PD-L1 expression and CD8+ infiltration shows heterogeneity in juvenile recurrent respiratory papillomatosis

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ABSTRACT

Introduction: Tumor immunotherapy have broadened therapeutic options for tumor treatment. The role of immune function in juvenile recurrent respiratory papillomatosis (JRRP) has not been investigated. Applying immunoblockade inhibitors as a novel disease treatment is unclear. Our study, for the first time, evaluates immune infiltration and immuno-suppressive molecule expression in JRRP. Our study provides insights in possibly treating this disease with tumor immunotherapies. We aimed to determine expression of programmed death-ligand 1 (PD-L1), a cancer escape protein, and presence of CD8+ T cell infiltration in tumor microenvironment.

Material and methods: Seven patients with JRRP (mean age: 7.43; age range 3–17) in this study routinely have their tumors surgical debulked at Massachusetts Eye and Ear Infirmary. Following surgery, samples were de-identified and sent to pathology where they were stained and analyzed.

Results: Six out of seven patients expressed PD-L1 on tumor cells to various extents. Three patients showed concurrent PD-L1 expression on tumor cells and abundant CD8+ tumor infiltrating lymphocytes as well as PD-L1+ stromal lymphocytes, while PD-L1 expression on tumor cells were not associated with CD8+ tumor infiltrating T cells nor PD-L1+ stromal lymphocytes in the other three patients. HPV 6/11 and p16 was detected in all the patients. There appeared to be no correlation between either PD-L1 expression and CD8+ infiltration and clinical severity as measured by both the number of surgeries per year or Derkay score.

Conclusions: Despite a small cohort, the expression of p16 and HPV 6/11 in all of the patients confirms the tissues were HPV tumor cells. PD-L1 expression was detected in the vast majority of tumor samples, while inflammatory cell compartments showed a higher degree of variation. Expression of PD-L1 on tumor cells but not inflammatory cells raises the possibility of a tumor cell intrinsic manner of PD-L1 expression. In contrast, a group of patients showed PD-L1 positivity in both tumor and inflammatory cells along with abundant CD8+ tumor infiltrating lymphocytes, suggesting adoptive immune resistance in these tumors and potential benefits from tumor immunotherapy.

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1. Introduction

Recurrent Respiratory Papillomatosis (RRP) is a human papilloma virus (HPV) associated disease that affects both adults and children [1]. It is the most common benign laryngeal neoplasm in children and it is characterized by the presence of squamous
papillomas within the airway [2].

Human Papilloma Virus (HPV) has over 90 different variants, with HPV-6 and HPV-11 being primarily responsible for the manifestation of RRP [3]. Although most cases of the Juvenile Recurrent Respiratory Papillomatosis (JRRP) remain benign, 3–5% of these tumors transform into malignancy with poor clinical outcome [4]. The mode of transmission of HPV is still unknown, however it is suspected that the vertical transmission of the virus occurs during birth, as the child pass through the infected birth canal [1].

The principle presenting symptoms of RRP is dysphonia [5] and other less prominent symptoms include acute respiratory distress, dyspnea, and chronic cough [1]. There currently is no single treatment that has been shown to effectively eradicate RRP [1]. The mainstay of treatment is recurrent surgical debulking with or without adjuvant medical treatment. Clinical severity is typically measured by a Derkay Score, which numerically quantifies clinical manifestations [6]. Because the Derkay score varies between visits, clinical severity is often measured solo by the frequency of surgeries needed to clear the airway [7]. The National Registry of Children with RRP has reported that the average number of procedures that a child undergoes per year is 4.4 [8]. The main concern with this treatment form of treatment is that repetitive anesthetic exposure has been associated with behavior changes and learning delays in children [9,10].

