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Molecular and immunologic analysis of laryngeal squamous cell carcinoma in smokers and non-smokers☆

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ABSTRACT

Background: Laryngeal squamous cell carcinoma (LSCC) is strongly associated with tobacco use, but recent reports suggest an increasing incidence of LSCC in patients without traditional risk factors, suggesting an alternative etiology of tumorigenesis. The purpose of this study is to characterize this non-smoking population and to compare immunohistochemical markers in tumor specimens from non-smokers and smokers with LSCC.

Methods: A retrospective chart review of patients with LSCC at Johns Hopkins Hospital (JHH) was performed. A tissue microarray (TMA) was constructed with tumor specimen from non-smokers with stage and age-matched smokers and stained for a variety of immunologic and molecular targets.

Results: In the JHH cohort of 521 patients, 12% (n = 63) were non-smokers. Non-smokers were more likely to be < 45 years old at time of diagnosis (OR 4.13, p = 0.001) and to have glottic tumors (OR 2.46, p = 0.003). The TMA was comprised of tumors from 34 patients (14 non-smokers, 20 smokers). Only 2 patients (6%) were human-papillomavirus (HPV) positive by high-risk RNA in situ hybridization (ISH). There was no correlation between smoking status and p16 (p = 0.36), HPV-ISH positivity (p = 0.79), phosphatase and tensin homolog (PTEN, p = 0.91), p53 (p = 0.14), or programmed death-ligand 1 (PD-L1, p = 0.27) expression.

Conclusions: Non-smokers with LSCC are more likely to be younger at the time of diagnosis and have glottic tumors than smokers with LSCC. In TMA analysis of stage and age-matched specimens from smoker and non-smokers with LSCC, the pattern of expression for common molecular and immunologic markers is similar. Further, HPV does not appear to be a major causative etiology of LSCC in either smokers or non-smokers in our cohort of patients.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common cancer of the head and neck. Demographically, it is more common in men with a median age of 65 years. Tobacco use is one of the strongest risk-factors for the development of laryngeal squamous cell carcinoma (LSCC), as nearly 95% of patients with LSCC have a smoking history. Changing societal habits in developed countries have thus caused a decrease in the incidence rate of LSCC [1]. However, there have been multiple reports suggesting that there is an emerging subset of patients with LSCC who present without the traditional risk factors and that the incidence rate within this cohort is increasing [2–5]. One possible etiology for the development of LSCC in patients without traditional risk factors is high-risk HPV infection, as multiple studies have demonstrated a low but detectable rate of HPV-positivity in LSCC [3,6].

The purpose of this study is two-fold: first, we sought to further define the demographic and clinical characteristics of non-smokers with LSCC using a large institutional cohort. Second, we analyzed the expression of several molecular and immunologic markers in tumor specimens from non-smokers with LSCC and their age and stage-matched smoking controls in order to evaluate the hypothesis that non-smokers represent a distinct etiologic and molecular cohort compared to smokers with laryngeal cancer.

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2. Methods

2.1. Data sets

This study was approved by the Johns Hopkins Institutional Review Board. A retrospective chart review was performed on all patients with a diagnosis of LSCC who were evaluated at Johns Hopkins Hospital (JHH) between the years 2003 and 2013. This cohort will be referred to as "JHH cohort" hereafter. Clinical characteristics of interest were retrospectively abstracted.

Patients were defined as young if they were < 45 years of age at the time of diagnosis [7]. Non-smokers were defined as never smokers and patients who reported a remote history of light smoking (< 10 pack year history with at least 20 years of cessation). Alcohol use was defined as a reported history of "heavy" or daily alcohol use. Tumor (T), nodal (N), and metastasis (M) categories were defined according the American Joint Cancer Commission (AJCC) 7th edition guidelines.

