Characterization of head and neck squamous cell carcinoma arising in young patients: Particular focus on molecular alteration and tumor immunity

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Abstract
Background: The purpose of this study was to comprehensively characterize head and neck squamous cell carcinoma (HNSCC) arising in young patients (<45 years old).
Methods: We performed immunohistochemistry, silver, and fluorescence in situ hybridization using samples obtained from 396 radically resected cases among 1787 HNSCCs.
Results: Young age HNSCCs occurred in 10.9% (194/1787) and were most common in the oral tongue (50.5%). They revealed distinctively lower frequency of p16 positivity, high c-MET expression, MET copy number gain, and lower pan-Trk expression. PD-L1 positivity in tumor cells and ICOS+ tumor infiltrating lymphocytes (TILs) were higher in the young age. Perineural invasion, PD-L1 positivity, and higher ratio of CD163+ tumor infiltrating macrophages to CD8 + TILs were determined to be independent factors for poor progression-free survival.
Conclusion: Characterizing these features of young age HNSCC may help to identify the underlying pathogenesis and to improve patient outcome through different treatment strategies.

KEYWORDS
cancer immunity, head and neck, molecular markers, squamous cell carcinoma, young age

1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a common cancer worldwide and occurs in various anatomical subsites, including the nasal cavity/paranasal sinuses, oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx. HNSCC usually occurs in late adulthood, with a mean age of 57-62 years old.1-4 HNSCCs of the oral (mobile) tongue and oropharynx, however, are increasingly prevalent in patients younger than 45 years old, although these young patients lack of significant exposure to tobacco and alcohol.5-6 However, factors underlying the increasing incidence of oral tongue and/or oropharynx SCC in young patients are not yet well known.

Conventionally, several toxins from tobacco smoking and alcohol consumption are known to be involved in the carcinogenesis of HNSCC by inducing genetic mutations and immune system impairment. Recently, high-risk human papilloma virus (HPV) infection was also identified to be involved in the carcinogenesis of HNSCC through deregulation of p16/INK4a-related cell cycle checkpoints and mutations of several molecules.7-9 Recent comprehensive genetic
analyses by The Cancer Genome Atlas Network show several known targets and driver oncogenes such as FGFR1 and CCND1 in HNSCC. Furthermore, recent studies show the implications of an impaired immune system for the growth and progression of HNSCC. Expression of immune-modulatory signals such as PD-1, ICOS, LAG3, and CTLA-4 deregulates tumor-infiltrating CD8+ cytotoxic T cells, FOXP3+ regulatory T cells (Tregs), and antigen-presenting dendritic cells/macrophages in HNSCC. HPV infection has been suggested to alter immune surveillance of HNSCC. Although genomic and immunogenic characteristics have been studied, further validation studies with large scale clinical samples using immunohistochemistry (IHC), in situ hybridization, and quantification assays of tumor-infiltrating immune cells are rare. Furthermore, validation studies are very rare focusing on young age HNSCCs, which are lack of significant exposure to tobacco and alcohol.

In the present study, we characterize HNSCC arising in young patients with particular focus on molecular alteration and tumor immunity.

2  MATERIALS AND METHODS

2.1  Case selection

From the database of Severance Hospital Cancer Registry Data, Seoul, Korea, 1787 cases of HNSCC were retrieved. These cases were histologically confirmed as SCC type at Severance Hospital between 2005 and 2012. Affected anatomical sites included the oral tongue (anterior two-thirds of the tongue; N = 332, 18.6%), other oral cavity (mouth floor, hard palate, buccal mucosa, and unspecified oral cavity; N = 258, 14.4%), oropharynx (tonsils, base of tongue, soft palate, and oropharynx; N = 361, 20.2%), hypopharynx (N = 222, 12.4%), larynx (lingual surface of the epiglottis, glottis, supraglottis, subglottis, and larynx; N = 515, 28.8%), and nasal cavity/paranasal sinuses (N = 99, 5.5%). Carcinomas of nasopharynx, ear cavity, salivary gland, or other anatomical sites were excluded from the present study. The median (mean) follow-up period was 64.5 (66.0) months.

