Soluble Human Leukocyte Antigen-G Expression in Patients with Ductal and Lobular Breast Malignancy

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Abstract. Background: Human leukocyte antigen-G (HLA-G) has been closely associated with diagnosis and prognosis in many types of human cancer. The current study aims to investigate soluble (s) HLA-G expression in patients with breast malignancy. Patients and Methods: sHLA-G plasma expression was determined in 120 patients with breast cancer and 40 healthy controls using enzyme-linked immunosorbent assay. Results: Plasma sHLA-G levels were significantly higher in breast cancer patients compared to healthy controls (p<0.001), with an area under the receiver operating characteristic (ROC) curve of 0.735 (95% Confidence interval=0.630-0.841, p<0.001). Significantly increased sHLA-G expression was detected in patients with mixed type of coexisting ductal and lobular breast lesions, compared to patients with pure ductal carcinoma or pure lobular neoplasia (p<0.05). Conclusion: sHLA-G expression is closely associated with the histological type of breast cancer. Our findings support the application of sHLA-G as a potential biomarker in body fluids for preoperative breast cancer detection and diagnosis.

Human leukocyte antigen-G (HLA-G) belongs to the non-classical HLA class I family of genes and was originally described to be selectively expressed at the maternal–fetal interface on cytotrophoblast cells, thereby contributing to maternal–fetal tolerance (1). Alternative splicing of the primary transcript generates seven different isoforms of the molecule, four of which are membrane-bound (HLA-G1, -G2, -G3 and -G4) while the other three are soluble (HLA-G5, -G6 and -G7) (2, 3). One additional soluble form of the molecule may be generated by shedding of the proteolytically cleaved surface HLA-G1 (4). Plasma sHLA-G derives from both the secretion of the soluble isoforms and the shedding form of HLA-G1 (5). Membrane-bound and soluble forms of HLA-G exhibit similar inhibitory functions by binding to their specific receptors (6). In normal tissue, HLA-G protein expression is restricted to fetal trophoblasts, endothelial precursors and mesenchymal stem cells, as well as to the thymus, cornea, nail matrix and pancreas (7-13). HLA-G expression has been closely associated with several pathological conditions, including pregnancy disorders, transplantation, autoimmune and inflammatory diseases, viral infections and cancer, with both beneficial and deleterious effects (14). In cancer, HLA-G has been implicated in a tumor-driven immune escape mechanism (14).

Aberrant up-regulated expression of HLA-G has been observed in many types of cancer. The first evidence came from Paul et al., who reported increased HLA-G protein expression in human melanoma cell lines (15). Subsequently, HLA-G expression was investigated in various tumor types, including glioma, renal cancer, lung cancer, lymphomas, leukemia and breast cancer (16-21). Based on these studies, it appears that HLA-G protein expression patterns are quite heterogeneous, potentially reflecting the different biology of individual tumors. In addition, HLA-G seems to be preferentially expressed in the tumor tissue, and only rarely in the adjacent normal tissue, suggesting that it may have a role in tumor growth and progression (22).

The potential application of HLA-G expression in the diagnosis of cancer has lately become the subject of intense investigation and was recently reviewed (23). HLA-G has been suggested as a tissue marker for the differential diagnosis of trophoblastic versus non-trophoblastic diseases, as well as for malignant versus benign neoplasms of epithelial origin (24, 25). Studies on breast cancer, chronic lymphocytic leukemia, hepatocellular carcinoma, non-small cell lung cancer and esophageal squamous cell carcinoma indicate that positive tissue expression of HLA-G is associated with poor disease outcome and worse prognosis (26-30). Moreover, correlations with advanced stage and metastatic disease have been
observed in hepatocellular, ovarian, non-small cell lung and endometrial cancer (31-34). Interestingly, contradicting data have been reported regarding tissue HLA-G expression in ovarian and gastric cancer, with some studies observing an association with favorable outcome (35, 36), while others with an unfavorable one (37, 38).

Regarding HLA-G expression in body fluids, scientific evidence suggest that soluble HLA-G (sHLA-G) may also be a surrogate tumor marker with clinical value in the discrimination of malignant versus benign ovarian, breast and colorectal disease (25, 39). Further experimental data propose that sHLA-G may be a useful preoperative diagnostic biomarker in renal, colorectal, gastric, esophageal and lung cancer (40, 41). Finally, a pronounced prognostic significance of plasma sHLA-G levels has been reported in lung and non-small cell lung cancer, with significant correlations observed between increased HLA-G expression and shorter survival (33, 42). Regarding breast cancer, few experimental data have so far been reported, indicating a diagnostic and prognostic role of tissue and sHLA-G expression.

The current study aimed to investigate plasma sHLA-G expression in patients with ductal and/or lobular breast malignancy, in an effort to gain further insight into the clinical significance of sHLA-G in breast cancer early diagnosis.

**Patients and Methods**

Study population. A total of 120 participants, aged between 35 and 80 years (mean=54.54±10.8 years), were recruited from the First Department of Propaedeutic Surgery, University of Athens, Greece.

