結案報告

檳榔嚼食相關之口腔癌的長期追蹤研究—從組織病理、生物標記、分子遺傳等方面探討

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Molecular Characterization of Angiogenic Properties of Human Squamous Cell Carcinoma Cells

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Running Title: Factors Influencing the Angiogenic Properties of Oral Cancer Cells.
Abstract

Tumor growth and metastasis are angiogenesis-dependent. Angiogenesis, formation of new blood vessels from existing endothelium, is a multi-step process involved in proliferation, migration and differentiation of endothelial cells. Dysregulation of angiogenic factors has been observed in many types of solid tumors including oral squamous cell carcinoma. However, little is known if the angiogenic property is specific to oral cancer cells but not normal oral keratinocytes and, if so, about the mechanism of regulation. Seven oral cancer cell lines regardless of their etiological difference but not normal human keratinocytes have angiogenic property in the aspects of promoting human umbilical vein endothelial cells (HUVEC) to undergo proliferation and migration. The amount of VEGF was 12-500 times higher than that of bFGF in the CM derived from oral cancer cells. Comparable level of VEGF to those detected in some oral cancer cells was also detected in CM derived from normal oral keratinocytes (NOK), suggesting the presence of angiogenic inhibitors in normal mucosa epithelium. Attenuation of the angiogenic properties of oral cancer cells by neutralizing antibodies to VEGF indicated that VEGF might in part play an important role in mediating these properties. Down regulation of TSP-1 indicated by western blotting analysis is not sufficient to account for the gain of these properties, suggesting the involvement of other factors. Inactivation of p53 either by mutations or overexpression of Mdm2 occurred in the majority of oral cancer cells including two cell lines established from patients with a history of betel-nut chewing. Although the etiology of tested oral cancers is different, loss of p53 functions appears to correlate with the gain of angiogenic properties in oral cancer cells. In summary, the angiogenic property of oral cancer cells is possibly mediated by many factors and among them VEGF and the status of p53 function may attribute significantly the mediation of angiogenic properties in oral cancer cells.

Introduction

Tumor growth and metastasis depends on angiogenesis, formation of new blood vessels from pre-existing endothelium [1]. Without proper nutrients and oxygen supplied by newly formed blood vessels, an avascular tumor cannot grow, expand and even metastasize to distant organs. The degree of angiogenesis thus provides a useful indicator for the presence of lymph node metastasis. The prognosis of several types of human cancer is found associated with intratumoral microvessel density[2-5]. This observation was further corroborated by the studies of angiogenic properties in a variety of human cancers including the squamous cell carcinoma of oral cavity [6-9].

Squamous cell carcinoma is the most common malignancy of the oral cavity. The incidence of oral cancer has been increasing particularly in the area where the betel quid chewing is prevalent. Approximately ninety percent of these oral cancer
patients showed a history of betel quid chewing. Others and we have shown there is a significant correlation between oral carcinogenesis and betel quid chewing [10-12]. Despite of introducing multiple treatment regimens in recent decades, the five-year survival rate of oral cancer patients still falls far below that of many other cancers such as breast and colon cancer [13]. Understanding the underlying mechanisms of oral cancer pathogenesis, therefore, will not only facilitate the development of an effective screening protocol for oral cancer but also improve the five-year survival rate of oral cancer that has plateaued for the past decades.

Angiogenesis is a multi-step process involved in proliferation, migration and differentiation of endothelial cells [14]. Several factors including interleukin-8 (IL-8), transforming growth factor-β1 (TGF-β1), acidic and basic fibroblast growth factors (aFGF, bFGF), and vascular endothelial growth factor (VEGF) are implicated to play an role in the angiogenic properties of certain oral cancer cell lines [15-17]. Moreover, human oral squamous cell carcinoma cells promote angiogenesis via endogenous expression of VEGF and increase the expression of VEGF receptor 2 (Flk-1/KDR) in bovine endothelial cells [18].

The balancing activity of angiogenic inducers and inhibitors controls this process [19, 20]. Among many angiogenic inducers, VEGF and bFGF are well characterized and promote endothelial cell proliferation and migration in vitro [21]. VEGF is the most intriguing factor with respect to that VEGF selectively acts on endothelial cells that express its receptors. VEGF is a glycoprotein with homology to platelet-derived growth factor. VEGF induces production of proteases, promotes endothelial cell proliferation, migration, differentiation and survival in addition to its ability to induce vascular permeability, which are known to be the angiogenic properties [22]. Several lines of evidence have suggested that VEGF may be an important factor in the angiogenesis and growth of human cancer. Expression of VEGF correlates with tumor aggressiveness and may serve as a marker for tumor invasion and metastasis in various cancers [8, 23-25].

In addition to the inducers, inhibitors like thrombospondin-1 (TSP-1) also involve the regulation of angiogenesis. TSP-1 is the first member of multifunctional extracellular matrix glycoprotein involved in proliferation, migration and adhesion. Endothelial cell sprouting is enhanced by the exposure to anti-TSP-1 antibodies [26, 27] and stable transfection of antisense TSP-1 construct enhanced the ability of endothelial cells to form tubes on a matrix gel [28]. Knockout of this gene results in hypervascularization of certain organs in mice [29-32]. The p53 protein is one of TSP-1 upstream regulators[33]. Mutations of p53 are frequently found in oral cancers and may be associated with oral carcinogenesis[34-38]. In addition to its role as a cell cycle regulator and DNA damage sensor, loss of p53 function is also
associated with the gain of angiogenic phenotype manifested by up-regulation of VEGF expression [39-41] and downregulation of TSP-1 [33]. However, the molecular mechanism by which oral squamous cells carcinoma cells and their normal counterpart, normal oral keratinocytes, regulate their angiogenic properties remains elusive. Furthermore, the etiology of oral cancers in Taiwan is different from those in western world where the major culprit for oral cancer is not betel-nut quid.

In this study, we first examined whether conditioned media (CM) derived from seven oral cancers including two established from betel-nut chewing oral cancer patients, and that from normal oral keratinocytes could promote proliferation and migration of human umbilical vein endothelial cells. ELISA assays measured the concentrations of VEGF and bFGF in these CM. Neutralizing antibody against VEGF was used to examine the role of VEGF in mediation of angiogenic properties. Expression of the antiangiogenic molecule, TSP-1, and the functional status and regulation of p53 in these oral cancer cells were also examined. Their role in regulating the angiogenic properties of oral cancer cells was also discussed.