For further analyses of clinical and histologic factors, genetic alteration, protein expression, immune cell infiltration, and immune checkpoint markers, 552 consecutive cases that underwent curative surgical resection without preoperative treatment were retrieved from the overall 1787 HNSCC cases. These patients underwent standard adjuvant chemotherapy and/or radiation therapy according to tumor stage after pathologic evaluation of the surgical specimen. Demographic features of the 552 cases were summarized in Supporting Information Table S1 and Supporting Information Figures S2 and S3. Cases with distant metastasis and unclear clinical information were excluded. For this cohort group, clinical and pathological parameters were characterized, including sex, age, smoking history, alcohol consumption, lymphovascular invasion (LVI), perineural invasion (PNI), pathologic T (tumor) classification, and pathologic N (lymph node) classification. Tumors were classified according to the Seventh American Joint Committee on Cancer TNM cancer classification system and the World Health Organization system. Never-smokers were defined as those with a lifetime smoking dose of fewer than 100 cigarettes, while former smokers were those who had not smoked for more than 1 year, and current smokers were those who were current smokers or who had quit smoking less than 1 year prior. Alcohol consumption status was determined by the amount of pure alcohol consumed which was calculated as grams per day, according to the average amount, frequency, and type. “Heavy drinkers” referred to people who consumed more than 30 g/day, and those who drank less were defined as “social drinkers.”

For IHC evaluation, dual-color silver in situ hybridization (SISH) and fluorescence in situ hybridization (FISH) were performed on archival formalin-fixed paraffin-embedded specimens obtained from HNSCC patients. However, samples that underwent decalcification or those with too few tumor cells were excluded. Cases of the nasal cavity/paranasal sinuses were also excluded because there were too few cases to perform appropriate statistical analyses. Finally, 396 cases of the oral tongue (N = 172), other oral cavity (N = 32), oropharynx (N = 122), hypopharynx (N = 28), and larynx (N = 42) were selected. The overall flow of case selection is summarized in Supporting Information Figure S4. The study was approved by the Institutional Review Board of Severance Hospital (protocol No.: 4-2015-0954). Histology as well as IHC, SISH, and FISH tests were interpreted by 4 experienced pathologists (S.O.Y, H.J.R, and E.K.K) blinded to clinical data.

2.2  IHC and microscopic analysis

Two or 3 different representative tumor areas per sample were selected for tissue microarray (TMA) construction. Core tissues 3 mm in diameter were obtained from donor tissue blocks and arranged in recipient TMA blocks using a trephine apparatus.

IHC was performed on 4-μm TMA sections with a Ventana Bench Mark XT Autostainer (Ventana Medical Systems, Tucson, Arizona), as described previously. Primary antibodies were p16 (RTU; Ventana), CD3 (dilution 1:200; LabVision, Fremont, California), CD8 (RTU; clone C8/144B; Dako, Glostrup, Denmark), CD163 (dilution 1:100; clone MRQ-26; Cell Marque, Rocklin, California), FOXP3 (dilution 1:100; Abcam, Cambridge, United Kingdom), ICOS/CD278 (clone M22402; dilution 1:50; Thermo Scientific, Rockford, Illinois), LAG-3 (clone EPR4392(2); dilution 1:100; Abcam), tumor infiltrating macrophage (TIM)-3 (dilution 1:50; polyclonal; Abcam), CTLA-4 (dilution 1:50; clone F-8; Santa Cruz Biotechnology, Dallas, Texas), PD-L1 (dilution 1:100; clone SP142; Ventana), PD-1 (dilution 1:100; clone NAT105; Cell Marque), e-Met (pre-dilution; clone SP44; Ventana), NUT (dilution 1:50; clone C52B1; Cell
Signaling Technology, Danvers, Massachusetts), TrkA (dilution 1:100; clone EP1058Y; Abcam), TrkB (dilution 1:300; polyclonal; Abcam), panTrk (Trk A+B+C[ABC]; dilution 1:100; clone EPR17341; Abcam), and cyclin D1 (dilution 1:100; clone SP4; Cell Marque).

c-Met, TrkA, TrkB, and panTrk protein expression was analyzed according to the semiquantitative H-score method; this method yields a total score range of 0-300 by multiplying the dominant staining intensity score (0, no staining; 1, weak or barely detectable staining; 2, distinct brown

