Evaluation of Human Papillomavirus 16 and Human Papillomavirus 18 in Saliva of Chronic Smokers in Malaysian Population: An In vitro Observational Study

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Abstract

Background: Although the health risks of tobacco smoking are well documented, there is increasing evidence that smokers have a low incidence of inflammatory and neurodegenerative diseases. Moreover, this reduced immunity may pave a way for increased relative risk of harboring these viral entities such as human papillomavirus (HPV) 16 and HPV 18 more specifically in oral cavity. Objective: The objective of the study was to evaluate whether tobacco smoking is associated with the prevalence of HPV 16 and HPV 18 in saliva of chronic smokers and nonsmokers. Methodology: A total of sixty participants constitute the study groups. Out of sixty participants, thirty were grouped with smoking habits, Group I (n = 30) and remaining thirty with no history of smoking, Group II (n = 30). DNA was extracted from saliva of all the participants using QIAamp “DNA mini kit.” Polymerase chain reaction was performed, using primer sequence specific for HPV 16 and HPV 18 obtained from NCBI database. The determination of HPV 16 and HPV 18 was done if and only if the bands were noticed on the ethidium bromide stained agarose gel at 120 bp for HPV 16 and 180 bp for HPV 18. Results: HPV 16 and HPV 18 were not found in both smokers and nonsmokers. Conclusion: The present study is in opinion that smoking is not associated with the HPV 16 and HPV 18 prevalence in saliva. However, the possibilities of synergistic effects of reduced immunity, as a result of smoking and increased prevalence of HPV 16 and HPV 18 in HPV-diagnosed individuals, should be addressed with a large sample.

Keywords: Carcinoma, human papillomavirus, real-time polymerase chain reaction, saliva, smoking

Introduction

Human papillomavirus (HPV) represents diverse papovavirus of epitheliotropic, nonenveloped, small, i.e., approximately 55 nm in diameter.[1] It is circular, closed, double-stranded DNA virus genome with a size of almost 8000 bp.[2] Various infections with HPV involvement were first presented by Syrjänen et al. in 1983.[3] Over the years, 200 different HPV subtypes[4] with different variations in their genetic and oncogenic potential are identified. Out of which forty types can be easily spread through direct skin-to-skin contact in both males and females. This virus can infect many types of epithelial cells, especially basal layers of squamous epithelium.[5] The DNA of virus can be integrated into host genome/nonintegrated/episomal/as a combination of these types in infected tissues.[6] Based on their ability to cause malignancies in humans, these viruses are classified as “low-risk” HPVs also known as “condylomata acuminata” which does cause cancer but cause skin warts, for example, HPV 6 and 11. “High-risk” (HR) oncogenic HPVs, i.e., HPV 16 and 18 can cause cancer[7] such as head and neck and cervical carcinoma.

Malaysia is a Southeast Asian country with multiethnic and multicultural backgrounds. Approximately one-third of men worldwide are active smokers and this country is no exception. Prevalence of smoking among Malaysian adults is becoming a worrying concern. From over the past decade, the percentage increased in spite of several population interventions.
Tobacco-related deaths in Malaysia were approximately 19% for men and 8.2% for women, a higher than average death rate than other middle-income countries.[9] Smoking-related disease such as cancers[10] is main cause of premature death globally. Various studies[2,4,5,7] showed that smoking has been associated with longer duration of oncogenic HPV infections as well as increased risk of cancers. Tobacco in cigarette releases nicotine. The concentration and duration of exposure to nicotine alter the antigen-mediated signaling pathways[9] which will influence the HPV infections by suppressing local immune function, interfering with HPV infection clearance, increased cellular proliferation, and cause host DNA damage resulting in increased susceptible to infections.[10,11] Tobacco-associated carcinogens also induce alterations in genetic events which may lead to molecular changes, making the individual susceptible to HPV infection. Since HPV appears to be highly prevalent in men, it is important to determine the potential effect of smoking on HPV infections. Hence, the present study was designed to compare the salivary adulteration with HPV 16 and 18 serotypes between smokers and nonsmokers by real-time quantitative polymerase chain reaction (PCR).

**Methodology**

This pilot study was done on sixty healthy adult population aged 20–40 years divided into thirty smokers and thirty nonsmokers. The study protocol was reviewed and approved by Craniofacial and Micro-Molecular Biology Research Laboratory of University Malaya of Dental Medical Science, Kuala Lumpur. The whole study process was described to patients, and informed consent was obtained. Individuals with medical and surgical history or with drug addictions are excluded from the study. An inclusion criterion was smoker’s group take tobacco in cigarette only, and nonsmokers should not take tobacco in any form.

**Saliva sample collection**

Healthy adult participants who agreed for the study were asked not eat or drink before 30 min before saliva collection. Saliva was collected by Oasis Diagnostics Super SAL™ Salivary Collection Device. Samples were stored in ice and transported to biomolecular laboratory for analysis.

