Salivary Human Papilloma Virus Detection in Oral Cavity Cancers in Malaysia: A Multicentre Study

Original Article

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ABSTRACT

Background: A steady rise in incidence of oral cavity cancer cases especially among young males with no history of tobacco smoking or alcohol consumption have given rise to a new risk factor, namely human papillomavirus (HPV).

Objective: This study was conducted to determine prevalence and association of HPV among oral cavity cancer patients and healthy local population. It also aims to identify the association of HPV and risk factors such as smoking, alcohol consumption and betel nut chewing.

Patients and Methods: This case-control study involves a test group (oral cavity cancer patients) and a control group (healthy individuals). HPV status was tested via salivary rinse samples collection and processed using Diacarta Quantivirus® HPV E6/E7 RNA assay. Data collection and salivary sample collection were done from July 2013 till June 2014 involving patients from 3 different institutions.

Results: This study involves 58 subjects, consisting of 29 test subjects and 29 control subjects. HPV prevalence was found to be 55.1% among test subjects and 3.4% among control subjects. This was found to be significant ($p=0.001$) with odds ratio of 33.90 (95% CI 3.88, 295.99). Among the risk factors, smoking habit was seen in 51.2% of test subjects and 13.8% of control subjects. This association was found to be significant ($p=0.041$) with odds ratio of 4.36 (95% CI 1.06, 17.86). Multi-colinearity and interaction term were checked and none found. Alcohol consumption and betel nut chewing were found to be insignificant in this study.

Conclusion: HPV prevalence among oral cavity patients was found to be high and this pivotal result demonstrates HPV infection is now an established risk factor in this country. Smoking habit was also found to be a significant risk factor among these patients and did not interact or confound the factor of HPV infection.

Key Words: Human papilloma virus, Oral cavity cancer.

Received: 26 June 2018, **Accepted:** 5 July 2020

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ISSN: 2090-0740, 2024

INTRODUCTION

Oral cavity cancers accounts to 2.64% of all cases worldwide in the year 2000.[1] Incidence of oral cavity cancer worldwide in the year 2000 was 267,000 cases while prevalence of this cancer was 707,000 cases.[1] Local epidemiology data also reveals high number of oral cancer cases in Malaysia. According to National Cancer Registry Report in 2007, there were a total of 353 cases of oral cancer cases consisting of lip, tongue and mouth cancers, in which 171 cases were male and 182 cases were female.[2] It was ranked at 21 among the list of cancers in general population, 17th in males and 16th in females. Further analysis revealed it was predominant among the Indian ethnic group as tongue and mouth cancers were among 10 most common cancers in both male and female. However, there were no specific local data on HPV-positive oral cancers.

In general, oral cavity cancers are mostly squamous cell carcinoma and have certain related risk factors consisting mainly of tobacco smoking, alcohol consumption and betel nut chewing. One study highlighted the prevalence of HPV at 24.5%, particularly HPV 16 & 18, among dysplastic and invasive cancers of oral cavity & oropharynx which supports the assumption that HPV infection occurs during early phase of these cancers.[3] Another study estimated proportion of HPV infection among oral and oropharygeal SCC at 35%.[4]

International Agency for Research on Cancer (IARC), in its monograph released 2007, have concluded there is sufficient evidence in humans for the carcinogenicity of HPV 16 in oral cavity and oropharynx but limited evidence in humans for the carcinogenicity of HPV 18 in oral cavity.[5]

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The aim of this study is to establish association of HPV infection and oral cavity cancers in Malaysian setting and ascertain any link between HPV infection and risk factors.

PATIENTS AND METHODS:

This case control study involves a test group (oral cavity cancer patients) and a control group (healthy individuals). Study involves walk-in patient who are seen in ORL & Dental clinic Hospital Universiti Sains Malaysia, Hospital Canselor Tuanku Muhriz & Hospital Tuanku Ja'afar Seremban for growths in oral cavity. A control group of the same amount took part in this study. Patients who have had HPV vaccination and whose definitive histopathology was non-cancerous were excluded from this study. Data collection and salivary sample collection commenced from July 2013 till June 2014. Patients with oral cavity cancers were first identified in ORL & dental clinic and were agreeable to participate in this study. Signed informed consent was first obtained from both groups followed by collection of their demographic data (age, gender, race, family history of cancer, site of oral cancer) and risk factor data (smoking, alcohol intake, betel nut chewing). Histopathology part of the checklist was filled up later once the result was available.

