Human Leukocyte Antigen (HLA)-E and HLA-F Expression in Gastric Cancer

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Abstract. *Human leukocyte antigen (HLA)-E and HLA-F are classified as non-classical HLA class Ib antigens. Ectopic HLA-E and HLA-F expression was recently detected in cancer cells; however, the clinical implication of their expression remains unknown. A total of 209 patients with gastric cancer were enrolled in this study. Immunohistochemistry was used to evaluate the expression of HLA-E and HLA-F in gastric cancer specimens. HLA-E and HLA-F expression were seen in the cell membrane. HLA-E and HLA-F expression significantly correlated with depth of invasion, nodal involvement, lymphatic invasion, and venous invasion. No significant correlation between HLA-E and HLA-F expression was found ($p<0.05$, $r=0.24$). The five-year survival rate of the HLA-E-positive group and HLA-F-positive group were significantly poorer than that of their respective negative groups. Combination of HLA-E and HLA-F made the p-value smaller than single analysis ($p<0.009$). This is the first report detailing a clinical implication of HLA-E and HLA-F expression simultaneously in gastric cancer. We identified that the HLA-E and HLA-F in gastric cancer independently affected clinical factors, including postoperative outcome. For HLA-E- or HLA-F-positive gastric cancer, we should settle on a treatment strategy that reinforces the host immune response.*

Gastric cancer is the second leading cause of cancer-related death worldwide and the primary cause of cancer death in Japan (1). Since the introduction of a mass screening program in Japan that utilizes double-contrast barium radiography for the early detection of gastric cancer, as well as developments in endoscopic equipment and improved diagnostic capability, gastric cancer is now being detected more often in the asymptomatic stage. In addition, with the improved detection rate of early gastric cancer in Japan, more minimally invasive treatments, such as endoscopic and laparoscopic procedures, have become widespread. However, despite its decreasing incidence in Japan, gastric cancer still remains a serious health concern.

Surgery is an important treatment option for some patients with gastric cancer; however, the postoperative outcome may depend upon the surgical curability (2). In addition, some patients who undergo curative surgery suffer unexpectedly from recurrence. Thus, molecular prognostic markers that can predict the aggressiveness and prognosis of gastric cancer have been the subjects of many studies. The tumor-suppressor genes, cell-cycle regulators, cell-adhesion molecules, and DNA-repair genes are representative prognostic markers in gastric cancer (3-7). Unlike these markers, tumor aggressiveness can be estimated by the impairments of the host immune system for the tumor (8). Dysfunction of antitumor immune surveillance causes tumor cells to evade lysis by host immunocytes. These defects in tumor surveillance range from loss of human leukocyte antigen (HLA) class I alleles (9), and dysfunction of the T-cell receptor (TCR) (10), to the abnormal expression of co-stimulatory ligands on tumor cells (11).

There are three non-classical HLA class I genes, namely, for HLA-E, HLA-F, and HLA-G. HLA-E is a ligand for CD94/NKG2 inhibitory receptors on natural killer (NK) cells, and for TCRs on NK cells and cytotoxic T-lymphocytes (CTLs) (12). In normal tissue, non-classical class Ib genes are all highly expressed in trophoblasts, and are thought to play key roles in implantation by controlling trophoblast invasion and maintaining a local immunosuppressive state (13-15). Recent studies have demonstrated the aberrant expression of HLA class Ib molecules in cancer cells (15-19). HLA-E and

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HLA-F expression in trophoblasts facilitates the resistance of trophoblast cells to NK- and CTL-mediated lysis in the maternal uterine environment (20). Thus, we can speculate that if HLA-E and HLA-F expression in cancer cells causes immune tolerance using aberrant HLA-class Ib molecules, this may be related to the biological aggressiveness of HLA-E- and HLA-F-positive cells. We previously examined HLA-G (21) and HLA-F (22) expression in gastric cancer and revealed a significant correlation with clinical factors, such as surgical outcome. HLA-E has only recently been studied in earnest, and there are few reports on HLA-E expression in gastrointestinal cancer cells.

Marín *et al*. examined the cell surface expression of HLA-E in the gastric cancer cell line AGS; they reported increasing HLA-E after Interferon (IFN) induction (23). In this study, we analyzed the correlation between HLA-E and HLA-F expression, and determined the clinical significance of HLA-E expression in gastric cancer.

