Increased HLA-DMB Expression in the Tumor Epithelium Is Associated with Increased CTL Infiltration and Improved Prognosis in Advanced-Stage Serous Ovarian Cancer

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Abstract Purpose: To evaluate the possible mechanisms influencing the infiltration of CD8 T lymphocytes into the tumor epithelium of advanced-stage serous ovarian cancers. Experimental Design: Immunohistochemical localization of CD8 T lymphocytes was done on a homogeneous population of 184 high-grade, advanced-stage serous ovarian cancer tissue specimens. Microarray analysis was done on microdissected tumor epithelium from 38 specimens to identify genes up-regulated or down-regulated in specimens with differing numbers of tumor-infiltrating CD8 T lymphocytes. Quantitative real-time PCR and immunohistochemistry were used to validate a candidate gene. Univariate and multivariate survival analyses were done combining CD8 T lymphocyte number and HLA-DMB expression with standard prognostic factors. Results: Marked CD8 T lymphocyte infiltration of the tumor epithelium is associated with a 20-month improvement in median overall survival. Additionally, when combined with cytoreduction status and age, CD8 T lymphocyte status is an independent prognostic factor for survival. Microarray analysis showed HLA-DMB, a component of the MHC II antigen presentation machinery, to be differentially expressed in specimens with an abundance of tumor-infiltrating CD8 T lymphocytes. This relationship was validated at both mRNA and protein levels. As well, high HLA-DMB expression in the tumor epithelium was associated with a significant improvement in median overall survival in both univariate and multivariate analyses. Conclusions: Tumor cell expression of HLA-DMB is associated with increased numbers of tumor-infiltrating CD8 T lymphocytes and both are associated with improved survival in advanced-stage serous ovarian cancer.

Epithelial ovarian cancer accounts for more deaths annually than all other gynecologic cancers combined (1). Although early-stage disease has a 5-year survival that approaches 95% (2), most cases are diagnosed at an advanced stage (2). With cytoreductive surgery and chemotherapy, greater than half of such patients will achieve remission, but most will experience recurrence and ultimately die of their disease (2). Therefore, therapies complementary to standard surgery and chemotherapy are sought to improve long-term survival in advanced-stage epithelial ovarian cancer. The immune system has long been noted to have an antitumor effect (3). Tumor-infiltrating CTLs (CD8) are associated with improved overall survival and have been described in several solid tumors including ovarian, endometrial, colon, and esophageal cancers (4–8). Zhang et al., analyzing CD3 T lymphocyte infiltration in 174 advanced-stage ovarian cancers, noted improved survival in tumors with infiltrating CD8 T lymphocytes (4). Equivalently, Sato et al. showed CD8 T lymphocyte infiltration in the ovarian tumor epithelium is associated with prolonged overall survival (5). Prior reports examining CD3 and/or CD8 T lymphocyte infiltrates in epithelial ovarian cancer included very heterogeneous populations, often incorporating all histologic subtypes...
and stages of disease. Furthermore, tumor intrinsic and extrinsic factors that may be associated with increased tumor-infiltrating CD8 T lymphocytes have not been completely identified.

The present study was undertaken to better understand the molecular mechanisms underlying differences in tumor-infiltrating CTLs. Differential transcription profiling was done by microarray analysis on mRNA isolated from the microdissected epithelium of serous ovarian cancer. HLA-DMB gene expression strongly correlated with the number of tumor-infiltrating CD8 T lymphocytes. HLA-DM protein is also directly related to antigen presentation and lymphocyte activation. The expression of HLA-DM was further evaluated. HLA-DRA was also analyzed on ovarian cancer cell lines and paraffin sections to study the possibility of an existing MHC II antigen presentation machinery in ovarian cancer cells.

