

Immune prognostic factors in ovarian cancer: Lessons from translational research

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1. Introduction

Tumor-host interactions and the tumor micro environment have emerged over the past decade as important factors in tumor growth and progression. Experimental evidence in animal tumor models has demonstrated the ability of the host to recognize and attack tumors, as well as the ability of tumors to evade such response [1]. It is now generally accepted that human cancer cells express unique tumor-specific antigens that in select patients may be recognized by the immune system. From previous research in melanoma we recognize that such antigens may be characterized as (i) tumor-specific proteins that are normally absent in adult normal tissues except for germline cells; (ii) tissue-specific differentiation proteins that are normally not expressed within a specific organ; (iii) mutated proteins; (iv) overexpressed proteins; or (v) unmasked protein epitopes. Recent work with adoptive T cell therapy in melanoma patients demonstrates that spontaneously occurring tumor-reactive T cells are directed against tumor antigens and that *ex vivo* expanded tumor-derived T cells can reject tumors when administered following host conditioning. In addition, the presence of tumor-infiltrating lymphocytes in melanomas has been demonstrated to be an independent prognostic factor for metastasis and survival [2].

Although antigen characterization has not been systematic in epithelial ovarian carcinoma (EOC), there is

sufficient evidence that tumor-associated antigens are present. Shared by ovarian cancer cells and cerebellar Purkinje cells, HLA A2-restricted onconeural protein cdr2 is the best differentiation tumor rejection antigen identified to date, and its recognition by cytotoxic lymphocytes (CTL) is associated with paraneoplastic cerebellar degeneration (PCD). Interestingly, in patients presenting with PCD, where CTL are effective, ovarian cancers are clinically occult [3]. Other antigens identified in ovarian cancer include HER-2 protein, the product of *c-erBb-2* oncogene; p53 tumor suppressor gene protein product; topoisomerase-II α ; folate binding protein; amino enhancer of split protein; sialylated TN (sTN), a mucin antigen; MUC-1; NY-ESO-1, a testis differentiation antigen; and mesothelin (reviewed in [4]). Universal tumor antigens such as the human telomerase reverse transcriptase (hTERT), cytochrome P450 CYP1B1 and survivin [5] are also expressed by EOC [6,7]. Tumor-specific T cells secreting interferon-gamma (IFN- γ) were recently reported in peripheral blood of patients with advanced stage ovarian carcinoma, indicating that tumor antigens are in fact recognized spontaneously *in vivo* [8].

Until recently, the role of the immune system in the natural course of ovarian cancer remained unknown. However, since there is evidence that ovarian cancers express antigens and that some cancers harbor a brisk leukocyte infiltrate [9–11], it is possible that immune mechanisms might affect the clinical outcome

of patients with ovarian cancer and provide important biomarkers for disease classification. Below we will summarize the work to date on immune biomarkers in ovarian cancer as they have been discovered from our laboratory and other investigators.

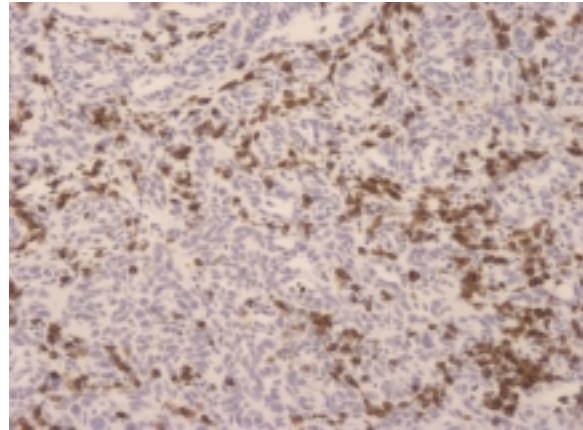
2. Cellular markers relevant to tumor immune recognition and attack

2.1. Effector T cells

T cells bearing the $\alpha\beta$ T cell receptor are the dominant adaptive immune effector cells that target human ovarian tumors. T cells have been detected in both solid tumor nodules and in ascites. In solid tumors, T cells have been observed within tumor cell islets (*intra-tumoral* or *intraepithelial* T cells) and/or the stroma surrounding tumor islets (Fig. 1) [12]. Approximately 50% of ovarian tumors lack intraepithelial T cells; however most of these tumors have T cells in the stroma surrounding tumor islets.

