The role of human papillomavirus infection in carcinogenesis of oral squamous cell carcinoma with evidences of prognostic association

Running title: HPV associated with OSCC

Keywords: oral squamous cell carcinoma; human papillomavirus; p16[INK4A]; p53; prognosis

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Abstract

BACKGROUND: Betel nut chewing, cigarette smoking, and alcohol drinking are thought to be major environmental risk factors responsible for the development of oral squamous cell carcinomas. Oncogenic human papillomavirus infections have a well-established association with uterine cervical carcinoma. However, little is known about the exact role of human papillomavirus infections in oral squamous cell carcinomas. This study is designed to elucidate the role of human papillomavirus infections in cancer development and prognosis of oral squamous cell carcinomas.

METHODS: Molecular techniques including *in situ hybridization* and immunohistochemistry of p16^{INK4A} and p53 for evidences of human papillomavirus in tissue micro-arrays were investigated.

RESULTS: 24 out of 65 cases of oral squamous cell carcinomas were found positive for *in situ hybridization* and 14 were found positive for p16^{INK4A}. The majority of cases without the evidence of human papillomavirus were related to p53 over-expression. There were statistically significant correlations between the results of human papillomavirus test and size or extent of the tumor ($p=0.003$) or the stage of oral squamous cell carcinomas ($p=0.015$). Kaplan-Meier plot analysis demonstrated a tendency of longer survival in cases of oral squamous cell carcinomas with the evidence of human papillomavirus or positive p16^{INK4A}.

CONCLUSION: Human papillomavirus infections may play a unique role in oral carcinogenesis. Our data strongly suggest that human papillomavirus-positive oral squamous cell carcinomas comprise a distinct clinical and pathological disease entity that appears related to a better outcome with longer survival and bears a causally associated relationship different from other carcinogenic mechanisms

Introduction

Oral squamous cell carcinomas (OSCC) are one of the most common and lethal head and neck malignancies with increasing morbidity and mortality in Taiwan (1). In spite of substantial improvements in both diagnosis and therapy in recent decades, the prognosis of OSCC remains generally poor (2). Oral habits like betel nut chewing, cigarette smoking, and alcohol drinking are thought to be major environmental risk factors responsible for the development of OSCC in Taiwan (3, 4). However, some patients develop OSCC without exposure to these three risk factors, which suggests that additional factors, such as genetic predisposition, diet, or oncogenic viruses, may help cells escape the physiological mechanisms of proliferation control (5, 6). The relevance of the role of human papillomavirus (HPV) infection in cervical and anal cancer is well established and, by way of analogy, oncogenic HPV viruses might play a role in malignant transformation of squamous epithelia in any body region (7). Although still controversial, such evidence implies that carcinogenesis by environmental chemical carcinogens is not the only cause of oral squamous cell carcinoma. Presence of HPV in variable proportions in the oral or oro-pharyngeal squamous cell carcinoma tissues other than the uterine cervix, especially those genotypes with known high oncogenic potential (such as HPV16 and 18), has been demonstrated by several worldwide studies (8-11) (8-13). Recent studies suggest that HPV, especially type 16, may be responsible for a small subgroup of oral squamous cell carcinoma and up to 50% of oro-pharyngeal squamous cell carcinomas, especially the tonsillar squamous cell carcinoma (11, 14). Some reports emphasize the influences of HPV in predicting a better disease prognosis in oro-pharyngeal and tonsillar cancers (15, 16). Furthermore, a strong association between the presence of HPV and expression of key cell cycle proteins has been recently reported in a series of tonsillar cancer studies (15, 16) (17-20). These data seem to strongly suggest that HPV-positive tonsillar squamous cell carcinoma represents a distinct molecular, clinical and pathologic entity.

