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Tumor-specific upregulation of human leukocyte antigen–G expression in bladder transitional cell carcinoma

Ling-Hong Gan [a], Li-Fu Huang [b], Xia Zhang [c], Aifen Lin [a,c], Dan-Ping Xu [a], Qing Wang [c], Tian-Ji Wang [b], Wei-Hua Yan [a,*]

[a] Medical Research Center, Taizhou Hospital of Zhejiang Province, Wenzhou Medical College, Linhai, Zhejiang, China
[b] Department of Urology, Taizhou Hospital of Zhejiang Province, Wenzhou Medical College, Linhai, Zhejiang, China
[c] Human Tissue Bank, Taizhou Hospital of Zhejiang Province, Wenzhou Medical College, Linhai, Zhejiang, China

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ABSTRACT

Human leukocyte antigen–G (HLA-G) is a potent immunosuppressive molecule that induces functional silencing of both innate and adaptive immune responses. The relevance of the aberrant HLA-G expression in malignant contexts has been intensively investigated. However, its expression status and clinical significance in bladder cancer remain to be elucidated. In the current study, HLA-G expression in 75 primary bladder transitional cell carcinoma (TCC) lesions was analyzed with immunohistochemistry, and relationship between HLA-G expression and clinical parameters, including disease stage was evaluated. Plasma soluble HLA-G levels were analyzed in 15 TCC patients and 109 normal controls. Data revealed that HLA-G was expressed in 68.0% (51/75) primary TCC lesions, whereas it was undetectable in adjacent normal bladder tissues. The proportion of HLA-G expression in TCC samples varied from negative to 100%, and no significant association was observed for the HLA-G expression status with the patient age, gender, and disease stage. Furthermore, no significance for sHLA-G levels was observed between the TCC patients and normal controls (median 10.75 vs 8.69 U/ml, $p = 0.578$). Given its immunotolerant properties, our finding suggested that lesion HLA-G expression upregulated in bladder TCC lesions might be an additional mechanism for tumor cells evading from host immunosurveillance; however, its clinical relevance needs further investigation.

1. Introduction

Abnormality in the expression of HLA class I antigens is one of the most common events during the procession of tumorigenesis [1]. Altered HLA phenotypes have been defined in human tumors, including HLA total loss, HLA haplotype loss, HLA-specific locus downregulation, HLA allelic losses, and a combination of these phenotypes [2]. Generally, structural and functional alterations of the HLA antigens in tumor cells serve to circumvent antigen-specific T-cell response but render the targets more susceptible to be eliminated by the natural killer (NK) cells [3]. In fact, despite HLA antigens loss, a nonclassical HLA class I antigen, human leukocyte antigen–G (HLA-G), is preferentially induced in tumor cells. It has now been well addressed that HLA-G expression is frequently detected in various malignancies. Accumulated evidence has suggested that the biologic role of HLA-G is in inactivating immune response, resulting in tumor cells continuing to grow and not being efficiently destroyed by immune defenders [4].

HLA-G was initially observed at the maternal–fetal interface on cytotrophoblast cells, and is believed to play a critical role in maintaining maternal–fetal immune tolerance during pregnancy [5,6]. Different from other HLA class I molecules, HLA-G is characterized by low polymorphism and restricted tissue distribution, and a particular expression patterns as seven proteins, including membrane-bound (HLAG1-G4) and soluble HLA-G (HLA-G5-G7) isoforms, were generated from alternative splicing [7]. In addition, another soluble HLA-G form could be generated by shedding of the proteolytically cleaved surface HLA-G1 (sHLA-G1) [8]. Plasma sHLA-G derives from the secretion of soluble isoforms, especially HLA-G5, as well as from the shedding form of HLA-G1 [9].

