Journal of Oral Biosciences 61 (2019) 190–194

Contents lists available at ScienceDirect

Journal of Oral Biosciences

journal homepage: www.elsevier.com/locate/job

Original Article

# Identification and characterization of novel human papillomaviruses in oral rinse samples from oral cavity and oropharyngeal cancer patients

Juliet Dang [a, *], Gregory A. Bruce [b], Qing Zhang [c], Nancy B. Kiviat [d]

[a] Oral Health Sciences, University of Washington, Seattle, WA, USA
[b] Seattle Children's Research Institute, Seattle, WA, USA
[c] Fred Hutchinson Cancer Research Center, Seattle, WA, USA
[d] University of Washington School of Medicine, Seattle, WA, USA

ARTICLE INFO

*Article history:*
Received 16 January 2019
Received in revised form
7 May 2019
Accepted 13 May 2019
Available online 24 May 2019

*Keywords:*
Oral cancer
Oropharyngeal cancer
Head and neck cancer
Human papillomavirus
Sequencing

ABSTRACT

*Objectives:* The objectives of this study were to: I) discover novel human papillomaviruses (HPVs) using next generation sequencing (NGS) technology in oral rinse samples collected from oral cavity cancer (OCC) and oropharyngeal cancer (OPC) patients; II) determine the prevalence of novel HPVs in archived OCC and OPC tissue samples; and III) examine the frequency of novel oncogenic HPVs in cancer and non-cancer oral rinse samples using real-time PCR.
*Methods:* Oral rinse samples were collected from 100 head and neck cancer patients, and 110 healthy individuals. NGS techniques were used to detect novel HPVs.
*Results:* Three potentially new types of HPV were discovered. Novel virus (NV) 14.4 was closely related to HPV76 with an 89% homology and is a member of the genus *Beta-papillomavirus* (β-PV); NV69.1 was distantly related to the genus *Alpha-papillomavirus* (α-PV), and NV95 was closely related to HPV147 with a 65–77% homology and is part of the genus *Gamma-papillomavirus* (γ-PV). In archived oral tissue samples, NV14.4 was detected in a single patient with OCC. Of the oral rinse samples, NV69.1 was more prevalent than the other two NVs.
*Conclusions:* Our results demonstrated that there are novel HPVs present in oral rinse samples that may be associated with OCC and OPC. These novel HPVs can be identified and characterized using NGS techniques.


## 1. Introduction

Human papillomavirus (HPV) has a circular, double-stranded DNA genome [1]. Approximately 79 million Americans are currently infected with HPV, and every year 14 million new infections occur [2]. HPVs that affect mucosal regions are classified as low-risk, which usually produce warts, or as high-risk, which are associated with cervical cancer [3,4]. High-risk HPVs contain the oncoproteins E6 and E7, which are responsible for inhibition of apoptosis, deactivation of tumor suppressor proteins, and creating a cellular environment that causes genome instability, thus increasing the risk of malignancy [4].

Annually, it is estimated that 263,000 oral and 135,000 pharyngeal cancers occur globally [5]. Grouping the two cancers together results in the sixth most-common cancer in the world [6]. Oral HPV infection is increasing at a considerable rate, and the projected number of HPV-positive oropharyngeal cancer (OPC) cases is expected to surpass the annual number of cervical cancer cases by 2020 [7]. The most prevalent type of HPV associated with oral infection is type 16 [8,9]. Both type 16 and 18 have been demonstrated to be oncogenic in OPC [10–12].

According to the Papillomavirus Episteme (PaVE) database there are almost 200 different HPV types (http://pave.niaid.nih.gov/#search/search_database). With modern techniques and methods currently available, it should not be impossible to identify novel HPVs involving the head and neck. The guidelines for determining whether an HPV isolate is novel can be found on the PaVE submission process page (http://pave.niaid.nih.gov/#explore/taxonomy/submission_process). When studying an unknown HPV, if there is a greater than 10% homology difference involving the viral L1 region, when compared to all existing HPVs, the unknown HPV is potentially new.

* Corresponding author.
*E-mail address:* jhtdang@gmail.com (J. Dang).

https://doi.org/10.1016/j.job.2019.05.001
1349-0079/

From our previous study, we identified and characterized three novel HPVs from oral rinse samples of healthy individuals [13]. HPVs 171, 172, and 173 are all of the γ-HPV type.