However, little is reported about the exact role of HPV in carcinogenesis and the associated

prognosis of OSCC. What is well known is that persistent high-risk HPVs infections may promote malignant transformation of stratified squamous lining mucosal epithelium which is mediated by inactivation of p53 and retinoblastoma (pRb) by the viral gene products E6 and E7 (21). The inactivation of pRb results in an over-expression of p16^{INK4A} (22). The p16 protein, encoded by the CDKN2A (MTS1, INK4A) tumor suppressor gene located on chromosome 9p21, decelerates the cell cycle by inactivating the function of cdk4 and cdk6-cyclin D complexes. These complexes regulate the G1 check-point by phosphorylation and subsequent inactivation of pRb, which in turn releases E2F allowing the cell to enter into the S phase (23, 24).In all HPV-positive neoplasms, pRb has been shown to be functionally inactivated as a consequence of HPV E7 protein expression, and as a result, a strong over-expression of p16^{INK4A} develops (25, 26).   p16^{INK4A} over-expression is easily detectable with immunohistochemical stains and has been proposed as a biomarker that would allow unambiguous identification of truly neoplastic cells and would help in the identification of a subset of tumors related to HPV infection in a variety of genital and extra-genital areas such as the penis, anus, tonsils, and rhino-sinusal cavities (26-30). It has even been shown that in particular settings, such as the oral cavity, immunohistochemical p16^{INK4A} detection is a simple, but not frequently used technique applicable for a histological diagnostic routine that is equivalent to HPV detection (29).   The aim of the current study is to elucidate the role of HPV in carcinogenesis of OSCC through *ISH* and IHC of p16^{INK4A} and p53 and to further correlate the clinical parameters with each individual case.

Materials and methods

Patients and tissue specimens

Tissue specimens of 88 patients with OSCC were collected and retrieved from the archives of the Department of Pathology, Tri-Service General Hospital, Taipei, Taiwan from January 2003 to June 2004. Tissue micro-arrays (TMA) with 1.5 mm-sized cores were then constructed and examined from the paraffin blocks. The majority of the cases were selected from the radically surgical specimens of OSCC. 23 cases were lost to follow-up or had insufficient clinico-pathological data for analysis and were therefore excluded. Thus, a total of 65 cases of OSCC were included in the study. All of the collection samples conformed to the ethical approval of Tri-Service General Hospital Institutional Review Board.

Immunohistocemistry

The formalin-fixed paraffin-embedded specimens were made in 4-μm-thick sections. After de-paraffinization and re-hydrated, antigen retrieval was carried out by incubation in a citrate buffer (10mM citrate acid, pH 6.0) at 95℃ for 40 minutes. Endogenous peroxidase and nonspecific antibody reaction were blocked with 3% H_2O_2 and 5% normal horse serum in PBS. For implementation of the labeled streptavidin–biotin method (LASB2 kit/HRP, DakoCytomation), primary mouse anti-Human p16^{INK4a} antibody (clone E6H4, DakoCytomation) and monoclonal mouse anti-human p53 antibody (clone Dc-7, DakoCytomation) were used at dilution of 1:100 and incubated overnight at 4℃. Subsequently, all sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted.

In situ hydridization

In situ hybridization was performed using labeled probes (for types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52) with wide spectrum HPV biotinylated DNA (DakoCytomation) and tyramide signal amplification system (DakoCytomation GenPoint^{TM}). Tissue sections 4μm thick were cut onto coating slides and dried overnight at 65° C. Tissues were dewaxed in two 10-minute

