

HPV infection and the alterations of the pRB pathway in oral carcinogenesis

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Abstract. Inactivation of the retinoblastoma (pRB) pathway is a common event in oral squamous cell carcinoma particularly through the aberrant expression of the components within this pathway. This study examines the alterations of molecules within the pRB pathway by looking at the presence of homozygous deletions in *p16^{INK4A}* and the expression patterns of pRB, cyclin D1 and CDK4, as well as the presence of human papillomavirus (HPV) in our samples. In our study, 5/20 samples demonstrated deletions of *p16^{INK4A}* exon 1 α . pRB overexpression was found in 20/20 samples, the expression was mainly observed in all layers of the epithelia, particularly in the basal layer where cells are actively dividing and aberrant pRB expression was found in 12/20 samples. Cyclin D1 and CDK4 overexpression was detected in 6/20 and 2/20 samples respectively in comparison to hyperplasias where both proteins were either not expressed or expressed at minimal levels (<10%). Strikingly, HPV was found to be present in all of our samples, suggesting that HPV plays a significant role in driving oral carcinogenesis. Notably, 17/20 of our samples showed more than one alteration in the pRB pathway, however, we did not find any significant relationship between the presence of HPV, homozygous deletion of *p16^{INK4A}* and overexpression of pRB, cyclin D1 and CDK4. Collectively, this data demonstrates that alterations in the pRB pathway are a common event and involve the aberration of more than one molecule within the pathway. Furthermore, the involvement of HPV in all our samples suggests that HPV infection may play an important role in oral carcinogenesis.

Introduction

The p53 and the retinoblastoma (pRB) pathways are two major tumour suppressor pathways that control cell proliferation

and senescence. The molecules involved in these two pathways are commonly abrogated in human malignancies including oral cancer. Oral cancer is a major cancer in Asian countries; it is the third most common cancer amongst Malaysian Indians and the incidence amongst Malaysian Indian females has been reported to be the highest in the world (1). In the West, cigarette-smoking has been reported to be the main cause for oral cancer whereas betel quid chewing is associated with a large fraction of oral cancer cases in Asia. Many studies have been conducted in the West on Caucasian cancer samples, however Asian and Caucasian oral cancers have distinctly different etiological factors and the genetic changes contributing to oral carcinogenesis in the Asian population are still unclear.

The pRB pathway comprises mainly of RB, *p16^{INK4A}*, cyclin D1 and cyclin-dependent kinase 4 (CDK4) molecules. There is mounting evidence that the pRB pathway is rendered dysfunctional in oral cancers by the dysregulation of upstream-positive (*cyclin D1* and *CDK6*) or -negative (*p21^{WAF1}*, *p27^{KIP1}*, *p16^{INK4A}* and *p19^{ARF}*) regulators (2). Inactivation of pRB has been reported to play an early role in head and neck cancer development (3) and also in the development of different types of human cancers (4). *p16^{INK4A}* is a tumour suppressor gene whose activity is attributed to its biochemical ability to bind to both CDK4 and CDK6 and inhibit the catalytic activity of the CDK4-6/cyclin D1 enzyme complex required for pRB phosphorylation and cell cycle progression (5). The inactivation of *p16^{INK4A}* has been reported to occur by three main mechanisms: homozygous deletion, hypermethylation of the 5' end of the gene, and mutation (6).

Molecular studies in head and neck squamous cell carcinomas (HNSCC) have indicated that *cyclin D1* residing in the 11q13 region is found to be amplified in approximately 30% of HNSCC (7). Cyclin D1 is a cell cycle regulatory protein that binds CDK4 and this complex promotes the phosphorylation of pRB; pRB phosphorylation is required for progression through the G1/S cell cycle check-point (8). By investigating the status of *p16^{INK4A}* and the expression pattern of pRB, cyclin D1 and CDK4 in this study, major changes in the pRB pathway in oral carcinomas will be elucidated.

