의학석사 학위논문

다중 면역형광 패널을 이용한 하인두암의 종양 내 침윤 림프구의 분포와 같이 발현되는 면역학적 지표

Validation of multiplex immunofluorescence panels to evaluate distribution of tumor infiltrating lymphocytes in hypopharyngeal cancer

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지도교수 남 순 열

이 논문을 의학석사 학위 논문으로 제출함

2018년 12월

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Summary

Background and Objectives: Immunological markers predicting treatment response to immunotherapeutic agents have not been revealed. Tumor infiltrating lymphocytes (TILs) are essential to activate innate or adaptive immunity against tumor. Rather than one type of TILs, the distribution of regulatory and cytotoxic T-cell markers may help to delineate the immune-profiling and to predict the response to immunotherapy. This preliminary study was aimed to evaluate the overall immunological tumor microenvironment by utilizing multiplex immunofluorescence (mIF) panels.

Materials and Methods: Formalin fixed paraffin-embedded tissue (FFPE) specimens of 10 cases of hypopharyngeal squamous cell carcinoma were prepared for conventional immunohistochemistry (IHC), uniplex and multiplex IF validation. The Opal™ Multiplex IHC Kit (PerkinElmer) was used for multiple IF validation and these findings were reviewed by a pathologist. The panel including pancytokeratin, PD-L1, CD4, CD8, CD68, and DAPI was developed and simultaneous marker expressions were quantified to evaluate the expression of tumor infiltrating lymphocytes (TILs) and programmed cell death ligand 1 (PD-L1).

Results: The mIF panel demonstrated the distribution of TILs and PD-L1 in the FFPE tissue. Compared to each conventional IHC, we could evaluate the distribution of PD-L1, $CD4^+$, $CD8^+$, $CD68^+$ cells in one slide. Quantitative analysis demonstrated that the types of TILs were distinct from each samples as well as the amount of aggregated TILs.

Conclusions: The mIF may give us an additional information presuming tumor tissue immune-profiling, elucidating main contributing TILs. This finding may help to evaluate immunological status and to choose adequate immunotherapeutic agents according to the crucial immune cells.

KEY WORDS: PD-L1, tumor infiltrating lymphocyte, multiplex immunohistochemistry, hypopharyngeal squamous cell carcinoma, head and neck

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Introduction

Programmed cell death protein 1 (PD1) is a receptor expressed on T cells that plays a central role in their activation and proliferation. Its ligand, programmed cell death-ligand 1 (PD-L1), binds to PD1 to induce apoptosis in activated T cells. PD-1/PD-L1 axis is thought to regulate a pathway that allows cancers to escape immune surveillance. Blockade of this interaction has been shown to strengthen the antitumor activity of T cells. PD-L1 is frequently overexpressed in many types of human cancer.[1)] Antibody blockade of PD-1with its ligand has shown favorable results against malignancies such as melanoma and non–small cell lung cancer.[2-4)] These recent results have led to the clinical testing of PD-1/PD-L1 inhibitors in head and neck squamous cell cancers (HNSCC).[5-7)]

Studies carried out on other tumors also found that clinical responses to immune checkpoint inhibitors are associated with increased immune inhibitory signals and with increased tumor infiltrating lymphocytes (TILs).[1,8-10)] Understanding this tumor microenvironment has become an important step in identifying evidence for the presence of distinct immunologic phenotypes, based on the presence or absence of certain immune cells.[11-12)] For example, high densities of myeloid cells, correlate with poor prognosis.[13)] However, the correlation between macrophage density and patient survival is less significant than that of T cells, particularly CD8+ T cells.[14)]

Patients with hypopharyngeal squamous cell carcinomas (HPSCC) present with advanced disease and have the worst prognosis among HNSCC despite the concurrent use of

treatments comprised of surgery and chemotherapy with or without radiotherapy.[15)] Currently, there is limited data on the prognostic values of PD-L1 or TILs expression in HPSCC patients.