**Peripheral blood samples** were collected upon diagnostic biopsy for mammographically suspicious breast lesions BI-RADS 4, by vacuum-assisted breast biopsy (VABB) under stereotactic guidance. In addition, blood samples were collected from 40 healthy individuals, aged between 42 and 78 years (mean=57.25±9.23 years) (Table I). Women with immune or lymphoproliferative disorders were excluded from the protocol. Patients had received no preoperative therapy or any other medication. The protocol was approved by the Institution’s Ethics Committee and written informed consent was obtained from all the participants.

**Sample analysis.** Blood samples were collected in EDTA-containing vacutainers and were left for 30 min at room temperature. Subsequently, the samples were centrifuged at 4400 × g for 15 min at 8˚C, and the plasma was separated into aliquots and stored at –80˚C until assayed. Plasma sHLA-G levels were quantified using a commercially available ELISA kit (Exbio, Prague, Czech Republic), according to the manufacturer’s instructions. The limit of detection (LOD) of the kit was 3 U/ml, the calibration range was 3.91-125 U/ml, and the intra-assay and inter-assay precision was 7.6% and 5.9%, respectively. All samples were assayed in double, without prior knowledge of the participants’ diagnoses.

**Statistical analysis.** All analyses were performed with the Statistical Package for Social Science (SPSS, Chicago, IL, USA), version 18.0. Normality of distribution was examined with Kolmogorov-Smirnov and Shapiro-Wilk’s tests. In cases where a normal distribution was indicated, paired or independent t-test was used for equality of means. Mann–Whitney U-test or Kruskal Wallis test was used for skewed data. The correlations of sHLA-G were investigated with Pearson and Spearman’s rho test coefficient. ROC curve analysis was employed to assess the performance of plasma sHLA-G levels in breast cancer diagnosis. A two tailed p-value less than 0.05 was considered to indicate statistical significance.

**Results**

sHLA-G plasma expression in breast cancer patients and healthy controls. sHLA-G was detectable in all samples. Plasma sHLA-G levels ranged between 12.55 and 256.54 U/ml, with a mean of 70.59 U/ml in patients with breast cancer, and between 5.14 and 183.39 U/ml, in healthy controls with a mean of 46.05 U/ml. Data showed that plasma sHLA-G expression was significantly higher in breast cancer patients compared to healthy controls (p<0.001). Data were further analyzed according to the histologic characterization of the
disease. Out of the 120 breast cancer patients, 40 patients were diagnosed with invasive ductal carcinoma (IDC) and 40 with ductal carcinoma in situ (DCIS), whereas the remaining 40 patients with lobular neoplasia (LN). sHLA-G levels were 77.60 U/ml in patients with IDC (range= 12.55-230.24 U/ml), 69.31 U/ml in patients with DCIS (range= 22.52-246.86 U/ml) and 64.84 U/ml in patients with LN (range= 21.20-256.54 U/ml). All three groups of patients exhibited significantly higher plasma levels of sHLA-G compared to healthy controls ($p<0.001$). No statistically significant differences in sHLA-G levels were observed between the three groups of patients. Plasma sHLA-G levels were not correlated to the age of patients or grade of the disease.

**ROC curve analysis.** ROC curve analysis was performed in order to evaluate the performance of plasma sHLA-G levels in discriminating between breast cancer patients and healthy controls. The area under the ROC curve (AUC) was 0.735 (95% CI=0.630-0.841, $p<0.001$) (Figure 1). Sensitivity and specificity ranged from 70.0-74.2% and 67.5-75.0%, respectively, for specified cut-off values of sHLA-G (Table II).

**Plasma sHLA-G expression in pure and mixed types of breast cancer.** Amongst the 80 individuals with ductal breast carcinoma, either invasive or in situ, the majority (67 patients) had pure IDC or DCIS, while the remaining 11 patients had ductal carcinoma coexisting with LN. Comparison of plasma sHLA-G levels between patients with pure IDC/DCIS or LN and those with a mixed type of IDC or DCIS coexisting with LN, revealed statistically significant differences. More specifically, plasma sHLA-G levels were significantly higher in patients with IDC or DCIS coexisting with LN (102.62 U/ml, range= 29.49-246.86 U/ml) compared to those who had pure IDC or DCIS (69.23 U/ml, range= 12.55-230.24 U/ml), as well as patients with pure lobular neoplasia (64.84 U/ml, range= 21.20-256.54 U/ml) ($p<0.05$) (Figure 2).