It has been well established that the presence of HPV causes mucosal cancers such as cervical and oral cancer. Genetic patterns in head and neck cancers associated with and without active HPV have been shown to be significantly

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Table I. The primer sequences of p16 exon 1 α , p16 exon 2, β -actin, GP5⁺/GP6⁺, HPV 16 and HPV 18 and the respective annealing temperatures.

| Name | Primer sequences | Annealing temperature (°C) |
|------------------------------------|---|----------------------------|
| p16 exon 1 α | p16 exon 1 α F: 5' GAG CAG CAT GGA GCC TTC 3' p16 exon 1 α R: 5' AAT TCC CCT GCA AAC TTC GT 3' | 51 |
| p16 exon 2 | p16 exon 2 F: 5' GGG CAC CAG AGG CAG TAA C 3' p16 exon 2 R: 5' GCG TGA GCT GAG GCA AGA 3' | 51 |
| β -actin | β -actin F: 5' CTG TGG CAT CCA CGA AAC TA 3' β -actin R: 5' AGG AAA GAC ACC CAC CTT GA 3' | 56 |
| GP5 ⁺ /GP6 ⁺ | GP5 ⁺ : 5' TTT GTT ACT GTG GTA GAT ACT AC 3' GP6 ⁺ : 5' GAA AAA TAA ACT GTA AAT CAT ATT 3' | 40 |
| HPV 16 | HPV 16 F: 5' GAA CAG CAA TAC AAC AAA CCC G3' HPV 16 R: 5' CCA TGC ATG ATT ACA GCT GG 3 \bar{a} | 63 |
| HPV 18 | HPV 18 F: 5' ACC TTA ATG AAA AAC CAC GA 3 HPV 18 R: 5' CGT CGT TTA GAG TCG TTC CTG 3' | 60 |

different (9). The HPV oncogenes E6 and E7 are known to disrupt the p53 and pRB pathways respectively (10) and the inactivation of the pRB pathway is an important event in oral carcinogenesis to allow cellular immortalization and continuous proliferation. It has been demonstrated that simultaneous ecotopic expression of CDK4 and HPV 16 E7 abrogate the tumour suppressive function of pRB and are equally efficient in extending the life span of normal oral keratinocytes (11). Interestingly, the involvement of HPV in human cancers has been reported to be dependent on geographical areas, and for oesophageal carcinomas in particular, HPV infection is more prevalent in Asian countries such as China and Japan in comparison to Europe and the USA (12). As an inverse correlation between HPV infection and p53 mutations has been reported (13), this trend is most likely similar in oral cancer where HPV can be expected to play a major role in oral carcinogenesis in Malaysia and Asian countries as the incidence of p53 mutations are lower in oral cancers seen in these countries (14). Thus, the aim of this study is to determine the involvement of HPV and the incidence of pRB pathway inactivation by analysing the homozygous deletion of *p16^{INK4A}*, expression pattern of pRB, cyclin D1 and CDK4 in Malaysian Indian patients which may contribute to oral carcinogenesis.

Materials and methods

Tumour specimens. Twenty archival formalin-fixed paraffin-embedded samples of well-differentiated oral squamous cell carcinomas were obtained from the Institute for Medical Research (IMR), Malaysia archival collection. Well-documented pathology reports were obtained from the same centre. The specimens collected were confirmed to be of the well-differentiated type according to the WHO classification. Betel quid chewing history of the patients was obtained from clinical records.

Tissue preparation, laser micro-dissection, pressure catapulting (LMPC) and DNA extraction. The specimens obtained were previously fixed in formalin and embedded in paraffin according to routine histological procedures. Five micron sections were cut onto clean glass slides. The slides were deparaffinized in 2 changes of xylene for 2 min, rehydrated in 100, 95 and 70% ethanol for 2 min each, rinsed in distilled water, stained with haematoxylin and eosin (H&E) for 1 min and 30 sec respectively, rinsed in distilled water, and finally immersed in 95 and 100% ethanol for 1 min each before the slides were dried at 37°C for 30 min. Epithelia cells were isolated using LMPC technology on the PALM Laser-Microbeam (P.A.L.M., Wolfratshausen, Germany) as described elsewhere (15). This procedure ensures that downstream analyses are performed on a pure population of epithelia cells without the interference of stromal cells. The micro-dissected cells were catapulted into the lid of a 0.5-ml reaction tube containing 25 μ l of ALT buffer (Qiagen, Germany). Between 3000-7000 cells were isolated for each DNA extraction using the QIAamp DNA micro extraction kit (Qiagen) according to the manufacturer's specifications, with slight modifications in the volumes of buffer used.

