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Characterization of three novel human papillomavirus types isolated from oral rinse samples of healthy individuals☆

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ABSTRACT

Background: Despite the strong evidence of HPV infection as the etiological agent in a subset of oral cancer, oral α-HPV detection is rare in healthy individuals, and little is known of the existing of novel HPV types in oral cavity.
Objective: We determined whether novel HPV types can be isolated from oral rinse samples collected from healthy individuals.
Study design: We performed rolling circle amplification (RCA) coupled with degenerated PCR assay on 48 oral rinse samples to amplify novel HPV types. Full length HPV DNA was cloned using long range PCR. Quantitative type specific Taqman assays were used to determine the prevalence of novel HPV types in 158 archived oral tissue samples.
Results: We were able to isolate four novel human papillomavirus types. Full length HPV DNA was cloned for three of the four novel HPV types. All four HPV types belong to the genus *Gammapapillomavirus* (γ-PV), where HPV 171 is most closely related to HPV 169, showing 88% similarity; HPV 172 is most closely related to HPV 156, showing 70% similarity; HPV 173 is most closely related to HPV 4, showing 73% similarity; oral sample lavage (OSL) 37 is most closely related to HPV 144, showing 69% similarity. Finally, we showed that HPV 173 was rarely present in oral tissues (2/158), HPV 172 was only detected in normal oral tissues (25/76), and HPV 171 was more prevalent in malignant oral tissues (17/82 vs. 10/76, $p = 0.21$).
Conclusions: Novel γ-HPV types are present in oral cavity of healthy individuals.

1. Background

Human papillomavirus (HPV) infection is the etiological agent for cervical cancer. Recent studies suggest that it is also the causal agent for a subset of head and neck cancer [1–3], the prevalence of HPV infection in oral squamous cell carcinoma (OSCC) ranges from 10 to 20% in oral cavity cancer to 6–60% in oropharyngeal cancer [4–11], with HPV 16 being the most prevalent type. Detection of HPV 16 infection in oral exfoliated cells increases the odds of oropharyngeal cancer more than 14 fold [3], and HPV integration and expression of E6 and E7 oncogenes have been detected in OSCC tissues [12–15]. In summary, HPV is a significant etiological factor for OSCC, with potential to influence the prevention, diagnosis and treatment of OSCC.

On the other hand, oral α-HPV detection so far is rare in healthy individuals. A recent systemic review of literature reported the prevalence of any oral HPV detected below 5%, and HPV 16 accounted for 28% of all HPV detected in the oral region [16,17]. We recently determined the incidence and prevalence of oral HPV infection in a cohort of male university students [18]. The prevalence at enrollment was 7.5% and 12-month cumulative incidence was 12.3%. However, our published study, like most others, was limited to testing for α-HPV types. As current evidence indicates that types from other genera infect oral epithelium [19], the true prevalence of HPV infection in oral cavity is likely underestimated.

Of over 160 HPV types identified so far, most are categorized into one of three genera based on sequence differences:

☆ *Nucleotide sequences*: Nucleotide sequences of full length new HPV types have been submitted to Genbank with following accession number: HPV 171: KF006398; HPV 172: KF006399; HPV 173: KF006400.

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Alphapapillomavirus (α-PV), *Betapapillomavirus* (β-PV), and *Gammapapillomavirus* (γ-PV). Thus far, only a few β-PVs [20–22] but no γ-PVs have been shown to participate in tumorigenesis. Recent studies suggest the presence of novel beta and gamma genra HPV types present in oral cavity. For example, Bottalico et al. identified 12 novel gamma and 8 beta HPV types in an HIV+ population, and a novel HPV type 120 was identified in the oral rinse sample that has shown a wide range of tropism [19].

To determine whether novel HPV types are present in oral rinse samples, we first used rolling circle amplification to enrich for full length circular HPV genomes then used degenerate PCR to identify novel HPV types present in oral rinse samples collected from healthy young men.

2. Objectives

We plan to isolate novel HPV types from oral cavity of healthy individuals and determine their prevalence in normal and malignant oral tissues.

3. Study design

3.1. Clinical samples

A total of 48 archived oral rinse samples from 41 subjects were selected for the isolation of novel HPV types. These samples were selected from a longitudinal study investigating the natural history of HPV infection in male population [18]. The demographics of the 41 subjects whose oral rinse samples were used in the current study was similar to the entire study population published before [18]. Briefly, oral specimens were collected via gargle/rinse and swabbing of the oropharynx. The median age of subjects was 20, and over 80% of them were Caucasians.

