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Potential immunotherapy targets in recurrent cervical cancer

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HIGHLIGHTS

- Immune cells were expressed in higher densities in the peritumoral stroma.
- Tumors demonstrated decreased expression of intratumoral cytotoxic T cells.
- There was increased expression of tumor associated macrophages in tumor cells.

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ABSTRACT

Objective. Our objective was to characterize the intra and peritumoral immune profile in recurrent cervical cancers to identify rational immunotherapy targets.

Methods. Archival pelvic exenteration specimens were examined using a validated multiplex immuno-fluorescent panel of antibodies against cluster of differentiation 8 (CD8), cluster of differentiation 68 (CD68), forkhead box P3 (FoxP3), programmed cell death protein 1 (PD1), and programmed death-ligand 1 (PD-L1, $N = 28$). Clinical data were abstracted from the electronic medical record.

Results. Cytotoxic T cells, macrophages, and regulatory T cells were found in higher densities in peritumoral stroma (CD8+ density 497.7 vs 83.5, $p < 0.0001$, CD68+ density 345.0 vs 196.7, $p = 0.04$, FoxP3+ density 214.5 vs 35.6, $p < 0.0001$). Antigen experienced T cells (PD1+) were higher in peritumoral compared to tumor tissue (median normalized fluorescence intensity 0.05 vs 0.0085, $p < 0.001$). Although there was a higher median density of intratumoral cytotoxic T cells and macrophages compared to regulatory T cells (median density CD8+ 83.5 vs 35.6, $p < 0.05$, median density 196.7 vs 35.6, $p < 0.05$), the presence of macrophages correlated with the presence of regulatory T cells in tumors ($r = 0.58$, $p = 0.001$).

Conclusions. While cytotoxic T cells are present in tumor tissue to varying degrees, their density is lower than in peritumoral stroma, suggesting intratumoral exclusion or destruction of T cells. Higher densities of intratumoral macrophages compared to regulatory T cells suggest macrophages may be important contributors to the immunosuppressive tumor environment. Future directions for combination therapy include altering T cell trafficking and targeting tumor associated macrophages (TAMs) to enhance intratumoral activated T cell density and effect a more robust immune response.

1. Introduction

Worldwide, there were 527,600 new cases of cervical cancer diagnosed in 2012 and over 265,700 deaths, making cervical cancer the fourth most common cause of cancer death among women [1,2]. Despite the development of effective vaccines for prevention, patients with advanced stage and metastatic disease continue to have a poor prognosis with response rates ranging from 35 to 50% with current therapeutic options [3,4]. Given the known causative risk factor of HPV infection in the development of cervical cancer, the utilization of immunotherapeutics in the treatment of this disease presents an attractive option for this patient population.

The human immune system is able to control the growth and elimination of foreign pathogens, and early tumors, through a process classically known as immunosurveillance. In this process, cytotoxic and helper T cells are activated when tumor associated antigens (TAA's) bound to major histocompatibility complex (MHC) class I and II

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molecules in tumor and antigen presenting cells (APCs) are presented in combination with multiple costimulatory signals. Following migration to the tumor site, activated T cells recognize specific tumor antigens, leading to T-cell mediated destruction of early tumors [5–7]. Consistent with this conceptual framework, it is estimated that 75–80% of women are infected with HPV during their lifetime, but the overwhelming majority of infections are cleared by the immune system [8]. However, "immune-editing," a process by which cells are able to escape immunosurveillance, can lead to selection of tumor clones capable of gaining resistance to immune detection and elimination, leading to ultimate tumor growth [9]. This resistance can be mediated by multiple immunosuppressive pathways that involve both the malignant cells and other constituents of the tumor microenvironment.

Yang and colleagues evaluated HPV status and expression of the PD-1/PD-L1 pathway in cervical intraepithelial neoplasia, and showed that upregulation of the PD-1/PD-L1 pathway was associated with HPV positivity and progression of precancerous lesions [10]. In addition, Heeren and colleagues evaluated PD-L1 expression in a cohort of primary tumor samples of squamous cell and adenocarcinomas of the cervix. They found that 54% of squamous cell and 14% of adenocarcinomas demonstrated intratumoral PD-L1 expression. In addition, all of the intra-tumoral and the majority of the peritumoral immune cell PD-L1 expression was seen in tumor associated macrophages [11]. Diffuse PD-L1 expression or negative expression was associated with worse disease specific survival when compared to patients whose tumor showed only marginal PD-L1 expression. The authors concluded that PD-1/PD-L1 therapy should be considered in the treatment of cervical cancers. Despite this, there remains a paucity of data regarding the contribution of immunosuppressive pathways to the immune evasion in recurrent cervical cancer, a group of patients where treatment options remain limited.