**Materials and Methods**

**Study patients.** One hundred eighty-four paraffin-embedded and frozen ovarian cancer tissue samples were used. All ovarian cancer tissues were of the serous subtype, high grade, and International Federation of Gynecology and Obstetrics stage IIIB to IV (advanced stage). Tissues were collected from patients undergoing primary cytoreductive surgery for ovarian cancer at the Brigham and Women’s Hospital between 1990 and 2006. After surgery, patients received platinum-based combination chemotherapy. All patients for the survival study received paclitaxel in combination with a platinum as the bulk of the cases accessioned were after 1999, when paclitaxel was already in common usage in clinical practice. Optimal surgical cytoreduction was defined by residual tumor ≤ 1 cm in diameter. The duration of overall survival was measured from the date of diagnosis to death or censored at the date of last follow-up. Clinical data including age, cytoreduction status (optimal versus suboptimal), and overall survival were abstracted from the medical record. All specimens and clinical data were collected under approval of the institutional review board.

**Sample size analysis.** Using 0.05 as \( \alpha \) value and 0.80 as desired power, 160 samples are required for the multivariate Cox proportional hazards model studies.

**Sample collection and preservation.** All samples were collected from primary surgeries and tumor tissues were processed with standard pathology paraffinization method to make paraffin-embedded blocks. Fresh tumor tissues from the same patients were also frozen in Tissue-Tek OCT compound (Sakura Finetek USA) and stored at -80°C till frozen sections were made for laser microdissection.

**Immunohistochemistry.** Immunolocalization of tumor-infiltrating CD8 T lymphocytes was done \((n = 184)\) using monoclonal mouse, anti-human CD8 (clone C8/144B, 1:50 dilution; DAKO). Paraffin-embedded sections were deparaffinized and dehydrated, and antigen

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**Fig. 1.** A, high-grade, advanced-stage serous ovarian cancer immunohistochemically stained for CD8 T lymphocytes and HLA-DMB. Marked CD8 T lymphocyte (red) infiltration in the tumor epithelium (>×10). B, scant CD8 T lymphocyte infiltration in the tumor epithelium (>×10). C, high expression of HLA-DMB (red) in the tumor cell cytoplasm (>×10). D, low expression of HLA-DMB (>×10).
retention was done in Target Retrieval Solution (DAKO) with the pressure cooker at 120°C for 20 min. Alternatively, frozen sections were fixed in methanol for 10 min. After blocking, sections were incubated with the primary antibody (anti-CD8) at room temperature for 90 min and then washed twice with 1× TBS (Boston BioProducts) and incubated with Polymer-AP (DAKO) for 30 min. CD8⁺ signals were visualized using Fast Red (DAKO).

Immunolocalization of tumor-infiltrating CD4 T lymphocytes was done on a subset (n = 24) of high-grade, advanced-stage serous ovarian cancer specimens using monoclonal mouse, anti-human CD4 (prediluted; Abcam). CD4⁺ signals were visualized using Envision G2 System/AP kit (permanent red; DAKO).

Immunolocalization of HLA-DM was done on a subset (n = 64) of paraffin-embedded, high-grade, advanced-stage serous ovarian cancer specimens. After deparaffinization and dehydration, antigen retrieval was done in Target Retrieval Solution (DAKO) with the pressure cooker at 120°C for 10 min. After blocking, tissue sections were incubated with monoclonal mouse, anti-human HLA-DMB (clone 6B3, 1:50 dilution; Novus Biologicals) at room temperature for 2 h and washed two times, and HLA-DMB⁺ signals were visualized with the Envision G2 System/AP kit (permanent red; DAKO).

Quantification of immunohistochemistry. The number of CD8 and CD4 T lymphocytes infiltrating the tumor epithelium was determined by counting manually the number of positive cells in four to eight high-power fields (x 20 objective) from each case. The counts from each high-power field were averaged, thus creating a mean number of tumor-infiltrating CD8 or CD4 T lymphocytes per specimen. Each specimen was analyzed by a single investigator (M.C.), with random specimens being evaluated by a second investigator (S.M.) for quality control.