2.2. Immunohistochemical analysis

A tissue microarray was created using primary tumor specimens from 14 non-smokers and 20 smokers with LSCC. The specimens were selected by including all available primary tumors from non-smokers, and then matching to smokers primarily through tumor location and stage, and then age and gender as secondary characteristics. To account for tumor heterogeneity, three 0.6 mm cores were included from each tumor. The slides were stained with a variety of immunologic and molecular targets, which were p16, high-risk HPV RNA, PTEN, p53, CD3, CD4, CD8, CD68, PD-1, and PD-L1.

For staining, TMA sections were cut at five-microns thickness, deparaffinized, and underwent antigen retrieval with 10 mM citrate buffer at 92 °C for 30 min. Immunostains for p16 (clone E6H4; prediluted; Ventana Medical Systems, Tucson, AZ), PTEN (clone 6H2.1, 1:100, Biocare Medical, Concord, CA), p53 (clone BP53-11, prediluted, Ventana), CD3 (polyclonal, 1:100; Dako, Carpinteria, CA.), CD4 (clone Sp35; prediluted; Ventana), CD8 (clone C8144B; prediluted, CellMarque/Sigma-Aldrich, St. Louis, MO), CD68 (clone KP-1, prediluted, Ventana), PD-1 (clone NAT105; 1:100; Cellmarque), and PD-L1 (clone 22C3, prediluted, Dako) as well as chromogenic in-situ hybridization (ISH) for a cocktail of 18 high-risk HPV types (HR-18 probe, Advanced Cell Diagnostics, Hayward, CA) were performed. Signals were visualized on a Ventana Benchmark XT autostainer using the Ultraview polymer detection kit (Ventana) in the presence of appropriate controls for each stain.

All staining was scored by a head and neck pathologist who was blinded to patient smoking status. p16 was scored manually as positive or negative on the basis of diffuse nuclear and cytoplasmic staining in > 70% of tumor cells. HPV ISH staining was also scored manually as positive or negative on the basis of punctate nuclear and cytoplasmic signals. PD-L1 staining was quantified manually as absent (< 1% expression), low expression (≥1% and < 50%), and high expression (≥50%). PTEN staining was evaluated for loss of expression in tumor cells, while p53 staining was evaluated for overexpression. Expression of CD3, CD4, CD8, and CD68 were quantitated using Halo image analysis software (Indica Labs, Corrales NM).

2.3. Statistical analysis

Stata version 14.2 (StataCorp, College Station, Texas) was used for statistical analysis. Chi square analysis was used to compare the patient characteristics of smokers and non-smokers. Logistic regression was used to calculate odds ratios (OR) and corresponding 95% confidence intervals (95% CI). Associations between IHC expression levels and clinical characteristics were performed by applying Fisher's exact test, chi square analysis, or *t*-test when applicable.

Table 1
Patient characteristics of Johns Hopkins Hospital (JHH) cohort.

	JHH cohort n (%)
Age at diagnosis in years (SD)	61.8 (11.2)
Sex	
Male	407 (78.1)
Female	114 (21.9)
Race	
White	372 (71.4)
African American	112 (21.5)
Other	24 (4.6)
Unspecified	13 (2.5)
Smoking use	
No	63 (12.1)
Yes	458 (87.9)
Alcohol use	
No	270 (51.8)
Yes	458 (87.9)
Unspecified	38 (7.3)
Primary site	
Supraglottic	219 (32.0)
Glottic	271 (52.0)
Subglottic	12 (2.3)
Unspecified	19 (3.7)
Initial T-category (AJCC-7ed)	
T1	175 (34.0)
T2	129 (24.8)
T3	128 (24.6)
T4	72 (13.8)
Unspecified	17 (3.3)
Initial N-category (AJCC-7ed)	
N0	352 (67.6)
N1	32 (6.1)
N2	106 (20.4)
N3	14 (2.7)
Unspecified	17 (3.3)
Initial M-category (AJCC-7ed)	
M0	497 (95.4)
M1	7 (1.3)
Unspecified	17 (3.3)