There is difficulty in setting a cut-off value representing the presence of TILs. Both location and density of TIL and their interrelationship with PD-L1 positive tumor microenvironment are important considerations.[12)] Its quantitation method need to be concise because the level of PD-1 on T cells may be associated with the state of differentiation and level of function of T cells.[16)]

This study analyzed PD-L1 expression, along with CD4+ and CD8+ T-cell density, CD68+ density in hypopharyngeal squamous cell carcinomas using quantitative multispectral imaging (MSI). Multiplex immunofluorescence (mIF) staining allows simultaneous detection of multiple markers in the same tissue section in formalin-fixed and paraffin-embedded (FFPE) tumor tissues for deeper understanding of the tumor microenvironment.[17] We aimed to verify mIF panels to apply to FFPE HNSCC tissues using a set of immune marker antibodies, including those against PD-L1 and TILs, multispectral microscopy and image analysis software..

Materials and Methods

1. Study patients and FFPE tissue specimens

This study enrolled 10 patients with newly diagnosed hypopharyngeal squamous cell carcinoma who underwent total laryngopharyngectomy at Asan medical center between 2004 and 2012. Sequential 4-μm-thick sections were prepared for mIF staining.

2. Multiplexed immunofluoresecence (mIF) and Quantification

Immunofluorescence multiplex staining was performed using PerkinElmer Opal kit (Perkin-Elmer, Waltham, MA). FFPE tissues were cut in 4 um thick sections. Slides were heated at least for 4 hours in a dry oven at 60°C and then rinsed with 100% xylene for 10 minutes, repeated 3 times to deparaffinize. The FFPE sections were rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was performed in citrate buffer (pH 6.0) using microwave treatment (MWT). Slides were washed and blocking was performed with 3% H2O2 blocking solution followed by Dako antibody diluent. The first primary antibodies for cytokeratin (M3515, Dako, USA, dilution dilution 1:500) were incubated for 1 hour in a humidified chamber at room temperature followed by detection using the OpalTM Polymer HRP Ms+Rb kit. Visualization of Cytokeratin was accomplished using Opal 690 TSA Plus (dilution 1:150), after which the slide was placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with primary antibodies for CD274 (PD-L1) (E1L3N, #13684, Cell Signaling, USA, dilution 1:300) for 1 hour in a humidified chamber at room temperature, followed by detection using the OpalTM Polymer HRP Ms+Rb kit. CD274

(PD-L1) was then visualized using Opal 650 TSA Plus (1:150), and the slide was placed in citrate buffer (pH 6.0) for MWT. The slide was then incubated with primary antibodies for CD4 (NCL-L-CD4-368, Leica, United Kingdom, dilution 1:100) for 1 hour in a humidified chamber at room temperature, followed by detection using the OpalTM Polymer HRP Ms+Rb kit followed by visualization using Opal 620 TSA Plus (1:150). The slide was again placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with primary antibodies for CD68 (, dilution 1:100) for 1 hour in a humidified chamber at room temperature, followed by detection using the OpalTM Polymer HRP Ms+Rb kit followed by visualization using Opal 570 TSA Plus (1:150). The slide was again placed in citrate buffer (pH 6.0) and heated using MWT. The slide was then incubated with the last antibody, CD8 (4B11, NB100-65729, Novusbio, USA, dilution 1:300), for 1 hour in a humidified chamber at room temperature, followed by detection using the OpalTM Polymer HRP Ms+Rb kit. CD8 was visualized using Opal 540 TSA Plus (1:150). The slide was again placed in citrate buffer (pH 6.0) and heated using MWT. Nuclei were subsequently visualized with DAPI, and the section was coverslipped using HIGHDEF® IHC fluoromount (ADI-950-260-0025, Enzo, USA).

3. Image collection and quantitative image analysis

The spectral information from a multiplexed panel of targets is captured through the Vectra 3.0 Automated Quantitative Pathology Imaging System. Each of the individually stained sections (Cytokeratin-Opal 520, PD-L1-Opal 650, CD4-Opal 620, CD8-Opal 540,

CD68-Opal 570 and DAPI) was used to establish the spectral library of fluorophores required for multispectral analysis (Fig 1A). This spectral library forms the reference of target quantitation, as the intensity of each fluorescent target is extracted from the multispectral data using linear unmixing. Immunostained sections were scanned using the Vectra 3.0 Automated Quantitative Pathology Imaging System 20 nm wavelength intervals from 420 n to 720 nm and combined these captures to create a single stack image which retained the unique spectral signature of all mIF markers. Image files created by Vectra were analyzed using InForm 2.2.1 image analysis software (Figure 1B). For analysis, 10 regions of interest were selected for each case to include at least 80% of malignant cells (Fig1C). Each cell was identified by detecting nuclear spectral element (DAPI). The total number of CD274 (PD-L1), CD4, CD8 and CD68 positive cells was considered identified as the total immune cell infiltrations in the tissue. The percentage of each immune cell subset was calculated by dividing the absolute number of each subset by the total numbers of all these cells.

