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Original Article

HLA-G impairs host immune response and predicts poor prognosis in pancreatic cancer

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Abstract: Human leucocyte antigen G (HLA-G) was shown to be associated with immune suppression and unfavorable prognosis in multiple types of cancers. However, its expression in pancreatic cancer (PC) was less investigated. Particularly, its roles in PC remain unknown. The present study aimed to address the issues. Expression of HLA-G was detected by Western blot and tissue microarray-based immunohistochemical staining in 10 and 158 patients with PC, respectively. In addition, tumor infiltrating lymphocytes (TIL) labeled by CD3 staining, as a marker of host immune response, were counted. Finally, immunohistochemical HLA-G expression was linked to clinicopathologic variables, TIL number and overall survival. It was found that HLA-G was overexpressed in 4 out of 10 patients. For staining, HLA-G expression was much higher in tumor than in non-tumor tissues. Tumoral expression of HLA-G was closely associated with T stage. Intratumoral CD3-positive TIL in tumors with diffuse HLA-G expression was less than that in those with negative or local HLA-G expression, but no significant differences for stromal TIL were observed. Univariate analysis found that diffuse HLA-G expression in tumor tissues and low intratumoral CD3-positive TIL number were of predictive significance for poor overall survival of PC. Furthermore, HLA-G expression and intratumoral CD3-positive TIL number were identified, by multivariate Cox regression test, as independent prognostic factors. Our data suggest that HLA-G impairs host immune response and predicts poor prognosis in PC.

Keywords: Human leucocyte antigen G, pancreatic cancer, host immune response, prognosis

Introduction

Thus far, pancreatic cancer (PC) remains one of lethal malignant tumors, because of its estimated mortality that is almost equal to its estimated incidence [1]. Bearing in mind the extremely poor prognosis, many clinicians paid much attention for prognostic factors of PC, and identified some clinicopathologic parameters influencing prognosis, including lymph node metastasis, neural invasion, tumor-node-metastasis (TNM) classification and CA19-9 level [2-5]. During recent years, biological prognostic factors, based on genes/proteins that have been demonstrated to be involved in tumorigenesis and progression PC, have been more and more found [6, 7]. However, further investigations for biomarkers in PC are quite expected.

Human leukocyte antigen G (HLA-G), a non-classical major histocompatibility complex (MHC) class I molecule mapped to 6p21.3 [8], plays crucial roles in immune modulation [9]. It has been proven that HLA-G inhibits many immune cells through binding to inhibitory receptors [10-12]. In many kinds of human cancers, such as colorectal cancer [13], gastric cancer [14, 15], lung cancer [16], esophageal cancer [17, 18], hepatocellular carcinoma [19, 20], ovarian cancer [21], nasopharyngeal carcinoma [22], breast cancer [23] and papillary thyroid cancer [24], HLA-G was shown to be associated with impaired host immune response, unfavorable clinicopathologic variables and poor prognosis. All these investigations support the role of HLA-G as a proto-oncogene. However, inconsistent reports can also be found. In colon cancer, HLA-G fails to act as a prognostic marker [25]. Surprisingly, this protein was even linked to improved survival in rectal and ovarian cancers [26, 27]. Therefore, further evidence needs to be accumulated. In PC, there has been a preliminary clue about HLA-G expression [28].

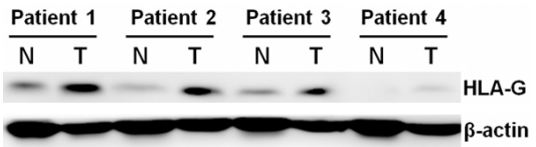

Figure 1. Expression of HLA-G in pancreatic cancer (PC), detected by Western blot. HLA-G, human leukocyte antigen G; N, non-tumor; T, tumor.

However, its impacts on clinicopathologic features, host immune status and patient survival have not been addressed.

The present study aimed to elucidate clinicopathologic, immunological and prognostic roles of HLA-G in PC.

