         |               |         |              |         |
| Negative                     | 73  | 83.6          | 0.003a  | 84.0          | 0.001a  | 76.3         | <0.001a |
| Positive                     | 35  | 60.0          |         | 61.0          |         | 56.6         |         |
| **HLA-G expression**         |     |               |         |               |         |              |         |
| Negative                     | 63  | 92.1          | <0.001a | 93.1          | <0.001a | 76.8         | <0.001a |
| Positive                     | 45  | 53.3          |         | 58.1          |         | 48.4         |         |
| **miR-148a tumor**           |     |               |         |               |         |              |         |
| ≤0.7                         | 62  | 69.4          | 0.005a  | 79.0          | 0.028a  | 69.7         | 0.070   |
| >0.7                         | 46  | 89.1          |         | 91.1          |         | 80.1         |         |
| **miR-148a non-tumor**       |     |               |         |               |         |              |         |
| <1                           | 62  | 88.7          | 0.006a  | 93.5          | 0.005a  | 89.6         | 0.021a  |
| ≥1                           | 46  | 65.2          |         | 76.1          |         | 64.6         |         |
| **miR-152 tumor**            |     |               |         |               |         |              |         |
| <1                           | 74  | 78.4          | 0.542   | 83.4          | 0.914   | 77.0         | 0.745   |
| ≥1                           | 34  | 82.4          |         | 85.3          |         | 78.6         |         |
In this study, we showed that the HLA-G protein was expressed at varying rates in 75.9% of colon cancer samples.

Table III. Continued.

| Category          | No. | 5-year DFS, % | P-value | 5-year DSS, % | P-value | 5-year OS, % | P-value |
|-------------------|-----|---------------|---------|---------------|---------|--------------|---------|
| miR-152 non-tumor |     |               |         |               |         |              |         |
| <1                | 80  | 77.5          | 0.347   | 79.3          | 0.200   | 70.1         | 0.385   |
| ≥1                | 28  | 82.1          | 84      | 77.0          |         |              |         |

*P<0.05. DFS, disease-free survival; DSS, disease-specific survival; HLA-G, human leukocyte antigen G; HR, hazard ratio; LVI, lymphovascular invasion; miR, microRNA; OS, overall survival; PNI, perineural invasion.

Table IV. Results of multivariate Cox regression analysis.

| Factors          | Disease-free survival | Disease-specific survival | Overall survival |
|------------------|-----------------------|---------------------------|------------------|
|                  | HR (95% CI)           | P-value                   | HR (95% CI)      | P-value |
|                  |                       |                           |                  |
| Nodal status     |                       |                           |                  |
| pN0              | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| pN1              | 1.10 (0.33-3.69)      | 0.876                     | 1.25 (0.32-4.81) | 0.749   |
| pN2              | 5.94 (1.92-18.42)     | 0.002                     | 2.09 (0.57-7.68) | 0.269   |
| HLA-G expression |                       |                           |                  |
| Negative         | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| Positive         | 6.07 (1.75-20.99)     | 0.004                     | 5.19 (1.49-18.11)| 0.010   |
| Tumor grade      |                       |                           |                  |
| I/II             | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| III              | 3.56 (1.20-10.58)     | 0.022                     | 1.55 (0.50-4.75) | 0.445   |
| LVI              |                       |                           |                  |
| Negative         | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| Positive         | 2.14 (0.41-11.16)     | 0.366                     | 3.79 (0.67-21.65)| 0.134   |
| PNI              |                       |                           |                  |
| Negative         | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| Positive         | 2.15 (0.40-11.49)     | 0.373                     | 1.60 (0.27-9.43) | 0.605   |
| miR-148a tumor   |                       |                           |                  |
| ≤0.7             | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| >0.7             | 0.62 (0.20-1.93)      | 0.407                     | 0.76 (0.25-2.36) | 0.636   |
| miR-148a non-tumor |           |                           |                  |
| <1               | 1 (Reference)         | 1 (Reference)             | 1 (Reference)    |
| ≥1               | 1.26 (0.52-3.09)      | 0.612                     | 1.86 (0.72-4.75) | 0.198   |

*P<0.05; Cox Regression Analysis (Method=Enter). 95% CI, 95% confidence interval; HLA-G, human leukocyte antigen G; HR, hazard ratio; LVI, lymphovascular invasion; miR, microRNA; PNI, perineural invasion.