changes of xylene and then washes by degraded (100–70%) ethanol. After rinsing in water, tissues were immersed in 10 mM sodium citrate, pH 6.0, at 95.0° C for 40 minutes. The slides were rinsed with water and air dried. Tissues were then digested at 25° C with 400μg pepsin (Sigma P-7012) in 0.2 N HCl for 10 minutes. Slides were rinsed three times for 5 minutes each in TBST and air dried. Endogenous peroxidase was blocked with 3% H_2O_2 at room temperature for 10 minutes. Pre-hybridization mixture was applied directly to the tissue section and comprised 50% formamide; 10% dextran sulfate; 10% 20x SSC, pH 7.0 ; 1000μ g/L salmon sperm signa-strade DNA (Sigma). Cover- slipped slides were placed into Terasaki plates (Nunc) containing 5x SSC at 95° C for 10 minutes to enable simultaneous denaturation of probe and target sequences. Tissues were hybridized overnight at 37° C. Samples were washed stringently three times (2x SSC at 55° C, twice for 5 min; 1x SSC at 37° C, twice for 5 min; 0.1x SSC at 37° C, twice for 5 min). Biotinyl-tyramide and secondary streptavidin– horseradish peroxidase conjugate were used as supplied in the GenPoint kit. Aminoethylcarbazole was (DakoCytomation) applied to sections for 10 minutes. Sections were counterstained with hematoxylin (DakoCytomation) and mounted in a glycerol gelatin (Sigma).

Histopathological evaluation

All of the histopathological slides on TMAs were concurrently reviewed and evaluated independently by two qualified pathologists using the same type of microscope without any prior knowledge of each patient's clinical details. When the opinions of the two evaluators differed, consensus was reached by discussion. Subsequent interpretations of cases of both *ISH* and IHC findings also followed the aforementioned procedure. In each TMA, a positive control with a case of cervical squamous carcinoma for p16^{INK4A} was included. Cases with more than 50% of tumor cells showing strong or moderate nuclear staining along with cytoplasmic staining were considered positive for p16^{INK4A} expression. In the meantime, a positive control with a case of poorly differentiated gastric adenocarcinoma for p53 was also prepared. Cases with more than

50% of tumor cells showing strong or moderate nuclear staining were considered positive for p53 expression. *ISH* was performed using individual plasmid probes including types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52. ~~Cases with a more than 10% integrated form (nuclear dots or punctuate staining) or episomal (diffuse staining) form throughout the nucleus were considered positive.~~ A discrete, punctate signal pattern within a cell nucleus correlated with integration, whereas a diffuse signal throughout the nucleus indicated episomal HPV (20, 31). The results were measured according to the previous study. Cases with more than 10% of tumor cells displaying punctate or diffuse signal throughout the nucleus were considered positive (11).

Statistical analysis

All statistical analyses were done using SPSS software v.13.0 (SPSS UK Ltd, Woking, UK) for windows. Chi-square (χ^2) tests (with adequate Yates' correction for 2x2 tables and d.f. =1, or with Fisher's exact test when expected value < 5 in > 20% table cells) were used to measure the significance between clinical parameters and ISH of HPV. Chi-square (χ^2) tests were also used to measure the significance between *ISH* of HPV and p16[INK4A] or p53. The log rank test was used to compare differences in survival between two groups and survival curves were obtained by the Kaplan-Meier method. $P < 0.05$ was considered to be significant.

Results

24 out of 65 cases of OSCC were positive for *ISH* showing evidence of HPV on TMA. Most of the *ISH*-positive cases demonstrated the integrated viral DNA by a dot-like or punctate merging signal within the nucleus. However, there were only four cases showing episomal viral DNA by a diffuse signal throughout the nucleus. Among these 24 positive cases, 14 were found positive for p16^{INK4A} by IHC. There was a close association between positive p16^{INK4A} immunostaining and positive *ISH* for evidence of HPV infection (14/24). However, there were 10 cases out of 24 that were positive for *ISH* but negative for p16^{INK4A} immunostaining. In addition, among these 24 positive cases, p53 immunostaining was variably expressed. There were 11 cases over-expressed for p53 including 3 positive p16^{INK4A} immunostaining and 8 positive *ISH*. There were 41 cases negative for *ISH* without evidence of HPV. The majority of *ISH*-negative cases were related to p53 over-expression (37/41). However, there were 4 cases negative both for p53 and for *ISH* (Fig. 1 and 2).