Materials and methods

Patients

There were 6 male and 4 female patients (40 to 72 years; median: 57 years) whose samples were used in Western blot. The cohort for staining comprised of 158 patients with PC, including 96 men and 62 women. Ages ranged from 35 to 85 (median: 62) years. Histological grade, peri-neural invasion (PNI), T and N stages were given by post-operational routine pathologic examinations. The Institutional Ethics Committee approval for the project was obtained.

Western blot

Proteins were extracted according to tissue protocols, and protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Meridian Rd, Rockford). Protein extracts were electrophoresed on polyacrylamide gels (SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) and blocking with 5% non-fat dry milk for 2 h. Membranes were incubated with primary antibodies (mouse anti-human HLA-G monoclonal and rabbit anti-human β-actin polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Secondary antibodies were incubated at 37°C for 1 h. Blots were washed with PBS for three times, exposed to chemiluminescence reagents (Merck, Darmstadt, Germany) and photographic films.

Tissue microarray (TMA) construction

Representative tumor and non-tumor areas, confirmed by re-identification, in formalin-fixed paraffin-embedded blocks of PC were marked. Then, two 1.5-mm cores of tumor and non-tumor tissues for each patient were punched out. The TMAs were constructed using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI).

Immunohistochemical staining and result evaluation

Two primary antibodies, i.e. mouse anti-human HLA-G monoclonal (Santa Cruz) and rabbit anti-human CD3 polyclonal (Dako, Carpinteria, CA) ones, as well as a two-step staining kit (EnVision™+kit, Dako) were used for staining. In brief, 4 μm-thick slides were first mounted, deparaffinized, rehydrated, and washed with phosphate buffered saline (PBS). Then, antigen retrieval was performed in an autoclave, using 0.01 M citrate buffer (pH 6.0), followed by incubation with 3% hydrogen peroxide to block endogenous peroxidase. Subsequently, slides were incubated overnight at 4°C with primary antibodies (HLA-G: 1:50; CD3: 1:100). After washing with PBS, horseradish peroxidase (HRP)-labeled secondary antibodies was added for reaction of 30 min. Diaminobenzidine was applied as a chromogen. Finally, slides were counterstained with hematoxylin. Non-immune mouse and rabbit sera at the same dilution served as the negative control.

Two pathologists who were independent to the clinicopathologic and follow-up data (Z.Y. L. and W.X. Z.) evaluated the slides respectively. A consensus was obtained by joint re-evaluation when they were divergent. The brown coloration in cells was defined as positive. According to the criteria reported in a previous paper [28], the ratio of HLA-G-positive cells was classified into three grades (Negative: <5%; local: 5-75%; diffuse: >75%), whereas its staining intensity was not considered. For quantification of tumor infiltrating lymphocytes (TIL), intratumoral and stromal CD3-positive cells were counted, respectively, in accordance with the definitions that were previously given [29]. Then, values were standardized to cells/mm^2, based on proportions of cancer cells in the cores and the area of cores. The median values served as the cut-off points.

Follow-up

A total of 143 patients (90.5%) underwent follow-up, ranging from 2.0 to 87.0 months (median, 13.0 months). One hundred and seven

