Frequent Loss of HLA-A2 Expression in Metastasizing Ovarian Carcinomas Associated with Genomic Haplotype Loss and HLA-A2-Restricted HER-2/neu-Specific Immunity

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Abstract
Defective expression of HLA class I molecules is common in tumor cells and may allow escape from CTL-mediated immunity. We here investigate alterations in expression of HLA class I and their underlying molecular mechanisms in ovarian cancer patients. The HLA class I and HLA-A2 expression levels on noncultured tumor cells of 12 patients diagnosed with ovarian carcinoma were investigated by flow cytometry. Molecular analyses of antigen-processing machinery (APM) components were done in metastatic cancer cells, and the HLA genotype was determined in both these and the primary tumor. HER-2/neu-specific immunity was evaluated by enzyme-linked immunospot assays. The metastatic tumor cells from all patients expressed low levels of HLA class I surface antigens. In six of nine HLA-A2+ patients, HLA-A2 expression was heterogeneous with a subpopulation of tumor cells exhibiting decreased or absent HLA-A2 expression. One patient-derived tumor cell line completely lacked HLA-A2 but exhibited constitutive expression of APM components and high HLA class I expression that was further inducible by IFN-γ treatment. Genotyping showed a haplotype loss in the metastatic tumor cells, whereas tumor tissue microdissected from the primary tumor exhibited an intact HLA gene complex. Interestingly, HLA-A2-restricted HER-2/neu-specific T-cell responses were evident among the lymphocytes of this patient. Abnormalities in HLA class I antigen expression are common features during the progression of ovarian cancer, and haplotype loss was, for the first time, described as an underlying mechanism. (Cancer Res 2006; 66(12): 6387-94)

Introduction
Ovarian cancer is the leading cause of death from gynecologic malignancy due to its abysmal prognosis. Most patients have distant metastasis at the time of diagnosis and a 5-year survival rate of only 29% (1). Therefore, novel therapeutic modalities for this disease are urgently needed. One promising area for the development of improved treatments of patients suffering from ovarian cancer is immunotherapy. Several different approaches of immunotherapy against ovarian cancer are currently being tested in the clinic, including infusion of monoclonal antibodies (mAb), adoptive transfer of T cells, or administration of tumor vaccines (2, 3).

Immunotherapy based on the activation of tumor-specific T cells is however severely limited by the appearance of resistant tumor variants lacking HLA class I molecules. Defective expression and presentation of HLA class I molecules is a common phenomenon observed in a variety of human tumors (4). The responsible mechanism seems to be immune selection mediated by tumor-specific T cells, induced by the growing tumor or by immunotherapy, favoring the selective outgrowth of HLA class I-negative variants (5–7). This will constitute an efficient tumor escape mechanism from T-cell–mediated immunity and a major challenge to T-cell–based immunotherapy. HLA class I antigens can be lost to various degrees and by many different molecular mechanisms (reviewed in ref. 8). Genomic deletions or translocations often cause loss of expression of one or several HLA alleles (9–11). Defects in one or several components of the antigen-processing machinery (APM) are frequently observed in tumor cells and can cause impairment of HLA class I surface expression (12–14). In these cases, HLA class I expression can often be restored by IFN-γ treatment (13, 15, 16).

Most T-cell–based immunotherapeutic strategies for cancer patients done with tumor vaccines or adoptively transferred T cells are based on CTLs with HLA-A2 as the restriction element (17–20). In addition, there is an overrepresentation of the HLA-A2 allele among patients with advanced stage ovarian cancer compared with patients with less advanced disease and healthy individuals (21, 22). Hence, HLA-A2 expression on the tumor cells is of critical importance for the effectiveness of immunotherapy and naturally occurring antitumor immunity in cancer patients in general and for patients suffering from ovarian carcinoma in particular.

The primary goal of this study was therefore to investigate the expression of HLA-A2 and total HLA class I surface antigens on metastatic tumor cells from patients with advanced ovarian cancer. To this end, HLA class I and HLA-A2 expression levels on freshly isolated noncultured tumor cells in the ascitic fluid of 12 patients were investigated by flow cytometry. We here show that tumor cells from the majority of patients with ovarian cancer undergo a progressive loss of HLA-A2 expression during the course of disease, resulting in heterogeneous HLA-A2 expression on metastatic tumor cells. In addition, we show for the first time that (a) haplotype loss
is an underlying mechanism for altered HLA class I expression in ovarian cancer and (b) that it was associated with a HLA-A2-restricted immunity to the HER-2/neu proto-oncogene. These data suggest a T-cell-mediated immune selection as an underlying mechanism for HLA class I abnormalities.