4. Analysis with Spotfire™ software and compartmentalization of the samples

The data obtained from InForm were sent to Spotfire™ software and threshold for positivity of each factor was determined based on IHC scoring methods by a pathologist (YSP) blinded to clinical data. Scores indicating positivity were > 2.5 for CK, > 0.7 for CD4, > 3.5 for CD8, > 0.7 for CD68, and > 0.3 for PD-L1. All cells in each slide were designated as positive or negative for each antibody, and the data were exported to an xls file for

analysis. The section was dichotomized into the tumor and the stroma in order to evaluate each component (Fig1D).

5. Statistical analysis

Continuous variables and proportions were compared using the Mann–Whitney U. P values < 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS version 24.0 (IBM Corp., Armonk, NY).

6. Ethics and Disclosures

This study was conducted in accordance with good clinical practice and the Declaration of Helsinki. Study permission was granted from the Institutional Review Board of our hospital (project ID 2017-1113) and informed consent requirement was waived. No private organizations or companies were involved.

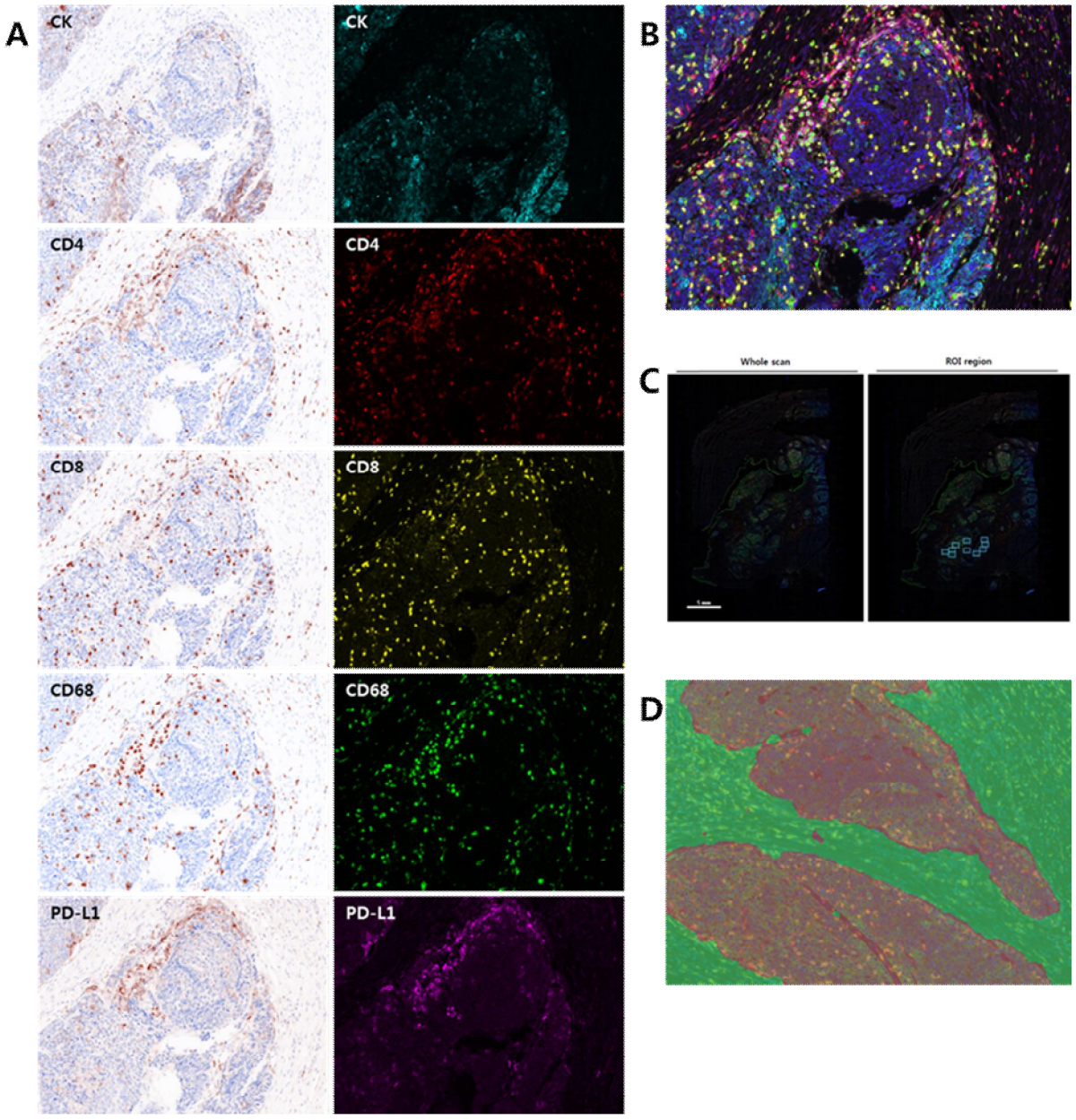

Figure 1. A. The results of Opal™ staining for CK, CD4, CD8, CD68, PD-L1 (right panel), and the results of corresponding IHC staining images (left panel). B. Composite image of multiplex staining in hypopharyngeal squamous cell carcinoma tissue sections. C Selection of intratumoral regions to include at least 80% malignant cells. D An example of a section dichotomized into the tumor and the stroma.

Results

1. Patient characteristics

The baseline demographic and clinicopathological characteristics of the 10 patients are shown in Table 1. All patients were male. Their median age was 72.5 years (range, 61–79). Nine of the patients had carcinoma arising from the pyriform sinus and 1 the posterior pharyngeal wall. Of the 10 patients, 9 of