HLA-DM protein expression was quantified using Image-Pro Plus 5.1.0.20 for Windows (Media Cybernetics). One to two sections per case were analyzed quantitatively. The staining saturation was measured from three fixed-size areas in the tumor epithelium and one background area in the tumor stroma. The difference between each epithelial and the stromal measurement was obtained and the values averaged yielding one score for each case.

Immunofluorescence. A subset of five high-grade, advanced-stage serous ovarian cancer specimens with high numbers of tumor-infiltrating CD8 T lymphocytes were selected for double immunofluorescent staining with rabbit, anti-human CD8 (prediluted; Abcam) and mouse, anti-human HLA-DR (clone LN-3, 1:50 dilution; Vision Biosystems, Novocastra). After deparaffinization, dehydrogenation, and antigen retrieval, blocking was done with 10% normal goat serum diluted in 1× TBS (Boston BioProducts). Next, the specimens were incubated with the first primary antibody (anti-CD8) for 60 min at room temperature and washed twice, and Alexa Fluor 647 (Molecular Probes) goat, anti-rabbit IgG (1:200 dilution) was applied for 60 min at room temperature. After washing, the specimens were incubated with the second primary antibody (anti-HLA-DR) for 60 min at room temperature and washed twice, and Alexa Fluor 546 (Molecular Probes) goat, anti-mouse IgG (1:200 dilution) was applied for 60 min at room temperature. Nuclear staining with 4,6-diamidino-2-phenylindole (Molecular Probes) was done. Fluorescent signals were visualized with a Leica IRE2 fluorescent microscope.

Microarray analysis. cRNA from 38 of the high-grade, advanced-stage ovarian cancer tissue samples (training set) was prepared according to the Affymetrix Expression Analysis Technical Manual (Affymetrix) and hybridized to the arrays as described previously (9). Global normalization at a target value of 500 was applied to all of the arrays under consideration using GeneChip Operating Software (Affymetrix), and the normalized data were uploaded into the National Cancer Institute Microarray Analysis Database (9). Biometrics Research Branch ArrayTools version 3.6 was used for statistical analysis of the array data. Probe sets scored as absent (A) at xᵢ = 0.05 or marginal (M) at xᵢ = 0.005 were excluded from the analysis. In addition, only those transcripts present in >50% of the arrays and displaying a variance in the top 50th percentile were evaluated. Using these filtering criteria, 14,657 probe sets were tested by a Pearson correlation test employing a random variance model. Signal intensities across the 38 specimens were correlated with the number of CD8⁺ cells per x 20 field. Probe sets possessing a significant correlation coefficient (P < 0.01) were considered further.

Quantitative real-time PCR. Quantitative real-time PCR was done to evaluate HLA-DMB (n = 32) and HLA-DRA (n = 32) mRNA expression in the microdissected high-grade, advanced-stage serous tumor epithelium of cases in the training set. Quantitative real-time PCR was done using the 7300 Real-time PCR System and TaqMan Gene Expression Assay probes (Applied Biosystems). For each quantitative real-time PCR, the primer of interest and cyclophilin A (an endogenous control) were multiplexed as follows: a total of 2 μL cDNA was added to 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL L1 TaqMan primer/probe, 1 μL cyclophilin A TaqMan primer/probe, and 6 μL autoclaved distilled water. Reactions started with a 10 min hold at 95°C followed by 40 cycles of denaturation at 95°C for 15 s followed by...
**Results**

**Tumor-infiltrating CD8 T lymphocytes are a predictor for improved overall survival.** A total of 184 high-grade, advanced-stage serous ovarian cancer samples were analyzed for tumor-infiltrating CD8 T lymphocytes (Fig. 1A and B). Cytoreduction data were available on 180 patients, 144 (80%) of which were optimally cytoreduced. The median age of the patients was 59.5 years (range, 20-95).

