Original Article - Comparative Studies

Does P16 Protein Expression Affect Treatment Prognosis in Oral Squamous Cell Carcinoma - A Comparative Study

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Abstract

Introduction: P16 is an independent and reliable surrogate for the detection of human papillomavirus (HPV) in oral squamous cell carcinoma (OSCC). The aim of this study was to assess the P16 expression as a marker for HPV infection in OSCC and its impact on the treatment outcome. Methods and Materials: A cross-sectional study was conducted on patients with a definite diagnosis of OSCC. Patients were assigned into two groups with and without recurrence or metastasis. Tumour resection was performed in the same manner for all patients. P16 expression was evaluated by immunohistochemical staining. Independent t-test and Chi-square tests were used to find significant differences in age, gender, stage of the disease, tumour size, lymph node involvement, and P16 expression between the two groups. Results: Of 50 patients, 37 did not show any recurrence or metastasis (group 1), while 13 had a relapse (group 2). There was no significant difference for age, gender distribution, stage of the disease, or lymph node involvement between the two groups (P > 0.05). A significant difference in tumour size was noted between the two groups (P = 0.001). The mean expression of P16 was 38.92 ± 24.36 in group 1 and 51.54 ± 33.63 in group 2. No significant difference was found between the two groups for the mean expression of P16 (P = 0.23). Discussion: A review of the recent literature revealed that the HPV role in OSCC treatment is controversial. According to the results of this study, there was no significant difference in terms of P16 expression between OSCC patients with and without recurrence or metastasis.

Keywords: Mouth neoplasms, P16 protein, papillomavirus infections, prognosis, treatment outcome

INTRODUCTION

Oropharyngeal squamous cell carcinoma (OPSCC) has the highest prevalence among head and neck cancers. Despite the advances in diagnosis and treatment modalities, including surgical and nonsurgical interventions, low survival rate, and high rates of morbidity are major concerns.1,2 The human papillomavirus (HPV) infection is associated with OPSCC in approximately 63% to 81% of the cases.3 HPV-related OPSCC has an evidently different disease profile than HPV-negative OPSCC, with a tendency for younger patients without known risk factors. However, the role of HPV in oral squamous cell carcinoma (OSCC) is controversial, and HPV has been reported in 5.9% to 6% of OSCCs.4,5 The majority of HPV-related OPSCCs and OSCCs are nonkeratinizing squamous cell carcinomas.6 Studies on the prognostic significance of HPV-positive OSCC variants are limited.7 Evidence suggests that some specific HPV-related morphological variants may have a better prognosis than non-HPV OPSCC.8 Identification of HPV in OPSCC may be clinically valuable for the prediction of disease prognosis. However, the role of HPV in OSCC is not well understood. P16 is an independent and reliable surrogate for the detection...

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of HPV in OSCC.\textsuperscript{[10]} The expression of P16 in OSCC may be helpful for the determination of treatment prognosis.\textsuperscript{[11]}

The purpose of this study was to address the following question: Does P16 expression affect treatment prognosis in OSCC? We hypothesized that P16 expression improves treatment prognosis in OSCC. Therefore, the aim of this study was to assess the P16 expression in OSCC and its impact on the treatment outcome.

**Subjects and Methods**

A cross-sectional and evaluative study was conducted on patients referred to 5 hospitals in Tehran, Iran, between September 1, 2017, and January 30, 2020. Patients with a definite diagnosis of OSCC according to their pathology report were enrolled. Patients with a history of radiotherapy, chemotherapy, or immunotherapy were excluded from the study. Patients who had an involved margin or a close margin (2 mm or less) in surgery were also excluded.

The design and objectives of the study were thoroughly explained to all participants. They were then requested to read and sign the written informed consent form for participation in the study. This study was performed according to the principles outlined by the World Medical Association’s Declaration of Helsinki on experimentation involving human subjects, as revised in 2000 and the protocol of the study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (code: IR SBMU.DRC.REC.1398.62).

Demographic data (including age and gender), clinical stage of the disease, tumour size and lymph node involvement based on the tumour node metastasis staging system, according to the American Joint Committee on Cancer, were recorded. Recurrence and metastasis were also evaluated during the follow-up period. The patients were divided into two groups based on the presence/absence of recurrence or metastasis: Group 1 patients had no recurrence or metastasis during the study period while group 2 patients experienced a recurrence or metastasis. Stage of OSCC, tumour size (T), lymph node involvement (N), age and gender were the variables of the study. P16 expression was considered as a predictive factor and relapse (recurrence or metastasis) was the outcome of the study.

All patients underwent tumour resection with a 1-cm safety margin and received 35 sessions of radiotherapy postoperatively with a total dose of 50 Gy. None of the patients received adjuvant or new adjuvant chemotherapy.