them had overall stage 4 disease. On pathology report, the median size of the tumor was 3.8 cm (range 2.1-5.5). Two cases showed well differentiation, 6 moderate, 2 poor and 1 unknown. Two cases had positive resection margins. Seven patients received postoperative radiation therapy and 1 patient received concurrent chemoradiation therapy. The other 2 patients were advised to undergo adjuvant radiation therapy but refused. There were 4 cases of recurrence during the median follow up period of 56 months (range 9-86). Two were locoregional recurrence and the other 2 were distant metastasis.

2. Multiplex IF Validation

After testing all of the markers by chromogenic IHC and uniplex IF staining, we validated the markers by multiplex IF staining. The panel (pancytokeratin, PD-L1, CD4, CD8, CD68, and DAPI) showed specific staining without background staining in the major part of the markers. Figure 2 shows the distribution of CD4+, CD8+, CD68+, PD-L1 markers according to tumor centers and stromata of the 10 hypopharyngeal squamous cell carcinoma samples.

3. TIL quantity and infiltration pattern

We divided the patients according to whether the tumor recurred during follow up; 6 were in the NED group and 4 in the recurred group. Then we assessed the density of TILs according to recurrence in the whole tumor section, the tumor, and the stroma. We found that CD4+ T-cell, CD8+ T-cell and CD68+ density tended to be higher in tumor centers of the NED group, however none showed statistical significance. Overall, TIL quantity and infiltration pattern did not show a consistent trend.

4. PD-L1 expression and PD-L1+CD8+ T-cell density

We also assessed the PD-L1 expression according to tumor recurrence. PD-L1 expression was higher in the NED group in the whole tumor section, the tumor, and the stroma. Also PD-L1+CD8+ T-cell density was higher in the whole tumor section, the tumor, and the stroma. However none showed statistical significance.