Apart from expression on trophoblast cells, in physiologic conditions, HLA-G is expressed in other tissues, such as adult thymic medulla, cornea, nail matrix, pancreatic islets, erythroid and endothelial precursors, and mesenchymal stem cells [10–15]. In the context of tumor biology, augmented HLA-G expression *in situ* was observed in more than 20 types of tumors. HLA-G was preferentially detected in tumor tissue and rarely in the adjacent normal tissue, suggesting its specific association with tumor growth and progression [4]. During the process of tumorigenesis, various microenvironmental factors, such as gene demethylation and histone acetylation, hypoxia, inflammatory stimuli (via nuclear factor–kB) and cytokines such as interleukin-10 and interferon-α or -γ could upregulate HLA-G transcriptional activation and protein expres-

* Corresponding author.
E-mail address: yanwhcom@yahoo.com (W.-H. Yan).

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sion [16–20]. However, the exact time point of HLA-G expression in tumor cells remains elusive.

Aberrant expression of HLA-G in cancer has been implicated both in establishment of antitumor immune responses and in tumor evasion [21]. Both the membrane-bound and sHLA-G isoforms share similar inhibitory functions by binding to the inhibitory receptors expression on immune competent cells, such as NK cells, both $CD4^+$ and $CD8^+$ T lymphocytes and macrophages [22]. Moreover, HLA-G–induced suppressor cells, such as regulatory T cells, dendritic cells, and NK cells, which could provide a long-term immune modulatory function [23–25]. Therefore, induction of HLA-G expression can arm the cancer cells with an additional powerful mechanism to overcome immune surveillance and attack.

To date, knowledge of the expression and clinical relevance of HLA-G in bladder transitional cell carcinoma (TCC) is limited. In this study, HLA-G expression in primary bladder cancer lesions was determined, and its correlation with clinical parameters was evaluated.

2. Subjects and methods

2.1. Study population

A total of 75 consecutive samples from patients with histologically confirmed TCC of the bladder between August 2004 and April 2008 were obtained at Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical College. Tumor diagnosis was based on World Health Organization (WHO) grading and tumor–node classification systems [26]. Patient data, including patient age, gender, date of initial diagnosis, and disease stage were collected. All tissue specimens underwent microscopic confirmation for pathologic features before inclusion in the study. For the plasma sHLA-G levels evaluation, 15 plasmas from bladder cancer patients and 109 age-matched, unrelated healthy blood donors were detected. This study was performed after an Institutional Ethics Review Board approval of a protocol to investigate molecular markers relevant to bladder cancer pathogenesis.

2.2. Immunohistochemistry

Four-micrometer-thick sections of the paraffin-embedded tissue blocks were cut and mounted on polylysine coated slides. The sections were dewaxed in xylene and rehydrated through a graded series of ethanol. After de-paraffinization, antigen retrieval treatment was performed at 120°C for 5 minutes in 10 mmol/l sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by using a 3% hydrogen peroxide solution at room temperature for 15 minutes. Anti–HLA-G mAb 4H84 (1:500, Exbio, Prague, Czech Republic) was then applied and the substance was incubated overnight at 4°C. After that, a thorough washing in a 0.01 mol/l phosphate-buffered saline (PBS) solution was performed. Subsequently, binding sites of the primary antibody were visualized using a Dako EnVison kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Finally, sections were counterstained with hematoxylin and mounted with glycerol gelatin. HLA-G staining in TCC samples was determined by 3 pathologists who were blinded to any clinical details related to the patients. Membrane or combined membrane and cytoplasmic expression of HLA-G were interpreted as positive. Cytotrophoblast from first-trimester human placenta served as an HLA-G–positive control and negative controls were achieved by an isotype matched IgG1 (1:500, Exbio, Prague, Czech Republic) in each of the immunostaining. HLA-G expression in partial samples ($n = 14$) was tested by Western blot using the mAb 4H84 in case-matched fresh bladder cancer lesions.

2.3. Staining evaluation

HLA-G staining in TCC samples was determined by 3 pathologists who were blinded to any clinical details related to the patients. Membrane or/and cytoplasmic expression of HLA-G were interpreted as positive. Percentage of HLA-G positive tumor cells was determined by each observer, and the average of 3 scores was calculated. At least 500 tumor cells were scored. Percentage of HLA-G expression was graded as negative for the absence of HLA-G staining, and the positive HLA-G staining was grouped as 1–25% (low percentage), 26–50% (intermediate percentage), 51–75% (high percentage), and >75% (very high percentage), respectively. The percentage of positive cells was assigned a value based on the presence or absence of HLA-G staining, irrespective of staining intensity.

