Determination of p16 Overexpression as an Indicator of Human Papillomavirus Infection in Oral Dysplasia and Carcinoma

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Abstract

**Context:** Oral and pharyngeal cancer, grouped together, is the sixth most common cancer in the world. In the past few years, human papillomavirus (HPV) infection has been suggested as a risk factor for oral cancer apart from traditional risk factors such as smoking, tobacco, and alcohol consumption.

**Aims:** The aim of this study was to determine HPV status of the tumors using polymerase chain reaction (HPV-DNA PCR) and p16 immunostaining and to correlate p16 overexpression as an indicator of HPV-associated oral dysplasia and carcinoma.

**Settings and Design:** A prospective study was conducted in fifty cases of suspected oral cancer.

**Materials and Methods:** PCR Amplification of extracted HPV-DNA was done for HPV-DNA status in fresh tissue of suspected oral cancer cases. Histomorphological features of the cases were analyzed, and p16 immunohistochemistry was performed on the same specimen after making paraffin blocks to study p16 overexpression.

**Statistical Analysis Used:** Chi-square test was used to analyze the differences between discrete variables.

**Results:** 5/6 (83.3%) HPV-DNA-positive cases were positive for p16 expression, whereas 26/44 (59.09%) p16-positive cases which were negative for HPV-DNA. Sensitivity and specificity of p16 as a surrogate marker for HPV-DNA were found to be 83.3% and 40%, respectively.

**Conclusions:** p16 immunostaining is a good first-line assay for eliminating HPV-negative cases from additional analysis, but other causes of p16 overexpression in oral tumorigenesis related to tobacco consumption in keratinizing squamous cell carcinoma needs to be explored further.

**Keywords:** Head and neck squamous cell cancer, human papillomavirus, p16 immunohistochemistry, polymerase chain reaction

Introduction

The suggestion of a causal relationship between human papillomavirus (HPV) and cervical cancer was made already in the 1970’s; although, it was only gradually accepted by the scientific community. Today, these viruses are recognized as carcinogenic infectious agents not only in cervical cancer but also in a proportion of head and neck squamous cell cancer (HNSCC), especially oropharyngeal cancer.

Most of the HPV-positive oral cancer is poorly differentiated nonkeratinizing tumor seen in sexually active young individual. These tumors are more likely to exhibit basaloid morphology. HPV-associated cancers are caused by the expression of HPV’s E6 and E7 proteins that bind to and inactivate tumor suppressor proteins p53 and retinoblastoma protein (pRb), respectively, leading to malignant transformation of HPV infected cells. There is not a standard HPV-DNA testing method, both in situ hybridization and polymerase chain reaction (PCR) are commonly used.

p16INK4a is a tumor suppressor protein and cyclin-dependent kinase (CDK) inhibitor that blocks CDK4- and CDK6-mediated pRb phosphorylation to inhibit E2F-dependent transcription and cell cycle progression. Functional inactivation of pRb by HPV E7 also results in hypomethylation of p16INK4a promoter leading to overexpression of p16INK4a and accumulation of the protein in the cell which can be demonstrated using immunohistochemistry (IHC).

Studies have postulated that patients with HPV-associated HNSCCs have a better response to treatment than their stage-matched non-HPV-related HNSCC. Thus, HPV-related HNSCCs define a unique population of patients with distinct biology...
that should be treated separately from non-HPV-related HNSCCs. p16 status is also reported as an independent prognostic factor for disease-free survival[7,8].

The aim of this study was 3-fold: first, to evaluate the histomorphology of suspected oral cancer tissue specimen; second, to determine HPV status of the tumors using PCR (HPV DNA PCR) and p16 immunostaining; and third, to determine p16 overexpression as an indicator of HPV-associated oral dysplasia and carcinoma.

**Materials and Methods**

**Study design**

A prospective study was conducted from January 2013 to July 2014.

**Samples**

In the present study, fifty random cases of suspected oral cancer were selected. Formalin-fixed, paraffin-embedded biopsy specimens were analyzed for histological morphology from Hematoxylin and Eosin stained slides. The specimens included 17 (34%) dysplasias, classified as mild, moderate, and severe following the WHO tumor classification, 33 (66%) invasive squamous cell carcinomas (SCCs) which were classified as well, moderately, and poorly differentiated. Of the total cases, 38 (76%) cases were from the oral cavity and 12 (24%) from the oropharyngeal lesion.

**Immunohistochemistry**

p16 monoclonal antibody (1:40 dilution) was used (Biogenex). Immunoperoxidase staining was done on formalin-fixed paraffin embedded, 4 μm tissue sections using the DAKO LSAB2 horseradish peroxidase system following the manufacturer’s instructions. Antigen retrieval was done by microwave heating for 10 min in 10 mM citrate buffer (pH 6.0). Cases were classified as either positive (any cells with nuclear and cytoplasmic staining) or negative. The staining intensity was graded as follows: 0 - no staining, 1 - weak staining intensity, 2 - intermediate, and 3 - strong staining intensity [Figures 1 and 2].

