Frequency and Role of HPV in the Progression of Epithelial Dysplasia to Oral Cancer

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Abstract. *Background: Human papillomavirus DNA (HPV DNA) and p16 and p53 protein expressions were investigated for their role in transforming dysplasia into squamous cell carcinoma of the oral cavity in a non-smoker and non-drinker patient group. Materials and Methods: A total of 56 oral biopsies from non-smoker and non-drinker patients were analyzed. The specimens were grouped into three categories: group 1 included 31 cases of hyperplastic mucosa and mild dysplasia, group 2 included 14 cases of moderate and severe dysplasia, while group 3 comprised 11 cases of invasive squamous cell carcinomas. In all cases, immunohistochemical methods were performed to detect p16 and p53 protein expressions. The nested polymerase chain reaction for HPV (nested HPV-PCR) and the catalyzed signal-amplified colorimetric DNA in situ hybridization (CSAC-ISH) methods were applied for HPV DNA detection and typing of high-risk genotype. Results: P16 protein, absent from all specimens of group 1, was especially noted in group 2 (92.86%) and in group3 (54.55%). Five out of 14 of group 2 cases (35.71%) and 3/11 (27.27%) of group 3 were HPV DNA positive. The HPVs detected were of both high-risk and low-risk genotype. The analysis of the relationship between HPV and p16 protein expression revealed that all the group 2 and 3 samples with HPV DNA, overexpressed p16 protein. Conclusion: The results suggest that HPV could be a molecular marker in group 2 and 3 specimens in non-smoker and non-drinker patients. The virus may play an etiological role in carcinogenesis in the oral cavity. The association between HPV and p16 overexpression suggests a molecular mechanism similar to that found in cervical cancer.*

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While tobacco and alcohol consummation are the primary risk factors for the development of head and neck squamous cell carcinoma (HNSCC), recent epidemiological studies report a strong association with human papillomavirus (HPV) in a subset of cases (1-4). Previous work evaluating different markers of exposure and viral activity in tumors indicates that HPV may play a role in some tumors of the oral cavity and oropharynx (5). However the prevalence of HPV detection varies broadly, depending on the population, combination of subsites, type of specimen, and detection method (6).

HPV is a small (55 nm), non-enveloped, icosahedral, epitheliotropic DNA tumor virus (7).To date, more than 120 genotypes have been identified. Because of the unequivocal implication of HPV in the etiopathogenesis of uterine cervical squamous cell carcinoma (SCC), HPV types in this context have been well studied and subdivided into two major groups based on their oncogenic potential in genital cancer: high-risk HPV (HR-HPV) and low-risk HPV (LR-HPV) types (8). The most frequent HR genotypes are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, and those of LR-HPV genotype are 6, 11, 43 and 43. LR-HPV genotypes have only rarely has been implicated in malignant transformation of the cervix (9). In the mouth, LR-HPV infection is associated with a variety of benign lesions including squamous cell papilloma, verruca vulgaris, condyloma acuminatum and focal epithelial hyperplasia, but can also be detected in potentially malignant epithelial lesions such as leukoplakia and erythroplakia (10, 11). The reported rates of HPV DNA detection, in potentially malignant and malignant oral lesions range from 0 to 100% (12, 13). This extreme variability is owing to differences in ethnicity, geographic location and sample size of the individuals examined, and to variations in the methods for HPV detection. HR-HPV genotypes, in particular 16 and 18, are those most frequently detected in malignant oral epithelial lesions. HPV-16 infection may be a risk factor for SCC of the head and neck (14).

In the cervix, the oncogenic potential of HR-HPV is attributable to the overexpression of viral oncogenic proteins E6/E7. In particular, E6/E7 viral proteins determine the alteration of various key functions of the host cells. The tumor suppressor factors of p53 and pRb pathways are suppressed, leading to defects in apoptosis, DNA repair mechanism, cell-cycle regulation and, finally, to cell malignant transformation The inactivation of pRb by E6/E7 genes causes p16 overexpression because p16 is regulated by negative feedback from pRb (15, 16). In (17) the oropharyngeal SCC, in where viral integration within the cellular genome was demostrated, unmutated p53, reduced expression of Rb and overexpression of p16 proteins were also demonstrated. However usually oral SCC and potentially malignant lesions, HPV DNA is not integrated into the host genome and alterations of the expression of p53 and p16 proteins is not unequivocally associated with the presence of HPV (17).

