Upregulation of human leukocyte antigen–G expression and its clinical significance in ductal breast cancer

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1. Introduction

During tumor development, immune cells infiltrate to tumors and make up a significant component of the multicellular cancer microenvironment; yet the immune system often fails to prevent tumor formation and progression [1]. A variety of strategies have been developed by tumor cells to avoid recognition by different kinds of immune effectors. Alteration of human leukocyte antigen (HLA) expression, structure, and function represents a frequent event in cancer and serves to circumvent T-cell and natural killer (NK) cell responses [2]. Other potent mechanisms, such as the presence of tolerance-promoting regulatory T cells (Tregs), would also benefit the tumor cells escaping from antitumor immune responses [3].

Aberrant upregulated expression of the nonclassical HLA class I antigen human leukocyte antigen–G (HLA-G) was observed in many types of cancers [4]. However, HLA-G expression in normal tissue is limited to fetal trophoblast cells, adult thymic medulla, cornea, nail matrix, pancreatic islets, erythroid and endothelial precursors, and in mesenchymal stem cells [5–11]. An increasing number of studies have highlighted the clinical relevance of HLA-G expression in certain pathologic conditions, such as those relating to oncology, autoimmunity, inflammation, infection, and transplantation [12]. In the context of malignancies, since Paul et al. [13] described for the first time the expression of HLA-G in melanoma, HLA-G expression in situ was observed nearly in 30 types of tumors. HLA-G was preferentially detected in the tumor tissue and rarely in the adjacent normal tissue, suggesting that it might play a role in tumor growth and progression, and could be a potential diagnosis marker and immunotherapy target for cancers [14–16].

Unlike classical HLA class I antigens, four membrane-bound (HLA-G1–G4) and three soluble HLA-G isoforms (HLA-G5–G7) are generated by alternative splicing of its primary transcript [17,18]. Another soluble form of HLA-G molecule, however, could be generated by shedding of the proteolytically cleaved surface HLA-G (sHLA-G). Therefore, plasma sHLA-G could derive from the secretion of soluble isoforms, especially HLA-G5, as well as from the shedding form of HLA-G1 [19]. Previous studies indicated that both membrane-bound and soluble HLA-G proteins could play profound role in the tumor microenvironment.
suppressing roles in every phase of cancer immunoeediting by suppressing the functions of various immune competent cells, such as NK cell, both CD4+ and CD8+ T lymphocyte and dendritic cells [20]. In addition, increasing evidence showed that HLA-G could induce the generation of suppressor/regulatory cells [21]. HLA-G induced regulatory T cells were first observed in vitro after allogeneic stimulation by HLA-G+APC. In that study, Lemautel et al. reported that HLA-G1+APCs could cause the differentiation of CD4+ T cells into suppressive cells and spread antigen-specific inhibition [22]. Selmani et al. reported that the soluble HLA-G protein HLA-G5 secreted from human mesenchymal stem cells could also induce CD4+CD25hiFOXP3+ Treg cells expansion in vitro [11]. The fact that Tregs can suppress both adaptive and innate antitumor immune responses, and increased CD4+CD25hiFoxp3+ Tregs have been found in the circulation or tumors of patients of various types of cancers, which was correlated with worse clinical outcomes [23]. However, the clinical significance of HLA-G and Treg need further investigation.

In our study, both lesion HLA-G expression and plasma sHLA-G levels from patients with breast cancer and their correlation with clinical parameters were evaluated. Furthermore, the correlation between HLA-G and Treg, and possible diagnosis significance of sHLA-G in breast cancer patients was also determined.

2. Subjects and methods

2.1. Tissue samples

Primary ductal breast cancer lesions and their adjacent nontumorous tissues were obtained from 58 consecutive patients between 12 November 2007, and 24 January 2009 at Taizhou Hospital of Zhejiang Province. None of the patients received preoperative anticancer treatment. Tumor staging was determined according to the sixth edition of the tumor–node–metastasis (TNM) classification by the International Union Against Cancer [24]. Patient data, including age, gender, date of initial diagnosis, histologic diagnosis, tumor grade, and clinical stage were documented. All tissue specimens underwent a microscopic confirmation for pathologic features before inclusion in the study. This study was performed after an Institutional Ethics Review Board approved the protocol and informed consent was obtained from all patients.