Materials and Methods

Materials
Recombinant VEGF and bFGF were purchased from R&D Systems (Minneapolis, MN). Most of growth media for cell cultures with the exception of the media for NOK and human umbilical vein endothelial cells (HUVEC) are from Life Technologies (Rockville, MD). EGM2 and KGM were from BioWittaker (Walkersville, MD). All chemicals are ordered from Sigma (St. Louis, MO). Antibodies to TSP-1 and p53 were from Oncogene Research Products (Boston, MA). Antibodies to Mdm2 and α-tubulin are from Neo Markers (Fremont, CA). Secondary antibodies conjugated with horseradish peroxidase were from Bio-Rad Laboratories (Hercules, CA). ELISA kits for VEGF and bFGF were from RD Systems (Minneapolis, MN). Oligonucleotide primers for PCR and sequencing analysis were ordered from MDbio, Inc. (Taipei, Taiwan).

Maintenance of cell culture and primary cells
Oral cancer lines including KB, CAL-27, SCC-9, SCC-15, and SCC-25 were obtained from American Tissue Cell Collection (Rockville, MD) and maintained as described. Two oral cell lines established from Taiwanese men with a history of betel quid chewing, OC-2 and OEC-M1 were obtained from Dr. R. Chang (Veteran General Hospital, Taipei, Taiwan) and Dr. C. Meng (National Defense Medical Center, Taipei, Taiwan), respectively [42]. Briefly, the cells were maintained in the appropriate growth medium at 37°C in a humidified atmosphere of 5% CO2 and 95% air and used over a restricted culture period of 10 passages. Gingival tissue from healthy
individuals was used as a source of normal mucosal epithelium [43]. Normal oral keratinocytes (NOK) isolated from these mucosal epithelia were then maintained in KGM and less than five passages of these cells were used for isolation of conditioned medium. HUVEC were isolated from umbilical veins [44] then grown in EGM2. All the studies using endothelial cells were restricted to the passage number no more than 6.

**Preparation of conditioned medium (CM)**

The established human oral cancer lines or NOK were seeded in the appropriate growth medium. When the cells reached 80-90% confluence, the medium was aspirated and washed twice with PBS. The washed tumor cells were then incubated with 10^3-10^6 cells/ml serum-free medium depending on the cell line at 37°C in a humidified atmosphere of 5% CO2 and 95% air. After 24-hr incubation, the conditioned medium was collected then centrifuged at 2,000 x g for 5 min. The CM was further filtered to remove cell debris using a Millipore filter (0.22μm). The cell-free conditioned medium was then stored at 4°C until use.

**Proliferation assay**

Subconfluent HUVECs were plated at a cell density of 5000 cells per well in 96-well plate. Eight hours after seeding, cells were serum-starved in M199 containing 1%FBS and 0.1% BSA for 12 hours. Then, serum-deprived HUVECs were treated with M199 alone or the indicated dilutions of CM with M199 in the presence of 2% FBS for 2 days. A tetrazolium-based nonradioactive CellTiter 96®AQeux One Solution Cell Proliferation Assay kit (Promega, USA) was used to measure the index of cell proliferation prior to and post treatment. This assay estimated the intracellular amount of formazan product, which was produced in proportion to the cell number. Each treatment was conducted in triplicates and each experiment was repeated twice. Student t-test was used to test the significance (p<0.05 or 0.01) of each treatment compared to control. For antibody neutralization experiments, HUVECs were cultured for 2 days in M199, or M199: CM (2:1) containing 2% FBS together with 500 or 1000 ng/ml preimmune control IgG (Jackson Immunoresearch Laboratories, West Grove, PA) or anti-VEGF antibodies prior to cell number counting.

**Cell migration assay**

HUVEC at a density of 2 x 10^5 cells/well in basal medium with 0.1% bovine serum albumin were plated in triplicate in the bottom of a modified Boyden chamber. The chamber was assembled and inverted, and cells were allowed to attach for 2 hours at 37 °C to polycarbonate membranes (8-μm pore size). The chamber was then reinverted, and CM with 3-fold dilution or M199 were added to the wells of upper chamber. After 4 hours of incubation at 37 °C, the cells that had migrated to the upper chamber were fixed, and stained with a diluted Giemsa solution. The numbers
of cells per 5 high power fields were counted. Each treatment was conducted in triplicates and each experiment was repeated twice. Student t-test was used to test the significance (p<0.05) of each treatment compared to control.

**Enzyme-linked immunosorbant assay of VEGF and bFGF in the conditioned media**

The levels of angiogenic inducers including bFGF and VEGF were quantified using solid phase enzyme-linked immunosorbant assay (ELISA) kits. Each conditioned medium was repeated twice in each ELISA assay. The concentrations of each factor in the conditioned media was calculated based on a standard curve made of human recombinant form of bFGF and VEGF at serial dilutions of known concentration. The protein concentration in each CM was also measured.

**Western blotting analysis**

Cancer cell lines were lysed in the boiled lysis buffer containing 1% SDS and 10 mM Tris-HCl (pH7.4). Supernatant containing total protein was collected by centrifugation at 13,000 rpm for 10 min following lysis. The protein concentration in each lysate was measured by Bradford protein assay. Equal amount of total protein was fractionated by SDS-PAGE. Following protein separation, the proteins were blotted onto PVDF membrane. The protein blot was then hybridized with the primary antibody of interest then secondary antibody followed by Super Signal Detection System (Pierce, USA). Protein expression of interest in oral cancer cells

**PCR and sequencing analysis of p53 gene**

Genomic DNA isolated from oral cancer lines serves as a template for PCR amplification using specific primers to the exons 4 to 9 of p53 gene. The PCR fragment was then column-purified followed by automated DNA sequencing analysis. The sequence was queried against the published p53 gene sequence.

**Result**

*CM derived from oral cancers but not normal oral keratinocytes (NOK) stimulate HUVEC proliferation and migration.* Tumor growth and metastasis rely on angiogenesis, a multi-step process involved in proliferation and migration of endothelial cells. We first tested whether CM derived from oral cancers or normal oral keratinocytes have any effects on HUVEC proliferation or migration. Serum-free CM derived from seven oral cancer lines and normal keratinocytes were serially diluted 2 to 11 folds with the M199. CM from KB, CAL-27, OEC-M1 and OC2 not only significantly promoted HUVEC proliferation but also the stimulatory effect was in a dose-dependent manner. Interestingly, complete CM attenuated the stimulatory effect (Fig. 1 A and B). Likewise, CM from three oral squamous carcinoma cells, SCC 9, 15 and 25 also promoted HUVEC proliferation but dose-dependently (Fig. 1C). Although the extent of stimulation and dilution folds appeared to depend upon
the origin of oral cancer cells, oral cancer cells regardless of their origin stimulated HUVEC proliferation. In contrast, CM derived from NOK had no effect on HUVEC proliferation (Fig. 1D).

