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ORIGINAL ARTICLE

# Clinicopathologic significance of human leukocyte antigen class I expression in patients with stage II and III gastric cancer

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## Abstract

Human leukocyte antigen class I (HLA I) molecules composed of alpha (heavy) chain, including HLA-A, -B, or -C encoded by *HLA* genes, and beta-2-microglobulin (β2M) are membrane proteins on all nucleated cells that display peptide antigens for recognition by CD8-positive cytotoxic T cells. Here, we examined the clinicopathologic signification of HLA I expression in patients with gastric cancer (GC). Immunohistochemistry was performed to detect HLA A/B/C, β2M, CD8, p53, and programmed death-ligand 1 (PD-L1) in the center and invasive margin of the tumor in 395 stage II and III GCs using tissue array method. Additionally, Epstein–Barr virus (EBV) infection and microsatellite instability (MSI) status were investigated. Negative expression of HLA A/B/C and β2M was observed in 258 (65.3%) and 235 (59.5%) of 395 stage II and III GCs, respectively. Negative HLA I expression was significantly associated with aggressive clinicopathologic features. Furthermore, negative expression of HLA A/B/C and β2M was inversely correlated with CD8-positive cytotoxic T cell infiltration, EBV-positivity, and PD-L1 expression (all $p < 0.001$). Patients with HLA A/B/C-negative GC had worse overall survival (OS) ($p = 0.019$) and combined analysis with both HLA A/B/C and β2M expression status significantly predicted OS in univariate ($p = 0.004$) and multivariate survival analysis ($p = 0.016$). Negative expression of HLA A/B/C and β2M was frequently observed in stage II and III GCs, particularly with the aggressive clinicopathologic features, and correlated with an unfavorable prognosis and host immune response status. These findings contribute to further development of immunotherapy.

**Keywords** Gastric cancer · Human leukocyte antigen · Beta-2-microglobulin · Programmed death-ligand 1 · Biomarkers

## Abbreviations

| | |
|---|---|
| AJCC | American Joint Committee on Cancer |
| β2M | Beta-2-microglobulin |
| CPS | Combined positive score |
| EBER | Epstein–Barr virus-encoded small RNA |
| EBV | Epstein–Barr virus |
| FFPE | Formalin-fixed paraffin-embedded |
| GC | Gastric cancer |
| HLA I | Human leukocyte antigen class I |
| IRB | Institutional review board |
| ISH | In situ hybridization |
| MSI-H | Microsatellite instability-high |
| MSI-L | Microsatellite instability-low |
| MSI | Microsatellite instability |
| MSS | Microsatellite stable |
| NCI | National Cancer Institute |
| TIL | Tumor-infiltrating lymphocyte |
| TMA | Tissue microarray |

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## Introduction

Gastric cancer (GC) is one of the most common human cancers worldwide and is the third leading cause of death [1]. In South Korea, GC is the leading cause of cancer deaths in both men and women [2]. The 5-year relative survival rate of patients with early GC is over 90%, but that of patients with stage II or III GC is approximately 55% [3]. Adjuvant therapy after D2 gastrectomy improves overall survival (OS) in patients with stage II and III GC, but more than 30% of those with stage III GC exhibit recurrence after surgery [4]. Therefore, with recent developments in targeted therapies for GC [5], molecular targeted therapies have become increasingly necessary based on the molecular understanding of GC, including cancer cell genetics and immune microenvironment.

Human leukocyte antigen class I (HLA I) molecules are found on all nucleated cell surfaces and are key molecules involved in the cell-mediated immune system driven by CD8-positive cytotoxic T cells. In cancer patients, neoantigens are presented via HLA I molecules on the cancer cell surface, thereby initiating a cell-mediated immune response to recruit CD8-positive cytotoxic T cells around the cancer cells. HLA I molecules consist of alpha (heavy) chain, including HLA-A, -B, or -C encoded by *HLA* genes and beta-2-microglobulin (β2M). HLA I expression on cancer cells has been reported to be heterogenous and often found to be reduced or lost in many cancers [6]. Additionally, the loss of HLA I heavy chain and β2M has been suggested as an immune escape mechanism in certain cancers [7, 8]. However, the clinical significance of HLA I expression has not been investigated in patients with GC.

Recent studies have investigated the immune escape mechanism in cancers [9] and clinical trials of checkpoint inhibitor therapy, especially various programmed cell death protein 1 (PD-1) inhibitors have shown beneficial outcomes in patients with solid tumors, including GC [10]. Although programmed death-ligand 1 (PD-L1) expression on the cancer cell surface, assessed by immunohistochemistry (IHC), was correlated with a therapeutic response [11, 12], predicting the therapeutic benefit and efficacy was not possible in all patients [13]. Therefore, identifying new biomarkers that can predict therapeutic effects in patients is currently under intense study [14]. Recent studies showed that inactivating mutations in β2M were associated with immunotherapy resistance [15] and suggested the importance of HLA I expression loss in predicting the therapeutic effect of immunotherapy [16, 17]. Therefore, the relationship between PD-L1 and HLA I expression must be verified to understand the immune microenvironment in GCs.

In this study, HLA I expression status was evaluated by IHC in patients with stage II and III GC to determine the clinicopathologic implications and prognostic significance of HLA I expression. We also investigated the immune microenvironment, such as PD-L1 expression and the density of CD8-positive cytotoxic T cells, and cancer cell molecular characteristics, such as Epstein–Barr virus (EBV) infection and microsatellite instability (MSI), to determine their relationship with HLA I expression status.

## Materials and methods

### Patients and samples

Three hundred and ninety-five patients with stage II and III GC who underwent curative radical surgery (R0 resection) with D2 lymph node dissection at Seoul National University Bundang Hospital (Seongnam-si, Republic of Korea) between 2006 and 2013 were registered in this study. Patients who received fluoropyrimidine-based adjuvant chemotherapy after surgical resection were included. Clinical and pathologic data were collected from medical records. OS was calculated as the period from the date of surgery until the death of any cause or censored observation.

Formalin-fixed paraffin-embedded (FFPE) tissue was collected from surgically resected GC specimens. In all 395 cases, two separate 2 mm cores were selected from both the center and invasive margin of the tumor. Tissue microarray (TMA) blocks were designed as described previously (SuperBioChips Laboratories, Seoul, Republic of Korea) [18].

