Human papilloma virus detection in oropharyngeal cancer with gargle samples

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Abstract. Human papilloma virus detection in oropharyngeal cancer with gargle samples. Objective: human papilloma virus (HPV) is a major risk factor for oropharyngeal squamous cell carcinoma (OPSCC) and knowledge of a patient’s HPV status is clinically important in terms of treatment and prognosis. The practicality of using oral gargle samples to reliably detect HPV in patients with OPSCC remains unclear. Therefore, we evaluated the feasibility of HPV detection in gargle samples of OPSCC patients using an HPV-dedicated nucleic acid amplification test (cobas 4800 HPV Test; Roche Diagnostics K.K.). Methodology: 15 patients with histologically proven OPSCC were evaluated from May 2014 to March 2015. Swab samples served as positive controls and were tested using both the Hybrid Capture II HPV Test (HC-II; Digene Corporation) and the cobas 4800 HPV Test. Oral gargle samples were tested using the cobas 4800 HPV Test. Five of the 15 patients were confirmed to be HPV-positive by a combination of p16 immunohistochemistry, HPV-DNA in situ hybridization and nucleic acid amplification. Results: the sensitivity and specificity of the gargling method were 60% and 100%, respectively. No false-positives were obtained. Detection of HPV in two very small tumours rising from the base of the tongue was difficult and these cases were overlooked as HPV-negative. Conclusions: use of the gargling method to determine HPV positivity in OPSCC patients appears feasible, except in patients with very small tumours. Real-time polymerase chain reaction using gargle samples may have greater clinical efficacy than the swabbing method.

Introduction

Human papilloma virus (HPV) infection is one of the major risk factors for developing oropharyngeal squamous cell carcinoma (OPSCC), equal in importance to smoking and alcohol consumption as risk factors.1 Incidence of HPV-positive OPSCC has risen in recent years. In the United States, HPV positivity in all cases of OPSCC was 16.3% from 1984 to 1989 and 71.7% from 2000 to 2004.2 In Japan, the prevalence of HPV positivity in all cases of OPSCC was reportedly 50.3%.3 The incidence of HPV positivity in patients with OPSCC in Japan is anticipated to rise in the future, as in the case of the United States. HPV-positive patients with OPSCC have a better therapeutic response to chemoradiotherapy and hence, better prognosis than do HPV-negative patients.4,5 As such, it has been stressed that rational diagnostic procedures employed to assess the patient’s HPV status should lead to identification of an optimal treatment strategy and the achievement of a reasonable prognosis.

Today, HPV vigilance in OPSCCs is exercised on a daily basis by employing liquid-phase cytology of scrubbed materials obtained directly from the tumour. The samples are taken by brushing the surface of the tumour and is applied to screening tests such as the Hybrid Capture II HPV Test (HC-II; Digene Corporation).6,7 The cobas 4800 HPV Test (Roche Diagnostics K.K.), an in vitro diagnostics test with which to detect HPV DNA from cervical cells, was credited for its performance in detecting HPV in head and neck squamous cell carcinoma (HNSCC) in a recent report.7 However, these methods may not work well when the targeted tumour is very small and located at the base of the tongue, because forcefully taking a swab from the tumour may elicit a severely painful vomiting reflux.
The above situation can be averted if the presence of HPV can be successfully verified in oral gargle samples from patients with these tumours. Some researchers have reported that oral rinsing can indicate the presence of high-risk HPV in healthy subjects. Additionally, a small number of studies have adopted oral gargle samples to detect HPV in patients with HNSCC. One study of oropharyngeal cancer transmission adopted the rinsing method to detect the prevalence of HPV, while another report discussed the utility of this method in patients with HNSCC. However, neither discussed the practicality of the gargling method in a routine setting. The practicality of the oral gargle method, therefore, has yet to be verified. This knowledge gap prompted us to perform a cross-sectional study to investigate the feasibility of detecting HPV in gargling samples using exclusive real-time polymerase chain reaction (PCR) (cobas 4800 HPV Test; Roche Diagnostics K.K.).

### Materials and methods

Fifteen patients were recruited for this cross-sectional study from May 2014 to March 2015. All patients had histologically proven OPSCC and no other simultaneous head and neck primary neoplasms (Table 1).

In this study, we investigated the HPV detection capability of an exclusive real-time PCR technique (cobas 4800 HPV Test, Roche Diagnostics K.K.) using oral gargle samples. Liquid-phase cytology specimens were prepared with both oral gargle and swabbed samples. The swab samples were assigned to undergo testing using both the HC-II and cobas 4800 HPV assays, and were designated as the positive controls. The oral gargle samples were assigned to undergo testing using the cobas 4800 HPV Test. Furthermore, formalin-fixed, paraffin-embedded sections from biopsy samples were used for HPV-DNA in situ hybridization (ISH) or immunohistochemistry (IHC) of p16 as a surrogate marker. The results of the cobas 4800 HPV Test using oral gargle samples were compared with those of the control studies.

