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HPV Genotyping in Squamous Cell Carcinoma of Upper Aerodigestive Tract

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Background : Smoking and alcohol consumption are the main risk factors for squamous cell carcinoma of the upper aerodigestive tract (SCCUAT). However, human papillomavirus (HPV) has been etiologically linked with tonsillar squamous cell carcinoma (TSCC). Therefore, we investigated the etiologic role of HPV in the context of SCCUAT in Korea. **Methods :** Archival paraffin block samples from 136 cases previously diagnosed as SCCUAT were randomly selected. A commercial HPV DNA chip was used for HPV genotyping. **Results :** One hundred and seventeen cases were available after checking β-globin (47 cases of tonsil and 70 of non-tonsil). A HPV-positive result (HPV 16 and 18) occurred in 13 cases of SCCUAT, and 12 cases were tonsil (25.5%, 12/47). Among the 12 HPV-positive patients with TSCC, nine were non-smokers and non-drinkers. Most HPV-negative patients with TSCC had a history of alcohol drinking and smoking (32/35, 91.4%). HPV infection status was not significantly associated with histological grade, clinical stage, or survival in patients with TSCC. **Conclusions :** HPV infection was significantly higher in patients with TSCC among those with SCCUAT. HPV may be independent risk factor in development of TSCC, such as smoking and alcohol drinking.

Key Words : Carcinoma; HPV; Tonsil

Head and neck cancer including cancer of the upper aerodigestive tract comprises over 600,000 incident cases each year. Cancer associated death is about 50% of worldwide incidence, and upper aero-digestive tract cancer (larynx, oropharynx, tonsil, oral cavity) is the fifth most common cancer worldwide.[1] The most frequent cancer type arising in the upper aerodigestive tract is squamous cell carcinoma. Squamous cell carcinomas of the upper aerodigestive tract (SCCUAT) are diseases that are largely attributed to environmental exposure. The use of tobacco products and alcohol have been confirmed to be the two major risk factors for the development of these cancers.[2-5] However, a small proportion of SCCUAT also occur in non-smokers and non-drinkers, suggesting the presence of other risk factors.

Human papillomavirus (HPV) is associated with the development of most anogenital carcinomas, including cervical cancer.[6] Recently, a study revealed that HPV was frequently detected in tonsillar squamous cell carcinoma (TSCC) among the Western population.[7] Numerous studies have shown that more than 50% of TSCCs are infected with HPV.[7-9] However, studies primarily concerned with Eastern populations have been limited, and the only documented HPV study on 16 Chinese patient with TSCC failed to identify HPV at all, and a study on 111 Taiwan TSCC patients showed that only 12.6% were infected with HPV.[10,11] Thus, HPV was etiologically linked with a small set of TSCC. Therefore, we investigated the etiologic role of HPV based on the tonsil cancer site.

MATERIALS AND METHODS

Patients

Archival tissue from 136 patients with primary SCCUAT diagnosed during 1999-2004 was randomly selected from the

Chonnam National University Hospital. These cases were classified by a tonsillar and non-tonsillar head and neck origin site. The non-tonsillar head and neck included the tongue, gingiva, palate, mouth floor, nasal cavity, and pharyngeal wall. Original slides from all patients were reviewed and the diagnosis was reconfirmed histologically by an expert pathologist. Squamous cell carcinoma was the histological type in all cases. The histological grades were divided into well, moderately, and poorly differentiated. There were 53 cases of tonsillar and 83 cases of non-tonsillar squamous cell carcinoma (NTSCC).

The charts of all patients were reviewed to extract data on patient's demographics, history of other diseases, carcinogenic risk factors (alcohol and/or tobacco), clinical stage, and survival status. When past or present tobacco use was reported, smoking was graded as positive or negative. Alcohol consumption was also graded as positive or negative when it was reported by the patient.

DNA extraction from paraffin sections

Selected paraffin-embedded SCCUAT tissues were cut into five 25 μm-thick tissue sections, using disposable microtome blades. Each sample was collected in a 1.5 mL Eppendorf tube and deparaffinized. Tissues were digested in 500 μL of buffer containing 500 μg/mL proteinase K, 0.45% Tween 20, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin to extract DNA. Tissues were incubated at 55℃ for 18-24 hours with intermittent mixing followed by boiling for 10 minutes to inactivate proteinase K.

Polymerase chain rection (PCR)-quality control

To avoid contamination leading to false positive results, all PCR-related work was conducted in specialized zones within a PCR laboratory that undergoes UV purification at least once every 24 hours. To detect crossover contamination, viral-free DNA and negative controls (PCR reagents containing no DNA) were included in each PCR amplification reaction. dUTP was used in place of 50% of dTTP to eliminate crossover contamination, if present.

To verify DNA integrity, each sample was amplified using the human β-globin primers GH20 and 04 (product length, 268 bases). All negative controls and viral-free DNA samples were negative for β-globin. Positive controls always amplified β-globin and HPV DNA, respectively.