2.4. Tissue protein extraction and Western blot analysis

For preparation of protein extracts, 14 case-matched fresh primary bladder cancer lesions were crushed with a mortar and pestle under liquid nitrogen. Harvested cells were washed 3 times with cold PBS. Cell pellets were collected and lysed with lysis buffer (pH 7.4, 50 mmol/l Tris-base, 150 mmol/l NaCl, 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, PMSF 1 mmol/l) with the final concentration of 1×10^7 cells/ml. After centrifugation at 15,000 *g* at 4°C for 30 minutes, cell lysate aliquots were separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. All samples were heated for 5 minutes at 100°C before loading. Proteins were then electroblotted onto PVDF membranes (Millipore, Bedford, MA) and blocked by incubation with PBS containing 5% nonfat dry milk for at least 4 hours. After blocking, membranes were washed in PBS containing 0.2% Tween-20 three times and then probed with the HLA-G–specific mAb 4H84 (1:1000, Exbio, Prague, Czech Republic) overnight at 4°C and washed with 0.2% Tween-PBS three times. The membranes were subsequently incubated for 30 minutes at room temperature with Peroxidase/DAB+Rabbit/Mouse (Dako, Glostrup, Denmark), washed thoroughly with 0.1% Tween–PBS. Finally, membranes were developed with Dako REAL EnVision Detection System (DAKO, Glostrup, Denmark) for 1–3 minutes. Samples from HLA-G isoform transfectants JAR-G1~G7 and JAR cells (ATCC, Rockville, MD) were used as positive and negative controls, respectively.

2.5. sHLA-G enzymed-linked immunosorbent assay

Plasma sHLA-G levels from 15 bladder cancer patients and 109 age-matched normal healthy individuals were determined with the sHLA-G specific immunosorbent assay (ELISA) kit (sHLA-G kit; Exbio, Prague, Czech Republic), which measures sHLA-G1 and HLA-G5. Each sample was measured in triplicate. The optical densities were measured at 450 nm (Spectra Max 250, Molecular Devices, Sunnyvale, CA). The final concentration was determined by optical density according to the standard curves. The detection limit of the kit was 1 U/ml. Details of the performance was according to the manufacturer's instructions.

2.6. Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL). Correlations between the degree of staining and clinical parameters were calculated with the Pearson χ^2 test. Difference of sHLA-G between groups was analyzed with the Mann–Whitney *U* test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Characteristics of patients with TCC

A total of 75 patients with bladder TCC (median age 65.0 years, range 43.0–83.0 years; 66 men and 9 women) were recruited.

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There were 15 patients with Ta, eight patients with Tis, 14 patients with T1, seven patients with T2a, 11 patients with T2b, three patients with T3a, seven patients with T3b, one patient with T3N2, and nine patients with T4. These patients were grouped as according to whether they had superficial bladder cancer (Tis+Ta+T1), deep bladder cancer (T2a+T2b+T3a+T3b), or metastatic bladder tumor (T3N2+T4) (Table 1).

3.2. HLA-G expression in TCC lesions

Heterogeneous staining was noted in TCC samples. In the TCC sections, the intensity of HLA-G staining varied from tumor to tumor and from one area to another within the same tumor. Some tumors showed focal patchy positive staining, and others displayed uniform staining pattern in tumor nests. Positive staining was observed in both the cell membrane and the sub-membranous cytoplasm region. The cytotrophoblasts were used as internal positive control for HLA-G expression. No staining was detected in the corresponding adjacent bladder tissue (Fig. 1). Overall, 68.0% (51/75) tumor lesions were determined as HLA-G positive. Statistic analysis revealed that HLA-G expression status was not associated with the patient clinical parameters such as patient age, gender, and TNM stage (Table 1).