The presence of intraepithelial CD3⁺ T cells within tumor cell islets was evaluated as a prognostic factor for overall survival [12]. This study included 174 evaluable patients who had stage III or IV ovarian carcinoma. Of these, 78% were grade 3; 30% of the patients were optimally debulked (< 1 cm residual tumor nodules); and 41% of these women had complete response to chemotherapy. The overall five-year survival rate for these patients was 25.3%. The survival curves for patients with and without intraepithelial T cells were significantly different (Fig. 2, top, $P < 0.0001$); the five-year overall survival rate was 38% in patients whose tumors had intraepithelial T cells and it was 4.5% in patients whose tumors had no intraepithelial T cells. Among patients with complete response to chemotherapy, the survival curve for patients with tumors that had intraepithelial T cells was also significantly different from the survival curve of those with tumors that did not have intraepithelial T cells (Fig. 2, bottom, $P < 0.0001$); the five-year overall survival rates for the patients with complete response and tumors with and without intraepithelial T cells were 73.9% and 11.9%, respectively. Among these patients, significant differences in progression-free survival and overall survival distributions were seen both in suboptimally and optimally debulked patients based on the presence or absence of intraepithelial T-cells ($P < 0.001$ all). Among patients with complete response to therapy following suboptimal debulking, five-year overall survival was

A



B

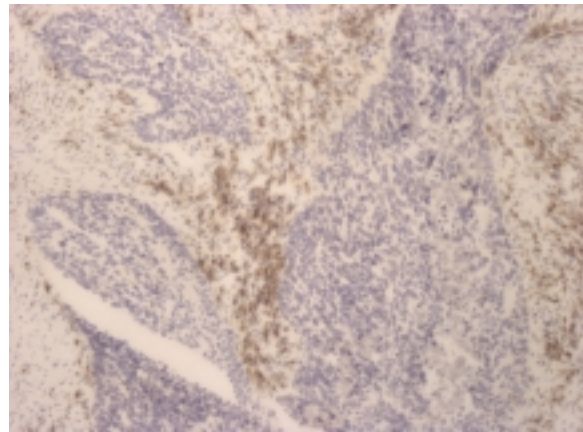


Fig. 1. Immunohistochemistry for CD8 reveals the presence or absence of intraepithelial T cells in stage III ovarian cancer. A: A brisk intraepithelial T cell infiltrate is detected in this tumor. B: No intraepithelial T cells are seen in this tumor, but a brisk infiltrate is seen in the stroma surrounding tumor islets. (40X magnification).

52.8% in the 14 patients whose tumors exhibited intraepithelial T-cells and 6.5% in the 22 patients whose tumors lacked intraepithelial T cells.

The presence of intraepithelial T cells was significantly associated with younger age (70% with intraepithelial T cells in those < 55 years old compared to 50% in those ≥ 55 year old; $P = 0.010$), and with residual tumor (52% with intraepithelial T cells in those with suboptimal debulking compared to 80% in those with optimal debulking; $P = 0.001$). Histotype (group of clear cell and undifferentiated vs. group of serous, mucinous and endometrioid), tumor grade, or the inclusion of paclitaxel in chemotherapy, were not associated with the presence of intraepithelial T cells. Based on the univariate Cox proportional hazards regression analyses,