In addition, we prepared frozen sections from fresh tumor tissue and matched non-neoplastic gastric mucosa tissue samples that were obtained immediately from surgical specimens isolated from six gastric cancer patients and FFPE sections were also produced. Both frozen sections and FFPE sections were produced on whole section slides.

### IHC for HLA I molecule and p53

We performed immunostaining of HLA I antigens, such as HLA A/B/C and β2M, and p53, using an automated immunostainer (BenchMark XT, Ventana Medical Systems, Tucson, AZ, USA) by following the manufacturer's instructions. The primary antibodies were as follows: Anti-HLA class I ABC antibody (EMR8-5, 1:8000, Abcam, Cambridge, UK); Anti-β2M antibody (B2M/961, 1:2000, Abcam, Cambridge, UK); and p53 antibody (DO-7, mouse monoclonal; Dako, Carpinteria, CA, USA).

HLA A/B/C and β2M expression was examined to determine the extent (%) and intensity of tumor cell membrane staining. The intensity was classified into the following three categories: 0, negative; 1+, weak positive; 2+, strong positive [19, 20]. For statistical analysis, we defined as positive

expression when 5% or more tumor cells showed strong intensity of staining.

Overexpression of p53 was observed in certain tumor cells and strong intensity of staining in more than 10% of tumor cell nuclei was defined as p53 overexpression/positive, while cases with less than 10% of tumor cell nuclei staining with strong intensity, including those expressing dispersed nuclear staining or partial tumor cell nuclei staining, were defined as negative [21].

### Immunostaining and interpretation of PD-L1

PD-L1 IHC was performed with a mouse monoclonal antibody (22C3 PharmDx kit, Dako, Carpinteria, CA, USA) using an automated immunostainer (Autostainer Link 48, Agilent Technologies, Santa Clara, CA, USA) by following the manufacturer's instructions.

PD-L1 was expressed in the membrane of tumor cells and membrane or cytoplasm of tumor-infiltrating lymphocytes and macrophages. PD-L1 expression was evaluated to determine both the number and intensity of PD-L1 staining cells, including tumor cells, lymphocytes, and macrophages. The combined positive score (CPS) was defined as the percentage of viable PD-L1-stained cells (tumor cells, lymphocytes, and macrophages) relative to total viable tumor cells. The cases were interpreted as PD-L1-positive if CPS was one or more [22, 23].

### Evaluation of CD8-positive cytotoxic T cell density

We performed immunostaining of CD8 (C8/114B, mouse monoclonal; Dako, Carpinteria, CA, USA) using an automated immunostainer (BenchMark XT, Ventana Medical Systems, Tucson, AZ, USA) by following the manufacturer's instructions. The CD8-immunostained TMA slides were digitally scanned with an X400 Aperio ScanScope CS instrument (Leica Biosystems, Wetzlar, Germany). The CD8-positive cytotoxic T cell densities (positive cell counts per $mm^2$) in each core of the TMA slides were counted using an Aperio image analysis system (Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions [20].

### MSI analysis

DNA extraction and polymerase chain reaction (PCR) of five NCI markers (BAT-26, BAT-25, D5S346, D17S250, and S2S123) were performed in both tumor tissues and matched non-neoplastic gastric mucosa. PCR products from the FFPE samples were evaluated with an automated sequencer (ABI 3731 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) according to a previously described protocol [24]. MSI status was interpreted by comparing the allele profiles of tumor cells with those in matched non-neoplastic tissues. Cases with two or more markers with unstable peaks were defined as MSI-high (MSI-H), with one unstable marker as MSI-low (MSI-L), and no unstable marker as microsatellite stable (MSS).

### EBV in situ hybridization

To determine the EBV status of tumor cells, EBV ISH was performed using a fluorescein-conjugated EBV-encoded small RNA (EBER) oligonucleotide probe (INFORM EBV-encoded RNA probe, Ventana Medical Systems, Tucson, AZ, USA) [24]. Cases with tumor cells positive for nuclear EBER were defined as EBV-positive GC.

### Statistical analysis

SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The association between HLA A/B/C and β2m expression and clinicopathologic characteristics was analyzed using Chi-squared distribution. The analysis of OS was performed by Kaplan–Meier method and compared with the log-rank test. We used Cox regression model for univariate and multivariate analyses of survival to determine the significance of independent prognostic factors. *P* values less than 0.05 were considered to suggest statistically significant difference.

## Results

### Patient characteristics

The characteristics of 395 patients enrolled in this study are shown in Table 1. The median age of the patients was 58 years (range 20–87 years); 251 (63.5%) were male and 144 (36.5%) were female (Table 1). There were 189 (47.8%) American Joint Committee on Cancer (AJCC) 7th TNM stage II GC cases and 206 (52.2%) stage III GC cases. According to Lauren classification, intestinal, diffuse, mixed, and intermediate type tumors accounted for 144 (36.5%), 219 (55.4%), 30 (7.6%), and 2 (0.5%) of total cases, respectively. According to Ming's classification, 320 (81.0%) cases were infiltrative type and 75 (19.0%) cases were expanding type.

### Clinicopathologic significance of HLA I and β2 M expression

To reveal the clinicopathologic significance of HLA I molecules, we performed IHC for HLA A/B/C and β2M. Representative pictures are shown in Fig. 1. Among the 395 cases, HLA A/B/C expression was negative in 210 cases