Table 1. Characteristics of 10 study patients

Characteristics	*N*	%
Age, median (range), y	72.5 (61−79)	
Sex		
Male/female	10/0	100/0
Subsite of primary tumor		
Pyriform sinus	9	90
Posterior pharynx	1	10
Smoking		
none /current, ex-smoker	0/10	0/100
>20/≤20 (pack-year)	8/2	80/20
Body mass index, kg/m		
Median (range)	20.9 (14.0−25.6)	
<18.5/18.5−25/>25	4/5/1	40/50/10
Clinical TNM stage		
T1/T2/T3/T4	1/0/2/7	10/0/20/70
N0/N1/N2/N3	2/2/6/0	20/20/60/0
Overall I/II/III/IV	0/0/1/9	0/0/10/90
Primary tumor size, cm		
Median (range)	3.8 (2.1−5.5)	
Differentiation		
Well/moderate/poorly/unknown	2/6/1/1	20/60/10/10
Lymphovascular invasion		
Positive/negative/unknown	4/6/0	40/60/0
Perineural invasion		
Positive/negative/unknown	1/6/2	10/60/20
Resection margin status		
Positive/negative	2/8	20/80
Adjuvant therapy		

None/RT alone/CCRT	2/7/1	20/70/10
Follow-up information		
Median follow-up (range), months	56 (9–86)	
Recurrence, local/regional/distant	1/1/2	10/10/20
Last status, NED/DOC/DOD	3/2/3	30/20/30

Abbreviations: RT, radiotherapy; CCRT, concurrent chemoradiation therapy; NED, no evidence of disease; DOC, death due to other cause; DOD, death due to disease

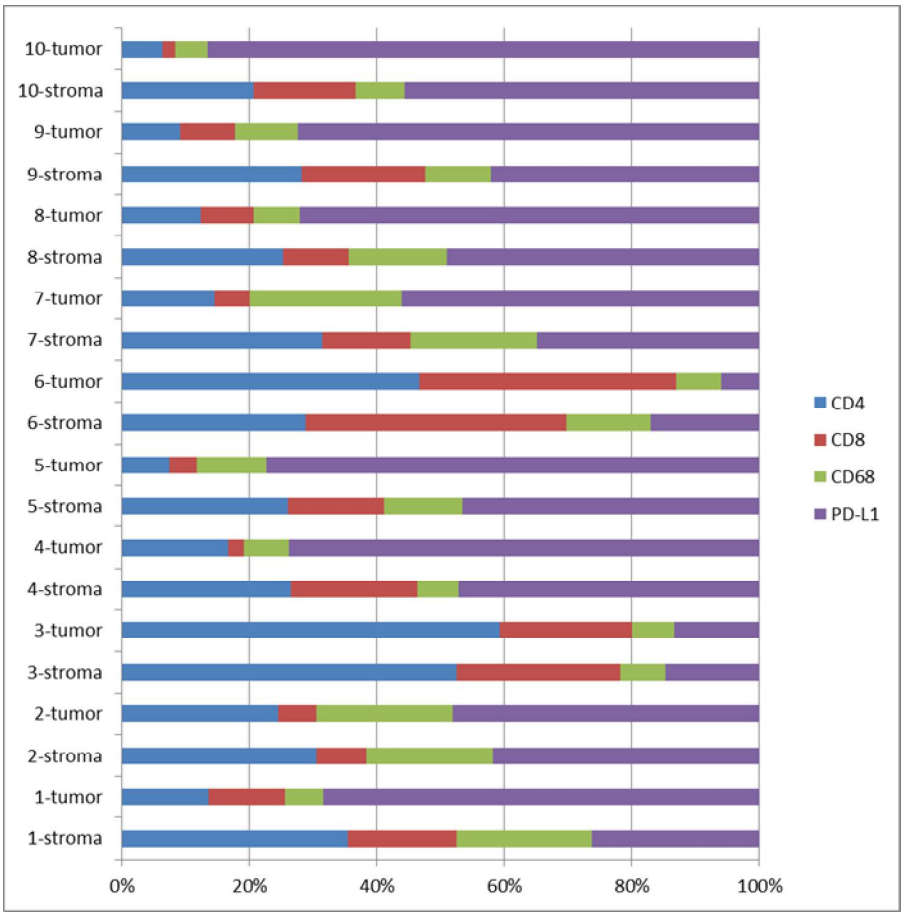

Figure 2. Distribution of CD4, CD8, CD68, PD-L1 according to tumor centers and stromata of 10 hypopharyngeal squamous cell carcinoma samples.