Salivary samples are collected by using Quantivirus® HPV salivary rinse sample collection kit (DiaCarta California, United States) and processed using Quantivirus® HPV E6/E7 RNA Assay (DiaCarta California, United States). The specimen mentioned above then undergoes a series of procedures to hybridize the nucleic acid and detect the presence of HPV by chemiluminescence. This is done using Quantivirus® HPV E6/E7 RNA Assay which detects the mRNA of oncogenes E6/E7 via branched DNA (bDNA) amplification technology. The prepared specimens undergo an assay procedure consisting of 2 major activities, which are i) hybridizing the probes to create branched DNA complex ii)measuring the light output via luminometer.

STEP 1- VIRAL RNA RELEASE FROM SALIVA SAMPLE

The lysis mixture is placed in a 37°C ± 2.5°C water bath for 20min or until any visible crystals dissolve. Mix the lysis mixture by inverting the bottle several times. It is then combined with Proteinase K and stored at room temperature.

Salivary sample in 50ml conical tube and spin for 5 min at 3,500 rpm. Supernatant is discarded and the pellet washed once with 400µL sample wash buffer. The pellet is resuspended via vortex. The sample is spun for 5 min at 3,500 rpm and supernatant discarded.

Each sample pellet is then added 305µL lysis mixture plus Proteinase K solution prepared earlier and then mixed by pipetting up and down 3 times. This mixture is then incubated at 65°C for 1 hour.

STEP 2- CAPTURING TARGET RNA FROM SALIVA SAMPLE

The lysis mixture is placed in a 37°C ± 2.5°C water bath for 20min or until any visible crystals dissolve. Then 50µL from each specimen are placed into the appropriate sample well of the capture plate which is pre-filled with 50µL working probe solution. Positive control well is also prepared, the plate sealed and incubated at 55°C for minimum 3.5 hours.

STEP 3 – HYBRIDIZING THE PRE-AMPLIFIER AND AMPLIFIER PROBES

The capture plate is removed from the incubator, seal removed and inverted to expel contents. Each well is then added 330µL of wash buffer and let soak for 30 seconds. The above step is repeated 2 more times. Then, 100µL of pre-amplifier working reagent is added to each well of the capture plate. The capture plate is then sealed and incubated at 55°C for 40 min. The capture plates are then washed again as mentioned earlier using the wash buffer and 100µL of amplifier working reagent is added to each well of the capture plate. The capture plate is then sealed and incubated at 55°C for 40min.

STEP 4 – HYBRIDIZING THE LABEL PROBE

The capture plate is washed using the wash buffer as in Step 3 and then 100µL of label probe working reagent is added to each well of the capture plate. The capture plate is then sealed and incubated at 50°C for 40 min.

STEP 5 – MEASURING THE LIGHT OUTPUT

The capture plate is washed using the wash buffer as in Step 3 and then 100µL of substrate working reagent is added to each well of the capture plate. The capture plate is then sealed and incubated at 46°C for 20 min. The plate is then removed from the incubator to room temperature and cooled down for 10 min. The plate is loaded onto the Diacarta Quantivirus® Reader system for reading (within 1 minute after 10 minute cool down period) and data analysis. The chemiluminescence emission results are recorded as relative light units (RLU) by a detector (Diacarta Quantivirus® Reader system).

TEST RESULT INTERPRETATION

In order to interpret the results, a test sample RLU signal cut-off reading has to be calculated. The formula to calculate is shown below as described in the manufacturer manual:

SIGNAL CUT-OFF = Patient sample RLU / Average RLU of Blank

If the signal cut-off equals or higher than 1.5, the result is positive. Otherwise, the result is negative.