The actual study protocol was as follows. The patients were given 15 ml of water and instructed to rinse their mouth for 30 seconds, then spit the water out into a paper cup. The sample was placed into a 50-ml Falcon tube and centrifuged at 3000 × g for 15 minutes. After removing the supernatant, the pellets were suspended in LBCTM (HOLOGIC). These gargle samples were then tested with the cobas 4800 HPV Test. This assay was conducted at Roche Diagnostics K.K. The samples were assigned a number and each assay was carried out anonymously. We analysed the measurement values as informed by the company.

After obtaining the gargle samples, two swab samples were obtained from each of the subjects. One swab sample was assigned to the cobas 4800 HPV Test and the other was assigned to the HC-II assay at Bio Medical Laboratories.

Since all patients had been diagnosed with OPSCC, formalin-fixed, paraffin-embedded
sections from biopsy samples were already available. These were used to examine the presence of HPV with HPV-DNA ISH and p16 IHC, as described below in detail.

**Ethics**

The Institutional Review Board of Miyagi Cancer Center approved the study protocol and informed consent was obtained from each subject prior to entry into this study. The study procedures were in accordance with the Helsinki Declaration.

**Detection of HPV using cobas 4800 HPV Test**

The cobas 4800 HPV Test is an exclusive system for HPV identification. This system can detect 14 high-risk HPVs with only one measurement and can specifically identify HPV type 16 and type 18. It automatically extracts nucleic acid, then amplifies and detects HPV DNA with real-time PCR. The assays of the present samples were anonymously conducted at Roche Diagnostics K.K.

**p16 IHC**

One of the recognized biomarkers of HPV-positive OPSCC is p16. Paraffin-embedded, formalin-fixed, 3-µm tissue sections were deparaffinized in xylene and rehydrated through ethanol to distilled water. Epitope retrieval was performed with heat-treatment and incubation of ethylenediaminetetraacetic acid (EDTA) solution (pH 8.5). The sections were incubated with primary antibodies to human p16 (CINTEC p16 Histology; Roche Diagnostics K.K.) for 16 minutes. Following counterstaining, the staining was visualized with Ultra View DAB substrate (Roche Diagnostics K.K.). HPV positivity of the tumour was considered when ≥70% of the tumour cells had strong and diffuse nuclear and cytoplasmic staining.

**HPV in situ hybridization**

For HPV in situ hybridization, paraffin-embedded, formalin-fixed, 3-µm tissue sections were deparaffinized in the same manner as for p16 IHC. Epitope retrieval was performed with heat treatment and incubation of a citric acid buffer (pH 6.0). Following enzymatic treatment with ISH Protease 3 (Roche Diagnostics K.K.), hybridization and detection were performed with HPV III iView Blue Plus (Roche Diagnostics K.K.), according to the manufacturer’s protocol.

**Results**

HPV positivity and negativity were determined by combining the results of the HC-II, cobas 4800 HPV Test using swab samples, HPV-DNA ISH and p16 IHC.

Five out of 15 enrolled patients with OPSCC were HPV-positive and 10 were HPV-negative. Representative results of HPV-DNA ISH and p16 IHC among the HPV-positive patients are shown in Figure 1. The patients’ characteristics are summarized in Table 1 and the results of each assay are similarly summarized in Table 2. The sensitivity of the HC-II and cobas 4800 HPV Test using swab samples was 60% (3/5) and 80% (4/5), respectively, and the specificity of each assay was
was difficult to obtain adequate swab samples in this case, too. Neither the HC-II nor cobas 4800 HPV Test using swab samples could detect HPV in this case. However, positivity for both HPV ISH and p16 IHC proved this case to be HPV-positive (Figure 1). Based on these results, we determined five of the 15 enrolled patients to be HPV-positive (Table 2).

The accuracy of HPV detection using oral gargle samples and the cobas 4800 HPV Test, the determination of which was the aim of the present study, was assessed according to the following results. Of the five HPV-positive cases, the positivity of which was determined from the aforementioned reference, the assay system detected three positives and overlooked two cases as negative. Therefore, the sensitivity and the specificity of the cobas 4800 HPV Test using oral gargle samples were 100% (10/10). The sensitivity of HPV-DNA ISH and p16-IHC was 80% (4/5) and 100% (5/5), respectively, and specificity was 100% (10/10) and 70% (7/10), respectively.