2.2. Immunohistochemistry

Sections (4 μm thick) of the paraffin-embedded tissue blocks were cut and mounted on polylysine coated slides. They were dewaxed in xylene and rehydrated through a graded series of ethanol. After deparaffinization, antigen retrieval treatment was performed at 120°C for 5 minutes in a 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 (1:300, Exbio, Prague, Czech Republic) was applied and incubated overnight at 4°C. After a thorough wash with 0.01 mol/l phosphate-buffered saline (PBS) solution, the binding sites of the primary antibody were visualized using a Dako EnVision kit (Dako, Glostrup, Denmark). Cytophoblast from first-trimester human placenta served as an HLA-G–positive control. Negative controls were achieved by including isotype matched IgG in each of the immunostainings.

2.3. Evaluation of staining

HLA-G staining in breast cancer tissues was determined by three pathologists. The pathologists were blinded to any clinical details related to the patients. Membrane and/or cytoplasmic expression of HLA-G was interpreted as positive. Percentage of the HLA-G positive tumor cells was determined by each observer, and the average of three scores was calculated. HLA-G expression was graded as follows: negative, 1 for 1–25%; 2 for 26–50%; 3 for 51–75%; and 4 for >75%. 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. sHLA-G enzymed-linked immunosorbent assay

Plasma sHLA-G levels from 92 breast cancer patients and 70 age-matched normal female healthy individuals were determined with the sHLA-G specific 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 procedure were as instructed by the manufacturer.

2.5. Flow cytometry analysis

Intracellular Foxp3 expression was detected with the eBioscience Foxp3 staining kit within 4 hours after the peripheral blood was taken. Briefly, peripheral blood mononuclear cells were first surface labeled with phycoerythrin–CD4 (BD Biosciences, San Jose, CA) and fluorescein isothiocyanate–CD25 (BD Biosciences, San Jose,

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of cases</th>
<th>HLA-G expression</th>
<th>p*</th>
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<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
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<tr>
<td>≤Median (49 y)</td>
<td>29</td>
<td>11 (37.9%)</td>
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</tr>
<tr>
<td>&gt;Median</td>
<td>29</td>
<td>6 (20.7%)</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td>12 (34.3%)</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>23</td>
<td>5 (21.7%)</td>
<td></td>
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<tr>
<td><strong>Progestosterone receptor status</strong></td>
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</tr>
<tr>
<td>Positive</td>
<td>13</td>
<td>2 (15.4%)</td>
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</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>4 (22.2%)</td>
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</tr>
<tr>
<td><strong>Estrogen receptor status</strong></td>
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</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>2 (14.3%)</td>
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<tr>
<td>Negative</td>
<td>18</td>
<td>4 (22.2%)</td>
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<tr>
<td><strong>Grade</strong></td>
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<td></td>
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<tr>
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<tr>
<td>III/IV</td>
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<td>9 (20.0%)</td>
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<tr>
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<td>15 (35.7%)</td>
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<tr>
<td>III/IV</td>
<td>16</td>
<td>2 (12.5%)</td>
<td></td>
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</tbody>
</table>

*p* Comparison of HLA-G expression status between or among variables using Pearson χ² test.
CA). Intracellular analysis of FoxP3 was then performed after fixation and permeabilization according to the manufacturer’s recommendation (eBioscience, San Diego, CA). Flow cytometry was performed on a FACSCalibur flow cytometer and analyzed using CellQuest (BD Biosciences, San Jose, CA) software.

2.6. Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL). Correlations between the HLA-G expression and clinical parameters were calculated with Pearson $\chi^2$ test. Differences in sHLA-G between groups was analyzed with the Mann–Whitney U test, and the frequency of Treg between groups was analyzed with two-sided Student’s $t$ test. The feasibility of using sHLA-G as a potential biomarker for differentiating breast cancer patients from normal controls was assessed using receiver operating characteristic (ROC) curve analysis. Linear regression was used to access the correlation between plasma sHLA-G level and Treg frequency. Values of $p < 0.05$ were considered significant.

3. Results

3.1. HLA-G expression in breast cancer lesions

Overall, 70.7% (41/58) of primary ductal breast cancer lesions were classified as HLA-G positive (Table 1). Heterogeneous HLA-G staining was observed in breast cancer lesions. The intensity of staining varied from tumor to tumor and/or from one area to another within the same tumor. Positive staining was observed in both cell membrane and cytoplasm region. Cytotrophoblasts were used as internal positive control for HLA-G expression. No staining was detected in adjacent normal breast tissues (Fig. 1).