Adjuvant medical treatment with cidofovir, avastin, or interferon has had limited success [11]. The lack of effective treatment options has prompted physicians and scientists to search for an alternative source of treatment. Within the past few decades, the field of Cancer Immune Escape has grown significantly, due in part to the discovery of programmed death 1 receptor (PD-1) in 1992 [11]. PD-1 is an immunoinhibitory receptor of the CD28 family, which is expressed on a number of immune cells including T cells, B cells, monocytes, and tumor-infiltrating lymphocytes [12]. Ligand of PD-1 (PD-L1) can be expressed on multiple cell populations in tumor environment, including tumor cells, fibroblasts and T cells [13] through several mechanisms, including innate immune resistance, in which PD-L1 expression is upregulated secondary to constitutive oncogenic signaling within tumor cells, adaptive immune resistance, amplification of PD-L1 and JAK2 on chromosome 9p21, up- or down-regulation of micro RNAs, and hypoxia [14]. In adaptive immune resistance, PD-L1 expression is induced on tumor cells and other types of cells by interferons and inflammatory cytokines that are secreted by CD8+ cytotoxic T lymphocytes (CTLs) and/or Th1 pathway activation, counterbalancing the CTL/Th1 microenvironment [15]. Thus, CD8+ T cell infiltration may predict patient response to immunotherapy [16]. The binding of PD-1 to PD-L1 can alter activity of multiple signaling pathways to suppress T cell functions by inducing T cell apoptosis, energy and exhaustion. Thus, PD-1/PD-L1 create a route for the cancer cells to evade T cell mediated antitumor effects [17].

The PD-1/PD-L1 pathway has been studied in a variety of cancers including melanoma, renal cell carcinoma, and lung, bladder, colon and gastric cancers [12]. This effort has led to a number of clinical trials that inhibit either PD-1 or PD-L1 [11]. Recent studies have shown that PD-1/PD-L1 pathway is present in HPV associated cancers including head neck squamous cell carcinoma [18]. Despite investigations on other types of HPV related cancer, little has been done to evaluate PD-L1 expression in JRRP tumors and the possibility of targeting the disease with immunotherapies. The purpose of this study was to analyze PD-L1 expression on tumor and inflammatory cells in respiratory papillomas. CD8+ T cell infiltration in tumor tissues, a marker for adaptive immune resistance, was also evaluated.

2. Methods

2.1. Human subjects

Approval was obtained by the Massachusetts Eye and Ear Human Studies Committee. During routine surgical debulking of laryngeal papilloma, samples were obtained, placed in bovine serum, and sent to the lab for further analysis.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 5-μm sections of formalin-fixed paraffin-embedded tissue samples using an automated stainer (Bond Rx, Leica Microsystems, Bannockburn, IL) and the following primary antibodies in accordance with the manufacturer’s recommendations: PD-L1 XP monoclonal antibody (E1L3N, 1:200, Cell Signaling Technology, Danvers, MA), CD8 monoclonal antibody (4B11, RTU, Leica Biosystems, Buffalo Grove, IL), T-bet monoclonal antibody (D6NBB, 1:100, Cell Signaling Technology, Danvers, MA), and p16 (E6H4, 1:100, Ventana Medical Systems, Tuscon, AZ). For the optimization of PD-L1, two known controls were used: HDLM2 (Hodgkin’s lymphoma cell line with high PD-L1 expression) as a positive control and PC3 (prostate cancer cell line with low PD-L1 expression) as a negative control.

2.3. In situ hybridization

The detection of nuclear HPV 6/11 was performed using a nuclear probe diluted 1:5 (Leica). Briefly, samples were heated at pH = 9.0 for 40 min and then denatured for 10 min. In-situ hybridization of HPV 6/11 probe were incubated with denatured samples for 4 h.

2.4. Evaluation of immunohistochemistry and in situ hybridization

A pathologist (M.M.-K.), blinded to the clinical and pathological data, evaluated the immunostains and in situ hybridization of each sample. Epithelial cell populations were confirmed to contain tumor cells after displaying positive nuclear HPV 6/11 expression in all samples. The percentages of tumor cells exhibiting membranous staining of PD-L1 were quantified, and the intensity of PD-L1 expression was scored with a 3-tierd system (0–2). In the tumor stroma, the percentages of PD-L1 positive lymphocytes compared with the total amount of inflammatory cells were assessed with 5% increments. Cytoplasmic expression of CD8 was semiquantitatively evaluated on a scale of 0–3 based on the extent of positive lymphocytes infiltrating within tumor cells (TILs) [19]. Each grade was defined based on the fraction of tumor cells on top of which positive T cells were present: 0, none or rare; 1, <5%; 2, ≥5% and <25%; 3, ≥25%. Given that CD8+ TILs were heterogeneously distributed within the tumor cells, the presence of score 2 or 3, even focally, was considered positive for abundant CD8+ TILs.

