RESEARCH COMMUNICATION

Detection of Human Papillomavirus in Oral Squamous Cell Carcinoma, Leukoplakia and Lichen Planus in Thai Patients

Siribang-on P Khovidhunkit¹*, Waranun Buajeeb², Sirima Sanguansin³, Sopee Poomsawat⁴, Woranut Weerapradist⁴

Abstract

Although tobacco, alcohol abuse and betel nut chewing habit are well recognized risk factors for oral squamous cell carcinoma (OSCC), there is evidence to indicate that human papillomavirus (HPV) may also play some inducing role. The purpose of this study was to investigate the presence of HPV in Thai patients with oral squamous cell carcinoma, leukoplakia and lichen planus using the polymerase chain reaction (PCR). Biopsies of oral squamous cell carcinoma, leukoplakia and lichen planus were obtained from 65 patients, 15 males and 50 females, aged between 30-88 years old. Extracted DNA was evaluated for HPV infections by PCR analysis using consensus primers specific for L1 region of HPV. Only one sample (1.54%) was positive, suggesting that HPV may not play an important role in this group of Thai patients.

Key Words: HPV - oral squamous cell carcinoma - oral premalignant conditions

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Introduction

While the oral squamous cell carcinoma (OSCC) has been found to be associated with tobacco, alcohol abuse and betel nut chewing habit, recent studies suggest that viral factors, including human papillomavirus (HPV), may contribute to the etiology of this malignant neoplasm (Scully et al., 2000; Hansson et al., 2005). Recently more than 100 human papillomavirus types have been identified in various lesions. In the oral cavity, many low-risk types of HPV, such as HPVs-6 and 11, have been shown to be associated with benign and some other types, especially HPVs-16, 18 and 33, were associated with malignant lesions (Chang et al., 1991; Yedall, 1992; Miller and Johnstone, 2001). Until now, HPV-16 is the most frequently identified HPV in OSCCs, and both HPV-16 and 18 play very important roles in malignant transformation (Boulet et al., 2007). The role of HPV infection has been of particular interest as HPV oncoproteins E6 and E7 inactivate p53 and pRB, respectively. More recently, the oncogene E5 has also been found to transform cells by modulating growth factor receptors (Chen et al., 2007). These data suggested that the HPV produces several oncoproteins which contribute in carcinogenesis.

Although many studies indicated high frequency of HPV in OSCC specimens, controversy still exists. The rate of HPV detection in OSCC has varied from 0-94% (Kashima et al., 1990; Watts et al., 1991; Cox et al., 1993; Woods et al., 1993; Miller and Johnstone, 2001). In some studies, HPV was identified with increasing frequency in normal oral mucosa, benign leukoplakia, intraepithelial neoplasia, squamous carcinoma and verrucous carcinoma (Miller and White, 1996). On the contrary, some studies, using microdissection and real-time quantitative PCR analysis, did not demonstrate the association between HPV infection and oral premalignant and malignant lesions (Ha et al., 2002). A large case-control study conducted in 9 countries also reported a low prevalence (3.9%) of HPV DNA in 766 biopsy specimens taken from patients with cancer of the oral cavity whereas the HPV DNA could be detected in 18.3% of specimens of oropharyngeal cancer (Herrero et al., 2003).

Until now, however, there have been few reports about oral HPV infections in malignant and premalignant lesions in Thai population. Therefore, the objective of this study was to investigate the presence of HPV in specimens obtained from patients with OSCCs and other precancerous lesion and condition including leukoplakia and lichen planus.

Materials and Methods

Specimens

The specimens used in this study were retrieved from 2 sources, Rajvithi Hospital and the Faculty of Dentistry.

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Mahidol University, Bangkok, Thailand. Thirty-two OSCC specimens were collected from Rajvithi Hospital, whereas, 17 specimens of oral leukoplakia and 16 specimens of oral lichen planus were collected from the Faculty of Dentistry, Mahidol University. Specimens from Rajvithi Hospital were resected oral tissues from 32 Thai patients presented with oral squamous cell carcinoma. Sample collection was carried out according to protocols approved by the Ethics Committee, Rajvithi Hospital. Clinical data and information related to lifestyle were obtained from clinical charts and personal communication. The resected tissues were frozen immediately after collection and stored at -80°C until used. Histopathological examinations were performed by a pathologist according to WHO-TNM classification of lip and oral cavity carcinoma (Pindborg, 1997).

Seventeen specimens of oral leukoplakia and 16 specimens of oral lichen planus were formalin-fixed paraffin embedded tissues from the Faculty of Dentistry, Mahidol University. All the specimens were subjected to pathological investigation from a pathologist at the Faculty of Dentistry, Mahidol University and the data of the patients were retrieved from the clinical charts.

**Extraction of DNA and PCR amplification**

DNA was extracted either from fresh frozen or formalin-fixed paraffin embedded tissues. Genomic DNA was extracted from fresh frozen OSCC tissues by proteinase K digestion, followed by salting out extraction (Miller et al., 1988). Specimens with more than 90% cancerous cells were used in this study. Genomic DNA from oral leukoplakia and oral lichen planus was extracted from formalin-fixed paraffin-embedded tissues using QIAamp DNA Mini Kit (Qiagen® GmbH, Hilden, Germany).

PCR primer sequences, conditions, thermocycling and the length of PCR products are demonstrated in Table 1. The PCR reaction was performed according to manufacture protocol using Taq PCR Core Kit (Qiagen®, Germany).

**Table 1. Primer Sequences and PCR conditions**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence</th>
<th>PCR cycle</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV DNA 150 bp</td>
<td>HPV1003: TTTTGTTACTGTGGTAGATA</td>
<td>*95°C 10, 94°C 1.5, 50°C 1.5: 72°C 2 (x40) 72°C 10</td>
<td>150</td>
</tr>
<tr>
<td>HPV DNA 150 bp</td>
<td>HPV1003: GAAAATTTAAGCTAAGTC</td>
<td>*95°C 10, 94°C 1.5, 50°C 1.5: 72°C 2 (x40) 72°C 10</td>
<td>150</td>
</tr>
<tr>
<td>HPV DNA 250 bp</td>
<td>HPV1003: GAAAATTTAAGCTAAGTC</td>
<td>*95°C 10, 94°C 1.5, 50°C 1.5: 72°C 2 (x40) 72°C 10</td>
<td>250</td>
</tr>
<tr>
<td>β-globin 250 bp</td>
<td>KM29: GGTTGGCCAA TCTACTCCCAGG</td>
<td>*95°C 10, 94°C 1.5, 50°C 1.5: 72°C 2 (x40) 72°C 10</td>
<td>250</td>
</tr>
</tbody>
</table>

**Figure 1. Upper panel: PCR analysis of HPV DNA** Lane 1: 100 bp marker; Lane 2: negative control of HPV-DNA; Lane 3: positive cervical cancer control; Lane 5: positive sample; Lane 6, negative sample 50 ml total reaction mixture containing 50 mM KCL, 10 mM Tris, pH 8.5, 4 mM MgCl2, 200 mM each dNTPs, 25 pmole of HPV L1 concensus primer (MY11-MY09 and HPV1003-HPV1004), 2.5 pmole of each β-globin primer (KM29-KM138), 1.25 unit of Taq polymerase and 200 ng of DNA was used in each reaction. The amplification of b-globin gene was examined for the DNA quality control in all specimens. All PCR products were subjected to 2% agarose gel electrophoresis. DNA bands were stained with ethidium bromide and photographed under UV transillumination (Geldoc, Biorad®, CA, USA).

**Results**

**Patient characteristics**

Patients were separated into 3 groups (Table 2) according to pathological diagnosis. Group 1 was composed of 32 patients presented with OSCCs and the specimens from these patients were retrieved from Rajvithi Hospital. The age of the patients in this group was between 30-88 years old (mean = 64.8 years) and the majority of the patients were female. Interestingly, the majority (97%) of the patients in this group either had a long history of betel nut chewing habit or smoking or alcohol consumption. The most frequent site of OSCCs was buccal mucosa (11 out of 32 or 34.4%) and almost 50% of the patients had stage IV OSCCs.

Group 2 was consisted of 17 patients presented with white patch in the oral cavity and the pathological diagnosis was hyperkeratosis with or without dysplasia. The range of the age of these patients was 36-80 years old with mean age of 53.1 years. Almost 50% of the patients had a history of smoking and some of the patients had betel nut chewing habit or alcohol consumption (Table 2). Group 3 encompassed 16 patients presented with white striation and erythematous areas and the pathological diagnosis was oral lichen planus. The range of the age of these patients was 32-72 years old with mean age of 50.53 years (Table 2). One of the patients in this group used to