We hypothesized that there may be unidentified novel HPVs in oral rinse samples of oral cavity cancer (OCC) and OPC patients.

## 2. Materials and methods

### 2.1. Study population

Between 2011 and 2013, we recruited 100 cancer patients from the Seattle Cancer Care Alliance (Seattle, WA), and 110 healthy subjects from the University of Washington Dental Clinic (Seattle, WA). The cancer cases included patients with OPC, OCC, laryngeal cancer, sinus cancer, and supraglottis cancer. We screened the schedules of five oncologists in order to identify eligible cancer patients, and discussed our study at their appointments. One patient declined to participate due to mouth sores and sensitivity. Twenty-one out of hundred cancer patients had already begun treatment: 18 patients had treatment less than 21 days before sampling, 2 had over 30 days of treatment, and one patient had undergone treatment for 7 months. Healthy subjects were randomly selected from within the student dental clinic; one patient declined to take part in the study. Inclusion requirements for the healthy population included being cancer-free, not pregnant, HIV-free, and over the age of 16. Each participant signed an informed consent form and answered a simple health questionnaire. Gender, age, race, smoking and alcohol history were recorded for all subjects. For all subjects, institutional review board (IRB) protocols and regulations were followed according to the Fred Hutchinson Cancer Research Center guidelines (IRB #7490 approved April 9, 2014).

### 2.2. Sample collection and DNA purification

For sample collection, all patients rinsed and gargled for 30 s with Scope Mouthwash Original Mint® mouthwash (Proctor and Gamble, Cincinnati, OH, USA). Four normal, healthy individuals requested to use Crest® Alcohol-free mouthwash (Proctor and Gamble) due to a history of alcoholism. Oral rinse samples were centrifuged at 2000 rpm for 15 min at 4 °C to form a pellet. The supernatant was discarded and the pellet was stored at −80 °C until further processing. The Puregene® DNA Purification Kit (Qiagen, Hilden, Germany #158467) was used to isolate genomic DNA from the buccal cell pellet of the mouthwash samples, according to the manufacturer's protocol.

### 2.3. HPV and analytic methods

#### 2.3.1. Identification of novel HPVs

We first determined whether our samples were infected with HPV16 and 18. Taqman real-time PCR (qPCR) assays were used for HPV detection on an ABI Prism 7900 Sequence Detection System with 40 amplification cycles (denaturation at 95 °C, annealing and extension at 60 °C), total run time was ~120 min. Absolute quantification was used to determine HPV16 and 18 viral load, and total human genomic DNA in the sample was determined using amplification of *Alu* sequences. Serial dilutions of human genomic DNA and of full length HPV16 and 18 plasmids of known concentrations were used to generate standard curves.

HPV16 E7 Primers
Forward: CGGACAGAGCCCATTACAATATT Reverse: CGCACAACCGAAGCGTAGA.

HPV16 E7 Probe: TAACCTT(T/C)TGTTGCAAGTGT.

HPV18 E7 Primers:
Forward: CCGACGAGCCGAACCA
Reverse: TGGCTTCACACTTACAACACATACA.

HPV18 E7 Probe: AACGTCACACAATGTT.

To increase the efficiency of HPV detection, we used the multiply-primed rolling-circular amplification technique (MP-RCA) to preferentially amplify unknown, circular HPV DNA. MP-RCA has been demonstrated to amplify circular DNA templates up to $10^7$-fold [14]. The TempliPhi 100 Amplification Kit (Amersham Biosciences, Little Chalfont, UK) protocol was followed.

The published fluorescent arbitrarily primed (FAP) PCR protocol was followed to detect a broad range of HPV types, where amplification primers were targeted to conserved viral L1 regions [15]. We only performed this technique on the cancer case samples since our main objective was to detect for oncogenic HPVs, and cancer patients would theoretically have a higher viral count for detection.

NGS was performed on pools of 7 samples using the Illumina HiSeq 2500 platform (Figure not shown). For supervised assembly, a total of 189 HPV L1 gene sequences and whole genome sequences were downloaded from the papillomavirus knowledge source at http://pave.niaid.nih.gov/. This data set includes a number of non-reference genomes. Short reads were aligned to the L1 gene region using BWA (http://bio-bwa.sourceforge.net/, version 0.7.12) with default settings. Subsequently, the whole-genome sequences were used as the reference database if a majority of the short reads did not map to the specific L1 gene region.