Materials and Methods

Patients. A total of 209 consecutive patients with gastric cancer who underwent resection (R0 resection) at Kagoshima University Hospital between 2003 and 2007 were enrolled in the present study. The patient group was composed of 140 men and 69 women, ranging in age from 31 to 82 years (mean age of 65 years). Total, distal, and proximal gastrectomies were performed in 93, 96, and 20 patients, respectively. None of the patients received preoperative chemotherapy. All patients underwent R0 resection with at least a D1 lymph node dissection. Patients received postoperative adjuvant chemotherapy according to the Japanese gastric cancer treatment guidelines 2010 (ver. 3) (24). The study was approved by the Institutional Review Board of Kagoshima University (authorization number 25-39). Clinical factors were assessed by the third English edition of the Japanese classification of gastric carcinoma (25).

Immunohistochemistry. HLA-E (16, 17) and HLA-F (18, 22) expression in the tumor was assessed immunohistochemically according to previous reports, and prepared specimens were visualized by the avidin-biotin complex (ABC) method. Namely, paraffin-embedded sections (4 μm), including tumor nests, obtained from the 209 patients with gastric cancer were deparaffinized and soaked in phosphate-buffered saline (PBS) prior to immunohistochemical analysis. Sections were treated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase, followed by treatment with bovine serum for 30 minutes to reduce nonspecific binding. Before treatment with primary antibody, sections were immersed in antigen retrieval buffer (1% citrate buffer) and autoclaved at about 120˚C for 10 minutes. HLA-E (MEM-E/02; Abcam, Cambridge, UK) and HLA-F antibodies (14670-1-AP; Proteintec Group, Chicago, IL, USA) were diluted at 1:200 and 1:500, respectively. MEM-E/02 recognizes denatured HLA-E. This antibody reacts with the denatured heavy chain of human HLA-E. It does not cross-react with HLA-A, -B, -C, or -G (27). Antibodies were incubated with the sections for 120 minutes at room temperature. Sections were rinsed in PBS and visualized using standard techniques for labeled avidin-biotin immunoperoxidase staining. HLA-E and HLA-F immunostaining was visualized using the DAB Substrate Kit (Vector, Burlingame, CA USA). The slides were briefly counterstained with hematoxylin and aqueously mounted. Positive controls for HLA-E and HLA-F expression were splenocytes and human trophoblasts, respectively. In addition, PBS with bovine serum albumin was used as a negative control for HLA-E and HLA-F.

Table I. *Association between clinicopathological features and human leukocyte antigen (HLA)-E in 209 patients with gastric cancer.*

Factor	Cancer HLA-E-positive tumor cells			Stromal HL-E-positive cells		
	Positive n=68	Negative n=141	*p*-Value	Many n=90	Few n=119	*p*-Value
Age, years	64	67	n.s.	62	63	n.s.
Sex						
Male	44	96	n.s.	60	70	n.s.
Female	24	45		30	49	
T Stage						
T1	8	85		38	55	
T2	31	28	<0.01	27	32	n.s.
T3	29	28		25	32	
N Factor						
N0	17	100	<0.01	50	67	n.s.
N+	51	41		40	52	
Ly Factor						
Yes	58	57	<0.01	50	65	n.s.
No	10	84		40	54	
V Factor						
Yes	49	38	<0.01	37	50	
No	19	103		53	69	
Histology						
Differentiated	34	57	n.s.	52	66	n.s.
Undifferentiated	34	55		38	53	
HLA-F						
Positive	34	56	n.s.			
Negative	34	85				

n.s.; Non significant.

Evaluation of HLA-E and HLA-F expression in gastric cancer. HLA-E-positive cells were evaluated according to previous reports (13, 16). Namely, HLA-E-positive expression was calculated in 10 representative high-power fields (×400) in each tumor nest and at the invasive tumor front. HLA-E-positive cells were identified in the cell membrane and cytoplasm (Figure 1). HLA-F-positive cells were also evaluated according to our previous reports (13, 16). All immunostained slides were evaluated by two independent observers (SI and AT), who were blinded to the clinical data and disease outcome.