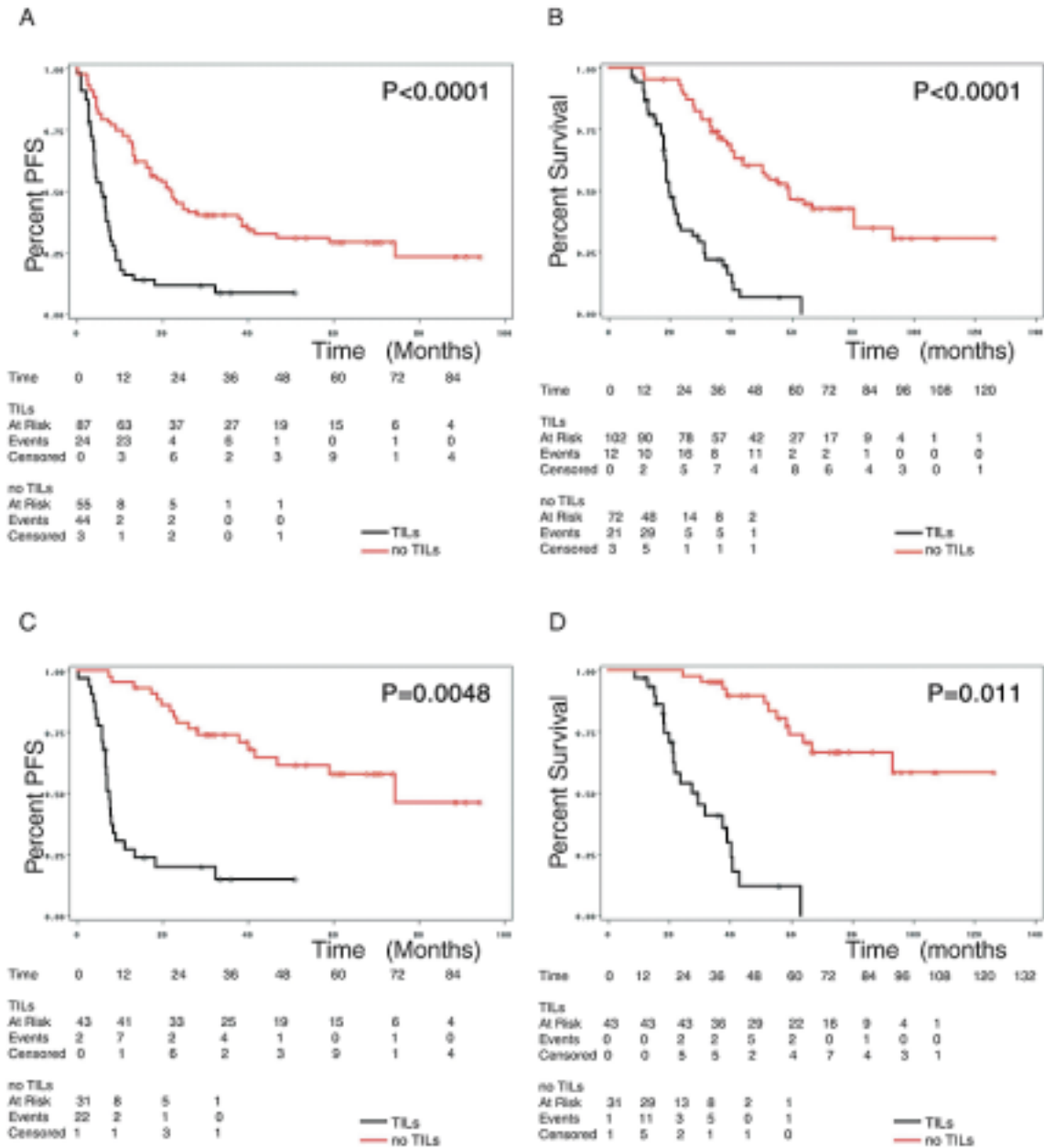


Fig. 2. Survival analyses in ovarian carcinoma based on intraepithelial T cells. A and B: Kaplan-Meier survival curves for progression-free (A) and overall survival (B) based on the presence or absence of intraepithelial T cells in 174 patients with stage III and IV epithelial ovarian cancer. The number of patients with events (death or progression), alive under follow-up and alive censored patients are provided at various times under the horizontal axis. C and D: Kaplan-Meier survival curves for progression-free (C) and overall survival (D) times based on the presence or absence of intraepithelial T cells for 74 patients with stage III and IV epithelial ovarian cancer and complete response to therapy. (TIL = Intraepithelial T cells). P-values indicated are for the log-rank statistics used to compare the survival curves.

the presence of intraepithelial T cells ($P < 0.0001$) and residual tumor ($P < 0.001$), but not grade, tumor histology, inclusion of paclitaxel in chemotherapy or age, were associated with overall survival. Similar results were obtained for the analysis of progression-free sur-

vival except for grade, where tumors classified as grade 1 had a better prognosis than those classified as grade 3 (1 vs. 3, $P = 0.047$; 2 vs. 3 $P = 0.76$). The multivariate Cox regression analysis demonstrated that intraepithelial T cells and debulking (optimal vs. suboptimal)

were the only two independent prognostic factors for both progression-free survival and overall survival.