The presentation and correlation of the clinico-pathological data and the results of HPV tests of 65 patients with OSCC is summarized in Table 1. When comparing the results of the HPV test with the clinico-pathological parameters, there was no significant correlation between the results of the HPV test and gender, age, location, status of lymph node metastasis and histological grading. However, there were statistically significant correlations between the results of the HPV test and size or extent of the tumor (*p*=0.003) or the stage of OSCC (*p*=0.015). Together with three main test parameters, there were statistically significant correlations between the results of the HPV test with p16^{INK4A} and/or p53 (Table 2).

Follow-up of 65 cases with OSCC from initial diagnosis to 60 months was performed and the overall survival rate estimated at 38.5%. There was a statistical significance between the patient's survival and results of *ISH* of HPV and IHC of p16^{INK4A} by the log rank test and Kaplan-Meier plots (Fig. 3). Cases of OSCC with positive HPV and/or positive p16^{INK4A} results were associated with better prognoses; however, cases of OSCC with over-expressed p53 were

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associated with worse prognoses.

Discussion

Oncogenic HPV infections have a well-established association with uterine cervical and ano-genital carcinomas. Their relationship to OSCC, however, is less clear. Based on our data, there were more than 1/3 (24/65) cases of OSCC showing evidence of HPV via *ISH*. Each positive *ISH* showing punctate or diffuse signals within the nucleus was variably expressed. Among these 24 positive cases, 14 were found positive for p16^{INK4A}. *ISH* for evidence of HPV infection in OSCC appeared more sensitive than immunohistochemical detection of p16^{INK4A}. The use of molecular assays between *ISH* by HPV probes and IHC for p16^{INK4A} comprised different sensitivities and specificities for viral or its oncoprotein detection. Nevertheless IHC for p16^{INK4A} detection is a simple technique applicable for histologically diagnostic tool only and exclusively indicative of E7 oncoprotein, this useful method thus results in less sensitive detection for HPV (28, 32). The majority (37/41) of HPV-negative cases of OSCC were related to over-expression of p53. In addition to 11 cases co-expressed with *ISH* for HPV and p53, there were a total of 48 cases of OSCC showing over-expression of p53 (48/65). It was generally agreed that the role of p53 mutations in the pathogenesis of certain head and neck cancers may be substituted by HPV infection because viral E6 protein can inactivate p53 by targeting the protein for ubiquitination and degradation (30, 32, 33). Mutant p53 proteins tended to accumulate and thus become over-expressed until carcinogenesis occurred in a certain percentage of all tumors. There was a marked difference in p53 mutation frequency between HPV-positive and HPV-negative tumors in OSCC. The inverse relationship between p53 mutation with over-expressions and HPV strengthens the etiologic role of HPV in OSCC. In addition, this inverse relationship further suggests two parallel or overlapping pathways of oral carcinogenesis: one was thought to be driven by environmental toxins (e.g., betel nut, tobacco and alcohol) and another driven by viral oncogens (e.g., high-risk HPVs). In addition, cases of HPV-positive OSCC were more likely to occur in patients without environmental risk factors

than those of HPV-negative OSCC (data not shown). As seen in other reports, the lifestyles of patients with HPV-related OSCC appeared relatively different and 'healthier' than those of patients with HPV-negative OSCC (6, 9, 11, 16). Nevertheless, viral infections may also act synergistically with environmental toxins effects. The coexistence of positive *ISH* and p53 over-expression seen in 11 cases, along with 4 cases negative for *ISH* and less expressed p53 immunostaining, may be explained by the variable sensitivity of certain p53 mutants to E6 degradation and/or by tumor promotion via p53-independent viral mechanisms such as disruption of pRb function by HPV E7 (33-36).In conjunction with recent studies, our data provide strong evidences that HPV are etiologically linked to a defined subset of OSCC. Compared to other previous reports where different methods conclude that the HPV-positive rate of OSCC ranges from 43 to 75%, our data with nearly 37% HPV-positive rate seems obviously lower than those in oro-phargnx and pharynx, as reported by others (11, 15-18).The difference strongly suggests anatomical site specificity. It also implies that the evidences of HPV in mucosal tissues of head and neck other than oral cavity are far more easily detected. The motile nature of oral cavity along with saliva secretion and cleaning perhaps, at least in part, is responsible for the lower detection rate of HPV.