The purpose of this research was to determine if HPV detection is a molecular marker of oral dysplasia and cancer in non-smoker and non-drinker patients and to determine if HPV in the oral cavity may play a role in the onset of oral dysplasia and into transition towards squamous cell carcinoma.

Materials and Methods

Samples. Fifty-six formalin-fixed, paraffin-embedded biopsy specimens of the oral cavity were obtained from the Milan-Bicocca University Department of Pathology, S. Gerardo Hospital, Monza, Italy. The specimens included 24 dysplasias, classified as mild, moderate and severe following the WHO tumor classification (18), 11 invasive squamous cell carcinomas and 20 normal or hyperplastic mucosa specimens. All the cases were limited to the oral cavity in its strictest definition (*i.e.* no tonsil, pharynx or larynx tumours were included), and matched control pairs. The sources of the oral biopsies and demographic characteristics are showed in Table I. For our purpose, as has been suggested (19), we grouped the specimens as follows: group 1 consisted of 31 patients with normal/hyperplastic mucosa and mild dysplasia (18 men and 13 women, age range 8 to 83 years); group 2 included 14 cases with moderate or severe dysplasia (8 men and 6 women, age-range 45 to 83 years); group 3 comprised 11 cases of invasive squamous cell carcinoma (8 men and 3 women, age range 50 to 80 years).

Immunohistochemistry. Immunohistochemical analysis was performed on paraffin sections mounted on sialinized slides incubated with the avidin-biotin-peroxidase complex and using commercially available antibodies: anti-p53 (clone DO7, Neomarkers, Union City, CA, USA; dilution 1:150), anti-p16 (clone E6H12; Novocastra, Newcastle, UK; dilution 1:40). For each antibody, positive and negative controls were used. As proposed by others (19, 20), p53 reaction was classified as positive or negative, attributing a score taking into account both the relative number of stained nuclei and their localization in the upper 2/3 of the epithelium in groups 1 and 2, while in invasive carcinomas (group 3), evaluation was made in the areas of infiltration, where cell proliferation was more active. With regard to p16 protein expression, immunostaining was considered positive when at least 10% of nuclei were stained in dysplastic epithelia (21).

Table I. *Pathological and demographic features of 56 patients studied.*

	Site		Gender	Age (years)
Group 1 (n=31)	Cheek	7 (22.5%)	18 M (58.1%)	8-83
No or mild	Floor	2 (6.45%)	13 F (41.9%)	
dysplasia	Lip	2 (6.45%)		
(mean=56)	Gingiva	8 (25.8%)		
	Tongue	12 (38.7%)		
Group 2 (n=14)	Cheek	4 (28.6%)	8 M (57.2%)	45-83
Moderate or	Floor	2 (14.3%)	6 F (42.8%)	(mean=70)
severe dysplasia	Lip	1 (7.1%)		
	Gingiva	3 (21.4%)		
	Tongue	4 (28.6%)		
Group 3 (n=11)	Cheek	3 (27.3%)	8 M (72.7%)	50-80
Invasive	Floor	1 (9.1%)	3 F (27.3%)	(mean=66)
carcinoma	Lip	1 (9.1%)		
	Gingiva	2 (18.2%)		
	Tongue	4 (36.3%)		

M: male; F: female.