Immunotherapeutics, and immune checkpoint inhibition specifically, have shown impressive efficacy leading to improved survival in a number of diverse cancers including melanoma, non-small cell lung cancer, renal cancer, and bladder cancer [12–15]. There are multiple ongoing studies to evaluate the role of immune checkpoint therapy in the treatment of cervical cancer in both the upfront and recurrent setting [16–19], however, the optimal timing for incorporation of immune checkpoint inhibitors into treatment and which patients may benefit from these therapies remain largely unanswered questions. Our objective was to characterize the intra and peritumoral immune profile in recurrent, therapy-resistant cervical cancer specimens, given that patients with such tumors are most likely to benefit from novel immunotherapies. We focused on large excisional specimens from pelvic exenterations to ensure access to representative areas of viable tumor without necrosis. Characterization of immune markers and cell types found in cervical cancer specimens and the surrounding microenvironment presents an opportunity to better understand the immune landscape of cervical cancer and provide important clinically relevant targets for the design of future immunotherapy trials.

2. Methods

Following Institutional Review Board approval (PA15-0286), patients were identified retrospectively through a departmental database (MDA 2008-0095). To obtain adequate tissue samples, we targeted specimens obtained at the time of pelvic exenteration for recurrent cervical cancer. All women with a diagnosis of squamous cell carcinoma or adenocarcinoma of the cervix who had a pelvic exenterative procedure at MD Anderson Cancer Center between 1994 and 2004 with available pathologic specimens were included. Clinical and pathologic data were abstracted from the electronic medical record.

Formalin-fixed, paraffin-embedded tumor samples were identified and specimens were reviewed on hematoxylin and eosin stain by a single gynecologic pathologist (A.Y.) to confirm the presence of tumor, adjacent normal tissue, and to select one representative paraffin block for each specimen. One slide for each sample was stained using a commercially available, validated multiplex immunohistochemistry PD1 Checkpoint Assay Panel, including cluster of differentiation 8 (CD8), cluster of differentiation 68 (CD68), forkhead box P3 (FoxP3), PD1, and PD-L1 (Table 1, PerkinElmer, PKI, Waltham, MA, USA). Up to five non-overlapping image fields for each slide were captured using the Vectra® system (PerkinElmer, PKI, Waltham, MA, USA) at 20× magnification and analyzed using inForm® software (PerkinElmer, PKI, Waltham, MA, USA). Human tonsil was stained with the PD1 Checkpoint Assay Panel as a positive control. Staining of human tonsil slides with isotype control antibodies was performed as negative controls (Table 1). PD1 and PD-L1 expression were measured on cell membranes and reported as normalized fluorescence in tumor and peritumoral tissue segments. Expression of CD8, CD68, and FoxP3 was measured as the total number of cells expressing each marker in tumor and peritumoral segments compared to total tissue area and was analyzed as phenotype density (counts/mm^2, Fig. 1). All staining and analysis was performed on de-identified specimens by NovaScreen Biosciences Corporation (PerkinElmer, PKI, Waltham, MA, USA).

In this retrospective study, descriptive statistics were calculated for variables of interest. Mann-Whitney *U* tests were utilized to compare expression of CD8, CD68, FoxP3, PD1, and PD-L1. Spearman's rank correlation was used to evaluate correlation between variables. Kruskal Wallis test was used for multiple comparisons. Kaplan-Meier survival curves were generated and log-rank analyses were performed. *P*-values <0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Demographics

A total of 28 patients with available pathologic specimens were treated with an exenterative procedure at MD Anderson between 1994 and 2004. Two patients had paired specimens available from original diagnosis and exenteration. The majority of patients were Caucasian ($n = 17$, 60.7%). Most patients presented with locally advanced disease ($n = 17$, 60.7%) and were treated with primary chemoradiation ($n = 19$, 67.9%). Two patients (7.1%) were treated primarily with a radical hysterectomy and 7 patients had both a hysterectomy and chemoradiation (25.0%). Most tumors were of squamous histology ($n = 19$, 67.9%). Seven patients (25.0%) were alive with no evidence of disease at last follow-up and 16 patients had died of their disease (57.1%, Table 2).

3.2. Immunoprofile in tumor versus peritumoral tissue

Antigen experienced T cells (PD1 +) were higher in peritumoral compared to tumor tissue (median normalized fluorescence intensity 0.05 vs 0.0085, $p < 0.001$). There was no difference in PD-L1 expression between peritumoral and tumor tissue segments (median normalized fluorescence intensity 0.31 vs 0.19, $p = 0.52$, Fig. 2A). Similar to antigen experienced T cells, cytotoxic T cells (CD8 +), tumor associated macrophages (CD68 +), and regulatory T cells (FoxP3 +) were found in higher densities in peritumoral tissue compared to tumor cells (CD8 + density 497.7 vs 83.5, $p < 0.0001$, CD68 + density 345.0 vs 196.7, $p = 0.04$, FoxP3 + density 214.5 vs 35.6, $p < 0.0001$). PD1 expression was correlated with the identification of cytotoxic T cells in both tumor and peritumoral tissue segments (tumor $r = 0.50$, $p = 0.007$, non-tumor $r = 0.71$, $p < 0.0001$, Fig. 3).

3.3. Immunoprofile in tumor alone

We then evaluated immune expression within the tumor itself. Tumors had higher PD-L1 compared to PD1 expression (median density 0.009 vs 0.24, $p < 0.001$). There was no difference in PD-1 or PD-L1

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