Absence of high-risk HPV 16 and 18 in Chinese patients with oral squamous cell carcinoma and oral potentially malignant disorders

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Abstract

Background: The critical role of human papillomavirus (HPV) in cancer has been recognized, but the involvement of HPV in oral squamous cell carcinoma (OSCC) and oral potentially malignant disorders (OPMD) is still controversial. The aim of this study was to identify and verify the prevalence of high-risk HPV infection (HPV16 and 18) in Chinese patients with OSCC or OPMD using real-time PCR and DNA sequencing.

Methods: Paired tissue and serum DNA samples were extracted from 40 Chinese patients with OSCC and 59 with OPMD. A SYBR Green-based real-time PCR assay was developed to detect the E6 gene of HPV16 and HPV18. Suspicious positive samples were then sequenced to eliminate false positives.

Results: We found that none of the tissue and serum samples of OSCCs and OPMDs were positive for HPV16 E6 or 18 E6, using both real-time PCR and DNA sequencing. Overall, 3 of 198 (1.52 %) and 7 of 198 (3.54 %) samples were false-positive for HPV16 E6 and HPV18 E6, respectively, using real-time PCR.

Conclusion: The lack of HPV16 and HPV18 detected in this study indicates that high-risk HPV 16 and 18 infections are uncommon in Chinese patients with OSCC and OPMD. Real-time PCR followed by DNA sequencing for HPV DNA detection is an effective strategy to rule out false positives.

Keywords: HPV16, HPV18, Oral squamous cell carcinoma, Oral potentially malignant disorders

Background

Head and neck squamous cell carcinoma (HNSCC), which includes squamous cell carcinomas of the oral cavity, oropharynx, larynx, and hypopharynx, is the sixth most common cancer worldwide [1, 2]. Two main risk factors related to HNSCC are tobacco use and alcohol consumption [3]. Recently, investigators have suggested that human papillomavirus (HPV) is a potential etiological factor of HNSCC in patients who do not smoke or drink alcohol, particularly in oropharynx squamous cell carcinoma (OPSCC) [4, 5]. The oncogenic proteins E6 and E7 of high-risk HPVs, such as HPV16 and HPV18, are considered to be associated with the carcinogenic process of OPSCC by inactivating the tumor suppressor genes p53 and Rb [6, 7]. However, the rate of detecting HPV in OSCC varies widely (0–100 %), and the role of HPV in oral carcinogenesis has long been controversial [8].

HPV has been detected in not only cervical cancer but in cervical premalignant lesions as well, and the detection rate is known to increase with the severity of disease abnormality [9]. Oral lesions and conditions associated with a risk of malignant transformation have been referred to as oral potentially malignant disorders (OPMD) and include oral leukoplakia (OLK), lichen planus, and erythroplakia [10]. Recent studies have revealed a varying rate of detected HPV in OPMD [8]. A better understanding of the true presence of HPV in OSCC and OPMD may thus contribute to further studies of these diseases.

Different techniques have been used to detect HPV, including in situ hybridization (ISH), Southern blot
hybridization, dot blot hybridization, hybrid Capture 2 (hc2), conventional PCR, and real-time PCR [11].ISH, Southern blot and dot blot hybridization are time-consuming procedures that require relatively large amounts of purified DNA [11]. Hc2 assay cannot genotype single HPV subtypes [11]. Of these methods, studies using PCR techniques have reported a higher sensitivity for HPV detection [12]. However, conventional PCR assays may have a lower sensitivity and specificity [11]. Real-time PCR has a sensitivity of 92 % and a specificity of 97 % in detecting HPV and is able to genotype and quantitate HPV viral load [13].

The aim of our study was to identify the detection rate of high-risk HPV types 16 and 18 in Chinese patients with OSCC and OPMD using real-time PCR and DNA sequencing.