Materials and Methods

Cells. Ascites was collected from 12 patients diagnosed with metastatic epithelial ovarian carcinoma from whom informed consent had been obtained. The ascitic cells were harvested as described previously (23). In addition, peripheral blood was obtained from two of these patients. Both ascitic and peripheral blood mononuclear cells (PBMC) were enriched by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) centrifugation removing RBCs and granulocytes. Tumor cells and lymphocytes were frozen at concentrations of 5 to 10 × 10⁶/mL in a solution containing 90% DMSO (Sigma-Aldrich, St. Louis, MO) and 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc., Grand Island, NY) and stored in liquid nitrogen until used in analyses. One homogenous tumor cell line (oval 11) was established from the ascites of patient 11 by continuous culture of the fresh ascitic cells for >3 months. Oval 11 cells were maintained in complete medium consisting of RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, and 25 μg/mL gentamicin (Life Technologies). For IFN-γ treatment, oval 11 cells were incubated in complete medium supplemented with 500 IU/mL recombinant IFN-γ (Boehringer Ingelheim, Ingelheim, Germany) for 48 hours. The renal cell carcinoma cell line MZ1257serving as positive control was subjected to reverse transcription-PCR (RT-PCR) analyses as described recently (14). The mRNA expression levels of the APM components and HLA-A2 were evaluated by using the following set of specific primer pairs: transporter associated with antigen processing (TAP) 1, 5'-GAGACATCTGGAACTGACCTC-3' and 5'-TTGATGGAATCTTGAGG-3'; TAP2, 5'-TTTATCCAGCAGCAGCGTCTC-3' and 5'-CTTCTTGCAACCTGTTGGA-3'; β2-microglobulin, 5'-CTTGCCTACCTCTCTCTT-3' and AAGACACGTCTCCTCCTGTA; and HLA-A2, 5'-AGACTCAACGCCGTGAGCTT-3' and 5'-TATTCGAGCAACCTCCTCAC-3'. Annealing temperatures ranged from 57°C to 60°C and between 26 and 30 amplification cycles were used. PCR products were separated by electrophoresis in 1.5% agarose gels together with molecular weight markers and visualized by ethidium bromide.

DNA extraction from microdissected tumor biopsies. DNA was extracted from microdissected tumor tissue samples from three slides of paraffin-embedded primary tumor material. For two steps of deparaffinization with xylole for 10 minutes in the first step and 96% ethanol for 10 minutes in the second step, the pellet was dried at 60°C until the ethanol had evaporated. The tissue pellet was incubated with 1 mL DNA extraction buffer (100 mmol/L NaCl, 10 mmol/L Tris-HCl, 25 mmol/L EDTA, 0.5% SDS) and 30 μL protease K solution at 37°C overnight followed by 48 hours of further incubation with the daily addition of 20 μL protease K solution. The DNA was finally isolated by phenol/chloroform/isooamylethanol extraction.

Genotyping of the HLA complex. The oval 11 tumor cell line and its autologous primary tumor and PBMCs were HLA genotyped. For the HLA-A*, HLA-B*, and DRB* low-resolution genotyping and for HLA-A*02 high-resolution genotyping, PCR-SSP kits (GenoVision, Vienna, Austria) were used in accordance with the manufacturer's instructions, with the addition of five extra amplification cycles due to the small amount of DNA. The PCR products were separated by standard 2% agarose gel electrophoresis and stained by ethidium bromide, and the results were documented with a photo printer.