The ability of these two biomarkers, intraepithelial T cells and residual tumor, to predict five-year survival is demonstrated in an analysis of 145 of the 174 women with at least five years of follow up or a death within five years of diagnosis [12]. Both biomarkers were significantly associated with five-year survival with odds ratios of 28.4 (95% CI = 3.7–216.0) and 10.5 (95% CI = 4.1–26.7) for the presence of intraepithelial T cells and optimal debulking, respectively. The absence of intraepithelial T cells and suboptimal debulking were both associated with poor prognosis and identified 51% and 88%, respectively, of those who died within five years of diagnosis. In contrast, the presence of intraepithelial T cells and optimal debulking associated with good prognosis and identified 96% and 68%, respectively, of those who survived at least five years.

An important aspect revealed by our data is that an improved clinical outcome depends on the infiltration of T cells specifically in tumor islets rather than stroma, as the mere presence of T cells in stroma alone did not predict better outcome. Indirect evidence of T cell activation selectively in tumors with intraepithelial T cells was provided by measurement of mRNA levels of two cytokines associated with T cell activation. IFN- γ and interleukin-2 (IL-2) mRNA levels were 10 ($P = 0.019$) and 26-fold ($P = 0.091$) higher in tumors with intraepithelial T cells as compared with tumors lacking intraepithelial T cells, and were undetectable in 7/10 (70%) and 9/10 (90%), respectively, of tumors lacking intraepithelial T cells.

A second study investigated various subtypes of intraepithelial T cells in ovarian tumors of 117 women with EOC. The women in this study was somewhat different from the women in the previous study on many characteristics. Median age was 58 in the Zhang study vs. 62 in the Sato study. Approximately 10% of patients were stage IV in both studies, but 11% of patients in the Sato study were stage I or II, while the Zhang study did not include any early stage patients. Grade and histology were also different. The Sato study included mainly grade 3 tumors (90% vs. 78% in the Zhang study) and mainly papillary serous tumors (78%). Residual disease was also different. In the Zhang study 30% of patients had <1 cm residual disease after surgery (optimally debulked), while 47% had no residual disease in the Sato study. However, women in the Sato study had a lower overall five year survival rate compared to the Zhang study (13.7% versus 25.3%). The Sato study was unable to find a predictive value of intraepi-

thelial CD3⁺ T cells (which comprises all T cells), but showed a strong predictive value of CD8⁺ T cells, the subset of T cells that comprises mainly cytotoxic lymphocytes (CTL). Patients with higher frequencies of intraepithelial CD8⁺ T cells (in the highest two tertiles of the distribution) demonstrated improved survival compared with patients with lower CD8⁺ T cell frequencies (median survival 55 months versus 26 months; hazard ratio = 0.33; $P < 0.001$) [13]. Interestingly, in the Zhang study CD4⁺ and CD8⁺ T-cell infiltrates correlated ($R^2 = 0.66$, $P < 0.001$; $n = 30$) and intraepithelial CD4⁺ and CD8⁺ cells were either present or absent by immunohistochemistry, similarly to total CD3⁺ cells. In summary, recruitment of T effector cells to tumors is associated with improved clinical outcome in advanced EOC.

2.2. Dendritic cells

Dendritic cells (DCs) play a critical role as regulators of adaptive immune response against tumors. They take up, process and present antigens to naïve T cells in major histocompatibility molecules (MHC) class I and/or class II-restricted fashion [14]. DCs are recognized as a diverse population of cells with remarkable plasticity. Depending on lineage and level of maturation they exhibit diverse phenotypes that can elicit potent type-1 T cell stimulation, promote type-2 responses or induce T cell tolerance [14–16]. At least two subsets of DCs with distinct phenotypic markers and functional properties, myeloid and plasmacytoid DCs, have been described in both humans and mice.