Methods
Subjects
A total of 99 patients including 40 OSCC and 59 OPMD patients were enrolled from the Department of Oral Mucosal Diseases and the Department of Oral Maxillofacial Surgery at the Shanghai 9th People’s Hospital, Shanghai Jiao Tong University School of Medicine. Paired tissue and serum samples were collected from each patient. Tissue samples were immediately frozen at −80 °C after surgery. Serum was obtained from the supernatant of the collected whole blood and stored at −80 °C until processing. Histological diagnoses were made by one pathologist who was on duty and confirmed by a superior pathologist according to the World Health Organization criteria [14, 15]. This study was approved by an Independent Ethics Committee of Shanghai Ninth People’s Hospital affiliated to Shanghai Jiao Tong University, School of Medicine (#200703), and signed informed consent was obtained from each patient. The baseline characteristics of the patients are presented in Table 1.

Cell culture
The CAL27 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MA, USA) and was grown in Dulbecco’s Modified Eagle Medium (HyClone, Logan, UT, USA) containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin solution at 37 °C in 5 % CO₂.

DNA extraction
Twenty 20-μm sections were cut from the frozen tissue samples, and DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Düsseldorf, Germany). Serum DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Düsseldorf, Germany). CAL27 cells were detached by trypsinization and extracted DNA with QIAamp DNA Mini Kit (Qiagen, Düsseldorf, Germany). The plasmid pB-actin 16 E6 and pB-actin 18 E6 were bought from Addgene (Cambridge, MA, USA). Plasmid DNA was extracted using the QIAfilter MidiKit (Qiagen, Düsseldorf, Germany). Purified plasmid DNA were sequenced and blasted with HPV16 E6 (NC_001526.2) and HPV18 E6 (NC_001357.1) NCBI reference sequence. The extracted DNA was stored at −80 °C until further use.

Real-time PCR and sequencing
Real-time PCR was performed by LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland) together with 0.5 μmol/L of each primer and 50 ng DNA in a 10 μl reaction were utilized. Positive controls were performed, which including HPV plasmid DNA, HPV containing cell line DNA and small amount of plasmids added to clinical sample DNA (Fig. 1). Negative controls were also performed, which including pure water, pure water instead of 2 x master mixture, pure water instead of positive control DNA (Fig. 1). A standard curve was developed for both HPV16 E6 (Fig. 2a) and HPV18 E6 (Fig. 2b) using a series of 10-fold diluted plasmid DNA 1 ng to 0.1 pg. The quantitated data was normalized by beta-actin (ACTB) using CAL27 genomic DNA. The reaction was performed by initiation at 95 °C for 5 min followed by 35 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s. Each sample was performed in triplicate. A sample was considered positive for HPV infection if two or three wells of the triplicate showed an amplifying curve. It was under suspicion if the amplifying curve was detected later than the 30th cycle of the reaction or had a deformed shape. The suspicious samples of HPV16 E6 or HPV18 E6 were then sequenced to rule out false positives. All primers are shown in Table 2.

Results
Real-time PCR was conducted to detect HPV16 E6 and HPV18 E6 DNA. We found that zero of the 99 tissue samples (0 %) showed a standard amplifying curve for HPV 16 E6, but a few samples showed late or deformed amplifying curves in one of the triplicates, which were clearly not considered to be positive (Fig. 3a). Thirty-nine of 40 OSCC and 57 of 59 OPMD serum samples did not show a standard amplifying curve for HPV 16 E6 using real-time PCR, but 1 OSCC and 2 OPMD serum samples had a late or deformed amplifying curve in two or three wells of the triplicate that was suspicious (Fig. 3b). In addition, 36 of 40 OSCC and all 59 OPMD tissue samples were negative for the standard amplifying curve of HPV 18 E6, but 4 OSCC tissue samples presented a late and deformed amplifying curve in two or three wells of the triplicate (Fig. 3c). Thirty-nine of 40 OSCC and 57 of 59 OPMD serum samples were negative for the standard amplifying curve of HPV 18 E6, but 1 OSCC and 2 OPMD serum samples had late and deformed amplifying curves in two or three wells of the triplicate (Fig. 3d). DNA sequence analysis
Table 1  The baseline characteristic of patients

