HLA-G Is a Potential Tumor Marker in Malignant Ascites


Department of Pathology [G. S., Y.-C. C., S. Z. A., D. W. C., C.-C. C., R. J. K., I.-M. S.], and Department of Epidemiology [H.-T. L., Johns Hopkins University Medical Institutions, Baltimore Maryland 21231]; Department of Immunology, University Hospital of Essen, Essen, Germany [V. R., H. G-W.]; Departments of Gynecology and Obstetrics [J. R., K. P.], and Department of Pathology [E. W.], University of Bonn, Bonn, Germany; and Department of Stomatology, University of California, San Francisco, California [M. T. M.]

ABSTRACT

Purpose: Molecular approaches as supplements to cytological examination of malignant ascites may play an important role in the clinical management of cancer patients. HLA-G is a potential tumor-associated marker and that one of its isoforms, HLA-G5, produces a secretory protein. This study is to assess the clinical utility of secreted HLA-G levels in differential diagnosis of malignant ascites.

Experimental Design: We used ELISA to assess whether secretory HLA-G (sHLA-G) could serve as a marker of malignant ascites in ovarian and breast carcinomas, which represent the most common malignant tumors causing ascites in women.

Results: On the basis of immunohistochemistry, 45 (61%) of 74 ovarian serous carcinomas and 22 (25%) invasive ductal carcinomas of the breast demonstrated HLA-G immunoreactivity ranging from 2 to 100% of the tumor cells. HLA-G staining was not detected in a wide variety of normal tissues, including ovarian surface epithelium and normal breast tissue. Reverse transcription-PCR demonstrated the presence of HLA-G5 isoform in all of the tumor samples expressing HLA-G. ELISA was performed to measure the sHLA-G in 42 malignant and 18 benign ascites supernatants. sHLA-G levels were significantly higher in malignant ascites than in benign controls (P < 0.001). We found that the area under the receiver-operating characteristic curve for sHLA-G was 0.95 for malignant versus benign ascites specimens. At 100% specificity, the highest sensitivity to detect malignant ascites was 78% (95% confidence interval, 68–88%) at a cutoff of 13 ng/ml.

Conclusions: Our findings suggest that measurement of sHLA-G is a useful molecular adjunct to cytology in the differential diagnosis of malignant versus benign ascites.

INTRODUCTION

Ascites is commonly associated with a variety of infectious diseases, inflammatory disorders, and cardiac, liver, and renal diseases as well as benign and malignant neoplasms (1–3). Cytological examination of ascites is performed in an effort to diagnose malignant tumors, but the sensitivity of cytology has been estimated to be 60% at best (4). The low sensitivity may be because of small numbers of tumor cells in the ascites or the presence of a large amount of leukocytes, mesothelial cells, and blood that can obscure the malignant cells. For example, inflammation that is often associated with a malignant ascites can result in reactive changes in mesothelial cells that make their morphological distinction from carcinoma cells extremely difficult (4). Thus, a molecular test that is able to distinguish malignant from benign ascites could have great diagnostic utility.

HLA-G is a nonclassical MHC class I antigen that interacts with natural killer cells (5). HLA-G expression has not been detected in normal tissues except in trophoblast in placentas from early gestation (6–8). In contrast, HLA-G expression has been detected in several human cancers including melanoma, renal cell carcinoma, breast carcinoma, and large cell carcinoma of the lung (9–14). HLA-G expression in cancer cells has been shown to be important for the escape of immunosurveillance by host T-lymphocytes and natural killer cells (6, 9–11, 15, 16). Recently, an HLA-G-specific ELISA was developed to measure sHLA-G, a product of an HLA-G5 isoform (17–19). Because HLA-G is not detected in normal adult tissues but is expressed by some carcinomas, we hypothesized that the detection of sHLA-G using the newly developed ELISA might be useful in the detection of cancer in ascites. In this study, we tested this hypothesis by assessing the expression pattern of HLA-G in women with ovarian serous carcinomas and invasive ductal carcinomas of the breast because these are the most common malignant tumors in women that produce ascites. We measured sHLA-G in peritoneal fluid supernatant to evaluate its potential as a marker for malignant ascites.

Received 2/24/03; revised 5/20/03; accepted 5/21/03.

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This work was supported in part by the United States Department of Defense OC 010017 Grant, the Richard TeLinde Research Fund from the Johns Hopkins University (JHU) School of Medicine, the JHU-American Cancer Society, and the Swiss National Science Foundation (to G. S.).

1 To whom requests for reprints should be addressed, at The Johns Hopkins Medical Institutions, 418 North Bond Street, Room B-315, Baltimore, MD 21231. Phone: (410) 502–774; Fax: (410) 502-7943; E-mail: ishih@jhmi.edu.