In this study, we showed that the HLA-G protein was expressed at varying rates in 75.9% of colon cancer samples.
EMIRZEOLU et al: ALTERED EXPRESSION LEVELS OF HLA-G, miR-148a AND miR-152 IN COLON CANCER

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HLA-G expression above 20%, which was found in 41.7% (n=45) of patients, was significantly correlated with patient survival (P=0.039). Previous studies have found that HLA-G expression varied between 50 and 80% in colon cancer and other gastrointestinal system cancers (26,27). Those studies also found no HLA-G expression in normal colon tissue, similar to our study.

Numerous studies have demonstrated that high HLA-G expression was associated with poor prognosis in gastrointestinal cancer patients (28-32). However, there are few studies showing that there is no significant relationship between HLA-G expression and the prognosis of gastrointestinal cancers (33-35). These differences between studies may be related to the autoantibody used. Lin et al (36) demonstrated that HLA-G expression was related to a poor prognosis when detected with antibody 4H84, but the results were inconsistent when using the 5A6G7 antibody. We used 4H84 antibodies for HLA-G detection in our study. The 4H84 antibody has been confirmed by international conferences as a reference tool for evaluating HLA-G expression in paraffin-embedded specimens (37). Limited studies of other antibodies could not justify the lack of prognostic value of HLA-G in colon cancer. Moreover, the invasive nature of the disease and the tumor microenvironment are different in gastrointestinal cancers, and the expression of HLA-G differs between different cancer types, which may explain the differing results between studies (38,39). The number of studies showing that HLA-G expression is a poor prognostic factor in colon cancer is increasing. Ye et al (40) studied HLA-G expression in colorectal carcinoma using immunohistochemistry and observed positive HLA-G expression in 64.6% of colorectal patients. Their multivariate analysis showed that HLA-G may serve as an independent prognostic factor for colorectal cancer patients. Guo et al (41) showed that HLA-G expression is significantly correlated with OS and can serve as an independent factor in OS. Zhang et al (39) showed that different rates of HLA-G expression in colorectal cancer patients affect patient survival and that a combination of HLA-G expression status with traditional clinical risk factors can improve the prediction of specific clinical outcomes in subpopulations of colorectal cancer patients. The results of current studies suggest that HLA-G expression is most likely associated with the prognosis of colorectal cancers. Similarly, our study showed that HLA-G-positive patients had worse outcomes in terms of both OS and DFS than HLA-G-negative patients.

In conclusion, we found that HLA-G is expressed in many colorectal carcinomas, and miR-148a levels in HLA-G-positive colon cancer tissues are lower than in HLA-G-negative tissues. This is the first study to investigate the relationship between miR-148a, miR-152 and HLA-G in colon cancer. Using the Kaplan-Meier analysis, we found a significant correlation between HLA-G expression and OS in colorectal cancer patients. Furthermore, our multivariate analysis results showed that the expression HLA-G can serve as an independent factor for OS. According to the results of the univariate Cox regression analysis, miR-148a levels were found to be a factor associated with DFS. Low expression of miR-148a in tumor tissue is associated with shorter DFS. Our results suggest that HLA-G and miR-148a may be new prognostic markers in colon cancer. Further investigation is needed to confirm these results.

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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.
Authors' contributions
LE and OO designed the study and prepared the manuscript. LE and OO confirm the authenticity of all the raw data. FNAM reviewed medical documents and collected patients' clinical characteristics and was involved in the analysis of data and validation. UB, IY, BBO, SC and MAO contributed to study design, data analysis and manuscript editing. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
All the procedures implemented in studies involving human participants were consistent with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients provided written informed consent for participation and patient anonymity was preserved. This retrospective study was approved by the ethics committee of GATA Haydarpasa Training Hospital (approval number 2016/37; Istanbul, Turkey).

Patient consent for publication
Written informed was obtained from the patients for publication of the data.

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

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