A *de novo* assembly strategy was applied for cases where the majority of the short reads did not align to HPV genome sequences. These short reads were first aligned to the human genome UCSC hg19 (GRCh37 build reference sequence, Feb. 2009). (http://support.illumina.com/sequencing/sequencing_software/igenome.html), and then unmapped reads were selected to run Velvet (https://www.ebi.ac.uk/~zerbino/velvet/, version 1.2.10) to construct high-quality unique contigs (short DNA fragments). Contigs with 200 bp or greater in length, and with a minimum 100-fold coverage were aligned (BLASTn) to both the HPV-specific database at http://pave.niaid.nih.gov/#search/pv_specific_blast, and to the NCBI GenBank nucleotide collection (nt) database at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.

The GenBank and PaVE databases were used to determine homology of the sequences to HPV. Homology of <90% to the viral L1 region indicates a novel HPV [3,16].

Ten samples that were FAP PCR-positive but not HPV16-positive, underwent Sanger DNA sequencing to determine if novel HPV sequences were present. Five samples had ambiguous and poor quality results, thus cloning was performed using the CloneJet PCR Cloning Kit from Thermo Scientific, Waltham, Massachusetts, USA), following the manufacturer's protocol. Sanger DNA sequencing by Genewiz was executed after cloning followed by BLASTn searching of GenBank and PaVE databases for similar HPVs. Chen et al. previously recommended using both databases in order to determine homology [17].

EMBL-EBI Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align multiple sequences and to produce a phylogenetic tree for observation of evolutionary relationships. The putative novel viruses (NVs) were compared to all HPVs in the PaVE database, and sequences from the L1 region were obtained.

#### 2.3.2. Determining prevalence of novel HPVs in oral rinse samples

OligoArchitect by Sigma (http://www.sigmaaldrich.com/technical-documents/articles/biology/oligoarchitect-online.html) was used to design custom primers and probes for the three putative novel HPVs. Using Taqman qPCR assays, we determined the

**Table 1**
MP-RCA preferential amplification. HPV16 acted as a positive control, and Siha a negative control.

| Sample | HPV16 gene | | Alu gene | | Enrichment[a] |
|---|---|---|---|---|---|
| | Before MP-RCA (Ct) | After MP-RCA (Ct) | Before MP-RCA (Ct) | After MP-RCA (Ct) | |
| HPV16 | 32 | 16 | 34 | 35 | 786,431 |
| Siha | 29 | 30 | 26 | 24 | 0.5 |

[a] Enrichment was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = [Ct_{HPV}(\text{after RCA}) - Ct_{HPV}(\text{before RCA})] - [Ct_{ALU}(\text{after RCA}) - Ct_{ALU}(\text{before RCA})]$.

**Table 2**
NGS data analysis. Mapped reads indicate NGS reads, which were mapped onto the extracted L1 region of all HPV types.

| Pooled sample | Total Reads | Mapped Reads | % Mapped Reads to L1 gene |
|---|---|---|---|
| 1. FAP PCR | 338,374,994 | 277,598,421 | 82.04 |
| 2. HPV16 RCA | 394,607,770 | 9 | 0 |
| 3. FAP PCR RCA | 380,204,724 | 67 | 0 |
| 4. RCA | 382,376,134 | 87 | 0 |
| 5. FAP PCR RCA | 196,273,326 | 61,518,757 | 31.34 |
| 6. FAP PCR RCA2 | 229,994,340 | 126,833,119 | 55.15 |
| 7. FAP PCR RCA16 | 199,750,516 | 101,856,187 | 50.99 |

**Table 3**
L1 sequence analysis. Matched OCL sample indicates patient sample where Sanger sequencing results coincided with results from NGS.

| Pooled sample | HPV type | NGS total reads | Matched OCL sample |
|---|---|---|---|
| 1. FAP PCR | 172 | 109,936,978 | 3, 40, 42 |
| | 8 | 39,473,771 | 14 |
| | 23 | 37,762,624 | 14 |
| | 76 | 4,134,700 | 14 |
| 5. FAP PCR RCA | 62 | 26,223,379 | 93 |
| | 152 | 15,993,736 | 69 |
| | 32 | 6,799,264 | 59 |
| | 122 | 461 | 93 |
| 6. FAP PCR RCA2 | 90 | 35,679,275 | 52 |
| | 23 | 26,865,855 | 14 |
| | 11 | 26,393,016 | 96 |
| | 8 | 23,967,471 | 14 |
| | 33 | 1,390,467 | 72 |
| | 105 | 1,112,699 | 63 |
| | 76 | 490,440 | 14 |
| | 147 | 15,910 | 95 |

