

Original Article

Detection of human papillomavirus types 16 and 18 in oral squamous cell carcinoma samples in Malaysia

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Abstract Infection of the oral cavity with high-risk human papillomavirus (HPV) has been implicated as one of the risk factors for the development of oral squamous cell carcinoma (OSCC). Among the high-risk HPV types, HPV 16 and 18 are the most common infective agents in oral cancers. This study aimed to compare the presence of high-risk HPV in genetic materials obtained from saliva, blood and tissues of OSCC patients in Malaysia. The genomic DNA was extracted from saliva (n=13), blood (n=59) and tissue (n=63) and subjected to polymerase chain reaction (PCR) amplification of human beta globin gene to confirm the presence and integrity of DNA. Positive amplification was then screened for high-risk HPV by nested PCR using MY11/09 and GP5+/6+ consensus primers, followed by a further confirmation by DNA sequencing of the positive samples. As a result, two saliva samples (2/13; 15.4%) were found to harbour HPV 16 and one tissue sample (1/63; 1.6%) was shown to be positive for HPV 18. However, none of the blood samples were positive for high-risk HPV. Thus, HPV is more likely to be found in the saliva of OSCC patients as compared to blood and tissue samples. The detection of high-risk HPV in OSCC patients is useful in deciding how to manage the patient as HPV-associated OSCC has better prognosis.

Keywords: HPV; nested PCR; OSCC; saliva; tissue.

Introduction

The etiological factors of OSCC are multifactorial; ranging from epigenetic, genetic, environmental factors and immunosuppression (Kumar *et al.*, 2016). Initially, the main risk factor of OSCC was etiologically related to betel quid chewing, heavy tobacco and alcohol consumption (Krishna *et al.*, 2014; Singh *et al.*, 2016). However, recent epidemiological and molecular studies have found human papillomavirus (HPV) as a new causative factor in OSCC cases where patients were not in contact or had been associated with the traditional risk factors. Several investigators have reported the presence of HPV DNA in OSCC (Krüger *et al.*, 2014; Erhart *et al.*, 2016) and even in healthy oral mucosa (Esquenazi *et al.*, 2010; Wimardhani *et al.*, 2015).

HPV is a non-enveloped DNA virus that has the capability to infect squamous epithelial cells (Stanley, 2012). Low-risk HPV such as HPV 6, 11, 42, 43 and 44 are commonly associated with benign lesions such as oral warts (Muñoz *et al.*, 2003). High-risk HPV such as HPV 16 and 18 are the most common infective agents in oral cancers and are known to cause malignant lesions and are often referred to as oncogenic types (Lee *et al.*, 2012; Hoste *et al.*, 2013; Kreimer, 2014; Andrade *et al.*, 2015). HPV 16 is highly associated with head and neck squamous cell carcinoma (HNSCC) where it is detected in approximately 90% of HPV positive HNSCC cases, 89.7% in oropharyngeal cancers and 95.5% in oral cavity cancers (Kreimer *et al.*, 2010; St Guily *et al.*, 2011). On the other hand, HPV 18 was only associated in less than 10% of oral cancer cases (Evans and Powell, 2010).

The reported prevalence of HPV varies due to the many factors which include sampling methods, HPV detection assay, variation of genetic materials used and anatomical location of the tumour. In Malaysia, the overall HPV detection rate was 28.6% in OSCC (Kerishnan *et al.*, 2016). In the detection of HPV, PCR-based assays are widely used since it is one of the most sensitive and flexible among all DNA analysis techniques. To monitor the HPV, saliva has been extensively used either in healthy individuals (Wimardhani *et al.*, 2015; Shigeishi and Sugiyama, 2016) or OSCC patients (Goot-Heah *et al.*, 2012; Dang *et al.*, 2015; Erhart *et al.*, 2016). Metastasized cancer cells or cell debris that are being shed from HPV in blood could also be used to investigate the HPV infection (Bodaghi *et al.*, 2005; Elshimali *et al.*, 2013).

