Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy in Sri Lanka, constituting 35-40% of all cancers (1). Epidemiological data attribute this high incidence to widespread betel quid chewing and smoking among patients (2). However, mutagenesis by components of betel quid and/or smoking may not be sufficient to cause oral cancer, suggesting a role for additional environmental and host cell genetic factors. Recent epidemiological and molecular biological studies have linked cervical cancer to human papillomavirus (HPV) (3-7).

HPV belongs to the papovaviridae family, and in humans more than eighty different subtypes have been identified (8,9). Some, such as types 16,18, 31, 33, 35, 39, 45, 50, 51, 53, 56, 59, 64 and 68, seem to play a role in development of cervical cancer and are characterized as high risk types (10). The two viral oncoproteins of high risk HPVs E6 and E7 promote tumor progression by inactivating p53 and retinoblastoma tumor suppressor gene products, respectively (11, 12). In addition, expression of high risk HPV E6 also increases telomerase activity (13).

HPV prevalences ranging from 0-100% have been reported in OSSC (14, 15). Moreover, there is now evidence for a connection between viral infection by HPV and the exist-
Prevalence of HPV in oral cancer from Sri Lanka

ence of OSCC, particularly of the oropharynx (16-18) and tonsils (19). However, in spite of the high incidence of OSCC in Sri Lanka, studies of patients with the habit of betel quid chewing are scarce (20).

Therefore, the aim of this study was to determine the prevalence of HPV and correlate the HPV status with clinico-pathological parameters of OSCC patients from Sri Lanka.

Materials and Methods

Sixty-eight formalin-fixed paraffin-embedded oral cancer specimens and thirty-four formalin-fixed unembedded biopsies were randomly selected from the archives of the Department of Oral Pathology, Faculty of Dental Sciences, University of Peradeniya and Maharagama National Cancer Institute, Sri Lanka, respectively, for this study. Written informed consent was obtained from the research committee of the Faculty of Dental Sciences, University of Peradeniya, Sri Lanka. According to the clinical information both samples consisted of males and females in a ratio of 4:1, with roughly an equal number of patients who were less or greater than 60 years old. Moreover, more than 50% of the sample consisted of cancers of the buccal mucosa in both groups. However, accurate history of habits such as betel chewing, smoking or alcohol use, as well as size of the local tumor (T classification), was available only from patients ing, smoking or alcohol use, as well as size of the local tumor (T classification), was available only from patients.

For histopathological analysis, 4 consecutive sections were cut from each block, with the first 4 m thick H&E stained sections were used to classify the lesions into well-differentiated squamous cell carcinoma (WDSCC), moderately-differentiated squamous cell carcinoma (MDSCC) or poorly-differentiated squamous cell carcinoma (PDSCC) in both groups of specimens according to Smith & Pindborg's criteria (21).

DNA template preparation and verification

From the paraffin-embedded specimens four consecutive sections were cut from each block, with the first 4 m thick section being stained with H&E to confirm the presence of cancerous tissue. The remaining three 10 m thick sections were collected in a 1.5ml sterile micro-centrifuge tube for DNA extraction. For each block a new microtome blade was used while the gloves were changed frequently to avoid contamination. DNA was isolated with a modified salt precipitation method using PUREGENE DNA isolation kit (Gentra, Minneapolis, USA). Briefly, the tissues were deparaffinized with xylene and placed in 300 l of cell-lysis solution and 3 l of 10mg/ml of proteinase K and incubated overnight at 37°C. Following the protein precipitation DNA was extracted with 100mM Tris HCl and 1mM EDTA. From the formalin-fixed unembedded biopsies, 10mg of tissues containing representative areas OSCC were minced (cut into small pieces) and washed under running water overnight. DNA was extracted using the PUREGENE-DNA isolation kit in a manner similar to paraffin-embedded specimens, except for the final step where the DNA pellet was dissolved in 100 l of DNA hydration solution (instead of 20 l).

Beta-globin primers PC03/04 or KM28/29 were used to confirm the presence of amplifiable DNA. The quantity of DNA extracted from each group of specimens was also measured using a spectrophotometer (DU64, Beckman, CA, USA). The quantities of DNA extracted from formalin-fixed paraffin-embedded and formalin-fixed unembedded specimens were 30-50 and 550-600 ng/l respectively. Moreover, amplification of the beta-globin gene with DNA extracted from paraffin-embedded specimens was seen in weaker bands by gel visualization compared to DNA from formalin-fixed unembedded specimens.