DATA MANAGEMENT & STATISTICAL ANALYSIS

Data collected is compiled into SPSS and basic analysis of demography and data statistics are done. Proposed methods of determining significance of data includes Chi-square test, Fisher's exact test and Multiple Logistic Regression.

RESEARCH ETHIC COMMITTEE APPROVAL

The Human Research Ethics Committee, Universiti Sains Malaysia has approved this study to be carried out (FWA Reg. No: 00007718; IRB Reg. No: 00004494).

RESULTS:

This study involves a total of 58 subjects, which consists of 29 test subjects who are patients with oral cavity cancer and 29 control subjects who are healthy. The test subjects were from 3 different centres, which are Hospital Universiti Sains Malaysia (HUSM), Hospital Canselor Tuanku Muhriz and Hospital Tuanku Ja'afar Seremban (HTJS). Test subjects from HUSM were 16 subjects, 6 subjects from Hospital Canselor Tuanku Muhriz and HTJS contributed 7 subjects.

AGE DISTRIBUTION

Age of subjects ranged from 22 years old to 83 years old with a mean age of 46 years old (SD 15.62). Among the test group, the highest number of subjects was found in 60-69 age group with 7 (24.1%) subjects followed by 40-49 age group with 6 (20.7%) subjects with a mean of 52.55 (SD=16.05). In comparison to control group, the majority of subjects were found in 20-29 age group with 9 (31.0%) subjects with a mean of 38.69 (SD=11.81). The age distribution of test and control subjects is shown in (Figure 1).

GENDER DISTRIBUTION

The number of subjects based on gender in overall is equally distributed at 29 male and 29 females. However, gender distribution differs among the test and control group. The male gender holds the majority in test group with 18 (62.1%) out of 29 subjects while females form the majority in control group with 18 (62.1%) out of 29 subjects.

RACE DISTRIBUTION

The majority of overall subjects are from the Malay race with 43 (74.1%) subjects followed by Chinese with 7 (12.1%) subjects and Indians with 3 (5.2%). Based on test subjects, the majority of subjects are the Malays at 19 (65.5%) subjects followed by 7 Chinese subjects (24.1%) and 1 Indian subject (3.45%). In the control group, majority of subjects are also by the Malays at 24 (82.8%) subjects followed by 2 Indians with no Chinese control subjects.

HPV PREVALENCE

Human papilloma virus (HPV) overall prevalence in this study sample was found to be at 29.3% in which 17 subjects were found to be positive. Among the positive subjects, 16 subjects were from the test group while 1 subject was from the control group originating from Hospital Canselor Tuanku Muhriz (Figure 2). This represents 55.1% HPV prevalence among the test subjects versus 3.4% among the control group. The high rate of prevalence among test subjects was verified using simple logistic regression and was found to be significant ($p=0.001$) with an odds ratio of 34.46 (95% CI 4.117, 288.449).

The highest number of HPV-positive test subjects was noted in the 40-49 age group with 5 (31.3%) subjects followed by the 50-59 age group and the 60-69 age group with 3 (18.8%) subjects in each group. The full distribution is shown in (Figure 3 below).

Gender distribution among HPV-positive test subjects consists of 11 (68.8%) males and 5 (31.2%) females while race distribution shows 11 (68.8%) subjects are Malays followed by Chinese with 4 (25%) subjects.

HISTOPATHOLOGY

The histopathology analysis of test subjects revealed that 28 (96.6%) out 29 test subjects had squamous cell carcinoma. The other subject was found to have spindle cell carcinoma.

RISK FACTOR: SMOKING HABIT

An overview of smoking habits shows that majority of the overall subjects do not smoke which consists of 39 (67.2%) subjects in comparison to 19 (32.8%) subjects who smoke. However, a more detailed look into this data revealed 15 (51.7%) out of 29 test subjects were smokers in comparison to 4 (13.8%) out of 29 control subjects (Figure 4). This correlation was verified using simple logistic regression and was found to be significant ($p=0.015$) with an odds ratio of 4.48 (95% CI 1.339, 14.991).