3. Results

From January 1, 2016 to May 30, 2016, 7 pediatric patients with JRRP had tissue debulking that was subsequently sent for analysis. Despite various intensities all of the patients expressed both p16 and HPV 6/11 (Table 2). This confirms that the sample were HPV infected tumor cells. Differences were found in the PD-L1 intensities between samples, as well as in the corresponding levels of CD8+ positive cells and total inflammatory cells—which included both lymphocytes and histiocytes as identified by cell morphology.

The results of PD-L1 expression on tumor cells amongst the cohort, when taken into account with the corresponding extent of
CD8+ tumor infiltrating lymphocytes (Table 1) led to the recognition of two categories which characterize the findings: 1. In the first group (patient 1, 2, and 3), PD-L1 positive tumor cells in association with abundant infiltrating lymphocytes (Figs. 1 and 4). In this group, PD-L1 was expressed on 1%–35% of tumor cells. Consistent with PD-L1 expression on tumor cell compartment, 5%–20% infiltrating lymphocytes also express PD-L1, suggesting the possibility of adoptive immune resistance. In the second group (patient 4, 5, and 6), PD-L1 was expressed on 1%–35% of tumor cells. In contrast with the tumor compartment, PD-L1 expression on infiltrating lymphocytes was low (Fig. 2). In addition, one patient (patient 7) sample was negative for PD-L1 expression in tumor compartment (Fig. 3A) and scant inflammatory cell infiltration (Fig. 3B). Due to the limited number of samples available, no correlation was found between clinical severity as noted by number of surgeries or Derkay Score and either PD-L1 expression or CD8+ infiltration (Table 1).

4. Discussion

Recent advances in the understanding tumor microenvironment and cancer immunity could lead to a more effective treatment for juvenile respiratory recurrent papillomatosis (JRRP). In HPV associated head and neck squamous cell carcinoma (HNSCC) tumors, previous research has shown HPV status correlated with profound differences in immune cell infiltration in tumor tissue and cytokine production as HPV positive tumors have significantly higher numbers of cytotoxic CD8 T cells and proinflammatory cytokine production relative to HPV negative tumors [20]. T cells infiltrating in HPV positive tumors also showed immune activation markers. HPV positivity and presence of immune cell activation markers positively correlated with favorable clinical outcome [21]. These results suggest HPV infection may elicit an immune reaction in tumor microenvironment and activation of these tumor-infiltrating lymphocytes (TILs) may mediate anti-tumor effects. In keeping with these findings, a recent study further unveiled the possibility of treating HPV infected cancer with immunotherapy. Stevanovic and colleagues extracted TILs from HPV positive metastatic cervical cancers and expanded these TILs ex vivo. Infusion of the expanded TILs achieved durable and complete cancer regression in a group of patients [22].

Table 1
Heterogenous Patterns of PD-L1 Expression and Immune cell Infiltration.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Sex</th>
<th>Age</th>
<th>Surgeries Per Year</th>
<th>Derkay Score</th>
<th>PD-L1 Tumor Cells</th>
<th>Stromal Inflammatory Cells</th>
<th>CD8 Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Extent</td>
<td>Intensity</td>
<td>Extent</td>
</tr>
<tr>
<td>Patient 1</td>
<td>M</td>
<td>3</td>
<td>12</td>
<td>22</td>
<td>35% Moderate to Weak</td>
<td>10% 0–2</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>F</td>
<td>4</td>
<td>4</td>
<td>14</td>
<td>1-5% Moderate</td>
<td>5% 1–2</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>F</td>
<td>17</td>
<td>10</td>
<td>14</td>
<td>1-5% Weak</td>
<td>20% 1–2</td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>M</td>
<td>12</td>
<td>10</td>
<td>17</td>
<td>1-5% Weak to Moderate</td>
<td>Rare 1</td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td>F</td>
<td>9</td>
<td>4</td>
<td>31</td>
<td>10% Weak</td>
<td>Rare 0–1</td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td>M</td>
<td>3</td>
<td>3</td>
<td>20</td>
<td>35% Weak</td>
<td>Rare 0</td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td>M</td>
<td>4</td>
<td>4</td>
<td>13</td>
<td>&lt;1% Weak</td>
<td>NE 0</td>
<td></td>
</tr>
</tbody>
</table>