**DNA isolation and human papillomavirus detection by polymerase chain reaction**

DNA was extracted from fresh frozen tissue using Pure Link™ Genomic DNA Mini Kit (Invitrogen, Cat# K1820-00) with reagents provided in the kit. The purified Genomic DNA was eluted in the tube. The DNA purity and concentration were evaluated with a spectrophotometer. At the completion of sample preparation, an amplification master mix was prepared with Taq polymerase, MgCl2 solution and oligonucleotide reagent containing primers, probes, and dNTPs along with extracted DNA diluted with molecular biology grade purified water to the final volume. The tube was kept in PCR machine for amplification. Agarose gel electrophoresis for analysis of amplified product was carried out, and fluorescent bands were observed under the Gel Documentation system.

**Statistics**

Data were compiled and analyzed using Statistical Package for the Social Sciences Version 16.0. Chi-square test was used to analyze differences between discrete variables. The value of $P < 0.05$ was considered statistically significant.

**Results**

Fifty cases of oral and oropharyngeal cancer/precancerous lesion were tested for HPV DNA status and p16 expression. Results of the study are summarized in Table 1.

HPV-DNA was present in only 12% of cases (6/50), of which included only 1/17 (5.8%) case of dysplasia and 5/33 (15.1%) cases of carcinoma. Most of the HPV–DNA-positive cases, 5/6 (83.3%), were located in the oropharynx compared to only 1/6 case (16.6%) in oral cavity ($P < 0.05$).

p16(INK4a) expression was seen in 31/50 (62%) of all fifty histological cases compared to 19/50 (38%) p16(INK4a)-negative ones. The overall score of p16 staining intensity in this study was 8%, 20%, and 34% of cases showing 1+, 2+, and 3+ positivity, respectively.

Within histological groups, the percentage of p16 positivity increased from 9/17 (52.2%) in dysplasias to 22/33 (66.6%) in carcinomas. Overall p16 staining intensity in carcinoma group was significantly higher than mild–moderate dysplasias. There was no significant difference between carcinoma and severe dysplasia groups. p16 expression was not seen in any cases of mild dysplasia or poorly differentiated carcinoma. In addition, p16 positivity was not significantly different in oropharynx 6/12 (50%) and oral lesion 25/38 (65%).
Table 1: Distribution of total cases in accordance to human papillomavirus DNA and p16 results

| Test                  | Dysplasia (n=17) | Lesion (n=50) |
|-----------------------|------------------|---------------|
|                       | Mild (n=3), n (%) | Moderate (n=6), n (%) | Severe (n=8), n (%) | WD (n=20), n (%) | MD (n=11), n (%) | PD (n=2), n (%) |
| HPV DNA (PCR)         |                  |                |                  |                |               |               |
| Positive              | 0                | 0              | 1 (12.5)         | 3 (15)         | 1 (9)         | 1 (50)        |
| Negative              | 3 (100)          | 6 (100)        | 7 (87.5)         | 17 (85)        | 10 (90.9)     | 1 (50)        |
| p16 IHC               |                  |                |                  |                |               |               |
| Negative              | 3 (100)          | 2 (33.3)       | 2 (25)           | 6 (30)         | 3 (27.2)      | 2 (100)       |
| +                     | 0                | 0              | 1 (12.5)         | 1 (5)          | 0            | 0             |
| ++                    | 0                | 1 (16.6)       | 3 (37.5)         | 4 (20)         | 2 (18.1)      | 0             |
| +++                   | 0                | 0              | 2 (25)           | 9 (45)         | 6 (54.5)      | 0             |

HPV=Human papillomavirus, PCR=Polymerase chain reaction, IHC=Immunohistochemistry, SCC=Squamous cell carcinoma, WD=Well differentiated, MD=Moderately differentiated, PD=Poorly differentiated, +, ++, +++ are scores for grading intensity of p16 IHC positivity.  

Figure 2: Pictomicrograph showing score of p16 staining intensity (×400). (a) 3+; (b) 2+; (c) 1+; (d) negative

Among six HPV-DNA-positive cases, 5 (83.3%) were positive for p16 expression assessed by IHC. There were also 26/44 (59.09%) p16-positive cases which were negative for HPV-DNA. These findings are summarized in Table 2.

Results from Chi-square demonstrated that overall HPV status and p16 expression were not significantly associated (P > 0.05). Sensitivity and specificity of p16 as a surrogate marker for HPV-DNA were found to be 83.3% and 40%, respectively.

**Discussion**

The proposal that HPV was involved in the etiology of the head and neck was reported as early as 1983 by Syrjänen et al. Since then, HPV has been recognized as a major etiological factor in oral cancer. Many head and neck studies have been published, but often the primary site is oropharyngeal rather than oral carcinoma. These studies have suggested that HPV-positive oropharyngeal cancers respond well to chemoradiotherapy and are associated with an improved prognosis. Therefore, knowing the HPV status for oropharyngeal carcinomas could impact on management and patient survival. In contrast, the primary treatment modality for oral cancer is surgery, so knowing the HPV status might not necessarily change the treatment, but it might impact on prognosis.

Many studies on head and neck cancer have utilized p16 as a surrogate marker for HPV DNA. Yet, the evidence for such a use for p16 as a surrogate marker of the presence of HPV-DNA in oral cancer is somewhat limited, unconvincing, and in some cases, controversial. Despite the volume of literature on this topic, methodological differences between studies make comparisons of results difficult, and any conclusions have to be interpreted with this caveat in mind.

In the present study, HPV-DNA prevalence was estimated in 5.8% of cases of dysplasias and 15% of cases of cancer. A large variation of HPV prevalence has been reported in the literature. The estimates of HPV infection have ranged from 0% to 41% in normal individuals, depending on the patient population as well as the sampling and assay methods used. HPV16 prevalence in oral dysplasia has been reported to be more than a 60% in few studies, but in another study, it was reported to be 14.6%. Within carcinomas, three studies reported a slightly higher percentage of high-risk HPV (HR HPV) prevalence ranging from 33% to 52%, whereas another study reported only 8% of HPV-positive cases of oral cancer.

Many different techniques have been used for detection, including PCR, Southern blot, dot blotting, and in situ hybridization. In a meta-analysis of the studies looking at these lesions, Miller and White noted that there was a significantly higher rate of HPV positivity reported by those studies using PCR techniques for either consensus or type-specific HPV sequences. This raises the possibility that nonquantitative PCR may allow not only for increased sensitivity as opposed to other techniques but may also indicate that nonquantitative PCR has a higher occurrence of false-positive results.
Higher prevalence of HPV-DNA was found in oropharyngeal cancer (83.3\%) compared to 16.3\% oral lesion which was HPV positive, and this result is statistically significant similar to other studies.\[31-33\]

A total of 31 of the fifty cases (62\%) were positive for p16 IHC. Fifty percent oropharyngeal lesion and 65% oral lesions expressed p16 positivity, whereas 52.2\% of cases of dysplasia and 66.6\% of cases of cancer expressed p16 positivity. Studies on p16 expression have reported that the percentage varied from 0\% to 90\% in oral dysplastic tissues and 13\%–94\% in oral cancer.\[3,25,33-38\] It could be argued the discrepancies, and a wide range of variation observed could be attributed to differences in sample size and the scoring scheme used for p16 assessment.

A meta-analysis involving 34 studies which included 5681 patients with HNSCC concluded that the overall HPV status was correlated with p16 expression.\[8\] In the present study, HPV DNA status and p16 expression were not significantly associated. Fifty-nine percent p16 IHC-positive cases did not show the presence of HPV DNA, but almost all (83.3\%) HPV-DNA-positive cases showed p16 overexpression. Differences with our results could be attributed to the different sample sizes and also additional contributing factors such as the site-specific proportion of the samples (oral samples was relatively much more than oropharyngeal samples), HPV detection system as only HR types were detected, and p16 expression methodology used.

A few studies have found results similar to the present study,\[36,39\] and Lewis et al. found a significant minority of tumors which were p16 positive and HPV negative, but the outcomes for p16-positive and HPV-DNA-negative oropharyngeal SCC were not significantly different from p16 positive, HPV DNA-positive tumors and were significantly better than for p16-negative tumors.\[40\] Chernock et al. documented all nonkeratinizing SCC were p16 positive, and 69\% were HR HPV positive. In contrast, 36\% of keratinizing SCCs were p16 positive, and only 8\% were HR HPV positive. Thus, he concluded that nonkeratinizing SCC was significantly more likely to be HPV-DNA and p16 positive than keratinizing SCC.\[41\] Another reason for p16 overexpression could be an alteration in pRb/p16 pathway which are early events in oral tumorigenesis and may be involved in the development of betel- and tobacco-related oral malignancies.\[39,42,43\]

In the present study, using p16 immunohistochemistry, a significant proportion (62\%) of oral cancer and dysplasias are found to be associated with HPV infection. However, overall HPV DNA prevalence was only 12\%. Results from Chi-square demonstrated that overall HPV DNA status and p16 expression was not significantly associated. Thus it can be concluded that p16 immunostaining is a good first line assay for eliminating HPV-negative cases from additional analysis. For HPV-DNA negative p16-positive cases, it is conceivable that HPV has still been involved in the carcinogenic process of these tumors. HPV might have been shed by the tumors as they progress genetically or the tumors have innate p16 overexpression or the current HPV-specific tests may not be recognizing HPV in such tumors. Bearing in mind the ever evolving nature of medical science, it is hence proposed that the scope of this study is immense with a larger number of cases, especially of keratinizing SCC of oral cavity and possibilities of exploring the correlation between betel quid and tobacco consumption and p16 overexpression.

### Conclusions

p16 immunostaining is a good first-line assay for eliminating HPV-negative cases from additional analysis, but other causes of p16 overexpression in oral tumorigenesis related to tobacco consumption in keratinizing squamous cell carcinoma needs to be explored further.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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