DNA isolation, and HPV detection by nested polymerase chain reaction (nested HPV-PCR). Genomic and viral DNA was obtained from four 10 μm-thick sections from the paraffin blocks. All sections were placed in 2 ml microcentrifuge tubes and dewaxed with multiple washes of xylene and absolute, 95% and 70% ethanol. After overnight digestion with sodium dodecyl sulphate (SDS) and 5% of 20 mg/ml of K proteinase (Sigma, Saint Louis, USA) at 37˚C, DNA was extracted by standard phenol-chloroform-isoamylic alcohol and sodium acetate-ethanol precipitation. The HPV-nested PCR method was applied to amplify the 139-150 bases of the HPV-L1 region or the 208-245 bases of the E6/E7 gene of the HR viral genotypes 16-18-33-35-52-58 (nested L1 and HR-PCR). Briefly, 2.5 μl of the DNA were used to amplify the target genes with the consensus primers (MY09/11) or HR type-specific primers (Nested HPV Kit, Experteam, Venice, Italy) in two-step nested PCR. All amplification reactions were performed in a total volume of 25 μl with 0.2 units of ExperTaq (Experteam). Cycling conditions for the amplification of the HPV-L1 region were 30 seconds at 95˚C, 30 seconds at 53˚C (first step) or at 45˚C (second step) and 30 seconds at 72˚C for 30 or 40 cycles. Cycling conditions for the amplification of the E6/E7 regions were 1 minute at 95˚C, 1 minute at 55˚C (first step) or at 52˚C (second step) and 1 minute at 72˚C for 30 or 40 cycles. Positive and negative controls were used for each amplification. Primers of the human β globin gene (Experteam) were used for testing the quality of the extracted DNA in order to exclude false negative results derived by DNA degradation of long-fixed samples.

Catalyzed signal-amplified colorimetric DNA in situ hybridization (CSAC-ISH) for HPV detection. CSAC-ISH was performed in 9 cases where nested PCR detected HPV-DNA. Briefly, a 5 μm-thick section of each formalin-fixed paraffin

embedded specimen was deparaffinized in xylene. Sections were treated by digestion with K proteinase (S 3020; Dako, Glostrup, Denmark) for 7 minutes. Ten µl of biotinylated probes for HR-HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (Y 1443, GenPoint™ HPV biotinylated DNA probe; Dako) were applied to each specimen, and then covered with coverslips. Target and probes were denatured by heating the slide to 92˚C in a hybridizer (S 2451; Dako). After washing and background quenching with 3% H_2O_2 for 10 minutes, the GenPoint kit (K 0620; Dako) was used following the manufacturer's instructions. First, sections were incubated for 15 minutes in a primary streptavidin- horseradish peroxidase medium (HRP) at 1:250 dilution. After washing, sections were incubated for 15 minutes in a biotin-tyramide solution and then in the secondary streptavidin-HRP medium at 1:250 dilution for 15 minutes. Positive hybridization signal was visualized by incubation with diaminobenzidine for 5 minutes. Counterstaining was performed with hematoxylin. Diffuse nuclear staining was indicative of episomal HPV, while point-form nuclear staining was characteristic of integrated HPV DNA.

Statistics. Statistical analysis was performed using the Chi-square and Fisher tests. The difference in values was considered significant when $p<0.05$.

Results

The immunohistochemical expression of p16 protein, absent from the group 1 cases, was especially noted in group 2 (92.86%, $p<0.001$) (Figure 1A) and in group 3 (cancer cases), (54.55%, $p<0.001$). In dysplastic epithelium, the expression of p16 increased as the grade of dysplasia progressed, becoming more evident in the middle and upper layers of the epithelium, and decreased from dysplasia to SCC. p53 protein expression was significantly associated with group 2 and 3 and increased with the severity of lesions (64.29% in dysplasia and 81.82% in carcinoma, $p<0.001$) (Table II). Figures 1A and B show an example of p16 and p53 overexpression in one case from group 2.

The association of different HPV markers (Table III) showed that the HPV- DNA was present in 1/31 (3.23%, n.s.) cases of group 1, 5/14 (35.71%, $p<0.01$) of group 2, and 3/11 (27.27%, $p<0.05$) of group 3. The Figure 1C shows an example of L1 and HR nested HPV-PCR for two cases of group 2. In particular, we found that both HR or LR genotypes were present: in the 60% and 40% of the cases of group 2 and in 1 case and 2 cases of group 3, respectively. For a case of SCC with an HR-HPV genotype, it was possible to demonstrate with CSAC-ISH the HPV DNA integration into genomic DNA (Figure 1D), while in two cases of dysplasia, the CSAC-ISH signal was episomic.

The analysis of the relationship between viral infection and p16 protein expression revealed that all the samples which were HPV positive were also p16 protein positive, but these data were not significantly associated.