For CD8 Kaplan-Meier analysis, patients were divided into two groups (marked versus scant CD8 T lymphocytes infiltration) using the 75th percentile of the CD8 counts as the cut point. Patients with marked infiltration of the tumor epithelium with CD8 T lymphocytes (n = 46) had a median overall survival of 50.0 months [95% confidence interval (95% CI), 23.9-76.1 months], whereas those with scant infiltration (n = 138) had a median overall survival of 30.0 months (95% CI, 23.6-36.4 months; P = 0.02; Fig. 2A).

In a proportional hazards model, with cytoreduction and age adopted as covariates, tumor-infiltrating CD8 T lymphocyte number was an independent prognostic factor for overall survival. This relationship held true regardless of whether CD8 T lymphocyte count was analyzed as a continuous or dichotomous variable (Table 1). The 50th, 66th, and 75th percentiles for tumor-infiltrating CD8 T lymphocyte number were analyzed separately as dichotomous variables in the proportional hazards model. The 75th percentile cut point for tumor-infiltrating CD8 T lymphocyte number produced the most clinically significant hazard ratio (data not shown for 50th and 66th percentiles) and thus was used in the above Kaplan-Meier analysis and Cox proportional hazards model. Additionally, the hazard ratio for the CD8 dichotomous variable was less than that of the continuous variable, implying there may be a critical threshold of CD8 T lymphocytes necessary in the tumor epithelium to meaningfully affect overall survival. Interestingly, older age was related to fewer numbers of tumor-infiltrating CTLs, but the correlation did not reach statistical significance (Spearman’s rs = -0.130; P = 0.08). Additionally, the number of tumor-infiltrating CD8 T lymphocytes was not related to achievement of optimal cytoreduction (P = 0.52, Mann-Whitney U test).

**Differential expression of HLA-DMB mRNA is positively correlated with tumor-infiltrating CD8 T lymphocytes.** Microarray analysis of microdissected tumor epithelium (n = 38) showed multiple genes whose expression significantly correlated with the tumor-infiltrating CD8 T lymphocyte number (Fig. 3). HLA-DMB, a gene whose protein product is involved in the MHC class II antigen presentation pathway, was selected for validation. Additionally, HLA-DMB expression in the samples with greater numbers of tumor-infiltrating CD8 T lymphocytes was a finding common to microarray analysis by all normalization methods (data not shown). Differential HLA-DMB mRNA expression in the tumor epithelium (training set), evaluated by quantitative real-time PCR, positively correlated with increased numbers of tumor-infiltrating CD8 T lymphocytes (Pearson’s rs = 0.741; P < 0.001). In a separate validation set of 25 microdissected high-grade, advanced-stage serous ovarian cancer specimens, HLA-DMB mRNA expression in the tumor epithelium positively correlated with

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 number as a continuous variable</td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
<td>1.013 (0.998-1.028)</td>
<td>0.092</td>
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<tr>
<td>Optimal cytoreduction</td>
<td>0.565 (0.370-0.862)</td>
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<tr>
<td>Tumor-infiltrating CD8 T lymphocytes (continuous)</td>
<td>0.962 (0.935-0.989)</td>
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<tr>
<td>CD8 number as a dichotomous variable, cut point at 75th percentile</td>
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<tr>
<td>Age</td>
<td>1.013 (0.998-1.028)</td>
<td>0.098</td>
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<tr>
<td>Optimal cytoreduction</td>
<td>0.604 (0.396-0.920)</td>
<td>0.019</td>
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<tr>
<td>Tumor-infiltrating CD8 T lymphocytes (dichotomous)</td>
<td>0.577 (0.373-0.893)</td>
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<tr>
<td>HLA-DMB expression as a dichotomous variable, cut point at 33rd percentile</td>
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<td></td>
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<tr>
<td>Age</td>
<td>1.004 (0.976-1.033)</td>
<td>0.775</td>
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<tr>
<td>Optimal cytoreduction</td>
<td>0.603 (0.265-1.373)</td>
<td>0.229</td>
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<tr>
<td>HLA-DMB expression (dichotomous)</td>
<td>0.470 (0.222-0.996)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