NE: not evaluable due to too scant inflammatory cells in the stroma.

Table 2
Confirmation of HPV Tumor Cells by HPV and p16 Stain.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Known HPV Status</th>
<th>HPV 6/11 Stain</th>
<th>p16 Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>Unknown</td>
<td>Positive mostly in the superficial layer</td>
<td>Focal expression mainly in the superficial layer</td>
</tr>
<tr>
<td>Patient 2</td>
<td>11</td>
<td>Diffusely positive</td>
<td>Focal expression in both surface and basal cell layer</td>
</tr>
<tr>
<td>Patient 3</td>
<td>6</td>
<td>Diffusely positive</td>
<td>Positive in 50% of the lesional cells</td>
</tr>
<tr>
<td>Patient 4</td>
<td>6</td>
<td>Positive in 50% of the lesional cells</td>
<td>Several scattered positive and in a few fragments a decent number of superficial cells are positive</td>
</tr>
<tr>
<td>Patient 6</td>
<td>6</td>
<td>Diffusely positive</td>
<td>Several scattered positive</td>
</tr>
<tr>
<td>Patient 7</td>
<td>6</td>
<td>Diffusely positive</td>
<td>Focal expression in both surface and basal cell layer</td>
</tr>
</tbody>
</table>

Fig. 1. Representative sample showing PD-L1 expression. A) PD-L1 in a tumor cell population. B) PD-L1 expression associated with CD8+ T cell infiltration.
Despite these promising results on other types of HPV associated tumors, few studies have been conducted to evaluate immune infiltration and immune response in JRRP. We performed a preliminary investigation to analyze total inflammatory cell and CD8+ cell infiltration in these tumors. In our findings, CD8+ cell infiltration in these papilloma samples presented two patterns with one group of samples showing higher immune cell infiltration than the other. Due to the small sample size, we could not statistically analyze implications of these two patterns, for example correlation between immune cell infiltration and disease recurrent rates. It would also be interesting to compare amount and types of intratumoral cytokine being produced in these two groups.

Our study shows that JRRP patients exhibit different expression patterns of programmed death ligand 1 (PD-L1) in tumor microenvironment. Previous research from other groups has shown PD-L1 expression in multiple cell populations in tumor

Fig. 2. Representative sample showing detection of PD-L1 in tumor cell compartment but lack of CD8+ cell infiltration. A) PD-L1 staining (brown) shows weak membranous expression on tumor cells as depicted in red circles. B) CD8 staining reveals rare CD8+ T cells in tumor tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Representative sample showing lack of PD-L1 expression in tumor cell population and scant CD8+ T cell infiltration. A) Detection of PD-L1 in tumor cell population is <1% and thus considered negative. B) CD8 staining in tumor tissue shows little to no infiltration by CD8+ T cells into the stromal or tumor compartments of cells.