Discussion

In this research, we found that the presence of HPV DNA appeared to be a molecular marker in dysplasia and SCC specimens of the oral cavity of a subgroup of non-smoker and non-drinker patients. Our findings indicate that HPV is positively correlated with high-grade (moderate/severe dysplasia) and invasive oral SCC. The association with HPV here, whose action appears not to be linked to that of other harmful factors such as alcohol and smoking, is interesting.

The presence of HPV, in the cases of dysplasia and SCC, suggests that the virus may play an etiological role in carcinogenesis in the oral cavity, in accordance with other reports that consider the presence of HR-HPV as an independent risk factor (22, 23). Although we did not find HPV in the normal mucosa of the oral cavity, its presence through the range of transformations of the epithelium, from mild dysplasia to infiltrating carcinoma, suggets that the viral infection is an important and independent event necessary for malignant transformation, and that it can be correlated with morphological alterations. However, no definitive conclusion may be drawn from our findings on the causal relationship between HPV infection and development of dysplasia.

In all cases of dysplasia and carcinoma in which we detected HPV, positive immunohistochemical expression of the p16 protein was also found. In patients with HR-HPV infection it has been hypothesized that p16 overexpression may be due to the removal of p16 inhibition by pRb (24, 25), and these findings indirectly support this hypothesis. In a 10 further cases, the expression of p16 protein was not associated with HPV infection. Direct compararision of p16 immunohistochemistry and HPV DNA reveals a high discrepancy rate of about 42%. Our data show that the p16 immunohistochemical method *vs*. HPV DNA tests (L1 and HR nested HPV-PCR, and CSAC-ISH) has a sensitivity of 100%, but a specificity of 80%. In head and neck SCC, the imperfection of p16 immunohistochemical detection as a HPV surrogate marker was documented (26). Immunohistochemical studies of p16 in oral squamous cancer and dysplastic lesions using p16 antibody have shown variable results. Non-expression (26, 27) and overexpression (28-30) have both been reported. Also variation in p16-positive staining criteria has been considered. Some studies evaluated only nuclear staining (31, 32), whereas another study utilized both nuclear and/or cytoplasmic staining (33). In accordance with other authors, we suggest that it is necessary that HPV detection strategies combine p16 immunohistochemistry and HPV DNA tests. Given a sensitivity that approaches 100%, p16 immunostaining is a good first-line assay for eliminating HPV-negative cases. HPV DNA testing can be run concurrently with p16 immunostaining or as a second-line assay following a positive p16 result (4).