Enzyme-linked immunospot assays. PBMCs were thawed, stimulated with 10 μg/mL of various peptides, and cultured at 37°C and 7.5% CO₂ in X-VIVO 15 (BioWhittaker, Walkersville, MD) with 2% autologous heat-inactivated plasma. U-bottomed 96-well tissue culture plates (Techno Plastic Products, Trasadingen, Switzerland) with 2 × 10⁶ cells per well were used, and 20 IU/mL interleukin (IL)-2 (PeproTech, Rocky Hill, NJ) was added on day 4. On day 8, 2 × 10⁵ irradiated (30 Gy) autologous PBMCs pulsed with the same peptides as used initially were added to each of the microcultures and incubation for another 24 hours followed. The numbers of IFN-γ-secreting cells were estimated by the use of an IFN-γ-specific enzyme-linked immunospot assay (ELISPOT) kit (Mabtech, Nacka Strand, Sweden). Cells were, after a thorough wash, transferred to ELISPOT wells (Millipore, Bedford, MA) coated with mAbs and allowed to secrete cytokines for 20 hours at 37°C. 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Sigma-Aldrich) was used, and the reaction was stopped with water. The number of spots was enumerated by using an automated ELISPOT reader system (Autoimmun Diagnostika, Strassberg, Germany).

Statistical analysis. The significance of the differences in the number of cytokine-secreting cells, measured by ELISPOT assays, between various peptide-stimulated cell populations were analyzed by the Student’s t test. A two-sided z-value of P < 0.01 was considered significant.

Results

Study population. Ascites and PBMCs from 12 patients diagnosed with metastatic ovarian cancer were used in this study. Characteristics, such as age, tumor histology, and stage of disease, are summarized in Table 1. The median age of patients was 67 years, the histopathology of all tumors was typical for ovarian carcinoma, and 11 of 12 patients had stage III or IV ovarian cancer.
malignancies at primary surgery. Nine of 12 patients were HLA-A2+ (Table 1).

**Low but constitutive surface expression of total HLA class I surface antigens on metastatic ovarian tumor cells.** HLA class I surface expression was determined on both lymphocytes and tumor cells in the same ascites sample. As shown in Fig. 1A, all lymphocytes displayed high expression levels of total HLA class I surface antigens. In contrast, tumor cells as defined by their constitutive HER-2/neu and epithelial antigen (stained by the anti-EpCAM mAb) surface expression and lack of the CD14 marker showed low expression levels of HLA class I antigens (Fig. 1A, left). HLA class I expression levels on the tumor cells, defined as the ratio between mean fluorescence intensities for the anti-HLA-ABC mAb and the isotype control antibody, were on average only 10% (median value, range 3-65%) of those exhibited by the respective autologous lymphocytes. Nevertheless, tumor cells lacking HLA class I antigens were rare and found only in a subpopulation of the tumor cells of patient 7 (Fig. 1A, left).

**Loss of expression of HLA-A2 on metastatic ovarian tumor cells.** As expected, the ascitic lymphocytes of the nine HLA-A2+ patients expressed high levels of HLA-A2 molecules at the cell surface (Fig. 1B, right). Interestingly, in six of these patients, the autologous noncultured tumor cells displayed heterogeneous HLA-A2 expression and could be divided into two different populations with regard to HLA-A2 expression levels (Fig. 1B, left). In three of these six patients (patients 1, 3, and 11), a subpopulation of the tumor cells completely lacked HLA-A2, whereas a fraction of cells from patients 6, 8, and 9 exhibited down-regulated but detectable HLA-A2 expression levels.

Ascites samples were obtained twice from patient 11 at an interval of 9 weeks. Although both samples showed a heterogeneous HLA-A2 expression, the frequency of HLA-A2+ tumor cells increased over time (Fig. 1B). Approximately half of the tumor cells lacked HLA-A2 surface expression in the first collected sample, whereas the HLA-A2+ population totally dominated in the ascites collected 9 weeks later.

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**Table 1. Characteristics of the ovarian cancer patients**

<table>
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<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Surgical staging</th>
<th>Histopathology</th>
<th>HLA-A2 status</th>
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<td>1</td>
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<td>Endometrioid</td>
<td>+</td>
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<tr>
<td>2</td>
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<td>Serous</td>
<td>+</td>
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<td>3</td>
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<td>Serous</td>
<td>+</td>
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<tr>
<td>4</td>
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<td>IIIC</td>
<td>Serous</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>6</td>
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<td>IV</td>
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<td>+</td>
</tr>
<tr>
<td>7</td>
<td>74</td>
<td>IIIC</td>
<td>Serous</td>
<td>–</td>
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<tr>
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<td>9</td>
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<td>74</td>
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<td>Serous</td>
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</tr>
<tr>
<td>11</td>
<td>57</td>
<td>IIIC</td>
<td>Endometrioid</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>48</td>
<td>Ic</td>
<td>Serous</td>
<td>+</td>
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</table>

*In years at the time of primary diagnosis.
†Defined at the time of primary diagnosis.
‡Patients were determined to be HLA-A2+ or HLA-A2− as defined by genotyping and/or serologic analysis of their lymphocytes.