The detection of HPV in oral cancer is important for diagnostic and prognostic information because HPV positive tumour has better clinical outcome as it react better to radiation therapy compared to HPV negative tumour (Ang *et al.*, 2010; Lassen *et al.*, 2018). Thus, in the present study, we aimed to detect the presence of high-risk HPV in different genetic materials obtained from saliva, blood and tissues of OSCC patients, followed by determination of HPV genotypes by sequencing.

Materials and methods

HPV viral strains

The high-risk HPV-type 16 (ATCC® 45113D™) control strain was obtained from the American Type Culture Collection (ATCC, USA) and was used as a positive control in this study.

Patients

A total of 135 samples comprising 13 saliva, 59 bloods and 63 tissues of OSCC patients. The samples (saliva, n=8; blood, n=7; tissue, n=7) were collected from the patients admitted to Hospital Universiti Sains Malaysia (Hospital USM) from 2015 to 2017. The rest of the samples (saliva, n=5; blood, n=52; tissue, n=56) were archived samples obtained from the Oral Cancer Research & Coordinating Centre (OCRCC), Faculty of Dentistry, Universiti

Malaya (UM). Only partial set of samples was available for some of the patient as a complete set (saliva, tissue, blood) was not possible due to limited availability of certain type of specimen.

Sample collection

Ethical approval was obtained from the Research Ethics Committee (Human), USM [USM/JEPEM/15020050] and Medical Ethics Committee, Faculty of Dentistry, University Malaya (UM) [OI DF1601/0072(P)]. A written consent was obtained from all participants prior to their participation.

Saliva samples

Prior to saliva collection, the patient's oral cavity was thoroughly rinsed with water to reduce contamination. After that, about ten ml of fresh saliva sample was collected in a sterile saliva collection container, kept on ice and immediately sent to a laboratory for further analysis.

Blood samples

Three ml of OSCC patients' blood withdrawn by clinician OSCC and 10 healthy saliva samples was kept in EDTA vacutainer tube and processed accordingly. The processed blood samples were stored at -80°C for further analysis.

Formalin-fixed tissue samples

A series of tissue samples were collected from OSCC patients who attended Hospital USM and collaborating centres of OCRCC, UM. The tissue samples were placed in 10% neutral buffer formalin (NBF) and kept at 4°C for further examination.

Formalin-fixed paraffin-embedded (FFPE) tissue samples

The FFPE samples were obtained from the Oral Pathology Laboratory, School of Dental Sciences, USM. The specimens were fixed in formalin and embedded in paraffin, followed by sectioning using microtome. Eight sections of 8 µm thick sample were subjected to DNA extraction for further HPV DNA detection.

DNA extraction

All clinical specimens obtained from the patients in Hospital USM were subjected to

DNA extraction using a commercial DNA mini kit (Qiagen, Germany) according to the manufacturer's protocol. The purity (A260/A280 ratio) and concentration of DNA samples were then determined using a spectrophotometer (Eppendorf, USA) and stored at -20°C for further analysis.

Beta-globin PCR

All DNA extracted were tested for the presence of β -globin with GH20/GH21 primer (Table 1) as described in a previous study (Al-Shabanah *et al.*, 2013) to check the quality, integrity and to test for the absence/presence of PCR inhibitors in the samples. β -globin amplification was carried out in a 20 μ l reaction mixture consisting of 2 μ l DNA template, 20 pmol primer, 10x PCR Buffer, 2.5 U *Taq* DNA polymerase, 25 mM MgCl₂ and 0.2 mM dNTPs. The amplification of β -globin gene was carried out as follows: 94°C for 4 min, 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. This was followed by a final extension of 72°C for 10 min and then storage at 4°C. A sample was considered positive if the PCR amplified a band with the expected size (408 bp). The positive samples were then subjected to HPV detection by nested PCR. DNA samples that were negative for β -globin amplification were re-extracted and PCR amplification was repeated.