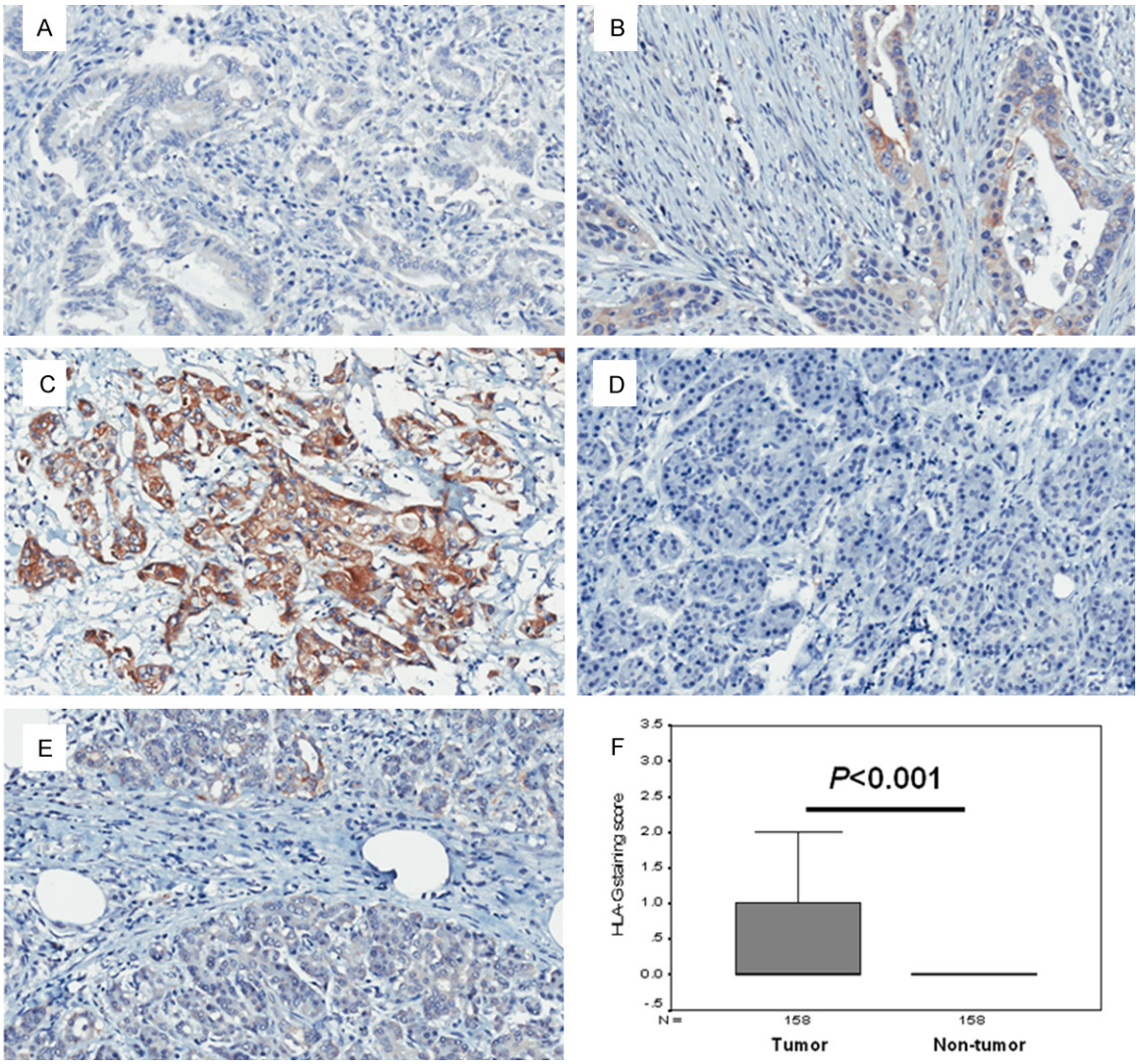

Figure 2. Expression of HLA-G in PC, detected by immunohistochemical staining. A. Negative expression in tumor tissue (original magnification ×200); B. Local expression in tumor tissue (original magnification ×200); C. Diffuse expression in tumor tissue (original magnification ×200); D. Negative expression in non-tumor tissue (original magnification ×200); E. Local expression in non-tumor tissue (original magnification ×200); F. Comparison of HLA-G staining ranks between tumor and non-tumor tissues (Mann-Whitney *U* test). HLA-G, human leukocyte antigen G.

patients have died, whereas 36 patients have lived 23.0 to 87.0 months, at the last follow-up.

