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RESEARCH ARTICLE

## Usefulness of human papillomavirus detection in oral rinse as a biomarker of oropharyngeal cancer

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### ABSTRACT

**Conclusion:** The detection of human papillomavirus (HPV)-DNA in oral rinse with auto-nested GP5+/GP6+ PCR is useful as a biomarker of oropharyngeal cancer.

**Background:** This study aimed to determine the usefulness of oral rinse to detect HPV-DNA as a biomarker of HPV-positive oropharyngeal cancer (OPC).

**Patients and methods:** One hundred and ten patients with various head and neck diseases, including 19 patients with OPC, were enrolled. Oral rinse and tonsillar swab were collected, and auto-nested GP5+/GP6+ PCR for HPV-DNA was performed. For oropharyngeal cancer, p16 immunostaining was also conducted.

**Results:** The rate of HPV-DNA detection in both oral rinse and tonsillar swab was significantly higher in OPC compared with non-OPC upper respiratory tract cancer and non-cancer diseases. HPV-DNA was detected in oral rinse in nine out of 12 p16-positive OPC cases, while none of the p16-negative OPC cases demonstrated detectable HPV-DNA. All p16-positive cases were also positive for HPV-DNA in tumor tissue. Based on p16 immunostaining, the sensitivity and specificity of HPV-DNA detection in oral rinse were 75% and 100%, respectively. Among eight of nine evaluable OPC cases positive for HPV-DNA in oral rinse at diagnosis, HPV-DNA was undetectable in oral rinse in seven cases after treatment.

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### KEYWORDS

Human papillomavirus; oral rinse; oropharyngeal cancer; biomarker; auto-nested PCR

### Introduction

Human papillomavirus (HPV) is a causative agent of cervical cancer [1]. On the other hand, it has become apparent that HPV infection is a principal cause of a distinct form of oropharyngeal cancer (OPC) [2]. According to epidemiological reports, the incidence of HPV-positive OPC increased from 16% in the 1980s to 72% in the early 2000s in the US [3,4]. Considering the trend in the US, the incidence of HPV-positive OPC is expected to increase in Japan.

In contrast to classic OPC, HPV-related OPC generally arises from deep crypts of palatine tonsils. Therefore, it is difficult to detect it in the early stage with regular examinations. In addition, anatomic change after surgery as well as fibrous scar formation after chemoradiotherapy make it difficult to visualize recurrent lesions. In consideration of those circumstances, HPV may be an attractive target to detect inconspicuous lesions of OPC. From this perspective, we focused on a simple and easy method, oral rinse, to detect HPV. Here, we report the usefulness of HPV-DNA detection in oral rinse as a biomarker of HPV-positive OPC.

### Patients and methods

#### Patients

One hundred and ten patients with various head and neck diseases including 19 OPCs treated in Kanazawa University Hospital (Kanazawa, Japan) and Ishikawa Prefectural Central Hospital (Kanazawa, Japan) were enrolled in this study. The clinical characteristics of the patients are shown in Table 1. This study was approved by the Ethics Committee of Kanazawa University School of Medicine, and Ishikawa Prefectural Central Hospital, and written consent was obtained from all enrolled patients.

#### Sample collection

With respect to the oral rinse sample, 20 mL of normal saline gargled for 20 s was collected and centrifuged at 3000 rpm for 10 min. The pellet was suspended in 2.0 mL of preservative solution for liquid-based cytology (Medical & Biological Laboratories, Nagoya, Japan) and stored at 4 °C until use. With respect to the tonsillar swab sample, scratching of the palatine tonsil using a swab for uterine cervix

cytodiagnosis was performed, and the sample was stored at 4°C until use after being suspended in a preservative solution.

### HPV-DNA detection

DNA was extracted from all samples before treatment using a DNA extraction kit (G&G Science, Fukushima, Japan) according to the manufacturer's protocol.  $\beta$ -Globin was first amplified by polymerase chain reaction (PCR) to confirm the adequacy of the extracted DNA in all samples. In  $\beta$ -globin-positive samples, PCR using GP5+ and GP6+ primers, which is widely used for the detection of a broad spectrum of mucosotropic HPV genotypes, was performed [5–7]. This generates an 140-bp-long fragment from the HPV L1 structural gene.

Samples that were negative in the first GP5+/GP6+ PCR were re-amplified using the same set of primers, which was defined as auto-nested PCR in this study. Thirty-six cycles and 20 cycles of amplification were performed in the first and second PCR, respectively. Each cycle consisted of 20 s at 94°C for denaturation, 30 s at 48°C for annealing, and 30 s at 72°C for extension. The amplified DNA was subjected to electrophoresis on 2% agarose gel, and then visualized by ultraviolet illumination using ethidium bromide. The negative control included all reagents except for DNA, while the p1203 PML2d HPV-16 plasmid, which was a gift from Peter Howley (Addgene plasmid # 10869), was used as a positive control.

