RESEARCH ARTICLE

Immunoregulatory Function of HLA-G in Gastric Cancer

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Abstract

__Background__: Human leukocyte antigen (HLA)-G-positive gastric cancers are associated with poor survival, but links with tumor escape mechanisms remain to be determined. __Materials and Methods__: We used immunohistochemistry to investigate HLA-G expression, tumor infiltrating CD8+ T lymphocytes, and Treg cells in 52 gastric cancer patients. __Results:__ There were 29 cancer-related deaths during the follow-up period. Kaplan-Meier analysis indicated that patients with HLA-G-positive (n=16) primary tumors had a significantly poorer prognosis than patients with HLA-G-negative tumors (n=36, p=0.008). The median survival time was 14 months and 47 months, respectively. Patients with high numbers of Tregs and low numbers of CD8+T lymphocytes in the primary tumor had a poorer prognosis than those with low numbers of Tregs and high numbers of CD8+T lymphocytes (p=0.034, p=0.043). Multivariate Cox proportional hazard regression analysis showed that HLA-G expression (hazard ratio: 2.662; 95% confidence interval: 1.242-5.723; p=0.012) and stage (hazard ratio: 2.012; 95% confidence interval: 1.112-3.715; p=0.041) were independent unfavorable factors for patient survival. __Conclusions:__ We found a significant positive correlation between HLA-G expression and the number of tumor infiltrating Tregs (p=0.01) and a negative correlation with the number of CD8+T lymphocytes (p=0.041). HLA-G may protect gastric cancer cells from cytolysis by inducing Foxp3+Treg lymphocytes and suppressing CD8+T lymphocytes.

Keywords: HLA-G - gastric cancer - CD8 T lymphocytes - treg cells

Introduction

HLA-G is an MHC class I antigen expressed primarily in the placenta. It modulates the immune tolerance of the mother (Guo et al., 2013). HLA-G expression is a valuable noninvasive embryo marker to assist in improving pregnancy outcomes (Kotze et al., 2013). HLA-G has been reported in several human cancers such as ovarian carcinoma, trophoblastic tumors, gastric cancer, melanoma, lung cancer, endometrial cancer, renal cell carcinoma, and breast cancer. HLA-G expression in malignant cells may represent one of the ways used by tumor cells to escape immunosurveillance. In addition to this, HLA-G has direct inhibitory effects on the functions of NK, DC and T cells, HLA-G could also induce tolerogenic regulatory CD4(+)CD25(+)FoxP3(+) T cells, DCs and NK cells, which provided these immune effectors with a long-term immunomodulatory functions (Lin et al., 2011). HLA-G expression is associated with aggressive tumor behavior and poor survival in patients with gastric cancer (Yie et al., 2007). The correlation between HLA-G expression and inflammatory infiltrating cells such as monocytes/macrophages or lymphocytes has been described in melanoma and gastric cancer (Ibrahim et al., 2004). HLA-G associated immune escape in gastric cancer is mediated by increasing local Foxp3+ regulatory T (Treg) cells (Du et al., 2011). Although the effects of HLA-G on immunocytes have been described, HLA-G expression, T lymphocytes, and Treg cells have not been evaluated together in gastric cancer patients. We investigated HLA-G expression and its relationship with tumor infiltrating CD8+ T lymphocytes, Tregs, and clinicopathologic parameters in patients with gastric cancer.

Materials and Methods

Patients and follow-up

Fifty-two gastric cancer patients (31 men, 21 women) who had undergone gastrectomy for gastric cancer at GATA Haydarpasa Training Hospital between 2003 and 2010 were enrolled in this retrospective study. Forty-two patients had undergone curative gastrectomy and the remaining 10 had undergone palliative gastrectomy. None of the patients received chemotherapy or radiation therapy before surgery. Patients who did not have primary gastric tumor samples were excluded. Histopathologic diagnoses were established according to the guidelines of the latest WHO classification. Cases were selected according to tissue availability not stratified for any known preoperative

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or pathological prognostic factor. Survival outcomes were calculated from the date of surgery to the date of death from any cause. Overall survival (OS) was defined as the interval between surgery and death or between surgery and the last observation for surviving patients. A local Ethical Committee approved our study.