2 The abbreviations used are: sHLA-G, secretory or soluble HLA-G; CI, confidence interval; RT-PCR, reverse transcription-PCR; ROC, receiver operating characteristic (curve).
MATERIALS AND METHODS

Tissue Samples and Ascites Specimens. The acquisition of paraffin tissues and ascites specimens was approved by the local Institutional Review Boards. A total of 180 formalin-fixed, paraffin-embedded tissue samples including 74 ovarian serous carcinomas, 88 breast invasive ductal carcinomas, 8 normal ovaries, and 10 benign breast tissues were retrieved from the surgical pathology files. Peritoneal fluid specimens (3 serous carcinomas, 88 breast invasive ductal carcinomas, 8) were obtained from the Cytopathology Division of the University of Bonn, Bonn, Germany, and from the Johns Hopkins Medical Institutions; they included 41 cytology-confirmed malignant ascites samples (24 ovarian serous carcinomas and 17 breast carcinomas) and 19 cytology-negative benign specimens in which the patients did not have concurrent malignant diseases. There was a cytology-false-negative specimen that was initially diagnosed by cytopathologists as benign, but the patients had stage III ovarian cancer, and the ascites sample contained ovarian serous carcinoma cells in culture (20). Thus, this sample was later classified into the ovarian cancer group in this study. The ascites samples were centrifuged at 2000 × g for 5 min within 6 h after collection. The supernatant and cell pellets were aliquoted and frozen until use. All of the specimens were obtained from female adult patients.

Immunohistochemistry and Western Blot Analysis. Expression of HLA-G was studied in surgical specimens using immunohistochemistry and Western blot analysis. Paraffin sections were used for immunohistochemistry with an HLA-G-specific monoclonal antibody, 4H84 (1:600), which reacted to the denatured HLA-G heavy chain (6), followed by the avidin-specific monoclonal antibody, 4H84 (1:600), which reacted to

The primer sequences for all of the HLA-G isoforms were:

\[
\text{5'}-\text{ggaagaggaacagcaagca-3'} \quad \text{and} \quad \text{5'}-\text{gcagctcaagtacagc-3'}.
\]

The primer sequences for HLA-G5-specific primers were:

\[
\text{5'}-\text{accgccttgtaaaggtc-3'} \quad \text{and} \quad \text{5'}-\text{caatgtgtaacaaaggag-3'}.
\]

Total RNA was purified and cDNA was synthesized using standard protocols. Briefly, frozen tissues were minced and placed in the TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated, and contaminating genomic DNA was removed using the DNA-free kit (Ambion, Austin, TX). cDNA was prepared using oligo(dT) primers and was diluted for PCR.

ELISA. sHLA-G was measured using ELISA, which has been described previously by us (18, 19). Briefly, soluble HLA-A, B, C, E molecules (sHLA-I) were selectively depleted from samples using immunomagnetic beads (Dynabeads M280; Dynal, Hamburg, Germany) coupled with the monoclonal antibody TP25.99. The remaining sHLA-G molecules were measured in an ELISA format using monoclonal antibody W6/32 [0.2 μg/ml in PBS (pH 7.2)] as the capture reagent. After the blocking of free binding sites with BSA in PBS (2%), diluted samples (1:2) were added and incubated for 1 h at room temperature. Unbound antigens were removed by intensive washing with PBS-Tween (0.05%). Bound sHLA-G heavy chains were detected by the sequential addition of pox-labeled antihuman β2-microglobulin (anti-HLA-A, B, C, E, Dakopatts, Hamburg, Germany), and substrate [0.075% H2O2, 0.1% ortho-phenylenediamine (0.035 M citrate buffer (pH 5.0)). The absorbance was measured at 490 nm (BIO-TEK Instruments, Winooski, VT). The intra- and interassay variations were 3.5 and 13.1%, respectively. The sensitivity of the assay in detecting sHLA-G was 3 ng/ml. ELISA was performed in a blinded fashion.

Statistical Analysis. The feasibility of using sHLA-G levels as a diagnostic tool for detecting malignant versus benign ascites was assessed using the ROC curve analysis. A ROC curve is a graphic presentation of the sensitivity against the false-positive rate (1-specificity), and the areas under the ROC curves were measured to evaluate test performance at different thresholds of a diagnostic measure. The χ2 test (one-sided) of the medians was used to analyze the difference in sHLA-G levels in malignant versus benign ascites samples. The CIs were estimated for the sensitivity of the HLA-G ELISA.