Fig. 4. Representative sample showing PD-L1 positivity in both tumor and inflammatory cell populations. A) The circled area shows tumor cells with weak to moderate PD-L1 expression. B) Inflammatory cells in the stroma from the same patient also stained positively for PD-L1.
microenvironment including tumor cells, fibroblasts cells, tumor-infiltrating lymphocytes (TILs) such as T cells, macrophages and dendritic cells [13]. PD-L1 expression on tumor cells has been linked with presence of TILs and T cell cytokine production and viewed as an adaptive response by tumor cells to attenuate immune reaction [13]. In our study, we found one group of patients samples staining positive for PD-L1 in both tumor cell compartment and inflammatory cell population. It is possible that upregulation of PD-L1 on tumor cells leads to resistance to immune challenges. Interestingly, our second group of patients exhibited PD-L1 expression on tumor cells but low to negligible PD-L1 expression on inflammatory cells. This seemingly paradoxical result suggests PD-L1 expression may be produced in a tumor cell intrinsic manner rather than being induced by extrinsic factors such as cytokines. Indeed, recent findings have associated PD-L1 expression with oncogenic kinase such anaplastic lymphoma kinase (ALK) in T cell lymphoma and mutations in oncogenic signaling pathway, such as loss of phosphatase and tensin homolog (PTEN) in glioma and triple negative breast cancer [23–25]. The relation between HPV infection and PD-L1 expression is not clear yet. One study found higher frequency and expression of PD-L1 in HPV + head and neck cancer [26] while another study found PD-L1 expression in majority of oesophageal squamous cell carcinoma regardless of HPV infection [27]. Given our results, it would be informative to investigate whether or not HPV infection can promote PD-L1 expression in JRRP cells and if there is any correlation between HPV viral load and PD-L1 expression level.

Once being expressed in the tumor microenvironment, PD-L1 and its receptor PD-1 can suppress T cell function by inducing T cell apoptosis, T cell anergy and thus provide tumor cells protection against T cell mediated cytotoxicity. Inhibition of PD-L1/ PD-1 axis could attenuate T cell apoptosis, rescue T cell anergy and restore T cell functions [28]. We have observed PD-L1 expression on all tumor samples but T cell infiltration varies from sample to sample. It is possible that expression of PD-L1 has eliminated infiltrating T cells. Therefore, it would be important to perform a longitudinal study to evaluate T cell population dynamics in the tumor microenvironment. It would also be interesting to compare PD-L1 expression level and T cell infiltration rates in a larger patient cohort.

PD-L1/PD-1 blockade therapies have been proven successful to reactivate immune cells, suppress disease progression and prolong patient survival in various cancers including melanoma and colorectal cancer [29,30]. Similar to these types of cancers, PD-L1 expression has been shown to modulate immune cell infiltration patterns and immune reactions in HPV positive cancers. In one study of HPV infected cervical cancer, PD-L1 positivity was correlated with HPV positivity and increase of cervical intraepithelial neoplasia (CIN) grade. Increase in CIN grade also correlated with increased anti-inflammatory cytokine IL-10 production and decreased interferon-gamma (IFN-γ) production, implying tumor expression of PD-L1 may negatively regulate immune cell function and ultimately contribute to cancer progression [31]. Such immunosuppression mediated by PD-1/PD-1 signaling pathway has also been suggested in HPV infected head and neck cancer [18]. Consistently, in vitro blockade of PD-1/PD-L1 signaling restored function of tumor-infiltrating T cells inhibiting and anti-PD-1 antibody synergized with HPV vaccine to induce tumor regression [21]. These findings suggest PD-1 blockade therapy as a promising approach to treat HPV related tumor. Efficacy of immune-noblockade inhibitors has yet to be evaluated in JRRP patients. Our analysis found a group of patients showed PD-L1 positive staining on both tumor and inflammatory cell compartments. These patients also had CD8 cell infiltrating in tumor tissues and may be most likely to benefit from PD-L1/PD-1 inhibitors.

5. Conclusion

Our study analyzed PD-L1 expression and CD8 infiltration in JRRP patients for the first time. Although the information may be limited due to the small size of our cohort, the results show heterogenous patterns of PD-L1 expression and immune cell infiltration in these HPV infected tumors. The findings provide new insights of immune cell function in JRRP tumors and potential strategies for treating this disease with therapies overcoming immune resistance.

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Conflict of interest

Mari Mino-Kenudson is supported by SU2C grant and is a pathology consultant for Merrimack Pharmaceuticals and H3 Biomedical. Jeffrey Engelman, David Kodack, and Tingyu Liu are employees of Novartis Institute of Biomedical Research. All other authors have no conflicts of interest.

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