We next examined whether the mitogenic dose of each CM at 3-fold dilution could promote HUVEC migration. In Fig. 2, we found that mitogenic dose of CM also significantly promotes HUVEC migration through porous membrane in response to treatment of CM. Consistent to the proliferation assay, CM from NOK also had no stimulatory effect on HUVEC, suggesting either the presence of the angiogenic inhibitors or absence of inducers. Stimulation of both HUVEC proliferation and migration by the treatment of these CM indicated the angiogenic properties of these oral cancer cells.

CM derived from both oral cancer cells and NOK contain VEGF and bFGF. VEGF and bFGF are implicated to be important angiogenic inducers among many cancer types [6, 15, 24, 45]. In addition, VEGF was expressed in human oral squamous cell carcinoma [9, 18]. We then measured by ELISA the amounts of either VEGF or bFGF protein in these CM. As shown in Table 1, all the CM including those from seven oral cancer cell lines and normal oral keratinocytes contain various degrees of both VEGF and bFGF. Different concentrations of VEGF and bFGF were found in the similar amount proteins per ml of CM. The amount of VEGF was at a range of 382 pg to 4524 pg per ml whereas bFGF was in the range of 9 to 31 pg per ml. The concentration of VEGF was 15 to 500 times higher than that of bFGF in the same volume of CM. Surprisingly, as high as 1303 pg/ml of VEGF was also detected in the CM derived from NOK. The inability of CM derived from NOK to promote HUVEC proliferation nor migration suggests the presence of angiogenic inhibitors rather than absence of VEGF in the normal oral keratinocytes.

Neutralizing VEGF antibodies attenuate the effect of CM on HUVEC proliferation and migration. Since the amount of VEGF was much greater than that of bFGF, we thus tested whether the stimulatory effects of these oral cancer cells-derived CM could be attenuated by VEGF neutralizing antibodies. In Figs. 3 and 4, excess VEGF neutralizing antibodies at hundreds to thousands folds dose-dependently attenuated the stimulatory effect on HUVEC proliferation and migration, respectively. The attenuation by the neutralizing antibody against VEGF suggests that VEGF might in part modulate the angiogenic effect of oral cells on endothelial cells.

Regulation of thrombospondin-1 (TSP-1), an angiogenic inhibitor, in oral cancer cells: Angiogenesis is multi-factorial event controlled by the balancing activity of angiogenic inducers and inhibitors. Recently, TSP-1 is shown to be a potent angiogenic inhibitor [31, 33, 46]. To examine the expression level of TSP-1 protein in these oral cell lines and NOK, we then used western blotting analysis to measure
the amount of TSP-1 in these cells. In Figure 5, the amounts of TSP-1 protein in CAL-27, SCC-25 and SCC-9 were compatible to that in NOK. Very little or no TSP-1 could be detected in KB, SCC-15, OEC-M1 and OC-2. The decrease of TSP-1 was only observed in four of seven oral cancer cells.

Protein expression and functional status of p53 in oral cancer cells. Mutations of p53 are frequently found in oral cancer cells and associated with oral carcinogenesis [33-35, 37]. Furthermore, loss of p53 may contribute to angiogenic switch during tumor angiogenesis via upregulation of VEGF[47] or downregulation of TSP-1 [33]. To determine the expression level of p53 in these oral cancers and NOK, we used western blotting analysis using an antibody specific to p53. This p53 antibody reacts with both mutant and wild types of p53 protein. The only difference between these two forms of p53 is that wild-type exhibits very low level of expression in cells due to its short half-life. As shown in Fig. 6, only three out of seven oral cancer lines expressed 2 to 3-fold higher level of p53 than that of NOK, indicating p53 in CAL-27, OEC-M1 and OC2 was mutated. Conversely, no or little p53 could be detected in KB and three other SCC lines (SCC-9, 15, and 25).

Sequence analysis of the p53 gene
The wildtype p53 has a short half-life. Several mechanisms explain the disregulation of p53 in cells. Among them, alteration of DNA sequence increases the stability of p53 protein. KB is previously infected with Human papilloma virus (HPV) type 18. The E6 of HPV degrades p53 via ubiquitin-mediated pathway whereas those from SCC-9, 15 and 25 carry frameshift mutations via insertion or deletion. None of p53 in CAL-27, OC-2 and OEC-M1 has been examined. We thus used genomic DNA from OC-2, OEC-M1 and CAL-27 as a template for PCR amplification followed by sequencing analysis of the exons 4 to 9 encoding the DNA binding domain of p53. As described in Table 2, the p53 genes from CAL-27 and OC-2 cell lines carried amino acid substitution at codons 193 and 132, respectively. Both mutations reside in the DNA binding domain of p53. Although the p53 expression of OEC-M1 was upregulated, no mutation was detected in this region.

Regulation of p53 expression by MDM2
The human homologue of the murine double minute 2 oncogene (Mdm2) binds to and forms an auto-regulatory feedback loop with normal p53. The major function of Mdm2 is to inhibit the transcriptional activity of p53 via proteasome-mediated degradation [48]. Our inability to detect mutations in the DNA binding region of p53 in the OEC-M1 oral cancer line overexpressing p53 suggests the regulation of p53 be disrupted. We then used western blotting analysis to examine the expression of Mdm2 among these cell lines. Overexpression of Mdm2 was seen in CAL-27, SCC-9 and OEC-M1. Low level of Mdm2 comparable to that in NOK was observed
in SCC-15 and OC-2. Little or no Mdm2 was detected in SCC-25 and KB oral cancer lines.

Discussion

Angiogenesis, a complex process involved in migration and proliferation of endothelial cells, plays a key role in tumor growth and metastasis. We have shown that serially diluted CM derived from seven oral cancer cells but not normal oral keratinocytes promoted both proliferation and migration of HUVEC. Interestingly, complete CM derived from KB, CAI-27, OEC-M1 and OC-2 have lower ability than the diluted CM to promote HUVEC proliferation, suggesting the presence of unknown inhibitors in the complete CM. This observation is consistent with the notion that the balancing activity of angiogenic inducers and inhibitors controls the on- and off-switch of angiogenesis. Up-regulation of two potent angiogenic molecules, VEGF and bFGF, have been implicated in their role in many types of solid tumors [6, 7, 15, 18, 23]. We also found their presence in the tested oral cancer cell lines including those two established from patients with a history of betel quid chewing. Higher level of VEGF than bFGF detected in these oral cancer cells suggests that VEGF might be more important than bFGF, although we could not completely rule out the effects of bFGF or other angiogenic inducers on the angiogenic properties of these cells [16, 49]. Consistent with VEGF being an important angiogenic factor, neutralizing against VEGF attenuated the stimulatory effect of CM derived from oral cancers on HUVEC proliferation and migration. Intriguingly, NOK also secreted comparable level of VEGF to those of oral cancer cells but had any effects to stimulate neither proliferation nor migration of HUVEC. Angiogenesis depends upon the presence of inducers and inhibitors of neovascularization in the environment around endothelial cells. The lack of angiogenesis is presumably due in part to the predominance of inhibitors. The inability of CM derived from NOK suggested the presence of VEGF is not sufficient to induce angiogenesis and predominant existence of angiogenic inhibitors in normal oral keratinocytes can downplay the positive effects of VEGF on endothelial cells.