**FIGURE 1**  Representative figures of gene expression and alteration (A) and tumor-infiltrating immune cells (B). A case of NUT midline carcinoma arising in the sinonasal cavity was used as the positive control. Figures were captured at ×100 to ×600 magnification fields, respectively [Color figure can be viewed at wileyonlinelibrary.com]
staining; 3, strong dark brown staining) by the percentage (0%-100%) of positive cells. NUT and cyclin D1 protein expression was analyzed by semiquantitatively measuring the percentage (0%-100%) of tumor cells showing positive nuclear expression; this method yields a total H-score range of 0-100. IHC for p16 overexpression has emerged as a robust surrogate biomarker for HPV-mediated carcinogenesis and it is conventionally considered positive when strong, dark, diffuse nuclear, and cytoplasmic expression is observed in >70% of tumor cells. All other staining patterns are scored as negative. Densities of tumor infiltrating T lymphocytes and macrophages were semiquantitatively scored by counting CD3+ T cells, CD8+ T cells, CD163+ macrophages, and FOXP3+ regulatory T cells (Tregs) as well as immune checkpoint markers of ICOS, LAG3, PD-L1, and PD-1+ immune cells within tumor cell nests. We additionally scored tumor cells (TC) expressing PD-L1 as a percentage of tumor area according to previously described method. Palatine tonsil tissues obtained from cancer-free individuals (N = 10) were used as the normal control of nontumorous squamous epithelium and lymphoid, immune cell tissues. Tonsillar SCC (N = 3) cases, which have been confirmed high-risk HPV-type 16-positive through HPV genotyping test (HPV DNA chip), were used as a positive control for p16 IHC.

2.3 | Dual-color SISH and interpretation

Using the MET DNA (Roche-Ventana) and Chromosome 7 enumeration (CEP7) probes (Roche-Ventana), dual-color SISH was performed on a Ventana BenchMark XT apparatus (Ventana Medical Systems). Signals were enumerated in 100 tumor cells with intact nonoverlapping nuclei per core under a light microscope with magnification of ×600. According to the interpretive guide provided for INFORM HER2 DNA probe staining of breast carcinoma (Ventana Medical Systems), tumor nuclei with clearly distinct color signals for MET (black signal) and CEP7 (red signal) were evaluated. Normal tissues including vessels, fibroblasts, lymphocytes or adjacent normal
squamous epithelial cells were considered as internal negative control. MET copy number gain (CNG) was defined as ≥2-4 MET signals per nucleus in ≥20% of tumor cells. MET amplification was defined as ≥5 MET signals per nucleus in ≥20% of tumor cells.19

## Table 1: Clinicopathological characteristics according to age in 552 radically resected head and neck squamous cell carcinoma (HNSCC) cases.

