RESEARCH ARTICLE

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

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Abstract

    <u>Background</u>: 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. <u>Objective</u>: This study aimed to investigate the viral load of HPV18 DNA in OSCC and potentially malignant lesions using saliva samples. <u>Materials and Methods</u>: Genomic DNAs of thirty saliva samples of normal subjects and thirty saliva samples compromised 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. <u>Result</u>: From thirty patients examined, only one of 30 (3.3%) cases was found to be positive for HPV18 in this study. <u>Conclusion</u>: 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

Introduction

    Oral cancer holds the eighth position in the cancer incidence ranking worldwide and is the 11th 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; Dissemond, 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°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'	
GP6+		5' GAA AAA TAA ACT GTA AAT CAT ATT C 3'	140
β-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 β-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 µl 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 95ºC for 3 minutes, followed by 45 cycles each at 95ºC for 30 seconds, annealing at 53ºC for 30 seconds and extension at 72ºC for 30 seconds. The final extension was at 72ºC for 7 minutes and 10 ºC 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 42.3ºC. 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 ng/µl 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 β globin primers as an internal control (Figure 1). β 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.
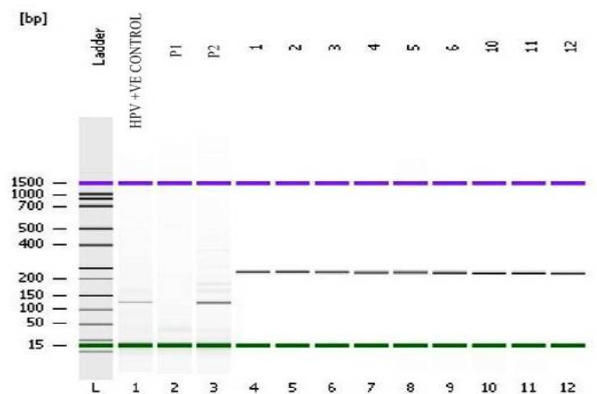
Figure 1. Representative PCR Products in Bioanalyzer Graph. Lane L show ladder marker. Lane 1 shows band the positive control of HPV18 clone positive (140 bp). Lane 2 shows sample P1 with negative in HPV18 detection. Lane 3 shows sample P2 with positive for HPV18 (140 bp). Lane 4-12 shows β globin PCR 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		Patients with HPV18	P value
		n	%	n	%	n	
Age range	20-39	26	86.7	0	0	0	
	40-59	4	13.3	11	36.70	0	
	60-79	0	0	18	60.00	1	
	≥80	0	0	1	3.30	0	P=0.508
Gender	Male	9	30.0	11	38.00	0	
	Female	21	70.0	19	62.00	1	P=0.633
Ethnicity	Malay	29	96.7	8	27.59	0	
	Chinese	1	3.3	3	10.34	0	
	Indians	0	0	19	62.07	1	P=0.762
Habits	No Habit	30	100	11	34.50	1	
	Smoking	-	-	6	20.70	0	
	Drinking	-	-	0	0	0	
	chewing	-	-	6	20.70	0	
	Drinking and Betel quid Chewing	-	-	4	13.80	0	
	Smoking and Drinking	-	-	1	3.40	0	
	Smoking, Drinking and Betel quid chewing	-	-	2	6.90	0	P=0.775
Pathological Diagnosis	Potentially malignant lesions	-	-	16	53.30	3.33	
	OSCC	-	-	14	46.70	0	P=1.000

* 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 β-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 hyperkerotosis 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.

Acknowledgements

References

An HJ, Cho NH, Lee SY, et al (2003). Correlation of cervical carcinoma and precancerous lesions with human papillomavirus (HPV) genotypes detected with the HPV DNA chip microarray method. *Cancer*, **97**, 1672-80.

Avon SL (2004). Oral mucosal lesions associatedwith use of quid. *J Can Dent Assoc*, **70**, 244-8

Badaracco G, Venuti A, Bartolazzi A, et al (2000). Overexpression of p53 and bcl-2 proteins and the presence of HPV infection are independent events in head and neck cancer. *J Oral Pathol Med*, **29**, 173-9.

Bouda M, Gorgoulis VG, Kastrinakis NG, et al (2000). High risk HPV types are frequently detected in potentially malignant and malignant oral lesions, but not in normal. *Oral Mucosa Mod Pathol*, **13**, 644-53.

Dissemond J (2004). Oral lichen planus: an overview. *J Dermatol Treat*, **15**, 136-40.

Dokianakis DN, Spandidos DA (2000). P53 codon 72 polymorphism as a risk factor in the developmentof HPV-associated cervical cancer. *Mol Cell Biol Res Commun*, **3**, 111-4.

D'Souza G, Kreimer AR, Viscidi R, et al (2007). Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med*, **356**, 1944-56.

Duensing S, Lee LY, Duensing A, et al (2000). The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *PNAS*, **97**, 10002-7.

Duensing S, Münger K (2004). Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins. *Int J Cancer*, **109**, 157-62.