Down-regulation of angiogenic inhibitors was also observed in many cancer types. Among these inhibitors, anti-angiogenic properties of TSP-1 have been reported in human bladder, melanoma and gastrointestinal tumors [50-52]. Moreover, overexpression of TSP-1 decreases vessel density and inhibits xenograph growth of human cutaneous squamous cell carcinoma [53]. TSP-2 with a high structural similarity to TSP-1 is also a potent endogenous inhibitor of tumor growth and angiogenesis [30]. The clinical association of TSP-2 with many types of human cancer remains to be clarified. We thus examined the role of TSP-1 in oral cancer cells using an antibody to the reduced form of TSP-1. Since both the structures and
molecular weights of TSP-1 and -2 are very similar, we cannot rule out the possibility of TSP-2 being detected by western blotting analysis. Very little or no TSP-1 was detected in four out of seven oral cancer cell, KB, SCC-15, OEC-M1, and OC-2. The loss of TSP-1 is thus not sufficient to account for the gain of angiogenic properties of these oral cancer cells and other factors may be involved in the regulation.

Loss of p53 function resulting from mutation or inactivation is frequently found in oral cancers [34-38]. Furthermore, the absence of p53 induces angiogenic properties possibly through two or more pathways. One is via the increased expression of angiogenic inducers such as VEGF [39-41]. The other is via the downregulation of angiogenic inhibitors like TSP-1 [33]. We have found overexpression of p53 in CAL-27, OEC-M1 and OC-2. Mutations resulting in the increase of protein stability or disrupted autoregulation of p53 are often observed in the cells expressing high level of p53. Sequencing analysis of exons 4 to 9 encoding the DNA binding domain of p53 in CAL-27, OEC-M1 and OC-2 indicated that there were indeed mutations in this protein from CAL-27, OC-2 but not OEC-M1. Little or none p53 were detected in the three SCC lines where p53 genes carry frame-shift mutations, resulting in an unstable, truncated version of p53 [36-38]. There was also no p53 detected in KB oral cancer line previously infected with HPV. E6 protein encoded by HPV genome inactivates p53 via ubiquitin-mediated degradation by proteasomes [38, 54, 55]. The absence of p53 protein in KB is likely due to the presence of E6 protein. Consistent with the previous finding, loss of p53 function either through mutations or inactivation by the E6 of HPV may be associated with oral carcinogenesis.

Mdm2 is a direct regulator of p53 and forms an auto-regulatory loop with p53. Overexpression of Mdm2 is considered to be an alternative mechanism of p53 inactivation and thus mimic tumorigenesis in the process of transformation. It has been found amplified in many human malignancies, thus abolishing the anti-proliferative function of p53 [56, 57]. Overexpression of Mdm2 was seen in CAL-27, OEC-M1 and SCC-9 despite of mutations found in p53 from CAL-27 and SCC-9. Accumulation of wild-type p53 protein in OEC-M1 result in overexpression of Mdm2, thus inactivating the wild-type function of p53 in these cells. Over-expression of Mdm2 in the absence of functional p53 observed in SCC-9 is possibly via p53-independent pathway. Recently, at least two homologues of p53, p63 and p73 were identified. The same canonical sites for DNA binding can be recognized by these homologues. All the tested oral cancer cells carried dysfunctional p53 via either mutation or disregulation of p53 or even infection of HPV. These data indicated that loss of p53 function is prevalent among oral cancer cells regardless of the etiological
difference.

In summary, we have showed that oral carcinogenesis is associated with the gain of angiogenic properties regardless of their etiology and the regulation of these properties are very complex and influenced by many factors. In particular, VEGF is an important mediator for oral cancer-induced angiogenesis. Downregulation of TSP-1 and disregulation of its regulator, p53, may account in part for angiogenic activity of CM derived from oral cancer cells. However, the involvement of other angiogenic factors cannot be completely ruled out. More studies are required to address this complex mechanism.

References
Table 1. Detection of VEGF and bFGF in the CM. The concentrations of VEGF and bFGF in CM derived from oral cancer cells and NOK were measured by ELISA using specific antibodies to VEGF and bFGF, respectively. The concentrations of VEGF and bFGF were represented with pg per ml under the condition of similar amounts of total proteins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>VEGF (pg/ml)</th>
<th>bFGF (pg/ml)</th>
<th>Total protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>2940</td>
<td>31</td>
<td>3300</td>
</tr>
<tr>
<td>CAL-27</td>
<td>1441</td>
<td>29</td>
<td>3200</td>
</tr>
<tr>
<td>OEClM</td>
<td>1584</td>
<td>10</td>
<td>4100</td>
</tr>
<tr>
<td>OC2</td>
<td>4524</td>
<td>19</td>
<td>3600</td>
</tr>
<tr>
<td>SCC9</td>
<td>1501</td>
<td>11</td>
<td>4400</td>
</tr>
<tr>
<td>SCC15</td>
<td>382</td>
<td>13</td>
<td>3900</td>
</tr>
<tr>
<td>SCC25</td>
<td>1432</td>
<td>9</td>
<td>4400</td>
</tr>
<tr>
<td>NOK</td>
<td>1303</td>
<td>ND*</td>
<td>ND</td>
</tr>
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</table>

Table 2. Status of p53 in oral cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Protein expression*</th>
<th>Mutations (exons 4-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOK</td>
<td>1</td>
<td>ND*</td>
</tr>
<tr>
<td>KB</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>CAL27</td>
<td>3.84</td>
<td>193, A→T, His→Leu</td>
</tr>
<tr>
<td>OEC-M1</td>
<td>2.02</td>
<td>Not found</td>
</tr>
<tr>
<td>OC2</td>
<td>2.51</td>
<td>132, A→G, Lys→Glu</td>
</tr>
<tr>
<td>SCC-9</td>
<td>-</td>
<td>274-285, 32bp deletion*</td>
</tr>
<tr>
<td>SCC-15</td>
<td>-</td>
<td>225-225, 5bp insertion*</td>
</tr>
<tr>
<td>SCC-25</td>
<td>-</td>
<td>208-209,2bp deletion,frameshift*</td>
</tr>
</tbody>
</table>

a, folds of p53 expression relative to NOK
b, not determined

Figure 1. CM stimulate HUVEC proliferation. Cell proliferation was assayed using HUVEC incubated for 2 days with the indicated diluted CM derived from (A)
KB and CAL-27; (B) OEC-M1 and OC-2; (C) SCC-9, 15 and 25; (D) NOK and 10 ng/ml bFGF. Student t-test was used to test the significance (p<0.05 or 0.01) of each treatment compared to control.