**Table 4**
Sanger sequencing results. BLASTn and PaVE database search results along with HPV types associated with the samples. % homology was the product of the query identity and the max identification multiplied by 100.

| Sample | Clone | HPV type | % Homology BLASTn | % Homology PaVE |
|---|---|---|---|---|
| OCL 3.1 | Yes | 172 | 97.02 | 96.51 |
| OCL 3.2 | Yes | 172 | 95.04 | 96.51 |
| OCL 3.6 | Yes | 172 | 96.00 | 96.70 |
| OCL 3.8 | Yes | 172 | 92.07 | 89.20 |
| OCL 3.10 | Yes | 172 | 97.02 | 96.51 |
| OCL 14 | No | 23 | 80.00 | 49.00 |
| OCL 14.1 | Yes | 8 | 95.04 | 95.63 |
| OCL 14.2 | Yes | 8 | 95.04 | 93.74 |
| OCL 14.3 | Yes | 76 | 91.08 | 86.67 |
| OCL 14.4 | Yes | 76 | 89.24 | 88.51 |
| OCL 14.6 | Yes | 76 | 87.40 | 86.70 |
| OCL 40 | No | 172 | 98.01 | 98.00 |
| OCL 42 | No | 172 | 98.01 | 95.60 |
| OCL 52 | No | 90 | 98.01 | 99.60 |
| OCL 63.2 | Yes | 105 | 96.03 | 96.42 |
| OCL 63.3 | Yes | 105 | 98.01 | 97.91 |
| OCL 63.4 | Yes | 105 | 96.03 | 94.01 |
| OCL 63.5 | Yes | 105 | 96.03 | 96.42 |
| OCL 63.6 | Yes | 105 | 96.03 | 96.43 |
| OCL 69.1 | Yes | 152 | 84.63 | 85.37 |
| OCL 69.2 | Yes | 152 | 89.18 | 89.18 |
| OCL 69.3 | Yes | 152 | 83.72 | 83.27 |
| OCL 69.4 | Yes | 152 | 89.18 | 89.55 |
| OCL 69.5 | Yes | 152 | 88.27 | 89.19 |
| OCL 93.1 | Yes | 62 | 95.04 | 94.94 |
| OCL 93.2 | Yes | 62 | 96.03 | 96.43 |
| OCL 93.3 | Yes | 122 | 99.00 | 99.30 |
| OCL 93.4 | Yes | 122 | 94.20 | 94.20 |
| OCL 93.5 | Yes | 122 | 96.03 | 96.72 |
| OCL 95 | No | 147 | 76.80 | 64.55 |
| OCL 96 | No | 11 | 98.01 | 99.60 |

frequency of the novel oncogenic HPVs in cancer and non-cancer oral rinse samples.

We categorized smoking history as follows: non-smoker (0 packs); light smoker (<1pack/week); moderate smoker (≥1 pack/week ≤ 1 pack/day); heavy smoker (≥1 pack/day). For those who smoked cigars or chewed tobacco, we calculated the equivalent of packages of cigarettes smoked. Alcohol history was categorized as follows: none (never drinks); rarely/occasionally (1 drink every 1–2 months); light (1–6 drinks/week for females, 1–13 drinks/week for males); moderate (7 drinks/week for females, 14 drinks/week for males); heavy (>7 drinks/week for females, >14 drinks/week for males).

### *2.3.3. Determining prevalence of novel HPVs in archived tissue blocks*

Using the same newly created primers and probes, the presence of the new viral sequences in 106 normal and 115 malignant (OCC/OPC) oral tissues was determined by HPV-type specific quantitative qPCR Taqman assays. Archived oral tissue blocks were accessed through the Department of Pathology's repository (University of Washington). A total of 80 μm of tissue was cut with a microtome, with a new blade for each tissue block to eliminate potential for contamination. Genomic DNA was isolated using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol.

All data analysis was conducted using Stata MP 13.1 (StataCorp LP, College Station, Texas, USA).