Mature myeloid DCs induce potent immune response against presented antigens, and their presence has been associated with improved clinical outcome in a number of tumors. DCs and memory lymphocytes were the focus of a retrospective case-control study of 18 patients with ovarian cancer (78% were clinical stage III or IV) who were followed from 10 to 37 months, where 9 cases had recurrence of their disease or died and 9 controls had no evidence of disease. A high frequency of tumor-infiltrating HLA-DR⁺CD1a⁺ Langerhan's type myeloid DCs was observed in the control patients who had no evidence of disease compared to cases who had recurrence of their disease; the frequency of cells expressing HLA-DR or CD1a in the control group (no evidence of disease) were 3.5 and 4.5 fold higher than in the case group (with recurrence), respectively [17].

3. Cellular markers relevant to tumor immune tolerance

3.1. Regulatory T cells

CD4⁺CD25⁺ regulatory T cells (Treg), a subset of T cells endowed with powerful suppressor activity, are an important mediator of peripheral immune tolerance. These cells prevent T cell-specific immunity by suppressing CD8⁺ T cell activation and secretion of IL-2 and IFN- γ ; inhibit specific cytotoxicity in a contact-dependent fashion and/or through contact-independent, paracrine mechanisms [18–20]; and affect the function of other immunosuppressive populations like tolerogenic antigen-presenting cells. The first evidence for the contribution of Treg to immune dysfunction in cancer in the human was presented in patients with ovarian cancer and lung tumors, where increased frequency of transforming growth factor-beta (TGF- β)-secreting Treg with potent immunosuppressive functions were identified in tumors, ascites and peripheral blood [21]. In human ovarian cancer, Treg were demonstrated to play an important immunopathogenetic role [22]. Tumor-infiltrating CD4⁺CD25⁺ T cells represented up to 25% of tumor-infiltrating CD4⁺ T cells and the percentage of CD4⁺CD25⁺CD3⁺ T cells in CD4⁺CD3⁺ T cells was higher in stage II, III and IV compared to stage I. In addition, approximately 75% of CD4⁺CD25⁺CD3⁺ T cells in the tumor mass were in proximity to infiltrating CD8⁺ T cells, suggesting that physical contact between CD4⁺CD25⁺ T cells and CD8⁺ cytotoxic T cells mediates regulatory functions [22].

Curiel et al. evaluated tumor Treg in a sample of 70 ovarian cancer patients [22]. The majority (80%) of these women had stage III or IV ovarian cancers; 79% had serous, mucinous or endometrioid ovarian carcinomas; 79% of all tumors were grade 3; 29% of the patients had \leq 1 cm residual disease and 33% of these women had complete response to chemotherapy. CD4⁺CD25⁺ T cells isolated from malignant ascites, solid tumor and blood from individuals with EOC express the transcription factor forkhead box P3 (FoxP3), which is crucial for the differentiation and function of CD4⁺CD25⁺ Treg cells in the mouse. Treg in each tumor were characterized by the mean and standard error of the number of FoxP3-positive cells by immunostaining in 10 high power fields. Three groups with an equal number of patients were defined based on the number of Treg (\leq 131, 132–363, \geq 364). A significant difference in survival curves was observed among the three

groups ($p < 0.001$). The approximate four-year overall survival rates for the three groups that were Stage III were 0%, 50% and 80% from highest to lowest Treg respectively; the four-year overall survival rates for the three groups that were Stage IV were approximately 0%, 60%, 65%, respectively [22, supplemental data]. Accumulation of Treg in the tumor was demonstrated to be a significant prognostic factor that was associated with poor prognosis based on both the univariate Cox proportional hazards regression model and the multivariate Cox proportional hazards model controlling for stage and debulking.