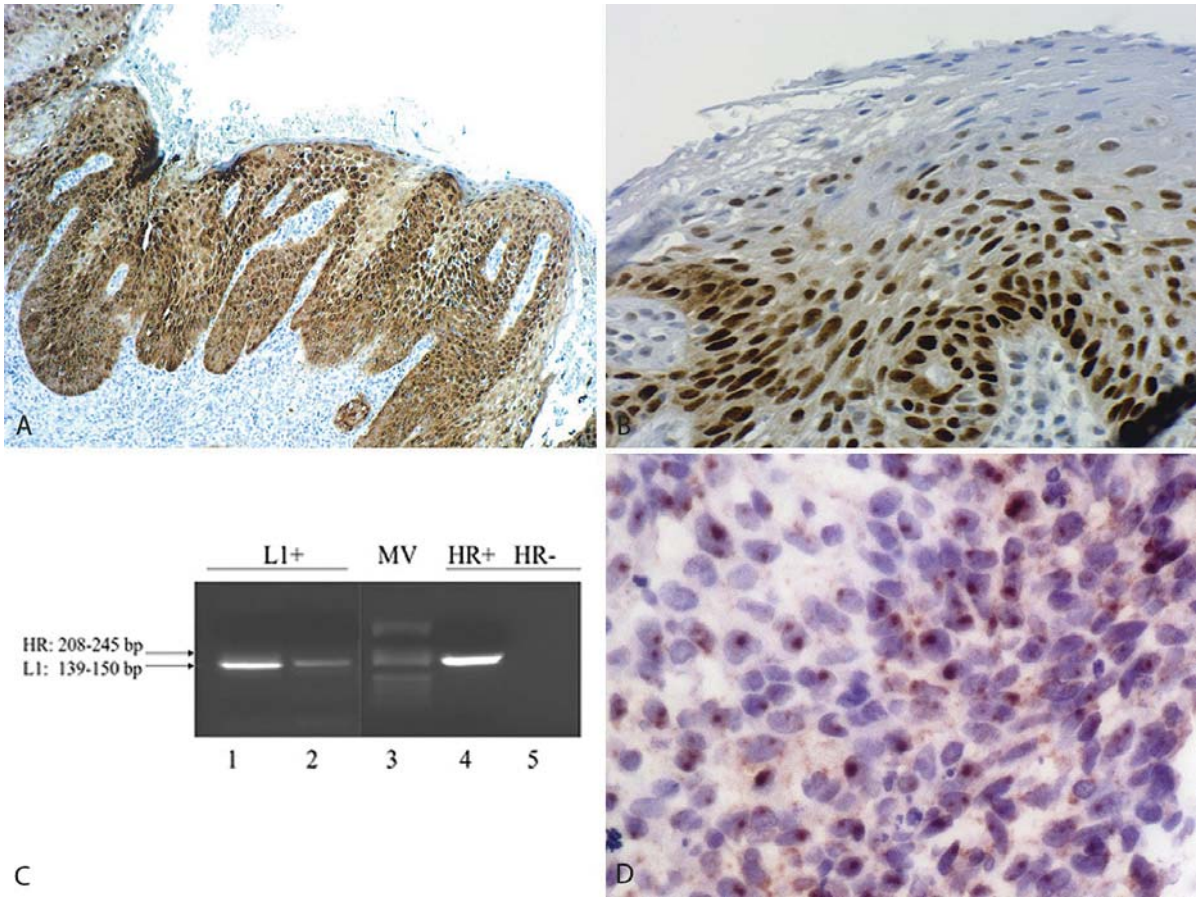

Figure 1. *A: Representative immunohistochemical pattern of expression of p16 protein in a case from group 2; ×200 original magnification. B: Immunohistochemical expression of p53 in the nuclei of the upper layers in a group 2 case; ×400 original magnification. C: HPV detection by nested PCR in two cases of dysplasia (group 2). Lanes 1 and 4: HPV L1- and HR-PCR positivity in a case of moderate dysplasia. Lanes 2 and 5: HPV L1-PCR positive and HR-PCR negative in a case of severe dysplasia. Lane 3: Molecular marker. D: Carcinoma (group 3) cells with integrated viral load detected by CSAC-ISH. Dot-like positive signals in the nuclei; ×600 original magnification.*

The CSAC-ISH data indicated that one case of carcinoma had viral DNA integrated. In this case, the HPV DNA integration was associated with p16 protein expression in one group. In one case of mild dysplasia, the signal was episomic and in one case the signal was not informative. For the five cases with probable LR- HPV, we did not investigate possible HPV DNA integration. The mechanisms whereby the expression of p16 protein may or may not accompanied by viral integration, differently from that in the uterine cervix (34), remain to be clarified.

With regard to the expression of p53, a progressive and significant increase was observed from mild dysplasia to infiltrating carcinoma, in agreement with previous studies (19, 35).

Our results suggest that HPV may be involved in inducing carcinogenesis. Although at the current state of knowledge this issue continues to be controversial, we may conclude that in the oral cavity a different pathway of carcinogenesis may exist, distinct from the known mechanisms in which the synergic action of alcohol and smoking is a determinant. Further studies on larger series will be necessary in order to clarify the role that HPV may play in carcinogenesis in the oral cavity.

Table II. *Immunohistochemical expression of p16 and p53 proteins in dysplasia and in carcinoma of oral cavity.*

Group	p16	p53
1 (n=31) No or mild dysplasia	0 (0%)	2 (6.45%)
2 (n=14) Moderate or severe dysplasia	13 (92.86%)	9 (64.29%)
3 (n=11) Invasive squamous cell carcinoma	6 (54.55%)	9 (81.82%)

Table III. *Analysis of HPV of the oral cavity and its relationship with p16 protein overexpression.*