3. Results

3.1. Demographic characteristics of the JHH population

The study population was composed of 521 patients with a diagnosis of LSCC. Demographic and clinical information are summarized in Table 1. Mean age at diagnosis was 61.8 years (standard deviation 11.2 years). The majority of patients were male (n = 407, 78.1%) or white (n = 372, 71.4%). Smoking use was common, as only 63 patients (12.1%) were characterized as non-smokers. With regard to subsite, 52% (n = 271) of tumors were glottic, 32% (n = 219) were supraglottic, and 2.3% (n = 12) were subglottic. The majority of tumors (n = 304, 58.8%) had T-stage of T1 or T2. Furthermore, most patients had N0 disease at the time of diagnosis (n = 352, 67.6%). Only 7 patients (1.3%) had distant metastases at the time of diagnosis.

We then isolated the non-smoking patients to better define their clinical characteristics (Table 2). In the JHH cohort, there was an association between smoking history and age. The mean age at diagnosis was slightly lower in non-smoking patients (59.1 vs 62.2, p = 0.04). Non-smokers were more like to have tumors of the glottis (OR 2.46, p = 0.003) that were less likely to be locally advanced (T3 OR 0.34, p = 0.006; T4 OR 0.26, p = 0.015). Non-smoking patients were more likely have a race recorded as "Other" (OR 4.36, p = 0.001). Non-smokers were also less likely to have a history of alcohol use (OR 0.14, p < 0.001). There were no associations between non-smokers and sex or nodal category. All patients with M1 disease at diagnosis had a history of smoking.

We then sought to create a model that identified characteristics that were associated with the non-smoker. Although there was only a small

Table 2
Comparison of non-smokers and smokers in the Johns Hopkins Hospital (JHH) cohort with associated odds ratios for a history of non-smoking.

	Non-smokers n (%)	Smokers n (%)	p-Value	Odds ratio (OR)	95% CI	p-Value
Number of Patients	63 (12.1)	458 (87.9)	–	–	–	–
Mean age at diagnosis (SD)	59.1 (12.7)	62.2 (11.0)	0.04	4.13[a]	1.84-9.30	0.001
Sex			0.80			
Male	50 (79.4)	357 (77.9)		Ref	–	–
Female	13 (20.6)	101 (22.1)		0.92	0.48–1.76	0.8
Race			0.001			
White	45 (71.4)	327 (71.4)		Ref	–	–
African American	8 (12.7)	104 (22.7)		0.56	0.26–1.22	0.29
Other	9 (14.3)	15 (3.3)		4.36	1.80–10.54	0.001
Unspecified	1 (1.6)	12 (2.6)		–	–	–
Alcohol use			< 0.001			
No	54 (85.7)	216 (47.2)		Ref	–	–
Yes	7 (1.1)	206 (45.0)		0.14	0.06–0.31	< 0.001
Unspecified	2 (3.2)	36 (7.9)		–	–	–
Primary site			0.026			
Supraglottic	16 (25.4)	203 (44.3)		Ref	–	–
Glottic	44 (69.8)	227 (49.6)		2.46	1.35–4.49	0.003
Subglottic	1 (1.6)	11 (2.4)		1.15	0.14–9.51	0.9
Unspecified	2 (3.2)	17 (3.7)		–	–	–
Initial T-category (AJCC-7ed)			0.015			
T1	32 (50.8)	143 (31.2)		Ref	–	–
T2	16 (25.4)	113 (24.7)		0.63	0.33–1.21	0.17
T3	9 (14.3)	119 (26.0)		0.34	0.16–0.74	0.006
T4	4 (6.3)	68 (14.8)		0.26	0.09–0.77	0.015
Unspecified	2 (3.2)	15 (3.3)		–	–	–
Initial N-category (AJCC-7ed)			0.92			
N0	45 (71.4)	307 (67.0)		Ref	–	–
N1	4 (6.3)	28 (6.1)		0.97	0.33–2.91	0.96
N2	10 (15.9)	96 (21.0)		0.71	0.35–1.46	0.35
N3	2 (3.2)	12 (2.6)		1.14	0.25–5.25	0.87
Unspecified	2 (3.2)	15 (3.3)		–	–	–
Initial M-category (AJCC-7ed)			0.61			
M0	61 (96.8)	436 (95.2)		–	–	–
M1	0	7 (1.5)		–	–	–
Unspecified	2 (3.2)	15 (3.3)				

[a] Denotes the odds ratio for patients < 45 years of age at diagnosis.

