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Co-expression of p53 and Ki67 and lack of EBV expression in oral squamous cell carcinoma

Anak Iamaroon[1], Udomrat Khemaleelakul[2], Surawut Pongsiriwet[1], Jarupan Pintong[3]

[1]*Department of Odontology and Oral Pathology,* [2]*Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand, and* [3]*Department of Medical Services, Institute of Pathology, Ministry of Public Health, Bangkok, Thailand*

BACKGROUND: The objectives of the study were to determine the association between Epstein-Barr virus (EBV) and oral squamous cell carcinoma (OSCC), to compare the expression of p53 and Ki67 between normal oral mucosa, oral hyperkeratosis, oral pre-malignant dysplasia, and OSCC, and to determine the correlation between the expression of p53 and Ki67 in OSCC.

METHODS: The expression of EBV mRNA was studied by *in situ* hybridization technique in 24 cases of OSCC, and the expression of p53 and Ki67 was investigated by immunohistochemical method in 19 cases of OSCC, 7 cases of oral pre-malignant dysplasia, 6 cases of oral hyperkeratosis, and 5 cases of normal oral epithelium.

RESULTS: None of OSCC cases expressed EBV-encoded RNA (EBER) transcripts. The labeling indices (LI) of p53- and Ki67-positive cells were significantly higher in OSCC than in oral pre-malignant dysplasia, oral hyperkeratosis, and normal oral mucosa ($P < 0.05$). A significant correlation between the LI of p53- and Ki67-positive cells was observed in OSCC ($r = 0.6$; $P = 0.01$).

CONCLUSIONS: These findings suggested that the co-expression of p53 and Ki67 may play roles in carcinogenesis of OSCC and p53 overexpression may promote cell proliferation in OSCC. Furthermore, EBV does not appear to be a risk factor for OSCC particularly in the population of northern Thailand.

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Correspondence: Dr Anak Iamaroon, DDS, MS, PhD, Department of Odontology & Oral Pathology, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand. Tel.: +6653 944451. Fax: +6653 222844. E-mail: iamaroon@yahoo.com
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Introduction

A series of genetic alterations of both oncogenes and tumor suppressor genes is an important step in oral carcinogenesis. The *p53* gene, a well-known tumor suppressor gene, is believed to serve as a gatekeeper against carcinogenesis (1). Under normal circumstances, the function of p53 protein is to prevent the propagation of genetically damaged cells (2). p53 assists in DNA repair by causing G1 arrest and inducing DNA repair genes or directs apoptosis in cells, which are genetically damaged beyond repair (1). Cells with loss of p53 function are speculated to undergo malignant transformation. To date, alterations of *p53* gene are the most common event in human cancers, including oral squamous cell carcinoma (OSCC; 3).

Ki67 is a nuclear non-histone protein expressed maximally in cells in G2 and M phases of the cell cycle, but absent in resting cells (4). Hence, Ki67 can be employed to measure the growth fraction of normal tissues and malignant tumors (5). Previous studies have shown that high indices of Ki67 were observed in OSCC and correlated with disease progression and poor prognosis (6–8). Recently, cell proliferation as measured by the expression of Ki67 at the invasive tumor front showed a strong positive relationship with histologic grading in OSCC (9). Collectively, these findings indicate that Ki67 be used as a prognostic marker for OSCC.

Epstein-Barr virus (EBV) is a DNA virus classified in a family of herpesviridae (10). EBV has long been known as a causative factor for infectious mononucleosis and found to be associated with a variety of human cancers including B and T cell lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma and smooth muscle tumors (11). EBV receptors are expressed in normal and malignant oral epithelium, suggesting that EBV can infect oral epithelial cells (12). Many investigators have attempted to study a role of EBV in oral carcinogenesis but with variable results. Some studies have shown association of EBV with OSCC (13–17), while the others have not (12, 18).

The aims of the present study were to determine the association between EBV and OSCC, to compare the expression of p53 and Ki67 between normal oral mucosa, oral

hyperkeratosis, oral pre-malignant dysplasia, and OSCC and to determine the correlation between the expression of p53 and Ki67 in OSCC.