Esquenazi D, Bussoloti FI, Carvalho MG, et al (2010). The frequency of human papillomavirus findings in normal oral mucosa of healthy people by PCR. *Braz J Otorhinolaryngol*, **76**, 78-84.

Fuju C, Stina S, Jari K, et al (1991). Human papillomavirus (HPV) infections and their associations with oral disease. *J Oral Pathol and Med*, **20**, 305-17.

Gichki AS, Buajeeb W, Doungudomdacha S, et al (2012). Detection of human papillomavirus in normal oral cavity in a group of pakistani subjects using real-time PCR. *Asian Pac J Cancer Prev*, **13**, 2299-304.

Iftner T, Villa LL (2003). Human papillomavirus technologies. *J Natl Cancer Inst Monogr*, **31**, 80-8

Joao M, Frederico SR, Gustavo J, et al (2006). Oral squamous cell carcinoma: Review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **102**, 67-76.

Khovidhunkit SP, Buajeeb W, Sanguansin S, et al (2009). Detection of human papillomavirus in oral squamous cell carcinoma, leukoplakia and lichen planus in Thai patients. *Asean Pac J Cancer Prev*, **9**, 771-5.

Kulkarni SS, Kulkarn SS, Vastrad PP, et al (2011). Prevalence and distribution of high risk human papillomavirus (HPV) types 16 and 18 in carcinoma of cervix, saliva of patients with oral squamous cell carcinoma and in the general population in Karnataka, India. *Asian Pac J Cancer Prev*, **12**, 645-8.

Lim GCC, Rampai S, Yahaya H (eds) (2008). Cancer Incidence in Peninsular Malaysia (2003-2005). The Third Report of the National Cancer Registry of Malaysia. National Cancer

Registry, Ministry of Health Malaysia, 1-179.

Malaysian National Cancer Registry (MNCR) (2003). Cancer statistics. Ministry of Health Malaysia.

Moore MA, Ariyaratne Y, Badar F, et al (2009). Cancer epidemiology in South Asia - past, present and future. *Asian Pac J Cancer Prev*, **11**, 49-66.

Munoz N, Bosch FX, de Sanjose S, et al (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*, **348**, 518-27.

Neville BW, Day TA (2002). Oral cancer and precancerous lesions. *Cancer J Clin*, **52**, 195–215.

Pindborg JJ, Reichart PA, Smith CJ, et al (1997). World Health Organization International Histological Classification of Tumours. Histological typing of cancer and precancer of the oral mucosa. 2nd ed. New York: Springer-Verlag, 33-40.

Pintos J, Black MJ, Sadeghi N, et al (2006). Human papillomavirus infection and oral cancer. *Oral Oncol*, **44**, 242-50.

Ragin CC, Modugno F, Gollin SM (2007). The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus. *J Dent Res*, **86**, 104-14.

Reichart PA, Philipsen HP (2005). Oral erythroplakia - a review. *Oral Oncol*, **41**, 551-61.

Riley RR, Duensing S, Brake T, et al (2003). Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Res*, **63**, 4862-71.

Robert A (2000). Types of Oral Cancer in Oral Cancer, the dentist role in diagnosis, management, rehabilitation, and prevention (Robert AO, Remy HB, eds). Quintessence Publishing.

SahebJamee M, Boorghani M, Ghaffari SR, et al (2009). Human papillomavirus in saliva of patients with oral squamous cell carcinoma. *Med Oral Patol Oral Cir Bucal*, **14**, 525-8.

Silverman S Jr (1998). Epidemiology. In: Oral Cancer (Silverman S Jr ed). pp1-6., 4th ed. Hamilton, Ontario, Canada: BC Decker Inc.

Saini R, Tan PK, Sarah AR, et al (2010). High-risk human papillomavirus in the oral cavity of women with cervical cancer, and their children. *Virology J*, **7**, 131-6.

Spielmann N, Wong DT (2011). Saliva: diagnostics and therapeutic perspectives. *Oral Diseases*, **17**, 345-54.

Tan BS, Rosman A, Ng KH, et al (2000). Profile of the betel/tobacco quid chewers in six Malaysian estates. *Annal Dent Univ Malaya*, **7**, 1-5.

Zain RB (2001). Cultural and dietary risk factors of oral cancer and precancer - a brief overview. *Oral oncol*, **37**, 205-10.

Zhao M, Jiang W, Carvalho AL, et al (2004). Feasibility of quantitative PCR-based saliva screening of HPV for head and neck cancer. *Otolaryngology – Head and Neck Surgery*, **131**, 180.

Zoltan S, Karoly P, L´aszlo T, et al (2005). Human papillomavirus in head and neck cancer: Molecular biology and clinicopathological correlations. *Cancer Metast Rev*, **24**, 19-34.

Zygogianni AG, Kyrgias G, Karakitsos P, et al (2011). Oral squamous cell cancer: early detection and the role of alcohol and smoking. *Head and Neck Oncol*, **3**, 1-12.

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