## 3. Results

We observed substantial amplification after MP-RCA with our HPV16 positive control, with enrichment of almost 800,000-fold, compared to only a 0.5-fold amplification with SiHa (ATCC® HTB-35™, a cervical cancer cell line with genome-integrated HPV16),

**Table 5**
Frequency of HPV infection in archived oral cavity and oropharyngeal tissue biopsies.

| HPV type | Oral cavity | | Oropharynx | |
|---|---|---|---|---|
| | Cancer- (n = 56) | Cancer+ (n = 65) | Cancer- (n = 50) | Cancer+ (n = 50) |
| HPV16 | 9, 16% | 6, 9% | 1, 2% | 39, 79% |
| HPV18 | – | – | – | – |
| NV14.4 | – | 1, 1.5% | – | – |
| NV69.1 | – | – | – | – |
| NV95 | – | – | – | – |

**Table 6**
Frequency of HPV infection in oral rinse samples.

| HPV type | No cancer (n = 110) | OPC (n = 76) | OCC (n = 16) | Other HNC (n = 8) |
|---|---|---|---|---|
| HPV16 | – | 19, 25% | 2, 12.5% | 2, 25% |
| HPV18 | 1, 0.9% | – | – | – |
| NV14.4 | – | 1, 1% | 1, 6% | – |
| NV69.1 | – | 10, 13% | 2, 12.5% | 1, 12.5% |
| NV95 | – | 1, 1% | 1, 6% | – |

OPC – oropharyngeal cancer; OCC – oral cavity cancer, HNC – head and neck cancer.

which was our negative control (Table 1). Thus, by MP-RCA, circular template DNA amplified substantially, whereas linear DNA exhibited minimal amplification.

With NGS, we only observed reads from sample 1, where 82% of the reads mapped onto the L1 gene of all 189 HPV types in the PaVE database (Table 2). For sample 2, we anticipated reads that mapped onto the HPV L1 gene, as this was our positive control, but no results were seen. We did not see any results for samples 3 and 4. Sample 5 had 31% of reads mapped onto the L1 gene; sample 6 had 55%; and sample 7 had 51% of reads mapped onto the L1 gene (Table 2).

With the results from Sanger sequencing of the ten pre-selected samples, we matched the HPV type results from the BLASTn and PaVE searches with the NGS mapped reads (Table 3). OCL3, 40, and 42 were positive for HPV172, which was one of the novel HPVs that our lab identified. OCL14 and 93 presented with multiple HPV infections. Our HPV16 control samples also demonstrated multiple HPV infection (data not shown).

Five samples that did not have clear Sanger sequencing results were cloned (Table 4). For each sample, we chose five different clones to be sequenced. The samples that we chose to investigate further were OCL14.4, 69.1, and 95 because their homology differences were greater than 10%.

Each potentially novel HPV was isolated only from males; the type of cancer was OPC, OCC, or laryngeal cancer; patients were aged 60 or over; two patients had a heavy smoking history while the other had a light history; alcohol history ranged from rarely to heavy (data not shown).

Phylogenetic tree analysis was completed for each of the novel NVs (Figures not shown). NV14.4 was closely related to HPV76 with an 89% homology and is part of the genus *Beta-papillomavirus* (β-PV). NV 69.1 was distantly related to the genus *Alpha-papillomavirus* (α-PV). NV95 was closely related to HPV147 with a 65–77% homology and is part of the genus *Gamma-papillomavirus* (γ-PV).

Newly designed primers and probes were created for the three NVs (data not shown). Of the three NVs, only NV14.4 was found to be positive in an OCC archived tissue biopsy (Table 5). With the oral rinse samples, NV14.4 was detected in 1 OPC and 1 OCC sample; NV69.1 was detected in 10 OPC, 2 OCC, and 1 HNC sample; NV95 was detected in 1 OPC and 1 OCC sample (Table 6).

## 4. Discussion

Through NGS we were able to identify HPVs in oral rinse samples. However, in order to obtain more accurate DNA sequences, Sanger sequencing was used. Regarding amplification with MP-RCA, our control seemed to exhibit significant enrichment; however, it is possible that other samples with HPV may not have amplified as well. MP-RCA has been demonstrated to amplify circular DNA templates up to $10^7$-fold [14]. Thus, if there was sufficient enrichment, NGS should have allowed us to discover the novel HPVs without resorting to Sanger sequencing. Pooling of samples may also have caused more confusion when analyzing, as the amount of data obtained was extremely large.

From the NGS results, OCL3, 40, and 42 were positive for HPV172, which was one of the HPVs our lab identified. What is interesting is that HPV172 was predominately seen in non-cancer tissue biopsies [13]. It could be possible that HPV172 is becoming more prominent in oral HPV infections in general.