Figure 2. CM promote HUVEC migration. Cell migration was assayed using HUVEC incubated for 4 h with a mitogenic dose of diluted CM (3-fold) derived from (A) control (M199) and seven oral cancer cell lines; (B) control (M199), NOK and 1 ng/ml bFGF. Student t-test was used to test the significance (p<0.05) of each treatment compared to control.

Figure 3. Neutralizing VEGF antibodies attenuate HUVEC proliferation. HUVEC were incubated with 3-fold diluted CM containing the indicated doses of control IgG or neutralizing antibodies to VEGF. Cell proliferation was assayed two days after treatment.

Figure 4. Neutralizing VEGF antibodies attenuate HUVEC migration. HUVEC were incubated with 3-fold diluted CM containing 1000 ng/ml of control IgG or neutralizing antibodies to VEGF. Cell migration was assayed 4 h after treatment.

Figure 5. Downregulation of TSP-1 in oral cancer cells. Protein lysates were harvested from NOK and seven oral cancer cell lines for a western blotting analysis using an antibody to the TSP-1 (top). α-tubulin serves as a loading control (bottom). NIH3T3 and HUVEC are negative and positive controls, respectively. The numbers underneath the gels are folds of TSP-1 expression relative to NOK.

Figure 6. Disregulation of p53 in oral cancer cells. Protein lysates were harvested from NOK and seven oral cancer cell lines for a western blotting analysis using an antibody to the p53 protein (top). α-tubulin serves as a loading control (bottom). NIH3T3 is a negative control. The numbers underneath the gels are folds of p53 expression relative to NOK.

Figure 7. Disregulation of Mdm2 in oral cancer cells. Protein lysates were harvested from NOK and seven oral cancer lines for a western blotting analysis using an antibody to the Mdm2 protein (top). Proteins stained with Comassie blue staining serves as a loading control (bottom). NIH3T3 is a negative control. The numbers underneath the gels are folds of Mdm2 expression relative to NOK.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>TSP-I</th>
<th>α-tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>JT3</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>HUVTEC</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>NK</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>KB</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>CAL-27</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>SCC-25</td>
<td></td>
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<td>SCC-15</td>
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<td>-</td>
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<tr>
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<tr>
<td>OC-2</td>
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</tbody>
</table>
\begin{figure}
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Graph showing expression levels of Mdm2 and C-G250 in different cell lines.}
\label{fig:expression_levels}
\end{figure}
### 行政院國家科學委員會補助國內專家學者出席國際學術會議報告

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<th>報告人姓名</th>
<th>斯應堂</th>
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<th>成功大學醫學院病理學科教授</th>
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<td>發表論文題目</td>
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<td>(英文) Pathologic Features of Betal Rubid-related Oral Epithelial Lesions in Taiwan -- with Special Emphasis on the Tumor Regression and HPV Association</td>
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報告內容應包括下列各項：

一、參加會議經過

二、與會心得

三、考察參觀活動（無需活動者省略）

四、建議

五、攜回資料名稱及內容

六、其他

表 Y04
2001 美加病理學會年會參加會議報告

國立成功大學醫學院病理學科

靳應臺教授

一、 參加會議經過

美加病理學會年會每年舉辦一次，本次研討會地點在美國亞特蘭大附近舉行，為期五天。主要討論的重點為下列幾個部份:

1. Annual AFIP seminar
2. Legal issue in dental informatics
3. Informatics and technology in oral and maxillofacial pathology education
4. Interactive multimedia patient simulation using IMPACT
5. Use of Information technology in the teaching of oral pathology and general pathology
6. Cutaneous adnexal tumors
7. Innovative therapies for mucositis and chronic pain
8. CT and MR imaging of cranial-facial lesions
9. Selected lesions of the head and neck
10. The clinical manifestations and management of the oral vesiculoerrosive diseases
11. Protecting human subjects in research: what oral pathologists need to know
12. Essay 1, Essay 2
14. Clinical pathological conference
口頭演講多為 review 或 Summary 的性質，節目相當緊湊共有 43 篇論文於會中發表，並引起與會者的熱烈討論。另外會期中有另闢壁報展示區。大會並安排時段讓所有與會者在壁報區面對面的討論，由談話中汲取寶貴的經驗。

二、與會心得及建議

1. 本次會議參加人數約三百人，共十多個國家，台灣參加人數不多，大概是距離較遠之故。

2. 口腔癌是台灣十大惡性腫瘤，國內研究 HPV 病毒的學者很多，成果也不錯，如果能結合臨床口腔癌的研究，應該在國際上更能擁有自己的一席之地。

3. 美國部分地區的物價、生活指數是本人到過所有地方（包括東京）較高的地方，國科會在生活補助費上可重新評量各國、地區之差異。
Pathologic Features of Betel Quid-related Oral Epithelial Lesions in Taiwan—with Special Emphasis on the Tumor Progression and HPV Association

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Running Title: Betel Quid-related Oral Epithelial Lesions

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Key Words: betel quid, oral epithelial lesion, pathologic features, HPV
Abstract

Betel quid (BQ) chewing has been a well-documented cause of oral epithelial lesions. Evolution from early hyperplastic lesions to late or carcinomatous stage has been recognized. The pathobiologic and molecular mechanism, however, remains to be elucidated. In this study, a total of 232 samples obtained from 153 cases of BQ-related oral epithelial lesions (OEL) were retrospectively evaluated for the expression of p53 and bcl-2 in comparison with 26 cases of BQ-unrelated lesions (n=29). The possible role of human papillomavirus (HPV) was also investigated. These BQ-related OELs included verrucous hyperplasia (VH, n=57, 24.6%), epithelial dysplasia (n=23, 9.9%), verrucous carcinoma (VC, n=5, 2.1%), and squamous cell carcinoma (SCC, n=106, 45.7%). Fifty-four cases (35.3%) had multiple lesions. In comparison with the BQ-unrelated OELs, the characteristics of BQ-related OELs were of younger age, male predilection, and multicentricity. In contrast to tongue in BQ-unrelated OELs, the most common site for all types of BQ-related lesions was buccal mucosa. Immunohistochemical studies of BQ-related lesions showed p53 staining in 30% of dysplasia and 38% of SCC, but consistent absence in VH and VC. The cases with p53-positive SCC had a higher recurrent rate than p53-negative. Bcl-2 expression was negligible for all types of lesions. HPV 6/11 was detectable in 10% of dysplasia and 13% of SCC, but in none of VH and VC. HPV 16/18, however, were consistently negative for all types of lesions. Our data suggest that p53, but not bcl-2, may play a role in tumor progression of BQ-related OELs, and that VH and VC are distinct and closely related histologic lesions. The consistent absence of the malignant type HPV in all BQ-related lesions suggests that HPV plays an insignificant role in the tumorigenesis of BQ-related oral cancers, although a cooperative role may exist between benign type HPV and BQ chewing.
Introduction