Materials and methods

Samples

Twenty-four cases of OSCC, collected from the archive of the Oral Pathology Laboratory, Faculty of Dentistry, Chiang Mai University, were used to detect the expression of EBV-encoded RNA (EBER) transcripts by *in situ* hybridization technique. Nineteen cases of OSCC with various differentiated features, seven cases of oral pre-malignant dysplasia, six cases of oral hyperkeratosis, and five cases of normal oral mucosa were used to study the expression of p53 and Ki67 by immunohistochemical method.

In situ hybridization

Epstein-Barr virus-encoded RNA transcripts in 24 cases of OSCC were detected by using the EBV probe *in situ* hybridization kit (NCL-EBV-K, Novocastra, UK). Briefly, deparaffinized sections were hydrated in 99% ethanol, 95% ethanol, and water. Sections were pre-digested by proteinase K for 30 min at 37°C. Subsequently, sections were immersed in water, dehydrated in 95% ethanol and 99% ethanol, and air-dried. Sections were added with EBV probe hybridization solution, cover-slipped, incubated for 2 h at 37°C, and washed in Tris-buffered saline (TBS) containing 0.1% v/v Triton X-100. Sections were incubated with blocking solution for 10 min, incubated with rabbit F (ab′) anti-fluorescein isothiocyanate/alkaline phosphatase (dilution, 1 : 100) for 30 min, washed in TBS, washed in alkaline phosphatase substrate buffer, pH 9.0, for 5 min, and incubated with enzyme substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) and levamisole at room temperature in the dark overnight. On the following day, sections were washed in running water for 5 min, counter-stained in hematoxylin, and mounted in aqueous mountant. The negative control section was processed identical to the above except that EBV probe solution was replaced by negative control probe solution, provided in the kit. The positive control section was EBV-infected lymphoid tissue, provided in the kit.

Immunohistochemistry

The expression of p53 and Ki67 was conducted by a technique of immunohistochemistry with the use of anti-p53 (clone DO-7, Dako, Denmark), and anti-Ki67 antibodies (clone MIB-1, Dako, Denmark). Both antibodies have previously been characterized (19, 20). Briefly, deparaffinized sections were immersed in 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase activity. Subsequently, sections were submitted to a pressure cooker (Kuhnrikon, Switzerland) treatment in citrate buffer (10 mM, pH 6.0) for 2.5 min, allowed to cool down, washed in Tris buffer (TB)/phosphate buffered saline (PBS)/0.1% Tween 20, and incubated with 2% bovine serum albumin for 20 min to block non-specific binding. Sections were then incubated with monoclonal mouse anti-p53 antibody (1 : 200 dilution), directed against both wild-type and mutant p53, or monoclonal mouse anti-Ki67 antibody (1 : 200 dilution), overnight at 4°C. On the following day, sections were washed in TB/PBS/0.1% Tween 20, incubated with biotinylated anti-mouse secondary antibody (1 : 400 dilution; Dako, Denmark) for 30 min, washed in TB/PBS/0.1% Tween 20, and reacted with streptavidin/horseradish peroxidase complex (1 : 800 dilution; Dako, Denmark) for 30 min. Chromogen was developed by using 1 mg/ml diaminobenzidine tetrahydrochloride (DAB) substrate for 10 min. Sections were counter-stained with hematoxylin and mounted using Permount (Fisher Scientific, NJ, USA). The slides were viewed and photographed under a light microscope. Negative control sections were processed identical to experimental sections except that the primary antibody was omitted and replaced with buffer. Positive controls for p53 and Ki67 were breast cancer tissues with previously known reactivity.

Quantitative analysis

Cells were considered to be positive for the p53 and Ki67 antigens if there was any staining of the nucleoplasm or nucleoli, regardless of the staining intensity. Labeling indices (LI) of p53 and Ki67 were determined by the number of positive nuclear profiles/mm^2 of epithelial cells, as described previously (9, 21). The p53 LI and Ki67 LI in the tissue of OSCC, pre-malignant dysplasia, hyperkeratosis, and normal oral mucosa per 10 fields at the magnification of $\times 400$ (0.1 mm^2 in size) were counted under light microscope, and the mean was determined. The expression of p53 was also semi-quantitatively determined by using the criteria that tumors with having more than 10% of positively stained cells were considered positive.

Statistical analysis

We assessed the statistical correlation between the LI of p53 and Ki67 by using the Spearman's correlation coefficient. To compare the LI of p53 and Ki67 between OSCC, pre-malignant dysplasia, hyperkeratosis, and normal oral mucosa, the Mann–Whitney test was used. *P*-values of <0.05 were regarded as significant.