| ID  | Diagnosis | Age | Gender | Smoking | Alcohol | Stage | Notes                                      | Tumor Site | Type     |
|-----|-----------|-----|--------|---------|---------|-------|--------------------------------------------|------------|----------|
| CXJ 1 | OSCC      | 77  | M      | Past    | Never   |       | real-time PCR                              | Tongue     |          |
| CXJ 2 | OLK       | 48  | F      | Never   | Sometimes |       | real-time PCR                              | Gingiva    |          |
| CXJ 3 | OLK       | 36  | M      | Current | Current |       | real-time PCR                              | Buccal     |          |
| CXJ 4 | OSCC      | 63  | M      | Never   | Past    |       | real-time PCR                              | Tongue     |          |
| CXJ 5 | OSCC      | 54  | M      | Current | Past    |       | real-time PCR                              | Buccal     | Papillary |
| CXJ 6 | OSCC      | 60  | M      | Current | Current |       | real-time PCR                              | Buccal     |          |
| CXJ 7 | OSCC      | 41  | M      | Current | Current |       | real-time PCR                              | Tongue     |          |
| CXJ 8 | OSCC      | 53  | M      | Past    | Current |       | real-time PCR                              | Buccal     |          |
| CXJ 9 | OSCC      | 41  | M      | Current | Current |       | real-time PCR                              | Floor of mouth |          |
| CXJ 10 | OSCC    | 69  | F      | Never   | Never   | T1M0N0 | real-time PCR & DNA sequencing (18 ZDNA)   | Gingiva    |          |
| CXJ 11 | OSCC    | 56  | M      | Current | Never   |       | real-time PCR & DNA sequencing (16 SDNA)   | Buccal     |          |
| CXJ 12 | OSCC    | 60  | F      | Never   | Never   |       | real-time PCR                              | Gingiva    |          |
| CXJ 13 | OSCC    | 58  | M      | Current | Sometimes |       | real-time PCR                              | Gingiva    |          |
| CXJ 14 | OSCC    | 57  | F      | Never   | Never   | T2N0M0 | real-time PCR                              | Tongue     |          |
| CXJ 15 | OSCC    | 55  | M      | Sometimes | Sometimes |       | real-time PCR                              | Hard palate | Papillary |
| CXJ 16 | OSCC    | 75  | M      | Never   | Sometimes |       | real-time PCR                              | Buccal     |          |
| CXJ 17 | OSCC    | 66  | F      | Never   | Never   | T4N1M0 | real-time PCR                              | Buccal     |          |
| CXJ 18 | OSCC    | 63  | M      | Never   | Never   | T4N0M0 | real-time PCR                              | Buccal     |          |
| CXJ 19 | OSCC    | 43  | M      | Current | Current | T4N0M0 | real-time PCR                              | Gingiva    |          |
| CXJ 20 | OLK      | 65  | M      | Never   | Sometimes |       | real-time PCR                              | Hard palate |          |
| CXJ 21 | OLK      | 56  | M      | Current | Sometimes |       | real-time PCR                              | Buccal     |          |