Polymerase chain reaction, *p16^{INK4A}* homozygous gene deletion and HPV detection. Extracted DNA was used for PCR amplification and β -actin was used as an internal control for both homozygous deletion and HPV analyses. For detecting the presence of HPV, consensus primer GP5/GP6 was used (16) and followed by specific HPV 16 (17) and 18 primers (18) in positive cases. For detecting the homozygous deletion of *p16^{INK4A}*, primers for *p16^{INK4A}* exon 2 were designed using the Primer Premier software (Premier Biosoft International, USA) and primers for *p16^{INK4A}* exon 1 α were obtained from Xing *et al* (19). Due to the low concentration of extracted DNA, two rounds of PCR were performed. The first round of PCR was performed in a 10- μ l volume mixture containing 3-5 μ l of

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NA, 1 μ M of each primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 3 mM MgCl₂, thermostable AccuPrime protein™, 10% glycerol, 200 μ M dNTPs, and 0.2 μ l AccuPrime™ 3' TaqDNA polymerase (Invitrogen Life Technologies, USA). Two microliters of PCR product from the first reaction was used as the template for the second PCR which was performed in a 50- μ l reaction mixture containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% TritonX-100, 2 μ l dNTPs and 2.5 U TaqDNA polymerase (Promega, Madison, USA). Both rounds of amplification were performed in the GeneAmp PCR System 9700 (Applied Biosystems, USA). PCR conditions were 95°C for 2 min, followed by 30 cycles of 95°C denaturing for 30 sec, annealing at respective temperatures (40-63°C) for 30 sec (Table I), 68°C (AccuPrime Taq polymerase) or 72°C (Promega Taq polymerase) elongation for 30 sec. The final extension was conducted for 4 min at the respective elongation temperatures. The resulting PCR products were electrophoresed on agarose gels.

Immunohistochemistry. Immunohistochemistry was performed using the Dakocytomation Envision® Dual Link System-HRP (DAB⁺) kit (Dako, USA) according to the manufacturer's protocol. Briefly, 5- μ m sections of paraffin-embedded tissue were placed in xylene for dewaxing and then graded ethanol for rehydrating as reported elsewhere (14). The tissue sections were treated with 10 mM Tris-HCl, 1 mM EDTA, pH 9.0 in a microwave for 20 min for antigen retrieval. The tissues were then subjected to endogenous enzyme block for 10 min followed by incubation with primary and secondary antibody for 30 min each (mouse monoclonal anti-human retinoblastoma antibody at 1:50 dilution, Dako; Mouse monoclonal anti-human cyclin D1 at 1:30 dilution, Dako; Mouse monoclonal anti-human CDK4 at 1:50 dilution, Santa Cruz, USA). The tissues were then incubated with diaminobenzidine (DAB) as a chromagen, counterstained with hematoxylin, and dehydrated with graded ethanol and xylene. Overexpression of the respective protein was scored if $\geq 10\%$ of the nuclei were brown in comparison to the hyperplasias. Negative controls for the immunostaining were performed by substituting the primary antibody with non-immune mouse serum.

Results

We have analyzed twenty patients with well-differentiated oral squamous cell carcinoma. Thirteen out of 20 patients were betel quid chewers and we were unable to confirm the betel quid chewing habits for the remaining 7. Twelve out of 20 (60%) of our patients had cancer of the buccal mucosa, while others had cancers on the mandible, cheek/lip, palate, alveolus, alveolar ridge or maxilla respectively (Table II).

p16^{INK4A} homozygous deletion. In our study, 5/20 samples demonstrated deletion of p16^{INK4A} exon 1 α , while no deletions were found for p16^{INK4A} exon 2 (Fig. 1). False negatives were negated with the amplification of the house-keeping gene in multiplex PCR.

Immunohistochemistry. pRB expression was found in 20/20 samples, and in 17/20 samples, staining was found in all layers of the epithelia including the basal layer where cells

Table II. The relationship between betel quid chewer and the site of cancer occurrence.

| | Site | |
|------------------------|-------------------|--------------|
| | Buccal mucosa (%) | Others (%) |
| Betel Quid chewer (13) | 9/13 (69.20) | 4/13 (30.80) |
| Unknown habit (7) | 3/7 (49.20) | 4/7 (57.10) |
| Total (20) | 12/20 (60) | 8/20 (40) |

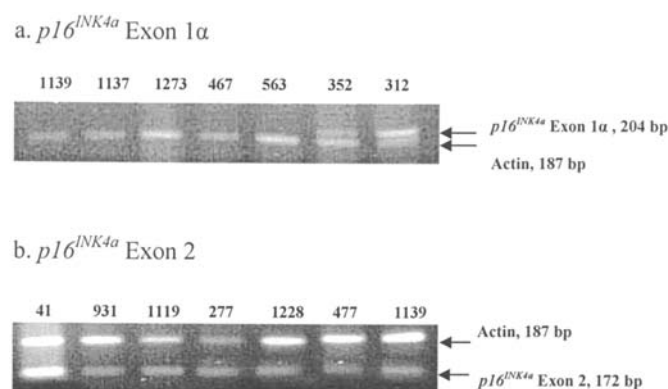


Figure 1. Homozygous deletion analysis of p16^{INK4A}. (a) Lanes 1-5, representative tumour samples indicating the deletion of p16^{INK4A} exon 1 α ; Lanes 7 and 8, representative tumour samples with no deletion. (b) Lanes 1-7, representative tumour samples demonstrating p16^{INK4A} exon 2 is intact.