Among the smokers, 5 subjects smoke between 1-19 sticks/day, 2 subjects smoke between 20-30 sticks/day and 1 subject smoke >40 sticks/day as shown in (Figure 5). Ex-smokers were put into the smokers' category as all subjects had stopped smoking recently after they were diagnosed with cancer and the increased risk of smoking is reduced only after 10 years of smoking cessation.[13]

RISK FACTOR: ALCOHOL CONSUMPTION

In terms of alcohol consumption, majority of the overall study subjects which was represented by 51 (87.9%) subjects, did not consume alcohol and only 7 subjects consumed alcohol. A detailed look into this data revealed that 5 (17.2%) out of 29 test subjects consumed

alcohol compared to 2 (6.9%) out of 29 control subjects (Figure 6). This correlation was verified using simple logistic regression but was found to be insignificant (*p=0.241*).

RISK FACTOR ; BETEL NUT CHEWING

Majority of the overall subjects (98.2%) do not have the habit of betel nut chewing. The only subject who has this habit is in the test group and insignificant in this sample group.

CORRELATION ANALYSIS OF HPV STATUS AND OTHER RISK FACTOR VARIABLES

Multiple logistic regression analysis was employed to help isolate which risk factors contribute significantly towards oral cavity cancer formation in these subjects and calculate its association separately. This analysis had revealed that both smoking and HPV are significantly associated with oral cavity cancer (Table 1) and no interactions among these factors were found. Alcohol consumption and betel nut chewing was not found to be significant in this study group.