The percentages of HLA-E-, and HLA-F-positive cells in the 209 gastric cancer patients ranged from 0 to 82% (average=11.4%) and from 0 to 60% (average=6.6%), respectively, and HLA-E positivity in tumor-infiltrating stromal cells ranged from 0 to 71% (average=7.4%). Therefore, we set the cut-off level at 10% for HLA-E-positive cells and 5% for HLA-F-positive cells. If the percentage

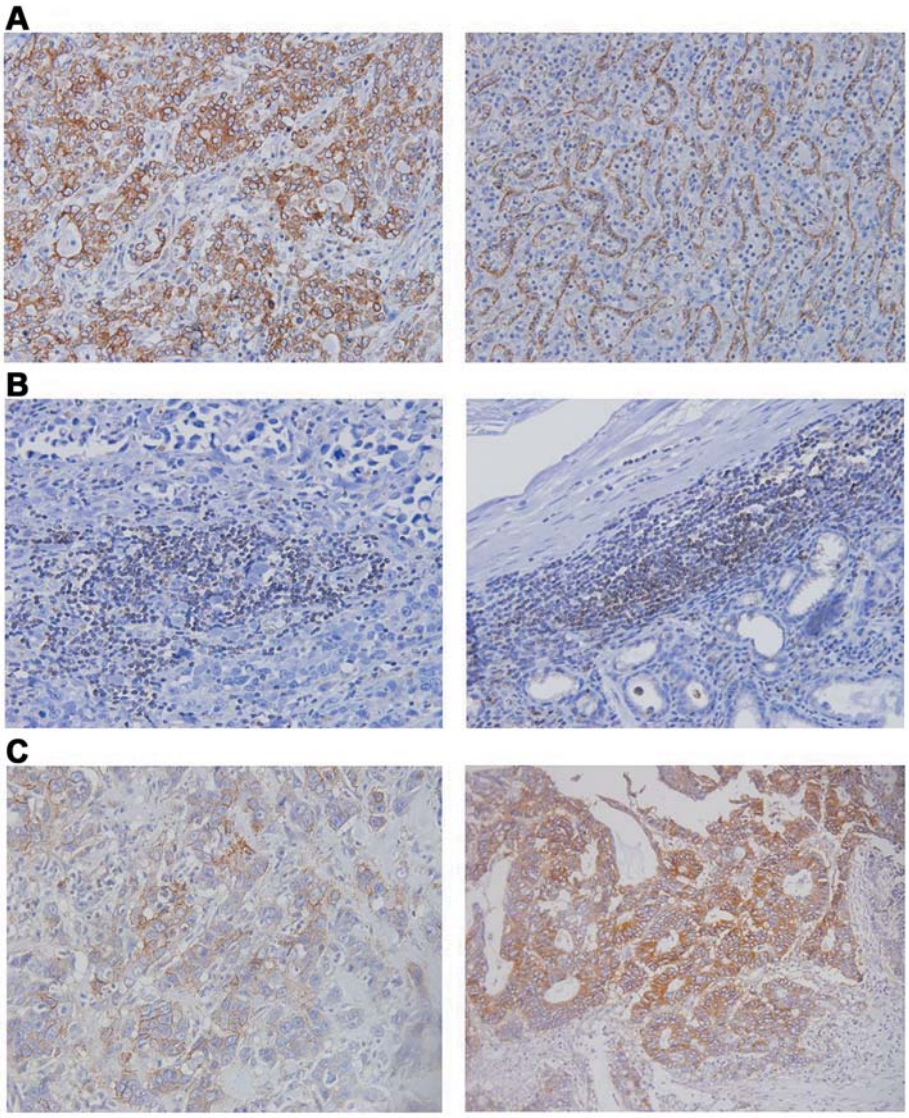

Figure 1. *Human leukocyte antigen (HLA)-E, and HLA-F positivity in gastric cancer tissue. A: HLA-E positivity in cancer cells. HLA-E expression was identified in the membrane and cytoplasm of cancer cells and infiltrating stromal cells. B: HLA-E positivity in the tumor stroma. HLA-E-positive cells partially infiltrated the tumor stroma and lymph follicle around the tumor nest. C: HLA-F positivity in gastric cancer. HLA-F expression was identified in the membrane of cancer cells and infiltrating stromal cells. Original magnification, ×400*

of HLA-E-positive cells exceeded 10%, patients were regarded as HLA-E-positive, while patients with more than 5% HLA-F positive cells were classified as HLA-F-positive. Similarly, patients with more or less than 10% HLA-E positivity in tumor-infiltrating stromal cells were regarded as the HLA-E-positive infiltration group and the HLA-E-negative infiltration group, respectively.