**Discussion**

The detection of up-regulated expression of HLA-G in a variety of tumors indicates that HLA-G activation is a frequent event associated with malignant transformation and cancer progression (43-46). Regarding breast cancer, although an initial report by Palmisano et al. failed to detect HLA-G expression both at RNA and protein levels (47), later studies revealed a selective expression of HLA-G within mammary tumor lesions. Lefebvre et al. found that HLA-G was expressed in 39% of breast cancer tissue lesions while expression appeared to correlate with high inflammatory grade (21). Similarly, a study on malignant breast carcinoma effusions and corresponding solid tumors observed HLA-G expression rates of 26% and 41%, respectively (26). Although breast cancer patients with HLA-G-positive tumor cells appeared to have shorter disease-free survival, HLA-G expression showed no correlation with clinicopathological parameters since it was only inversely correlated with protein expression of the tyrosine kinase receptor 2 gene ($ERBB2$). More recent studies have observed positive HLA-G expression in a significantly higher percentage of malignant breast tissues. He et al. analyzed 235 primary breast cancer tissue specimens and found that approximately
66% of them were HLA-G-positive (48). HLA-G expression was significantly correlated with tumor size, nodal status and advanced disease stage, while patients with positive HLA-G expression had a lower overall survival rate than those with negative expression. Similar results were obtained by Chen et al., who observed positive HLA-G expression in 71% of primary breast cancer lesions, significantly associated with tumor histological grade and stage (49). A comparative analysis of surface and cytoplasmic HLA-G expression on breast tissue specimens using flow cytometry showed that surface (membrane-bound) HLA-G was expressed in 40% of malignant specimens, while cytoplasmic in 24.4% (50). Elliott et al. observed significantly higher tissue HLA-G expression both in the membrane and cytoplasm of breast cancer tissue compared to normal breast tissue (51). Finally, evidence that tissue expression of HLA-G, in combination with HLA-E, can assist in the prediction of the clinical outcome of patients with early breast cancer have recently become available (52).

Peripheral blood analysis has lately attracted research interest since it provides an ideal tool during diagnosis and monitoring of cancer disease. To date, very few studies have investigated the potential clinical implications of circulating HLA-G expression in invasive breast cancer, detecting markedly higher levels of sHLA-G in breast cancer patients compared to healthy controls (48-50). According to these data, circulating sHLA-G levels are not significantly correlated to the age of the patients or the disease clinicopathological characteristics, such as tumor size and grade, and disease stage (48-50). In agreement with the above, our findings also indicate that plasma sHLA-G levels are statistically significantly higher in patients with breast cancer. We also observed no correlation between sHLA-G expression and the age of patients or grade of the disease, however, since our samples were obtained during diagnostic biopsy procedures, additional correlations with disease characteristics are not available. Further analysis of our results reveals that increased sHLA-G expression is not only detected in patients with IDC but also in those with preinvasive lesions of the breast, namely with DCIS and LN, an observation made for the first time. Within our study, we performed ROC curve analysis affirming that sHLA-G quantitative assessment may serve as an efficient tool in breast cancer diagnosis, not necessarily alone but potentially when performed within a panel of routinely applied biomarkers.

He et al. observed no significant differences in plasma sHLA-G levels between patients with invasive ductal (IDC) or invasive lobular (ILC) breast cancer (48). However, previous data on breast carcinomas indicated that HLA-G is preferentially expressed in IDC, rather than ILC (22). Our study is the first to investigate plasma sHLA-G expression in patients with pure or mixed types of breast carcinoma. It is not uncommon for IDC or DCIS to coexist with LN. Evidence from core biopsy specimens indicate a 14-21% incidence of either atypical ductal hyperplasia or LN coexisting with IDC or DCIS (53-56). LN is defined as a proliferation of generally small and often loosely cohesive cells and it encompasses all atypical epithelial proliferations that originate in the terminal duct-lobular unit, namely atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS) (57). It has been suggested that the presence of concurrent proliferative lesions associated with in situ or infiltrating breast carcinoma may reflect underlying perturbations of cancer-related pathways, with potential significance for disease risk, occult disease or tissue response (58). Thus, women with DCIS and concurrent LN were found to be at a more than two-fold greater risk for developing ipsilateral recurrence, compared to those with pure DCIS (58). Similarly, a 10-year cumulative incidence rate of 29% for ipsilateral breast tumor recurrence has been recorded in women with invasive breast carcinoma coexisting with LCIS, as opposed to 6% in patients with pure invasive carcinoma (59). According to our data, plasma sHLA-G levels are significantly higher in patients with ductal breast carcinoma coexisting with LN compared to those with pure IDC, DCIS, or LN. Taking into consideration that the presence of concurrent LN in early-stage breast cancer appears to have a prognostic significance regarding the course of the disease, the quantitative assessment of sHLA-G levels in breast cancer patients may have important clinical implications during diagnosis and management of the disease.

In conclusion, our study provides evidence in further support of the application of sHLA-G as a biomarker in body fluids for preoperative breast cancer detection and diagnosis. Most importantly, we demonstrate that plasma sHLA-G levels are closely associated with the histological type of breast cancer, since significantly elevated sHLA-G expression is detected in patients with mixed type of coexisting ductal and lobular breast lesions. Taking into account the heterogeneity of breast cancer and its associated implications in prognosis and clinical outcome, the integrated use of sHLA-G measurement may provide a novel approach in management of the disease.

Conflict of Interest

All Authors declare they have no conflict of interest.

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