HLA-G expression in 14 case-matched fresh bladder TCC lesions was performed with Western blot to test the types of HLA-G isoform expressed. The cell lysates from the transfectants JAR-HLA-G1–G7 was used as positive controls (Fig. 2A). Data showed that only the HLA-G1 was expressed in these bladder TCC samples. Of note, two HLA-G–positive samples (4.0% and 10.0%) were undetectable by Western blot assay, but the Western blot results were highly consistent with those of immunohistochemistry (Pearson correlation $r = 0.645$, $p = 0.013$; Fig. 2B).

3.3. Plasma sHLA-G expression in bladder cancer patients

Median plasma sHLA-G levels were 10.75 U/ml (range 0–59.53) for bladder cancer patients and 8.69 U/ml (range 3.98~70.72) for normal controls. No significance was observed for the sHLA-G levels between bladder cancer patients and normal controls ($p = 0.578$) (Fig. 3).

4. Discussion

Since Paul et al. [27] reported for the first time that HLA-G was expressed in melanoma lesions in 1998, upregulation of HLA-G expression *in situ* or elevated sHLA-G levels was observed in more than 20 types of malignances, such as ovarian cancer, retinoblastoma, hepatocellular carcinoma, breast carcinoma, leukemia, renal cell carcinoma, and lung cancer [28–34]. Recently susceptibility for certain kinds of malignancies such bladder transitional cell carcinoma with HLA-G polymorphism was examined [35]. In that study, the G*0104 group was associated with progression to high-grade tumors irrespective of smoking habit, whereas the G*0103 allele conferred high-grade tumor susceptibility only to smoking patients once the tumor was established. HLA-G polymorphisms were also observed to be associated with cervical squamous intraepithelial lesions [36]. These studies provide new evidence that the HLA-G locus, combined with environmental variables, may be associated with susceptibility to tumor development and progression.

HLA-G expression in tumor cells could dramatically inhibit natural killer (NK) cell cytotoxicity in an HLA-G–dependent manner, indicating that HLA-G could compromise the host antitumor immune response, consequently favoring tumor cell evasion of immune surveillance [28,32,34,37]. Furthermore, HLA-G–involved suppressor cells, such as HLA-G–induced regulatory T cells, dendritic cells (DCs), and the HLA-G–bearing antigen presenting cells (APC), mesenchymal stem cells (MSCs), T and NK cells, or even the HLA-G–positive tumor cells, have long-term immune modulatory function and can induce the generation of suppressive/regulatory cells and block the immune effectors [25]. HLA-G–induced regulatory T cells were first observed *in vitro* after allogeneic stimulation by HLA-G+ APC. In that study, LeMaoult et al. [38] reported that HLA-G1+ APCs could cause the differentiation of $CD4^+$ T cells into suppressive cells and spread antigen-specific inhibition. Selmani et al. documented that the soluble HLA-G protein HLA-G5 secreted from human mesenchymal stem cells could also induce $CD4^+$ CD25highFOXP3+ Treg cells expansion *in intro* [15]. Thus HLA-G expression presented in malignancies and its potential role in the induction of Treg may contribute to the escape of tumor cells from immune surveillance.

The clinical relevance of HLA-G in malignancies was evaluated in many studies. HLA-G expression in primary non–small-cell lung cancer was significantly associated with the poor prognosis and shorter survival time [39]. Our recent study on non–small-cell lung cancer indicated that the HLA-G expression status was significantly associated with tumor stage, which is preferentially observed in patients with more advanced tumor stage [34]. In hepatocellular carcinoma, HLA-G expression was found to be strongly correlated with advanced disease stage and to be more frequently observed in older patients [30]. This finding was further strengthened in a study by Cai et al. [40], which found that high level of HLA-G expression in hepatocellular carcinoma, especially in early-stage disease, was independently associated with shortened overall survival and increased tumor recurrence. However the clinical significance of HLA-G expression in B-cell chronic lymphocytic leukemia (B-CLL) remains under debate. Nückel et al. [41] documented that B-CLL patients with 23% or fewer HLA-G–positive cells had a significantly longer progression-free survival time than patients with more than 23% positive cells, and HLA-G was hypothesized to be a better