**Table 2. Characteristics of Patients with OSCCs, Oral Leukoplakia and Oral Lichen Planus**

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>N</th>
<th>Age (Mean)</th>
<th>Male</th>
<th>Female</th>
<th>Betel nut chewing</th>
<th>Smoking*</th>
<th>Alcohol consumption</th>
<th>No data</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSCC</td>
<td>32</td>
<td>30-88 (Mean=64.8)</td>
<td>6</td>
<td>26</td>
<td>24 (75.0)</td>
<td>9 (28.1)</td>
<td>14 (43.8)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Leukoplakia</td>
<td>17</td>
<td>36-80 (Mean=53.1)</td>
<td>7</td>
<td>10</td>
<td>4 (23.5)</td>
<td>8 (47.1)</td>
<td>2 (11.8)</td>
<td>3 (17.6)</td>
</tr>
<tr>
<td>Lichen planus</td>
<td>16</td>
<td>32-72 (Mean=50.5)</td>
<td>2</td>
<td>14</td>
<td>0 (0.0)</td>
<td>2 (12.5)</td>
<td>0 (0.0)</td>
<td>3 (18.8)</td>
</tr>
</tbody>
</table>

* Smoking status includes both former and current smokers.
smoke for 30 years but had already quit smoking for 4 years. Another patient used to smoke but had already quit smoking for more than 20 years. None of the patients in this group had history of betel nut chewing or alcohol consumption. Some of them had systemic diseases such as hypertension or type II diabetes mellitus (type II DM).

Presence of HPV

In this study, the presence of HPV DNA was investigated using 2 primer sets including HPV1003-HPV1004 and MY11-MY09. Both primer sets recognized the L1 region of HPV. All the DNA samples were subjected to the PCR of β-globin gene as a control of DNA quality. The DNA extracted from Hela cells and a cervical carcinoma biopsy specimen which is known to contain HPV DNA was used as positive control. As shown in Figure 1, out of 65 samples studied, only 1 DNA sample (1.54%) from OSCC was positive for HPV.

Discussion

The aim of this study was to investigate the presence of HPV DNA in oral epithelial lesions from patients attending Rajvithi Hospital and the Faculty of Dentistry, Mahidol University. The frequency of 1.54% of HPV positivity observed was very low among the enormous range (5-80%) described in the literature (Syrjanen et al., 1988; Chang et al., 1991; Holladay and Gerald, 1993; Fouret et al., 1995; Franceschi et al., 1996; Bouda et al., 2000; Sand et al., 2000). This is less likely due to the result of poor DNA quality since all of the samples were found to be positive for β-globin PCR. Moreover, in every PCR reaction, PCR products could be amplified from both positive controls, i.e., Hela cells and the cervical cancer specimen suggesting that the low frequency of the HPV positivity in this study was not due to technical problem. Comparable to one of the studies in laryngeal cancer, PCR analysis was performed using 3 sets of primers universally accepted for HPV detection. Although all positive controls were accomplished as expected, only 1 of 30 laryngeal carcinoma specimens was positive for HPV DNA. It was stated that the incredible range of HPV detection in laryngeal carcinoma in the publications could only be explained by a high frequency of false positive results. In addition, HPV could be present in some laryngeal carcinomas, but it is not a common event in the group of patients with laryngeal cancer investigated (Lindeberg and Johansen, 1990).

The role for HPV in head and neck cancer pathogenesis was investigated by several researchers (Miller and White, 1996, Miller and Johnstone, 2001). Because of the wide range of the rate of HPV DNA detection, it has been stated that this difference could be depending on the population studied, combination of sites of cancer, type of specimen and detection method (Scully, 2005). In a meta-analysis of HPV as a risk factor for OSCC, HPV detected in normal oral mucosa (10%) was significantly less than that in benign leukoplakia (22.2%), intraepithelial neoplasia (26.2%), verrucous carcinoma (29.5%) and OSCC (46.5%) and the probability of detecting HPV in oral carcinoma was approximately 4.7 times higher than in normal oral mucosa (Miller and Johnstone, 2001). Contrarily, low prevalence of HPV DNA was reported in some studies. Using microdissection and real-time quantitative PCR analysis, HPV DNA was detected in 1 of 102 (0.98%) microdissected premalignant head and neck lesions (85 from the oral cavity) and 1 of 34 (2.9%) invasive oral cavity carcinomas (Ha et al., 2002). Some investigators reported the specificity of the HPV association to oropharyngeal rather than oral cancers. For example, some particular oropharyngeal sites such as palate and lingual tonsils were more likely to have HPV infection (Genden et al., 2003). Furthermore, it has been reported that HPV appeared to play a definite etiologic role in a substantial fraction of cancers of the oropharynx but possibly a minor role in a small subgroup of cancers of the oral cavity (Herrero et al., 2003).