Statistical analysis. Statistical analysis of clinical features was performed by chi-squared test, and the percentages of HLA-E- and HLA-F-positive cells were evaluated using the Pearson correlation coefficient. Survival curves were produced using the Kaplan–Meier method, and the statistical significance of differences between two groups was calculated using the generalized Wilcoxon test. Multivariate analysis was performed to determine the independent prognostic markers for survival. *p*-Values less than 0.05 were considered statistically significant.

Results

HLA-E and HLA-F expression in gastric cancer and stroma -infiltrating cells. HLA-E positivity was found in the cellular membrane and cytoplasm of gastric cancer cells (Figure 1A), and it was also identified in the membrane and cytoplasm of infiltrating cells in cancer stroma (Figure 1B). HLA-F positivity was detected in the membrane and cytoplasm of

gastric cancer cells, similarly to HLA-E (Figure 1C). As previously reported, we set the cut-off level at 10% offor HLA-E-positive cells and 5% for HLA-F-positive cells. Therefore, the 209 patients were divided into two groups: 68 in the HLA-E-positive group and 141 in the HLA-E-negative group, as well as 62 in the HLA-F-positive group and 147 in the HLA-F-negative group.

Clinicopathological features of 209 patients with gastric cancer according to HLA-E-/HLA-F-positive gastric cancer. We evaluated the correlation between seven significant clinical factors and HLA-E-/HLA-F-positivity in gastric cancer by chi-square test (Table I). HLA-E and HLA-F positivity in cancer cells was significantly associated with increased tumor invasion ($p<0.05$ and $p<0.01$, respectively), nodal involvement ($p<0.01$), lymphatic invasion ($p<0.01$), and venous invasion ($p<0.01$). However, there was no association between tumor histology and HLA positivity.

Association between HLA-E and HLA-F positivity in gastric cancer. Percentages of HLA-E and HLA-F positivity for each patient were plotted, and the correlation between HLA-E and HLA-F was analyzed. No association was found between cancerous HLA-E and HLA-F expression in cancer tissue ($r=0.24$, $p<0.05$) (Figure 2).

Survival analyses of 209 patients with gastric cancer according to HLA-E, HLA-F and the combination of HLA-E and HLA-F. The five-year survival rate of patients in the HLA-E-positive group was 59%, which was significantly poorer than that of the HLA-E-negative group ($p<0.05$) (Figure 3). On the other hand, as well as HLA-E positivity, the five-year survival rate of patients in the HLA-F-positive group was 61%, which was significantly poorer than that of patients in the HLA-F-negative group ($p<0.05$) (Figure 4). Combination analysis of HLA-E and HLA-F for survival was performed. Eighty-five and 34 patients were classified as being in the HLA-E-positive/HLA-F-positive and HLA-E-negative/HLA-F-negative groups, respectively. Five-year survival of the HLA-E-positive/HLA-F-positive group was 58% and the *p*-value was 0.009 (Figure 5).

We performed multivariate analysis for surgical outcome using six clinical factors HLA-E, HLA-F, tumor depth, nodal involvement, lymphatic invasion, and venous invasion which were significant prognostic markers by univariate analysis. However, neither HLA-E nor HLA-F positivity was an independent prognostic marker in gastric cancer.

Discussion

Although HLA-E expression patients with cancer was recently reported (25-27), as far as we are aware this is the first study to evaluate its prognostic value in gastric cancer. We showed that the percentage of HLA-E-positive expression in gastric cancer was 45%, similar to that in hepatocellular carcinoma (27), but lower than that in ovarian (89%), cervical (83%), and breast (50%) cancer (28). In this study, HLA-E positivity was defined as there being more than 10% HLA-E-positive cells in the tumor, according to the average positivity of HLA-E expression. On the other hand, de Kruijf *et al*. simply evaluated HLA-E positivity in terms of whether HLA-E-positive cells were present or not (28). The above findings may also depend on differences in tumor histology among these types of cancer. In breast cancer, there is little histological diversity, so a monotonous population of HLA-E-positive cells was found. Therefore, the selected cut-off level of HLA-E expression in each type of cancer may differently alter the percentage positivity.