Cases 2, 3 and 14 were positive by the HC-II and cobas 4800 HPV Test using swab samples and these cases were verified to be HPV-positive with histological specimens. Case 12 was a small OPSCC arising from the base of the tongue (Figure 2a, 2b) and it was difficult to obtain swab samples by brushing the tumour. Therefore, the discrepancy in the results between the HC-II and cobas 4800 HPV Test may have been caused by the inadequacy of the samples in this case. In fact, HPV ISH; and p16 IHC were positive, supporting the evidence of HPV-positivity in this case. Similarly, Case 13 was a very small cancer arising from the base of the tongue (Figure 2c, 2d) and it was difficult to obtain adequate swab samples in this case, too. Neither the HC-II nor cobas 4800 HPV Test using swab samples could detect HPV in this case. However, positivity for both HPV ISH and p16 IHC proved this case to be HPV-positive (Figure 1). Based on these results, we determined five of the 15 enrolled patients to be HPV-positive (Table 2).

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60% (3/5) and 100% (10/10), respectively. The two cases overlooked as negative included very small tumours arising from the base of the tongue. Detection of HPV in these cases was difficult, even when using swabs for the HC-II and cobas 4800 HPV Test. This difficulty may have been because of sample inadequacy, i.e., due to the anatomical location and small size of the tumours. We expected the oral gargle samples to resolve this problem, but they did not.

Discussion

We conducted the present study to examine the feasibility of an HPV screening test in patients with OPSCC using gargling samples instead of swabbed materials obtained directly from the tumour. Using the gargling method, the cobas 4800 HPV Test detected three of five HPV-positive cases and 0 of 10 HPV-negative cases (i.e., no false-positives). In contrast, using swabbed samples, the HC-II and cobas 4800 HPV Test detected three and four of five HPV-positive cases, respectively, and both tests showed HPV negativity in all 10 HPV-negative patients. Notably, both the HC-II and cobas 4800 HPV Test detected HPV in no cases, and in only one case, respectively, of the two cases (Cases 12 and 13) (Figure 2) in which swabbed samples were obtained with great difficulty from the small tumours (T1) at the base of the tongue. Thus, exclusive real-time PCR using gargling samples competes well with the swabbing method.

The availability of an adequate amount of DNA in the gargling sample was confirmed by the presence of a sufficient amount of β-actin, a housekeeping gene (data not shown). However, the amount of DNA from HPV-positive cancer cells could be diluted, because many cells are sloughed from the normal mucosa.13 Three conceivable solutions to this problem of HPV DNA dilution are as follows: 1) reduce the gargling fluid to <15 ml to concentrate the HPV DNA; 2) alter the centrifugation setting; 3) change the PCR setting. With respect to PCR settings, HPV detection data in the present study were obtained with the cobas 4800 HPV Test with the assay level set at “default.” Modification of the cycles of replication reaction, annealing and others may lead to a better outcome of HPV-DNA

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<th>Case Number</th>
<th>p16</th>
<th>HPV ISH</th>
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<th>Cobas (swab)</th>
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HPV = human papilloma virus; IHC = immunohistochemistry; ISH = in situ hybridization.
detection. However, the aim of this study focused on the performance of the cobas 4800 HPV Test; changes in PCR settings will be considered in future studies.

Several modalities are now employed for detecting HPV, including p16 IHC and HPV ISH using tissue sections and liquid-phase cytology using swabbed materials. Typically, p16 IHC has high sensitivity and low specificity, while HPV ISH has low sensitivity and high specificity; these characteristics were also confirmed in the present study. Furthermore, small OPSCCs may defy proper swabbing. More sophisticated methods for detecting HPV in OPSCCs are therefore required.

The present study suggests that the use of the gargling method to detect HPV in OPSCCs is feasible on a routine basis and except for very small tumours, detectability seems satisfactory in a practical sense. From this experience, we consider that the gargling method may have at least two benefits within the clinical setting. After further confirmation of its reliability, this method may complement p16-IHC or HPV-ISH from biopsy samples in the initial screening process of HPV among patients with OPSCC. Another benefit may be the early detection of recurrence, likely in combination with state-of-the-art imaging modalities such as high-resolution magnetic resonance imaging (MRI) and 2-deoxy-2-fluoro-D-glucose positron emission tomography-computed tomography (FDG PET-CT), following definitive treatment for HPV-associated OPSCC. Rettig et al.\textsuperscript{15} recently investigated the prevalence of HPV in patients with OPSCC using oral gargling samples before and after treatment, and reported that the prevalence of HPV in the samples following treatment was associated with a poor prognosis. They claimed that the gargling method for HPV detection can be a potential tool for tumour surveillance. Their results thus support the additional clinical value of the gargling method.

Conclusion

Use of the gargling method to determine HPV positivity in OPSCC patients appears feasible, except in patients with very small tumours. Although the present study is preliminary in the sense that the number of patients involved therein was limited, additional studies may prove that the gargling method has significant clinical value in detecting HPV-positive OPSCC.

Disclosure statement

The cobas 4800 HPV Test assay was conducted by Roche Diagnostics K.K. without any clinical information and LBC\textsuperscript{TM} (HOLOGIC) was supplied by the same corporation. However, the authors have no financial relationship with Roche Diagnostics K.K. that supported the research.

References

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