Table 3
Relationship of age of diagnosis and non-smoking history within the Johns Hopkins Hospital cohort.

	Odds ratio (95% CI)	p-Value
Age < 35	1.46 (0.17–12.71)	0.73
Age < 40	3.38 (1.01–11.33)	0.05
Age < 45	4.13 (1.84–9.30)	0.001
Age < 50	2.82 (1.51–5.28)	0.001
Age < 55	1.72 (0.98–2.99)	0.06
Age < 60	1.54 (0.91–2.61)	0.11

difference in the mean age between the smokers and non-smokers, a clear trend emerged when we divided the JHH cohort based on age at diagnosis in clustered 5-year increments (Table 3). The largest correlation between age and a non-smoking history was seen when we isolated patients that were < 45 years of age at diagnosis (OR 4.13, p = 0.001). To this model we added primary tumor location, and found that patients with glottic cancer who were < 45 years old were even more likely to be non-smokers (OR 5.91, p < 0.001).

3.2. Tissue microarray analysis

Given the low rate of non-smokers, we only had available tumor specimen from 14 non-smokers to include in our TMA. Thus, the TMA was composed of 14 non-smokers with 20 age and stage-matched smokers (Table 4). Our cohorts were very well matched, as expected by the study design. The average age of the non-smoking and smoking cohort was 58.4 and 60.0 (p = 0.9), respectively. The majority of both cohorts were male. There was no statistical between the cohorts in regards to tumor stage (p = 0.50), nodal stage (0.87), and primary tumor site (0.59).

Table 4
Demographic and clinical characteristics of patients included in TMA.

	Non-smokers N = 14	Smokers N = 20	p-value
Age (SD)	58.4 (4.5)	59.0 (2.2)	0.91
Gender (% male)	71	65	0.50
T stage, n (%)			0.50
T1	5 (36)	8 (40)	
T2	3 (21)	1 (5)	
T3	4 (29)	6 (30)	
T4	2 (14)	5 (25)	
N stage, n (%)			0.87
N0	9 (64)	12 (60)	
N1	2 (14)	3 (15)	
N2	3 (21)	4 (20)	
N3	0	1 (5)	
Site, n (%)			0.59
Transglottic	0	1 (5)	
Glottic	9 (64)	14 (70)	
Supraglottic	5 (36)	5 (25)	

In order to evaluate possible molecular and immunologic differences between these cohorts, we performed a wide range of immunohistochemical stains that have been reported to have diagnostic utility or predict oncologic outcomes in head and neck [8–12]. Of the 34 total patients, only 5 (3/14 non-smokers, 2/20 smokers) were positive for p16 expression (Table 5), a commonly used surrogate marker for HPV positivity in oropharyngeal cancer [13]. Only two tumors were positive for high-risk HPV via RNA in situ hybridization, and both of these were also p16+. The positive predictive value for high-risk HPV

Table 5
Expression patterns of various molecular and immunologic targets.

	Non-smokers N = 14	Smokers N = 20	p-Value
p16, n (%)			0.63
Positive	3 (21)	2 (10)	
Negative	11 (79)	18 (90)	
HPV ISH, n (%)			1
Positive	1 (7)	1 (5)	
Negative	13 (93)	19 (95)	
PTEN, n (%)			1
Loss of expression	6 (43)	9 (45)	
Sustained expression	8 (57)	11 (55)	
p53, n (%)			0.14
Normal expression	12 (86)	12 (60)	
Pathologic expression	2 (14)	8 (40)	
PD-L1 expression, n (%)			0.51
Absent	4 (29)	5 (25)	
Low	7 (50)	7 (35)	
High	3 (21)	8 (40)	

positivity based on p16+ in the larynx is therefore only 40%. There was no correlation between smoking status and p16 (p = 0.63) or HPV-ISH (p = 1.0) positivity. PTEN expression did not correlate with smoking status (p = 1.0) or p16 (p = 0.36) positivity. Similarly, p53 staining did not correlate with smoking status (p = 0.14) or p16 (p = 1.0) positivity.