These findings were recently confirmed by two other groups. A study from Innsbruck, Austria, included 99 women with ovarian carcinoma, of which 75% were stage III and IV; 66% were serous or mucinous; 40% were grade 3; and 37% were optimally debulked [13]. A cut-point for FoxP3 expression was established that maximized the difference between survival curves of two groups with higher and lower FoxP3 expression based on the log-rank statistic. High FoxP3 mRNA expression identified a patient subgroup (that included the 19 patients with highest FoxP3 expression) with significantly lower overall survival ($P = 0.003$) as well as significantly lower progression-free survival ($P = 0.004$) [23]. As in the Curiel study, all patients in the high FoxP3 group died within four years of diagnosis; the four-year survival rate for the low FoxP3 group was approximately 50%. In the multivariate Cox proportional hazard regression model, high FoxP3 expression was demonstrated to be an independent prognostic factor for both overall survival ($P = 0.004$) and progression-free survival ($P = 0.004$).

A second study included 117 women treated at Roswell Park Cancer Institute. Among these women 88% had stage III or IV ovarian cancers; 78% had serous carcinomas; 90% of all tumors were grade 3; 47% had no residual disease; and 48% had complete response to chemotherapy. Treg were identified as the number of CD25⁺ FoxP3⁺ T cells. T cells were examined by CD25/FoxP3 double immunohistochemistry and three groups were defined based on the tertiles of the number of Treg. The median survival for those with low Treg ($n = 36$) was 55 months compared to 42 months for the others with higher Treg ($n = 81$). In contrast to the previous studies, Treg was not associated significantly with survival based on either the univariate or multivariate model that controlled for age, stage, grade, histological type and residual disease. However, the ratio of CD8⁺ cells to CD25⁺FoxP3⁺ cells was found to be a significant predictor of survival. The me-

dian survival for patients with high CD8⁺/Treg ratios was 58 months, whereas patients with low CD8⁺/Treg ratios had a median survival of 23 months (hazard ratio = 0.31; $P = 0.0002$) [13].

4. Antibodies

B cells and humoral immunity are involved in antitumor immune response. Tumor-specific antibodies may provide sensitive biomarkers that are readily accessible in serum, even though autoantibodies against specific tumor antigens may be ineffective in controlling tumor growth or may even promote tumor progression [24]. HER-2/neu overexpression in primary tumor is associated with production of HER-2 – specific antibodies, and in patients with colon and breast cancer the accumulation of p53 in primary tumor cells is associated with the presence of serum p53-specific antibodies [25,26]. Serum autoantibodies to p53, Her2/neu, and topoisomerase II-alpha proteins were evaluated as prognostic factors for overall survival in a recent study of 104 patients with EOC. Eighty-percent of these women were stage III or stage IV, 87% of their tumors were serous, mucinous, or endometrioid. Blood samples were taken at the time of surgery and a biomarker was considered positive if its value was above the 95-th percentile of the control samples ($n = 175$) for each antigen. Of the three biomarkers, only the proportion of women with p53 antibody immunity increased significantly with stage ($P = 0.006$), with 6% in early stage disease and 30% in late stage disease. There was a significant association between overall survival and the presence of p53 antibodies ($P = 0.01$). Women with p53 autoantibodies had increased overall survival (median = 51 months) compared to those who did not have them (median = 24 months) [27]. Multivariate analysis showed the presence of p53 autoantibodies to be an independent variable for prediction of overall survival in advanced-stage EOC patients. Interestingly, there was no survival benefit related to humoral immunity to other oncogenic proteins evaluated including HER-2/neu and topoisomerase II α .

Previous studies had failed to demonstrate a similar relationship [28,29]. One study included 83 women with EOC where 76% had stage III or stage IV tumors, and 56% were grade 3. Anti-p53 autoantibodies were more frequently present in patients whose tumors overexpressed p53 or were moderately or poorly differentiated. In univariate or bivariate analysis, p53 antibody-positive patients were at an increased risk for relapse

but not death. In the multivariate analysis, there were no significant differences in either disease-free or overall survival for patients who were p53 antibody-positive compared to those who were negative [28,29].

Another interesting study tested the hypothesis that autoantibodies against tumor-associated antigens are detected in normal subjects and predict disease risk. High levels of autoantibodies against epithelial mucin 1 (MUC-1) were associated with decreased risk for ovarian cancer. These autoantibodies targeted MUC-1 expressed on the surface of several types of polarized epithelial cells. Factors predicting the presence of antibodies included, among others, previous use of oral contraceptives, pelvic surgery and non-use of talc in genital hygiene. There was a significantly higher incidence of anti-MUC-1 antibodies in women with five or more conditions (51.4%) than in women with 0 or 1 condition (24.2%). The risk for ovarian cancer was inversely associated with number of conditions predisposing to anti-MUC-1 antibodies ($P < 0.0001$) [30].