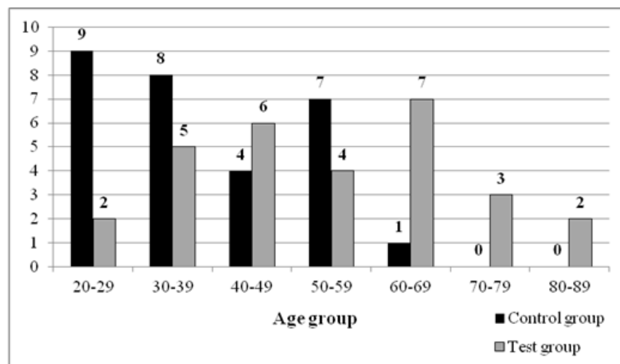

Figure 1: Age distribution of test and control subjects

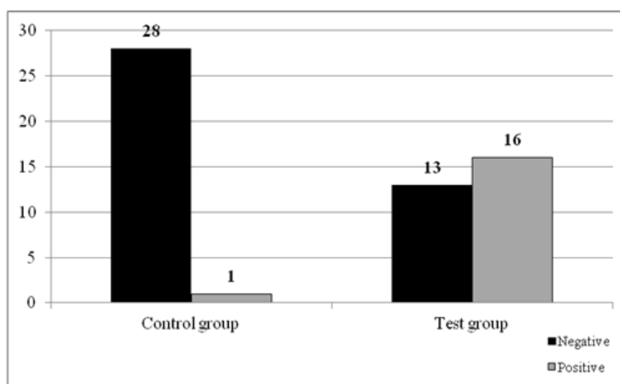

Figure 2: HPV prevalence among test and control subjects

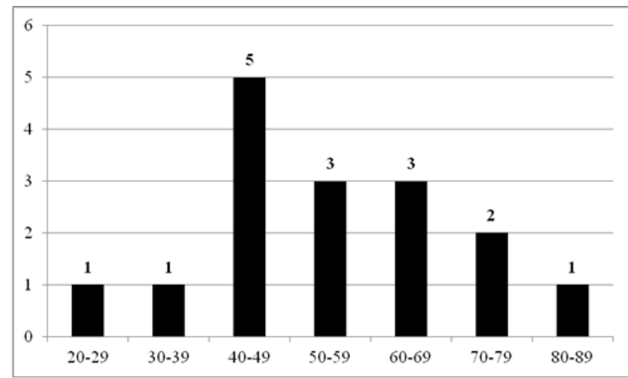

Figure 3: Age distribution among HPV-positive test subjects

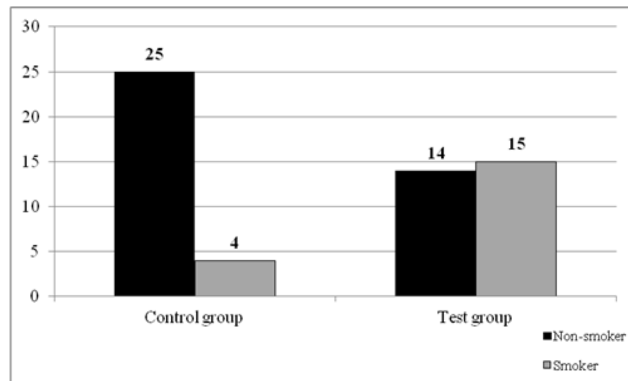

Figure 4: Smoking habit among test and control subjects

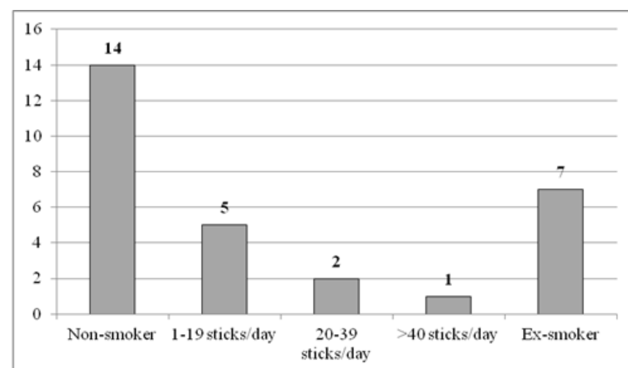

Figure 5: Smoking amount among the test subjects

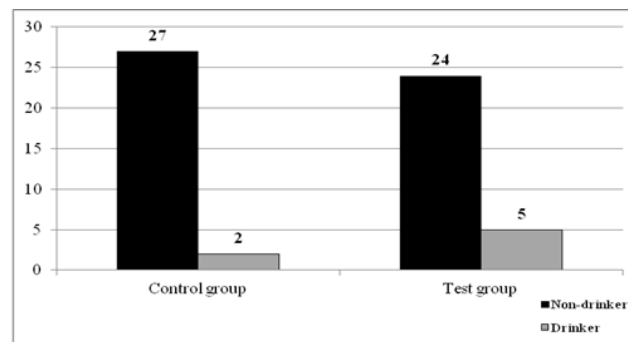

Figure 6: Alcohol consumption among test and control subjects

Table 1: Associated factors of oral cavity cancer by Multiple Logistic Regression

Variable	Regression coefficient (b)	Adjusted Odds Ratio[a] (95%CI)	Wald statistic	*p-value*
HPV Status				
- Negative	0	1		
- Positive	3.52	33.90 (3.88,295.99)	10.16	0.001
Smoking habit				
- Non-smoker	0	1		
- Smoker	1.47	4.36 (1.06,17.86)	4.18	0.041

a Forward LR Multiple Logistic Regression model was applied. Multicolinearity and interaction term were checked and not found. Hosmer-Lemeshow test, ($p=0.078$), classification table (overall correctly classified percentage = 81%) and area under the ROC curve (83.9%) were applied to check the model fitness.

DISCUSSION

This study presents findings of HPV prevalence among population of Malaysia which is very scarce in the local setting. This study also explores use of salivary rinse in HPV detection among oral cavity cancer patients.

The HPV prevalence rate of 55.1% among oral cavity cancer patients in this study is concordant with findings of various studies done internationally with number of positive cases ranging from 10% to 74%.[6,7,8,9,10] Prevalence rates of HPV in oral cavity cancer from 24% to 74% have been reported in the United States of America, which is caused by genotype HPV 16.[6,7] Studies done in European countries such as Italy and Sweden revealed rates of 36% and 26% respectively.[8,9] A similar study done in China revealed HPV prevalence rate of 40%.[10] In terms of HPV-positive test subjects, most are seen in younger age group of 40-49 (31.3%), majority of which were males (68.8%) which supports similar findings in certain studies.[11,12] The evidence procured from this study shows that HPV prevalence among oral cavity cancer patients in this country is at par with the international community and is not to be taken lightly. The lack of similar studies done locally undermines the actual scenario that is occurring internationally and is a risk factor that needs to be taken into consideration seriously.