A number of common immunologic markers were also studied, again, those that have demonstrated associations with clinical outcomes in head and neck cancer [14–16]. The majority of tumors showed some degree of PD-L1 staining, although this did not correlate with smoking status (p = 0.51) or p16 positivity (0.23). Interestingly, both tumors that were HPV-ISH positive had high levels of PD-L1 expression. Tumors from the non-smoking patients did show a higher ratio of CD4+ tumor infiltrating lymphocytes (TIL) (p = 0.05), although there was no difference in the expression patterns of CD3, CD8, or CD68 cell lineages (Fig. 1).

4. Discussion

The demographics of HNSCC have changed substantially in the last two decades due to the emergence of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC). While the overall incidence rate of HNSCC has decreased, the rate of OPSCC has increased, most notably in younger men without a smoking or drinking history. This increase in OPSCC has been driven by HPV. Within this context, recent reports have identified a subset of patients with LSCC without a smoking history and have suggested that the incidence of LSCC within this population is increasing [4,5]. We therefore sought to characterize this population and to determine whether there was differential staining of various molecular and immunologic markers in this non-smoking population.

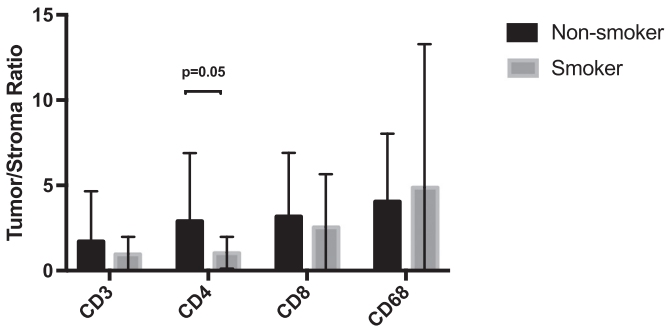

Fig. 1. Tumor infiltrating lymphocyte populations within non-smoking and smoking populations.

There is a strong association of LSCC with smoking, as historically 95% of patients with this disease have a smoking history. While the vast majority of LSCC can be attributed to smoking, the etiology behind the development of LSCC in non-smoking patients has yet to be determined [17]. This has led many to further characterize this population in retrospective analyses. Hamzany et al. published their experience in Israel with LSCC, and 3.8% (55 patients) of their cohort were classified as non-smokers, which they defined as “never-smokers” and “past-smokers” (stopped > 12 years prior to diagnosis) [4]. The tumors from the never-smokers were exclusively glottic and more likely to be early stage (T1). There was, however, no statistically significant difference in the age of diagnosis between non-smokers and smokers. Zeitels recently described his experience with glottic LSCC, reporting that he was seeing a sharp increase in the rate of non-smokers with LSCC [5]. He characterized these tumors as exophytic, papillary, and vascular and suggested that his findings demonstrated an emerging epidemiologic shift for glottic LSCC, presumably related to HPV.

In our cohort of patients evaluated at a tertiary care institution, 12.1% of patients with LSCC were non-smokers. Our analysis showed that the non-smokers were also younger on average (p = 0.04) by a few years, but there was a stronger association with non-smoking when divided into age cohorts, a cut-off value of < 45 years old best separated non-smokers from smokers with LSCC. Furthermore, non-smokers were more likely to have locally confined glottic disease when compared to patients with a smoking history.