HPV-DNA detection by PCR

A commercially available HPV DNA chip was used (MyGene Co., Seoul, Korea). The HPV DNA chip contained 15 types of high-risk HPV and nine types of low-risk HPV strains. The PCR products from all samples were detected by electrophoresis using 2.5% agarose gels, and the HPV DNA product size was 150 base pairs. After 10 μL of the HPV-amplified product was denatured at 95℃ for 5 minutes, it was mixed with a hybridization solution, and then applied to the DNA chip. Hybridized HPV DNA was visualized using a DNA chip scanner (ScanArray Lite, GSI Lumonics, Ottawa, ON, Canada). HPV amplicons were hybridized with the corresponding type of specific oligonucleotide probe and visualized on HPV DNA chip slides as double positive spots.

Statistical analysis

The chi-square test was used to compare categorical variables between groups. Survival rates between groups were calculated and compared by the Kaplan-Meier method. All calculated p-values were two sided, and p-value < 0.05 was considered statistically significant.

RESULTS

When we verified the β-globin values in the 136 cases, only 117 cases were available (47 cases of tonsil and 70 cases of non-tonsil). Among the 117 patients with SCCUAT, 13 were positive for HPV. Only one HPV-positive case was found in a patient with NTSCC (1/70, 1.4%) and the remaining cases were TSCC (12/47, 25.5%). The HPV infection rate was significantly higher in the TSCC than in the NTSCC cases ($p < 0.05$) (Table 1), and most were HPV 16 and HPV 18 (Fig. 1).

The 70 NTSCC cases consisted of 68 men and two women. Among them, only one woman was positive for HPV. Except for two cases with a loss of social history, most patients with NTSCC had an alcohol drinking and/or smoking history (67/68, 98.5%). The 47 TSCC cases consisted of 45 men and two women. Among these cases, 11 men and one woman tested positive for HPV. The average age of the 12 HPV-positive patients with TSCC was 58.4 years (range, 49 to 74 years), and that of the 35 HPV-negative patients with TSCC was 66.7 years. Of all TSCC cases, patients with a history of tobacco or alcohol consumption comprised 74.5% (35/47). Thirty cases had a history

Table 1. Prevalence of human papillomavirus (HPV) genotypes in patients with squamous cell carcinoma of the upper aerodigestive tract as determined by an HPV DNA chip

Site	HPV positivity (%)	HPV type (n, %)
Tonsil (n = 47)	12 (25.5)	HPV 16 (11, 23.4) HPV 16 &18 (1, 2.1)
Non-Tonsil (n = 70)	1 (1.4)	HPV 16 (1, 1.4)
Oral cavity (n = 22)	1	
Nasopharynx (n = 27)	0	
Larynx (n = 21)	0	
Total (n = 117)	13 (11.1)	HPV 16 (12, 10.3) HPV 16 &18 (1, 0.8)

Table 2. Human papillomavirus infection and alcohol and smoking history in patients with tonsillar squamous cell carcinoma (n = 47)

	Alcohol			Smoking		
	Positive (%)	Negative (%)	p-value	Positive (%)	Negative (%)	p-value
HPV (+) (n = 12)	2 (16.7)	10 (83.3)	< 0.05	3 (25.0)	9 (75.0)	< 0.05
HPV (-) (n = 35)	31 (88.6)	4 (11.4)	< 0.05	30 (85.7)	5 (14.3)	< 0.05

of both smoking and alcohol consumption. The remaining patients with TSCC (12/47, 25.5%) did not have a history of smoking or alcohol consumption. Among the 12 HPV-positive patients with TSCC, nine did not have a history of smoking or alcohol consumption, the other three had a history of smoking, and, of those, two also had a history of alcohol consumption. Among the 35 HPV negative patients with TSCC, most also had a history of alcohol drinking or smoking (32/35, 91.4%). Only three patients did not have a smoking or alcohol consumption history (Table 2). HPV infection status was not significantly associated with histological grade or clinical stage. Survival data were available in 36 of the 47 TSCC cases. No statistical significance was observed between survival and HPV infection rate (p = 0.128).