Results

By using *in situ* hybridization technique, we were unable to find EBER transcripts in any cases of OSCC. The positive and negative controls were stained appropriately. The negative results of OSCC in comparison with the positive control tissue are shown in Figs. 1 and 2.

The results of the immunohistochemical analysis of p53 and Ki67 expression in OSCC, pre-malignant dysplasia, hyperkeratosis, and normal oral mucosa are graphed in Fig. 3. The means of the p53 LI for OSCC, pre-malignant dysplasia, hyperkeratosis, and normal oral mucosa were 1508 ± 1116, 456 ± 309, 282 ± 231, and 100 ± 117 nuclear profiles/mm^2, respectively. By using Mann–Whitney analysis, the mean p53 LI was significantly higher in OSCC than in pre-malignant dysplasia ($P = 0.005$), hyperkeratosis ($P = 0.002$), and normal oral mucosa ($P = 0.002$). The mean p53 LI was significantly higher in pre-malignant dysplasia than in normal oral mucosa ($P = 0.028$). Of all OSCC cases studied for p53 expression, 15 cases showed to have more than 10% of positively stained cells.

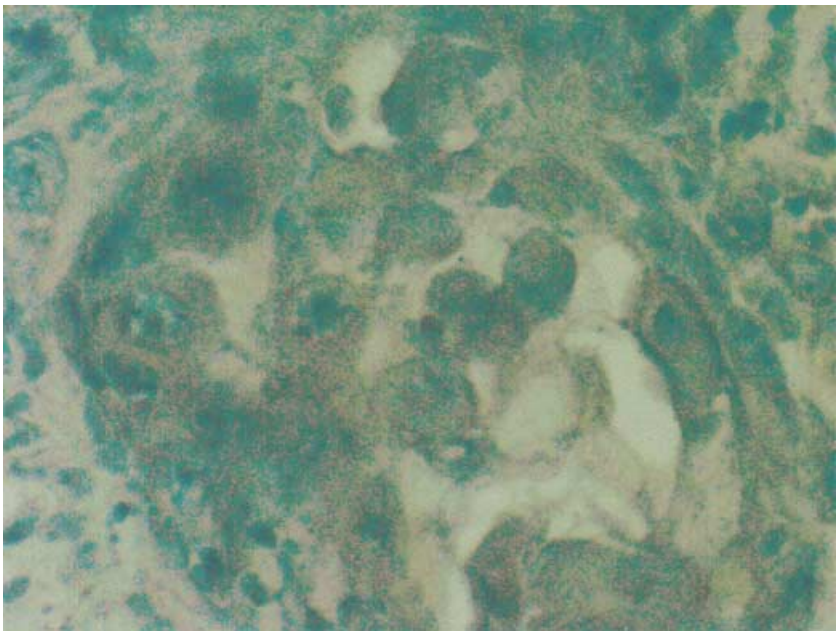

Figure 1 OSCC shows EBV-negative staining by *in situ* hybridization technique (original magnification, ×400).

The means of the Ki67 LI for OSCC, pre-malignant dysplasia, hyperkeratosis, and normal oral mucosa were 813 ± 551, 339 ± 257, 132 ± 159, and 116 ± 94 nuclear profiles/mm^2, respectively. By using Mann–Whitney analysis, the mean Ki67 LI was significantly higher in OSCC than in pre-malignant dysplasia ($P = 0.030$), hyperkeratosis ($P = 0.002$), and normal oral mucosa ($P = 0.003$).

The distribution patterns of p53 and Ki67 labeling in hyperkeratosis and pre-malignant dysplasia were mainly in the basal and suprabasal cell layers, while in normal oral mucosa, they were mainly in the basal cell layer. In OSCC, most malignant cells were intensely stained with anti-p53 and -Ki67 antibodies. The histopathologic appearances of OSCC stained with anti-p53 and -Ki67 antibodies are shown in Figs. 4 and 5, respectively, and of hyperkeratosis with anti-p53 and -Ki67 antibodies are shown in Figs. 6 and 7, respectively. The positive and negative controls stained appropriately. By using Spearman's correlation coefficient, the mean p53 LI was significantly correlated with the mean Ki67 LI ($r = 0.6$; $P = 0.01$) in OSCC (Fig. 8).