Statistical analysis

The ranks of HLA-G staining in tumor/non-tumor tissues and intratumoral/stromal TIL numbers were compared by Mann-Whitney *U* test. Chi-square test was applied to show the relationship between HLA-G staining and clinicopathologic variables. Overall survival was analyzed using Kaplan-Meier method and log-rank test. Cox regression (Proportional hazard model) was adopted for multivariate analysis of prognostic factors. Statistical software package SPSS11.5 (SPSS Inc, Chicago, IL) was used for all the analyses. The statistical significance was defined when a *P* value less than 0.05.

Results

HLA-G expression in PC and its clinicopathologic significance

HLA-G overexpression in tumor tissues, in contrast to non-tumor ones, detected by Western blot, was found in 4 out of 10 patients (**Figure 1**). However, there was no up-regulatedly expressed HLA-G in non-tumor tissues. According to aforementioned criteria [28], negative, local and diffuse expressions in tumor

Table 1. Relationship between HLA-G expression and clinicopathologic features of PC

Variables	Number (n)	Tumoral HLA-G expression			*P**
		Negative	Local	Diffuse	
Gender					0.206
Male	96	59	20	17	
Female	62	37	19	6	
Age					0.279
≥65 years	54	32	11	11	
<65 years	104	64	28	12	
Tumor size					0.234
>4 cm	69	46	13	10	
≤4 cm	86	47	26	13	
Histological grade					0.225
G1-2	108	68	27	13	
G3-4	34	20	6	8	
PNI					0.977
Present	80	49	20	11	
Absent	70	44	17	9	
T stage					0.033
T1-2	90	51	29	10	
T3	66	43	10	13	
N stage					0.980
N0	85	51	22	12	
N1	65	40	16	9	

HLA-G, human leucocyte antigen G; PC, pancreatic cancer; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; G4, undifferentiated; PNI, peri-neural invasion; T, tumor; N, lymph node. *Chi-square test.

tissues were observed in 96, 39 and 23 patients, respectively (**Figure 2A-C**). In non-tumor ones, 135 and 23 patients were defined as negative and local expressions (**Figure 2D**, **2E**). The staining rank of HLA-G was significantly higher in tumor than in non-tumor tissues (P<0.001, Mann-Whitney U test, **Figure 2F**), indicating its upregulated expression in PC. Using Chi-square analysis, the positive association between tumoral HLA-G expression and T stage was found (P=0.033, **Table 1**), but no other clinicopathologic parameters were of implication (P>0.05, **Table 1**).

Impact of tumoral HLA-G expression on host immune response in PC

There were 156 patients whose TIL could be evaluated. Specimens with low and high intratumoral and stromal TIL numbers were shown in **Figure 3A-D**. It could be discovered that the intratumoral TIL number in tumors with diffuse HLA-G expression was significantly less than that in those with negative or local expressions (All P<0.05; **Figure 3E**), while no such differences were observed for stromal TIL numbers (P>0.05; **Figure 3F**).

Prognostic factors for overall survival of PC in this cohort

Univariate analysis showed that diffuse expression of HLA-G in tumor tissues and low intratumoral TIL number were significantly associated with gloomy overall survival (P<0.001; **Figure 4** and **Table 2**). Besides, gender, perineural invasion, T and N stages were also prognostic (All P<0.05; **Table 2**). Using multivariate Cox regression analysis, male gender, peri-neural invasion, N1 stage and diffuse HLA-G expression were identified as independent risk factors for prognosis of PC (All P<0.05; **Table 2**), whereas high intratumoral TIL number was proven to be an independent protective variable for patient survival (P<0.05; **Table 2**).