HLA-E has the capacity to bind to the inhibitory CD94/NKG2A receptor, which is expressed by NK cells and a subset of $CD8^+\delta\gamma$ T-cells. HLA-E is therefore a ligand not only for NK cells, but also for T-cells in various healthy tissues (29). HLA-E expression contributes to the protection of tumor cells from lysis by NK cells. Monaco *et al*. showed that the inhibition of lysis by HLA-E-positive tumor cells involved NKG2A-mediated protection using several tumor cell lines (30). In this context, the up-regulation of HLA-E expression counteracts susceptibility to NK-mediated lysis, allowing tumor cells to escape destruction by the host's immune system. Moreover, Gooden *et al*. suggested that CD8 αβ T-cells may express the CD94/NKG2A inhibitory receptor, which is frequently the case for tumor-infiltrating T-cells in gynecological cancer (31). Thus, HLA-E expression in tumor cells may contribute to the ability to escape from host immune defenses for NK cells and $CD8^+$ T-cells, suggesting anergy of antitumor activity through NK or CTL tolerance from HLA-E or HLA-F.

HLA-E expression significantly influences the post-operative outcome in patients with gastric cancer. Specifically, studies have shown that HLA-E positivity correlates with a worse outcome in lung (18), breast (28), and colorectal (32) cancer, as well as glioblastoma (33), which is in accordance with the data in our study. In contrast, controversial data regarding the impact of HLA-E expression on the overall survival of patients with cancer have been reported. Spaans *et al*. showed that HLA-E positivity correlated with a better outcome in cervical (16) and colorectal (17) cancer, which is surprising since they used the same anti-HLA-E antibody (MEM-E/02) as used in our study. It is possible that the differences in results are related to organ specificity; however, this should be confirmed in a multi-institutional study.

HLA-F and HLA-E have different ligands and are differentially distributed in normal tissue; however, HLA-E and HLA-F on different tumor cells may have similar functions (34). To clarify the differences in the clinical impact of HLA-E and HLA-F, we simultaneously analyzed HLA-E and HLA-F expression. We found no correlation

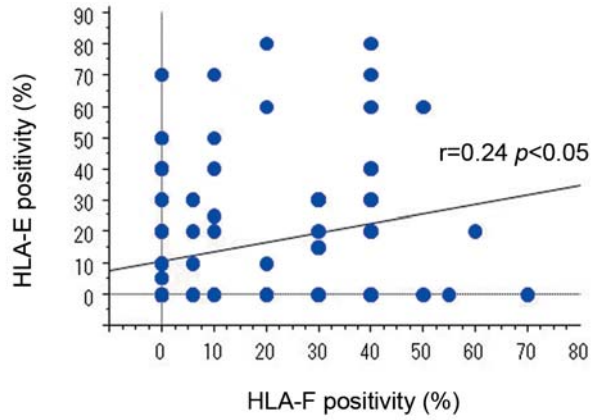

Figure 2. *Association between tumor HLA-E and HLA-F expression in gastric cancer. There was no significant association between HLA-E and HLA-F expression (r=0.24, p<0.05).*

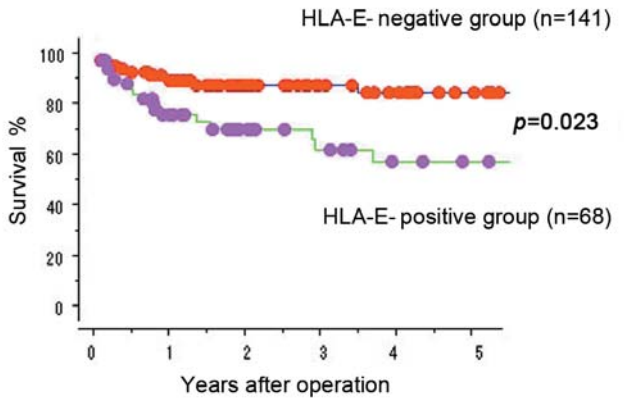

Figure 3. *Surgical outcomes of 209 patients with gastric cancer according to HLA-E expression. The five-year survival rate of the HLA-E-positive group was 53%, which was significantly poorer than that of the negative group (p<0.05).*

