International Journal of Pediatric Otorhinolaryngology 95 (2017) 133–138

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: http://www.ijporlonline.com/

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

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ARTICLE INFO

Article history:
Received 17 December 2016
Received in revised form
13 February 2017
Accepted 17 February 2017
Available online 21 February 2017

Keywords:
Juvenile recurrent respiratory papillomatosis (JRRP)
PD-L1
CD8+

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.

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

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http://dx.doi.org/10.1016/j.ijporl.2017.02.022
0165-5876/

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 current 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 [1]. 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 (D6N8B, 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