**Table 1** Correlation between clinicopathological features and human leukocyte antigen (HLA) A/B/C and beta-2-microglobulin (β2M) status in stage II, III gastric cancer

| Characteristics | Total | HLA A/B/C | | | β2M | | | HLA A/B/C or β2M | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Loss | Intact | *P* | Loss | Intact | *P* | Loss | Intact | *P* |
| Age (years) | | | | 0.312 | | | 0.307 | | | 0.400 |
| < 65 | 261 (66.1%) | 175 (67.8%) | 86 (62.8%) | | 160 (68.1%) | 101 (63.1%) | | 186 (67.4%) | 75 (63.0%) | |
| ≥ 65 | 134 (33.9%) | 83 (32.2%) | 51 (37.2%) | | 75 (31.9%) | 60 (36.9%) | | 90 (32.6%) | 44 (37.0%) | |
| Sex | | | | 0.049 | | | 0.047 | | | 0.033 |
| Male | 251 (63.5%) | 155 (60.1%) | 96 (70.1%) | | 140 (59.6%) | 111 (69.4%) | | 166 (60.1%) | 85 (71.4%) | |
| Female | 144 (36.5%) | 103 (39.9%) | 41 (29.9%) | | 95 (40.4%) | 49 (30.6%) | | 110 (39.9%) | 34 (28.6%) | |
| pTNM | | | | 0.604 | | | 0.127 | | | 0.373 |
| II | 189 (47.8%) | 121 (46.9%) | 68 (49.6%) | | 105 (44.7%) | 84 (52.5%) | | 128 (46.4%) | 61 (51.3%) | |
| III | 206 (52.2%) | 137 (53.1%) | 69 (50.4%) | | 130 (55.3%) | 76 (47.5%) | | 148 (53.6%) | 58 (48.7%) | |
| Tumor size | | | | 0.201 | | | 0.022 | | | 0.116 |
| ≤ 5 cm | 222 (56.2%) | 139 (53.9%) | 83 (60.6%) | | 121 (51.5%) | 101 (63.1%) | | 148 (53.6%) | 74 (62.2%) | |
| > 5 cm | 173 (43.8%) | 119 (46.1%) | 54 (39.4%) | | 114 (48.5%) | 59 (36.9%) | | 128 (46.4%) | 45 (37.8%) | |
| Location | | | | 0.120 | | | 0.111 | | | 0.066 |
| Upper third | 184 (46.6%) | 112 (43.4%) | 72 (52.6%) | | 98 (41.7%) | 86 (53.8%) | | 121 (43.8%) | 63 (52.9%) | |
| Middle third | 84 (21.3%) | 61 (23.6%) | 23 (16.8%) | | 56 (23.8%) | 28 (17.5%) | | 62 (22.5%) | 22 (18.5%) | |
| Lower third | 104 (26.3%) | 70 (27.1%) | 34 (24.8%) | | 65 (27.7%) | 39 (24.4%) | | 74 (26.8%) | 30 (25.2%) | |
| GEJ | 6 (1.5%) | 2 (0.8%) | 4 (2.9%) | | 3 (1.3%) | 3 (1.8%) | | 3 (1.1%) | 3 (2.5%) | |
| Entire | 17 (4.3%) | 13 (5.1%) | 4 (2.9%) | | 13 (5.5%) | 4 (2.5%) | | 16 (5.8%) | 1 (0.9%) | |
| WHO | | | | 0.001 | | | < 0.001 | | | < 0.001 |
| WD | 5 (1.3%) | 3 (1.2%) | 2 (1.5%) | | 1 (0.4%) | 4 (2.5%) | | 3 (1.1%) | 2 (1.7%) | |
| MD | 124 (31.4%) | 68 (26.4%) | 56 (40.9%) | | 64 (27.2%) | 60 (37.5%) | | 77 (27.9%) | 47 (39.5%) | |
| PD | 164 (41.5%) | 112 (43.4%) | 52 (38.0%) | | 95 (40.4%) | 69 (43.1%) | | 115 (41.7%) | 49 (41.2%) | |
| PCC (SRC) | 96 (24.3%) | 74 (28.7%) | 22 (16.1%) | | 74 (31.5%) | 22 (13.8%) | | 80 (29.0%) | 16 (13.4%) | |
| UD | 1 (0.3%) | 0 (0.0%) | 1 (0.7%) | | 0 (0.0%) | 1 (0.6%) | | 0 (0.0%) | 1 (0.8%) | |
| GCLS/ADSQCA | 5 (1.2%) | 1 (0.3%) | 4 (2.8%) | | 1 (0.5%) | 4 (2.5%) | | 1 (0.3%) | 4 (3.4%) | |
| Lauren classification | | | | 0.001 | | | 0.002 | | | 0.002 |
| Intestinal | 144 (36.5%) | 81 (31.4%) | 63 (46.0%) | | 71 (30.2%) | 73 (45.6%) | | 90 (32.6%) | 54 (45.4%) | |
| Diffuse | 219 (55.4%) | 161 (62.4%) | 58 (42.3%) | | 148 (63.0%) | 71 (44.4%) | | 169 (61.2%) | 50 (42.0%) | |
| Mixed | 30 (7.6%) | 15 (5.8%) | 15 (10.9%) | | 15 (6.4%) | 15 (9.4%) | | 16 (5.8%) | 14 (11.8%) | |
| Indeterminate | 2 (0.5%) | 1 (0.4%) | 1 (0.8%) | | 1 (0.4%) | 1 (0.6%) | | 1 (0.4%) | 1 (0.8%) | |
| Ming's classification | | | | < 0.001 | | | < 0.001 | | | < 0.001 |
| Infiltrative | 320 (81.0%) | 227 (88.0%) | 93 (67.9%) | | 207 (88.1%) | 113 (70.6%) | | 243 (88.0%) | 77 (64.7%) | |
| Expanding | 75 (19.0%) | 31 (12.0%) | 44 (32.1%) | | 28 (11.9%) | 47 (29.4%) | | 33 (12.0%) | 42 (35.3%) | |
| Lymphatic invasion | | | | 0.171 | | | 0.822 | | | 0.411 |

**Table 1** (continued)

| Characteristics | Total | HLA A/B/C | | | β2M | | | HLA A/B/C or β2M | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Loss | Intact | *P* | Loss | Intact | *P* | Loss | Intact | *P* |
| Absent | 121 (30.6%) | 85 (32.9%) | 36 (26.3%) | | 73 (31.1%) | 48 (30.0%) | | 88 (31.9%) | 33 (27.7%) | |
| Present | 274 (69.4%) | 173 (67.1%) | 101 (73.7%) | | 162 (68.9%) | 112 (70.0%) | | 188 (68.1%) | 86 (72.3%) | |
| Vascular invasion | | | | 0.884 | | | 0.754 | | | 0.419 |
| Absent | 333 (84.3%) | 217 (84.1%) | 116 (84.7%) | | 197 (83.8%) | 136 (85.0%) | | 230 (83.3%) | 103 (86.6%) | |
| Present | 62 (15.7%) | 41 (15.9%) | 21 (15.3%) | | 38 (16.2%) | 24 (15.0%) | | 46 (16.7%) | 16 (13.4%) | |
| Perineural invasion | | | | 0.096 | | | 0.013 | | | 0.121 |
| Absent | 137 (34.7%) | 82 (31.8%) | 55 (40.1%) | | 70 (29.8%) | 67 (41.9%) | | 89 (32.2%) | 48 (40.3%) | |
| Present | 258 (65.3%) | 176 (68.2%) | 82 (59.9%) | | 165 (70.2%) | 93 (58.1%) | | 187 (67.8%) | 71 (59.7%) | |
| Total | 395 (100%) | 258 (65.3%) | 137 (34.7%) | | 235 (59.5%) | 160 (40.5%) | | 276 (69.9%) | 119 (30.1%) | |