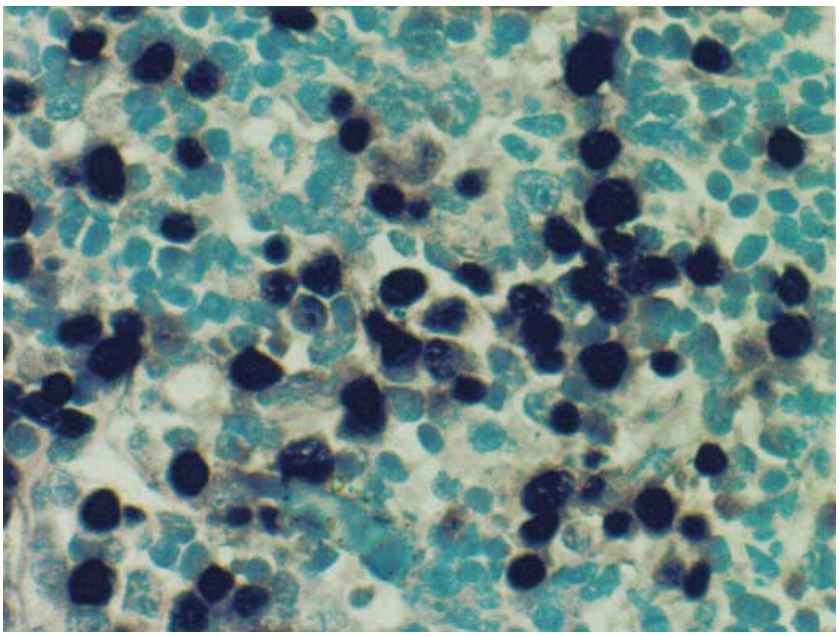

Figure 2 The positive control tissue demonstrates intense staining of the EBV-infected lymphoid cells by *in situ* hybridization technique (original magnification, ×400).

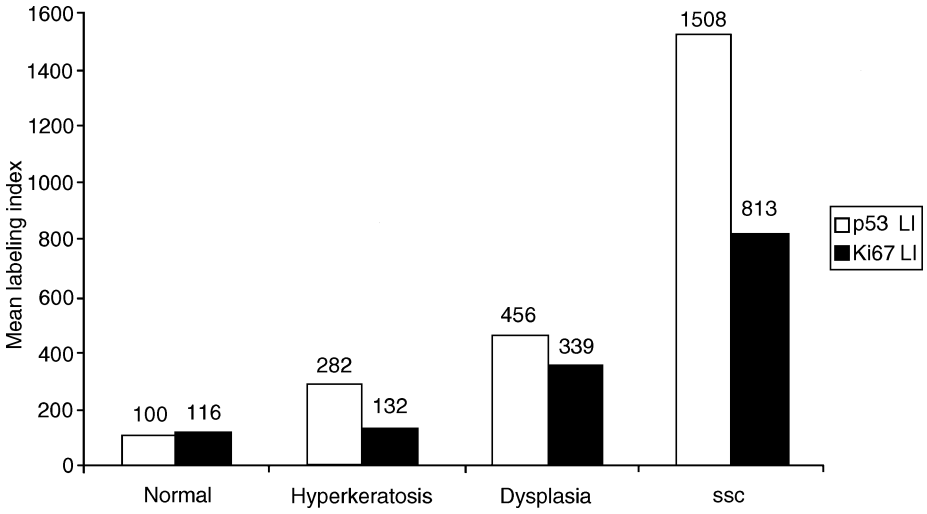

Figure 3 Histograms of the mean LI of p53 and Ki67 (shaded) in normal oral mucosa, hyperkeratosis, premalignant dysplasia, and OSCC.