5. Major histocompatibility and related molecules

Target cell recognition by effector cytotoxic CD8⁺ T cells requires surface expression of class I human leukocyte antigen (HLA) molecules, which carry epitopes recognizable by CD8⁺ T cells. Thus, downregulation or genetic loss of HLA class I molecules is a common mechanism of immune evasion during tumor progression or metastasis. In a recent study of 51 primary EOC, HLA class-I antigen expression was determined by immunohistochemistry. HLA class-I antigen expression was not associated with tumor grade but was significantly associated with stage ($p = 0.003$). The odds ratio for HLA class-I antigen loss in stage III EOC compared to stage I or II was 7.6 (95% confidence interval, 1.9–30.5; $P = 0.007$). The multivariate survival Cox model based on a subset of these patients ($n = 39$) demonstrated that grade and stage were independent prognostic factors but HLA class-I expression downregulation was not [31], suggesting that multiple overlapping mechanisms may account for tumor recognition and that HLA expression is not sufficient to predict activation of immune rejection mechanisms.

A second study examined whether HLA-A2 gene status predicts outcome in ovarian cancer. HLA-A2 was examined by PCR on paraffin-embedded tissue in a prospective cohort of 88 patients with EOC seen over a 1 year period with sufficient tissue for analysis. Among 88 patients evaluated, 57% were grade 3, and 73% were

HLA-A2 positive. In the subset of stage III and IV patients ($n = 47$), none of the HLA-A2 positive patients survived 5 years, compared to more than 50% of the HLA-A2 negative patients. In the multivariate analysis including all patients both stage and HLA-A2 positivity, but not grade, were independent prognostic factors. The adjusted hazard ratio for HLA-A2 positivity was 1.7 (C.I. = 1.02–2.9; $P = 0.04$) [32].

In peripheral tissues, effector cell function is positively regulated by the expression of ligands for the NKG2D immunoreceptor. A new MHC-I related ligand for the NKG2D receptor named Lymphocyte Effector Cell Toxicity Activation Ligand (Letal) was up-regulated in advanced EOC with intraepithelial T cells, suggesting an important role for Letal in the homeostasis of peripheral CD8⁺ effector T-cells and the immune defense against tumors in the human [33, 34]. Letal expression was evaluated in the tumors of 43 women with EOC [33,34]. The presence of Letal was associated with intraepithelial T cells ($p = 0.06$). Residual disease, the presence of intraepithelial T cells and the presence of Letal were each significantly associated with overall survival. The 5-year overall survival rate was 52% among patients whose tumors expressed Letal, but only 21% among patients whose tumors were Letal-negative. In a multivariate analysis, Letal expression, optimal debulking, and the presence of intraepithelial T cells were found to be important prognostic factors associated with prolonged overall survival in stage III ovarian cancer, demonstrating a potential protective role for Letal in ovarian carcinoma [34].

6. Conclusions

Investigation of tumor-host interactions has enhanced our understanding of immune mechanisms underlying tumor progression and outcome. This investigation promises to yield important therapeutic approaches. At the same time, it is evident that immunologic investigation has already produced important biomarkers. The clinical application of these biomarkers is not yet clear, but potential applications include disease prognosis and perhaps classification for selection of therapy. Additional work needs to be undertaken by cooperative groups to validate the predictive value of these biomarkers with respect to outcome in the context of phase III trials. An important direction of the near future will be to test the usefulness of these biomarkers in the selection of patients for biological therapies, including vaccine, adoptive lymphocyte

therapy or therapy neutralizing Treg cells. It is possible that these therapies, similar to any other cancer therapy will be useful only in a subset of patients. As clinical testing is designed to test biological and immune therapeutics, it will be important to take advantage of the biomarkers already identified to define the populations of patients that may benefit from one or another form of therapy.

Acknowledgments

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