The nucleotide searches accomplished using BLASTn and PaVE coincided with each other for the majority of the time. For OCL95, there was a notable difference in results, where BLASTn demonstrated 77% homology compared to PaVE, which showed 65% homology to HPV147. We observed an even greater difference between the two databases (GenBank and PaVE) for OCL14: 80% versus 49%. Each database may contain different HPV variants, which could result in inconsistencies between the two.

Each of the novel HPVs belonged to separate genera, although OCL69.1 was possibly distantly related to α-PVs. Obtaining the entire genome would give us a clearer picture of its overall HPV homology.

In the archived oral tissue biopsies, we only detected OCL14.4 in one of the OCC samples. DNA degradation within the tissue blocks is possible for older samples, which may have limited our results. The oral rinse samples, on the other hand, demonstrated more frequent infection by the NVs compared to the tissue block samples. All tissue and oral rinse samples were quantified for human genomic DNA, and all samples had adequate amounts for detection (Figures not shown). Whether these NVs are indeed oncogenic is still to be seen, and further research will need to be accomplished.

One major limitation to our study was budget. NGS is expensive, thus we opted to minimize the cost of sequencing runs by pooling our samples. However, if budget was not an issue, less pooling would obviously be preferred.

Through NGS, we were able to identify HPVs in oral rinse samples. With Sanger sequencing, we were able to identify and characterize three different novel HPVs in oral rinse samples. We obtained similar results with both sequencing platforms when detecting HPVs, and propose that NGS is a more modern technique, which should be investigated further.

## Conflicts of interests

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

## Sources for funding

Warren G. Magnuson Scholarship.
University of Washington Royalty Research Fund.
ITHS TL1 Training Program.

## Ethics approval

Fred Hutchinson Cancer Research Center IRB #7490, approved April 9, 2014.

## Acknowledgements

Oral Health Sciences Department, University of Washington, Seattle, WA.
Qinghua Feng, PhD - study design, consulting.
Dolphine Oda, BDS, MSc – manuscript review.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2019.05.001.

## References

[1] Dell G, Gaston K. Human papillomaviruses and their role in cervical cancer. Cell Molr Life Sci 2001;58:1923–42.
[2] Genital HPV infection - Fact Sheet [https://www.cdc.gov/std/hpv/stdfact-hpv.htm].
[3] Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology 2010;401:70–9.
[4] Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The biology and life-cycle of human papillomaviruses. Vaccine 2012;30:F55–70.
[5] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–917.
[6] Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009;45:309–16.
[7] Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol 2011;29:4294–301.
[8] Gillison ML, Broutian T, Pickard RK, Tong ZY, Xiao W, Kahle L, et al. Prevalence of oral HPV infection in the United States, 2009–2010. JAMA 2012;307:693–703.
[9] Hariri S, Unger ER, Sternberg M, Dunne EF, Swan D, Patel S, et al. Prevalence of genital human papillomavirus among females in the United States, the national health and nutrition examination Survey, 2003–2006. J Infect Dis 2011;204:566–73.
[10] Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709–20.
[11] Mineta H, Ogino T, Amano HM, Ohkawa Y, Araki K, Takebayashi S, et al. Human papilloma virus (HPV) type 16 and 18 detected in head and neck squamous cell carcinoma. Anticancer Res 1998;18:4765–8.
[12] Michaud DS, Langevin SM, Eliot M, Nelson HH, Pawlita M, McClean MD, et al. High-risk HPV types and head and neck cancer. Int J Cancer 2014;135:1653–61.
[13] Martin E, Dang J, Bzhalava D, Stern J, Edelstein ZR, Koutsky LA, et al. Characterization of three novel human papillomavirus types isolated from oral rinse samples of healthy individuals. J Clin Virol 2014;59:30–7.
[14] Nelson JR, Cai YC, Giesler TL, Farchaus JW, Sundaram ST, Ortiz-Rivera M, et al. TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. Biotechniques 2002 Jun;(Suppl):44–7.
[15] Forslund O, Antonsson A, Nordin P, Stenquist B, Hansson BG. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol 1999;80:2437–43.
[16] de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology 2004;324:17–27.
[17] Chen Z, Freitas LB, Burk RD. Evolution and classification of oncogenic human papillomavirus types and variants associated with cervical cancer. Methods Mol Biol 2015;1249:3–26.