Discussion

In the present study, we aimed to investigate a possible role of EBV in the pathogenesis of OSCC. However, none cases expressed EBER transcripts by *in situ* hybridization method. Several previous studies from different parts of the world have demonstrated widely variable results with various techniques, including polymerase chain reaction (PCR), *in situ* hybridization, Southern blot analysis, and immunohistochemistry. For example, the studies from the UK, Spain, Sweden, and the Netherlands revealed that EBV was detected in 0, 19.2, 37.9, and 100% of all OSCC cases, respectively (12–15). Although the high prevalence of EBV in OSCC was found in the study from the Netherlands, it was suggested that these may be because of increased EBV shedding, possibly because of associated immunodepression in these patients rather than its clonal presence in the neoplastic cells (15). In Asia, several studies have come from different parts of Japan and again have shown variable findings from 0 to 76.6% (16–18, 22). Interestingly, it was found that OSCC patients with EBV infection had a better prognosis, suggesting a favorable role of EBV (17). Furthermore, some previous studies have also included non-malignant, non-viral associated lesions and normal oral mucosa used as control groups, and have demonstrated that a considerable proportion of those tissues as high as 25–42% were EBV positive (23, 24). Taken together, the differences of the previous and our results may be because of geographic variations of the subjects investigated and the variation of the sensitivity of various techniques used to detect the

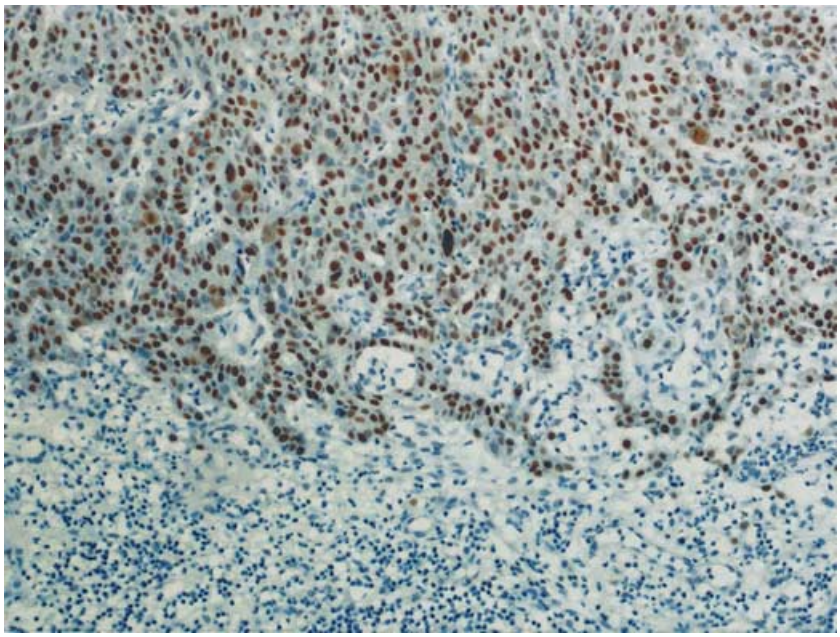

Figure 4 Histologic appearances of p53-positive cells at the invasive tumor front of OSCC (original magnification, ×100).

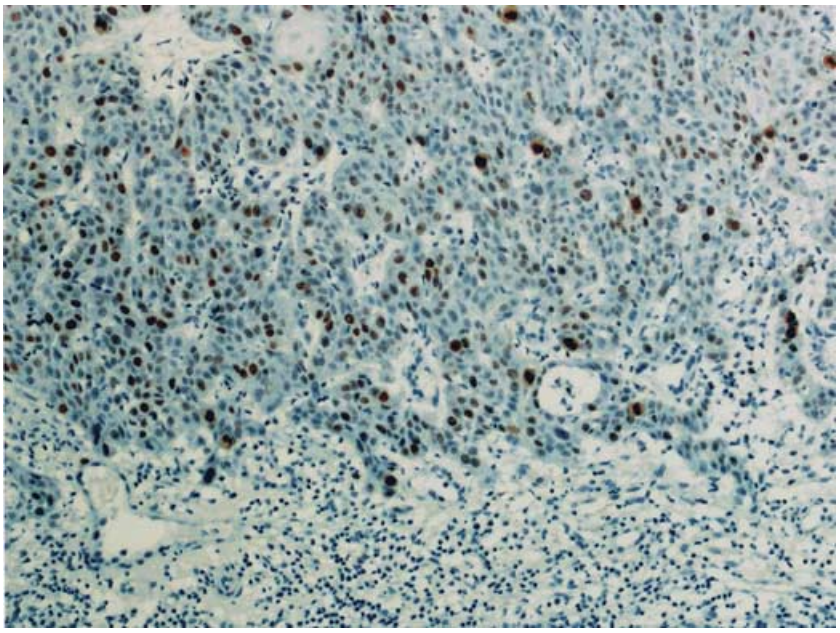

Figure 5 Histologic appearances of Ki67-positive cells at the invasive tumor front of OSCC (original magnification, ×100).

presence of EBV. The role of EBV as a major risk factor in oral carcinogenesis therefore remains inconclusive. Further investigation of a larger sample size is required to determine whether EBV has an etiologic role in OSCC of the Thai population or EBV is merely a coincidental passenger in OSCC as described elsewhere.