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Characterization of an ovarian tumor cell line derived from the ascites of patient 11. To do functional analyses and to determine molecular mechanisms of the HLA-A2 loss observed in the metastatic tumor cells, a long-term tumor cell line (oval 11) was established by culturing ascitic cells from patient 11 for >3 months. The homogeneous tumor line showed a positive expression of both epithelial antigen and the HER-2/neu proto-oncogene. However, despite high levels of HLA class I surface expression, this cell line completely lacked HLA-A2 expression (Fig. 2).

**Intact and functional APM in the oval 11 tumor cell line.** To delineate the underlying molecular mechanism for the selective loss of HLA-A2 at the cell surface, the constitutive expression of APM components was determined by RT-PCR. As shown in Fig. 3A, constitutive TAP1, TAP2, and β2-microglobulin, but not HLA-A2 mRNA, were found in oval 11 cells. The levels of all the APM components were however lower in the oval 11 tumor sample than in the renal cell carcinoma control sample despite that similar amounts of β-actin were present in both samples (data not shown). The lower levels of APM components were accompanied by lower HLA class I and higher HER-2 cell surface expression on oval 11 cells than on MZ1257RC cells (data not shown).

We next investigated whether the defect in HLA-A2 expression was reversible on IFN-γ treatment that represents a potent method to induce MHC class I antigens and the main APM components (29). Addition of 500 IU/mL IFN-γ to the culture medium for 48 hours significantly increased the levels of total HLA class I antigens but was not able to induce HLA-A2 surface expression in the oval 11 cells (Fig. 3B).

**Haplotype loss in the tumor cells of patient 11.** The lack of constitutive as well as IFN-γ-inducible HLA-A2 expression on the oval 11 cells suggests a structural alteration in the HLA gene complex as the underlying mechanism. Analysis of the HLA genotype shows a complete haplotype loss and lack of the HLA-A*02, HLA-B*08, and DRB1*03 alleles in the line derived from ascitic tumor cells when compared with autologous PBMCs (Table 2).

To determine whether the primary tumor also would exhibit a haplotype loss similar to the one found in the metastatic cancer, the paraffin-embedded autologous primary tumor cells were microdissected and then analyzed for the presence of the HLA-A*02, HLA-B*08, and DRB1*03 alleles by PCR-SSP. There was no evidence of haplotype loss in the primary tumor as intact genes for all six HLA alleles were detectable (Table 2). However, it cannot be excluded that this primary tumor contained a subpopulation of tumor cells, which had lost the expression of a haplotype as was shown to be the case in the ascitic tumors.

**Loss of HLA-A2 is associated with a natural immunity to the HER-2/neu proto-oncogene.** It has been suggested that the outgrowth of tumor escape variants in cancer patients might be caused by CTL-mediated immunologic selection of rare HLA class I loss variants (7, 30). To investigate this possibility, we analyzed the PBMCs of patient 11 for the presence of HLA-A2-restricted T cells recognizing tumor antigens expressed on the autologous metastatic tumor cells. As HER-2/neu was expressed on oval 11 (Fig. 2) and is an immunodominant antigen in patients with ovarian cancer (31–33), our analysis focused on detecting immunity to some of the previously defined MHC class I epitopes from this molecule (34, 35). Indeed, naturally occurring HER-2/neu-specific, HLA-A2-restricted T-cell responses were found in PBMCs from patient 11 as analyzed with peptide-specific IFN-γ ELISpot assays (Fig. 4). Both the two evaluated HER-2 epitopes, HER-2/369 and HER-2/689, were recognized compared with the unstimulated and
control (HIV derived) peptide-stimulated cultures, and importantly, the responses against the proto-oncogene were of the same magnitude as those against an influenza-derived recall peptide. Identical analyses were also done for the only other patient from whom matched PBMCs were available. The ascitic tumor cells from this patient (patient 12) expressed high levels of both total MHC class I and HLA-A2 (Fig. 1). In the PBMCs of patient 12, there was no immunity versus HER-2/neu despite the ability of these cells to

