Detection of p53, Bax, Rb, p16 and Human Papillomavirus DNA in Betel Quid Associated Oral Squamous Cell Carcinoma in Myanmar

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Chronic betel quid chewing has a strong correlation with oral cancer. In order to examine relationship of p53 and p16, and the prevalence of human papillomavirus (HPV), we studied p53, Bax, p16INK4a, Rb and HPV prevalence using forty-seven cases of betel quid associated oral squamous cell carcinoma (OSCC) in Myanmar. HPV was detected by polymerase chain reaction (PCR), using primers MY09 / MY11 and L1C1 / L1C2 and L1C2m. Immunohistochemically, 59.6% were p53-positive and Bax-negative, and 68.0% were p16INK4a-negative and Rb-positive. HPV DNA was detected in only 14.9% of all OSCCs.

The expression of p53/Bax relation (P<0.01) and p16INK4a/Rb relation (P<0.001) were found in betel quid associated OSCCs when compared with normal mucosa. Therefore, betel quid plays a critical role in oral carcinogenesis in Myanmar.

Key words: oral squamous cell carcinoma; betel quid; p53; p16INK4a; human papillomavirus

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Introduction

The chronic habit of betel quid chewing has a strong correlation with oral cancer in Myanmar and other Southeast Asian countries (1, 2). In Myanmar, betel quid is betel leaf wrapped with lime and areca nut, with or without tobacco products. Carcinogenesis associated with the betel quid habit is a multistep process, and an accumulation of several mutations causes the progression of cancer (2, 3). p53 mutation is the most commonly identified in human malignancies, including oral squamous cell carcinoma (OSCC) (4-7). The p53 has a direct effect on apoptosis by upregulating Bax expression (8). p16 inhibits the binding of cyclin-dependent kinases (CDKs) 4 and 6 and cyclin D to form a CDK4-cyclin D complex. This complex promotes the phosphorylation of Rb and the release of transcriptional factor, which accelerates the cell cycle (4, 9, 10).

The E6 and E7 proteins from high-risk types of HPV cause degradation of p53 and inactivation of Rb (11-13). Thus, increased risk of oral cancer development may be associated with high-risk HPV infection in combination with other established risk factors such as betel quid chewing (14-16).

In this study, we evaluated p53, Bax, Rb, p16INK4a and HPV DNA expressions and their relations in betel quid chewing associated OSCCs in Myanmar.

Materials and Methods

Tissue sample collection

Samples from 47 previously untreated OSCC patients were collected at the Department of Oral Medicine and Pathology, Institute of Dental Medicine, Yangon, Myanmar, during January 2000 to August 2001. All cases involved the betel quid chewing habit. Twenty-one cases of normal gingival tissues were used as normal control. The biopsy specimens were fixed in 10% buffered formalin solution and embedded in paraffin.

Immunohistochemistry

An immunohistochemical study was performed on
the paraffin sections by streptavidin-biotin-immunoperoxidase staining methods. Four μm sections were deparaffinised in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide in absolute methanol for 30 min, followed by washing in phosphate-buffered saline (PBS). For better detection of p53, Bax, Rb and p16INK4a, sections were pretreated with microwave oven heating (three cycles of 5 min in 0.01M citrate buffer, pH 6.0). Nonspecific bindings were blocked by incubating the slides in 10% normal rabbit serum for 10 min. After an overnight incubation with the primary antibodies, anti-p53 (DO-7, Dako, Glostrup, Denmark, 1:75), Bax (B-9, Santa Cruz, CA, USA, 1:50), anti-human retinoblastoma gene product (Rb1, Dako, Glostrup, Denmark, Denmark, 1:50) and p16INK4a/MTS1 (Chemicon, CA, USA, 1:50), secondary biotinylated anti-mouse antibody was applied followed by streptavidin-biotin-peroxidase complex. The color was developed by 3,4-diaminobenzidine (DAB), after which the sections were counterstained with methyl green. For the negative control, PBS was used instead of the primary antibodies. Each specimen was observed under magnification of 400× and was graded as follows: negative (<10%), 1+ (10-50%), 2+ (50-75%) and 3+ (>75%).