Discussion

HLA-G belongs to MHC class I and has been demonstrated to be pivotal in immune inhibition via several mechanisms, such as blocking effector cells and generating regulatory cells [9]. It was also suggested that HLA-G was associated with progressive phenoypes in a line of solid malignancies [13, 14, 16-18, 22, 24], indicating its role as a proto-oncogene. Thus far, expression pattern of HLA-G in PC remains unclear, because its expression in tumor and non-tumor tissues was not compared, although it was shown that HLA-G was positively expressed in PC tissues [28]. Moreover, its clinical and pathologic significances in PC has not been investigated. The present study first found, by Western blot, that HLA-G was overexpressed in many PC tissues, compared with non-tumor ones (**Figure 1**). In addition, no case with opposite trend was observed. Using the staining evaluation ctiteria same with the previous literature [28], it was further revealed that HLA-G expression was

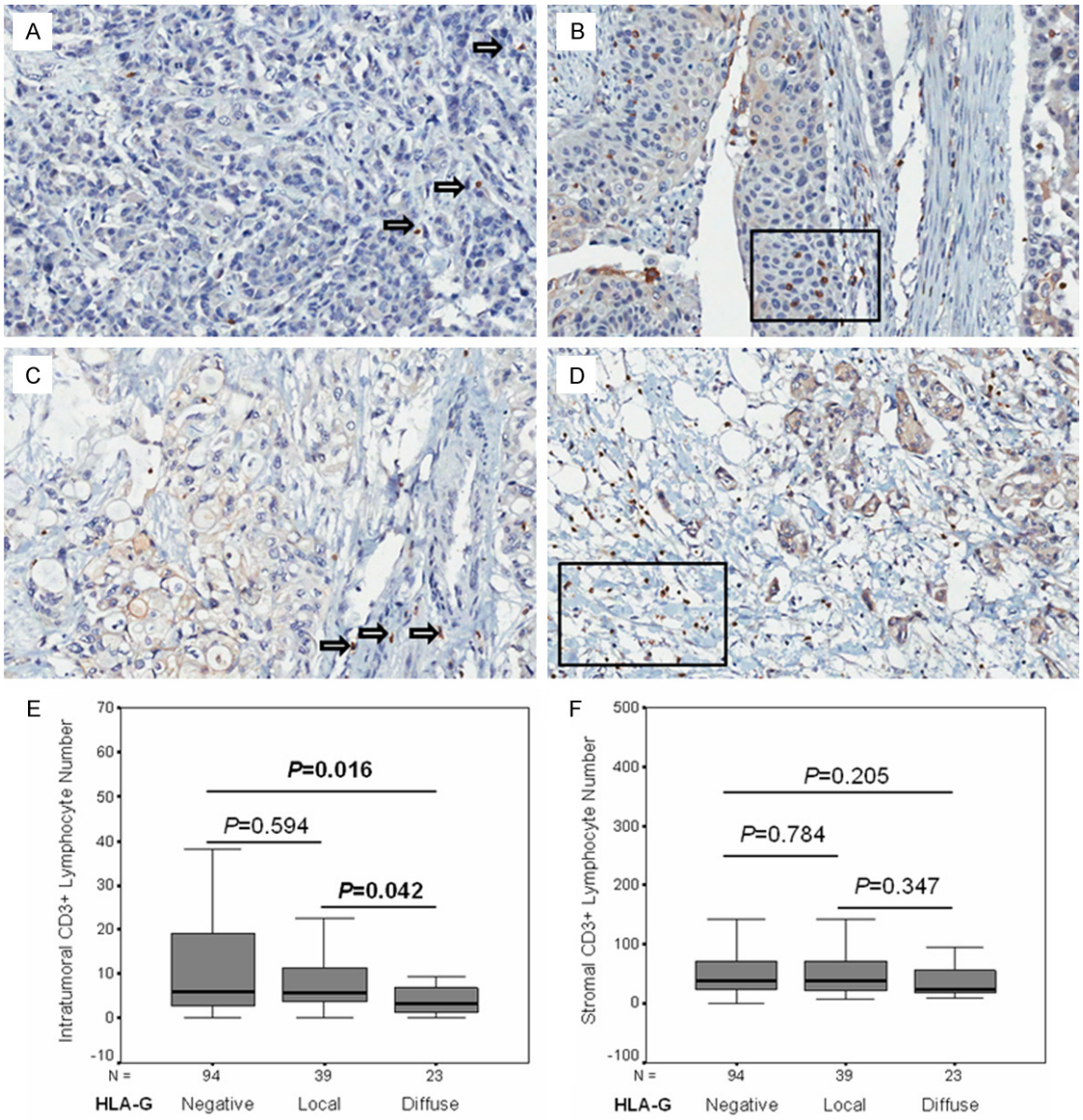

Figure 3. Influence of tumoral HLA-G expression on tumor infiltrating lymphocytes (TIL) in PC. A. Low intratumoral TIL number (original magnification ×200); B. High intratumoral TIL number (original magnification ×200); C. Low stromal TIL number (original magnification ×200); D. High stromal TIL number (original magnification ×200); E. Comparison of intratumoral