Table 1
Association of human leukocyte antigen–G (HLA-G) expression in primary bladder cancer with clinicopathological parameters

Variables	No. of cases	HLA-G expression					*p* Value*
		Negative (%)	1–25%	26–50%	51–75%	>75%	
Age							
≤Median (65 y)	43	15 (34.9%)	12	8	5	3	0.514
>Median	32	9 (28.1%)	6	10	6	1	
Gender							
Male	66	21 (31.8%)	16	17	8	4	0.448
Female	9	3 (33.3%)	2	1	3	0	
TNM stage							
Tis+Ta+T1[a]	37	12 (32.4%)	12	8	3	2	0.110**
T2a+T2b+T3a+T3b[b]	28	8 (28.6%)	4	9	7	0	
T3N2+T4[c]	10	4 (40.0%)	2	1	1	2	

*Comparison of HLA-G expression status between or among each variable using the Pearson χ^2 test.
**Overall *p* value among TNM stage group a, b, and c is 0.110; *p* values for comparison between a and b, between a and c, and between b and c are 0.118, 0.541, and 0.084, respectively.

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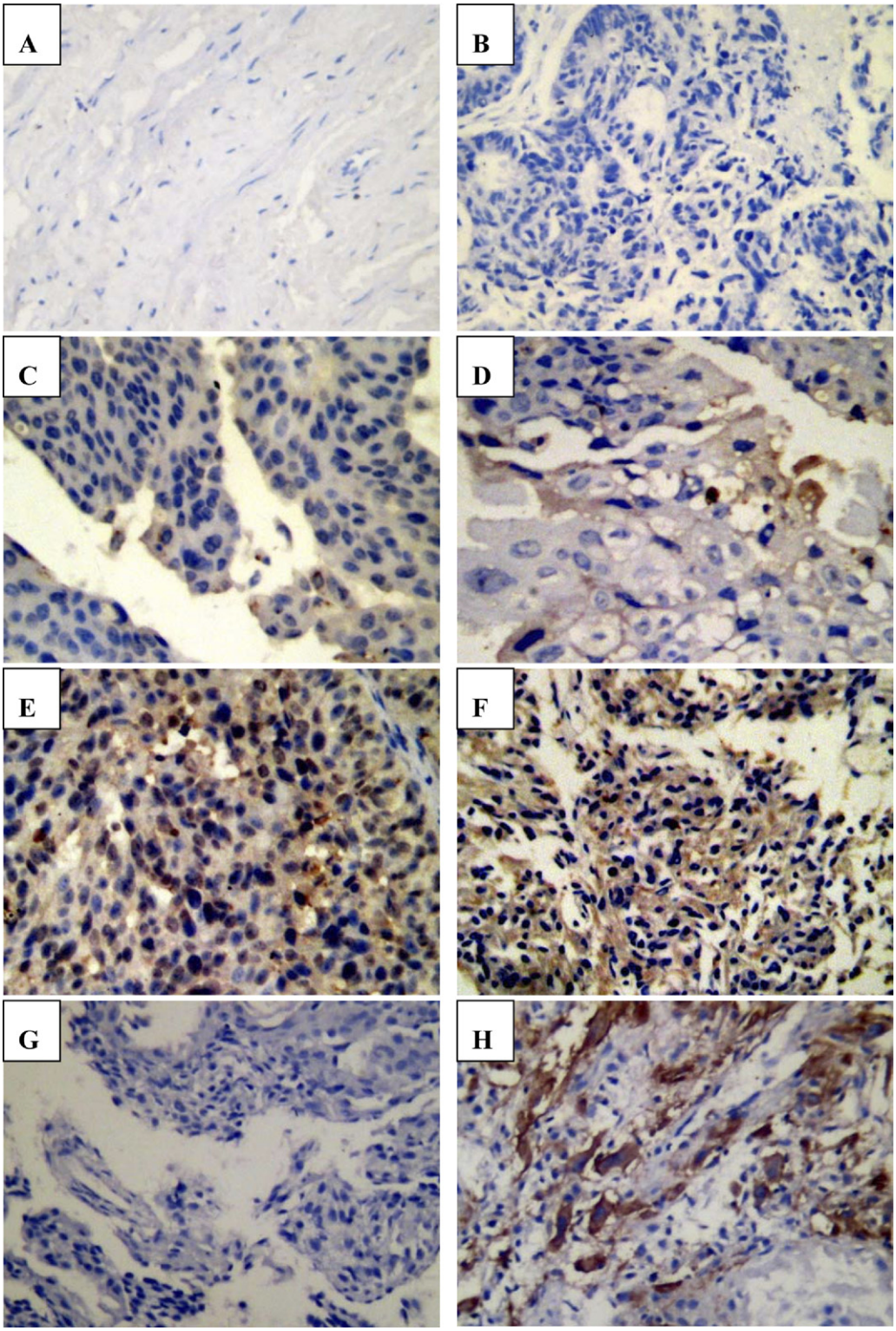

