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Systematic evaluation of multiple immune markers reveals prognostic factors in ovarian cancer

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HIGHLIGHTS

- Cytotoxic CD8+ T cells and CD20+ B cells play a critical role in ovarian cancer.
- Markers for tumor infiltrating lymphocyte homing and function have survival benefits.
- Presence of HLA Class II staining translates directly to ovarian cancer prognosis.

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ABSTRACT

Objective. Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. Several factors prognostic for survival have been identified including the presence of certain lymphocyte markers. Tumor-infiltrating lymphocytes (TILs), particularly cytotoxic CD8+ TILs, have been shown to be most favorable for prognosis in ovarian cancer, although other immune cells including CD3+ T-cells, CD4+ T-cells, and B-cells have also demonstrated survival benefits. Although data for these markers exists, results are not uniform in the literature. Furthermore, other immunomodulatory protein markers that have been targeted in effective immunotherapies for other malignancies may prove to be favorable in ovarian cancer.

Methods. Here, extensive immunohistochemical analysis was performed on a tissue microarray, containing 135 ovarian cancer cases obtained during tumor debulking detecting 15 key lymphocyte markers such as CD3, CD4, and CD20, as well as activation and immunomodulatory molecules such as TIA-1 and PD-L1. Samples were analyzed for expression of markers in tumor islets or stroma and expression was correlated with overall survival, histotype, stage, age, debulking grade, and response to chemotherapy.

Results. Our results confirm the presence of CD8+ and CD20+ TILs is positively correlated with overall survival, with further multivariate modeling replicating that prognostic benefit. Additional markers of significant prognostic importance, including TIA-1, CD103 and HLA Class-II were also revealed.

Conclusions. Our results further support the vital role of cytotoxic T-cells in defense against ovarian cancer and reveals new questions as to the role of B-cells in tumor control as well as the potential benefits of immunotherapy involving other immune modulating molecules.

1. Introduction

Ovarian Cancer is the most lethal gynecologic malignancy in the United States with over 14,000 deaths annually [1]. Outcomes remain quite poor despite optimal therapy as most patients present in advanced stages of disease. There are, however, a number of positive prognostic factors in ovarian cancer. For example, large-scale analyses of late stage ovarian cancer treated with chemotherapy in 428 and 1895 patients found that age, serous histology, and extent of residual disease were predictors of overall survival [2,3]. In addition to patient, cancer and treatment related factors, the presence of distinct leukocyte subsets has been linked to positive prognosis in late stage ovarian cancer. Tumor infiltrating lymphocytes (TILs) are leukocytes that infiltrate a tumor in attempt to survey, target and destroy tumor cells. CD8+ cytotoxic TILs have been shown to be the immune cell subset that is most favorably associated with improved prognosis in ovarian cancer [4,5], although other immune cell subsets including CD3+ T-cells [6],

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CD4 + T-cells [7], and B-cells [8,9] have demonstrated survival benefits. While these survival benefits are well-noted, a number of contradictory studies show no survival benefit or worse outcomes with the presence of these immune cells [8].

Morphological evidence of active immunity in ovarian cancer is supported by multiple, yet oft anecdotal, observations that leukocyte activation-associated molecule expression is associated with patient survival. Naïve T-cells experiencing antigens undergo dynamic phenotypic and functional change including the up-regulation of activation-associated molecules and mobilization of effector molecules. For instance, CD45RO is widely accepted as a surface marker for antigen-experienced memory lymphocytes [10] that can be found on a subset of T-cells in ovarian cancer [8]. CD103, a marker for resident memory TILs, as well as a subset of dendritic cells in lymph nodes and gut mucosa, has been shown to correlate with increased survival in high-grade ovarian cancers [11]. Additionally, CD137, a TNF receptor family member, is up-regulated upon T-cell activation and is a biomarker of tumor-reactive TILs with capacity for tumor destruction [12] though its prognostic importance is not known. Granzyme-B is a protease found in cytotoxic lymphocytes that can mediate target apoptosis and granzyme-B infiltrating lymphocytes may be associated with improved outcomes in advanced, treated ovarian cancer [13]. Similarly, TIA-1 is a cytoplasmic granule-associated protein expressed on cells with cytolytic potential found in the ovarian cancer microenvironment that has also shown prognostic value [8].