| CXJ 22 | OLK      | 78  | F      | Never   | Never   |       | real-time PCR                              | Buccal     |          |
| CXJ 24 | OSCC    | 59  | M      | Current | Current |       | real-time PCR                              | Gingiva    |          |
| CXJ 25 | OSCC    | 54  | F      | Never   | Never   | T1N0M0 | real-time PCR                              | Tongue     |          |
| CXJ 26 | OSCC    | 72  | M      | Never   | Never   | T3N0M0 | real-time PCR                              | Tongue     |          |
| CXJ 27 | OSCC    | 75  | F      | Never   | Never   | T1N0M0 | real-time PCR                              | Tongue     |          |
| CXJ 28 | OSCC    | 40  | M      | Sometimes | Never   |       | real-time PCR                              | Gingiva    |          |
| CXJ 30 | OLK      | 56  | F      | Never   | Never   |       | real-time PCR & DNA sequencing (16 SDNA)   | Gingiva    |          |
| CXJ 31 | OLK      | 60  | M      | Current | Never   |       | real-time PCR                              | Gingiva    |          |
| CXJ 32 | OSCC    | 44  | M      | Current | Current |       | real-time PCR                              | Floor of mouth |          |
| CXJ 33 | OLK      | 65  | M      | Never   | Never   |       | real-time PCR                              | Buccal     |          |
| CXJ 34 | OSCC    | 81  | M      | Never   | Never   |       | real-time PCR                              | Lip        |          |
| CXJ 35 | OLK      | 63  | M      | Never   | Never   |       | real-time PCR                              | Gingiva    |          |
| CXJ 36 | OSCC    | 58  | F      | Never   | Never   |       | real-time PCR                              | Tongue     |          |
| CXJ 37 | OLK      | 72  | M      | Never   | Never   |       | real-time PCR                              | Gingiva    |          |
| CXJ 38 | OLK      | 75  | M      | Never   | Never   |       | real-time PCR                              | Buccal     |          |
| CXJ 39 | OLK      | 73  | M      | Past    | Never   |       | real-time PCR                              | Tongue     |          |
| CXJ 40 | OSCC    | 60  | F      | Never   | Never   |       | real-time PCR                              | Buccal     |          |
| CXJ 41 | OLK      | 36  | M      | Never   | Sometimes |       | real-time PCR                              | Gingiva    |          |
| CXJ 42 | OLK      | 57  | F      | Never   | Never   |       | real-time PCR                              | Buccal     |          |
| CXJ 43 | OLK      | 51  | M      | Past    | Never   |       | real-time PCR                              | Tongue     | Verrucous |
| CXJ 44 | OLK      | 54  | M      | Past    | Never   |       | real-time PCR                              | Gingiva    |          |
| CXJ 45 | OLK 56 M | Never | Sometimes | real-time PCR | Tongue |
| CXJ 46 | OLK 66 F | Current | Never | real-time PCR | Gingiva |
| CXJ 47 | OLK 62 M | Never | Past | real-time PCR | Tongue |
| CXJ 48 | OLK 50 F | Never | Never | real-time PCR | Gingiva |
| CXJ 49 | OSCC 63 M | Current | Sometimes | real-time PCR | Buccal |
| CXJ 50 | OLK 53 F | Never | Never | real-time PCR | Tongue |
| CXJ 51 | OLK 54 M | Current | Past | real-time PCR | Soft palate |
| CXJ 52 | OLK 30 M | Current | Sometimes | real-time PCR | Tongue |
| CXJ 53 | OLK 62 M | Current | Sometimes | real-time PCR | Soft palate |
| CXJ 54 | OLK 64 F | Never | Never | real-time PCR | Buccal |
| CXJ 55 | OSCC 70 M | Past | Current | real-time PCR | Buccal |
| CXJ 56 | OLK 50 F | Never | Never | real-time PCR & DNA sequencing (16 SDNA, 18 SDNA) | Tongue |
| CXJ 57 | OSCC 73 F | Never | Never | real-time PCR & DNA sequencing (18 SDNA) | Buccal |
| CXJ 58 | OLK 59 F | Never | Never | real-time PCR | Tongue |
| CXJ 59 | OLK 62 F | Never | Never | real-time PCR | Gingiva |
| CXJ 60 | OLK 57 F | Current | Never | real-time PCR | Tongue |
| CXJ 61 | OLK 51 M | Current | Never | real-time PCR | Tongue |
| CXJ 62 | OLK 50 F | Never | Never | real-time PCR | Tongue |
| CXJ 63 | OSCC 67 M | Current | Never | real-time PCR | Buccal |
| CXJ 64 | OLK 64 M | Never | Never | real-time PCR | Tongue |
| CXJ 65 | OLK 45 F | Never | Never | real-time PCR | Gingiva |
| CXJ 66 | OLK 60 M | Never | Never | real-time PCR | Buccal |
| CXJ 67 | OLK 66 F | Never | Never | real-time PCR | Tongue |
| CXJ 68 | OSCC 38 M | Current | Sometimes | real-time PCR | Tongue |
| CXJ 69 | OSCC 61 M | Past | Past | real-time PCR & DNA sequencing (18 ZDNA) | Buccal |
| CXJ 70 | OLK 52 F | Never | Never | real-time PCR | Tongue |
| CXJ 71 | OLK 35 M | Past | Sometimes | real-time PCR | Buccal |
| CXJ 72 | OLK 58 F | Never | Never | real-time PCR | Buccal |
| CXJ 73 | OLK + EK 37 M | Past | Past | real-time PCR | Tongue |
| CXJ 74 | OSCC 34 M | Current | Current | real-time PCR | Tongue |
| CXJ 75 | OSCC 53 M | Current | Current | real-time PCR | Tongue |
| CXJ 76 | OLK 71 F | Never | Never | real-time PCR | Tongue |
| CXJ 77 | OSCC 58 F | Never | Never | real-time PCR | Tongue |
| CXJ 78 | OLK 58 F | Never | Never | real-time PCR | Buccal |
| CXJ 79 | OLK + EK 37 F | Never | Never | real-time PCR | Tongue |
| CXJ 80 | OLK 53 M | Past | Current | real-time PCR | Tongue |
| CXJ 81 | OSCC 58 M | Past | Sometimes | real-time PCR | Tongue |
| CXJ 82 | OLK 55 F | Never | Never | real-time PCR | Tongue |
| CXJ 83 | OLK 53 M | Current | Current | real-time PCR | Tongue |
| CXJ 84 | OLK 53 M | Current | Current | real-time PCR | Hard palate |
| CXJ 85 | OLK 54 F | NA | NA | real-time PCR | Tongue |
| CXJ 86 | OLK 54 F | Never | Never | real-time PCR | Tongue |
| CXJ 87 | OLK 63 M | Sometimes | Current | real-time PCR | Tongue |
was then performed on the suspicious samples, which found that all of the samples sequenced were negative for HPV16 and HPV18. Overall, 3 of 198 (1.52%) and 7 of 198 (3.54%) samples were false-positive for HPV16 E6 and HPV18 E6, respectively, using real-time PCR. Overall, none of the OSCC or OPMD cases were positive for HPV 16 or 18 in our study.

**Discussion**

In the past few decades, there has been speculation worldwide about the role of HPV in the pathogenesis of HNSCC. The most commonly detected HPV, HPV16, accounts for 90% of the HPV DNA-positive cases in HNSCC, followed by HPV18 and other high-risk subtypes [16]. However, the detection rate of HPV in OSCC and OPMD varies widely and remains controversial [8, 17]. This variation may due to differences in the types of sample, detection methods or geographic locations [8, 18]. Therefore, confirming the HPV infection rate in OSCC and OPMD cases may contribute to the study of carcinogenesis in the oral cavity [19, 20]. In this study, we used real-time PCR to detect HPV16 and HPV18 in paired

![Fig. 1 Positive and negative controls for HPV16 and HPV 18 with real-time PCR](image_url)

**Table 1** The baseline characteristic of patients (Continued)

| Patient | Sex | Age | Smoking Status | Drinking Status | Detection Method | Site |
|---------|-----|-----|----------------|----------------|-----------------|------|
| CXJ 88  | OLK | 72  | M              | Never          | real-time PCR   | Gingiva |
| CXJ 89  | OLK | 79  | F              | Never          | real-time PCR   | Buccal |
| CXJ 90  | OLK | 55  | M              | Past           | real-time PCR   | Tongue |
| CXJ 91  | EK  | 45  | F              | Never          | real-time PCR   | Buccal |
| CXJ 94  | OLP | 54  | F              | Never          | real-time PCR   | Buccal |
| CXJ 95  | OLP | 54  | F              | Never          | real-time PCR & DNA sequencing (18 SDNA) | Buccal |
| CXJ 96  | OLP | 29  | M              | Current        | real-time PCR   | Buccal |
| CXJ 97  | OLP | 40  | F              | Never          | real-time PCR   | Buccal |
| CXJ 98  | OLP | 58  | F              | Never          | real-time PCR   | Buccal |
| CXJ 99  | OLP | 28  | M              | Current        | real-time PCR   | Buccal |
| CXJ 100 | OSCC| 28  | M              | Current        | real-time PCR & DNA sequencing (18 ZDNA) | Buccal |
| CXJ 101 | OSCC| 62  | F              | Never          | real-time PCR   | Buccal |
| CXJ 102 | OSCC| 68  | M              | Never          | real-time PCR   | Buccal |
| CXJ 103 | OSCC| 59  | M              | Never          | real-time PCR & DNA sequencing (18 ZDNA) | Buccal |

OSCC oral squamous cell carcinoma, OLK oral leukoplakia, OLP oral lichen planus, EK oral erythroplakia, ZDNA tissue DNA, SDNA serum DNA, NA data not available

*a* Union for International Cancer Control; T, tumor size; N, lymph node; M, Metastasis

**Fig. 1** Positive and negative controls for HPV16 and HPV 18 with real-time PCR.

A: Positive and negative controls for HPV16. B: Positive and negative controls for HPV18. Standard curve 1–5, 10-fold diluted HPV16 E6 or HPV18 E6 plasmid DNA ranging from 1 ng/well to 0.1 pg/well. Positive control 1, clinical DNA sample added with 0.1 pg HPV16 E6 or HPV18 E6 DNA. Positive control 2, 50 ng Hela cell DNA. Negative control 1, pure water. Negative control 2, pure water instead of 2 × master mixture. Negative control 3, pure water instead of positive control DNA.
tissue and serum samples of Chinese OSCC and OPMD patients [21]. We conducted complementary analyses to verify the results of the real-time PCR with DNA sequencing. We found that none of the patients with OSCC or OPMD demonstrated existence of high-risk HPV16 or HPV18. The absence of HPV DNA in our sample implies that HPV infection may not be common in Chinese patients with OSCC and OPMD.

A critical step in malignant transformation is the integration of high-risk HPV DNA into the human cellular genome, followed by the expression of the oncoproteins E6 and E7, which promote tumor progression [21]. In a previous study, although the reported detection rate of high-risk HPV DNA in OSCC was 6.6 %, HPV mRNA was only detected in 5.9 % [22]. These findings indicated that the mRNA or oncoproteins of HPV E6 and E7 were less commonly found than the DNA, as the presence of HPV in the genome differed from the HPV-related etiology [23, 24]. The gold standard to identify the presence of HPV was therefore suggested to be detecting HPV DNA [25].