TIL number between tumors with different HLA-G expression levels (Mann-Whitney U test); F. Comparison of stromal TIL number between tumors with different HLA-G expression levels (Mann-Whitney U test). Arrowheads and square frames emphasize concentrated TIL. HLA-G, human leukocyte antigen G.

much higher in tumor tissues than in non-tumor ones in PC (**Figure 2**). In addition, tumoral HLA-G expression was correlated with T stage (**Table 1**), a key factor of widely accepted TNM classification and a powerful prognostic marker in PC [30-34]. These findings indicated the upregulation of HLA-G in PC, and expanding the spectrum of cancers in that HLA-G has the potential to present oncogenic properties.

Previous studies showed the influence of HLA-G on TIL, a mark of host immune response, in several types of cancers [13, 14, 16, 17, 23], but no such data were presented in PC. In the current work, the authors attempted to answer the question according to the method that focused on both intratumoral and stromal TIL [29], using a antibody against CD3, a glycoprotein expressed in all human T lymphocytes [35].

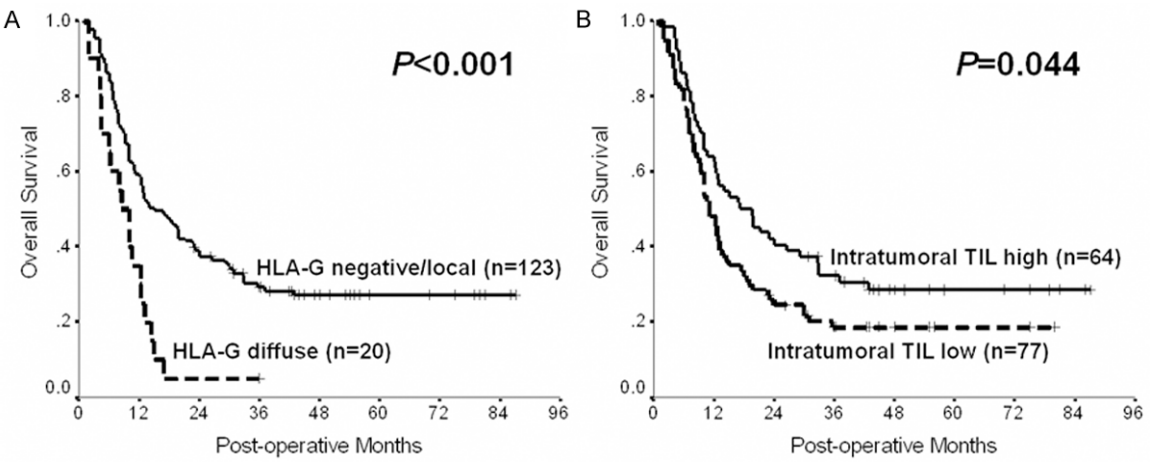

Figure 4. Influence of tumoral HLA-G expression and intratumoral tumor infiltrating lymphocytes (TIL) number on overall survival of PC. A. Tumoral HLA-G expression; B. Intratumoral TIL number. HLA-G, human leukocyte antigen G.