DISCUSSION

There are differences in head and neck squamous cell carcinoma epidemiology among countries. Korea has a relatively high rate of SCCUAT compared with China but a low rate compared with Western countries.[10] Although females in Ecuador and other countries such as France have similar rates compared with males, a much higher incidence for males than females has been

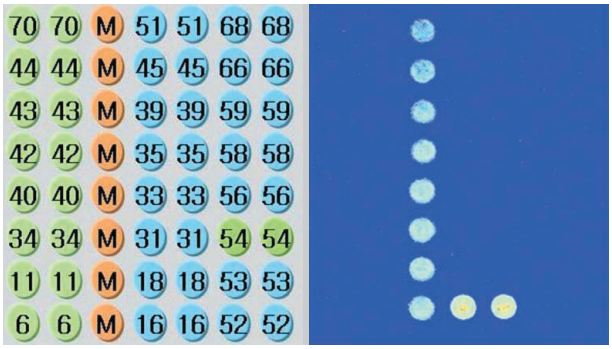

Fig. 1. Human papilloma virus (HPV) scanning of HPV 16 in patients with tonsillar squamous cell carcinoma.
M, position marker.

reported in Korea.[12] This could be due to different cultural or social habits that affect cigarette smoking and alcohol consumption. Our results showed that smoking and alcohol consumption are significant causes of SCCUAT.

The results showed an association between HPV and cases of SCCUAT in Korea. Alcohol and/or tobacco are known risk factors for the development of SCCUAT,[13] and recent studies have shown that HPV may also be a independent risk factor in a subset of patients with oropharyngeal squamous cell carcinoma in Western populations.[5,8,12-16] Some studies have reported that the HPV positive rate in all SCCUAT cases including oral cavity, oropharynx, and larynx SCC is between 21.9 and 38.7%.[17,18] Another Western study showed that HPV infectivity occurs in 40% of oral cavity SCC, 33% of tongue, 43% of larynx, and 100% of tonsil.[19] In contrast, our study showed only a 1.4% rate of HPV infectivity in 70 NTSCC cases and an 11.1% rate of HPV positivity in all SCCUAT cases. We could not analyze the HPV positive rate in NTSCC, compared with other Asian data, because there are no studies about them yet.

Many studies have shown that TSCC has a higher HPV infection rate among patients with SCCUAT.[8,15,20] Our results are similar with previous reports demonstrating that the HPV infection rate is higher in patients with TSCC than those with NTSCC.[21] However, the HPV infection rate of patients with TSCC is quite different between countries; several reports have revealed that none of 16 tonsillar cancer specimens in a Chinese population and 12.6% patients with TSCC were positive for HPV DNA in a Taiwan population.[10,11] Our results revealed that the rate of HPV-positive TSCC was 25.5%. One Korean study showed that HPV 16 integration may be related initially to TSCC carcinogenesis in tonsillar crypts through a cell-cycle abnormality associated with p16 overexpression and *c-myc* amplification.[21]

Consistent with our findings, a recent study reported that a greater incidence of HPV infection in patients with TSCC is associated with non-smokers and non-drinkers.[22] In our study, HPV-positive TSCC without a smoking and alcohol consumption history comprised 75% of all HPV-positive TSCC cases. It has been proposed that HPV infection has an independent carcinogenic effect in relation to other risk factors such as smoking and alcohol, but it has been unclear and unconfirmed as of yet.[23]

Previous data have indicated that patients with HPV-associated TSCC tend to be younger.[8] Our data showed that HPV-infected patients with TSCC tended to be younger than non-HPV infected patients with TSCC. One epidemiological study showed that exposure to HPV could precede the occurrence of TSCC by 10 years or more.[23] As HPV is a sexually transmitted disease, sexual behaviors such as multiple sexual partners and/or practicing oral sex may affect the incidence of HPV infection in patients with SCCUAT, and is associated with the risk of head and neck cancer.[24] Our study found a much lower HPV infection incidence; it may be that Koreans have a much lower incidence of oral sex and oral sex partners.

HPV 4, 6, 11, 16, 18, and 33 in patients with TSCC have been reported in other studies and HPV 16 is the most predominant type in patients with SCCUAT.[16,17,25] In our study, HPV 16 was the most common type and occurred in 91.7% (11/12) of HPV-positive TSCC cases, and only one case was positive for both HPV 16 and HPV 18. HPV 16 and HPV 18 are the high risk group HPVs that existed in most of the patients who were positive for HPV.

Although HPV has a potent carcinogenic effect for the development of TSCC, some reports have documented no significant association between HPV infection status and tumor, node and metastasis stage, tumor differentiation, or risk of death.[6,11,26] However, another study noted that an HPV positive state is an important prognostic factor with a better outcome after chemoradiation therapy.[15,27] Although the number was too small, our study showed that HPV infection status was not significantly associated with histological grade, clinical stage, or survival rates. We expect to study more cases in the future and we may suggest a better treatment plan for patient with HPV-positive TSCC.

In conclusion, HPV infection was significantly higher in patients with TSCC than those with NTSCC. The role of HPV in the development of TSCC is very likely independent of risk factors such as smoking and drinking. As HPV-infected TSCC has a higher prevalence in men, HPV vaccination, which is a cervical cancer preventive in women, may also be useful in men from the Western population to prevent TSCC. However, as in China and Taiwan, Korea has a low rate of HPV-positive TSCC. We must consider the usefulness of an HPV vaccination in the Korean population for preventing HPV-positive TSCC.

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