| Variables                        | Total No. (%) | Age < 45 No. (%) | Age ≥ 45 No. (%) | P value |
|----------------------------------|---------------|------------------|------------------|---------|
| No. of patients (%)              | 552 (100)     | 81 (100)         | 471 (100)        |         |
| Sex                              |               |                  |                  | <.001   |
| Male                             | 434 (78.6)    | 50 (61.70)       | 384 (81.50)      |         |
| Female                           | 118 (21.4)    | 31 (38.30)       | 87 (18.50)       |         |
| Smoking                          |               |                  |                  | .001    |
| Never smoker                     | 178 (32.2)    | 39 (48.10)       | 139 (29.50)      |         |
| Ex-smoker                        | 118 (21.4)    | 7 (8.60)         | 111 (23.60)      |         |
| Current smoker                   | 256 (46.4)    | 35 (43.20)       | 221 (46.90)      |         |
| Alcohol                          |               |                  |                  | .33     |
| No or social                     | 179 (32.4)    | 30 (37.00)       | 149 (31.60)      |         |
| Heavy                            | 373 (67.6)    | 51 (63.00)       | 322 (68.40)      |         |
| Anatomical sites                 |               |                  |                  | <.001   |
| Oral tongue                      | 175 (31.7)    | 57 (70.40)       | 118 (25.10)      |         |
| Oral cavity, other               | 40 (7.2)      | 5 (6.20)         | 35 (7.40)        |         |
| Oropharynx                       | 120 (21.7)    | 14 (17.30)       | 106 (22.50)      |         |
| Hypopharynx                      | 70 (12.7)     | 1 (1.20)         | 69 (14.60)       |         |
| Larynx                           | 138 (25.0)    | 3 (3.70)         | 135 (28.70)      |         |
| Nasal/paranasal                  | 9 (1.6)       | 1 (1.20)         | 8 (1.70)         |         |
| Age                              |               |                  |                  | .007    |
| Negative                         | 275 (49.8)    | 66 (81.30)       | 312 (66.30)      |         |
| Positive                         | 123 (22.3)    | 15 (18.70)       | 159 (33.70)      |         |
| Not evaluable                    | 154 (27.9)    | -                | -                |         |
| Lymphovascular invasion          |               |                  |                  | .006    |
| Negative                         | 442 (80.1)    | 74 (91.40)       | 368 (78.10)      |         |
| Positive                         | 110 (19.9)    | 7 (8.60)         | 103 (21.90)      |         |
| Perineural invasion              |               |                  |                  | .01     |
| Negative                         | 478 (86.6)    | 63 (77.90)       | 415 (88.10)      |         |
| Positive                         | 74 (13.4)     | 18 (22.20)       | 56 (11.90)       |         |
| pT classificationb               |               |                  |                  | .21     |
| pT1-2                            | 405 (73.4)    | 64 (79.00)       | 341 (72.40)      |         |
| pT3-4                            | 147 (26.6)    | 17 (21.00)       | 130 (27.60)      |         |
| pN classificationb               |               |                  |                  | .07     |
| pN0-1                            | 354 (64.1)    | 59 (72.80)       | 295 (62.60)      |         |
| pN2-3                            | 198 (35.9)    | 22 (27.20)       | 176 (37.40)      |         |
| Progression                      |               |                  |                  | .12     |
| No                               | 382 (69.2)    | 62 (76.50)       | 320 (67.90)      |         |
| Yes                              | 170 (30.8)    | 19 (23.50)       | 151 (32.10)      |         |
| Death                            |               |                  |                  | .03     |
| No                               | 426 (77.2)    | 70 (86.40)       | 356 (75.60)      |         |
| Yes                              | 126 (22.8)    | 11 (13.60)       | 115 (24.40)      |         |

Figures in boldface indicate statistical significance.

* Information for p16 expression status of 154 cases was unavailable due to lack of samples.

b Pathologic T and N classification were categorized according to 7th American Joint Committee on Cancer guidelines.

2.4 | FGFR1 FISH and interpretation

FGFR1 probes labeled with the fluorophore Spectrum Orange (red) that hybridizes to the 8p12-8p11.23 region and probes labeled with the fluorophore Spectrum green that recognizes the centromere region of chromosome 8 (CEP 8) were used for FISH following the manufacturer’s instructions (Abbott Molecular, Abbott Park, Illinois). Tumor tissue was scanned for amplification hot spots using an ×40 or ×63 objective. If FGFR1 signals were homogeneously distributed, random areas were used to count the signals. Twenty contiguous tumor cell nuclei from 3 hot spots or random areas, resulting in a total of 60 nuclei, were individually evaluated with the ×100 objective by counting red FGFR1 and green CEP8 signals. Normal tissues including vessels, fibroblasts, lymphocytes, or adjacent normal squamous epithelial cells were considered as internal negative control. High amplification of FGFR1 was defined based on modification of a previous protocol28,29 as follows: FGFR1/CEP8 ratio is ≥2.0; or average number of FGFR1 signals per tumor cell nucleus ≥6 or; percentage of tumor cells containing ≥6 FGFR1 signals or large clusters is ≥10%. Representative figures of each marker of protein expression, gene alteration, and infiltrated immune cells are presented in Figure 1A,B.

2.5 | Statistical analysis

Chi-square test, Fisher exact test, two-sample t test, or one-way analysis of variance tests were used to analyze differences between evaluated variables. Overall survival was measured from date of initial diagnosis to date of death or last follow-up. Progression-free survival was measured from the date of initial diagnosis to that of disease progression, defined as cancer recurrence, progressive disease without complete remission, or cancer-related death during the study period. The Kaplan-Meier method was used to analyze survival rates, and differences were compared using the log-rank test. The Cox proportional hazards model was implemented for multivariate analysis. Two-sided P values <.05 were considered statistically significant. Statistical analyses were conducted using IBM SPSS 23 software for Windows (IBM Corp, Armonk, New York).