As shown in Figure 3, like previous reports, significant differences were found between the presence or absence of HPV and survival in OSCC (15, 16, 37, 38). The reason for the favorable prognoses with longer survival in patients with HPV-positive OSCC is still unclear. A recent *in vitro* study disclosed that HPV oncoproteins E6 and E7 mediate suppression of NF-*k*B transcriptional activity and may further contribute to HPV escaping from the immune system in cases of HPV-related carcinogenesis (39). Evidences also revealed that HPV-positive cancers may have an intact apoptotic response to radiation and chemotherapy (40). Aforementioned theoretical statements may also be referred to the statistical significance between the results of HPV test and tumor size as well as the clinical stage as seen in our study. The reason why other clinico-pathological parameters are not involved still needs further investigation and additional

cases.

Along with other recent reports, our study suggests that HPV-positive OSCC should be regarded as a unique subset of all squamous cell carcinomas that arise in the head and neck (23, 37). As seen in recent reports for prevention of persistent high risk HPV-induced cervical dysplasia and cancer an alternative strategy using prophylactic vaccine for disease prevention of HPV-related OSCC, although still in animal model experiments, may be served the similar protective effect in the near future (41).

In our study we investigated and analyzed two molecular techniques including *ISH* and IHC via TMAs which provide powerful tools for high-throughput in situ analysis allowing the simultaneous evaluation of tumor by histology. All slides on TMAs are prepared and stained under the same conditions and analyzed accordingly. ~~In addition, *ISH* is an emerging technology that allows sensitive direct visualization of HPV at the light microscopic level. Comparatively, *ISH* test for HPV appears qualitatively better than an assay of PCR as seen in our study.~~ *ISH* is an emerging technology that allows direct visualization of the viral particles of HPV in the nucleus with satisfactory sensitivity and specificity at the light microscopic level. Many studies for detection of HPV are measured by PCR and the test result of PCR is based on repeated amplification of fragments of viral DNA resulting in the highest sensitivity and HPV detection rate. Nevertheless this technique may also inevitably cause unreliable data following repeated amplification. Comparatively, *ISH* test for HPV appears qualitatively better than an assay of PCR as shown by other reports. (11, 31)

In conclusion, HPV associated tests including *ISH* and IHC comprise distinct molecular techniques which may serve as clinically accessible markers. HPV infections may play a unique role in oral carcinogenesis distinct from other carcinogenic mechanisms. HPV-positive OSCC appears related to a better outcome with longer survival.

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Acknowledgements

Table 1. Correlations and analysis of clinico-pathological parameters with the test results of HPV in 65 patients with OSCC.

|	n (%)	HPV (+)	HPV (-)	P^a
Clinico-pathological features	**65**	**24**	**41**	
Age				
Median	53 (30~81)			
＜53	32 (49)	9 (38)	23 (56)	0.148
≧53	33 (51)	15 (62)	18 (44)	
Gender				
Male	52 (80)	17 (71)	35 (85)	0.157
Female	13 (20)	7 (29)	6 (15)	
Location				
Tongue	35 (54)	16 (67)	19 (46)	0.158
Buccal mucosa	20 (30)	4 (17)	16 (39)	
Gingiva	2 (3)	0 (0)	2 (5)	
Hard palate	1 (2)	0 (0)	1 (2)	
Floor of mouth	7 (11)	4 (16)	3 (8)	
T				
I/II	28 (43)	16 (67)	12 (29)	*0.003
III/IV	37 (57)	8 (33)	29 (71)	
N				
No	58 (89)	21 (88)	37 (90)	0.731
Yes	7 (11)	3 (12)	4 (10)	
M				
No	64 (98)	24 (100)	40 (98)	0.441
Yes	1 (2)	0 (0)	1 (2)	
Stage				
I/II	23 (35)	13 (54)	10 (24)	*0.015
III/IV	42 (65)	11 (46)	31 (76)	
Histology				
Well differentiated	26 (40)	14 (59)	12 (29)	0.070
Moderately differentiated	31 (48)	8 (33)	23 (56)	
Poorly differentiated	8 (12)	2 (8)	6 (15)	

[a] Data were analyzed with Chi-square test.