*GEJ* gastroesophageal junction, *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated, *PCC(SRC)* poorly cohesive carcinoma (signet ring cell carcinoma), *GCLS* gastric carcinoma with lymphoid stroma, *UD* undifferentiated carcinoma, *ADSQCA* adenosquamous carcinoma

(53.2%) in the tumor center and 210 cases (53.2%) in the invasive margin. HLA A/B/C expression in the tumor center was correlated with the invasive margin ($k = 0.502$, $p < 0.001$) and negative expression in either the center or invasive margin was observed in 258 cases (65.3%). β2M expression was negative in 177 (44.8%), 193 (48.9%), and 235 (59.5%) of cases in the center, invasive margin, and center or invasive margin, respectively. The kappa value between the center and invasive margin of β2M expression was 0.482 ($p < 0.001$). Both HLA A/B/C and β2M expression showed no definite predilection for the tumor center or invasive border. Additionally, HLA A/B/C expression in the center or invasive margin was closely associated with β2M expression in the center or invasive margin ($k = 0.683$, $p < 0.001$). In the FFPE sections and frozen sections of six cases, when HLA A/B/C expression in the non-neoplastic gastric mucosa was compared with expression in tumor tissues, HLA A/B/C expression was found to be negative or lower in non-neoplastic gastric mucosa in frozen sections of all six cases and FFPE sections of five cases. The intensity and extent of HLA A/B/C expression was generally similar between the frozen and FFPE sections, but one case showed focal expression of HLA A/B/C in the frozen section and negative in the FFPE section derived from tumor tissue (supplementary Figs. 1 and 2).

The correlations between clinicopathologic variables and HLA I expression are summarized in Table 1. Negative expression of both HLA A/B/C and β2M was significantly associated with more aggressive clinicopathologic features, including poorly differentiated/poorly cohesive carcinoma (HLA A/B/C, $p = 0.001$; β2M, $p < 0.001$), diffuse histologic type by Lauren classification (HLA A/B/C, $p = 0.001$; β2M, $p = 0.002$), and infiltrative tumor border (all $p < 0.001$). Negative β2M expression was significantly correlated with perineural invasion ($p = 0.013$), while negative HLA A/B/C expression did not show a significant correlation ($p = 0.096$). Additionally, we combined the expression status of HLA A/B/C and β2M and negative expression of either HLA A/B/C or β2M was significantly associated with poorly differentiated/poorly cohesive carcinoma ($p < 0.001$), diffuse histologic type by Lauren classification ($p = 0.002$), and infiltrative tumor border ($p < 0.001$).

## Correlation of HLA I expression with tumor microenvironment and molecular characteristics

We performed PD-L1 IHC and calculated CPS in each core to correlate the HLA I expression status with the tumor microenvironment. Among the 395 cases, PD-L1 IHC was positive in 197 cases (49.9%) for the tumor center, 158 cases (40.0%) for the invasive margin, and 226 cases (57.2%) for either the center of tumor or invasive margin. When HLA A/B/C or β2M expression status was

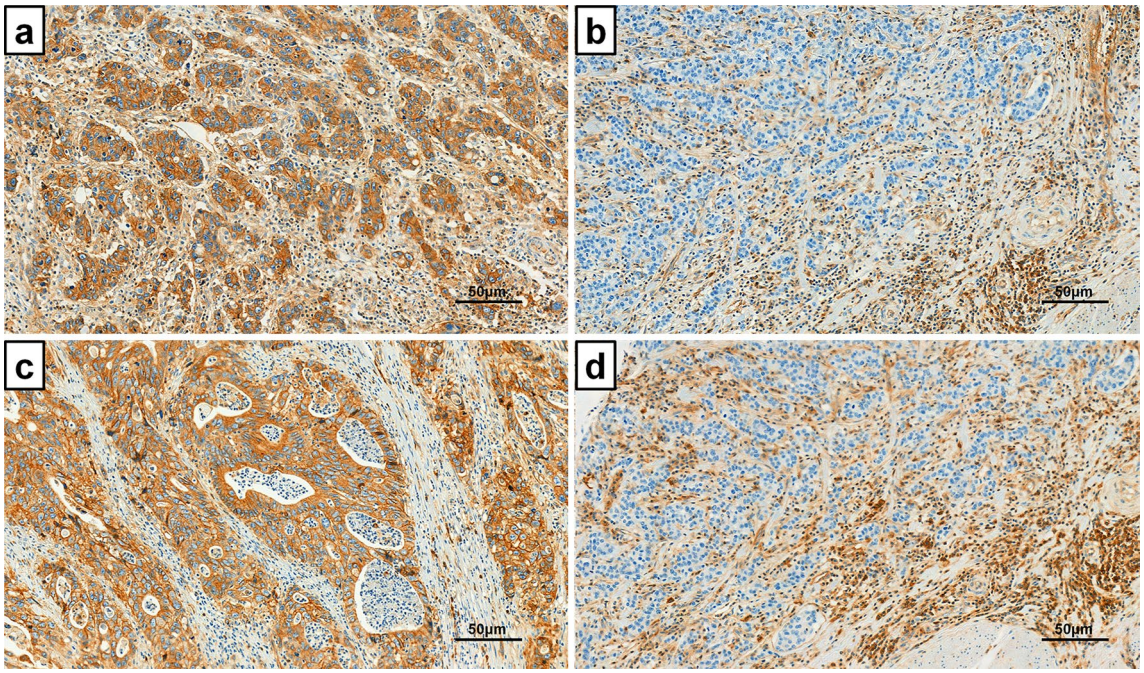


**Fig. 1** Representative figures showing intact expression of human leukocyte antigen (HLA) A/B/C (**a**), beta-2-microglobulin (β2M) (**c**) and expression loss of HLA A/B/C (**b**), β2M (**d**) (20× magnification)

correlated with the PD-L1 expression status, HLA A/B/C and β2M expression was significantly correlated with PD-L1 expression ($p < 0.001$, Table 2). However, among 226 PD-L1-positive GC cases, HLA A/B/C or β2M expression was negative in 138 cases (61.1%).