![Figure 1](https://cancerres.aacrjournals.org/article-pdf/66/12/6389/11078712/6389.pdf)

**Figure 1.** Low levels of total HLA class I molecules and heterogeneous expression of HLA-A2 antigens on metastatic ovarian tumor cells. Ascites samples were stained for the cell surface expression levels of HLA class I molecules and analyzed by flow cytometry as described in Materials and Methods. A, left, HLA-ABC antigens (W6/32 FITC; black) versus isotype control (IgG2a FITC; gray) on tumor cells, defined as the large and granular cells positive for epithelial antigen and HER-2 whereas negative for CD14 for all the 12 patients; right, HLA-ABC antigens (W6/32 FITC; black) versus isotype control (IgG2a FITC; gray) on ascitic lymphocytes, defined by forward/side scatter gate, EpCAM, HER-2, and CD14 negativity and CD45 positivity for all the 12 patients. For patient 11, two sequentially collected ascites samples were available. B, HLA-A2 antigens (BB7.2 FITC; black) versus isotype control (IgG2b FITC; gray) on tumor cells (left) and lymphocytes (right) for the nine HLA-A2+ patients. Two ascites samples sequentially collected from patient 11 at an interval of 9 weeks were analyzed and clearly showed a substantial increase in the proportion of HLA-A2+ tumor cells over time.
recognize the HLA-A2-restricted influenza-derived positive control peptide (Fig. 4, bottom). Quite heterogeneous immunologic responses were found in identically treated microcultures, it is however clear that HER-2/neu-specific CTLs were present in patient 11 whereas absent in patient 12. To verify and compare the observed HLA-A2-restricted HER-2/369 and HER-2/689 responses in the PBMCs of patient 11, a modified IFN-γ ELISPOT assay was done. By pooling all identically treated microcultures before redistributing the cells in the ELISPOT plate, the variation between the individual ELISPOT wells decreased (data not shown). In this analysis, the HER-2/369-specific response was found to be significantly stronger than that obtained for unstimulated and control peptide-stimulated cultures ($P < 0.001$), whereas the HER-2/689-specific response did not differ significantly from these controls ($P > 0.01$).

**Discussion**

The present study was motivated by the possibility of conducting T-cell–based immunotherapy trials in patients with advanced ovarian cancer and by recent evidence suggesting that T cells may play a role in the clinical course of ovarian carcinoma (36). As CTL immunity against cancer is severely compromised by HLA class I abnormalities in general (37, 38) and by loss of HLA-A2 in particular, due to the high frequency of this allele among patients (21) and its dominating position as the restriction element in most T-cell–based immunotherapy trials, the study focused on defects in the expression of these molecular complexes. We show for the first time a preferential loss of HLA-A2 expression on the metastatic tumor cells in the majority of ovarian cancer patients and provide the first report describing haplotype loss in this type of cancer.

Only scanty information is available about the mechanisms responsible for an overall poor MHC class I expression on ovarian carcinomas. Soluble factors present locally in the peritoneal cavity and produced by infiltrating lymphocytes or myelomonocytic cells could account for the observed phenotype of most metastatic tumor cells. A recent report described that ascitic plasmacytoid dendritic cells induced IL-10 producing CD8+ regulatory T-cells in ovarian carcinoma patients (39). In addition, a selective expression of IL-10 in ovarian cancer biopsies, as well as significantly elevated levels of this cytokine in ascites fluid, was described (40, 41). Furthermore, IL-10 negatively interferes with the MHC class I pathway by down-regulating both MHC class I antigen and TAP1/2 expression (42, 43). Therefore, IL-10 represents a possible candidate for the observed phenotype of the ascitic tumor cells in some of the patients. We have however failed to observe down-regulation of the surface expression of HLA class I molecules in oval 11 cells as a result of culturing this tumor line in the presence of autologous (patient 11) or allogenic (patients 7 and 8) ascites fluids compared with autologous plasma or human AB-sera from normal donors (data not shown).