Immunohistochemistry

The pathology of all tissue specimens was confirmed by microscopy before IHC. Formalin-fixed and paraffin-embedded (FFPE) tissues were used for IHC., Sections of FFPE cancer and adjacent normal tissue blocks were cut (4 mm) and placed on poly-lysine coated slides. Tissues were deparaffinized with xylene and ethanol in 56ºC and rinsed in distilled water. Antigen retrieval was applied in a microwave oven (700 watt) for 5 min in EDTA. Peroxidase activity was blocked with 3% H_2O_2 at room temperature for 20 min. Antibody blockage was performed with Blocking Reagent-Ultra V Block (Lab Vision) and phosphate-buffered saline(PBS). Immunohistochemical staining was performed with primary antibodies to FoxP3 (Abcam ab10563), CD8 (Abcam ab4055), and HLA-G (Abcam ab76869).

Evaluation

One pathologist who was unaware of the clinical data evaluated tumor infiltrating Tregs, CD8+ T lymphocyte, and HLA-G expression under a light microscope. HLA-G positivity and frequency of tumor infiltrating Tregs and CD8 lymphocytes were evaluated as described (Ishigami S et al., 2012). HLA-G positivity was determined in 10 representative high-power fields (HPFs). Percentages of HLA-G positivity exceeding 10% were regarded as HLA-G positive. The absolute number of Foxp3- and CD8-positive lymphocytes was counted in 10 HPFs at 400× and averaged. Patients with more than 10/HPF-positive cells were regarded as having a high density of Tregs and CD8 lymphocytes.

Statistical analysis

Statistical analysis of clinicopathological parameters and HLA-G status was performed by Pearson's chi-square test. The Kaplan-Meier method was used to estimate overall survival. Survival differences were analyzed using the log-rank test. The Cox proportional hazard model was used for univariate and multivariate analysis of prognostic factors. Association between HLA-G positivity and degree of Foxp3- and CD8-positive cell infiltration was compared by Pearson's correlation coefficient. p values <0.05 were considered significant.

Results

Clinical and demographic characteristics

A retrospective examination was performed of patients who underwent surgery for gastric cancer from 2003 to 2010 at a single institution. Patient characteristics are shown in Table 1.

HLA-G expression in primary gastric cancer

HLA-G immunoreactivity was observed in 31% (16

of 52) of malignant specimens; however, no staining was detected in corresponding adjacent normal gastric tissue (Figure 1).

Association of HLA-G expression with clinicopathologic parameters and factors related to survival in gastric cancer patients

To identify the clinical relevance of HLA-G expression in gastric cancer, we examined the correlation between HLA-G expression and clinicopathological parameters such as grade of tumor differentiation, histological type, nodal status, and disease TNM stage. HLA-G expression was significantly correlated with nodal status, tumor differentiation, histological type, and disease TNM stage; there was no significant association between HLA-G expression and age or gender (Table 2).

There were 29 cancer-related deaths during the follow-up period. Kaplan-Meier analyses indicated that patients with HLA-G-positive gastric cancer had a poorer prognosis than those with HLA-G negative cancer (P=0.008) (Figure 2). The median survival was 14 months for HLA-G-positive gastric cancer patients and 47 months for HLA-G negative patients.

HLA-G, Foxp3, CD8, TNM staging, and tumor

Table 1. Patient Characteristics

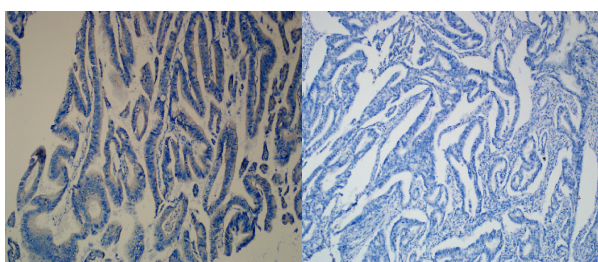| Variables	No. of Cases	Variables	No. of Cases
Age		Lymph Node	
Maximum	87	Positive	26
Minimum	33	Negative	26
Median	63	Surgery	
Gender		Total	34
Female	21	Subtotal	18
Man	31	Stage *AJCC 7th*	
Hist. Grade		I	4
Low	10	II	15
Intermediate	24	III	23
High	18	IV	10

Figure 1. Immunohistochemistry of HLA-G Expression in Gastric Cancer and Normal Tissue
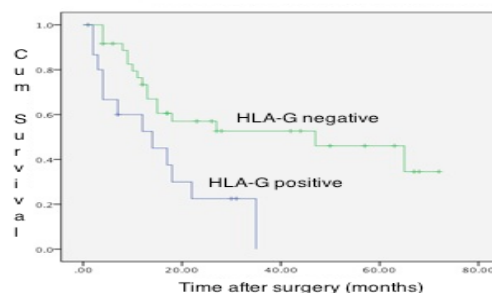

Figure 2. Overall Survival of Patients with Gastric Cancer According to HLA-G Positivity