are actively dividing in contrast to the hyperplastic samples where staining was minimal (Fig. 2a and b). Cyclin D1 and CDK4 overexpression were detected in 6/20 and 2/20 samples respectively, in comparison to hyperplasias where both proteins were either not expressed or expressed at minimal levels (<10%) (Fig. 2c-f).

The presence of human papillomavirus. By amplifying the HPV L1 region, we found the presence of HPV DNA in all of the OSCC samples (Table III and Fig. 3). Further PCR analysis demonstrated that 6/20 (30%) specimens were infected by HPV 16, 15/20 (75%) by HPV 18 and 4 specimens had the presence of both these subtypes (Fig. 3).

Discussion

The inactivation of tumour suppressor pathways is crucial in carcinogenesis as it leads to uncontrolled cell growth and the formation of tumours. In this study we investigated alterations in the pRB pathway, specifically of its constituents p16^{INK4A}, cyclin D1, CDK4 and RB. Hypophosphorylated pRB inhibits S phase entry by physically associating with members of the E2F transcription factor family forming the pRB-E2F complex which represses the transcription of target genes that regulate DNA synthesis, thus inducing apoptosis in cells (2). Upon pRB phosphorylation, pRB is unable to bind E2F, thus permitting transcription and leading to differentiation. In cancer, the ability of retinoblastoma protein to bind with E2F can be

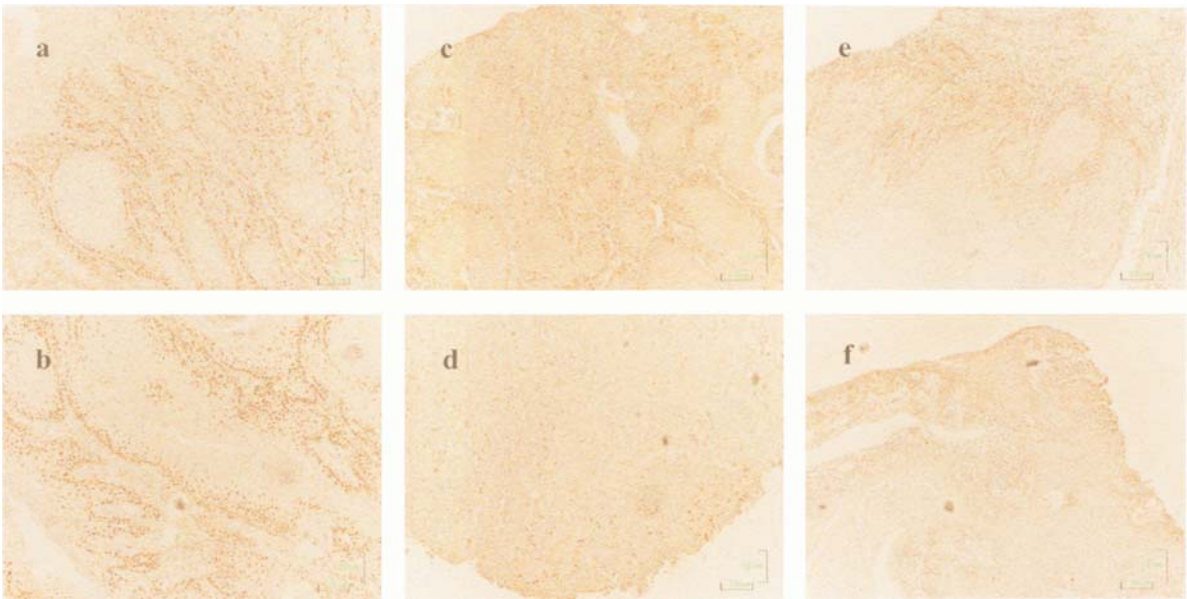


Figure 2. Immunohistochemistry demonstrating the expression of pRB, cyclin D1 and CDK4 in OSCC. (a, b) pRB expression. (c, d) Cyclin D1 expression. (e, f) CDK4 expression. Original magnification x100.