*Multivariate analysis of prognostic factors for overall survival in high-grade, advanced-stage serous ovarian cancer (CD8 number as a continuous variable).  
†Multivariate analysis of prognostic factors for overall survival in high-grade, advanced-stage serous ovarian cancer (CD8 number as a dichotomous variable, cut point at 75th percentile).  
‡Multivariate analysis of prognostic factors for overall survival in high-grade, advanced-stage serous ovarian cancer (HLA-DMB expression as a dichotomous variable, cut point at 33rd percentile).
tumor-infiltrating CD8 T lymphocyte number (Spearman’s $\rho = 0.416$; $P = 0.04$).

**Increased HLA-DMB protein expression in the tumor epithelium is associated with increased overall survival.** Using Kaplan-Meier analysis, patients with high HLA-DMB expression in the tumor epithelium had a significantly greater median overall survival than patients with low expression of HLA-DMB [59.0 months (95% CI, 33.4-84.6) versus 27.0 months (95% CI, 10.9-43.1); $P = 0.003$; Fig. 2B].

In a proportional hazards multivariate analysis with cytoreduction status and age adopted as covariates, high HLA-DMB expression was associated with an improved overall survival (hazard ratio, 0.47; 95% CI, 0.22-0.996; $P = 0.05$; Table 1).

**Expression of HLA-DMB and invariant chain mRNA in the tumor epithelium correlate positively with HLA-DRA mRNA expression.** After showing a differential expression of HLA-DMB in advanced-stage serous ovarian cancers with greater numbers of tumor-infiltrating CD8 T lymphocytes, we investigated the expression of other genes involved in the MHC class II antigen presentation pathway. Expression of HLA-DRA mRNA in the tumor epithelium correlated positively with HLA-DMB mRNA expression in the tumor epithelium of high-grade, advanced-stage serous ovarian cancers (Spearman’s $\rho = 0.415$; $P = 0.02$). Additionally, a subset of cases was evaluated for invariant chain (II) mRNA expression and its expression positively correlated with HLA-DRA mRNA expression (data not shown).

Tumor-infiltrating CD8 T lymphocyte number correlated positively with tumor-infiltrating CD4 T lymphocyte number (Spearman’s $\rho = 0.428$; $P = 0.04$) and HLA-DRA mRNA expression (Spearman’s $\rho = 0.353$; $P = 0.05$). However, tumor-infiltrating CD4 T lymphocyte number did not correlate with the mRNA expression level of any of the above genes.

**HLA-DR is expressed by the tumor epithelial cells and also colocalizes to CD8 T lymphocytes.** Using immunofluorescence technique in a subset of five high-grade, advanced-stage serous ovarian cancer cases, HLA-DR was noted to be expressed by the tumor epithelial cells and localized to the cell membrane (Fig. 4). Additionally, tumor-infiltrating CD8 T lymphocytes were noted to express HLA-DR, indicating their status as activated CTLs (Fig. 4; refs. 12, 13).

**Discussion**

The present study confirms that increased tumor-infiltrating CTLs are associated with improved overall survival and also presents a new candidate gene, which might have a role in increasing the number of the tumor-infiltrating lymphocytes and therefore improve survival in serous ovarian cancer patients.