3 | RESULTS

3.1 | Demographic pictures

The median (mean) age of overall 1787 HNSCC patients was 60.2 (61.0) years old. When dividing the age group into young age (age < 40, and 40 ≤ age < 45 years old) and late adulthood (age ≥ 45 years old), the majority (89.1%, 1593/1787) of HNSCC cases occurred in late adulthood, and only 10.9% (194/1787) of HNSCC cases occurred in young age (age < 45 years old). Among the 194 young age HNSCC cases, SCC occurred mostly in the oral tongue (50.5%, 68/194), followed by the oropharynx (17.5%,...
34/194) and other sites of the oral cavity (11.3%, 22/194). However, hypopharynx SCC was very rare (2.6%, 5/194) in young age (P < .001) (Figure 2A). In univariate survival analysis according to anatomical sites, hypopharynx SCC revealed the worst overall survival rate while oral tongue SCC revealed the best overall survival rate (P < .001; Figure 2B). In univariate survival analysis according to age, young age group showed superior overall survival rate compared to that of late adulthood group (P < .001; Figure 2C).

3.2 | Young age versus late adulthood:
Clinicopathological factors, molecular alteration, and tumor immunity

When comparing the clinicopathological features of HNSCC between young age (<45 years old) and late adulthood (≥45 years old) groups (Table 1) in 552 radically resected HNSCC, young age HNSCC (81 cases; 14.7%) was more associated with female sex, never smoker, oral tongue site, p16 (HPV) negativity, absence of LVI, and presence of PNI compared to late adulthood SCC. Other factors, especially pathologic T or N classification, were not different between these 2 groups.

The mean values for molecular alteration and tumor immunity according to patient age are summarized in Figure 3. HNSCCs of young age patients revealed lower frequency of p16 positivity (19% vs 34%, P = .01) indicating lower HPV infection rate than HNSCCs of late adulthood patients. Most p16 positive young age HNSCCs were located in the oropharynx (14 of 15; 93.3%). C-MET expression (73.5% vs 56.2%, P = .01) was higher and MET gene CNG (31% vs 17%, P = .01) was more frequently observed in the young age group than the late adulthood group. Cyclin D1 expression (31.5% vs 27.6%), TrkA (90.7% vs 92.1%) or TrkB (33.7% vs 37.3%) expression, and FGFR1 amplification (5% vs 5%) were not different between the 2 groups, although pan-Trk expression (65.3% vs 78.9%, P = .02) was higher in the late adulthood group.

Regarding tumor immunity, PD-L1 (SP142) positivity was higher in the young age group (28% vs 16%, P = .01). Numbers of infiltrating CD3+ T cells, CD8+ cytotoxic T cells, CD163+ macrophages, FOXP3+ Tregs, and tumor infiltrating lymphocytes (TILs) expressing ICOS, PD-L1, and PD-1 were not different between the 2 groups except ICOS+ TILs. There were more ICOS+ TILs in the young age group (9.1% vs 6.2%, P = .008). Although the number of CD8+ T cells was not significantly different, the ratio of FOXP3+ Tregs to CD8+ T cells (85.3% vs 46.6%, P < .001) and ICOS+ TILs to CD8+ T cells (65.9% vs 30.9%, P < .001) was significantly higher in the young age group than the late adulthood group.

3.3 | Analysis of survival outcomes

In univariate Cox analysis for overall survival in young age HNSCC patients (Table 2), pT and pN classification types were significantly related to inferior overall survival rates. High pN classification types (pN2-3 vs pN0-1) were determined to be independent factors for poor overall survival rate in multivariate Cox analysis. High expression of cyclin D1 protein showed a tendency related to inferior overall survival rates in the univariate Cox analysis (Table 2).