The number of tumor-infiltrating CD8-positive cytotoxic T cells ranged from 2.79 to 1222.93 cells/mm$^2$ with a median value of 219.92 cells/mm$^2$ at the center of the tumor and ranged from 6.90 to 1374.94 cells/mm$^2$ with a median value of 195.75 cells/mm$^2$ at the invasive margin. CD8-positive cytotoxic T cell density was significantly higher in cases with positive expression of HLA A/B/C and β2M. In contrast, the density was significantly lower, when HLA A/B/C or β2M expression was negative (all $p < 0.001$, Fig. 2).

Negative expression of HLA A/B/C and β2M was inversely correlated with EBV positivity in GCs (all $p < 0.001$, Table 2). HLA A/B/C and β2M expression tended to be positive in EBV-positive GCs. Negative expression of HLA A/B/C and β2M showed no significant correlation with p53 overexpression (HLA A/B/C, $p = 0.971$; β2M, $p = 0.559$) or MSI status (HLA A/B/C, $p = 0.432$; β2M, $p = 0.748$). Among the 37 MSI-H cases, HLA A/B/C expression was negative in 22 (59.5%) cases and β2M was negative in 21 (56.8%) cases. Although negative HLA I expression was infrequently found in EBV-positive GCs (5 of 26 cases, 19.2%), MSI-H GCs frequently showed negative expression of HLA I molecules (25 or 37 cases, 67.6%).

## Univariate and multivariate survival analyses

According to Kaplan–Meier survival analysis, patients with negative expression of HLA A/B/C and β2M had significantly worse outcomes (HLA A/B/C, $p = 0.019$; Fig. 3a; β2M, $p = 0.009$; Fig. 3b). Negative expression of either HLA A/B/C or β2M was also significantly associated with worse OS compared to the HLA A/B/C and β2M positive group ($p = 0.003$, Fig. 3c).

Univariate analysis indicated that negative expression of either HLA A/B/C or β2M and established prognostic factors, including age, tumor size, pathologic stage, vascular invasion, and perineural invasion, were significantly associated with OS (Table 3). By multivariate logistic regression analysis, negative expression of either HLA A/B/C or β2M was identified as an independent unfavorable prognostic factor for OS (hazard ratio 1.946; 95% confidence interval 1.131–3.350; $p = 0.016$). Age, tumor size, pTNM stage, vascular invasion, and perineural invasion were also independent prognostic factors for OS (age, $p = 0.046$; size, $p = 0.019$; pTNM, $p = 0.009$; vascular invasion, $p = 0.002$; perineural invasion, $p = 0.028$).

## Discussion

HLA I molecules are critical mediators of cytotoxic T cell responses. HLA I expression is downregulated or lost in various carcinomas and is considered as an immune escape

**Table 2** Correlation between human leukocyte antigen (HLA) A/B/C and beta-2-microglobulin (β2M) status and PD-L1 expression and molecular characteristics in stage II, III gastric cancer

| Characteristics | | HLA A/B/C | | | β2M | | | HLA A/B/C or β2M | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Loss | Intact | *P* | Loss | Intact | *P* | Loss | Intact | *P* |
| PD-L1 CT or IM | | | | | | | | | | |
| Negative | 169 (42.8%) | 127 (49.2%) | 42 (30.7%) | < 0.001 | 122 (51.9%) | 47 (29.4%) | < 0.001 | 138 (50.0%) | 31 (26.1%) | < 0.001 |
| Positive | 226 (57.2%) | 131 (50.8%) | 95 (69.3%) | | 113 (48.1%) | 113 (70.6%) | | 138 (50.0%) | 88 (73.9%) | |
| PD-L1 CT | | | | | | | | | | |
| Negative | 198 (50.1%) | 148 (57.4%) | 50 (36.5%) | < 0.001 | 139 (59.1%) | 59 (36.9%) | < 0.001 | 160 (58.0%) | 38 (31.9%) | < 0.001 |
| Positive | 197 (49.9%) | 110 (42.6%) | 87 (63.5%) | | 96 (40.9%) | 101 (63.1%) | | 116 (42.0%) | 81 (68.1%) | |
| PD-L1 IM | | | | | | | | | | |
| Negative | 237 (60.0%) | 178 (69.0%) | 59 (43.1%) | < 0.001 | 166 (70.6%) | 71 (44.4%) | < 0.001 | 190 (68.8%) | 47 (39.5%) | < 0.001 |
| Positive | 158 (40.0%) | 80 (31.0%) | 78 (56.9%) | | 69 (29.4%) | 89 (56.3%) | | 86 (31.2%) | 72 (60.5%) | |
| EBV | | | | | | | | | | |
| Negative | 369 (93.4%) | 253 (98.1%) | 116 (84.7%) | < 0.001 | 231 (98.3%) | 138 (86.3%) | < 0.001 | 271 (98.2%) | 98 (82.4%) | < 0.001 |
| Positive | 26 (6.6%) | 5 (1.9%) | 21 (15.3%) | | 4 (1.7%) | 22 (13.8%) | | 5 (1.8%) | 21 (17.6%) | |
| p53 | | | | | | | | | | |
| Negative | 285 (72.2%) | 186 (72.1%) | 99 (72.3%) | 0.971 | 167 (71.1%) | 118 (73.8%) | 0.559 | 198 (71.7%) | 87 (73.1%) | 0.780 |
| Positive | 110 (27.8%) | 72 (27.9%) | 38 (27.7%) | | 68 (28.9%) | 42 (26.2%) | | 78 (28.3%) | 32 (26.9%) | |
| MSI | | | | | | | | | | |
| MSS/MSI-L | 358 (90.6%) | 236 (91.5%) | 122 (89.1%) | 0.432 | 214 (91.1%) | 144 (90.0%) | 0.722 | 251 (90.9%) | 107 (89.9%) | 0.748 |
| MSI-H | 37 (9.4%) | 22 (8.5%) | 15 (10.9%) | | 21 (8.9%) | 16 (10.0%) | | 25 (9.1%) | 12 (10.1%) | |
| Total | 395 (100%) | 258 (65.3%) | 137 (34.7%) | | 235 (59.5%) | 160 (40.5%) | | 276 (69.9%) | 119 (30.1%) | |