Polymerase chain reaction (PCR)

Consensus primers GP5+/6+, which amplify 140 base pairs in the highly conserved L1 region of the HPV genome, were used in the PCR as it allows detection of most important HPV sub types. In order to determine the sensitivity of the primers, 100ng/l of HPV 16 DNA from CaSki cells were subjected to 10 fold dilution and tested with PCR as previously reported (22), and the PCR amplimers were separated on 2% agarose gel (ME-agarose, Iwaiakagaku Co., Tokyo, Japan). Thereafter, 0.5 l of PCR products containing 1pg to 100 ng of viral DNA from the first reaction were again subjected to PCR under similar conditions for 25 cycles in order to determine whether it would improve the sensitivity of the GP5+/6+ primers. To rule out the possibility of contamination, negative control containing all reaction components (except template DNA) with milliQ water was used in the first round PCR. Subsequently, the negative control of the second round PCR contained 0.5 l from the negative control of the first round PCR. The results revealed detection of HPV DNA at the 100 fg level by gel electrophoresis as previously reported (22). However, only a faint band was detected at the 100 fg dilution level during first round PCR. During second round PCR, stronger bands were visualized at the 100 fg level. No HPV products were detected in the first or second round negatives controls, which confirmed the absence of contamination. Thereafter, hot start PCR was performed in a final reaction volume of 50 l containing 3-5 l of DNA sample (5 l from paraffin-embedded specimens, 3 l formalin-fixed unembedded specimens), 50 mM KCl, 10 mM Tris HCl- pH 8.3, 2 mM of each dNTP, 3.5 mM MgCl2, 1U DNA polymerase (AmpliTaq, Applied Biosystems, New Jersey, USA) and 50 pmol of each primer as previously reported (22). The
Condition for PCR was as follows: initial denaturation step at 94°C for 15 min, followed by 40 cycles at 94°C for 1 min, 40°C for 2 min, 72°C for 1.5 min with a final elongation step at 72°C for 4 min. Each PCR experiment was performed with a negative control (Milli Q water) and positive control (DNA from CaSki cells). PCR amplimers were separated on 2% agarose gel, visualized under UV light, and recorded on Polaroid film. Subsequently, the samples that resulted in faintly positive bands were again subjected to PCR, using 0.5 μl of PCR products as the DNA template from the previous reaction. Other components and PCR conditions were similar to the first reaction except for the reduction of number of cycles from 40 to 25.

**Direct cycle sequencing**

GP5+/6+ amplimers from the 38 positive cases were subjected to direct sequencing in order to determine the subtype. The sample preparation for the sequencing reaction was carried out in three stages as follows:

Initially, PCR products were purified using QIAquick nucleotide removal kit (QIAGEN, Victoria, AUSTRALIA) according to the manufacturer’s instructions. Thereafter, sequencing reaction was performed in a final reaction volume of 10 μl with 4 μl of Big Dye Terminator ready reaction mix (Applied Biosystems, USA), 50-100 ng of DNA template (purified PCR products), 25 pmol of primer (GP5+ or 6+). Sequencing reaction for each positive sample was carried out twice, using the forward or reverse primer in each reaction, as the readable sequence length from one reaction was only 70 bp. Conditions for PCR were as follows: rapid thermal ramp to 96°C followed by 30 cycles of 96°C 10 s, 50°C 5 s, 60°C 4 min. The extension products were then purified and precipitated using ethanol in microcentrifuge tubes (according to the protocol provided with the ABI Prism Big Dye Terminator cycle sequencing kit). The dried precipitated extension products were then resuspended in 12 μl of template suspension region (TSR, Applied Biosystems, USA) and denatured at 96°C for 3 min. The samples were run on an ABI 310 prism genetic analyzer and analyzed with ABI 310 prism collection software (Applied Biosystems, USA). The sequences thus obtained were compared with the known HPV sequences using returned results from the GenBank on-line BLAST server at http://www.ncbi.nlm.nih.gov/BLAST/.

Finally, the distribution of clinico-pathological parameters by HPV status was compared using Pearson’s Chi-square test at 5% level of significance.

**Results**

**HPV prevalence**

The HPV prevalence of the overall samples was 38/102 (37.2%). However, the HPV prevalence detected from paraffin-embedded vs formalin-fixed non-paraffin-embedded tissue was 17/68 (25%) and 21/34 (61.7%), respectively (p<0.0003). Direct sequencing using PCR products of the 38 HPV positive samples by consensus primers resulted in identification of known HPV sequences in 30 samples. Predominantly high risk HPV types were identified from both groups of specimens (Table 1). From the 102 OSCC, HPV 16, 18, 45 and 66 were detected in 18, 3, 3 and 1 OSCC while HPV 6 and 11 were detected in 3 and 2 OSCC. However, the remaining 8 positive samples by PCR did not yield identifiable HPV sequences and could not be subtyped.

Correlation of HPV positivity with clinical and pathological information and its statistical significances are summarized in Tables 2 and 3. Similar correlations were also observed for paraffin-embedded and formalin-fixed unembedded samples when analyzed separately. Accordingly, there were no statistically significant correlations between HPV detection and clinico-pathological parameters, except for the size of the local tumor.

**Site & histopathological diagnosis**

Thirty seven percent of the cancers derived from the buccal mucosa and 50% of tongue cancers were HPV positive while no HPV was detected from the cancers of the lower lip. HPV positivity was 39, 40 and 33% for well, moderate- and poorly-differentiated OSCC, respectively. Poorly-differentiated cancers were less likely to be associated with HPV than well- or moderately-differentiated OSCC. However, these associations were not statistically significant.