In addition, a total of 158 oral tissue blocks were selected from University of Washington Department of Pathology, including 76 normal oral tissue blocks (56 from oral cavity and 20 from oropharynx), 82 malignant oral tissue blocks (66 from patients with oral squamous cell carcinoma (OSCC), 16 from patients with oropharyngeal squamous cell carcinoma (OPSCC)). Cancer patients on average were significantly older than normal patients (58.5 vs. 45.1, $p < .0001$). The majority of the population was male (63.5%) and Caucasian (65.7%).

3.2. Genomic DNA isolation

Genomic DNA was extracted from oral rinse samples by the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Tissue sections were prepared by the Department of Pathology of University of Washington. Special care was taken to minimize cross-contamination between tissue blocks: microtome was cleaned and blade was replaced after processing each block. Total DNA was extracted from 80 μm oral tissue block sections using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

3.3. Multiply primed rolling circle amplification (MP-RCA)

MP-RCA was performed on each oral rinse DNA sample with the TempliPhi 100 amplification kit (GE Healthcare, Piscataway, NJ) with modifications optimized for papillomavirus amplification [23]. Specifically, 1 μl purified sample DNA (~100–300 ng) was denatured at 95 °C for 3 min in 5 μl Sample Buffer, then cooled to 4 °C. Subsequently, TempliPhi premix containing 5 μl Reaction Buffer, 0.047 μl of 50 mM dNTPs, and 0.2 μl enzyme mix was added to each denatured sample. Reaction was performed at 30 °C for 16 h, then heat inactivated at 65 °C for 10 min. For the first 33 oral rinse samples, every three RCA reactions were pooled for subsequent consensus PCR reaction, while consensus PCR was performed for individual RCA samples for oral rinse samples 34–48. Therefore, a total 26 RCA samples went through consensus PCR reactions.

Table 1
Primers and probes used in this study.

	Primers and probe sequences
HPV171	
Long range PCR	AAGCAGGATTATGCCCTCCT GGACCACCTCTGTCAATTTCA
Real time PCR	F: TGTGTGTGTAGTTGCTTCAGAAGGA Probe: 6FAM-CAATACACCTGCTGCAGC-MGB R: GGAGAGATCGGTCGACAAAAGT
HPV172	
Long range PCR	TGGCCTGTTGCTCCTATACC CACACCACCAACAGGACAGT
Real time PCR	F: AACCAGCACCGGTTACTATAAAAGA Probe: 6FAM-TGTTCTGCAAAGCTC-MGB R: CCAGCCATCGAAAAGAGAGAA
HPV173	
Long range PCR	CCTAAGGGTCCTCCTCTGCT CCAGTTATTGGAGAGCATTGG
Real time PCR	F: AGGAGGTGTTTTCTATTTAGTTCGAAGT Probe: 6FAM-AGTGGAAAGGGTGTTGCA-MGB R: GTCCCTCTCATTGTTCAATCATACA

Both primers used to clone new HPV types as well as primers and probe for Taqman assays were listed.

3.4. HPV detection by consensus PCR amplification

Four consensus PCR assays were performed on MP-RCA amplified samples, following published protocols: nested PGMY/GP5+/6+ PCR assay [24], FAP [25], CP [26], and a newly described broad spectrum (BS) PCR assay [27]. PCR product was cloned using TA cloning kit (Invitrogen, Life technologies, Grand Island, NY) and at least two clones from each PCR product were sequenced. The presence of HPV sequence was determined through BLAST search (http://blast.ncbi.nlm.nih.gov/).

3.5. Full length HPV cloning by long range PCR and sequencing

The cloned HPV fragment sequence was used to design primers for long range PCR reaction using Stratagene kit (Stratagene, La Jolla, CA). The presence of full length HPV PCR product (~6–7 kb) was subsequently cloned and sequenced. The primers used for long range PCR are listed in Table 1.

3.6. Phylogenetic analysis of novel HPV types

Maximum likelihood tree was estimated by PhyML v3 [30]. ModelTest v3.7 [29] reported GTR + I + G substitution model as the best fit model. HPV16, an α-HPV type was used as the out group for the tree analysis, and the tree was based on L1 sequences of 50 gamma HPV reference clones (www.hpvcenter.se).

3.7. HPV detection by real time PCR in oral tissue samples

Type-specific Taqman assays were designed for HPV 171, 172 and 173 based on the HPV E6/E7 region. Primers and probe sequences were listed in Table 1. These Taqman assays were extensively validated using plasmids containing individual HPV type E7 gene. Each assay can specifically detect down to 1 copy HPV but did not detect nonspecific HPV types present at 10,000 copies. Normalization of input DNA was performed using the quantitative Taqman assay on Alu sequence. Absolute quantification was performed on