*CT* center of the tumor, *IM* invasive margin, *MSS* microsatellite stable, *MSI-L* microsatellite instability-low, *MSI-H* microsatellite instability-high

mechanism of tumor cells [25, 26]. The mechanisms of HLA I downregulation or loss include mutation, deletion, or loss of heterozygosity of β2M and HLA I heavy chain genes, inhibition of transcription or translation of HLA antigen heavy chain, defects in HLA antigen processing regulatory mechanisms, and defects in antigen processing machinery components [27]. HLA I abnormalities are classified into total HLA I antigen loss, HLA I downregulation, and selective loss or downregulation of HLA I allospecificities [27]. In recent studies, HLA I downregulation or loss in GC was reported to vary from approximately 19 to 75%. This may be due to differences in the preparation of tissue sections, primary antibodies, and scoring systems used [19, 28–30]. In previous studies, HLA I expression by IHC was interpreted as positive when the membrane of tumor cells was stained [19, 28–30]. The same method was used in this study.

Although downregulation or loss of HLA I expression has been reported in various type of cancers, including GC, HLA I expression patterns were complex in gastric cancer and autologous gastric mucosa. Previous studies have shown that autologous gastric mucosa lacked the expression of HLA I and HLA II antigens before becoming malignant. In addition, GC showed increased HLA I expression when compared with autologous gastric mucosa [31]. Similar to the previous studies, we observed higher HLA A/B/C expression in GC tissues than autologous mucosa tissues, which showed predominantly negative expression in frozen sections and lower expression in FFPE sections. However, HLA I expression was heterogenous in a large cohort of GC patients; HLA I-positive GCs tended to have higher T cell response and HLA I-negative GCs had lower T cell response with poor prognosis. Therefore, negative HLA I expression in GC was also suggested to be associated with immune escape and poor outcome similar to expression loss of HLA I molecules in other cancers.

Since HLA I molecules play an important role in restricting carcinoma-specific antigen recognition by CD8-positive cytotoxic T cells, loss of HLA I is the most important escape pathway from CD8-positive cytotoxic T cell surveillance [26]. Additionally, loss or downregulation of HLA I expression was reported to be associated with poor prognosis in various carcinomas, including non-small cell lung cancer [32], endometrial cancer [33], colorectal cancer [34], primary laryngeal squamous cell carcinoma [35], biliary tract cancer [36], and bladder cancer [37]. In contrast, the patients with downregulated HLA I expression were reported to have good prognosis in some studies, including colorectal cancer [38] and breast cancer [39]. In a study of GC, two conflicting