Table 2. Factors associated with overall survival of patients with PC

		OS (Univariate)			OS (Multivariate)		
Variables	Number (n)	Median ± SE	95% CI	P^*	RR	95% CI	$P^\#$
Gender				0.015			0.022
Male	92	12.5±1.3	10.0-15.0		1.694	1.080-2.655	
Female	51	18.9±5.8	7.5-30.3		1		
Age				0.634			
≥65 years	48	12.3±2.9	6.6-18.0				
<65 years	95	13.2±1.2	10.9-15.5				
Tumor size				0.902			
>4 cm	62	12.5±1.3	9.9-15.1				
≤4 cm	78	13.7±3.0	7.9-19.5				
Histological grade				0.054			
G1-2	95	15.0±2.8	9.4-20.6				
G3-4	33	10.0±0.8	8.4-11.6				
PNI				0.009			0.026
Present	73	11.2±1.1	9.1-13.3		1.623	1.058-2.488	
Absent	63	18.5±2.6	13.5-23.5		1		
T stage				0.009			0.072
T1-2	85	18.0±3.2	11.7-24.3		1		
T3	56	12.0±1.8	8.5-15.5		1.464	0.967-2.216	
N stage				0.004			0.017
N0	80	18.9±2.8	13.3-24.5		1		
N1	55	11.0±1.7	7.6-14.4		1.681	1.099-2.570	
Tumoral HLA-G				<0.001			0.011
Negative/Local	123	15.0±2.9	9.2-20.8		1		
Diffuse	20	8.5±1.5	5.6-11.4		2.135	1.187-3.839	
Intratumoral TIL				0.044			0.026
Low	77	11.0±1.2	8.6-11.4		1		
High	64	17.0±4.2	8.9-25.1		0.611	0.396-0.944	
Stromal TIL				0.648			
Low	69	12.5±1.2	10.2-14.8				
High	72	13.2±3.3	6.8-19.6				

PC, pancreatic cancer; OS, overall survival; SE, standard error; RR, relative risk; CI, confidence interval; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; G4, undifferentiated; PNI, peri-neural invasion; T, tumor; N, lymph node; HLA-G, human leucocyte antigen G; TIL, tumor infiltrating lymphocytes. *, Log-rank test; #, Multivariate Cox regression test.

It was revealed that intratumoral, rather than stromal, TIL was remarkably decreased in tumors with diffuse HLA-G expression, in contrast to those with negative or local ones (**Figure 3**). These results support the more extensive inhibition of HLA-G for intratumoral TIL in PC. Some investigators has found and summarized that HLA-G exerts its inhibitory functions through interactions with immune cells [9, 10-12]. Because intratumoral TIL and tumor cells are closer, it could be speculated that HLA-G suppresses this type of TIL more easily. Of course, the hypothesis needs to be validated, although detailed relative mechanisms might be of particular interest in the future.

It was well recognized that PC carried very dismal prognosis. Thus, its prognostic indicators have been the hotspot of research. Except for clinicopathologic ones [2-5], biomarkers for prognosis in PC have recently been identified and summarized [6, 7]. This study provided a novel candidate, i.e. HLA-G. Our univariate test found that diffuse HLA-G expression was associated with significantly shorter survival (**Figure 4A** and **Table 2**). More importantly, its prognostic value was confirmed in multivariate analysis (**Table 2**). These results, similar with those showing its correlations with unfavorable outcomes in other cancers [13-22], suggest that HLA-G expression is a valuable predictor of long-term prognosis, and verifying its tumor-promoting effects, in PC. On the other hand, low intratumoral, not stromal, TIL number was also of adverse prognostic relevance in multivariate test (**Figure 4B** and **Table 2**). The finding defines this immunological factor as an independent prognosticator of PC, on the basis of evidence derived from univariate analyses by us and in the aforementioned article [29]. In addition, the inverse impacts of these two variables on patient survival corroborate the inhibition of host immune response by HLA-G again.

In conclusion, our data show that HLA-G expression is upregulated and is associated with T stage in PC. Besides, diffuse HLA-G expression correlates with decreased intratumoral TIL number and worse overall survival of PC. Therefore, HLA-G impairs host immune response and predicts poor prognosis in PC.

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Disclosure of conflict of interest

None declared.

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