**DNA extraction**

“QIAamp DNA Blood Mini Kits” [Figure 1] are used for DNA extraction. The Qia gen extraction protocol was as follows:

One milliliter saliva was collected in a 15 ml falcon tube. Four milliliters phosphate-buffered saline (PBS) was added to 1 ml samples and centrifuged at 8000 rpm for 5 min. Discard the supernatant and suspended the pellet into 180 µl PBS. Qia gen protease 20 ml and 200 µl Buffer AL were added to the sample. Close the tube lid and mix immediately by vertexing for 15 s. The tubes are incubated at 56°C for 10 min. Two hundred milliliters ethanol (96%–100%) was added to the sample and mix again by vertexing. The mixture of ethanol (96%–100%) is added to QIAamp Spin Column without moistening the rim.

![QIAamp DNA Blood Mini Kit](image)

The QIAamp spin column was carefully opened, and 500 µl of Buffer AW1 was added and centrifuge it at 8000 rpm for 1 min. The QIAamp spin column was opened, and 500 µl of Buffer AW2 was added then centrifuge it at full speed for 3 min.

The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing filtrate is discarded. QIAamp spin column was carefully opened then eluted the DNA with 100 µl of buffer AE. Then, incubated it at room temperature for 3 min and centrifuge at 8000 rpm for 1 min. The quantitative and qualitative of DNA was tested by NanoDrop 2000 spectrophotometer with ratio A250/A280 [Figure 2].

**Real-time polymerase chain reaction [Figure 3]**

The real-time PCR was carried out according to manufacturer instructions. HPV 16, 18 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as targets for the primers. The volume of primers for sixty samples was prepared. The DNA from each sample was used to perform PCR using following target primers - HPV 16, 18 and GAPDH. For PCR reaction mix, 21 ml of master mix + the volume of GoTaq Green Master mix for forty samples is measured and mixed with each individual DNA template and each individual specific SdH20.

The DNA samples were transferred to thermal cycler device to amplify segments of DNA through the PCR [Table 1].

Sterile water is used as a negative control and error control of the kit - (21 µl master mix + DNA 200 ng + 9 µl sdH20).

**Gel electrophoresis**

PCR products were analyzed using ethidium bromide stained 2% agarose gel for 30 min. The bands were visualized by ultraviolet illumination of ethidium bromide-stained gels and captured using Kodak Gel Logic 1000 Imaging System and Image Analysis Software. PCR product was purified and sequenced for confirmation of HPV 16, HPV 18 and GAPDH gene [Figures 4 and 5].
RESULTS

Sixty healthy adult populations who participated in the study were divided into thirty patients in smoking group and thirty patients in the nonsmoking group. Samples of saliva were collected from the patients between October and December 2015. The smokers have a mean age of 37.93 ± 10.81 years whereas the nonsmokers have 28.9 ± 7.52 years [Table 2]. The patients from whom samples were collected were not statistically significant. Smokers were selected based on patients who smoke more than five cigarettes in a day at least for 5 years were considered as a smoker. The extracted DNA was subsequently screened for the presence of HPV 16 and 18 using real-time PCR. Results showed that all samples in the two groups had negative results for HPV 16 and 18 serotypes [Table 3].

DISCUSSION

The result of our study showed smoking does not increase the prevalence of HPV infection. This study was consistent with a study by Arbabi-Kalati et al. [12] In their study, 100 healthy adult Iranian males are compared for salivary contamination between smokers and nonsmokers. Results showed that no HR types of virus found in both groups.

Although HPV infections are transient and asymptomatic or subclinical, 6 million new infections occur annually in the USA. [13,14] As smoking damages immune system, along with HPV, it may lead to head and neck cancers. [11] The carcinogens released from cigarette such as tar and nicotine cause immune suppression, susceptibility to infections, and also contribute to carcinogenesis. Studies have shown 60% of head and neck cancer cases have HPV genomic DNA was detected by PCR-based methods. [15,16] HPV genes take control over the cellular proliferation that leads to uncontrolled cell division. Recent epidemiological evidence also suggests that HPV may be an independent risk factor for oropharyngeal cancers, revealing HPV is 3 times more potential to cause precancerous oral lesions and almost 5 times oropharyngeal

Figure 2: Spectrophotometer

Figure 3: Real-time polymerase chain reaction

Figure 4: M: marker Lane 1: Negative control Lane 2-7: Human papillomavirus 16-Glyceraldehyde-3-phosphate dehydrogenase multiplex polymerase chain reaction samples, Glyceraldehyde-3-phosphate dehydrogenase amplicons present only