**Habits**

Of the 31 OSCC patients for whom history of hab-

<table>
<thead>
<tr>
<th>Table 1: The percentage of different HPV types identified in the two groups of OSCC</th>
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</thead>
<tbody>
<tr>
<td>Paraffin-embedded</td>
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<tr>
<td>OSCC</td>
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<tr>
<td>High risk HPV</td>
</tr>
<tr>
<td>Low risk HPV</td>
</tr>
<tr>
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</table>

High risk HPV types identified- 16,18,45,66
Low risk HPV types identified- 6,11
its were available, 25 were habitual betel quid chewers, and four patients had a history of smoking or alcohol consumption without betel quid chewing. Only two patients did not have any risk habits (Table 3). OSCC of 64% of patients who were habitual betel quid chewers were infected with HPV while only 36% were uninfected (p>0.05). However, the sample of patients without any risk habits or risk habits other than betel quid chewing was too small for accurate statistical analysis.

**Tumor size**

HPV detection rate was higher in smaller local tumors (T1&T2) compared to large tumors (T3 & T4) (p<0.025)

**Discussion**

Using PCR based techniques, HPV DNA has been detected in a variety of head and neck tissues and sites (14, 15). The overall prevalence of HPV infection detected in our study was 37.2%. However, statistically significant differences existed between prevalence of HPV detected from formalin-fixed unembedded (61.7%) vs paraffin-embedded tissues (25%) (p<0.0003). The observed difference may be due to differences in the quantity of DNA extracted from the two types of tissue, as higher DNA yield was obtained from formalin-fixed unembedded tissue compared to paraffin-embedded tissue. DNA degradation during tissue processing may account for the low DNA yield (23) from paraffin-embedded tissues. Although DNA degradation can occur during formalin fixation, previous studies (24) have shown DNA extracted after one month of fixation to successfully amplify 270 bp segments. However, as our specimens were subjected to DNA extraction within two weeks of fixation, effect of formalin fixation may have not influenced the ability of HPV amplification of formalin-fixed unembedded specimens. Furthermore, previous studies have reported low HPV prevalence in tumors with advanced T classification (T3/4) (25). Therefore, the size of the tumor may have also contributed to this difference as most of the paraffin-embedded OSCC specimens were randomly selected from patients subjected to surgery who may have had rela-

<table>
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<th>Variable</th>
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<tr>
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</tr>
<tr>
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<td>2</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>Tumor size</td>
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<td>20</td>
<td>15</td>
<td>5</td>
<td>p&lt;0.025</td>
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<td></td>
<td>T3&amp;T4</td>
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<td></td>
<td>unknown</td>
<td>4</td>
<td>3</td>
<td>1</td>
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</tbody>
</table>

WD- well differentiated, MD-moderately differentiated, PD-poorly differentiated OSCC

*p Pearson’s Chi-square test

Table 2: Association of HPV positivity in OSCC with clinical and pathological variables

Table 3: Correlation of HPV positivity of formalin-fixed unembedded OSCC with habits and size of the local tumor

T1&T2 - Size of primary tumor <4cm, T3&T4 - Size of primary tumor >4cm.
tively large local tumors because all the patients had more than one paraffin embedded block of tumor.

In the present study, 61.7% of formalin-fixed unembedded OSCC contained HPV. This prevalence is comparable to previous studies in India and confirms the observation of presence of higher HPV infection rate in betel quid associated OSCC from South East Asia (20) compared to OSCC from Western countries (14, 15). On the other hand, HPV detection rate for paraffin-embedded specimens in the same population was only 25%. Therefore, formalin-fixed unembedded biopsies may be more suitable for DNA extraction when using PCR based techniques for HPV detection.

Upon sequencing, the well-known genital high risk HPV types 16, 18, 45 were also identified from OSCC. Therefore, the high risk types in both oral and genital setting seem to be similar (20). However, 20% of the HPV positive samples by PCR did not give an identifiable HPV sequence. Presence of multiple HPV infection, amplification of cellular DNA resulting in multiple bands in gel electrophoresis, or false positive PCR results may have resulted in unidentifiable HPV sequences and need further investigations. In our study, HPV detection rate was significantly higher in small local tumors compared to large tumors in accordance with studies by Mellin (25). However, we did not find any differences in HPV detection by age, gender, site, habits or tumor differentiation (Table 2 & 3) similar to previous studies (26).

Previous studies have already suggested an association of HPV with cancers of the oropharynx and tonsils (16-19). However, our sample did not contain oropharyngeal carcinoma, and the highest frequency of OSCC was found in the buccal mucosa. Therefore, the detection of HPV in relatively high rates, despite the absence of oropharyngeal carcinoma, may indicate a role for HPV in the oral carcinogenesis, especially in populations where oral cancer is associated with habitual betel quid chewing. The high HPV incidence may also support a multifactor model of oral cancer causation. However, further molecular biological studies are necessary to determine the exact role of HPV in oral carcinogenesis.

References


(Accepted for publication December 2, 2002)