**Polymerase chain reaction (PCR)**

For DNA extraction, three 10 μm thick sections were cut and DEXPAT (TAKARA, Tokyo, Japan, DNA extraction kit) was used. The HPV-specific PCR was performed using a set of primers, MY09 / MY11 (MY-PCR) and L1C1 / L1C2 and L1C2m (L1-PCR) in all cases (17, 18). Both consensus primers were used, which were directed to the L1 coding region of the HPV genome responsible for expression of late capsid protein of this virus. To amplify HPV DNA, as for MY-PCR, 40 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min) were done on a DNA Thermal Cycler (Perkin Elmer, CA, USA). By using L1-PCR, 40 cycles of denaturation (94°C, 1.5 min), annealing (48°C, 2 min) and extension (72°C, 2 min) were done on a DNA Thermal Cycler. After amplification, electrophoretic analysis was done on 2% agarose gel stained with ethidium bromide and visualized under ultraviolet transillumination. The sequences of these primers were:

MY09: 5’CGTCCMARGGAWACTGATC3’
MY11: 5’GCMCAGGGWCATAAYAATGG3’
L1C1: 5’CGTAAACGTTTTCCCTATTTTT3’
L1C2: 5’TACCCTAAATACTCTGTATTG3’
L1C2m: 5’TACCCTAAATACCCCTATATTG3’

![Fig. 1: Immunohistochemical staining of anti-p53 antibody in OSCC. Positive cells are seen in the basal cell layer in the tumor nests. (100)](image1)

![Fig. 2: Positive for anti-Bax antibody staining in OSCC. Positive cells are diffusely seen. (100)](image2)

**Table 1: Relationship between p53 and Bax expression**

<table>
<thead>
<tr>
<th>p53/Bax</th>
<th>Normal mucosa</th>
<th>OSCC</th>
<th>HPV (+) OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53+/Bax+</td>
<td>15 (71.4)</td>
<td>19 (40.4)</td>
<td>5 (10.6)</td>
</tr>
<tr>
<td>p53+/Bax−</td>
<td>4 (19.0)</td>
<td>28 (59.6)</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>p53−/Bax+</td>
<td>1 (4.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>p53−/Bax−</td>
<td>1 (4.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

**Table 2: Relationship between Rb and p16 expression**

<table>
<thead>
<tr>
<th>p16/Rb</th>
<th>Normal mucosa</th>
<th>OSCC</th>
<th>HPV (+) OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16+/Rb+</td>
<td>17 (81.0)</td>
<td>8 (17.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>p16+/Rb−</td>
<td>1 (4.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>p16−/Rb+</td>
<td>1 (4.8)</td>
<td>32 (68.0)</td>
<td>7 (14.9)</td>
</tr>
<tr>
<td>p16−/Rb−</td>
<td>2 (9.5)</td>
<td>7 (15)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
The amplified products were sequenced for HPV typing.

To check the integrity of the extracted DNA, all specimens were subjected to PCR amplifying of a 262bp fragment of \( \beta \)-globin with \( \beta \)-globin primers (18).

Statistical analysis
Relation between OSCC cases and normal control cases on the expression of the p53, Bax, p16\(^{INK4a} \) and Rb and prevalence of HPV was statistically evaluated by the chi-square test (\( \chi^2 \) test).

Results
Immunohistochemical study
p53, Bax, Rb and p16\(^{INK4a} \) immunoactivities were indicated by brown nuclear staining. The data were shown in Tables 1 and 2. Of forty-seven cases of OSCC associated with betel quid in Myanmar, all of cases were positive for anti-p53 (Fig. 1) and 40.4% were positive for anti-Bax antibodies (Fig. 2). Also, 59.6% of OSCC were p53-positive but Bax-negative. Among OSCC cases, 85.1% were positive for anti-Rb (Fig. 3) and 17.0% were positive for anti-p16\(^{INK4a} \) antibodies (Fig. 4), whereas 68.0% of OSCC cases showed p16\(^{INK4a} \)-negative and Rb-positive.

The cell count value with the corresponding grade for each antibody used were as follows: anti-p53 antibody 2+ was 42.5%, anti-Bax antibody negative was 59.6%, anti-Rb antibody 3+ was 36.2% and anti-p16\(^{INK4a} \) negative was 83.0%.

In twenty-one cases of normal gingival tissue, 90.4% were positive for anti-p53 and 71.4% were positive for anti-Bax. Moreover, 85.8% were positive for anti-Rb and 85.8% cases were positive for anti-p16\(^{INK4a} \).

Detection of HPV
HPV DNA was detected in seven (14.9%) out of forty-seven OSCC cases (Fig. 5, Tables 1 and 2), although no-positive band was detected using MY-PCR. HPV typing was done on the HPV positive cases. Three cases were HPV type 18, one was HPV type 16 and three cases were unknown. HPV was not detected in normal gingival tissue.