Figure 5: Human papillomavirus 16 polymerase chain reaction positive control M: Marker Lane 1: Negative control Lane 2-3: Human papillomavirus 16 polymerase chain reaction using Caski cell line DNA (200 ng) Lane 4-5: Human papillomavirus 16 polymerase chain reaction using Caski cell line DNA (1000 ng)
Table 1: Thermo cycle process

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3 mins</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>20 second</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C</td>
<td>60 second</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>30 second</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>5 mins</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Demographic Data of study population

<table>
<thead>
<tr>
<th>Demographic Data</th>
<th>Smoker (30)</th>
<th>Non-smoker (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Age</td>
<td>37.9±10.81*</td>
<td>28.9±7.52*</td>
</tr>
<tr>
<td>Marital Satus</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>Alcoholic</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Years of smoking</td>
<td>18.43±10.09</td>
<td>N/A</td>
</tr>
<tr>
<td>Packs/day</td>
<td>&lt;1</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>&gt;1</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3: Results of HPV 16 and HPV 18 genotypes

<table>
<thead>
<tr>
<th>HPV Genotypes</th>
<th>PCR Result</th>
<th>Smoker n=30</th>
<th>Non-smoker n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 16</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>HPV 18</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>

cancers compared to normal mucosa. Oral cancer is the sixth most common malignancy in worldwide causing severe illness to deaths. The estimated prevalence of oral cancer ranges from 38% to 100% in various studies. This percentage varies from country to country depending on various habits practiced, lifestyles, cultural backgrounds, socioeconomic status, personal awareness, and preventive strategies. In Malaysia, the National Cancer Registry reported oral cancer as the 22nd and 15th most common cancer type among males and females, respectively.

Saini et al. reported that HPV 16 was the predominant type found in Malaysian patients with oral squamous cell carcinoma. In their study, HPV 16 was the most frequent type of HR-HPV found in cases and control group and HPV 18 was not detected in the samples. The difference of HPV prevalence could be due to detection methods, i.e., universal primers MY09/MY11 and GP5+/GP6+ in nested PCR method and specimens were collected using buccal swab sampling. Kreimer et al. evaluated 1680 healthy men in Brazil, Mexico, and the USA and reported the prevalence rates of 4% and 3.1% for oral HPV infection and HR-HPV, respectively. Their study concluded that smoking increases the prevalence of oral HPV infection. Gillison et al. evaluated 5579 healthy patients of 14–69 years and reported the prevalence rates of 6.9% and 1% for oral HPV infection and HPV 16 serotypes, respectively, in the USA. Schabath et al. determined baseline risk of HPV infection associated with smoking and results suggested that current smokers are associated with an increased risk for oncogenic HPV infection. Our results cannot be compared with above study as they considered sexual activity also.

Syrjänen concluded that HPV infection in utero, i.e., prenatal transmission can occur in 20% of cases. The mother seems to be the main transmitter of HPV to her newborn, but subsequently HPV infections are acquired horizontally through saliva or other contact. The role of HPV infection in the development of oral pathologies is essential considering the important role of infection in pediatric population. A study done by Smith et al. in 1235 healthy children and teenagers showed that overall prevalence rates range from 1.2% to 3.3%. This shows dentists should have proper knowledge regarding this issue and pay attention to lesions located in oral mucous membrane. Xi et al. evaluated the relationship between cigarette smoking and HPV types 16 and 18 DNA load in 1050 women who were current smokers. High baseline HPV 16 and 18 DNA load was associated with current smokers but not former smokers. A study by Kumar et al. in the northeast region of India demonstrated that tobacco may act as risk factors for HR-HPV infection of about 31.13%. Their study investigated HR-HPV infection and its association with lifestyle habits such as tobacco and alcohol consumption. Jatin et al. analyzed for HPV 16 and 18 infection of oral cancer due to tobacco chewing. Out of 110 cases, 37 patients (33.6%) have HPV 16 and 18 serotypes, respectively. Limited data exist on the association between HPV infection and smoking in the Malaysian population. Dentist, however, should remember that HPV is associated with the development of head and neck carcinoma. As it is an epitheliotropic virus, it shows affinity and differentiates in stratified squamous epithelium.

From the results of our study, a lack of relationship between cigarette smoking and viral load may indicate a low threshold for the effect of smoking on HPV load. Smoking causes host DNA damage, which results in increased susceptibility to HPV infection.

**Conclusion**

We conclude from our present study that smoking alone is not associated with the HPV 16 and 18 prevalence in the saliva. However, the possibilities of synergistic effects of reduced immunity as a result of smoking and increased prevalence of HPV 16 and 18 in human papilloma virus diagnosed individuals should be addressed with large samples.

**Limitations of the study**

Some limitations such as sexual practices were not designed as critical to the initial goals due to cultural constraints. A large sample size and detailed history of irritants should be allowed in the future.
Goud, et al.: HPV in Malaysian population

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES