

## RESEARCH ARTICLE

# Human Papilloma Virus 18 Detection in Oral Squamous Cell Carcinoma and Potentially Malignant Lesions Using Saliva Samples

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### Abstract

**Background:** Oral cancer has become one of the most prevalent cancers worldwide and human Papillomavirus is one of the risk factors for developing oral cancer. For this study HPV18 was chosen as it is one of the high risk HPV types and may lead to carcinogenesis. However, prevalence of HPV18 infection in Oral Squamous Cell Carcinoma in Malaysia remains unclear. **Objective:** This study aimed to investigate the viral load of HPV18 DNA in OSCC and potentially malignant lesions using saliva samples. **Materials and Methods:** Genomic DNAs of thirty saliva samples of normal subjects and thirty saliva samples comprised of 16 samples from potentially malignant lesions and 14 of OSCC patients were amplified for HPV18 DNA using a nested polymerase chain reaction analysis. All PCR products were then analyzed using the Bioanalyzer to confirm presence of HPV18 DNA. **Result:** From thirty patients examined, only one of 30 (3.3%) cases was found to be positive for HPV18 in this study. **Conclusion:** The finding of this study revealed that there is a low viral detection of HPV18 in Malaysian OSCC by using saliva samples, suggesting that prevalence of HPV18 may not be important in this group of Malaysian OSCC.

**Keywords:** HPV 18 - oral squamous cell carcinoma - nested PCR - Malaysia

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### Introduction

Oral cancer holds the eighth position in the cancer incidence ranking worldwide and is the 11<sup>th</sup> most common form of cancer in Malaysia (Lim et al., 2008; Moore et al., 2009). Of all head and neck cancers, Oral Squamous Cell Carcinoma (OSCC) is the most common malignant epithelial neoplasm of oral cavity (90%); they represent approximately 5% in men and 2% in women considering all malignancies (Silverman, 1998; Robert, 2000). Several potentially malignant lesions that are linked to oral cancer include leukoplakia, lichen planus, and erythroplakia (Neville and Day, 2002; Dissemmond, 2004; Reichart and Philipsen, 2005).

In Malaysia, incidence of oral cancer varies between gender and ethnic groups (MNCR, 2003); for males it is the sixth while for females it is the third most common cancer (MNCR, 2003). OSCC is common among Malaysian Indians especially in the lower socio-economical groups

mainly because of their habit of chewing betel quid. As for the Malays and Chinese, smoking and alcohol consumption are the major risk factors (Tan et al., 2000; Zain, 2001).

A well-established risk factor of OSCC is tobacco use, including pipes, cigars, cigarettes, and chewing tobacco. Meanwhile betel quid chewing is a common habit widespread in some parts of the world. Another established risk factor for causing OSCC is alcohol consumption, especially when combined with cigarette smoking it has been proven to be a strong risk factor as both factors interact synergistically (Joao et al., 2006; Zygogiann et al., 2011). Human Papillomavirus (HPV) infections on the other hand, may present another factor for causing oral cancer. OSCC was found to be significantly associated with oral HPV infection among patients both with and without a history of heavy tobacco and alcohol use (Munoz et al., 2003; Pintos et al., 2006; D'Souza et al., 2007; Ragin et al., 2007). Recently, more than 100

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HPV types have been classified into low and high risk groups according to their viral expression in malignancies. HPV16 and 18 are categorized into the high-risk group as they are associated with several malignancies including cervical and oral cancers (Gichki, 2012).

With regards to squamous cell carcinomas, HPV is implicated as a causative agent in anogenital tract, skin, conjunctiva, larynx, tracheobronchial mucosa, esophagus and oral cavity SCCs (Fuju 1991; An et al., 2003). The HPV gene contains regions of early open reading frame genes (E6 and E7) exhibiting their oncogenic effect by disrupting the function of tumor suppressor genes (pRb and p53) and therefore allowing the virus to overcome regulatory mechanism preventing excessive cell growth. As a result, it causes defective cell apoptosis, DNA repair and cell cycle control (Duensing and Münger, 2004; Ragin et al., 2007). Molecular studies suggested that HPV infection may be associated with the oral cancer (Munoz et al., 2003; Zoltan et al., 2005; Saini et al., 2010). At present, there have been very scarce studies looking at HPV18 infection in OSCC and potentially malignant lesions in Malaysia. Thus, our work targeted the viral load of HPV18 in Malaysian OSCC and potentially malignant lesions by using saliva samples, to investigate its putative role in oral carcinogenesis.

## Materials and Methods

### Sample population

Thirty normal subjects without any oral and systemic diseases were recruited from Faculty of Dentistry, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor. They practiced a healthy life styles which includes no habits of smoking, quid chewing and alcohol consumption. They also had no history of head and neck cancer. Another 30 subjects consisted of 16 patients who have potentially malignant lesions and 14 OSCC patients were recruited from Oral Cancer Research and Coordinating Centre (OCRCC), University Malaya. They were selected after obtaining an informed consent. Ethics approvals were obtained from the Ethics Committee of UiTM and Faculty of Dentistry, University of Malaya. Histopathology of samples was confirmed by an oral pathologist, according to WHO-TNM classification of oral cavity carcinoma (Pindborg, 1997). All selected OSCC patients were new cases and have not previously undergone any anticancer treatment.

### Sample preparation

Saliva samples from normal subjects and patients were collected and transported immediately to the laboratory for processing. DNA extraction was performed by using the Saliva DNA Isolation Kit (Norgen, Canada) as described by the manufacturer. The purity (A260/A280 ratio) and concentration of DNA samples were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The DNA products were stored at  $-20^{\circ}\text{C}$  until further use.

### Nested polymerase chain reaction protocol

Nested PCR with specific primers (MYII/MY09 and

**Table 1. Primers Used in HPV DNA PCR Amplification with the Primer Sequences and PCR Products Length (bp)**

Primer name	Primer Sequence	Products Length (bp)
MY09	5' CGT CCM ARR GGA WA C TGA TC 3'	450
MY 11	5' GCM CAG GGW CAT AAY AAT GG 3'	
GP5+	5' TTT GTT ACT GTG GTA GAT ACY AC 3'	140
GP6+	5' GAA AAA TAA ACT GTA AAT CAT ATT C 3'	140
$\beta$ -globin Forward primer	5' GAA GAG CCA AGG ACA GGT AC 3'	250
Reverse primer	5' CAA CTT CAT CCA CGT TCA CC 3'	

GP5/GP6) for L1 region of the HPV genome were used (Table 1). The  $\beta$ -globin gene was used as an internal control and a HPV18 positive clone as positive control were concurrently run with each test. PCR was performed using MYII/MY09 primer in a total volume of 20  $\mu\text{l}$  containing 100 ng of DNA sample and 1X Master Mix (Qiagen Taq Master Mix kit, Germany). Amplification was carried out in Mastercycler gradient (Eppendorf, Germany) with an initial denaturation step at  $95^{\circ}\text{C}$  for 3 minutes, followed by 45 cycles each at  $95^{\circ}\text{C}$  for 30 seconds, annealing at  $53^{\circ}\text{C}$  for 30 seconds and extension at  $72^{\circ}\text{C}$  for 30 seconds. The final extension was at  $72^{\circ}\text{C}$  for 7 minutes and 10  $^{\circ}\text{C}$  for holding time. Then a second PCR protocol was carried out using GP5/GP6 primer that will produce an amplicon size of 140 bp. The annealing temperature was at  $42.3^{\circ}\text{C}$ . The PCR products were detected using Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

### Statistical analysis

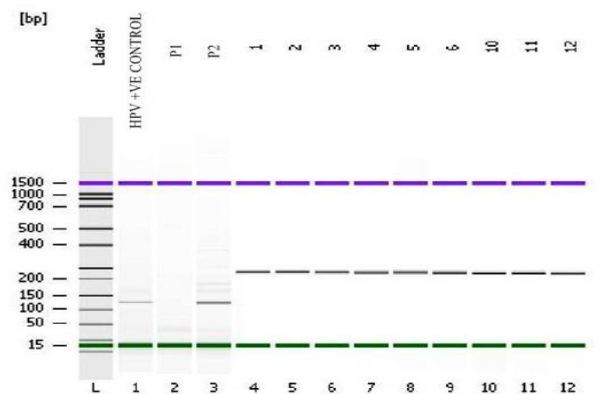
Statistical analysis was performed using the SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Chi-square or Fisher Exact tests were employed to determine an association between the patients' demographic profiles (age range, gender, ethnicity, and habits) and pathological diagnosis with HPV18 detection in OSCC. A p value of less than 0.05 was considered statistically significant.

## Results

Saliva samples were collected from 30 normal subjects, 9 males and 21 females aged between 20-59 years old, and 30 saliva samples comprising of 16 potentially malignant lesions and 14 OSCC patients (11 males and 19 females aged between 42-95 years old). Patients' details are shown in Table 1. The range of saliva' DNA concentration ranged from 4.66-39.53  $\text{ng}/\mu\text{l}$  and the purity of DNA ranged from 1.66-2.22.

Nested PCR was performed on 60 DNA samples of normal subjects and patients with  $\beta$  globin primers as an internal control (Figure 1).  $\beta$  globin primer was applied in the PCR to show the DNA integrity of the samples.

Only one out of 30 (3.33%) patients' saliva samples was positive for the HPV18. This HPV18 positive sample was from a 61 year old Indian female patient with hyperkerotosis mild dysplasia with drinking and chewing habits. Furthermore, none of the normal subjects was positive for HPV18.



**Figure 1. Representative PCR Products in Bioanalyzer Graph.** Lane L show ladder marker. Lane 1 shows band the positive control of HPV18 clone positive (140 bp). Lane 2 shows sample P1 with negative in HPV18 detection. Lane 3 shows sample P2 with positive for HPV18 (140 bp). Lane 4-12 shows  $\beta$  globin PCR products with good DNA integrity (250bp)

**Table 2. Demographic Profile of Normal Subjects and Patients with OSCC and Potentially Malignant Lesions**

		Normal subjects (Frequencies)		Patients		P value
		n	%	n	%	
Age range	20-39	26	86.7	0	0	P=0.508
	40-59	4	13.3	11	36.70	
	60-79	0	0	18	60.00	
	$\geq 80$	0	0	1	3.30	
Gender	Male	9	30.0	11	38.00	P=0.633
	Female	21	70.0	19	62.00	
Ethnicity	Malay	29	96.7	8	27.59	P=0.762
	Chinese	1	3.3	3	10.34	
	Indians	0	0	19	62.07	
Habits	No Habit	30	100	11	34.50	P=0.775
	Smoking	-	-	6	20.70	
	Drinking	-	-	0	0	
	chewing	-	-	6	20.70	
	Drinking and Betel quid Chewing	-	-	4	13.80	
	Smoking and Drinking	-	-	1	3.40	
	Smoking, Drinking and Betel quid chewing	-	-	2	6.90	
Pathological Diagnosis	Potentially malignant lesions	-	-	16	53.30	P=1.000
	OSCC	-	-	14	46.70	
		-	-	0	0	

\* There was no statistical significance of association between the HPV18 with demographic profile of patients' age range (P=0.508), gender (P=0.633), ethnicity (P=0.762), habits (P=0.775) and pathological diagnosis (P=1.000) using Chi-square or Fisher Exact tests of SPSS version 17.0 (p value >0.05)

The demographic profile and pathological diagnosis of the patients are summarized in Table 2. The demographic profile included in this study is age range, gender, ethnicity, and habits. The ethnicity was included in this study because Malaysia is a multiethnic country. In view of the age range; 60% of the patients are between 60-79 years old, 36.7% are between 40-59 years old and 3.3% age 80 and above. The gender and ethnicity categories for the patients consisted of 11 males and 19 females. In the male patient category are four Malays, two Chinese and five Indians, while in the female category are four Malays, one Chinese and 14 Indians. Most of the patients (65.5%) have habits of alcohol drinking, tobacco smoking and/or betel

quid chewing while only 34.5% of the patients have none of the above mentioned habits. In the group of patients with risk factor habits, 20% are smoking, 20% are chewing betel quid, 13.3% are drinking alcohol and chewing betel quid, 3.3% are smoking and drinking and 6.7% have all three habits (smoking, drinking and chewing). There was no statistical significance of association between the HPV18 with demographic profile of patients' age (P=0.508), gender (P=0.633), ethnicity (P=0.762), habits (P=0.775), and pathological diagnosis (P=1.000) using Chi-square or Fisher Exact tests of SPSS version 17.0 (p value >0.05).