In Taiwan, BQ chewing is a prevalent habit and a major problem in public health [1]. The "quid" for chewing consists of areca nut and pieces of unripe Piper betel fruit or areca nut wrapped into a piece of betel leaf together with white or red lime. It was defined as "betel quid" (BQ) according to the consensus in Kuala Lumpur workshop [2]. BQ chewing has a strong association with oral cancers [3-6], which arise predominantly from surface epithelium with evolution from early premalignant lesions [7]. The preneoplastic lesions may last for years before invasion, and may behave persistently and progressively after abstinence of BQ chewing. Although the histopathologic features have been well studied, the molecular mechanism underlying the progression from benign epithelial lesion to cancer still remains to be elucidated.

p53 protein is a 53-kd nuclear phosphoprotein and acts as "the guardian of the genome" to regulate cell growth and proliferation [8]. Mutations of the p53 gene play an important role in multistep carcinogenesis [9]. Overexpression of p53 protein was found to be associated not only with oral cancers and premalignant lesions [10-12], but also with the histologic grade of malignancy [13]. The carcinogenic pathway of overexpressed p53 protein has been suggested to synergize with bcl-2 overexpression, which occurs early in oral carcinogenesis resulting in defective apoptosis and subsequent tumor progression [14].

HPV is well known to be strongly associated with SCC. Besides the anogenital cancer, the virus has also been recently demonstrated to be associated with SCC in head and neck regions in Taiwan [15]. Multiple lesions of HPV infection had also been found in the patients with oral cancer and BQ chewing, and it was proposed that viral infection was an important etiological component, with BQ probably causing
additional mutagenic steps in the carcinogenic process [16].

In this study we desire to clarify the following issues: (1) Is there any histopathologic feature or biomarker specific for BQ-related OELs? (2) Are p53 and bcl-2 proteins correlated with the pathobiologic progression of oral lesion? (3) Is the role of HPV significant in the oral carcinogenesis in Taiwan?

‘p’ (petit) Material and Methods

Patients and samples

Two hundred and nine patients with oral epithelial lesions at the National Cheng Kung University Medical Center (Tainan, Taiwan) between February 1989 and January 1997 were collected in this study. All histologic sections were taken from surgical specimens. The pathologic diagnosis was retrospectively reviewed based on the WHO classification of oral mucosa [17]. The clinical data obtained by chart review included the following: sex, age, lesion site and number, and status of BQ chewing, alcohol drinking, and cigarette smoking, including the duration and daily amounts consumed. BQ-related oral epithelial lesions (OELs) were defined as the lesions in a patient with habitual BQ chewing (one quid or more per day for at least one year). Cigarette smoking was defined as one cigarette or more per day for at least one year, and alcohol drinking as drinking more than four days a week. The follow-up information of recurrent or new lesions was traced until July 2000.

Technique

All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Hematoxylin and eosin sections were available for histologic review.
Immunohistochemical studies by the streptavidin-biotin-peroxidase method were performed on paraffin-embedded tissues using 2 monoclonal antibodies, p53 (BioGenex, San Ramon, CA, diluted 1:100), and bcl-2 (BioGenex, San Ramon, CA, diluted 1:100). A pulmonary carcinoma and a normal lymph node with strong immunoreactivity to p53 and bcl-2 were used as positive controls. Positive stain was defined as distinct and strong nuclear (for p53) or cytoplasmic (for bcl-2) staining in more than 10% of the lesional cells. Adequate and optimal tissue sections were selected for immunohistochemical studies and HPV detection.

HPV DNA was detected from paraffin blocks by using polymerase chain reaction (PCR) as previously described [15]. In brief, after extraction, desiccation, and rehydration, each specimen was amplified with β-actin primers (BA2, 5'-TACATGGCTGGGGTGTTGAA-3'; and BA3, 5'-AAGAGAGGCACTCCTCACCACT-3') to verify the integrity of genomic DNA [18]. The pU-1M/pU-2R primer pair, detecting HPV-16 and -18, yields PCR products 238 and 268 bp and the pU-31B/pU-2R primer pair, detecting HPV-6 and -11, yields 228-bp products [19]. The primer sequences were as follows: pU-1M, 5'-TGTCAAAAACCGTTGTGTCC-3'; pU-31B, 5'-TGCTAATTCCGGTGCACCTG-3'; pU-2R, 5'-GAGCTGTCGCTTAATTGCTC-3'.

Each 50 μl of reaction mixture was prepared containing 1 mmol/L each of dATP, dCTP, dGTP, and dTTP, 25 pmol of each oligonucleotide primer, 0.5 U of Taq polymerase (Perkin Elmer, Oak Brook, IL) and 10 μl of specimen DNA in a buffer solution. The buffer solution consisted of 50 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 10 mmol/L Tris/hydrochloride, pH 9.0, and 0.1% Triton X-100. The mixtures were overlaid with a drop of mineral oil. The PCR was performed in a Perkin-Elmer Cetus thermal cycler. Amplification consisted of 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and
extension at 72°C for 1 minute. The initial denaturation step was extended to 2 minutes, and the final extension step was carried out for 5 minutes. The positive controls for PCR analysis consisted of DNA from formalin-fixed, paraffin-embedded SiHa cells (HPV-16), HeLa cells (HPV-18), and HPV-6/11 carrier perianal condyloma acuminatum. Distilled water in place of the DNA templates was used as the negative control. The PCR products were directly separated by electrophoresis on 2% agarose gel followed by staining and analyzed later.

Statistical analysis

Univariate analyses using chi-square test or Fisher’s exact test when appropriate were performed to test the correlation of categorical and clinicopathological variables with BQ chewing, whereas an unpaired t-test was used for interval variables.