Table 2. Correlations between the results for HPV by *ISH* and IHC for p16^{INK4A} and p53 in 65
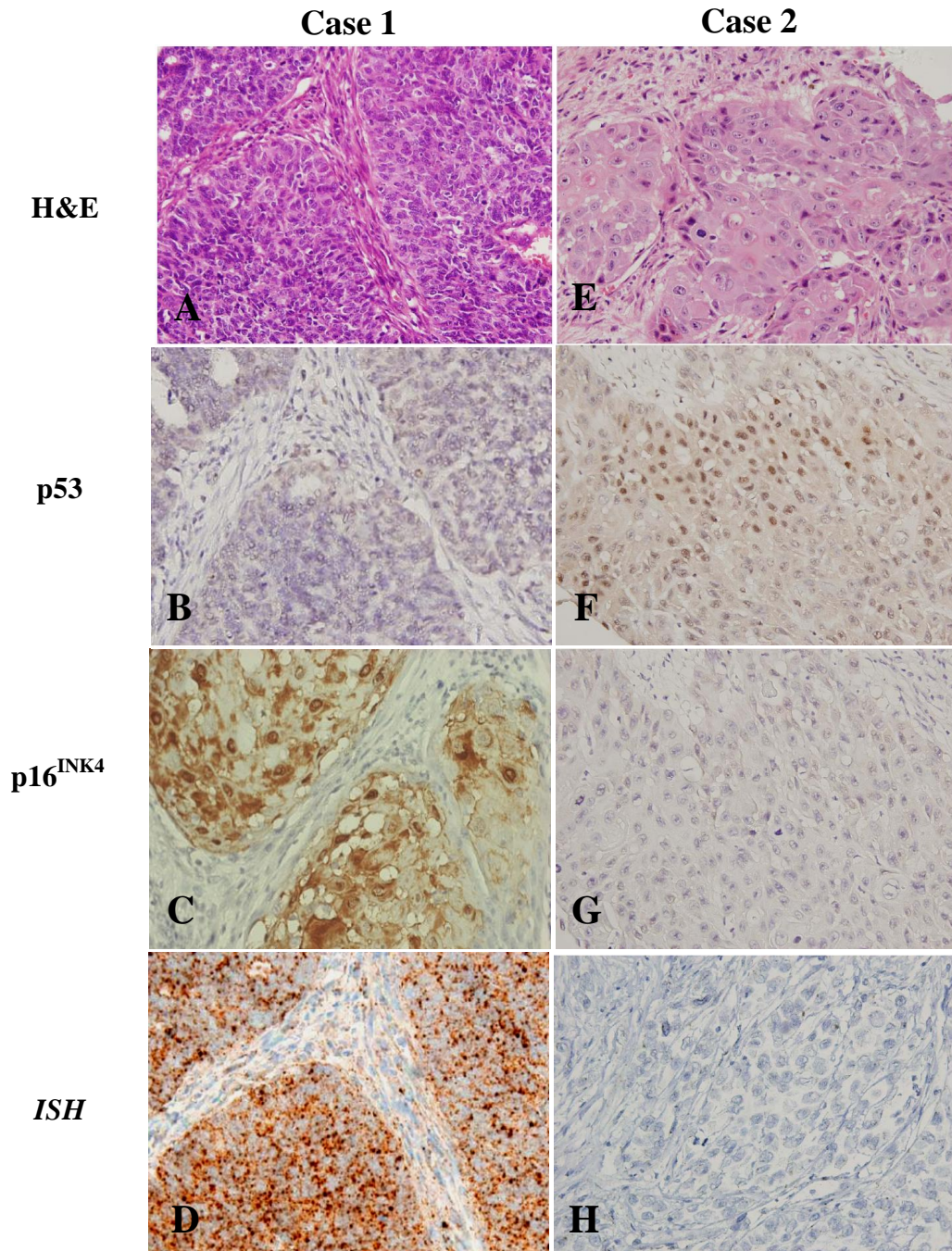
patients with OSCC.