## Discussion

In our findings, the HPV18 detection was one out of 30 cases of OSCC and potentially malignant lesions using saliva samples. In this study we couldn't detect HPV18 in any of the 14 OSCC patients; however one out of 16 potentially malignant lesions was positive for HPV18 DNA. According to Khovidhunkit et al. (2009) study, 1.54% of Thai OSCC patient and premalignant lesions of DNA-derived fresh or formalin-fixed paraffin embedded tissues was positive for HPV, which is similar to our study. A similar result was also reported by Saini et al. (2010) who showed a low percentage (5.71%) of women with cervical cancer to be positive for HPV in patients' oral cavity. However, study conducted by Kulkarni et al. (2011) found that 54.2% patients to be positive of HPV18 and 4.18% of HPV16 and 18 in multiple infections of OSCC in India (Kulkarni et al., 2011). It is difficult to compare the various published data on HPV with regards to oral cancers because of variations in parameters, such as type of sample (DNA extracted by biopsy tissues, scraping or saliva, preparation method (fresh, frozen or fixed), sensitivity of the methods used, ethnic differences between patients, status of the disease, and geographic regional differences (Iftner et al., 2003; Esquenazi et al., 2010). The saliva samples were used in our study mainly based on its non-invasive sample collection technique and as a diagnostic fluid with high sensitivity and specificity of detection (Zhao et al., 2004; Spielmann and Wong, 2011).

The low prevalence of HPV18 in patients in the present study is unlikely due to poor DNA quality, since all of the samples were shown to have good DNA integrity for  $\beta$ -globin. Moreover, all PCR products of positive controls were amplified during the PCR process (Khovidhunkit et al., 2009). Therefore, the low viral load of HPV18 observed in this study is unlikely due to experimental problems.

In this study, the 61 years old female Indian patient with HPV18 positivity has been diagnosed as hyperkeratosis with mild dysplasia associated with drinking and chewing habits. According to the meta-analysis study done by Bagnardi et al. (2001) on alcohol drinking and cancer risk, alcohol can be one of the risk factors for head and neck cancer. Another study done by Avon (2004) stated that betel quid chewing is one of the risk factor for oral leukoplakia, oral submucous fibrosis, and also OSCC. This study suggested that risk factors like alcohol drinking, betel quid chewing and HPV18 infection may

have a synergistic effect in increasing the risk of creating potentially malignant lesions.

In view of the demographic profile of the patients, 36.7% of them with age between 40-59 years old and 60% of patient's ages between 60-79 years old. These results showed that the risk of developing oral cancer increases with age, and may not be associated with betel quid chewing, alcohol consumption or smoking habits (Munoz et al., 2003). However, majority of patients in our study had history of alcohol consumption, tobacco smoking and betel quid chewing. Therefore, infection of HPV may not play an important role in this group of patients. In the ethnic category, Indians patients (66.7%) occupied the largest group followed by Malay (23.3%) and Chinese (10.0%). These results were in concordance with Zain (2001) study that identified Indians as one of the ethnic groups with high risk of developing oral cancer.

This study was unable to provide support for the suggested association between viral detection of HPV18 with patients' demographic profile and pathological diagnosis of potentially malignant lesions and OSCC (p value>0.05). Moreover, various studies have investigated the role of HPV in OSCC but the results are highly controversial (Khovidhunkit et al., 2009; Saheb et al., 2009; Saini et al., 2010; Kulkarni et al., 2011). In addition, analysis of viral genome has revealed the 2 early genes, E6 and E7, of high risk HPV16 and 18 are transforming genes that are responsible for maintenance of carcinogenic phenotype (Duensing et al., 2000), are associated with anogenital and oral invasive tumour (Riley et al., 2003). Moreover, a research team reported the probable correlation between p53 polymorphism and HPV-associated carcinogenesis (Dokianakis et al., 2000), where genetic polymorphism of the genes involved in carcinogenesis may determine individual susceptibility to cancer.

In conclusion, in the present study, HPV18 DNA was detected at a low percentage (3.33%) of OSCC and potentially malignant cases in Malaysia, suggesting that HPV18 infection may not play a role in the development and progression in this group of OSCC. Since our study was unable to prove any correlation between HPV18 and OSCC in a relatively small sample size, more samples should be recruited for future prospective studies and to confirm the feasibility of early HPV18 detection using quantitative real-time PCR. In addition most reports have shown that HPV16 is one of the most prevalent types in both oral and genital tumors, thus we propose to further investigate the HPV16 prevalence in Malaysia.

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