It has been reported that HPV-positive cancers were less likely to have tobacco-associated p53 mutations, less likely to occur in smokers and alcohol drinkers, and had a distinct basaloid histopathology. HPV positive tumors tend to have wild-type p53, because p53 is functionally inactivated by the E6 oncoprotein (Balz et al., 2003, Haufkamp et al., 2003, Wiest et al., 2002). The pRB function is also inactivated by viral E7 protein in the HPV-positive tumor, therefore, the pRB pathway is disrupted. On the contrary, HPV-negative tumor tends to be disrupted by other mechanisms such as inactivation of the p16$$^{\text{ink4a}}$$ protein either by deletion, mutation or promotor methylation and cyclin D1 amplification (Wiest et al., 2002). In addition, patients with HPV-positive tumor tend to be younger when compared with HPV-negative patients. A significant association between HPV presence and age was established. Patients older than 60 years showed a lower prevalence of the virus (29.4%) compared with patients below this age (77.8%) (Cruz et al., 1996). Ringstrom and co-workers also demonstrated that the HPV DNA was more frequently detected in tonsil and oropharyngeal tumors than that of the oral cavity and the mean age of subjects with HPV16-positive tumors was younger than the patients with HPV-negative tumors.

With regards to the association of HPV and sexual behavior of the patients, in male patients, oral cancer risk increased with self-reported decreasing age at first intercourse, increasing number of sex partners, and a history of genital warts (Schwartz et al., 1998). Recently, a review of the association of oral cancer and the evidence of sexual transmission demonstrated that chances of oral HPV infection increase with age, male sex, and HSV-2 seropositivity. Epidemiologic studies have shown that exposure to HPV increases the risk of head and neck squamous cell carcinoma suggesting that it may be sexually transferred (Scully, 2005). Specific sexual behaviors have been more strongly associated with risk of an HPV-positive tumor, including a history of performing oral sex and oral-anal contact (Herrero et al., 2003, Smith et al., 2004). In our study, when the characteristics of the patients were considered, most of the patients with oral cancer were elderly patients with a long history of betel nut chewing habit, alcohol consumption or smoking. Furthermore, the majority of the tumors were located in the oral cavity, therefore, the
low prevalence of HPV DNA in this study was not unexpected and this indicated that HPV infections may not play an important role in this group of patients.

The presence of HPV has also been investigated in oral leukoplakia, a precancerous lesion, and oral lichen planus, a precancerous condition, in many studies (Sugiyama et al., 2003; Campisi et al., 2004; Lodi et al., 2005). Campisi et al demonstrated an increased risk of HPV infection in oral leukoplakia and oral lichen planus (Campisi et al., 2004). When the clinical variant of oral leukoplakia, i.e., homogeneous versus non-homogeneous leukoplakia, were taken into account, no difference of the prevalence of HPV was demonstrated in both groups. Similarly, when non-atrophic/erosive and atrophic/erosive forms of lichen planus were considered, no significant association with clinical variant was depicted. In contrast, a current review of controversies in oral lichen planus indicated that the detection of HPV in specimens of oral lichen planus ranged from 0-100% (Lodi et al., 2005).

Furthermore, the detection of HPV DNA does not prove a casual relationship, since its presence in the lesional tissue may be casual or result from the disease process or immunosuppressive therapy, as shown by a recent case report of HPV reactivation following treatment of penile erosive lichen planus with topical corticosteroids (von Krogh et al., 2002). In this study, the HPV DNA was not detected in any oral leukoplakia or lichen planus specimens. When the characteristics of the patients in the leukoplakia group were considered, most patients either had a history of smoking, alcohol abuse or betel nut chewing habit. Although most of the patients in the lichen planus group did not have a history of smoking or alcohol consumption, they had a history of systemic diseases such as hypertension and/or type II DM and a long history of using several medications which might induce oral lichenoid drug reaction. Hence, the undetectable HPV DNA was possible in these groups of patients.

In conclusion, the prevalence of HPV-positive sample in this study was 1.54% which was very low compared to other studies. The majority of patients in this study had a long history of smoking, alcohol consumption or betel nut chewing habit, therefore, it is concluded that the HPV-infections may not play an important role in this group of patients. It would be interesting to investigate the prevalence of HPV infections in younger patients with OSCCs who do not have any history of smoking, alcohol abuse or betel nut chewing habit. Because of the low number of the specimens investigated, more specimens should be recruited for future study. In addition, the molecular pathogenesis such as mutation of p53 or aberration in the pRB pathway should be further investigated in these groups of patients.

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References


HPV and Oral Squamous Cell Carcinoma, Leukoplakia and Lichen Planus in Thailand