Detection of HPV by nested PCR assay

The detection of HPV in the clinical specimens was made using a standard nested PCR with MY11/09 and GP5+/6+ primers (Table 1) as described previously (Erhart *et al.*, 2016). The combination of these primers that amplify L1 region of viral genome were frequently used for clinical and histological HPV detection. MY11/09 and GP5+/6+ primers have been widely used in several HPV detection studies (Moussavou-Boundzanga *et al.*, 2017; Shaikh *et al.*, 2017).

DNA amplification was carried out in the same way as described above with 20 μ l reaction mixture consisting of 2 μ l DNA template, MY11/09 or GP5+/6+ primers (20 pmol each), 10x PCR Buffer, 2.5 U *Taq* DNA polymerase, 25 mM MgCl₂ and 0.2 mM dNTPs. Primary amplification was carried out using MY11/09 primer set (20

pmol each) with an expected size of about 450 bp using a thermal cycler machine (Applied Biosystems, USA) according to the following conditions: 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. This was followed by a final extension of 72°C for 5 min and then storage at 4°C.

In the second round of PCR, the reaction mixture was similar as the primary PCR. Two μ l of the primary PCR products were amplified using GP5+/6+ primer set which resulted in a ~140 bp product. The amplification was performed using the following conditions: 95°C for 5 min, 40 cycles of 94°C for 1 min, 40°C for 2 min and 72°C for 1 min and a final extension of 72°C for 5 min followed by 4°C storage. The PCR products were electrophoresed on 1.5% agarose gel in 0.5X TBE buffer with FloroSafe DNA stain (Axon Scientific Sdn Bhd, Malaysia) at 80 V for 50 minutes. The DNA bands were then visualized under ultraviolet light illumination using an image analyser (Bio-Rad, USA).

DNA sequencing

The positive PCR products were submitted to First Base Laboratories Sdn. Bhd. (Malaysia) for sequencing using BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems, USA). The sequences were compared with HPV genomes database that are available in the NCBI-GenBank using the BLAST program (NCBI, USA).

Statistical analysis

The data were analysed using IBM Statistical Product and Service Solutions (SPSS) version 24. The determination of the presence of high-risk HPV in each OSCC samples was analysed using Chi-square (χ^2) test. The *p* value of <0.05 was considered as statistically significant.

Results

Beta-globin PCR

A total of 135 extracted DNA from saliva, blood and tissue were tested in a PCR with β -globin primer to detect the presence of DNA prior to HPV detection (Fig. 1). β -globin DNA was successfully detected in all samples.

Detection of HPV in saliva, blood and tissue specimens by PCR

Three out of 135 (2.2%) samples were found positive with consensus primer MY11/09 and GP5+/6+ and were the HPV genotypes for all positive samples were confirmed through DNA sequencing. Out of three positive samples, two saliva samples (15.4%) were found to harbour HPV 16 (Fig. 2) and one tissue sample (1.6%) was shown to be positive for HPV 18 (Fig. 3). However, none of the blood samples were positive for high-risk HPV.

Identification of HPV genotype by DNA sequencing

The sequencing results of the positive high-risk HPV were analysed using BLAST analysis from NCBI website in order to identify the genotype. Both saliva samples showed 100% similarities to HPV type 16. Meanwhile, tissue sample showed 100% similar identity with HPV type 18. The representative hits from BLAST analysis are shown in Table 2.

Statistical analysis

High-risk HPV was detected in 2 out of 13 saliva samples (15.4%) and 1 out of 63 tissue samples (1.6%). Chi-square (χ^2) analysis showed the HPV positivity in both samples were statistically significant (Table 3).

Table 1 List of primers used in this study

Primers	5' → 3' sequence	Size (bp)	References
First step outer primer			
MY11	GCA CAG GGA CAT AAC AAT GG	~ 450	(Shikova <i>et al.</i> , 2009)
MY09	CGT CCA AAA GGA AAC TGA GC		
Second step inner primer			
GP5+	TTT GTT ACT GTG GTA GAT ACT AC	~ 140	
GP6+	GAA AAA TAA ACT GTA AAT CAT ATT C		
Human β-globin gene			
GH20	GAA GAG CCA AGG ACA GGT AC	~ 408	(Al-Shabanah <i>et al.</i> , 2013)
GH21	CAA CTT CAT CCA CGT TCA CC		

Table 2 BLAST analysis of high-risk HPV genotypes

Sample No.	Homology (%)	Genotype	GenBank accession no.
Saliva 1	100	Human papillomavirus type 16 L1 (L1) gene, partial cds	MF134647.1
Saliva 2	100	Human papillomavirus type 16 L1 (L1) gene, partial cds	MF134647.1
Tissue 1	100	Human papillomavirus type 18 isolate CC108 L1 protein (L1) gene, partial cds	MF066886.1

Table 3 Determination of the presence of HPV in OSCC samples

OSCC samples	HPV positive (%)	χ^2	df	p value
Saliva	2 (15.4%)	6.231 ^a	1	0.013*
Tissue	1 (1.6%)	59.063 ^a	1	0.000*

^a 0 cells (0%) have expected frequencies less than 5.

* Significant level was set at 0.05.

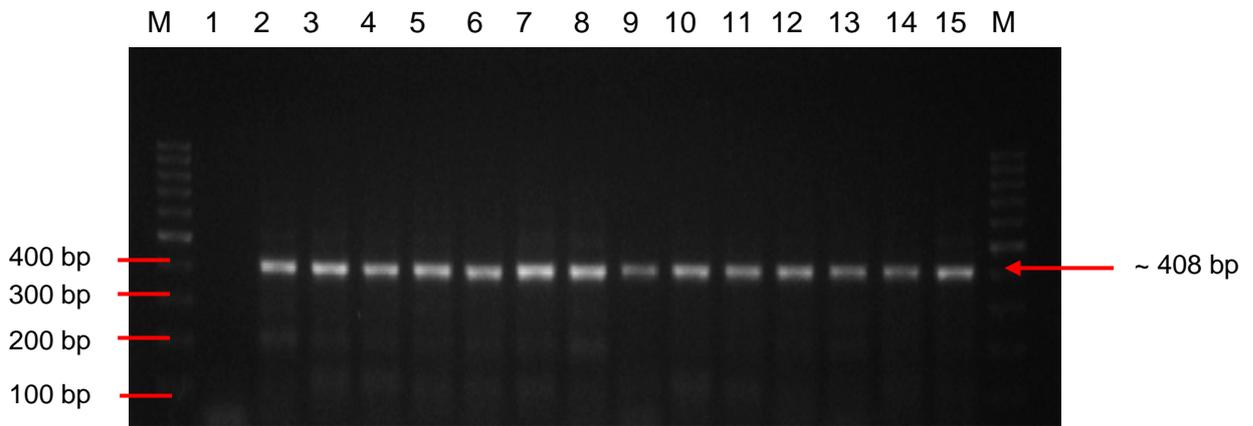


Fig. 1 Electrophoretic gel analysis resolved on 1.5% agarose gel for human β -globin amplification. M; 100 bp ladder (Axon Scientific), 1 to 15; DNA samples.

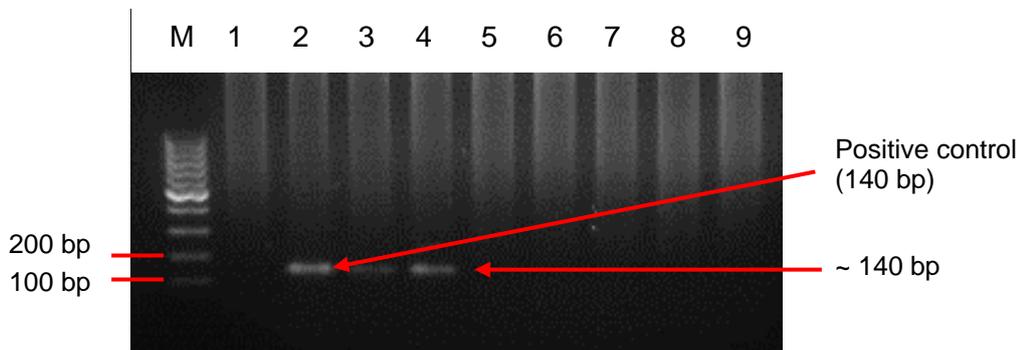


Fig. 2 Electrophoretic gel analysis resolved on 1.5% agarose gel for detection of high-risk HPV in saliva samples by nested PCR. M; 100 bp ladder, 1; negative control, 2; positive control (HPV 16), 3 to 9; DNA samples.

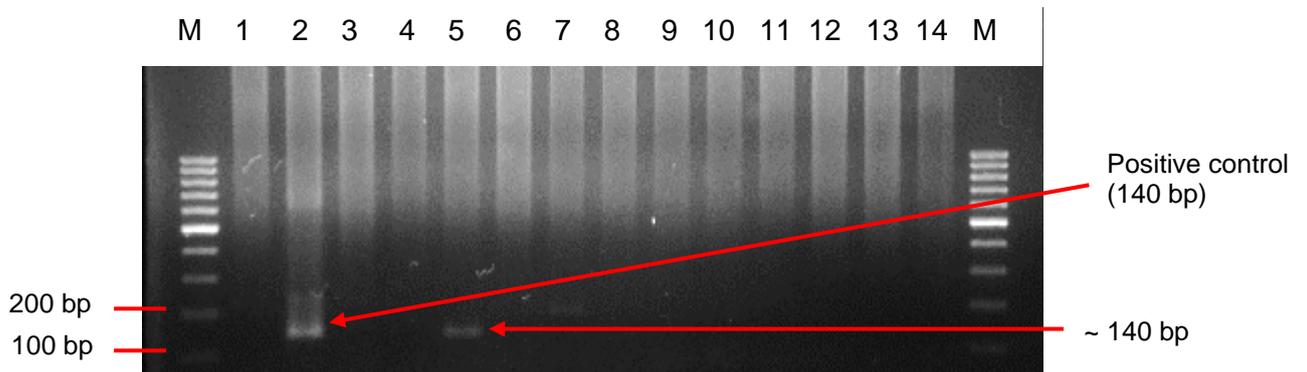


Fig. 3 Electrophoretic gel analysis resolved on 1.5% agarose gel for detection of high-risk HPV in tissue samples by nested PCR. M; 100 bp ladder, 1; negative control, 2; positive control (HPV 16), 3 to 14; DNA samples.

Discussion

In the present study, the presence of high-risk HPV in different genetic materials obtained from saliva, blood and tissues of OSCC patients was compared using nested PCR. HPV detection and typing by nested PCR with consensus primer is a simple yet sensitive method. The consensus PCR primers used in nested PCR are developed specifically to target and amplify the highly conserved region in HPV genome and it is potentially capable to detect a broad spectrum of HPV types (Asadi-Amoli *et al.*, 2011). The combination of these primers has been extensively used in epidemiological studies and have contributed to an effective detection of HPV in clinical samples. Previous studies have demonstrated that the use of MY11/09 and GP5+/6+ primer pairs increases the sensitivity and rate of HPV detection and hence promises better results in nested PCR assay when compared to a single PCR assay (Cosser *et al.*, 2011; Jalouli *et al.*, 2015; Erhart *et al.*, 2016). The highly sensitive nature of nested PCR has enabled the detection of low level HPV DNA in easily degraded samples such as formalin-fixed tissue and less-preserved specimens (Kristoffersen *et al.*, 2012).

The prevalence of high-risk HPV-related OSCC in this study varied based on the different sources of biological materials. In the present study, the detection rate of HPV in saliva was 15.4%. This result was in accordance with other studies that reported the prevalence of HPV 16 in saliva of OSCC patients was approximately 19.2% (Kaminagakura *et al.*, 2012). A systematic review on the prevalence of oral HPV infection in about 9,541 oropharyngeal squamous cell carcinoma patients from different countries and continents reported that HPV has higher detectable rate in the saliva of OSCC patients when compared to blood and tissue samples (Stein *et al.*, 2015).