The down-regulation or loss of HLA-A2 antigen expression on the metastatic tumor cells in ~70% of the ovarian carcinoma patients motivated an analysis of the underlying mechanism of these deficiencies. The heterogeneity in HLA-A2 surface expression on the tumor cells within the same ascites sample is compatible with a selective outgrowth of tumor variants, which have lost or down-regulated this molecule, as a result of immune selection. In particular, the variation in size of the HLA-A2+ versus the HLA-A2− subpopulations found in the autologous ascites samples sequentially collected from patient 11 supports this hypothesis because the proportion of cells lacking HLA-A2 clearly has increased during disease progression (Fig. 1B).

A potential role of different APM molecules in the observed HLA class I abnormalities of tumor cells from patient 11 was considered. Defects in individual APM components have been described in various types of human tumors, including ovarian carcinoma, renal...
cell carcinoma, and melanoma (14, 37, 44). They include, but are not limited to, defects in expression of TAP1, LMP2, and LMP7 and are mainly caused by regulation of expression rather than structural genetic alterations (14, 45). Because IFN-γ treatment failed to up-regulate HLA-A2, but not total HLA class I antigen expression in the oval 11 cell line, this argues against a deficient APM expression as the underlying mechanism of the impaired HLA-A2 expression. Indeed, constitutive TAP1, TAP2, and β2-microglobulin mRNA expression was found in oval 11 cells, whereas no HLA-A2 mRNA could be detected (Fig. 3). The levels of transcripts of the APM components were in all cases lower in oval 11 cells than in the renal cell carcinoma cell line MZ1257RC. As HER-2 overexpression often correlates with decreased expression of APM molecules (46), we investigated the possibility that the relatively lower levels of APM components in oval 11 correlated with higher HER-2 expression. Indeed, the oval 11 tumor cells expressed significantly higher levels of HER-2 than the MZ1257RC cells (data not shown).

To further define the non-APM-associated mechanism underlying the lack of HLA-A2 expression on the tumor line, we investigated the HLA gene complex. One entire haplotype, including the HLA-A2 locus, was missing in the oval 11 tumor line compared with the autologous PBMCs (Table 2). Among the various mechanisms described to account for loss of MHC class I expression, total or partial deletion of one chromosome 6 has been reported to generate loss of heterozygosity in a variety of human tumors, including melanomas, colorectal, laryngeal, cervical, pancreatic, head and neck squamous cell, and lung carcinomas (9–11, 47, 48). Our finding is the first example of ovarian carcinomas showing haplotype loss, underlining the importance of this molecular mechanism as a tumor escape strategy also for this type of cancer. However, to what extent this relates to the rapid progression of the disease in this patient with steep off-scale increase in CA125 values and death a couple of months after this material was collected remains a speculation.

It has to be addressed whether the autologous primary tumors from these patients have a similar HLA-A2 loss as shown in the metastasizing tumor cells. We therefore did HLA genotyping of primary tumor cells by PCR-SSP. To avoid contamination of the tumor tissue by normal cells (e.g., stromal cells or infiltrating lymphocytes that are highly positive for the HLA class I molecules), all analyses were done on pure tumor cell samples prepared by laser microdissection of the paraffin-embedded primary tumor lesion. The PCR analysis showed that the HLA-A*02, HLA-B*08, and DRB1*03 alleles were all present in the primary tumor of patient 11; hence, it exhibited an intact HLA class I and II gene complex. An enumeration of the proportion of cells in the primary tumor that have lost or down-regulated HLA-A2 would require an analysis at the single-cell level. However, only paraffin-embedded material was available from the primary tumors in this study, and to the best of our knowledge no HLA-A2-specific antibodies, which can be used on these fixed tumor samples, are available.

Because the overall low expression of total MHC class I observed on the majority of ovarian carcinoma tumors seems to be reversible by IFN-γ treatment (Fig. 3; refs. 49, 50), this phenomenon seems to be unrelated to the loss of HLA at the genomic level observed in patient 11. In a recent report, the expression of HLA class I antigens and of TAP1 and TAP2 proteins was investigated on formalin-fixed paraffin sections of primary ovarian carcinoma lesions using Table 2. Haplotype loss in metastatic but not primary tumor cells

<table>
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<tr>
<td>DRB1*</td>
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<td>04</td>
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</tbody>
</table>

NOTE: HLA genotyping of autologous blood mononuclear cells, primary tumor tissue, and metastatic tumor cells (oval 11) from patient 11 was done as described in Materials and Methods.