**Immunohistochemical staining**

Fifty paraffin blocks were collected during the study period. The Envision standard method was used for immunohistochemical staining and 4-μm thick sections were deparaffinized in xylene and rehydrated sequentially in methanol for 30 min. The sections were incubated with fresh 3% H\textsubscript{2}O\textsubscript{2} to inhibit endogenous peroxidase and were then autoclaved at 120°C for 15 min in 10 μm of citrate buffer (pH = 6) and cooled at room temperature for 30 min.

The sections were incubated at 25°C for 60 min with the primary antibody for P16 (anti-p16 (INK4); Clone G175–405, Biogenix, Fremont, USA). Then, they were rinsed with Tris buffer saline (TBS) followed by incubation with a secondary antibody. The Avidin-Biotin Complex reagent was used for slides and rinsed with TBS. Rinsing with TBS was performed for 2–5 min each time. Finally, the slides were immersed in 3, 3’-diaminobenzidine tetrahydrochloride (peroxidase activity substrate, RE7169, Novocastro, England) and rinsed with water. They were counter-stained with Mayer’s hematoxylin and mounted. We used a known p16 expressing head and neck squamous cell carcinoma (HNSCC) specimen as the positive control. The primary antibody was omitted from one of the specimens for the negative control.

The cases were reviewed independently by two pathologists under a light microscope (Zeiss, Germany). Positive results were obtained when the P16 marker was stained in nuclei and cytoplasm of tumoural cells. They were observed at low magnification to find areas with high staining (×40 to ×100). Stained tumoural cells were counted at ×400 magnification. At least 1000 cells were counted in each sample, and almost 10 fields were counted. The average number was considered for the whole sample [Figure 1].

**Statistical analysis**

The statistical analyses were performed using SPSS version 21 (SPSS Inc., IL, USA). The independent \textit{t}-test was used to compare the mean age and P16 expression between the two groups. The Chi-square test was applied to compare the stage of the disease, tumour size, and lymph node involvement between the two groups. \( P < 0.05 \) were considered statistically significant.

**Results**

Fifty patients were studied with a mean age of 60.94 ± 10.80 years. Thirty-seven patients did not show any recurrence or metastasis during the study period (Group 1), while 13 patients had a relapse (Group 2). The mean age of

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**Figure 1:** Immunohistochemical staining of P16 marker in nuclei (×400)
patients in groups 1 and 2 was 59.86 with 10.96 standard
deviation (SD) and 64 with 10.15 SD years, respectively,
with no significant difference \( (P = 0.23) \). The stage
of OSCC, tumour size, lymph node involvement, and
gender distribution were compared between the two
groups [Table 1]. Analysis of the data demonstrated a
significant difference in tumour size between groups 1
and 2 \( (P = 0.001) \). There were no significant differences
in the OSCC stage, lymph node involvement or gender
distribution between the two groups \( (P > 0.05) \). The results
did not demonstrate any difference in the number of smokers
between the two groups \( (P = 0.17, \) Table 1). The mean P16 expression was 38.92 with 24.36 SD in group 1
and 51.54 with 33.63 SD in group 2. Analysis of the data did
not indicate any significant difference between the two groups
for the mean P16 expression \( (P = 0.23) \).

**Discussion**

HPV is an additional risk factor for OSCC as smoking and
alcohol consumption.\(^{[10]}\) Irrespective of its morphological type,
HPV is believed to be positively associated with a favorable
treatment outcome.\(^{[12]}\) Identification of HPV as a risk factor in
these variants is, therefore, not only of academic interest but
also has a potential clinical significance in treatment planning
and determination of the treatment outcome.\(^{[10]}\) In 1983, the
presence of HPV infection in OPSCC was reported for the
first time. Since then, a significant increase in the incidence
of HPV positivity, especially in oropharyngeal tumours, has
been reported, such that approximately 60% of the cases over
the past decade were HPV positive.\(^{[1,13,14]}\)

According to the results of this study, the rate of p16
expression was not different in OSCC patients with/without
recurrence. It seems that tumour size is a more important
factor than p16 expression in OSCC treatment outcome.
The number of smokers was not significantly different in
the recurrence and nonrecurrence groups. Fakhry \textit{et al.}
reported that p16 expression status did not have any
impact on treatment prognosis in non-OPSCC of the head
and neck, which was similar to our findings.\(^{[15]}\) Salazar
\textit{et al.} published a contradictory report on the prognosis of
HPV-related non-OPSCC of the head and neck.\(^{[16]}\) Stephen
\textit{et al.} studied the p16 expression in site-specific HPV
positive and negative cases with head and neck squamous
cell carcinoma (HNSCC). They concluded that patients with
p16 negative/HPV16 negative status had the worst survival
for all sites combined, as well as for the oropharyngeal site.
They believed that p16 status is a significant prognostic
indicator for both OPSCC and non-OPSCC.\(^{[17]}\)