The etiology of LSCC in the non-smoking population remains unknown. p53 is a well-studied gene in tumorigenesis and loss of this protein prevents cell cycle arrest and apoptosis in the setting of DNA damage. Previous work by Koch et al. compared the molecular characteristics of tumors from non-smokers and smokers with HNSCC [18]. In their study, they found that smokers were more likely to have tumors with p53 mutations and loss of chromosomal heterozygosity. Their cohort was composed of 308 patients, but had only 4 non-smoking patients with laryngeal tumors, limiting its application to LSCC. More recently, Karpathiou et al. published a cohort of 120 patients with HNSCC (39 laryngeal) and found that p16 and p53 expression were significantly associated with non-smokers [10]. They did not, however, perform a subgroup analysis looking specifically at the LSCC population. In our cohort, we did not find a differential pattern of p53 expression between smokers and non-smokers with LSCC.

PTEN is a tumor suppressor gene that is mutated in a variety of malignant tumors. Two recent studies have used immunohistochemistry to expression pattern of PTEN in LSCC. They both showed that a majority of tumors from the larynx demonstrated decreased PTEN expression [11,12]. Poorly differentiated tumors and glottic tumors were more likely to have decreased PTEN expression [11]. Given the differential staining pattern within LSCC, we hypothesized that there may be an association with PTEN expression and smoking history. However, we did not find an association between smoking and PTEN loss in our cohort.

Due to the increasing prevalence of HPV+ OPSCC, many have suggested that HPV may play a role in tumors of the larynx. Several studies have reported varying rates of HPV positivity in LSCC and a recent meta-analysis calculated the rate of HPV, as detected by PCR, in LSCC to be 23.6% [19]. However, the presence of HPV DNA does not indicate that the virus is transcriptionally active and driving tumorigenesis. Thus, there is much debate over the true rate of oncogenic HPV infections within the larynx. Immunohistochemistry of p16 has been used as a powerful surrogate for HPV+ identification in OPSCC, but a recent study showed that it is not specific for high-risk oncogenic HPV infections of the larynx [8,9]. RNA ISH or PCR for E6/E7 mRNA are considered the “gold standard” for identifying a tumor as HPV-related [13]. In our cohort, a total of 5 patients (3 non-smokers, 2 smokers) were p16+, but only 2 patients were positive by high-risk RNA ISH (1 non-smoker, 1 smoker). Our results demonstrate that the true rate of HPV-driven oncogenesis in the larynx is low and that it is

unlikely to be a major causative factor of LSCC in the non-smoking population.

PD-L1 expression has been well characterized in HNSCC and is currently a therapeutic target for treatment in a variety of solid tumors [16,20,21]. A recent study analyzed the expression profile of PD-L1 in primary LSCC and its association with survival [14]. They hypothesized that tobacco-induced HNSCCs bear a large number of mutations which may render them more immunogenic. Their study did show that tumors with increased PD-L1 expression and TILs were associated with superior disease-free survival. While the cohort did contain a small number of non-smokers, they did not comment on differential expression patterns of the non-smoking and smoking population. Thus, we sought to compare the immune microenvironment of the non-smoking and smoking population by analyzing PD-L1 expression and TIL populations. We also included a macrophage marker, CD68, which has been associated with improved survival in oral cavity squamous cell carcinoma [22]. In our cohort, there was no difference in the expression profile of PD-L1 between the smoking and non-smoking population. There was, however, a statistically significant difference in the infiltrating CD4+ population, which was higher in the non-smoking population, although this was not seen in the other cell lineages.

A limitation of our analysis is the relatively small sample size included in our TMA. As our study is retrospective, we were limited by available banked tumor specimen and the relatively low rate of non-smokers with LSCC. Nonetheless, our analysis is strengthened by our inclusion of stage and age-matched controls. Likewise, as with any TMA-based study, the potential for sampling error was present. However, this construction design allowed the staining of multiple tumors simultaneously under identical conditions.

Our study provides clear evidence that non-smokers with LSCC tend to be younger at diagnosis and are more likely to have early stage glottic disease. Furthermore, there is no clear causative role of HPV in the development of LSCC in the non-smoking population within the cohort at our institution. The molecular and immunologic characteristics of tumors from non-smokers are similar to that of the smoking population.

Conflict of interest

No conflict of interest to disclose.

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