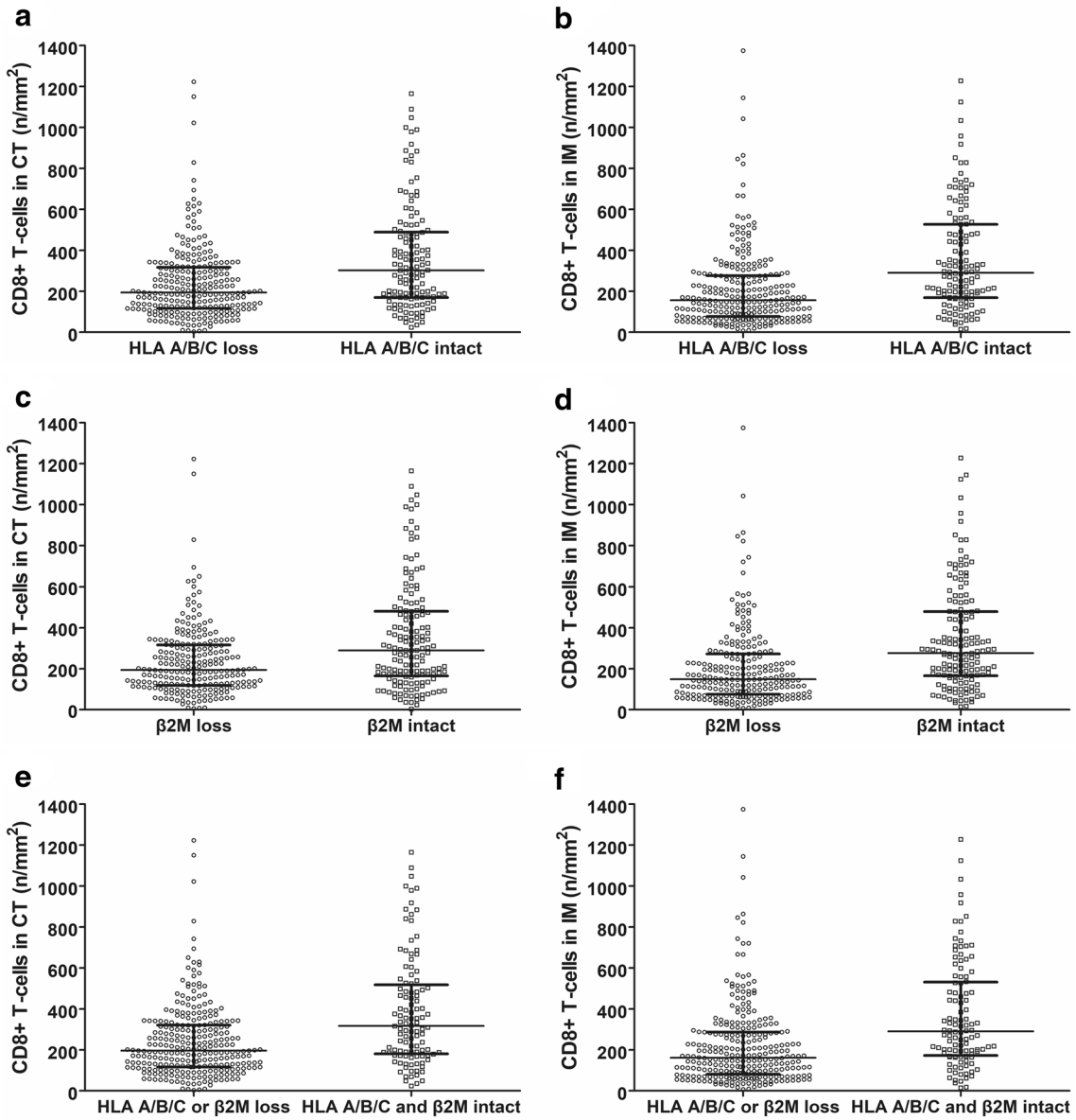


**Fig. 2** Association between CD8-positive cytotoxic T cell infiltration and expression of human leukocyte antigen (HLA) A/B/C (**a**, **b**) and beta-2-microglobulin (β2M) (**c**, **d**) and either HLA A/B/C or β2M (**e**, **f**) in the center of the tumor (CT) (**a**, **c**, **e**) and invasive margin (IM) (**b**, **d**, **f**). CD8-positive cytotoxic T cell density was significantly higher in cases with intact expression of HLA A/B/C and β2M. In contrast, it was significantly lower when HLA A/B/C or β2M expression was lost in either center of the tumor or invasive margin ($p < 0.001$ in all cases)

prognostic outcomes for negative HLA I expression have been reported [19, 28–30]. In our study, negative expression of HLA I was found to be an independent poor prognostic factor by univariate and multivariate survival analyses. Previous studies were performed in a heterogeneous GC population [19, 28–30], whereas our study was performed in a relatively homogeneous cohort of patients with stage II and III GC who were treated with fluoropyrimidine-based adjuvant chemotherapy after curative surgical resection. In addition, our study revealed the clinicopathologic importance of the negative expression of HLA I molecules in the largest GC cohort to date. Therefore, we cautiously suggest that the results of survival analysis in this study are relatively more reliable compared to those of the previous studies.

HLA I not only mediates the CD8-positive cytotoxic T cell response, but also inhibits NK cells by binding to inhibitory receptors. Therefore, HLA I loss could lead to escape from CD8-positive cytotoxic T cell response, but may activate cytotoxic NK cell response. Cancer cells are believed to regulate the level of HLA I expression to establish a balance

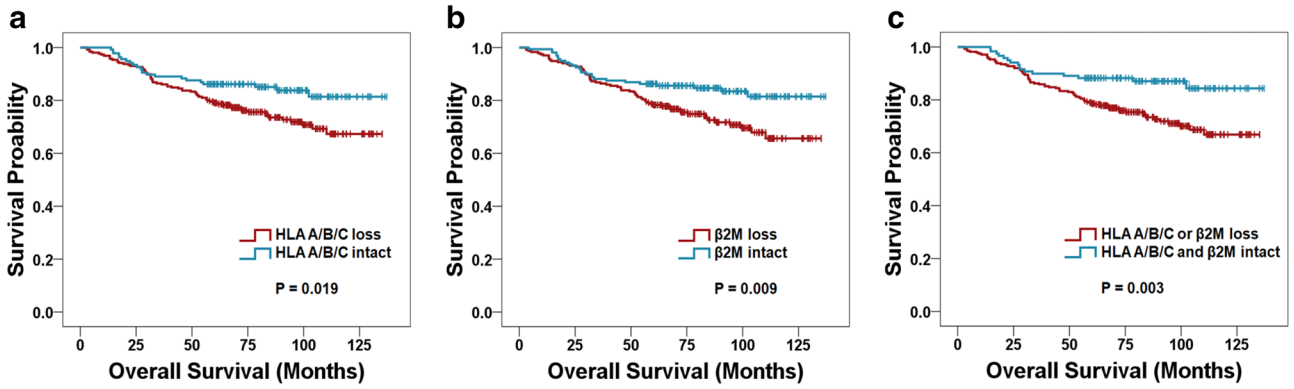


**Fig. 3** Kaplan–Meier survival curves showing the prognostic value of human leukocyte antigen (HLA) A/B/C and beta-2-microglobulin (β2M) expression in gastric cancer. Loss of HLA A/B/C (**a**) and β2M (**b**) was associated with poor overall survival ($p = 0.019$ and $p = 0.009$, respectively). Loss of either HLA A/B/C or β2M (**c**) expression was also significantly associated with poor overall survival when compared with intact HLA A/B/C and β2M expression ($p = 0.003$)

**Table 3** Univariate and multivariate analyses for the predictors of overall survival in patients with stage II, III gastric cancer by proportional hazards model

| Variable | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* | HR | 95% CI | *P* |
| Age (years) | | | | | | |
| ≥ 65 vs. < 65 | 1.878 | 1.243–2.839 | 0.003 | 1.540 | 1.131–3.350 | 0.046 |
| Sex | | | | | | |
| Female vs. male | 0.767 | 0.506–1.163 | 0.212 | – | – | – |
| Tumor size | | | | | | |
| > 5 cm vs. ≤ 5 cm | 2.617 | 1.702–4.026 | < 0.001 | 1.718 | 1.094–2.699 | 0.019 |
| pTNM stage | | | | | | |
| III vs. II | 3.706 | 2.255–6.089 | < 0.001 | 2.076 | 1.202–3.584 | 0.009 |
| Lauren classification | | | | | | |
| Non-intestinal vs. intestinal | 1.429 | 0.906–2.252 | 0.124 | – | – | – |
| Ming classification | | | | | | |
| Infiltrative vs. expanding | 1.844 | 0.982–3.464 | 0.057 | – | – | – |
| Lymphatic invasion | | | | | | |
| Present vs. absent | 1.528 | 0.938–2.489 | 0.088 | – | – | – |
| Vascular invasion | | | | | | |
| Present vs. absent | 3.519 | 2.278–5.437 | < 0.001 | 2.097 | 1.326–3.317 | 0.002 |
| Perineural invasion | | | | | | |
| Present vs. absent | 3.187 | 1.803–5.634 | < 0.001 | 1.969 | 1.077–3.600 | 0.028 |
| Loss of HLA A/B/C or β2M | | | | | | |
| Loss vs. intact | 2.191 | 1.277–3.761 | 0.004 | 1.946 | 1.131–3.350 | 0.016 |