|		N (%)	HPV (+)	HPV (-)	P^a
		65	24	41	
p16	(-)	51(78)	10(42)	41(100)	*0.001
	(+)	14(22)	14(58)	0(0)	
p53	(-)	17(26)	13(54)	4(10)	*0.001
	(+)	48(74)	11(46)	37(90)	

[a] Data were analyzed with Chi-square test.
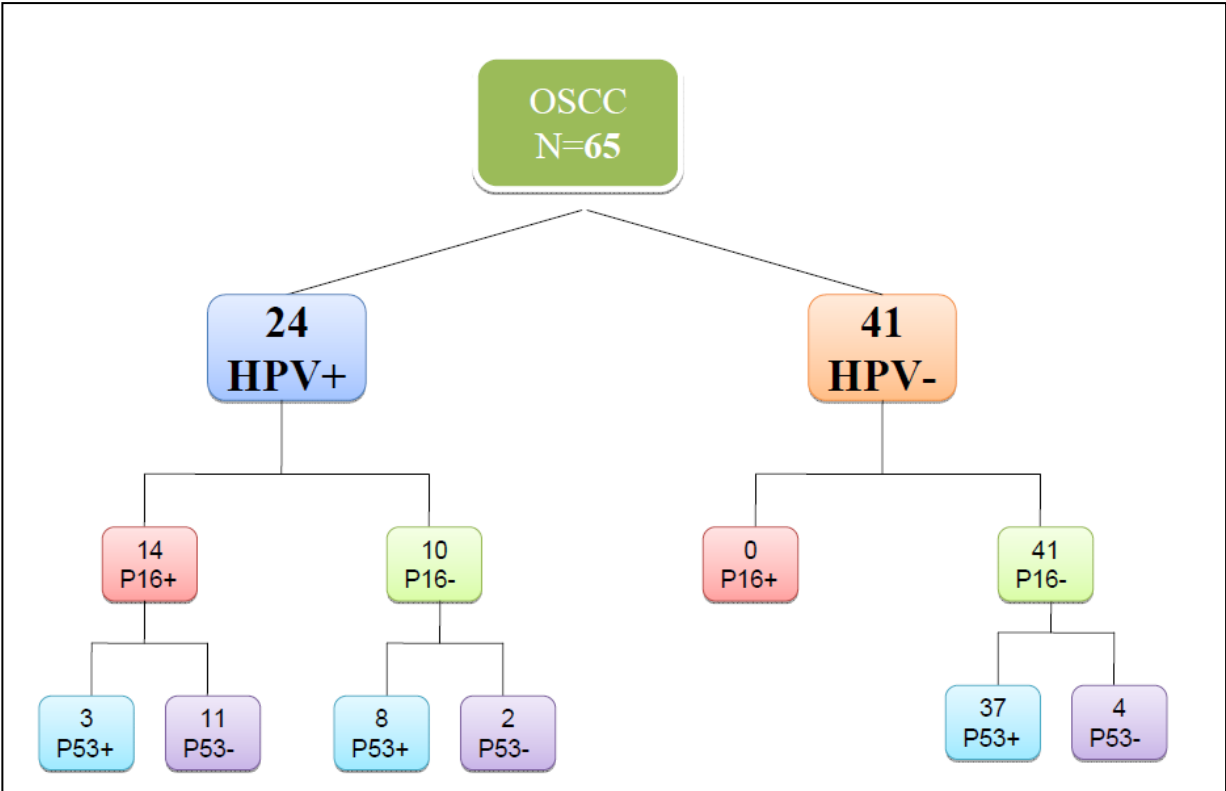
FIGURE 1. Distributions of the test results of HPV by *ISH* and IHC of p16^{INK4A} and p53 in 65 patients with OSCC.