Case	Group	p16 protein	Nested HPV-PCR		CSAC-ISH		HPV genotype
			L1-DNA*	HR-DNA	Episomic signal	Punctate signal	
1	1	NEG	POS	NEG	NEG	NEG	LR-HPV
2	2	POS	POS	POS	POS	NEG	HR-HPV 16-18-31-33-35-52-58
3	2	POS	NEG	POS	NEG	NEG	HR-HPV POS**
4	2	POS	POS	NEG	NEG	NEG	LR-HPV
5	2	POS	POS	NEG	POS	NEG	HR-HPV 39-45-51-56-59-68
6	2	POS	POS	NEG	NEG	NEG	LR-HPV
7	3	POS	NEG	POS	NEG	POS	HPV-DNA integration
8	3	POS	POS	NEG	NEG	NEG	LR-HPV
9	3	POS	POS	NEG	NEG	NEG	LR-HPV

*L1-DNA were from both high- and low risk HPV genotypes. **ISH not informative due to insufficient biopsy material.

References

1 Ragin CC and Taioli E: Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis – review. Int J Cancer *121(8)*: 1813-1820, 2007.

2 Ragin CC, Modugno F and Gollin SM: The epidemiology and risk factors of head and neck cancer: a focus on human papillomavirus – review. J Dent Res *86(2)*: 104-114, 2007.

3 Curado MP and Hashibe M: Recent changes in the epidemiology of head and neck cancer. Curr Opin Oncol *21(3)*: 194-200, 2009.

4 Adelstein DJ, Ridge JA, Gillison ML, Chaturvedi AK, D'Souza G and Gravitt PE *et al*: Head and neck squamous cell cancer and the human papillomavirus: summary of a National Cancer Institute State of the Science Meeting, November 9-10, 2008, Washington, D.C. Head Neck *31(11)*: 1393-1422, 2009.

5 Ritchie JM, Smith EM, Summersgill KF, Hoffman HT, Wang D and Klussmann JP *et al*: Human papillomavirus infection as a prognostic factor in carcinomas of the oral cavity and oropharynx. Int J Cancer *104(3)*: 336-344, 2003.

6 Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S and Viscidi R: Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst *100(6)*: 407-420, 2008.

7 Letian T and Tianyu Z: Cellular receptor binding and entry of human papillomavirus. Virol J *7(1)*: 2, 2010.

8 Feller L, Khammissa RA, Wood NH and Lemmer J: Epithelial maturation and molecular biology of oral HPV. Infect Agent Cancer *25*: 4-16, 2009.

9 Monk BJ and Wiley DJ: Will widespread human papillomavirus prophylactic vaccination change sexual practices of adolescent and young adult women in America? Obstet Gynecol *108(2)*: 420-424, 2006.

10 Nielsen H, Norrild B, Vedtofte P, Praetorius F, Reibel J and Holmstrup P: Human papillomavirus in oral premalignant lesions. Eur J Cancer B Oral Oncol *32B(4)*: 264-270, 1996.

11 Reichart PA and Philipsen HP: Oral erythroplakia – a review. Oral Oncol *41(6)*: 551-561, 2005.

12 Hennessey PT, Westra WH and Califano JA: Human papillomavirus and head and neck squamous cell carcinoma: recent evidence and clinical implications. J Dent Res *88(4)*: 300-306, 2009.

13 Ha PK and Califano JA: The role of human papillomavirus in oral carcinogenesis. Crit Rev Oral Biol Med *15(4)*: 188-196, 2004.

14 Mork J, Lie AK, Glattre E, Hallmans G, Jellum E and Koskela P *et al*: Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med *344(15)*: 1125-1131, 2001.

15 Ghittoni R, Accardi R, Hasan U, Gheit T, Sylla B and Tommasino M: The biological properties of E6 and E7 oncoproteins from human papillomaviruses. Virus Genes *40(1)*: 1-13, 2010.

16 Huh WK: Human papillomavirus infection: a concise review of natural history. Obstet Gynecol *114(1)*: 139-143, 2009.

17 Nemes JA, Deli L, Nemes Z and Márton IJ: Expression of p16(INK4A), p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *102(3)*: 344-352, 2006.

18 Gale N, Pilch BZ and Sidramsky D (eds.): Epithelial precursor lesions. *In*: World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours. Lyon: IARC Press pp. 177-179, 2005.

