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Composition of inflammatory cells regulating the response to concurrent chemoradiation therapy for HPV (+) tonsil cancer

Yoon Se Lee [a], Ji Young Park [b], Kyung Ja Cho [b], Sung Bae Kim [c], Sang Wook Lee [d], Seung-Ho Choi [a], Jong-Lyel Roh [a], Soon Yuhl Nam [a], Sang Yoon Kim [a,*]

[a] Department of Otolaryngology, University of Ulsan, College of Medicine, Asan Medical Center, Seoul, Republic of Korea
[b] Department of Pathology, University of Ulsan, College of Medicine, Asan Medical Center, Seoul, Republic of Korea
[c] Department of Internal Medicine, University of Ulsan, College of Medicine, Asan Medical Center, Seoul, Republic of Korea
[d] Department of Radiation Oncology, University of Ulsan, College of Medicine, Asan Medical Center, Seoul, Republic of Korea

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SUMMARY

Background: Human papillomavirus (HPV) (+) tonsil squamous cell carcinoma (TSCC) responds well to concurrent chemoradiation therapy (CCRT) and demonstrates a favorable prognosis. However, cases of HPV (+) TSCC-related death remain unresolved. We evaluated the distribution and prognostic value of inflammatory cells in HPV (+) TSCC.
Methods: We reviewed the medical records of 53 patients who were diagnosed with TSCC. HPV (+) TSCC was confirmed using HPV DNA PCR and immunohistochemical p16 overexpression. The numbers of CD4 (+), CD8 (+), and CD68 (+) stained cells were used to evaluate peritumoral lymphocyte infiltration. Patients were divided into two groups according to the mean numbers of stained cells and the mean ratios of each cell type.
Results: HPV (+) was noted in 39 patients. During the follow-up period, 27 patients had no evidence of disease, 2 patients showed disease, and 10 patients died of disease. In this group, advanced T and N stages were not related to overall or disease-specific survival outcomes. The overall survival rate was affected by a high CD68 (+) (HR = 19.8; $P = 0.040$) and low CD8/CD4 ratio (HR = 7.7, $P = 0.025$). The disease-specific survival rate was affected by a high number of CD68 (+) cells (HR = 15.2; $P = 0.03$) and low CD8 (+)/CD4 (+) ratio (HR = 3.3; $P = 0.04$).
Conclusions: The number of CD68 (+) cells and the distribution of cytotoxic or immunosuppressive T lymphocytes could be determining factors for CCRT outcomes in HPV (+) TSCC patients.
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Introduction

Prognostic factors for head and neck squamous cell carcinomas (HNSCCs), such as tonsillar squamous cell cancer (TSCC), include age, smoking, TNM stage, margin status, metabolic status, and extracapsular spread [1–3]. Recently, rather than these factors, human papillomavirus (HPV) infection in combination with p16 expression in TSCC has also been significantly associated with TSCC prognosis [4]. HPV infection-related TSCC is frequently encountered, and it presents different patient characteristics and better treatment results than HPV (−) TSCC [5]. Given the functional and cosmetic morbidity associated with surgical treatment and the good response to nonsurgical therapies, concurrent chemoradiation therapy (CCRT) with or without induction chemotherapy is a good alternative treatment method for HPV (+) TSCC. CCRT is widely used for TSCC due to organ preservation and locoregional control rates comparable to those of surgical treatment [6–8]. Despite the favorable prognosis of TSCC patients treated with CCRT, HPV (+) tonsil cancer-related deaths, which are accompanied by distant metastasis, have yet to be completely resolved, and treatment-induced toxicities also should not be overlooked.

The tumor microenvironment is an emerging concept that proposes that the survival, growth, proliferation, and metastasis of tumor cells are determined by their interactions with the surrounding milieu, rather than genetics alone [9]. Among the participants in the microenvironment, immune-related stromal cells play significant roles in the therapeutic response. Inflammatory cells, such as T and B lymphocytes, are involved in regulating the bioactivity of myeloid cells, including macrophages, monocytes,

* Corresponding author at: Department of Otolaryngology, Asan Medical Center, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea. Tel.: +82 2 3010 3710; fax: +82 2 489 2773.
E-mail address: sykim2@amc.seoul.kr (S.Y. Kim).

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and mast cells, as well as the response to CCRT. In addition to T and B lymphocytes, tumor-associated macrophages (TAMs) in human breast, ovarian, and non-small-cell lung cancers are related to poor prognosis [10–13]. These macrophages are responsible for radioresistance and chemoresistance in various types of cancers [14–16]. In contrast, the infiltration of CD8 (+) lymphocytes is related to favorable treatment outcomes of breast cancer. The absence of CD8 (+) cytotoxic T lymphocytes and the presence of CD4 (+) T effector lymphocytes enhance the protumor bioactivity of TAMs and the metastasis of mammary adenocarcinoma [17]. In addition, in HNSCCs, immunosuppressive cells or molecules and chronic inflammation promote the development of the tumor-promoting microenvironment. HPV-associated SCCs that arise from the deep crypts of the oropharynx, which is surrounded by lymphoid tissue, demonstrate lymphocyte infiltration into the stoma and tumor nests [18]. Sustained inflammatory conditions influence this type of tumor and affect the capacity of tumor-infiltrating lymphocytes to act as effector cells during tumor progression.