While immune activation is evident in some ovarian cancers, a host of immunosuppressive elements exist to thwart immune attack and promote tumor progression. FOXP3 is the defining marker for immunosuppressive regulatory T-cells [14], cells responsible for enforcing host tolerance and capable of dampening antitumor immunity, and is associated with poor prognosis in ovarian cancer [15,16]. CD68 + tumor-associated macrophages are associated with poor or no survival benefit in ovarian cancer [8,17]. Suppressive ligands and receptors are also prevalent in the tumor microenvironment. PD-1 is a negative immunoregulatory molecule with activation-induced expression on T-cells that binds to its ligands, PD-L1 and PD-L2, on tumors and accessory cells, that can suppress and inhibit the ability of TILs to destroy tumors [18–20]. Similarly, CTLA-4 is a key negative regulator of immune responses, maintaining peripheral tolerance in T-cell responses and has been used as a target in a number of immunotherapies [21]. TIM-3 has been shown to play a role in suppressing antitumor activity and is a negative prognostic indicator in renal cell carcinoma [22], and its blockade may improve anti-tumor activity in an ovarian cancer murine model [23]. B7-H4 is over-expressed in ovarian cancers and acts as an immunemodulatory protein arresting T-cell proliferation and cytokine secretion; its expression on tumor-associated macrophages and tumor cells is linked to poor staging and negative outcomes [24,25]. Similarly, BTLA, a B and T-lymphocyte attenuator, has been shown to be a negative co-stimulatory molecule in melanoma; however, its expression is associated with TIL-mediated tumor regression [26].

There are a number of proteins expressed by tumor cells, tumor-associated stroma or induced by tumors that can interact with immune cells and lead to changes in immune attack against tumors. Major histocompatability complex (MHC) Class-I molecules are found on nearly every nucleated cell in the human body, and act to display endogenous antigens to T-cells. Ovarian tumors are prone to down-regulation of MHC Class-I as a mechanism of escaping immune system recognition [27]. Higher MHC Class-I expression on ovarian tumor cells correlates with TIL infiltration and improved ability to expand TILs ex-vivo [28]. MHC Class-II molecules are expressed on antigen presenting cells to present exogenous antigens, and high Class-II expression is correlated with increased survival and number of TILs in ovarian cancer specimens [29]. Beyond cancer cells themselves, endothelin-B receptor serves as an endothelial barrier to T-cell homing into tumors and may contribute to failures of immunotherapy [30]. Finally, indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the degradation of tryptophan and induces immune tolerance; high expression in ovarian tumors is correlated with a reduced number of CD8 + TILs and poor survival [31].

We hypothesize that expression of cytotoxic T-cell markers (namely CD8, CD137, CD103 and TIA-1) will correlate with survival while markers of immune suppression and evasion (e.g. PD-1 and CTLA-4) will correlate with poor patient survival. To address this hypothesis and address the limited available data in the literature on markers vital to future immunotherapies, we performed immunohistochemical analysis to assess expression of the aforementioned immune markers on ovarian cancer patient samples obtained during debulking. Expression of each immune marker was analyzed and assessed for its correlation with 1, 5 and 10-year survival, overall survival, time to recurrence, histotype, stage, age, debulking grade, and response to chemotherapy.

2. Methods

2.1. Immunohistochemistry (IHC) analysis

Formalin-fixed, paraffin embedded (FFPE) tumor blocks from a total of 135 primary epithelial ovarian carcinoma patient samples with 80 case-matched metastases obtained under an IRB-approved tumor collection protocol of University of Turin, Italy, from December 1991–December 2005 (Table 1). The analysis was performed with IRB approval from previously untreated patients undergoing debulking surgery. All specimens were processed in compliance with institutional review board of University of Turin and Health Insurance Portability and Accountability Act (HIPAA) requirements. Both primary tumor samples and matched metastatic deposits from the same patients were included in a tumor tissue microarray (TMA). For each block, triplicate 0.6-mm cores of tumors and metastases were placed on a TMA slide with control tissues from colon, kidney, ovary, tonsil, and spleen (Fig. 1).

IHC staining of FFPE tissue was performed on a Leica Bond™ instrument using the Bond Polymer Refine Detection System or standard manual techniques as described in Supplementary Methods. Antibodies specific for the following 21 proteins were selected for the use in IHC: CD3, CD4, CD8, CD20, CD45RO, CD68, CD103, CD137, granzyme-B, FOXP3, PD-1, IDO, HLA-ABC, HLADR, TIA-1, PD-L1, CTLA-4, B7-H4, endothelin-B, BTLA, and TIM3.

Table 1
Demographic data for 135 patients enrolled in study analysis.

Number of patients	135
Median age at surgery (years)	58 (range 33–88)
Median survival (months)	63 (range 0–194)
FIGO stage	
I	31 (23%)
II	8 (6%)
III	87 (64%)
IV	9 (7%)
Surgical debulking status	
Optimal debulking	80
Suboptimal debulking	50
Histology	
Serious	89
Endometroid	38
Clear cell	8
Response to chemotherapy	
CR	94
PR	29
NC	1
PD	8

Note that 1 patient is missing debulking status and 3 patients are missing status of response to chemotherapy.