Table 2. HLA-G Expression in Gastric Cancer and Clinicopathologic Parameters

Variables		No. of cases	HLA-G Negative	HLA-G Positive	p
Age	<63	26	19	7	0.48
	≥63	26	17	9	
Gender	Male	31	19	12	0.13
	Female	21	17	4	
Nodal status	Positive	26	14	12	0.01
	Negative	26	22	4	
Tumor differantion	Low Intermediate	34	29	5	0.001
	High	18	7	11	
Histological type	Diffuse	19	9	10	0.01
	Intestinal	33	27	6	
Stage *AJCC* 7th	I+II	19	17	2	0.01
	III+IV	33	19	14	

Table 3. Univariate and Multivariate Analysis of Factors Associated with Overall Survival

Variables	p	HR	95%CI
HLA-G	0.008	3.122	1.226-6.223
Foxp3	0.034	1.328	1.116-2.834
CD8	0.043	0.715	0.642-0.912
Age	0.64	0.829	0.374-1.459
Gender	0.42	1.714	0.693-3.642
Stage	0.002	2.914	1.325-4.116
T. differation	0.041	0.893	0.654-0.945
Variables	p	HR	95%CI
HLA-G	0.012	2.662	1.242-5.723
Foxp3	0.73	2.088	0.934-4.670
CD8	0.94	0.475	0.199-1.134
Age	0.234	0.629	0.294-1.349
Gender	0.56	1.266	0.573-2.798
Stage	0.041	2.012	1.112-3.715
T. differation	0.81	0.614	0.352-1.213

Figure 3. Immunohistochemistry of High and low-Density Foxp3+ Treg Lymphocytes in Gastric Cancer Patients

Figure 4. Overall Survival of Patients with Gastric Cancer According to Treg Density

Figure 5. Immunohistochemistry of High and Low-density CD8+T Lymphocyte in Gastric Cancer

differentiation showed prognostic significance for overall survival on univariate analysis. Gender and age were not associated with overall survival (Table 3).

The significant factors observed in univariate analysis were applied to a multivariate Cox proportional hazard regression analysis, which showed that HLA-G expression (hazard ratio: 2.662; 95% confidence interval: 1.242-5.723; p=0.012) and stage (hazard ratio: 2.012; 95% confidence interval: 1.112-3.715; p=0.041) were independent unfavorable factors for overall survival (Table 3). These results indicate that HLA-G expression in gastric cancer is closely associated with poor prognosis.

Association of Treg and CD8+ T lymphocytes and survival in gastric cancer patients

Tumor-infiltrating Tregs were quantified by counting Foxp3-positive cells in gastric cancer tissues. Patients with intratumoral Tregs ≥10 were defined as high-density Treg; others were defined as low-density Treg (Figure 3). High-density Tregs were detected in 26 of the 52 (50%) gastric cancer patients. Patients with high-density Tregs (n=26) in the primary tumor had a significantly poorer prognosis than those with low-density Tregs (n=36, p=0.034) (Figure 3). The median survival time was 14 months for gastric cancer patients with high-density Tregs and 47 months for those with low-density Tregs (Figure 4).
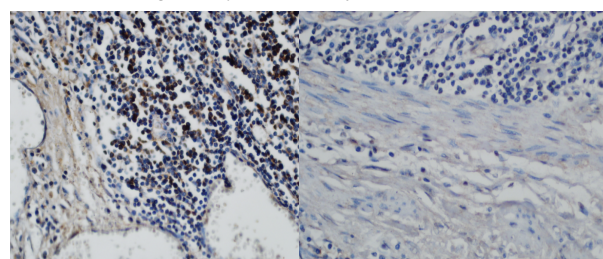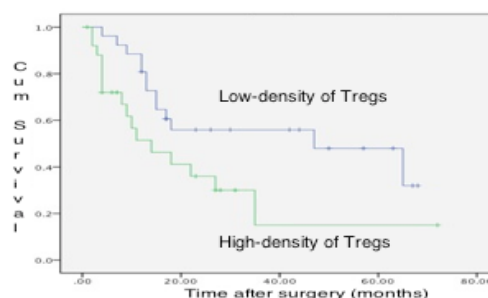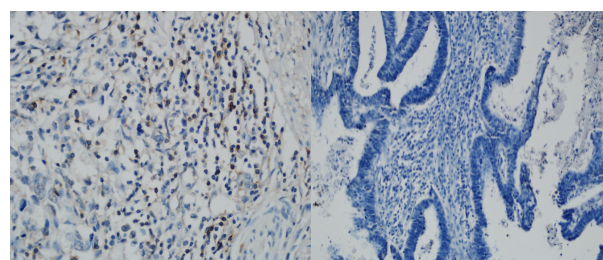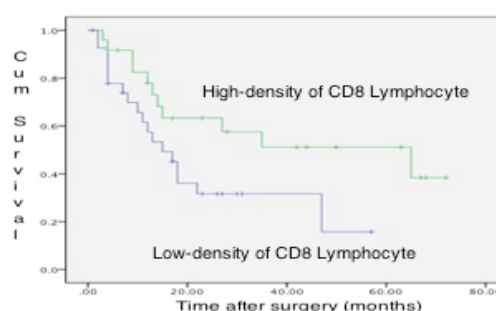