19 Cruz IB, Snijders PJ, Meijer CJ, Braakhuis BJ, Snow GB and Walboomers JM *et al*: p53 expression above the basal cell layer in oral mucosa is an early event of malignant trasformation and has predictive value for developing oral squamous cell carcinoma. J Pathol *184*: 360-368, 1998.

20 Pirog EC, Baergen RN, Soslow RA, Tam D, DeMattia AE and Chen YT: Diagnostic accuracy of cervical low-grade squamous intraepithelial lesions is improved with MIB-1 immunostaining. Am J Surg Pathol *26*: 70-75, 2002.

21 Tanaka R, Wang D, Morishita Y, Inadome Y, Minami Y and Iijima T: Loss of function of *p16* gene and prognosis of pulmonary adenocarcinoma. Cancer *103*: 608-615, 2005.

22 Strome SE, Savva A, Brisett AE, Gostout BS, Lewis J and Clayton AC *et al*: Squamous cell carcinoma of the tonsils: a molecular analysis of HPV associations. Clin Cancer Res *8*: 1093-1100, 2002.

23 D'souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C and Koch WM *et al*: Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med *356*: 1944-1956, 2007.

24 Strati K, Pitot HC and Lambert PF: Identification of biomarkers that distinguish human papillomavirus (HPV)-positive versus HPV-negative head and neck cancers in a mouse model. Proc Natl Acad Sci USA *103(38)*: 14152-14157, 2006.

25 Yamakawa-Kakuta Y, Kawamata H, Doi Y, Fujimori T and Imai Y: Does the expression of HPV16/18 E6/E7 in head and neck squamous cell carcinomas relate to their clinicopathological characteristics? Int J Oncol *35(5)*: 983-988, 2009.

26 Pande P, Mathur M, Shukla NK and Ralhan R: pRb and p16 protein alterations in human oral tumorigenesis. Oral Oncol *34*: 396-403, 1998.

27 Buajeeb W, Poomsawat S, Punyasingh J and Sanguansin S: Expression of p16 in oral cancer and premalignant lesions. J Oral Pathol Med *38(1)*: 104-108, 2009.

28 Gologan O, Barnes EL and Hunt JL: Potential diagnostic use of $p16^{INK4A}$, a new marker that correlates with dysplasia in oral squamoproliferative lesions. Am J Surg Pathol *29*: 792-796, 2005.

29 Paradiso A, Ranieri G, Stea B, Zito A, Zehbe I and Tommasino M *et al*: Altered $p16^{INK4a}$ and FHIT expression in carcinogenesis and progression of human oral cancer. Int J Oncol *24*: 249-255, 2004.

30 Pai RK, Erickson J, Pourmand N and Kong CS: p16 (INK4A) immunohistochemical staining may be helpful in distinguishing branchial cleft cysts from cystic squamous cell carcinomas originating in the oropharynx. Cancer Cytopathol *117(2)*: 108-119, 2009.

31 Ralhan R, Chakravarti N, Kaur J, Sharma C, Kumar A, Mathur M *et al*: Clinical significance of altered expression of retinoid receptors in oral precancerous and cancerous lesions: relationship with cell cycle regulators. Int J Cancer *118*: 1077-1089, 2006.

32 Anderson KM, Stoner GD, Fields HW, Chacon GE, Dohar AL, Gregg BR *et al*: Immunohistochemical assessment of Viadent-associated leukoplakia. Oral Oncol *41*: 200-207, 2005.

33 Bradley KT, Budnick SD and Logani S: Immunohistochemical detection of $p16^{INK4a}$ in dysplastic lesions of the oral cavity. Mod Pathol *19*: 1310-1316, 2006.

34 Queiroz C, Silva TC, Alves VA, Villa LL, Costa MC, Travassos AG *et al*: P16 (INK4A) expression as a potential prognostic marker in cervical pre-neoplastic and neoplastic lesions. Pathol Res Pract *202*: 77-83, 2006.

35 Ögmundsdóttir HM, Björnsson J and Holbrook WP: Role of TP53 in the progression of pre-malignant and malignant oral mucosal lesions. A follow-up study of 144 patients. J Oral Pathol Med *38*: 565-571, 2009.

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