Mutation of the tumor suppressor gene *p53* is the most common genetic abnormality detected in human cancers, including OSCC. Several investigators have reported high frequencies of *p53* gene alteration in head and neck cancer and OSCC varying from 34 to 100% (25–31). These data were in line with ours, in which most cases of OSCC (79%) expressed p53. Furthermore, we found that p53 and Ki67 appeared to be reliable indicators for OSCC development, as the LI of Ki67 and p53 showed a trend to increase with disease progression. Previous studies of p53 and/or Ki67 with an emphasis on the oral cancer and pre-cancerous lesions also revealed similar findings (8, 32, 33). In our study, the distribution patterns of p53 and Ki67 labeling were mainly localized in the basal cell layer in normal oral mucosa, while the expression of p53 and Ki67 extended into the parabasal and suprabasal cell layers in hyperkeratosis

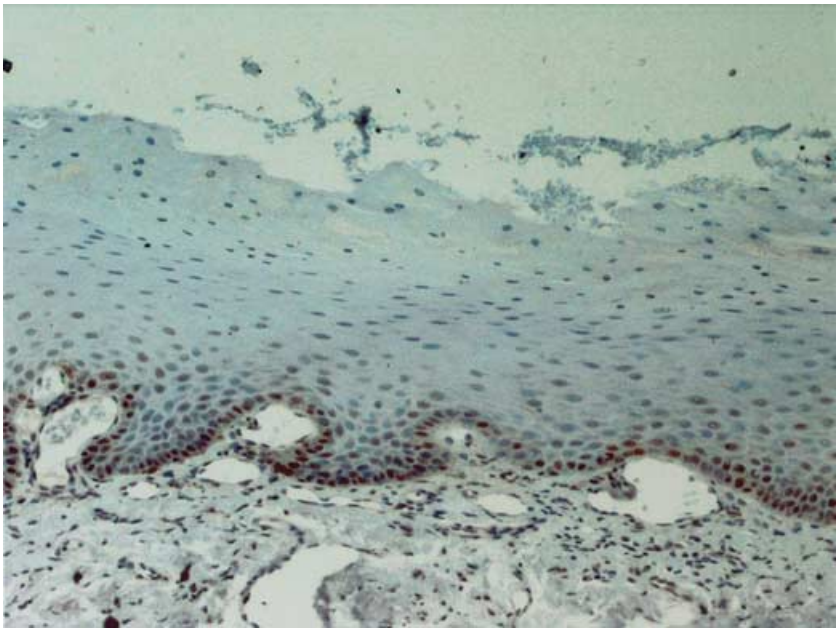

Figure 6 Oral hyperparakeratosis shows p53-positively stained epithelial cells in the basal and parabasal cell layers (original magnification, ×100).