Table III. The involvement of HPV and alterations of the pRB pathway in oral cancer.

| Alterations | HPV 16 | | HPV 18 | | p16 deletion | | Cyclin D1 expression | | CDK4 expression | |
|-------------------|----------|----------|----------|----------|--------------|----------|----------------------|----------|-----------------|----------|
| Status | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| BQ chewing | | | | | | | | | | |
| Chewer (13) | 5 | 8 | 9 | 4 | 4 | 9 | 3 | 10 | 2 | 11 |
| Habit unknown (7) | 1 | 6 | 6 | 1 | 1 | 6 | 3 | 4 | 0 | 7 |
| Total (%) | 6 (30) | 14 (70) | 15 (75) | 5 (25) | 5 (25) | 15 (75) | 6 (30) | 14 (70) | 2 (10) | 18 (90) |

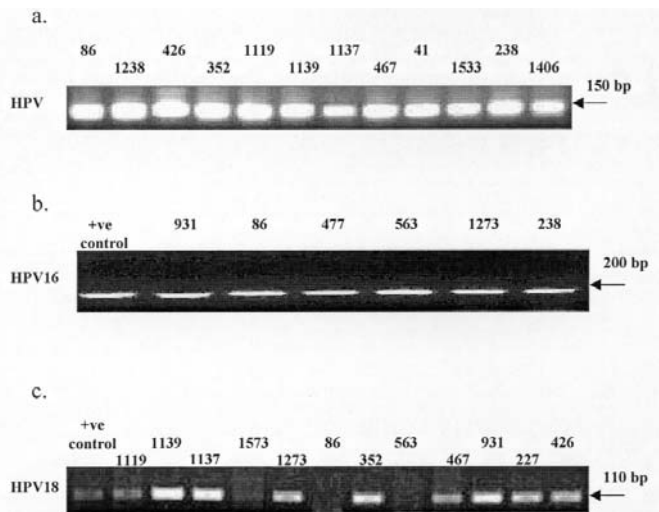


Figure 3. Analysis of the presence of HPV DNA by PCR. (a) HPV DNA detected using GP5/GP6 universal primers. Lanes 1-12, representative specimens demonstrating HPV positivity. (b) Detection of HPV 16 DNA. Lanes 2-7, tumour samples positive for HPV 16. Lane 1, positive control for HPV 16. (c) Detection of HPV 18 DNA. An amplicon of ~110 bp indicates the presence of HPV 18 DNA.

disrupted either by overexpression of CDK4 and cyclin D1, which promote the phosphorylation of retinoblastoma protein, or by inactivation of *p16^{INK4A}* through homozygous deletion or hypermethylation, since wild-type *p16^{INK4A}* inhibits the formation of the CDK4-cyclin D1 complex.

The *p16^{INK4A}* gene was analyzed for homozygous deletions by examining both exon 1 α and exon 2 of *p16^{INK4A}*. Homozygous deletion in *p16^{INK4A}* exon 2 was not seen in our samples but *p16^{INK4A}* exon 1 α was deleted in 5/20 (25%) samples, of which four were betel quid chewers and one was of unknown habit, however the betel quid chewing habit has shown to have no significant relationship with the homozygous deletion of *p16^{INK4A}* after analysis with Fisher's exact test ($P < 0.05$) (Table III). The frequency of *p16^{INK4A}* deletion is consistent with a previous report by Kresty *et al* who reported *p16^{INK4A}* exon 1 α homozygous deletion was more prevalent than homozygous deletion of exon 2 and found in 11.5% (3/26) of severe oral epithelial dysplasia (20). The low deletion rate, however does not take into consideration the inactivation of *p16^{INK4A}* by other mechanisms such as hypermethylation that may render *p16^{INK4A}* inactive.



SPANDIDOS codes a nuclear phosphoprotein which is involved in cell control and cell differentiation, the pRB protein is mutated or absent in a variety of human malignancies (21). We studied pRB expression by immunohistochemistry. The interpretation of pRB expression in squamous cell carcinoma was made following previously published criteria by Geradts *et al* (21), where normal pRB expression was considered when definite nuclear staining was identified in all tumour cells; and abnormal pRB expression was scored if the staining intensity was not uniform, and not all nuclei, either normal or tumour were stained. All of our samples (20/20) showed overexpression of pRB (>10%) and the expression of pRB was most intense at the hyperproliferative basal layer and minimal in the differentiated cells. Aberrant pRB expression was found in 12/20 (60%) of our samples where pRB expression was found in some part of the tumour cells but their neighbouring cells were pRB-negative.