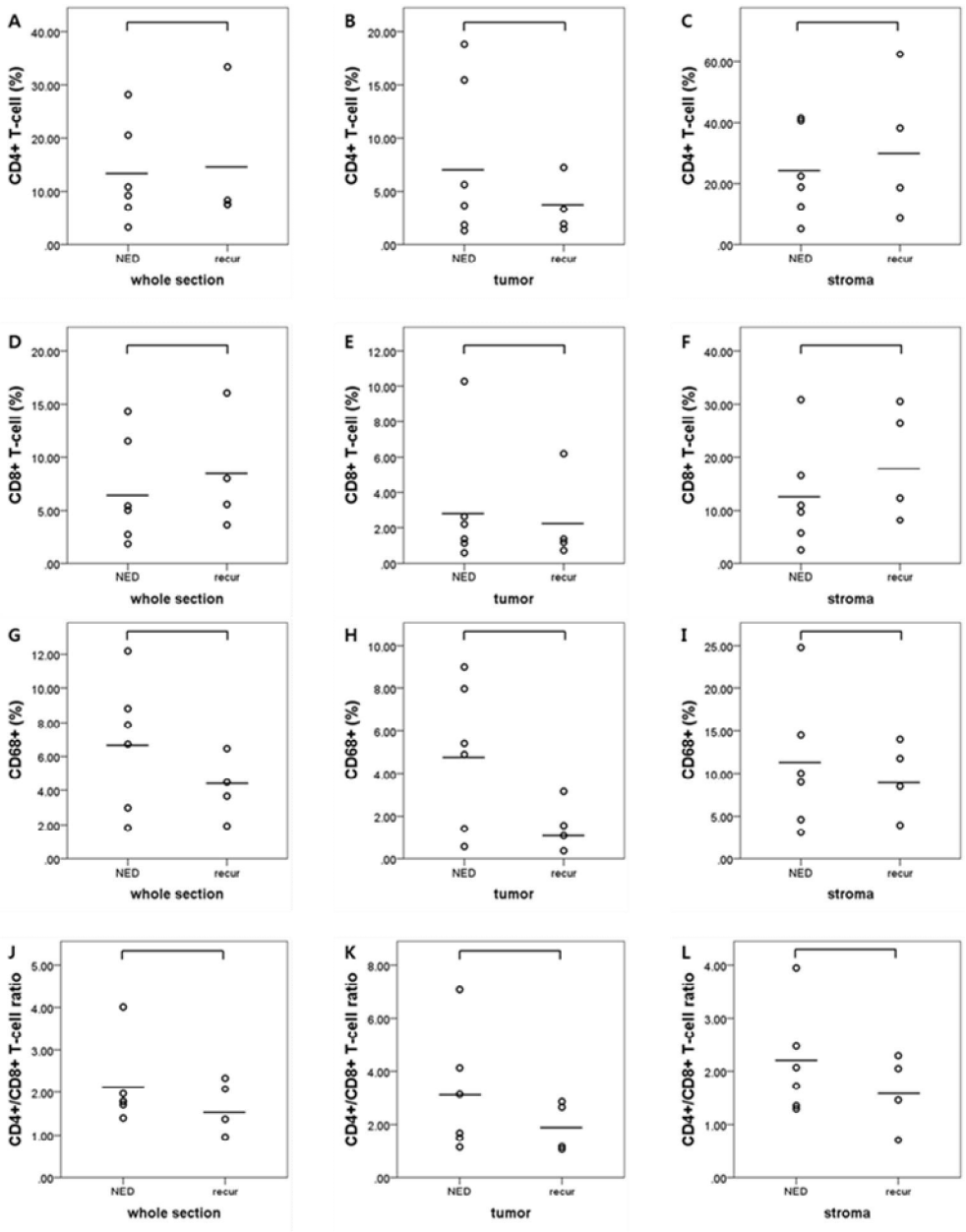

Figure 3. TILs quantity and infiltration patterns. CD4+ T-cell densities in A whole sections, B tumor centers, and C stroma of hypopharyngeal squamous cell carcinoma samples. CD8+ T-cell densities in D whole sections, E tumor centers, and F stroma of hypopharyngeal squamous cell carcinoma samples. CD68+ T-cell densities in G whole sections, H tumor centers, and I stroma of hypopharyngeal squamous cell carcinoma samples. CD4+/CD8+ T-

cell ratios in J whole tumor sections, K tumor centers, and L stroma of hypopharyngeal squamous cell carcinoma samples