*HLA* human leukocyte antigen, *β2M* beta-2-microglobulin

between escaping from CD8-positive T cell cytotoxicity and activating NK cell cytotoxicity. The situation of cancer cells under the pressure to maintain this balance could be reflected in the heterogeneous loss of HLA I [26]. Heterogenous loss of HLA I has been shown to correlate with clinicopathologic features including stages or grades and either good or poor prognosis in previous studies [28–30, 33, 34, 36, 38, 40, 41]. In our study, heterogenous expression of HLA I was associated with histologic grades and considered to affect poor prognosis. These results suggest that heterogeneous expression of HLA I may more potently inhibit CD8-positive cytotoxic T cell response than activating NK cells. Further studies on HLA I expression and the relationship between CD8-positive cytotoxic T cells, NK cells, and tumor cells are needed.

Recent studies on the relationship between PD-L1 expression and HLA-I expression in lung cancer have been unable to establish a significant correlation, but loss of HLA-I

expression was associated with high-grade primary tumor size in PD-L1-positive lung cancer [42]. In our study, HLA I expression was significantly correlated with PD-L1 expression. It might be because our study enrolled a larger cohort of patients with stage II and III GC and HLA I and PD-L1 interpretation was also different from those of the previous studies. Therefore, further studies are needed to confirm the relationship between HLA I expression and PD-L1 expression.

The previous studies have demonstrated that EBV-positive and MSI-H GCs are two distinct and important subtypes according to molecular and clinicopathologic characteristics, especially tumor immune microenvironment [43]. In our study, an association was observed between HLA I expression and EBV infection, but no association with MSI was observed. There is no previous report on the association between HLA I and EBV infection or MSI. However, recent studies have shown that the immune signature of MSI-H GCs was not consistently high in most cases, whereas the immune signature of EBV-positive GCs was consistently high in all cases [44]. Unlike EBV-positive GCs, the immune signature of MSI-H GCs was heterogeneous. Since HLA I expression was highly correlated with T cell response, MSI-H GCs showing heterogeneity in immune signature had no significant association with HLA I expression, as observed in EBV-positive GCs. According to a previous study, overexpression of wild-type p53 was associated with increased HLA I expression [45], but we did not find a significant correlation with HLA I expression due to the limitations of IHC in detecting p53 overexpression. Further studies are needed to investigate the relationship between these parameters and HLA I expression.

Transition from positive to decreased or negative expression of HLA I in tumors has been reported to be directly correlated with the degree of T cell infiltration; thus, it is considered as one of the escape mechanisms from T cell response [46]. In addition, the association between the loss or downregulation of HLA I expression and a low density of tumor-infiltrating lymphocytes (TILs) has been demonstrated in colorectal cancer [47] and pancreatic cancer [48] and low TIL densities have been reported to have an unfavorable prognostic impact. Our study also revealed higher CD8-positive cytotoxic T cell density in GCs with positive HLA A/B/C and β2M expression, positive PD-L1 expression, and EBV positivity. Recent studies showed that CD8-positive cytotoxic T cells, among the types of TILs, were important for the function of immune checkpoint inhibitors [49] and heavy infiltration of TILs in EBV-positive GCs was related to a favorable response to immune checkpoint blockade [50, 51]. HLA I expression can be considered as a new predictive biomarker for immune checkpoint inhibitor therapy. Recently, a phase III KEYNOTE-061 trial reported that Pembrolizumab did not reach the pre-specified level of significance for improving OS compared to Paclitaxel as a second-line therapy for advanced/metastatic GC or gastroesophageal junction cancer [22]. In this study, HLA I expression was negative in 61.6% of PD-L1-positive GCs, 19.2% of EBV-positive GCs, and 67.6% of MSI-H GCs. EBV-positive GCs had a tendency of positive expression of HLA I when compared to PD-L1-positive GCs and MSI-H GCs. Therefore, in addition to PD-L1 expression status, assessment of other biomarkers such as TIL density and expression of HLA I molecules would be helpful for predicting the effects of PD-1 inhibitors.

This study had some limitations. This was a retrospective study conducted at a single institution and sampling bias might have included owing to the TMA slides used. The absence of interpretation and scoring guidelines for HLA A/B/C and β2M expressions was another limitation of this study. However, compared to the previous studies, the population used in our study was large and homogenous with restricted confounding factors. Therefore, further comprehensive studies and clinical trials are needed to confirm the implications of HLA A/B/C and β2M expressions as prognostic and predictive biomarkers in managing GC patients. Additionally, a consensus of interpretation and scoring guidelines is also necessary.

In summary, we evaluated the clinicopathologic significance of HLA I expression status through HLA A/B/C and β2M IHCs in a large homogeneous cohort of patients with stage II and III GC. Negative expression of HLA A/B/C and β2M was frequently observed in stage II and III GCs, and particularly correlated with the aggressive behavior of GC in addition to unfavorable prognosis. Moreover, we found that HLA A/B/C and β2M expressions were significantly correlated with host immune response status such as PD-L1 expression and CD8-positive T cell density as well as EBV infection. Therefore, our study suggests that negative expression of HLA I molecules can serve as a poor prognostic factor for GC patients and should be further analyzed as a predictive biomarker for immunotherapy.

**Author contributions** YP and HSL conceived and designed the study. SHA, DJP, and HHK provided clinical data and interpretation. YP, JK, YK, WHK, and HSL collected, analyzed, and interpreted pathologic data. YP and HSL wrote the manuscript. All the authors read and approved the final manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors report no conflict of interest.

**Ethical approval and ethical standards** All human tissue samples were obtained from the archive of the Department of Pathology, Seoul National University Bundang Hospital and clinicopathologic data including patients' survival were obtained from medical records. This study was approved by the institutional review board (IRB) of Seoul National University Bundang Hospital (IRB number: B-1606/349-308 and B-1402/240-004).

**Informed consent** Written patient consent and the consent process were waived by the IRB under the condition of anonymization and no additional intervention to the participants.

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