The risk of oral cavity cancer associated with smoking is both dose and duration dependent and the risk dissipates after cessation of smoking for 10 or more years.[13,14] Based on the latter fact, 7 ex-smokers in the test group who recently stopped smoking had been included as smokers in bivariate categorical analysis of smoking. The prevalence of smoking in the test group is slightly higher compared to other studies on influence of smoking alone on oral cavity cancer. Odds ratio was noted to increase with higher amounts of cigarettes and gave an odds ratio range of 1.2 - 2.8 for males and 1.8 – 6.2 for females depending on number of cigarettes per day.[14] One study looked into the interaction between HPV and smoking and found no synergistic effect, rather more to an additive effect.[6]

The risk of oral cavity cancer associated with alcohol consumption increases linearly with dose and total amount ingested and this is more important than type of alcoholic drink.[13] However, in this study, alcohol consumption among test subjects was 17.2% and all were occasional drinkers (< 1 drink/week). One study have shown odds ratio of 1.0 for non-smoking, alcohol drinkers of < 1 drink/ week and odds ratio of 1.2 – 8.8 in males and 1.2 – 9.1 for females who were non-smokers with odds ratio increasing with amount of alcohol.[14] In view of this, alcohol consumption was not expected to be a contributing factor in this study.

Over the past few years, a lot of interest has been shown in detecting HPV from saliva or salivary rinse. Studies have clearly shown the usefulness and consistency of salivary detection of HPV via salivary rinse by isolation of genetic material from exfoliated oral mucosal cells.[6,15,16] The viral type most commonly isolated is HPV 16, followed by HPV[18], both of which are high risk types of HPV.[3,15,16] The salivary rinse method has good potential as a screening tool due to ease of collection and flexibility in comparison to biopsy acquired HPV detection. Another advantage is that HPV were also detected in salivary samples of patients post treatment and those with recurrence of the malignancy. In terms of technique used in salivary detection of HPV, PCR methods were used in some of the studies. Quantitative PCR looks for HPV E6 & E7 regions and specific primers & probes were used to amplify these regions. Another method is in-situ hybridization which is also commonly used method.

HPV can be classified as low-risk and high risk types based on established cervical oncogenicity.[17] Low risk HPV types (eg. type 6 and 11) induce benign lesions with minimum risk of progression to malignancy such as in laryngeal papilloma.[18-21] On the other hand, high risk HPV have higher oncogenic potential with HPV 16 being the most prevalent followed by types.[18, 31 & 33.18,19]

This study proves the actual prevalence of HPV among oral cavity cancer patients in Malaysia. However, the findings of this study is limited by the small sample size which mainly affected the possible association of alcohol and betel nut chewing. A more elaborate study needs to be undertaken to confirm these findings, to identify the link between all the relevant risk factors and to HPV in local setting and the potential use of salivary rinse screening in detection of HPV. The effectiveness of HPV vaccine in preventing HPV-induced oral cavity cancer must be explored in order to arrest the escalating incidence of these cancers.

CONCLUSION

Our study has shown that HPV prevalence among oral cavity cancer patients was found to be 55.1% versus 3.4% in the control group which was found to be significant (*p=0.001*) with odds ratio of 33.90 (95% CI 3.88, 295.99). Smoking was found to be a significant risk factor (*p=0.041*) with odds ratio of 4.36 (95% CI 1.06, 17.86). No interaction was found between HPV and smoking in this study. Salivary rinse proved to be a viable method for detecting HPV and also as a possible screening method for HPV in the future.

CONFLICT OF INTEREST

There are no conflicts of interest.

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