In univariate Cox analysis for progression-free survival in young age HNSCC patients (Table 3), PNI, PD-L1
positivity in tumor cells (TC2 + TC3), and higher ratio of CD163+ TIMs to CD8+ TILs were significantly related to progression-free survival rates; and these three variables were determined to be independent factors for poor overall survival rate in multivariate analysis. High expression of cyclin D1 and pan-Trk protein showed a tendency related to inferior overall survival rates in the univariate Cox analysis (Table 3).

4 | DISCUSSION

In this study, we comprehensively characterize HNSCC arising in the young age (<45 years old) patients compared to the late adulthoods (≥45 years old), with focus on molecular alteration and tumor immunity. This may be the first comprehensive validation study using diverse molecular and immune markers in a large number of clinical samples that compares patient age groups.

Young age HNSCCs occurred in 10.9% (194/1787) and showed different clinicopathological features compared to late adulthood HNSCCs. Young age SCC was more associated with female sex, never-smoker status, p16 (HPV) negativity, and oral tongue site. The exact incidence of young age HNSCC still remains unclear, as most of the previous studies on young age HNSCCs have focused on the oral cavity or oropharyngeal SCC, which show relatively higher incidence of young age, rather than SCC of various anatomical locations of head and Table 2

| Variables | Univariate analysis | Multivariate analysis |
|-----------|---------------------|----------------------|
|           | P value | HR | 95% CI | P value | HR | 95% CI |
| Sex       | .65     | 0.76 | 0.2 | 2.3 | - |
| Smoking   | .17     | 3.24 | 0.6 | 17.7 | - |
| p16       | .55     | 1.48 | 0.4 | 5.5 | - |
| Lymphovascular invasion | .40     | 0.42 | 0.1 | 3.3 | - |
| Perineural invasion | .19     | 2.3 | 0.7 | 7.7 | - |
| Resection margin | .19     | 2.43 | 0.6 | 9.2 | - |
| pT category | .02     | 3.77 | 1.2 | 12.4 | .16 | 2.4 | 0.7 | 8.4 |
| pN category | .005    | 5.9 | 1.7 | 20.2 | .01 | 4.7 | 1.3 | 17.0 |
| PD-L1 positivity | .18     | 2.2 | 0.7 | 7.3 | - |
| CD3+ TILs | .21     | 0.4 | 0.1 | 1.7 | - |
| CD8+ TILs | .21     | 0.4 | 0.1 | 1.7 | - |
| CD163+ TIMs | .79    | 1.2 | 0.4 | 3.8 | - |
| FOXP3+ TILs | .93    | 1.0 | 0.3 | 3.2 | - |
| ICOS+ TILs | .91     | 1.1 | 0.3 | 3.5 | - |
| LAG3+ TILs | .13     | 0.2 | 0.0 | 1.6 | - |
| PD-L1+ TILs | .69     | 0.7 | 0.2 | 3.4 | - |
| PD1+ TILs | .23     | 0.3 | 0.0 | 9.7 | - |
| Ratio of ICOS to CD8+ TILs | .83     | 1.1 | 0.3 | 3.7 | - |
| Ratio of FOXP3 to CD8+ TILs | .74     | 1.2 | 0.4 | 4.0 | - |
| Ratio of LAG3 to CD8+ TILs | .90     | 0.9 | 0.2 | 3.5 | - |
| Ratio of PD-L1 to CD8+ TILs | .79     | 0.8 | 0.2 | 3.8 | - |
| Ratio of PD-1 to CD8+ TILs | .83     | 0.9 | 0.2 | 3.9 | - |
| Ratio of CD163 to CD8+ TILs | .07     | 2.9 | 0.9 | 9.6 | - |
| c-MET protein | .77    | 1.2 | 0.4 | 3.9 | - |
| MET copy number gain | .72    | 1.2 | 0.4 | 4.3 | - |
| FGFR1 amplification | .54    | 0.0 | 0.0 | 3.9 | - |
| Cyclin D1 protein | .07     | 3.4 | 0.9 | 12.8 | - |
| TrkA protein | .75     | 1.2 | 0.4 | 3.9 | - |
| TrkB protein | .71     | 0.8 | 0.2 | 2.9 | - |
| panTrk protein | .10     | 2.7 | 0.8 | 8.9 | - |

Abbreviations: CI, confidence interval; HR, hazard ratio; TC, tumor cell; TIL, tumor infiltrating lymphocyte. Figures in boldface indicate statistical significance.