FIGURE 2. The microscopic illustrations with comparisons of two representative cases of OSCC were shown in left panel from A-D (case 1) and right panel from E-H (case 2). The histological findings of case 1 elicited a poorly differentiated squamous cell carcinoma in A, compared with the case 2 presenting a moderately differentiated squamous cell carcinoma in E (X200); The corresponding immunohistochemical stains for p53 showed a negative result (B) in case 1, compared with over-expressed p53 (F) in case 2 (X200); while p16^{INK4A} stains demonstrated a positive result in case 1 (C) (X400) and a negative result in case 2 (G) (X200); *In situ hybridization* studies for evidence of HPV showed a positive result with nuclear punctate pattern in case 1 (D) (X400) and a negative result in case 2 (H) (X200).
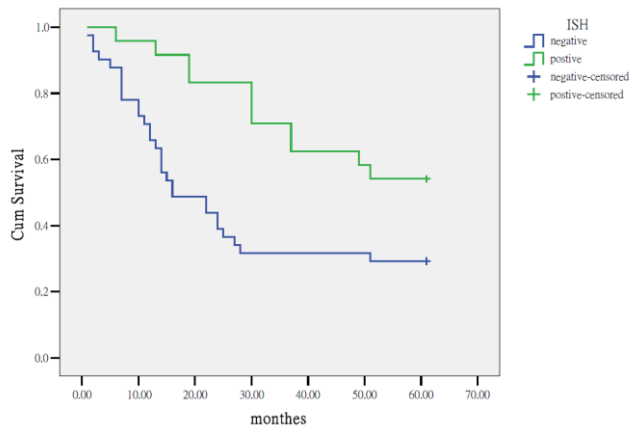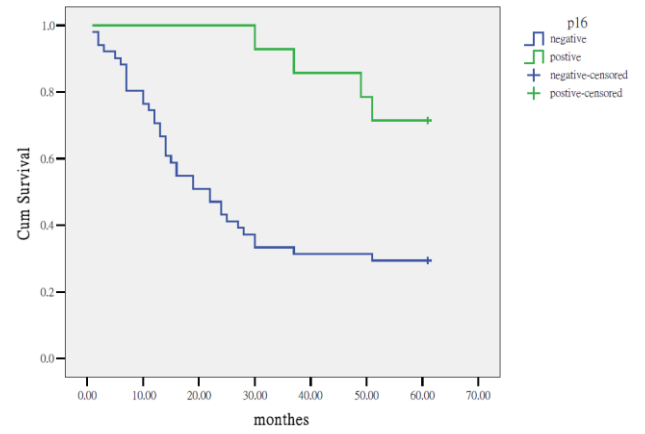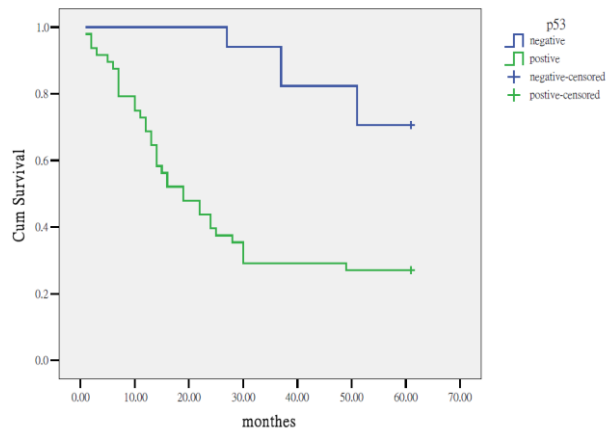
FIGURE 3. Relationship between overall survival and the test results of HPV by *ISH* (A), IHC of p16^{INK4A} (B) and p53 (C) in 65 patients with OSCC by Kaplan-Meier analysis.