Results

Clinicopathologic features

The total 209 cases were divided into BQ-related (n=153) and BQ-unrelated (n=26) groups with 30 cases excluded due to unknown history. The age at presentation ranged from 20 to 82 years (mean 50.0) in BQ-related group, and from 43 to 72 years (mean 60.3) in BQ-unrelated group. The male to female sex ratio was 151:2 and 18:8 in each group. The clinicopathological features between BQ-related and -unrelated OELs are listed in Table 1. The case (patient) numbers of multiple lesions were 54 and 3 in the BQ-related and -unrelated groups, respectively. Accordingly, there were total of 232 and 29 lesions (and so many sites) in each group. In contrast to the tongue in BQ-unrelated OELs, the most common site for all types of BQ-related lesions was
the buccal mucosa (53% in SCC, 61% in VH, 80% in VC, and 61% in dysplasia). In the BQ-related group, the patients had a younger mean age by 10 years, a strong male predilection, a higher rate of cigarette smoking and alcohol drinking, predominantly buccal mucosal involvement, and higher prevalence for multiple lesions, which frequently presented as concurrent lesions or metachronous lesions in the same (recurrence) and/or other sites (new lesions). Based on the pathologic classification, there was a strong association between verrucous hyperplasia and BQ chewing. All of the aforementioned characteristics were significant statistically.

**Immunohistochemistry**

The number of each lesion that we selected for immunohistochemical and HPV studies was as the following: SCC (n=104), VH (n=25), VC (n=6), dysplasia (n=13), epithelial hyperplasia (EH, n=7), keratosis (n=10), squamous papilloma (n=10), and verruca vulgaris (n=5). Overexpression of p53 protein was found in 36% (37/104) of SCC, 23% (3/13) of dysplasia, 14% (1/7) of EH, and 10% (1/10) of keratosis, but consistently negative for VH, VC, squamous papilloma, and verruca vulgaris. p53 positivity (Fig 1) was significantly higher in SCC than in VH and squamous papilloma (p<0.05, unpaired t-test). In each disease category for p53 staining, BQ-related group constantly had a higher rate than BQ-unrelated group (38% vs 24% in SCC, 30% vs 0 in dysplasia, 14% vs 0 in EH, and 11% vs 0 in keratosis), although there was no statistical significance.

Bcl-2 staining was observed in only one (1%) of 104 SCC and the other lesions were all negative (Fig 2).

**Detection of HPV by PCR**
Only the DNA samples showing the expected control 214-bp β-actin band after amplification with β-actin primers were included for PCR analysis (Fig 3). HPV 6/11 DNA was detected in 11% (9/79) of SCC, 17% (2/12) of dysplasia, 11% (1/9) of papilloma, and 20% (1/5) of verruca vulgaris, but not in VH (n=16), VC (n=3), EH (n=6), and keratosis (n=9). All types of lesions were negative for high-risk type HPV 16/18: SCC (n=77), VH (n=15), VC (n=3), dysplasia (n=8), EH (n=6), keratosis (n=5), papilloma (n=6), and verruca vulgaris (n=4). The results of immunohistochemical and HPV studies associated with the status of BQ chewing are summarized in Table 2.

Follow-up Results with p53 Status

In the cases of SCC including unknown BQ status, 7 out of 39 p53-positive and 3 of 56 p53-negative cases had recurrent or new carcinomas. It is significant statistically (P<0.05, χ²=3.9). In the cases of dysplasia, there is no difference between p53 positive (1 of 3) and negative (2 of 10) status in the association with SCC.

Discussion

The carcinogenic effects of BQ chewing have been widely investigated [3-6]; however, the association between pathologic classification and BQ-chewing has not been well elucidated. In this study, we found significant differences between the BQ-chewing and -non-chewing groups. The patients in the former group had a younger age by 10 years, a strong male predisposition, higher rates of smoking and drinking; and these lesions occurred mainly in buccal mucosa, and often presented as multiple lesions. Some of these features had also been observed in oral SCC patients with BQ chewing [20]. Our data reflected that most BQ chewers also carry habits of smoking
and drinking. The practice of BQ chewing has gained popularity among the teenaged population in Taiwan [21, 22], which may account for the younger age of patients. In this study, OELs associated with BQ chewing were prone to development in buccal mucosa. It is a typical site for BQ chewers [23], but different from the retromolar region for smoking and the mouth floor for drinking [24, 25]. The microtrauma by thick fibers of areca nut may result in the development of oral lesions in combination with the effect of the BQ ingredient [26, 27]. The practice to change chewing sites may contribute to the higher rate of multiple lesions, especially in bilateral buccal mucosa. Another mechanism for multiplicity was the field cancerization theory [28, 29]. This theory tells well the development of subsequent lesions after quitting chewing habits; however, it may not explain the predisposition to buccal mucosa involvement in our cases.

In this study the BQ-related group displayed a higher incidence of VH. It has been found to be associated with proliferative verrucous leukoplakia, epithelial dysplasia, VC, and SCC, and HPV may play a role in some VH [30, 31]. The association between VH and BQ chewing has not yet been found before. The incidence of VH in BQ chewers may be underestimated because it tends to be overlooked in concurrence with the aforementioned lesions or to be misinterpreted as VC. Since VH shared the persistent and irreversible features, some authors considered it an early form of VC and treated it accordingly [31-33]. Pathogenetically, we found that VH together with VC showed no etiological linkage with p53, bcl-2, or HPV, distinct from epithelial dysplasia and SCC. Other mechanisms, such as the expression of carbohydrate sialosyl-Tn or glutathione S-transferase isoenzyme may play a role [34, 35].

Overexpression of p53 protein and/or mutations of p53 gene have been found in varying proportions of precancerous and malignant oral lesions, and most of them
were associated with consumption of BQ and tobacco [10-12, 36-37]. It was, therefore, proposed that p53 alternations induced by carcinogens derived from BQ or tobacco were involved in the early development of oral cancer and may be helpful in identifying premalignant lesions [37, 38]. In our previous publication, we had stated that p53 immunoregionality is not always associated with gene mutation and it is therefore not appropriate to employ p53 immunohistochemistry (IHC) alone to predict alteration of the p53 gene [10]. Nevertheless, IHC is a much simpler and easier method than DNA sequencing, and it can facilitate the identification of p53 expression in specific cell types that molecular techniques cannot provide. We have performed DNA sequencing in limited cases to correlate the genetic and functional status, and 4 of 5 IHC-positive and 1 of 5 IHC-negative cases harbored p53 gene mutation (data not shown). In our series, there was a stepwise increase of p53 immunoreactivity in the sequence of EH-dysplasia-SCC. Thus, the role of p53 protein may account partially for the oral carcino genesis of SCC but not of VC. Interestingly, the follow-up data showed that the cases with p53-positive dysplasia didn’t bear a higher incidence for SCC development, whereas that with p53-positive SCC had a higher recurrent rate. Ogden et al. had also found that the expression of p53 protein in normal or precancerous oral mucosa did not necessarily predict its malignant potential [39]. However, more cases of BQ chewers with p53-positive dysplasia should be studied to clarify the issue of lesional progression. Clinically, the patients with p53-positive SCC should be regularly followed up for the recurrent lesions.