Relationship between p53 / Bax and p16 / Rb expression, and HPV infection
All HPV positive cases were positive for anti-p53, five cases were positive and two cases were negative for anti-Bax. All HPV positive cases were negative for anti-p16\(^{INK4a} \) and positive for anti-Rb. There was a relationship between p53/Rb expression and HPV infection, as

Fig. 3: OSCC positive for anti-Rb antibody. Positive cells are seen in the basal cell layer of the tumor nests. (100)

Fig. 4: Immunohistochemical staining of anti-p16\(^{INK4a} \) antibody in OSCC. Some positive cells are seen in the tumor nests. (100)

Fig. 5: Detection of HPV by L1-PCR. M: 100bp marker, N: negative control, P: positive control. Arrow: cand. 240 bp. The others are case numbers and No. 2, 3, 4, 5, 6, 8 and 13 are positive.
Table 3: Relationship between p53/Rb expression and HPV

<table>
<thead>
<tr>
<th>p53 / Rb expression</th>
<th>No. of HPV (+) cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (+)</td>
<td>7/7</td>
</tr>
<tr>
<td>Rb (+)</td>
<td>7/7</td>
</tr>
</tbody>
</table>

shown in Table 3. A low relationship was found between p53/Rb expression and HPV infection. The significance was found in the phenotype of p53+/Bax- (P<0.01) and p16NTK4a+/Rb+ (P<0.001) in betel quid associated OSCCs.

Discussion

In this study, p53 expression was found in all of the OSCC cases and in 90.5% of normal control samples. The prevalence of anti-p53 positive cases reported in other South East Asia countries ranged from 10 to 70% (2, 3, 19, 20). The p53 monoclonal antibody clone, DO-7, used in this study could recognize both wild and mutant-type p53. Mutant-type p53 had a longer half-life than wild-type p53 and could be detected by immunohistochemical methods, and positive p53 staining has been proposed as an indirect indicator for mutations of p53 (21, 22). However, wild-type p53 protein could be over-expressed without mutations, and frameshift and nonsense mutations were not sufficiently detected by immunohistochemical analysis (23-26). The p53 regulated cell proliferation and apoptosis had a direct effect on apoptosis by upregulating Bax expression (8). Therefore, we used both of the antibodies against p53 and Bax to detect the p53/Bax relation in betel quid associated OSCC.

In this study, positive stain for anti-Bax was detected in 40.4% of OSCC cases and 71.4% of normal control samples. In normal oral mucosa, positivity of anti-Bax was higher than in OSCC cases, but some negative cases were also found. The remaining 59.6% of OSCC cases showed positive for anti-p53 but negative for anti-Bax. These results suggested that betel quid might inactivate the function of a p53 tumor suppressor gene.

The p16 gene product inhibited cell cycle progression by binding of CDK4 and 6 and cyclin D to form a CDK4-cyclin D complex, which promoted Rb phosphorylation (4, 9, 10). In this study, 85.1% of OSCC cases were positive for anti-Rb and 83.0% were negative for anti-p16INK4a. 17.0% of OSCC cases were positive for both of anti-Rb and p16INK4a. Negative for anti-p16INK4a in this study reflected the absence of p16INK4a, which induced acceleration of cell cycle. It was suggested that betel quid might also cause the degradation of p16.

Etiologically, an important risk factor for causing OSCC was HPV infection and HPV types 16 and 18 were known as high-risk types (27-29). High prevalence of HPV was identified in the betel quid associated OSCC cases (14, 16). The E6 and E7 proteins from HPV high-risk types could induce degradation of p53 and inactivation of Rb (11-13). In this study, seven HPV positive cases (14.9%) were detected by L1-PCR, one case was HPV type 16, and three cases were HPV type 18. In three other cases, types were unknown. In this study, HPV DNA was detected by L1-PCR but it was not detected by MY-PCR. This might be due to the bp length detection specificity as the fragment length for each detection primer differs from each other as follows: 240 bp for L1-primer and 450 bp by MY-primer respectively (17, 30). All HPV positive cases were positive for anti-p53 and Rb. The prevalence of HPV had a low correlations between OSCC and the betel quid chewing habit. Recent immunohistochemical studies had shown that high p16 and Rb expression were associated with HPV infection in pre-cancerous and cancerous cervical lesions (31,32). But in this study, all HPV positive cases were negative for anti-p16INK4a and positive for anti-Rb. In conclusion, it could be postulated that (a) HPV might have caused the inactivation of p16INK4a and phosphorylation of Rb, (b) betel quid chewing habit might have caused the abnormal p16INK4a function, and (c) inactivation of p53 function is independent of HPV infection among betel quid associated OSCC cases from Myanmar.

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References


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