The prevalence of HPV infection contributed to the increasing trends of HPV-positive OSCCs. HPV transmission to the oral cavity could be due to sexual activities (oral sex or open-mouthed kissing) or poor oral hygiene (Cook *et al.*, 2014; Dahlstrom *et al.*, 2014). Consequently, the oral mucosa is constantly exposed to infections and trauma which make it susceptible for HPV to enter

the basal cells and replicate. Once HPV infects the host tissue, its genome is integrated into the host genome. The delivery of viral DNA into host cells is further regulated by the early gene proteins (E6 and E7). E6 protein leads to degradation of p53 tumor suppressor protein, thereby inhibiting apoptosis, while E7 protein that disturbs the pRb tumor suppressor gene which leads to the increase of DNA synthesis (Chocolatewala and Chaturvedi, 2009; Prabhu and Wilson, 2013).

The ability of saliva to serve as a main mode of virus transmission from individuals with high infectious dose could be one possible reason to this finding (Montaldo *et al.*, 2007). The detectable state of HPV DNA in saliva results either from the cells that are actively producing viral particles or when the viruses are released back to saliva once the infected cells die (Wasserman *et al.*, 2017). Saliva has serum components that could be utilized as a diagnostic tool to detect and monitor the interested biomarkers especially among the low-cooperative patients; furthermore it is easy to collect in a non-invasive and inexpensive way (Chiappin *et al.*, 2007). A high throughput of HPV DNA in saliva was also seen in other studies that finally concluded that salivary rinse has a potential to be developed as a molecular screening for HPV-related head and neck squamous cell carcinoma (Zhao *et al.*, 2005; Wasserman *et al.*, 2017).

Similar to the findings of a study by Albano *et al.* (2017), we also found a very low prevalence of HPV in OSCC tissue which was only 1.6%. A low prevalence of HPV in tissue could also be affected by overall DNA concentration, pH of the solution, temperature and duration and type of tissue storage (Noori *et al.*, 2012). These factors may lead to a poor tissue stability and quality for further HPV testing. On the other hand, blood circulation is one of the possible routes of HPV transmission to the site of cancer. However in this study, no HPV DNA was detected in the blood of OSCC patients. An absence of high-risk HPV in OSCC was also found in a Chinese study which reported zero positivity of HPV 16 and 18 in serum samples and concluded that HPV infection is uncommon within their population (Chen *et al.*, 2016). Nevertheless, several studies have found a high prevalence of high-risk

HPV in the plasma of OSCC patients which suggested that the plasma evaluation is comparable to that of the saliva (Wang *et al.*, 2015).

Oral HPV prevalence obtained in this study was comparable to the results of other published studies. Similar to previous studies (Parshad *et al.*, 2015; Awan *et al.*, 2017), the overall detection rate of HPV showed that HPV 16 is the most detectable subtype, followed by HPV 18. Consistent with the previous studies, HPV 16 was the predominant type found among Malaysian OSCC patients (Saini *et al.*, 2011) and also the OSCC patients worldwide (Kreimer *et al.*, 2005; Gillison *et al.*, 2012). Meanwhile, HPV 18 is the second most common oncogenic subtype found in the literature (Ahmed *et al.*, 2012). These two high-risk subtypes of HPV are responsible for most HPV-associated cancer. The predominance of HPV 16 can be attributed to the remarkably high rate of persistent infection when compared to HPV 18.

The prevalence of HPV 16 and HPV 18 would be more appropriate if all types of samples (saliva, blood, formalin-fixed tissue and FFPE) were collected from each patient for better comparison. However, in this study, we were unable to collect all four types of samples in some critically ill patients.

Conclusion

HPV is more likely to be detected in the saliva of OSCC patients in Malaysia when compared to blood and tissue samples and HPV 16 was the most predominant subtype found.

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Conflict of interest

The authors deny any conflict of interest related to this study.

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