Table 1. Patient characteristics.

	Number of patients	Male	Female	Mean age (years)
Upper respiratory tract cancer	66	56	10	65.3
Oropharynx	19	15	4	65.3
Larynx	15	15	0	65.3
Hypopharynx	13	13	0	
Oral cavity	9	7	2	
Nasopharynx	7	5	2	
Others	3	1	2	54.5
Non-cancer	44	18	26	
Sinonasal disease	15	3	12	60.4
Thyroid tumor	8	2	6	
Benign laryngeal disease	7	6	1	60.4
Ear disease	7	4	3	
Salivary gland tumor	3	2	1	
Others	4	1	3	
Total	110	74	36	

### HPV genotyping

HPV genotyping using HPV Geno Array Test Kits (HybriBio, Hong Kong, China) was performed according to the manufacturer's protocol for HPV-DNA-positive samples [8]. This can detect 37 HPV genotypes, consisting of 15 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68), six low-risk types (6, 11, 42, 43, 44, and CP8304), and 16 probably low-risk types (26, 34, 40, 54, 55, 57, 61, 67, 69, 70, 71, 72, 73, 82, 83, and 84), by flow-through hybridization using HPV-DNA amplified by PCR.

### Immunohistochemistry

Immunostaining for p16 was also performed in 19 OPC cases as described previously [9]. A mouse monoclonal antibody against human p16 (Santa Cruz Biotechnology, Dallas, TX) was used at a dilution of 1:200. Immunohistological p16 positivity was evaluated as described previously [10].

### Statistical analysis

Differences in the age and HPV detection between p16-positive and -negative OPC were analyzed using the Mann-Whitney U-test. The sex, smoking status, T stage, N stage, and subsite were analyzed using two-tailed Fisher's exact test. All analyses were carried out using SPSS19.0 software (SPSS Inc., Chicago, IL). In all tests,  $p < 0.05$  was considered significant.

## Results

### HPV-DNA detection in head and neck diseases

$\beta$ -Globin was detected in all oral rinse and tonsillar swab samples. With respect to HPV-DNA detection, adding the second PCR clearly increased the detection threshold, as shown in Figure 1. In accordance, most HPV-DNA-positive samples were identified using the second PCR in both oral rinse and tonsillar swab in the present study, as shown in case 2 of Figure 2.

One hundred and ten cases were divided into three groups: OPC, upper respiratory tract cancer other than OPC (URTC), and non-cancer. In oral rinse, HPV-DNA was detected in nine of 19 cases (47.4%) with OPC, eight of 47 cases (17.0%) with URTC, and seven of 44 cases (15.9%) with non-cancer (Table 2). In tonsillar swab, HPV-DNA was

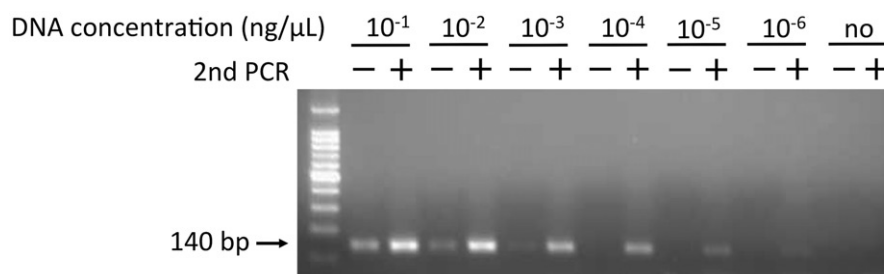


Figure 1. Detection of HPV-DNA in the first and second PCR using gradient concentrations of the positive control.

detected in 10 of 19 cases (52.6%) with OPC, four of 47 cases (8.5%) with URTC, and four of 44 cases (9.1%) with non-cancer (Table 2). The concordance of HPV-DNA detection between oral rinse and tonsillar swab was 94.7% in OPC, 85.1% in URTC, and 79.5% in non-cancer.

**HPV-DNA detection and p16 immunohistochemistry in oropharyngeal cancer**

Because almost half of the cases with OPC were positive for HPV-DNA in both oral rinse and tonsillar swab, we next investigated the association with p16 immunostaining in OPC. The clinical characteristics of OPC patients are shown in Table 3. Twelve of the 19 OPC cases were positive for p16, while the remaining seven were negative. There were no significant differences with respect to the sex, smoking status, T stage, N stage, or tumor sub-site between p16-positive and -negative cases.

In addition, we also performed PCR using GP5+/GP6+ primers to detect HPV-DNA in samples obtained from formalin-fixed paraffin-embedded tissue of OPC, and demonstrated that all p16-positive cases were also positive for HPV-DNA. Therefore, there was no p16-positive case who was negative for HPV in the present study.