Figure 4. Naturally occurring T-cell responses specific for the two HLA-A2-restricted HER-2 epitopes, HER-2/369 and HER-2/689, in the PBMCs of patient 11. One of two representative IFN-γ ELISpot assays done as described in Materials and Methods with PBMCs from patients 11 and 12. Reactivities versus the HER-2/369 and HER-2/689 epitopes as well as HLA-A2-restricted positive (influenza derived) and negative (HIV derived) immunogenic control peptides were investigated. The numbers of spots are per the 200,000 PBMCs added to each well.
immunohistochemistry (44). Of interest, this study found down-regulation of HLA class I antigens in 37% of the samples and it was associated with the disease stage. Together with our results, this would motivate an analysis to define whether HLA class I and/or HLA-A2 abnormalities correlate with the presence of CD8+ T cells in primary or metastatic ovarian carcinomas.

An in vitro model to study mechanisms, by which ovarian tumor cells overexpressing HER-2/neu can escape recognition by CD8+ T cells isolated from ovarian carcinoma tumor-infiltrating lymphocytes or tumor-associated lymphocytes, was developed by our group (50). In this model, HLA-A2 surface expression was markedly decreased on the majority of tumor variants produced by in vitro coculturing the ovarian tumor line with autologous tumor-specific CD8+ T-cell clones. It is therefore possible that the decreased HLA-A2 expression levels observed here is dependent on CD8+ T-cell–mediated selection in vitro in analogy with our in vitro model. Tumor-specific CTLs have been detected both in the peripheral blood and locally in the ascites fluid of patients with ovarian carcinoma (51, 52). Of these, HER-2-specific CTLs seem to be a predominant component (23, 53), and HER-2 is an antigen expressed on the majority, if not all, metastatic ovarian tumors (31). Because HLA-A2 expression was lost to various degree in the majority (67%) of the patients, our analyses of the T-cell responses focused on the two HLA-A2-restricted immunodominant HER-2 CTL epitopes, HER-2/369 and HER-2/689, which are naturally presented on ovarian tumor cells (23, 35, 53). Interestingly, HLA-A2-restricted HER-2/369 and HER-2/689-specific T-cell immunity was evident in PBMCs from patient 11 whose tumor cells lacked HLA-A2. Quite heterogeneous immunologic responses were found in identically treated microcultures. Presumably, this was due to the low precursor frequency of peptide-specific CTLs in the lymphocytes obtained from the patients. This lead to a stochastic variation dependent on whether T cells with any particular specificity was present in any given individual microculture. Because each culture initially consisted of 200,000 PBMCs, we can, from the distribution of responses in the microcultures, conclude that the precursor frequency of HER-2/369 and HER-2/689 probably were in the order of one specific T cell in 100,000 PBMCs. Due to the variable responses detected in individual but identically treated cultures, the HLA-A2-restricted HER-2/neu-specific immunity of patient 11 was further investigated. By the use of a modified IFN-γ ELISPOT assay, the HER-2/369-specific response, but not the HER-2/689-specific response, was confirmed to be significantly stronger than those obtained with unstimulated and control peptide-stimulated cultures. In contrast to the situation in patient 11, no HER-2-specific CTL responses were present in PBMCs from patient 12, whose tumor cells had normal total MHC class I and HLA-A2 surface expression. Hence, the T-cell responses were associated with loss of the HLA-A2 expression caused by the haplotype loss. These findings suggest that MHC class I loss variants are directly selected for by tumor-specific CTLs during tumor progression.

Our findings underline the importance of characterizing the HLA class I expression on tumor cells from patients to be included in immunotherapy trials. The majority of peptide-based trials use HLA-A2-restricted epitopes, and our study suggests that most HLA-A2+ patients with advanced ovarian carcinomas would not benefit from this type of treatment. However, a detailed characterization of the molecular mechanism responsible for the HLA loss in each patient is essential. Strategies aimed at correcting regulatory defects in HLA class I expression on ovarian cancer cells, which do not have structural alterations of the HLA class I gene complex, should improve T-cell–based immunotherapy.

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Håkan Norell, Mattias Carlsten, Tomas Ohlum, et al.


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