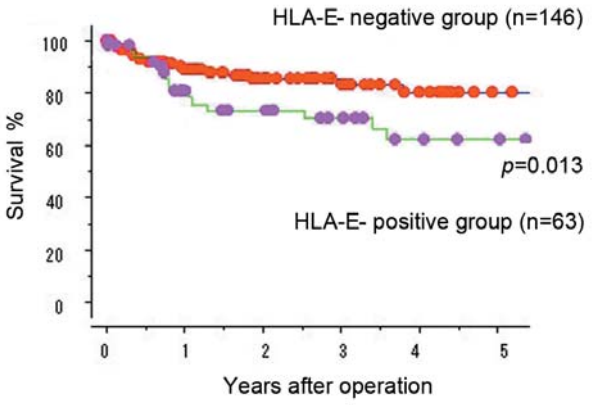

Figure 4. *Surgical outcomes of 209 patients with gastric cancer according to HLA-E expression. The 5-year survival rate of the patients with HLA-F positivity was 61%, which was significantly poorer than that of the patients with HLA-F negativity (82%) (p<0.05).*

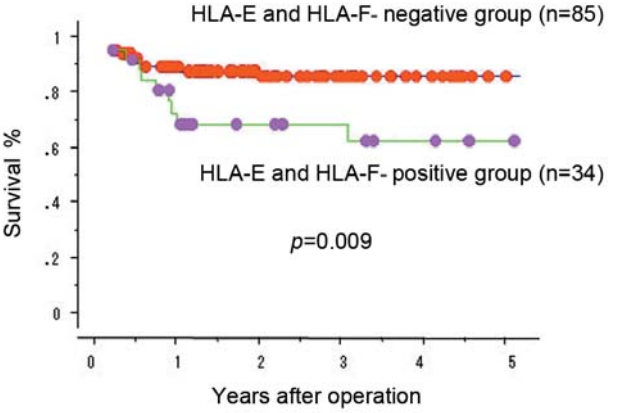

Figure 5. *Combination survival analysis of HLA-E and HLA-F expression. Both HLA-E and HLA-F positive patients (n=34) had significantly poorer outcome than both HLA-E and HLA-F negative patients (n=85). The significance was getting higher (p=0.009) by the combination analysis.*

between HLA-E and HLA-F positivity. de Kruijf *et al*. simultaneously examined HLA-G and HLA-E expression in breast cancer and found that their expression independently correlated with surgical outcome, which is similar to our results. Moreover, the authors suggested the clinical utility of a combination of non-classical HLA Ib molecules as independent prognostic markers in breast cancer (28). Here, we analyzed the combination of HLA-E and HLA-F expression as a prognostic marker and clarified it as a significant parameter for postoperative outcome. Gastric cancer is concomitant with inflammation of the gastric mucosa due to exogenous stimuli such as *Helicobacter pylori*. HLA expression may be altered in gastric cancer (9), however, combination of the HLAs reinforced to be value of immunological prognostic marker in gastric cancer.

HLA-E expression can serve not only as a prognostic marker, but also as a biomarker and a target for treatment to restore the host immune system. Allard *et al*. measured the serum level of HLA-E in patients with cancer and found it to be a significant clinical marker of patients with melanoma as well as kidney, colorectal, and breast cancer (35). IFNγ, IFNα were found to up-regulate serum HLA-E production by tumor cells. Kren *et al*. demonstrated the aberrant activation of HLA-E expression by IFNs in renal cell carcinoma; thus, determination of HLA-E status could contribute to a better selection of patients with renal cell carcinoma who could

benefit from more tailored neoadjuvant immunological therapy (36). Enqvist *et al*. demonstrated that selenite induced the post-transcriptional blockade of HLA-E expression, and increased the sensitivity of tumor cells to CD94/NKG2A-positive NK cells (37). By evaluating HLA-E expression from a resected specimen or endoscopic biopsy, the malignant behavior and appropriate treatment strategy can be determined.

In conclusion, this is the first report detailing clinical implication of HLA-E and HLA-F expression simultaneously in gastric cancer. We identified that HLA-E and HLA-F in gastric cancer independently affected clinical factor including postoperative outcome. For patients with HLA-E or HLA-F expression, we should settle on their treatment strategy to reinforce their immune response.

Conflicts of interest

The Authors declare that they have no conflicts of interest in regard to this study.

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