Yadav et al. showed that the HPV DNA detection limit for conventional PCR was 200 copies, whereas for real-time PCR, which has a higher sensitivity, detecting HPV DNA required only 1 copy [26]. Lingen et al. detected high-risk HPV DNA in 9.8 % of OSCC cases using consensus primer PCR, but the positive rate was 6.6 % using real-time PCR [22]. Scapoli et al. found the detection rate of HPV16 to be 2 % in OSCC with real-time PCR [27]. Real-time PCR shows a higher sensitivity and specificity than conventional PCR assays [12, 22, 26]. In the current study, we utilized real-time PCR and found that 3 of 198 samples showed late and deformed amplifying curves of HPV 16 E6 and 7 of 198 samples had late and deformed amplifying curves of HPV 18 E6. To rule out false positives, we performed subsequent sequencing and found that the rate of false positives using real-time PCR to detect HPV16 E6 and HPV18 E6 DNA was 1.52 and 3.54 %, respectively. Ha et al. found a 2 % false-positive rate for real-time PCR using the minimum criteria of HPV DNA copy number, which was similar to our results [12].

The population has also been considered to be another factor affecting rate diversification. Several countries have revealed a zero detection rate of OSCC, including India [28–30], Brazil [31], Japan [32] and Mozambique [33]. Other reported detection rates have been 1.54 % in Thailand [34], 6.6 % in America [22], 5 % in Mexico [35], 39.4 % in Spain [36] and 66.7 % in Sudan [37]. Studies performed in China have yielded varied results using conventional PCR assays, ranging from 2.2 to 74 % [38–42]. However, real-time PCR data for OSCC has not been reported in China. Our study revealed a zero detection rate of HPV16 and 18 in OSCC by combining real-time PCR and DNA sequencing, which was a reliable method and provided further understanding of HPV infection in Chinese patients.

HPV infection has been identified in cancers of the cervix [43], vulva [44], vagina [44], anus [44], penis [45] and oropharynx [46]. It is widely accepted that OPSCCs, especially tonsillar cancers, are frequently associated with HPV infection [17]. The recent reported prevalence of HPV in OPSCC was approximately 60-70 % [47], but the corresponding rate was substantially lower and significantly varied in OSCC [8, 17]. HPV prevalence in OPSCC has been suggested to be an independent prognostic factor [47]. HPV-positive OPSCC has been shown to be distinct from HPV-negative OPSCC with regard to prognosis [48–50]. However, there have been no direct correlations between HPV infection and oral carcinogenesis [23, 27, 51].

HPV has been detected not only in cancer but also in premalignant lesions, such as in lesions of the cervix and breast [9, 52, 53]. In contrast, there was a lack of HPV in premalignant lesions of the colon [54–56]. Interestingly,
Ha et al. demonstrated a low prevalence (1.1%) of HPV16 in OPMD [12]. Similarly, we detected no presence of HPV 16 and 18 in Chinese patients with OPMD.

Conclusion
Overall, we demonstrated a prevalence rate of 0 % of HPV 16 and 18 in Chinese patients with OSCC and OPMD. Our data suggests that high-risk HPV16 and HPV18 infection may not be common in Chinese patients with OSCC and OPMD. Combining real-time PCR and DNA sequence for HPV DNA detection is an effective strategy to eliminate false positives.

Abbreviations
HNSCC: head and neck squamous cell carcinoma; HPV: human papillomavirus; OPSCC: oropharynx squamous cell carcinoma; OSCC: oral squamous cell carcinoma.

Acknowledgements
The authors thank the Department of Oral Pathology at the Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, for its support in diagnosis. This study was also supported by the General Program of the National Natural Science Foundation of China (no. 30872887), the Natural Science Foundation of Shanghai Municipality (no. 15ZR1424700) and the National Clinical Key Specialized Subject Construction Project ([2013]544-03).

Authors’ contributions
XJC extracted the DNA, performed real-time PCR, and drafted the manuscript. KS extracted the DNA and collected tissue and serum samples. WWJ designed the study and reviewed the manuscript drafts. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 14 February 2016 Accepted: 11 April 2016
Published online: 20 May 2016

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Fig. 3 Amplification curves for HPV16 and HPV 18 with real-time PCR. a Detection of HPV16 E6 in tissue samples; b Detection of HPV16 E6 in serum samples; c Detection of HPV18 E6 in tissue samples; d Detection of HPV18 E6 in serum samples.
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