Expression of bcl-2 protein was nearly absent in all types of oral lesions. The lack of bcl-2 expression, distinct from some other studies [40, 41] may result from methodological or geographic differences, such as positive cut-off value (5% or 10%), antibody source, or antigen retrieval. The model of anti-apoptosis of bcl-2 cannot
apply in our cases and other gene products in the cell-cycle regulation may be alternatively involved, such as enhanced translation of *mdm2*-P2 transcripts [42, 43]. Importantly, the progression mechanisms of premalignant lesions to cancers are quite complex, and in addition to p53, there are several biomarkers providing useful information, including aneuploid DNA content [44], loss of *p16* (MTS1/CDKN2) [45], and CD44 expression [46, 47]. Molecular analysis, such as microsatellite markers may also provide genetic models for progression of precancer [28, 48].

HPV infection has been considered as an important factor of oral cancers in Indian BQ chewers [16]. In west countries, the detection rate of HPV in OELs was varied, ranging from inconsistency to 54% [49-53]. The differences were considered to be geographical and/or methodological [54]. The consistent absence of the high-risk type HPV 16/18 in all BQ-related lesions suggests that HPV plays an insignificant role in the tumorigenesis of BQ-related oral cancers in Taiwan, although a cooperative role may exist between low-risk type HPV and BQ chewing. Other high-risk HPV types, e.g. 31, 33, 52b, 58, in our and other authors’ experience are far less prevalent in OELs (0~10%) and if present, usually show co-infection with 16/18 [15, 55-56].

In conclusion, our study reports in detail the specific features of BQ-related OELs in Taiwan where the chewing habit is a major problem in epidemiology. The characteristics are well recognized. The progression model of EH-dysplasia-SCC was found at least partially through the pathway of p53 overexpression, whereas the roles of *bcl-2* protein and HPV were insignificant. VH and VC are distinct and closely related pathologic entities, and further studies with more cases are warranted for the clarification of the pathogenesis.
Acknowledgement

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<th>BQ-unrelated</th>
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<td>Total case number (209)*</td>
<td>153 (100%)</td>
<td>26 (100%)</td>
<td>P=0.041</td>
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<td>Mean age (years)</td>
<td>50.0 (20–82)</td>
<td>60.3 (43–72)</td>
<td>P&lt;0.05</td>
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<td>Sex (M:F)</td>
<td>151:2</td>
<td>18:8</td>
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<tr>
<td>Smoking history</td>
<td>117/126 (93%)</td>
<td>11/26 (42%)</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Drinking history</td>
<td>77/105 (73%)</td>
<td>5/24 (21%)</td>
<td>P&lt;0.01</td>
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<tr>
<td>Cases of multiple lesions</td>
<td>54 (35.3%)</td>
<td>3 (11.5%)</td>
<td>P&lt;0.05</td>
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<tr>
<td>Sites</td>
<td>232 (100%)</td>
<td>29 (100%)</td>
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<tr>
<td>buccal mucosa</td>
<td>134 (57.8%)</td>
<td>7 (24.1%)</td>
<td>P&lt;0.003</td>
</tr>
<tr>
<td>tongue</td>
<td>57 (24.6%)</td>
<td>15 (51.7%)</td>
<td>P&lt;0.003</td>
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<tr>
<td>lip</td>
<td>13 (5.6%)</td>
<td>3 (10.3%)</td>
<td>NS</td>
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<tr>
<td>gingiva</td>
<td>13 (5.6%)</td>
<td>1 (3.5%)</td>
<td>NS</td>
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<td>palate</td>
<td>10 (4.3%)</td>
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<td>tonsil</td>
<td>1 (0.4%)</td>
<td>1 (3.5%)</td>
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<tr>
<td>others†</td>
<td>4 (1.7%)</td>
<td>1 (3.4%)</td>
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<td>Pathologic classification</td>
<td>232 (100%)</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>106 (45.7%)</td>
<td>17 (58.6%)</td>
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<td>Verrucous carcinoma</td>
<td>5 (2.1%)</td>
<td>1 (3.4%)</td>
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<tr>
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<td>57 (24.6%)</td>
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<tr>
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<tr>
<td>Others†</td>
<td>7 (3.0%)</td>
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<td>NS</td>
</tr>
</tbody>
</table>

NS: no significance (P>0.05). * Thirty cases with unknown history are excluded. † Other sites include retromolar region, alveolar ridge, oral vestibule, and sublingual area. ‡ These lesions include ulcer and chronic inflammation. § Unpaired t-test. † Fisher's exact test. § Chi-square test.
Table 2. Results of p53, bcl-2, and HPV positivity in association with BQ-related and –unrelated oral epithelial lesions

<table>
<thead>
<tr>
<th>lesions (No.)</th>
<th>p53</th>
<th>bcl-2</th>
<th>HPV 6/11</th>
<th>HPV 16/18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>BQ + (87)</td>
<td>33* (38%)</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>carcinoma (104)</td>
<td>BQ - (17)</td>
<td>4* (24%)</td>
<td>13</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Verrucous</td>
<td>BQ + (23)</td>
<td>0</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>hyperplasia (25)</td>
<td>BQ - (2)</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Verrucous</td>
<td>BQ + (5)</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>carcinoma (6)</td>
<td>BQ - (1)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Dysplasia (13)</td>
<td>BQ + (10)</td>
<td>3 (30%)</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Epithelial</td>
<td>BQ - (3)</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>hyperplasia (7)</td>
<td>BQ + (7)</td>
<td>1 (14%)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Keratosis (10)</td>
<td>BQ - (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Squamous</td>
<td>BQ + (9)</td>
<td>1 (11%)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>papilloma (10)</td>
<td>BQ - (1)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Verruca</td>
<td>BQ + (5)</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>vulgaris (5)</td>
<td>BQ - (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* p53 positivity is significantly higher in SCC than in VH and papilloma (p<0.05, t-test).
Legends

Fig 1. Oral squamous cell carcinoma shows strong nuclear staining for p53 protein.

Fig 2. Oral squamous cell carcinoma shows negative staining for bcl-2 protein, while the tumor infiltrating lymphocytes are stained in cytoplasm.

Fig 3A. Example of PCR detection of HPV by primer pair pU-1M/pU-2R on oral lesions. M, 100-bp marker; 1, positive control (HPV-16); 2, positive control (HPV-18); 3, negative control; 4-13, cases. 3B. Example of PCR detection of HPV by primer pair pU-31B/pU-2R on oral lesions. M, 100-bp marker; 1, positive control (HPV-6/11); 2-6, cases; 7, negative control.