* Cutoff for high versus low level was based on mean values for protein expression and tumor-infiltrating immune cell numbers in overall HNSCC cases.
A systemic review of oral and oropharynx cancer in young age patients reported a significantly different incidence between North America (5.5%) and both Africa (17.2%) and Asia (12.1%). With respect to the finding that young age HNSCC showed lower p16 positive rates compared to late adulthood HNSCC, it is considered that young age HNSCC is composed of mostly oral tongue cancer which is usually p16 negative. In fact, most p16 positive young age HNSCCs were located in the oropharynx (14/15; 93.3%) in the present study.

Although pathologic T or N classification types were not different, less frequent LVI and more frequent PNI were observed in young age SCC. More frequent PNI in young age HNSCC seems to be related to anatomical characteristics of oral tongue where young age HNSCC occurs prevalently. Oral tongue is highly innervated with peripheral nerve systems, and PNI correlates with recurrence and survival. In fact, we also noted that PNI was significantly related to tumor progression in young age HNSCC. These findings should be considered for the management of young age HNSCC patients.

Regarding molecular alteration, higher expression of c-MET protein and higher frequency of MET gene CNG was more characteristic in young age SCC. Although the alterations

| Variables                           | Univariate analysis |          |          |          |          | Multivariate analysis |          |          |          |          |
|-------------------------------------|---------------------|----------|----------|----------|----------|-----------------------|----------|----------|----------|----------|
|                                     | P value  | HR  | 95% CI  |          |          | P value  | HR  | 95% CI  |          |          |
|                                     |          |     | Lower   | Upper   |          |          |          |           |          |          |
| Sex                                 | .32      | 0.6 | 0.3     | 1.6     |          |          |          |           |          |          |
| Smoking                             | .57      | 1.6 | 0.3     | 7.3     |          |          |          |           |          |          |
| p16                                 | .62      | 1.3 | 0.5     | 3.3     |          |          |          |           |          |          |
| Lymphovascular invasion             | .68      | 1.3 | 0.3     | 5.8     |          |          |          |           |          |          |
| Perineural invasion                 | .02      | 2.9 | 1.2     | 7.3     |          | .01      | 3.3 | 1.3     | 8.4     |          |
| Resection margin                    | .28      | 1.8 | 0.6     | 5.5     |          |          |          |           |          |          |
| pT stage                            | .19      | 1.9 | 0.7     | 5.0     |          |          |          |           |          |          |
| pN stage                            | .07      | 2.3 | 0.9     | 5.6     |          |          |          |           |          |          |
| PD-L1 positivity                   | .02      | 2.8 | 1.1     | 7.1     | .007     | 4.1 | 1.5     | 11.3    |          |
| CD3+ TILs                           | .17      | 0.5 | 0.2     | 1.4     |          |          |          |           |          |          |
| CD8+ TILs                           | .42      | 0.7 | 0.2     | 1.8     |          |          |          |           |          |          |
| CD163+ TIMs                         | .20      | 1.8 | 0.7     | 4.7     |          |          |          |           |          |          |
| FOXP3+ TILs                         | .34      | 0.6 | 0.2     | 1.7     |          |          |          |           |          |          |
| ICOS+ TILs                          | .93      | 1.0 | 0.4     | 2.6     |          |          |          |           |          |          |
| LAG3+ TILs                          | .37      | 0.6 | 0.2     | 1.8     |          |          |          |           |          |          |
| PD-L1+ TILs                         | .58      | 1.3 | 0.5     | 3.7     |          |          |          |           |          |          |
| Ratio of FOXP3 to CD8+ TILs         | .94      | 1.0 | 0.4     | 2.5     |          |          |          |           |          |          |
| Ratio of ICOS to CD8+ TILs          | .41      | 1.5 | 0.6     | 3.7     |          |          |          |           |          |          |
| Ratio of LAG3 to CD8+ TILs          | .22      | 1.8 | 0.7     | 4.6     |          |          |          |           |          |          |
| Ratio of PD-L1 to CD8+ TILs         | .82      | 1.1 | 0.4     | 3.4     |          |          |          |           |          |          |
| Ratio of PD-1 to CD8+ TILs          | .67      | 0.8 | 0.2     | 2.6     |          |          |          |           |          |          |
| Ratio of CD163 to CD8+ TILs         | .01      | 3.4 | 1.3     | 8.6     | .002     | 5.1 | 1.8     | 14.4    |          |
| c-MET protein                       | .98      | 1.0 | 0.4     | 2.5     |          |          |          |           |          |          |
| MET copy number gain                | .54      | 0.7 | 0.2     | 2.2     |          |          |          |           |          |          |
| FGFR1 amplification                 | .47      | 0.0 | 0.0     | 219.6   |          |          |          |           |          |          |
| Cyclin D1 protein                   | .05      | 2.7 | 1.0     | 7.1     |          |          |          |           |          |          |
| TrkA protein                        | .68      | 1.2 | 0.5     | 3.1     |          |          |          |           |          |          |
| TrkB protein                        | .72      | 0.8 | 0.3     | 2.3     |          |          |          |           |          |          |
| panTrk protein                      | .07      | 2.3 | 0.9     | 5.9     |          |          |          |           |          |          |