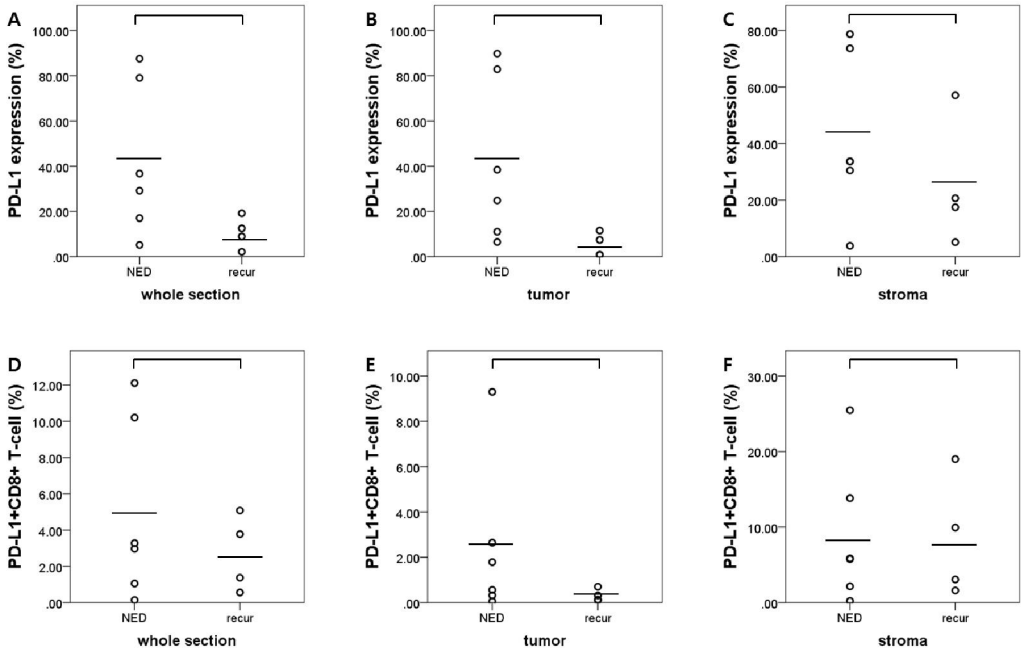

Figure 4. PD-L1 expression in hypopharyngeal squamous cell carcinoma, determined by a quantitative multispectral imaging system (Opal™) in A whole sections, B tumor centers, and C stroma.

Discussion

In this study, we were able to demonstrate the distribution of CD4+, CD8+, CD68+, PD-L1 markers according to the tumor center and stroma of 10 HPSCC cases using multiplex immunofluorescence staining. Although the results showed no statistical significance, we could observe a diverse infiltrating pattern of the tumor microenvironment.

CD8+ cytotoxic T cells are known to play an essential role in cell-mediated antitumor immune responses.[18-19] A previous study conducted on HNSCC patients who underwent surgical resection found out that tumor-infiltrating CD8+ TILs are independent predictors of beneficial overall survival.[20] Other studies on advanced head and neck cancer patients undergoing definitive chemoradiotherapy[21] and neoadjuvant chemotherapy[22] showed similar results that patients with larger number of tumor-infiltrating CD8+ TILs tend to survive longer. There were also suggestions that a further subgroup analysis of PD-L1 expression could help predict the prognosis of patients with higher CD8+ TIL density and may be used as a predictive biomarker.[20,22-23] However, immunological hot tumor and cold tumor showed different absolute number for TILs including CD4+, CD8+, CD68+ and NK cells rather than absolute number of TILs. Thus, relative distribution of these immune cells would be more helpful to delineate the prognosis irrespective of immunological milieu.