RESULTS

Expression of HLA-G in Ovarian and Breast Cancer Tissues. Immunohistochemical analysis of ovarian and breast carcinomas revealed HLA-G immunoreactivity in 45 (61%) of 74 high-grade ovarian serous carcinomas and in 22 (25%) of 88 invasive ductal carcinomas of the breast (Fig. 1). The positive tumor cells showed a discrete membranous staining pattern, and the proportion of positive cells varied from 2 to 100% in any given specimen. HLA-G staining was not detected in low-grade ovarian serous carcinomas and normal tissues including ovarian surface epithelium, mammary ducts, and lobules. We also assessed HLA-G immunoreactivity in benign ovarian and breast lesions. HLA-G expression was not detected in 8 ovarian serous cystadenomas, 12 ovarian borderline tumors (atypical proliferative serous tumors and noninvasive micropapillary serous carcinomas), nor 10 intraductal hyperplasias of the breast. Only rare HLA-G-positive tumor cells were identified in 2 of 10 intraductal carcinomas of the breast. The specificity of HLA-G immunostaining was confirmed by Western blot analysis and RT-PCR as shown in Fig. 2. A 39 kDa band corresponding to the HLA-G protein was identified in five ovarian serous carcinomas but not in two ovarian cystadenomas, ovarian surface epithelium, and stroma. RT-PCR was performed in ovarian...
HLA-G: A Potential Tumor Marker in Malignant Ascites

Serous carcinomas and invasive ductal carcinomas of the breast using primers that amplified all HLA-G isoforms. The PCR products were isolated by electrophoresis to reveal a predominance of the HLA-G1 and G5 isoforms. HLA-G5 RNA transcript, a secretory isoform, was specifically amplified using the HLA-G5-specific primers in all five of the representative ovarian serous carcinomas and in the two invasive ductal carcinomas of breast that expressed HLA-G (Fig. 2). This finding prompted us to assess whether sHLA-G could be detected in peritoneal fluid samples in ovarian and breast cancer patients.

**Measurement of sHLA-G in Ascites Specimens.** sHLA-G levels were measured in the supernatant of ascites from 60 samples using ELISA. All but one malignant ascites specimen contained detectable sHLA-G, including one specimen that had been missed on cytology (20). In contrast, 7 of 18 benign ascites specimens contained detectable but low levels of sHLA-G. As shown in Fig. 3, the levels of sHLA-G were significantly higher in malignant as compared with benign ascites (P < 0.001). Among 11 benign ascites specimens with undetectable sHLA-G, 2 were obtained from patients with ovarian serous cystadenomas that could be confused with ovarian cancer on clinical examination. The remaining seven benign specimens with detectable sHLA-G were from patients with non-neoplastic diseases including liver, cardiac, and renal diseases.

ROC curves were used to evaluate the performance of sHLA-G in detecting ovarian and breast cancer in ascites using multiple cutoff values. The area under the ROC curve was 0.95 in assessing sHLA-G levels as the diagnostic tool to detect ovarian and breast cancer. More specifically, the areas under the ROC curve were 0.99 and 0.90 for ovarian cancer versus benign samples and breast cancer versus benign samples, respectively (Fig. 4). Given 100% specificity, the highest sensitivity achieved to detect cancer was 78% (95% CI, 68–88%) at a cutoff of 13 ng/ml. The sensitivity to diagnose ovarian cancer and breast cancer was 84% (95% CI, 70–98%) and 65% (95% CI, 42–87%), respectively, at this arbitrary cutoff. With a specificity of 94.4%, the sensitivity was 100% (95% CI, 100%) and 71% (95% CI, 49–93%) for ovarian cancer and breast cancer, respectively.

Correlation of HLA-G expression in tissue or ascites cell pellets and the sHLA-G level in ascites supernatants was performed in 31 patients as the corresponding surgical specimens or cell pellets were available for analysis. In 21 malignant ascites samples with detectable sHLA-G, HLA-G expression was demonstrated in 12 tissue specimens and ascites cell pellets by immunohistochemistry or Western blot analysis (data not shown).
polypeptide-specific antigen, soluble interleukin-2 receptor protein markers have been studied including CA125 (22), tissue associated markers in diagnosing malignant ascites? Several cancers.

ovarian cancers express HLA-G more frequently than breast cancer is consistent with our immunohistochemical findings that sHLA-G in detecting ovarian cancer as compared with breast versus benign samples, respectively. The better performance of (23), soluble aminopeptidase N/CD13 (24), /H9251 for ovarian cancer examination. The areas under the ROC curve were 0.99 and 0.90 was ultimately shown to be a false-negative case by cytological malignant cells, identified by cytology, had detectable sHLA-G in ascites cell pellets using Western blot analysis.