In oral rinse, HPV-DNA was detected in nine of 12 p16-positive cases, while none of the seven p16-negative cases demonstrated HPV-DNA, showing a significant difference ( $p=0.003$ ) (Table 4, Figure 2). Similarly, in tonsillar swab, HPV-DNA was detected in 10 of 12 cases, while none of the seven p16-negative cases demonstrated HPV-DNA, showing a significant difference ( $p=0.001$ ) (Table 4, Figure 2). Based on p16 immunostaining, the sensitivity, specificity, positive predictive value, and negative predictive value of HPV-DNA detection in oral rinse and tonsillar swab were 75, 100, 100, and 70%, and 83, 100, 100, and 78%, respectively.

**HPV-DNA detection in oral rinse after treatment in oropharyngeal cancer**

Eight of nine OPC patients positive for HPV-DNA in oral rinse at diagnosis were further investigated after treatment (Table 5). In these eight, oral rinse samples were collected 5–15 months (mean=9.6 months) after the completion

of treatment. As shown in Table 5, HPV-DNA was undetectable in oral rinse in seven of eight cases after treatment, while the remaining case was positive for HPV-DNA. Four were treated with chemoradiotherapy, and four were

**Table 2.** Positive rate of HPV-DNA detection in oral rinse and tonsillar swab.

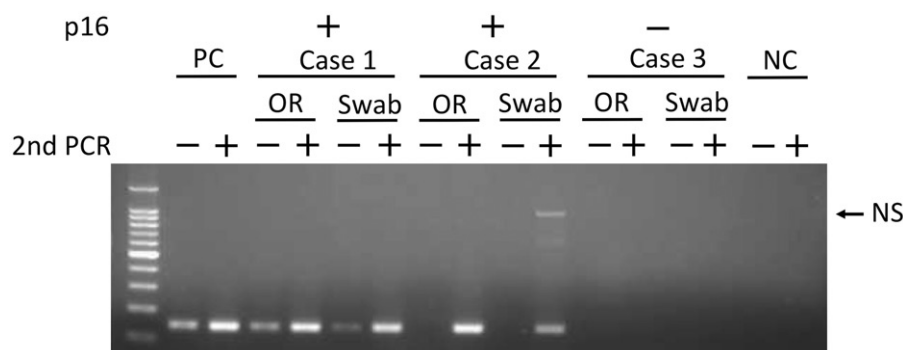
	Oral rinse	Tonsillar swab
Oropharyngeal cancer	9/19 (47.4%)	10/19 (52.6%)
Non-oropharyngeal upper respiratory tract cancer	8/47 (17.0%)	4/47 (8.5%)
Non-cancer	7/44 (15.9%)	4/44 (9.1%)

**Table 3.** Characteristics of oropharyngeal cancer patients.

	p16 immunostaining		p-value
	Positive	Negative	
Number of patients	12	7	
Mean age (years)	63.7	68.0	0.432
Sex			0.245
Male	8	7	
Female	4	0	
Smoking status			0.594
Current	4	4	
Former	5	2	
Never	3	1	
T stage			0.266
T1	3	1	
T2	6	3	
T3	3	1	
T4	0	2	
N stage			0.956
N0	4	2	
N1	1	1	
N2	7	4	
N3	0	0	
Subsite			0.326
Tonsil	9	3	
Base of tongue	3	4	

**Table 4.** Comparison between HPV-DNA detection and p16 immunostaining.

HPV-DNA	p16 immunostaining		p-value
	Positive	Negative	
Oral rinse			0.003
Positive	9	0	
Negative	3	7	
Tonsillar swab			0.001
Positive	10	0	
Negative	2	7	



**Figure 2.** HPV-DNA detection in oropharyngeal cancer cases. Cases 1 and 2 are p16-positive, while case 3 is p16-negative. PC: positive control; OR: oral rinse; NC: negative control; NS: non-specific band.

**Table 5.** Comparison of HPV-DNA detection in oral rinse before and after treatment in p16-positive oropharyngeal cancer.

Case	Subsite	TNM	HPV-DNA detection in oral rinse		State
			Before	After	
1	Tonsil	T3N2cM0	+	–	CR
2	Tonsil	T1N2bM0	+	–	CR
3	Tonsil	T3N0M0	+	–	CR
4	Tonsil	T1N2bM0	+	–	CR
5	Tonsil	T2N2cM0	+	+	CR
6	Tonsil	T2N2bM0	+	–	CR
7	Tonsil	T2N0M0	+	–	CR
8	Tonsil	T2N2bM0	+	–	CR

CR: Complete response.

treated with surgery. All patients achieved a complete response after treatment, and were without any evidence of disease at sample collection.