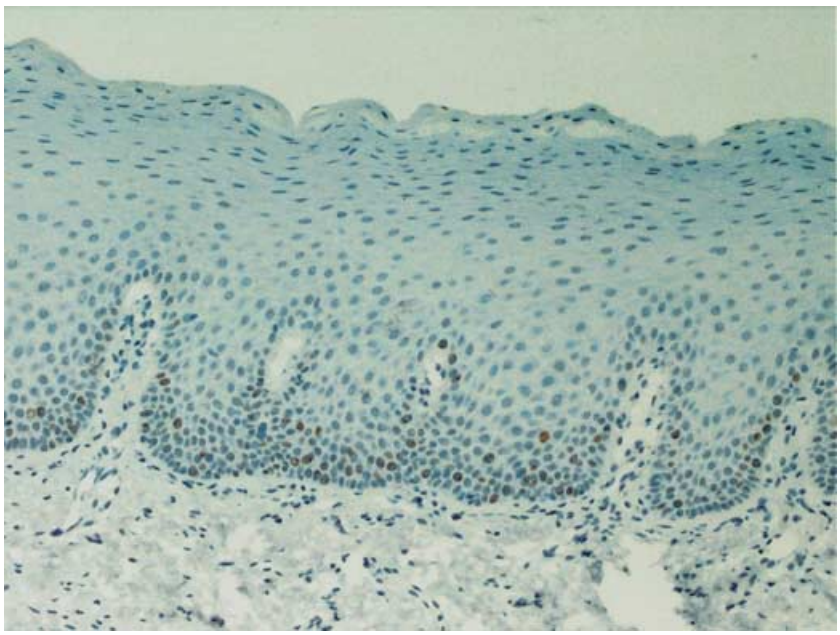

Figure 7 Oral hyperparakeratosis shows Ki67-positively stained epithelial cells in the basal and parabasal cell layers (original magnification, ×100).

and pre-malignant dysplasia. Previous studies have also shown similar observations (33, 34) and suggested that increased LI and suprabasal labeling especially of Ki67 in oral dysplastic and neoplastic lesions correlated with poor clinical outcome (3 years post-treatment) such as recurrence of the disease or cervical lymph node metastasis (33). Recently, the Ki67 LI measured particularly at the invasive tumor front of OSCC, suggested to have different molecular characteristics compared with superficial areas of the tumor, showed to have a strong positive relationship with histologic grading in OSCC (9). Concerning the prognostic values, the Ki67 LI in particular was found to be a valid indicator of treatment failure in a large series of SCC of the oropharynx and oral cavity, while there was no relationship between p53 or proliferating cell nuclear antigen (PCNA) status and tumor prognosis (35). Similar results were found in the study of Ki67 in SCC of the tongue (7).

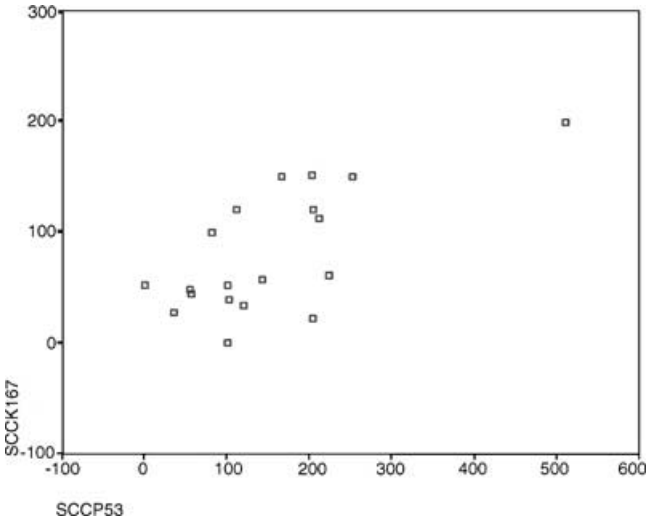

Figure 8 The Spearman's correlation coefficient showing a significant correlation between the LI of p53 and Ki67 in OSCC ($r = 0.6$; $P < 0.01$).

The co-expression of both p53 and Ki67 was clearly demonstrated in OSCC in the present study. By using Spearman's correlation coefficient, we found a significant correlation between these two markers in OSCC. Under normal circumstances, p53 regulates the cell cycle by inducing G1 arrest or apoptosis in cells that are genetically damaged by ultraviolet light or chemical carcinogens (36). Mutations of *p53* lead to uncontrolled cell growth (37). Taken together, the significant correlation between the expression of p53 and Ki67 in our study suggested that alterations of *p53* lead to increased cell proliferation in OSCC. Recently, it was found that both Ki67 and mutant p53 were overexpressed in the pre-malignant and malignant oral epithelial cells *in vitro* (38). Moreover, some but not all chemopreventive agents could suppress Ki67 and mutant p53 levels, suggesting that the suppression of Ki67 and mutant p53 were good indicators of the effectiveness of agents in those pre-malignant and malignant cells.

In conclusion, (1) lack of the expression of EBV in OSCC in the present study suggested that EBV not play a pathogenetic role of OSCC in this Thai population. (2) The expression of p53 and Ki67 increased with disease progression, indicating that p53 and Ki67 be used as markers for OSCC development. (3) The expression of p53 was significantly correlated with that of Ki67 in OSCC suggested that altered *p53* gene promoted cell proliferation in OSCC.

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