DISCUSSION

The results of this study provide evidence that HLA-G is expressed in ovarian and breast carcinomas and that measurement of sHLA-G using ELISA is a highly sensitive technique to diagnose malignant ascites. Ninety-eight % of specimens with malignant cells, identified by cytology, had detectable HLA-G levels. In addition, sHLA-G was detected in one specimen that was ultimately shown to be a false-negative case by cytological examination. The areas under the ROC curve were 0.99 and 0.90 for ovarian cancer versus benign samples and breast cancer versus benign samples, respectively. The better performance of sHLA-G in detecting ovarian cancer as compared with breast cancer is consistent with our immunohistochemical findings that ovarian cancers express HLA-G more frequently than breast cancers.

How does HLA-G compare with other soluble tumor-associated markers in diagnosing malignant ascites? Several protein markers have been studied including CA125 (22), tissue polypeptide-specific antigen, soluble interleukin-2 receptor α (23), soluble aminopeptidase N/CD13 (24), α-fetoprotein (25), carcinoembryonic antigen, CA 19–9, CA 15–3 (25), and several cytokines (26), but none are specific enough for cancer diagnosis because a variety of normal tissues, benign tumors, and nonneoplastic diseases also express these markers (24, 27–28). Using a cutoff value to achieve >90% specificity in detecting malignant ascites, the sensitivity of these markers was generally very low and, therefore, unacceptable for clinical application. In contrast, HLA-G has very limited tissue distribution because only a subpopulation of trophoblast (intermediate trophoblast) is known to express this molecule (7, 8) suggesting that sHLA-G would be more specific for cancer diagnosis. The findings in this study confirm this impression because the HLA-G ELISA achieved a sensitivity of 78% and a specificity of 100% for diagnosing malignant ascites at a cutoff of 13 ng/ml.

The finding that almost all of the malignant ascites samples contained detectable sHLA-G contrasted with the lower rate of HLA-G expression in the tumors based on immunohistochemistry, because 61% of ovarian and only 25% of breast cancer tissue specimens were positive. In addition, some malignant ascites supernatants contained elevated sHLA-G levels, whereas HLA-G immunoreactivity was not detected in the corresponding tissue specimens and cell pellets from ascites by immunohistochemistry. This discordant finding can be explained by the fact that HLA-G is only focally expressed in most tumors and, therefore, may be undetected in representative tissue specimens selected for immunostaining or immunoblotting. It is likely that carcinoma cells in ascitic fluid secrete sHLA-G, resulting in high sHLA-G levels in ascites. If only a few tumor cells are present in the peritoneal fluid they may not be detected by cytology. Although these are our favorite explanations, other possibilities, albeit unlikely, should be also pointed out. For example, sHLA-G is expressed by other tissues in response to malignant diseases. The low level of sHLA-G in benign ascites may be attributable to nonspecific binding (background noise) of the antibody used in the ELISA. Alternatively, there may be unknown tissue resources that express sHLA-G and contribute to the low level of sHLA-G in ascites samples.

In summary, HLA-G is a tumor-associated molecule that is expressed by ovarian serous carcinoma and ductal carcinomas of the breast, the most common malignant tumors that produce ascites in women. Malignant ascites specimens contained much higher levels of sHLA-G than the benign ascites specimens. The detection of sHLA-G in ascitic fluid may provide a novel molecular approach to supplement cytological examination in the evaluation of ascites. It should be noted that the sensitivity of sHLA-G ELISA to diagnose malignant ascites may not be as high as shown in this study because the threshold to distinguish benign and malignant ascites could be higher than 13 ng/ml after a larger number of benign samples are analyzed. In order for this new marker to have clinical utility, several issues must be addressed. Although the sensitivity of sHLA-G ELISA in diagnosing malignant ascites in this study was 78% with 100% specificity, higher sensitivity would be desirable. Sensitivity could be improved by combining the measurement of sHLA-G with other tumor-associated markers (20, 29). It will be necessary to compare the performance of the sHLA-G ELISA and routine cytological examination by testing a large number of cytology-negative but biopsy-positive samples. It will also be important to address how age, menopausal status, histological grade and other clinical parameters affect HLA-G levels in ascites. Lastly, the potential use of sHLA-G in other body fluids such as plasma should be further investigated.

ACKNOWLEDGMENTS

We thank John Lauro at the Johns Hopkins Medical Institutions for his excellent technical support. This study is in memory of Dr. Frederick J. Montz of the Kelly Gynecologic Oncology Service at Johns Hopkins Hospital.
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HLA-G Is a Potential Tumor Marker in Malignant Ascites


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