### HPV genotyping

HPV genotyping was further performed in 42 samples with HPV-DNA detection. A certain HPV genotype was detected in only 14 (35.0%) samples, while no types of HPV were detected in the remaining samples. In p16-positive OPC, HPV type 16 (HPV16) was detected in four of nine oral rinse samples, and four of 10 tonsillar samples, while HPV type 58 was detected in one tonsillar swab sample. On the other hand, HPV genotyping from tissues of OPC patients identified HPV16 in 11 of 12 p16-positive OPC cases and HPV58 in the remaining case. In non-oropharyngeal URTC samples, HPV16 was detected in one of eight cases which were HPV-positive in oral rinse samples, and one of four tonsillar swab samples. In non-cancer samples, HPV16 was detected in three of 15 cases which were HPV-positive in oral rinse samples, and three of eight tonsillar swab samples. Only a single sub-type of HPV could be detected in all evaluable samples.

### Discussion

The prevalence of oral HPV infection among healthy persons was reported to be 5.7–8.3% in the US [2]. On the other hand, the HPV-positive rate in oral rinse in non-cancer patients was 15.9% in the present study, being higher than in previous reports. Adding a second PCR may increase the detection threshold, while a difference in the patient background may affect the positive rate. Accordingly, the availability of auto-nested GP5+/GP6 + PCR to detect HPV-DNA was described by Remmerbache et al. [7]. Furthermore, the increased sensitivity of auto-nested GP5+/GP6 + PCR compared with conventional single-round GP5+/GP6 + PCR was demonstrated [11]. Thus, auto-nested PCR may be a useful method to investigate samples with low viral loads [11]. This was also supported by the fact that only one third of the samples with HPV-DNA detection identified certain HPV subtypes in the present study, which suggests the higher detection sensitivity of auto-nested PCR than the regular genotyping kit.

There have been several reports with respect to HPV-DNA detection in oral rinse, showing positive rates from 39–54% in HPV-related OPC [12–14]. In comparison with these reports, the present study demonstrated a higher positive rate of 75% in p16-positive OPC. This is in accordance with the favorable result of auto-nested PCR as described above.

HPV-DNA was not detected in oral rinse in three p16-positive OPC cases. Among them, two involved OPC arising at the base of the tongue, and showing a submucosal appearance. Thus, the HPV-DNA detection rate in p16-positive OPC was 89% (eight of nine) at sub-sites of the tonsil, and 33% (one of three) at the base of the tongue. Therefore, it is considered that HPV-DNA in oral rinse could be more detectable in OPC in the tonsil than at the base of the tongue. Wang et al. [14] similarly reported a higher detection rate in HPV-related OPC of the tonsil (67%) than at the base of the tongue (24%).

In the present study, the concordance rate of HPV-DNA detection between oral rinse and tonsillar swab was 94.7% in OPC, 85.1% in URTC, and 79.5% in non-cancer. In addition, based on p16 immunostaining, the sensitivity and specificity of HPV-DNA detection were 75% (nine of 12) and 100% (seven of seven) in oral rinse, and 83.3% (10 of 12) and 100% (seven of seven) in tonsillar swab, respectively. A high concordance rate suggests the usefulness of unobstructive sample collection, namely oral rinse, being in accordance with a study comparing oral rinse and cytology brush sampling [15].

Interestingly, HPV-DNA in oral rinse after treatment became negative in seven of eight evaluable cases. Although we did not observe any recurrent cases among these eight patients, HPV-DNA in oral rinse after treatment could be a biomarker of the disease status. Rettig et al. [12] demonstrated that persistent HPV16 DNA in oral rinse after treatment was associated with poorer disease-free survival and overall survival. Further research with respect to the prevalence of HPV-DNA in oral rinse after treatment detected with our strategy is warranted.

One of the limitations of the present study is the small number of cases of OPC. However, we could at least demonstrate the superiority of our method to detect HPV-DNA in oral rinse. Another limitation is the relatively short period of post-treatment observation. Actually, it is unclear whether persistent HPV-DNA in oral rinse after treatment detected with our procedure is associated with persistent disease, or whether HPV-DNA will be re-detected in cases of disease recurrence at the primary site. Another limitation is the low rate of HPV genotyping in oral rinse samples. The HPV genotyping procedure included one round of PCR consisting of 40 cycles according to the manufacturer's protocol, while the second PCR was added after the first PCR to identify HPV-DNA in oral rinse samples in the present study. Therefore, we consider that the procedural difference between genotyping and auto-nested PCR was a major reason for the low rate of HPV genotyping in oral rinse samples. Further study is planned to answer these limitations.

## Conclusion

In conclusion, HPV-DNA detection in oral rinse is useful as a biomarker of HPV-related OPC. Auto-nested GP5+/GP6+ PCR was a helpful method for this purpose.

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## Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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