Fig. 1. Immunohistochemistry analysis of HLA-G expression in normal bladder tissues and TCC lesions. (A) Negative HLA-G staining in normal bladder tissues. (B) Negative HLA-G staining in bladder TCC lesions. (C, D, E, and F) Representative positive HLA-G staining in bladder TCC lesions, with 1–25% (low percentage), 26–50% (intermediate percentage), 51–75% (high percentage), and >75% (very high percentage), respectively. HLA-G immunoreactivity is localized both in the cell membrane and in cytoplasma of transitional cell carcinoma (TCC) tumor cells. Cytotrophoblast tissues were used as internal controls (G, with an IgG1 isotype antibody, HLA-G–negative control) and (H, with mAb 4H84, HLA-G–positive control), respectively. HLA-G mAb 4H84 (1:500, Exbio, Prague, Czech Republic) was used to detect the HLA-G expression. Original magnification ×100.

prognostic factor than ZAP-70 or CD38 status. The prognostic value of HLA-G in B-CLL was supported by Erikci et al. [42], who observed a statistically significant correlation between HLA-G positive (>12%) expression and progression free survival ($p < 0.045$, $n = 20$). It is noteworthy that the prognostic irrelevance of HLA-G in B-CLL was examined in a larger cohort ($n = 169$) study that did not find HLA-G expression to be correlated with any clinical or biologic features, including survival [43].

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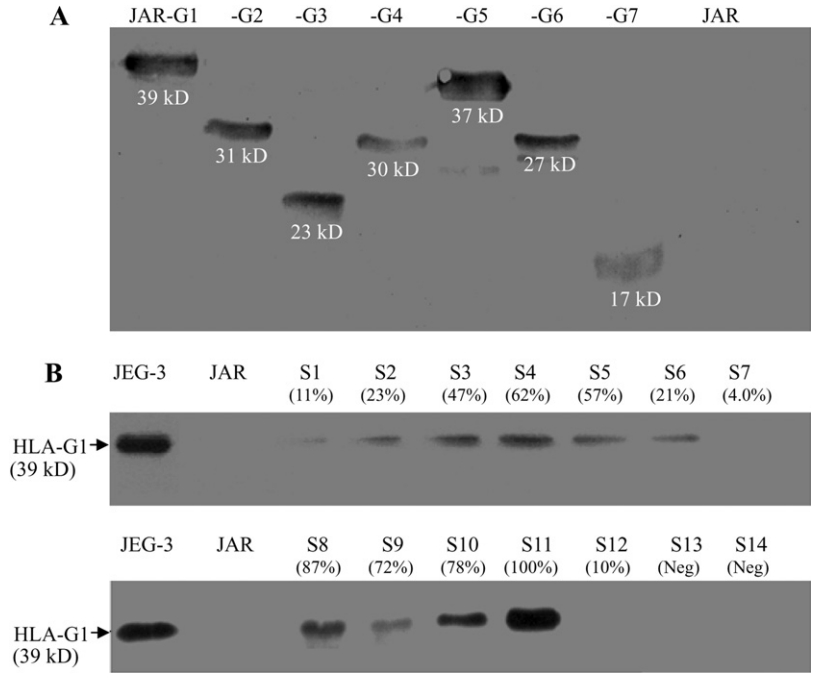