The expression of P16ink4a (p16 positive) is highly correlated
with HPV infection in the HNSCC; however, p16 positivity
is not limited to HPV positive tumours and therefore, is not
a perfect surrogate for HPV. The p16 survival outcomes are best
documented for the oropharyngeal site, and nonoropharyngeal
sites such as the oral cavity, larynx, and hypopharynx are
understudied. The goal of this study was to evaluate p16 in
the context of HPV16 and examine p16 survival outcomes in
HPV16 positive and HPV16 negative site-specific HNSCC. The p16 and HPV16 status were determined by
immunohistochemistry and quantitative polymerase chain
reaction, respectively, on 80 primary HNSCC specimens from
four sites: oral cavity, oropharynx, larynx, and hypopharynx.
The p16 expression was different across the sites \( (P < 0.001) \);
it was more frequent in oropharyngeal than nonoropharyngeal
sites \( (P < 0.001) \), and was different between Caucasian
Americans and African Americans \( (P = 0.031) \), similar to
HPV \( (P = 0.013) \). The p16 expression was associated with
marital status \( (P = 0.008) \) and smoking \( (P = 0.014) \). Also,
p16-positive patients had improved survival (similar to HPV16
positive cases).

Ringström \textit{et al.}\(^{[2]}\) reported 20% of HPV-positive OPSCC
cases in their study population. The mean age of patients with
HPV-positive tumours was significantly lower compared with
others (8.4 years younger); moreover, alcohol consumption
and smoking, as well-known risk factors, were less frequent
in the HPV-positive group, which was similar to other reports
on the head and neck region.\(^{[18]}\) In this study, there was no
significant correlation between tumour staging and lymph
node involvement. Gillison \textit{et al.}\(^{[19]}\) also reported 25% HPV-positive cases among patients with head and neck cancers
and 90% high-risk HPV-16 cases in HPV-positive tumours.
They showed that poor tumour grade and oropharyngeal sites
were correlated with HPV infection, and were less likely to
be observed among heavy smokers and alcoholics. Some
relevant studies showed that the overall treatment outcome and
disease-specific survival rate were better in the HPV-positive
group, and a smaller percentage of HPV-positive tumours
experienced recurrence. Interestingly, up to 59% reduction in
risk of death from the tumours was observed in HPV-positive

| Variables            | Group 1 \( (n=37) \) | Group 2 \( (n=13) \) | \( P \) |
|----------------------|----------------------|----------------------|------|
| OSCC stage           | S1 (3), S2 (13), S3 (14), S4 (7) | S1 (0), S2 (1), S3 (5), S4 (7) | 0.05 |
| Tumour size          | Td 1 (4), T2 (19), T3 (14), T4 (0) | T1 (0), T2 (2), T3 (5), T4 (6) | <0.001* |
| Lymph node involvement | Ne 0 (24), N1 (10), N2 (3) | N0 (6), N1 (7), N2 (0) | 0.16 |
| Gender distribution  | 18 males, 19 females | 7 males, 6 females | 0.5 |
| Smoking              | 18 smokers, 19 nonsmokers | 9 smokers, 4 nonsmokers | 0.17 |

\*Significant difference, \#Group 1: With recurrence or metastasis, \#Group 2: Without recurrence or metastasis, \#Stage, \#Tumour size, \#Lymph node involvement. OSCC=Oral squamous cell carcinoma

\( *=0.05, \)
cases, compared with HPV-negative HNSCC patients.\(^{[2,3,19]}\) Krüger et al.\(^{[10]}\) reported that only about 6% of OSCC cases were HPV positive. In this study, there were no significant differences in terms of age, gender, tumour staging, smoking or alcohol consumption between the two groups.

Delayed diagnosis is the main reason that contributes to the low survival rate of OSCC. Also, similar to many other diseases, poor early screening and lack of biomarkers play a critical role in this respect. According to the literature, only one-third of patients with HNSCC are in the early stages of the disease. The main risk factors that are repeatedly mentioned in the literature include a history of tobacco use and alcohol consumption, which are both times-and dose-dependent. Moreover, epidemiological and cell line data show that a considerable portion of patients with HNSCC represent HPV infection, especially type 16.\(^{[19,20]}\)

A review of the recent literature on HNSCC patients suggests a distinct management approach for HPV-positive tumours in the head and neck region, considering the significantly superior treatment outcome and disease-specific survival rate in HPV-positive cases.\(^{[1]}\) While HPV infection is considered as an independent risk factor for HNSCC, the role of HPV in carcinogenic activity and tumourigenesis in the oral cavity and more importantly, its effects on the treatment outcome and tumour behavior are still uncertain.\(^{[6,18]}\)

Despite the improvements in HPV detection as an independent risk factor for HNSCC and monitoring the clinical and pathological features of HPV infection, further studies with a larger sample size on OSCC and long-term follow-ups in case of local relapse and metastasis will be beneficial, especially on the etiologic role of HPV and its interactions with treatment modalities.

### Conclusion

According to the results of this study, there was no significant difference in terms of P16 expression between patients who did not show any recurrence or metastasis during the study period and those with relapse. Except for tumour size, which was significantly larger in patients who experienced recurrence during the study period, age, gender, tumour stage and neck involvement did not show any correlations.

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### Conflicts of interest

There are no conflicts of interest.

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