3.2. HLA-G expression in breast cancer lesions relative to clinicopathologic parameters

HLA-G expression was observed in 38.46% (5/13) of tumors histologic grade I/II and in 80.0% (36/45) of tumors grade III/IV, respectively. HLA-G expression in primary ductal breast cancer

![Image](https://example.com/image1.png)

**Fig. 1.** Immunohistochemistry analysis of HLA-G expression in normal breast tissues and breast cancer lesions. (A) Negative HLA-G staining in normal breast tissues. (B) Negative HLA-G staining in breast cancer lesions. (C, D) Positive HLA-G staining in breast cancer lesions. HLA-G immunoreactivity is localized both in the cell membrane and in cytoplasm of breast cancer tumor cells (inset, ×400). Cytotrophoblast tissues were used as internal controls (E, with an IgG1 isotype antibody, HLA-G negative control) and (F, with mAb 4H84, HLA-G–positive control), respectively. HLA-G mAb 4H84 (1:300) was used to detect the HLA-G expression. Original magnification ×100.
lesions was significantly associated with tumor grade, with an overall p value of 0.021. Furthermore, HLA-G expression was observed in 64.29% (27/42) of the patients with TNM I/II, and 87.50% (14/16) of the patients with TNM III/IV, respectively. HLA-G expression in primary breast cancer lesions was also associated with disease stage, with an overall p value of 0.044. However, the percentage of lesion HLA-G expression was not significantly associated with clinical parameters, such as patient age, tumor location, or progesterone receptor or estrogen receptor expression status (Table 1).

3.3. Plasma sHLA-G expression in breast cancer patients

Concentration of the plasma sHLA-G was with the median of 82.19 U/ml (range 13.50–191.37) for breast cancer patients, and 9.65 U/ml (range 4.38–69.89) for normal controls, respectively. HLA-G levels in breast cancer patients were significantly higher than in normal controls (p < 0.001) (Fig. 2(A)). Plasma sHLA-G levels were 83.3 U/ml (range 15.13–182.92 U/ml), 90.77 U/ml (range 13.5–191.37 U/ml), and 74.02 U/ml (range 21.06–97.83 U/ml) in patients with TNM I, II, and III, respectively (Fig. 2B). With respect to tumor grade, plasma sHLA-G levels were 124.83 U/ml (range 43.61–180.28 U/ml), 104.49 U/ml (range 22.31–177.84 U/ml), and 101.01 U/ml (range 21.16–191.37 U/ml) in patients with tumor grade I, II, and III, respectively (Fig. 2C). Unlike lesion HLA-G expression, sHLA-G levels were not associated with the tumor grade and disease TNM stage (all p>0.05).

ROC curve analysis was used to determine the feasibility of sHLA-G as a diagnosis marker for breast cancer. The area under the ROC curve for plasma sHLA-G from breast cancer patients versus normal controls was 0.953 (95% CI = 0.926–0.981, p < 0.001) (Fig. 3). Given 100% specificity, the highest sensitivity achieved for sHLA-G to detect the patients with breast cancer was 65.9% at a cut-off of 68.82 U/ml.

Fig. 2. Plasma sHLA-G levels in normal controls and breast cancer patients, and their correlation with clinical stage and tumor grade. (A) Comparison of plasma sHLA-G levels between breast cancer patients (n = 92) and normal controls (n = 70). (B) Comparison of patient plasma sHLA-G levels between different clinical disease stages (nI = 36, nII = 44, nIII = 11). (C) Comparison of patient plasma sHLA-G levels between different tumor grades (nI = 4, nII = 33, nIII = 21). Bar in box indicates median.

Fig. 3. Receiver-operating characteristic (ROC) curve analysis to assess the performance of plasma sHLA-G levels for diagnosis between breast cancer patients (n = 92) and normal controls (n = 70). The area under the ROC curve is 0.953 (95% CI = 0.926–0.981, p < 0.001). When specificity was set to 100%, the highest sensitivity achieved for sHLA-G to distinguish the patients with breast cancer from normal controls was 65.9% at a cut-off of 68.82 U/ml.
3.4. Frequency of Treg and its correlation to sHLA-G in breast cancer patients

We then identified the subpopulation of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells as regulatory T cells (Treg) with flow cytometry (Fig. 4A). The frequency of Treg was significantly higher in breast cancer patients (n = 65) when compared with that in normal controls (n = 23) (4.46 ± 1.36% vs 2.67 ± 1.45%, p < 0.001). Moreover, correlation between proportion of Treg and plasma sHLA-G levels in 30 case-matched samples was analyzed. The proportion of Treg was strongly correlated with plasma sHLA-G levels (R = 0.582, p = 0.001, n = 30) (Fig. 4B and Fig. 5). However, no significant association was observed between the frequency of Treg, tumor grade, or clinical stage (data not shown).