Abbreviations: CI, confidence interval; HR, hazard ratio; TC, tumor cell; TIL, tumor infiltrating lymphocyte. Figures in boldface indicate statistical significance.

a Cutoff for high versus low level was based on mean values for protein expression and tumor-infiltrating immune cell numbers in overall HNSCC cases.
were not exclusive in young ages when compared to late adulthood, high cyclin D1 expression and FGFR1 amplification were noted with similar frequencies as previously reported in smoking-related HPV-negative HNSCC. Considering young age SCC is more related to both HPV-negativity and never-smoker, alterations of these oncogenes might involve in the tumorigenesis of young age SCC through somewhat different manners from the usual HNSCCs occurring in late adulthood. Furthermore, high cyclin D1 expression showed a tendency related to poor prognosis of young ages in the present study. From these findings, targeting therapy to MET and CCND1 may be useful in the management of young age HNSCCs.

In the present study, we investigated trophomyosin-related kinases (Trk), TrkA, TrkB, and pan-Trk(A+B+C). The expression level of TrkA and pan-Trk were not low in both young age and late adulthood patients. Furthermore, young age cases showing high pan-Trk expression showed a tendency related to inferior progression-free survival rate. These findings suggest that Trk inhibitors such as entrectinib (panTrk inhibitors) could be applied to disease control for young age HNSCC patients.

In the previous study, the prognostic implications of PD-L1 positivity in tumor cells remain controversial in overall HNSCC. When limited to young age HNSCC patients, however, PD-L1 positivity in tumor cells (TC2 + TC3) was not uncommon and was determined to be a poor prognostic factor for tumor progression. In addition to higher PD-L1 (SPI42) positivity in tumor cells (TC2 + TC3), higher infiltration of ICOS+ TILs, and higher ratio of FOXP3+ Tregs and ICOS+ TILs relative to CD8+ T cells were also more distinct in young age HNSCC. Furthermore, higher ratios of CD163+ TIMs relative to CD8+ TILs were determined to be independent factors for poor progression-free survival rate.

Expression of immune blockade signals such as PD-1, PD-L1, ICOS, LAG3, and CTLA-4 deregulates tumor-infiltrating CD8+ cytotoxic T cells, and upregulation of these signals alters immune surveillance of cancers. FOXP3+ Tregs and CD163+ M2 type macrophages are also known to be generally protumorigenic and immunosuppressive. The prognostic value of the expression of immune blockade signals on tumor-infiltrating lymphocytes, FOXP3+ Tregs, or CD163+ M2 type macrophages is controversial; they are known to alter CD8+ T cells and ICOS+ TILs, and higher ratio of FOXP3+ Tregs and ICOS+ TILs relative to effector CD8+ T cells were also more associated with the young age patients. PD-L1 positivity in tumor cells and higher ratio of CD163+ M2 type macrophages relative to effector CD8+ T cells revealed a prognostic impact in young age HNSCC patients. Although these clinical, molecular, and immunologic pictures are described in overall HNSCC regardless of age group in our previous studies, the present results might help to understand the tumorigenesis of young age HNSCC, especially in oral tongue sites and to improve patient outcome through different treatment strategies.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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