In our study, using Kaplan-Meier analysis with a 75th percentile cut point, patients with marked numbers of tumor-infiltrating CD8 T lymphocytes had a 20-month improvement in overall survival. The improvement in overall survival held in a multivariate analysis indicates that tumor-infiltrating CD8 T lymphocytes are an independent prognostic factor in advanced-stage serous ovarian cancer.
As shown in this and previous studies, the presence of tumor-infiltrating T lymphocytes, specifically CTLs (CD8), confers an improved overall survival in many different epithelial tumors (4–8). However, the underlying molecular mechanisms that promote or inhibit the infiltration of CTLs is not fully understood. In our sample of 38 microdissected advanced-stage serous ovarian cancers, HLA-DMB was positively differentially expressed in the tumor epithelium of patients with an abundance of tumor-infiltrating CTLs. Furthermore, a significant positive correlation between the differential expression of HLA-DMB mRNA in the tumor epithelium and the number of tumor-infiltrating CD8 T lymphocytes was noted both in the training set and in a separate validation set of specimens. This relationship held true at the protein level as well, implicating ovarian cancer epithelial cells in the process of antigen presentation.

Normal adult epithelial cells are not thought to express MHC class II molecules, such as HLA-DR and HLA-DM, both of which are composed of α and β subunits. However, various epithelial tumor cells have been shown to express HLA-DR molecules and their presence is associated with an improved prognosis (14–16). Additionally, in breast cancer, expression of HLA-DM and its cooperating molecules, HLA-DR and Ii, is associated with a helper CD4 T lymphocyte-associated response and improved survival (16). HLA-DM enhances antigen presentation by catalyzing the removal of class II-associated Ii peptide from the antigen-binding groove of HLA-DR molecules (17). Class II-associated Ii peptide is the product of cleavage of the Ii, which occupies and stabilizes HLA-DR molecules after their synthesis and assembly (18). By catalyzing the dissociation of class II-associated Ii peptide from the binding groove of HLA-DR, HLA-DM allows efficient antigen peptide loading onto HLA-DR for presentation at the cell membrane to helper CD4 T lymphocytes (18, 19).

A robust and effective cytotoxic CD8 T lymphocyte immune response requires the aid of helper CD4 T lymphocytes (20). In vitro and in vivo experiments have shown that tumor cells may be converted into efficient antigen-presenting cells by expression of MHC class II components and inhibition of the Ii (21). Consequently, they are able to present endogenous tumor antigen to CD4 helper T lymphocytes (21). The present study shows that advanced-stage serous ovarian cancers with an abundance of tumor-infiltrating cytotoxic CD8 T lymphocytes differentially express increased levels of HLA-DMB in the tumor epithelium. HLA-DM serves to catalytically replace class II-associated Ii peptide (cleavage product of Ii) with antigen peptide. Hence, increased expression of HLA-DM may be functionally similar to inhibition of the Ii and enhance antigen peptide presentation by HLA-DR. Additionally, we found that HLA-DMB and Ii mRNA expression both correlate positively with HLA-DRA mRNA expression in the tumor epithelium and HLA-DR localizes to the tumor epithelial cell membrane. Consequently, it may be hypothesized that certain serous ovarian cancer epithelial cells, coordinately expressing these three critical components of the MHC class II antigen presentation pathway, are able to present endogenous tumor antigen to helper CD4 T lymphocytes and, by extension, activate an efficient and robust CD8 cytotoxic immune response.

However, the present study cannot define whether the differential expression of HLA-DMB by the tumor epithelial cells leads, by extension, to a marked CD8 T lymphocyte response, or vice versa. It is plausible that tumor-infiltrating CD8 T lymphocytes may induce the expression of MHC class II components, such as HLA-DM, HLA-DR, and Ii, in tumor epithelial cells by the production and secretion of IFN-γ (22). Therapeutic up-regulation of the IFN-γ production of T lymphocytes might increase HLA-DM, HLA-DR, and Ii expression in ovarian tumor cells, which might contribute to better survival in selected patients.
In summary, the present study serves to begin to elucidate the molecular mechanisms that may underlie the differential numbers of tumor-infiltrating CD8 T lymphocytes in advanced-stage serous ovarian cancer. Further study will be necessary to evaluate the potential antigen-presenting capabilities of serous ovarian cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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