Fig. 2. Western blot analysis of HLA-G expression in bladder TCC lesions. (A) HLA-G isoform transfectants JAR-G1–G7 and JAR lysates were used as HLA-G–positive and –negative controls, respectively. Expected molecular weigh of each HLA-G isoform is indicated under corresponding band. (B) Degree of HLA-G expression is shown in brackets according to case-matched immunohistochemistry data. Analysis was performed with HLA-G mAb 4H84 (1:1000, Exbio, Prague, Czech Republic).

To the best of our knowledge, few studies have focused on the HLA-G expression in bladder TCC. EI-Chennawi et al. [44] reported that 16.7% bladder cancer sections (7/42; TCC, $n = 23$; squamous cell carcinoma, $n = 19$) expressed HLA-G as detected by immunohistochemistry. No significant association was observed for HLA-G expression between TCC (5/23, 21.7%) and squamous cell carcinoma (2/19, 10.5%) sections. Furthermore HLA-G expression in these bladder cancers was not associated with patient age, gender, or tumor grade. Although no clinical relevance of HLA-G expression was found, the percentage of HLA-G positive TCC samples in our study was dramatically different. In our study, 68.0% of the TCC samples were positive for HLA-G despite the fact that the proportion of HLA-G–positive transitional cell carcinoma (TCC) cells varied from negative to 100%. Methodology differences may explain the discrepant findings between the two studies. In the EI-Chennawi et al. report, details of the immunohistochemistry protocol and the staining evaluation procedure were not provided. The antibodies applied for probing HLA-G expression in that study were not clearly described by EI-Chennawi et al. and the positive and negative controls to guarantee the HLA-G detection was not included. Unlike other malignancies, such as lung cancer and hepatocellular carcinoma, HLA-G expression in bladder TCC is unrelated to disease stage [30,34,39,40]; furthermore, no significant difference in sHLA-G levels was observed between the TCC bladder cancer patients and normal controls in our study, whereas elevations of sHLA-G expression was found in patients with various other cancers [34,45–47], raising the hypothesis that the clinical relevance of HLA-G is tumor type dependent and varies among different types of malignancies.

Together, our finding show that HLA-G expression is induced in a high percentage of bladder TCC (68.0% in our study), and that HLA-G expression in TCC is not associated with clinical stage. Given the immunotolerant properties of HLA-G, our finding suggest that expression induced in TCC cells might be an additional mechanism for tumor cells evading host immunosurveillance. However, the clinical relevance of HLA-G in TCC needs further investigation.

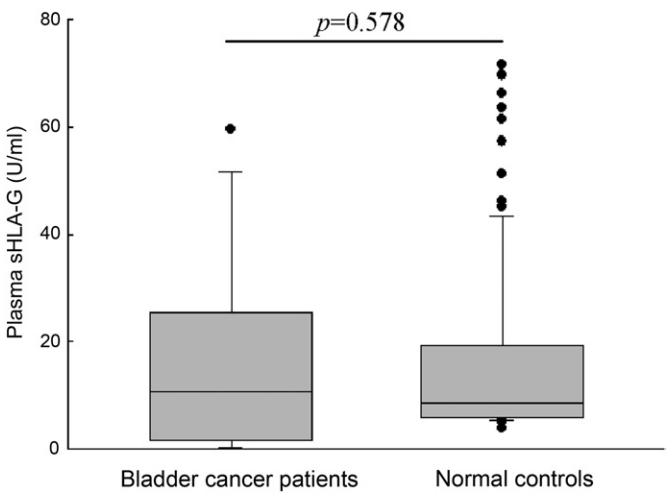

Fig. 3. Comparison of plasma sHLA-G levels between bladder TCC patients ($n = 15$) and normal controls ($n = 109$). Bar in box indicates the median.

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