Cyclin D1 and CDK4 are downstream proteins/effectors of *p16^{INK4A}* which catalyze the phosphorylation of pRB, leading to the dissociation of pRB from E2F (22). Cyclin D1 is often overexpressed at both transcriptional and translational levels, and associated with a more rapid and frequent recurrence of cancer (23). Protein overexpression may also occur via other mechanisms which precede gene amplification such as translocations, inversions or yet unknown causes of transcriptional activation. Moreover, deregulation of cyclin D1 either by overexpression or mutation may contribute to the resistance of head and neck squamous cell carcinomas to EGFR inhibitors and other known drugs for cancer, thus the role of cyclin D1 as a prognostic marker warrants additional analysis (24). Cyclin-dependent kinases (CDKs) are critical regulators of cell cycle progression and RNA transcription (25). A variety of genetic and epigenetic events cause universal overactivity of CDKs in human cancer, and their inhibition can lead to both cell cycle arrest and apoptosis (25). Cyclin-dependent kinase 4 (CDK4) is involved in regulation of G1 to S transition by interaction with cyclin D1, the level of this complex has been reported to be significantly higher in OSCC than in oral precancer lesions and normal oral mucosa (26). In our samples, 14/20 (70%) demonstrated minimal or undetectable cyclin D1 expression and 18/20 (90%) demonstrated minimal or undetectable CDK4 expression (Table III), however there was no significant relationship between both cyclin D1 and CDK4 overexpression and betel quid chewing habit (Fisher's exact test, $P < 0.05$, Table III). Infrequent expression of cyclin D1 and CDK4 in this study, suggested that the activation effects of this gene may be accomplished by alternative mechanisms. One possibility would be the involvement of the human papillomavirus E7 protein in the inactivation of the RB protein which would substitute for the activation effect of the cyclin D1/CDK4 complex, as expression levels in our samples were relatively low compared to previously reported data.

In contrast to the lack of CDK4 and cyclin D1 overexpression, HPV infection appears to have a more significant association with OSCC. The role of HPV in oral carcinogenesis is still unclear, but it is believed that the expression of E6/E7 oncogenes of HPV in tumours may indicate an active role of HPV in the development of oral cancer since the E6 oncoprotein can disrupt the p53 pathway by targeting the

p53 protein for ubiquitination and degradation, while the E7 oncoprotein can inactivate pRB. In our study, we found the presence of HPV in all of our samples (20/20) when using consensus primers which target the L1 region of all HPV types, whereas no HPV DNA was detected in DNA from 20 normal individuals (data not shown). These results were confirmed by an independent laboratory. Amongst the 20 samples, 15 were confirmed to carry HPV 18 by using HPV 18-specific primers, while 6 carried HPV 16 detected by HPV 16-specific primers. HPV 16 and 18 are the most prevalent HPV types and have been reported in premalignant lesions and squamous cell lesions (27). The significantly high prevalence of HPV in our cohort has not been presented by other groups, which mostly report HPV presence ranging from 11-74% (28,29). The high prevalence of HPV is most likely due to our relatively small sample size, as well as our tight selection criteria, where the majority of the patients were Indian females who chew betel quid and do not smoke. This is consistent with reports of HPV-positive head and neck cancers shown to be more commonly observed in patients who do not smoke (30).

In conclusion, the high prevalence of HPV suggests that HPV might be involved in oral carcinogenesis, possibly by the binding of the E7 oncoprotein to pRB, thus decreasing the ability of pRB to bind to the transcription factor E2F. This is also supported by our findings that while pRB expression is high in our cohort, expression of cyclin D1 and CDK4 are relatively low. However, our results also suggest that multiple events have to take place simultaneously to inactivate this tumour suppressor pathway as 17/20 (85%) of our samples showed more than one alteration within the same pathway (Table III). As this is the first preliminary report of the significance of the involvement of HPV in oral carcinogenesis in Malaysian patients particularly among the Indian ethnic group where a majority of these OSCC lesions are associated with betel quid chewing, HPV studies need to be performed in a larger cohort with equal numbers of healthy individuals to identify the baseline presence of this virus in the oral cavity of Malaysians. The role of HPV in oral carcinogenesis and the stimulation of immune response caused by HPV warrant further research in order to better understand the role of HPV in oral cancer. Viral infection in combination with existing chemical carcinogens may be the causative agents for the development of oral cancer in Malaysia.

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