4. Discussion

The potential clinical relevance of HLA-G expression in malignancies was previously postulated in various studies. Functionally, both membrane-bound and sHLA-G isoforms share similar inhibitory functions via binding to their specific receptors [20]. Furthermore, HLA-G–dependent suppressor cells, such as HLA-G–induced regulatory CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells, DCs, and NK cells, or even the HLA-G–positive tumor cells, have a long-term immune modulatory function to block immune effectors [21]. Recently, induction of regulatory T cells by HLA-G gains its interest. LeMaoult et al. reported that HLA-G1<sup>+</sup>APCs could cause the differentiation of CD4<sup>+</sup> T cells into suppressive cells and spread antigen-specific inhibition [22]. In another study, HLA-G5 secreted from human mesenchymal stem cells was observed to induce CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> T reg cells expansion in vitro [11]. 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. Indeed, HLA-G expression in various malignancies, and its correlation with poor prognosis was addressed previously [25–29].

In the context of HLA-G expression in breast cancer patients, in an initial report, Palmisano et al. noted that neither HLA-G transcripts nor protein was detected in 25 breast cancer lesions [30]. Later, studies revealed that 25–66% breast cancer lesions sele-
tively expressed HLA-G [31–34]. Breast cancer patients with HLA-G-positive tumor cells had a shorter disease-free survival or a lower survival rate than patients with negative expression [32,34].

In the current study, HLA-G was expressed in 70.7% (41/58) of primary breast cancer lesions. HLA-G expression status was significantly associated with tumor histologic grade and tumor stage, and lesion HLA-G expression is preferentially observed in patients with more advanced tumor stage. Similar findings were reported in other studies [31,34], indicating that HLA-G status might be involved in tumor progression and yields unfavorable prognostic factors for patients with breast cancer. Meanwhile, we found that plasma sHLA-G in breast cancer patients were dramatically increased over that in normal controls. It should be noted that the clinical diagnostic importance of HLA-G was emphasized by measuring sHLA-G in ascites samples of breast and ovarian cancer patients. sHLA-G levels were significantly higher in malignant ovarian and breast carcinoma ascites than that in benign ones. ROC analysis showed that the under curve area for sHLA-G was 0.95 for malignant versus benign ascites specimens [33]. In this study, the area under the ROC curve for the plasma sHLA-G level was 0.953. Interestingly, a recent study by He et al. also indicated that plasma sHLA-G levels were significantly higher in breast cancer patients, with the area under the ROC curve being 0.95 [34]. These studies provided further evidence that measurement of plasma sHLA-G levels is a highly specific and sensitive technique to differentially diagnose breast cancer patients from healthy subjects.

Increasing evidence suggests that Tregs protect tumors from the potentially effective immuneresponses and Tregs within the tumor, ascites, and peripheral blood of patients with cancer are associated with poor prognosis [3]. Recently, the relationship between HLA-G expression and T-regulatory cells has been investigated. In HCC patients, no correlation between HLA-G expression and Tregs was found, but a positive correlation was discovered between tumor HLA-G expression and Tregs/CD8⁺ ratio [35]. Cell surface HLA-G expression in B-cell chronic lymphocytic leukemia cells was also found to be irrelevant to the frequency of CD4⁺ CD25⁺FoxP3⁺ Treg, whereas an inverse correlation between the frequency of Treg and the serum sHLA-G levels was observed [36]. However, in the current study, a significant positive correlation between the plasma sHLA-G levels and CD4⁺ CD25⁺FoxP3⁺ Treg frequencies was observed in breast cancer patients (R = 0.582, p = 0.001). Our findings may be strengthened by the evidence that a positive correlation was found between sHLA-G and CD4⁺ CD25⁺ CD152⁺ Treg frequency in allogeneic peripheral blood hematopoietic stem cell–transplanted patients, and that CD4⁺ CD25⁺ CD152⁺ Treg is correlated with increased sHLA-G expression in vitro mixed leukocyte reaction cultures [37]. The controversial findings for the relationship between HLA-G and Treg frequencies may be partially explained by the samples used for sHLA-G detection. Plasma sHLA-G was almost invariably higher than those from serum for the same individual was addressed [38]. In that study, serum sHLA-G was detected in B-CLL patients, and the sHLA-G levels were extremely low, as reported [36]. In addition, although remains uncertain yet, the type of malignancies, such as leukemia and solid cancer may contribute the difference. Therefore, more studies on the relationship between HLA-G expression and Treg frequencies are necessary.

In conclusion, our findings revealed that both lesion HLA-G expression and plasma sHLA-G levels were upregulated in breast cancer patients, and that a strong relationship between sHLA-G and CD4⁺ CD25⁺ FoxP3 Treg may provide a pivotal role in the immune escape of breast cancer cell from immune surveillance and attack. Furthermore, sHLA-G levels in breast cancer patients might be a useful preoperative biomarker for diagnosis.

Acknowledgments

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References