Accordingly, we surmised that the peritumoral distribution of TAM and T lymphocytes, which compose the tumor microenvironment, may affect the treatment outcomes of HPV (+) TSCC. In our current study therefore, we evaluated the distribution of inflammatory cells in HPV (+) TSCC and their prognostic value in these lesions.

Materials and methods

Patients

We reviewed the medical records of 53 patients who were diagnosed with TSCC and completed CCRT with or without induction chemotherapy with curative intent at our hospital between January 2008 and December 2011. This study was approved by our Institutional Review Board. Patients with distant metastasis at the initial diagnosis, a history of other malignant disease, failure to compete CCRT, or a lack of full medical records for ⩾12 months were excluded. HPV (+)-related TSCC was confirmed using HPV-DNA PCR, and p16 overexpression was confirmed using immunohistochemistry (IHC) analysis of tumor tissue obtained from the primary site.

Treatment

CCRT consisted of RT with three doses of cisplatin (100 mg/m^2) per week. RT was administered to the primary tumor bed using a total dose of 70 Gy (range = 62–76 Gy), and RT was administered to levels I–V of the cervical region using a median dose of 50.4 Gy delivered using a single daily dose of 1.8–2.4 Gy/fraction. Upon CCRT, RT was continued 5 days/week in order to administer the total dose over 30 fractions for 6 weeks, in conjunction with chemotherapy. The induction chemotherapy regimen consisted of FP (1000 mg/m^2 5-fluorouracil intravenous [IV] + 60 mg/m^2 cisplatin IV), DP (70 mg/m^2 docetaxel IV + 75 mg/m^2 cisplatin IV), or DFP (70 mg/m^2 docetaxel IV + 750 mg/m^2 5-fluorouracil IV + 75 mg/m^2 cisplatin IV), which was administered two or three times before definitive therapy.

Follow-up

After completing treatment, patients were evaluated by physical examination, endoscopy, and imaging studies (e.g., computerized tomography, magnetic resonance imaging, or positron emission tomography/computerized tomography) every 2–3 months for the first year according to the protocols of the institution, which were mainly based on the guidelines of the National Comprehensive Cancer Network. Follow-up intervals were increased to 3–6 months in the second year and 6–12 months after that.

HPV PCR

Detection and subtyping of HPV were performed using a Pap Type HPV PCR detection test (Genera Biosystems, Scoresby, Australia). This assay detects 14 high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 2 low-risk (HPV 6 and 11) HPV subtypes. Formalin-fixed, paraffin-embedded (FFPE) tissue sections were assessed by a pathologist to determine the tumor percentage. DNA was then extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). The extracted DNA was assessed using PCR with general primers in order to detect all HPV subtypes. The PCR reaction mix was then hybridized to genotype-specific (i.e., HPV subtype) hybridization beads, which were analyzed using a BD FACSArray (BD Biosciences, San Jose, CA) in order to determine the HPV status of each sample.

p16 IHC

IHC analysis of p16 was performed using FFPE tissue sections using immunoperoxidase staining and a Ventana Autostainer and ultraView DAB detection kit (Ventana Medical System Inc., Tucson, AZ) in accordance with the manufacturer's instructions. A monoclonal p16INK4 antibody was used (1:10; Pharmingen, NJ). p16 expression was semiquantitatively scored by two independent observers (K.J. Cho and J.Y. Park) who were blinded to any clinical information. The scores were based on the proportion of nuclear-immunoreactive tumor cells relative to the total number of tumor cells/nuclei (thus, the scores ranged from 0% to 100%). The scores from both observers were then averaged. p16 expression was scored as positive if there was strong and diffuse nuclear and cytoplasmic staining present in >70% of the malignant cells. All other staining patterns were scored as negative.

Lymphocyte infiltration

Biopsy samples were taken from the FFPE sections corresponding to the tumor and peritumoral areas, which were previously marked. For IHC analysis, 4.0-μm-thick paraffin sections were used. Under high magnification (400×), two pathologists (K.J. Cho and J.Y. Park) without clinical knowledge of the enrolled patients counted the number of CD4 (+)-, CD8 (+)-, and CD68 (+)-stained cells to quantify the expression of the three markers (Figs. 1 and 2). We used the mean number of stained cells to compare the number and distribution of cells. Patients were divided into two groups (high vs low) according to the mean number of stained cells: CD4 (+) (mean = 360 cells), CD8 (+) (299), and CD68 (+) (147). To evaluate the distribution of these cells, we divided the patients into two groups (high vs low) using the mean ratio for each cell: CD8 (+)/CD4 (+) (mean ratio = 0.82) and CD8 (+)/CD68 (+) (1.81).

Statistical analysis

Statistical analyses were performed using SPSS software (version 18.0; SPSS, Chicago, IL). Kaplan–Meier analysis and the log-rank test were used to illustrate differences in overall survival and disease-specific survival (DSS) according to the CD4 (+), CD8 (+), and CD68 (+) expression profiles and CD8 (+)/CD4 (+) and CD8 (+)/CD68 (+) ratios. Univariate analysis was used to estimate the relationship between overall and disease-free survival and clinicopathological parameters (i.e., age, sex, tumor stage, nodal stage, nodal status, p16 expression, and immune profiles [CD4, CD8, and

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