Figure 6. Overall Survival of Patients with Gastric Cancer According to CD8+ T Lymphocyte Density

Tumor-infiltrating CD8+T lymphocytes were divided: patients with ≥ 10 intratumoral CD8+T lymphocytes were defined as high-density and others were defined as low-density (Figure 5). High-density CD8+T lymphocytes were detected in 28 of 52 (53.8%) gastric cancer patients. Low-density CD8+T lymphocytes (n=24) in the primary tumor was associated with a poorer prognosis in comparison to high-density CD8+T lymphocytes (n=28, p=0.043) (Figure 6). The median survival time was 65 months for gastric cancer patients with high-density CD8+T lymphocytes, and 15 months for those with low-density CD8+T lymphocytes.

Association of HLA-G expression with intensity of Treg and CD8+ T lymphocytes in gastric cancer

We analyzed the association between the prognostic significance of HLA-G expression and the intensity of Tregs and CD8+T lymphocytes; we found a significant positive correlation between HLA-G expression and tumor infiltrating Tregs (p=0.01) and a negative correlation with the intensity of CD8+T lymphocyte (p=0.041).

Discussion

HLA-G, a non-classic MHC class I protein was first observed on extravillous cytotrophoblasts, thymic epithelial cells that play an important role in immune tolerance during pregnancy. HLA-G expression was demonstrated in melanoma in 1998 (Paul et al., 1998). Since then, HLA-G expression has been detected in various human malignancies such as ovarian cancer, colorectal cancer, lymphoma, breast cancer, and gastric cancer. Recent studies have shown that HLA-G is rare in normal or adjacent non-tumorous tissues. Thus, HLA-G expression mediates tumor growth and progression by suppressing immune regulation, similar to maternal immune tolerance (Yan, 2011). Many studies have reported the association between HLA-G-positive gastric cancer patient and poor survival, although few studies have investigated the tumor escape mechanism of this disease cells (Du et al., 2011). We observed HLA-G expression in 31% (16/52) of primary gastric cancer lesions, but not in corresponding adjacent non-tumorous gastric tissue. A study by Elliott et al. indicated HLA-G expression only in breast cancer tissue. Paul et al. identified HLA-G expression in primary and metastatic melanoma but not in healthy skin (Paul et al., 1999). HLA-G is detected in tumor tissue but rarely in normal tissue, suggesting its specific association with tumor growth and progression. HLA-G expression varies between 0% and 100% for different types of cancer. In our study, HLA-G expression was observed in 31%; others have reported 49%, 58%, and 71% (Elliott et al., 2011). These differences may be related to tumor biology or varying research methodology and antibody use.

We found that HLA-G expression was associated with clinical parameters such as disease stage, tumor differentiation, and nodal status. In our study, high-density Foxp3+ Tregs detected in 50% of gastric cancer patients were associated with poor prognosis. Similar to Du et al., high-density Foxp3+ Tregs correlated with HLA-G expression. We also identified low-density CD8+T lymphocytes in 54% of gastric cancer patients; this was also associated with poor prognosis and negatively correlated with HLA-G expression. Peng et al. (2012) revealed that an increase in tumor-infiltrating CD8+Foxp3+ T lymphocytes is associated with tumor progression in human gastric cancer (Peng et al., 2012). Both of these indicated a strong relationship between HLA-G and CD8+T, Foxp3+Treg lymphocytes in gastric cancer. HLA-G expression may therefore protect gastric cancer cells from cytolysis by increasing Foxp3+Treg lymphocytes and decreasing CD8+T lymphocytes. In our study, HLA-G expression was identified immunohistochemically; therefore, we do not yet know whether transcript expression is related to IHC status. Limitation of our study, we did not look at natural killer (NK) in this study. HLA-G expression is thought to contribute to the escape in immune surveillance by suppressing NK cell function (Zeng et al., 2013).

We identified significant correlations between HLA-G expression, high-density Tregs, and low-density of CD8+T lymphocytes. HLA-G has an immunosuppressive role in gastric cancer and may be a useful prognostic marker in this disease.

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