A previous investigation carried out on patients with extrahepatic bile duct cancer exhibited that patients with high PD-1+/CD8+ TILs were associated with a significantly worse prognosis compared to those with low PD-1+/CD8+ TILs.[24] Another study by Kansy

et al on HNSCC patients found HPV+ patients had a significant lower fraction of the PD-1 high-expressing cells in comparison with the HPV- patients. In contrast, the PD-1 low fraction was significantly higher in HPV+ patients in comparison with HPV- patients.[25] These findings suggest that PD-1 high-expressing CD8+ TILs may stand for an weakened state of effector T cells so the level of PD-1 expression on CD8+ TILs may be necessary to differentiate functional from nonfunctional CD8+ TILs.[22,25]

The association between PD-L1 expression and the prognosis of patients with HNSCC still seems unclear. Some studies show that higher levels of PD-L1 expression are related to a poor prognosis in various types of cancers.[26-30] However, other studies question these results.[31-33] The present study also did not find a significant association between PD-L1 expression and the recurrence of patients with HPSCC. A possible explanation for this could be that HPSCCs do not contain high enough densities of TILs for it to demonstrate significance according to its expression.[11] This result seems to attribute to the different role of TILs in each immunological hot and cold tumor.

CD4+ T-lymphocyte is also known to contribute to a favorable prognosis when detected in higher numbers.[34] The effect of FoxP3+CD4+T-reg cells does not seem unified among studies[34-39] which might account to different behavior in different anatomic sites.[40]

Our study was a retrospective review on a small number of patients from a single tertiary center. Some factors not regarded as meaningful in this study may show significance in larger populations. We included patients who underwent total laryngopharygectomy for

newly diagnosed HPSCC which implied that the patients were at advanced stage at the time of diagnosis. More meaningful results could be derived from a larger population of patients representing all stages of disease. However, this study demonstrated the advantages of the MSI technique, which can simultaneously examine cells positive for multiple markers and test whole section slides, thereby facilitating an outline of the whole landscape of tumor and immune cell populations.

Conclusion

This study demonstrated the distribution of CD4+, CD8+, CD68+, PD-L1 markers according to the tumor center and stroma of 10 HPSCC cases using multiplex immunofluorescence staining which can simultaneously analyze cells positive for multiple markers. This technique might help to get an overview of the whole landscape of tumor and immune cell populations.

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국문 요약

면역 치료제에 대한 치료 반응을 예측하는 면역 학적 지표는 아직 밝혀지지 않았다. 종양 내 침윤 림프구는 종양에 대한 선천성 또는 후천성 면역을 활성화시키는 데 필수적이다. 단일 유형의 종양 내 침윤 림프구보다는 조절 및 세포 독성 T 세포의 전반적 분포가 면역 프로파일을 묘사하고 면역 요법에 대한 반응을 예측하는 데 도움이 될 수 있다. 이 예비 연구는 다중 면역 형광 패널을 이용하여 전반적인 면역학적 종양 미세 환경을 평가하기 위해 시행하였다.

포르말린 고정 파라핀 포매된 하인두 편평세포암종 10 례의 검체를 기존 면역 조직 화학 염색법, 단일 및 다중 면역 형광 염색법 검증을 위해 준비하였다. Opal ™ Multiplex IHC Kit (PerkinElmer)이 다중 면역 형광 염색법에 사용되었으며, 위 결과는 병리학자가 검토하였다. Pancytokeratin, PD-L1, CD4, CD8, CD68 및 DAPI 를 포함하는 패널을 개발하여 종양 내 침윤 림프구 및 PD-L1 의 발현을 평가하였다.

다중 면역 형광 패널은 포르말린 고정 파라핀 포매 조직에서 종양 내 침윤 림프구와 PD-L1 의 특징적인 분포를 보여 주었다. 각각의 전통적인 면역 조직 화학 염색법과 비교하여 한 슬라이드에서 PD-L1, CD4 +, CD8 +, CD68 + 세포의 분포를 동시에 평가할 수 있었다. 정량 분석은 종양 내 침윤 림프구의 유형이 각 샘플마다 뚜렷한 차이가 있음을 보여 주었고 집계 된 종양 내 침윤 림프구의 양도 나타냈다.

다중 면역 형광 패널은 종양 조직의 면역 프로파일을 추정하는 정보를 제공하여 주요 종양 내 침윤 림프구를 밝히는 데 사용할 수 있다. 이는 종양의 면역 상태를 평가하고 중요한 면역 세포에 따라 적절한 면역 치료제를 선택하는 데 도움이 될 수 있다.