

# Prevalence of human papillomavirus 16 DNA and p16<sup>INK4a</sup> protein in oral squamous cell carcinoma: A systematic review and meta-analysis

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## Abstract

**Background and Aim:** Indian patients with oral squamous cell carcinoma (OSCC) are etiologically associated with the use of tobacco and alcohol; yet, a proportion of tumors that may harbor human papillomavirus (HPV) infections cannot be neglected. The following meta-analysis was conducted to address the association of p16<sup>INK4a</sup> and HPV DNA with OSCC. In addition, the study also provides the updated prevalence of HPV-induced OSCC.

**Materials and Methods:** Literature survey was performed using databases such as PubMed with the help of the following keywords – “HPV infection,” “oral squamous cell carcinoma,” “p16<sup>INK4a</sup>,” “HPV DNA,” “E6,” “E7,” “L1,” “L2” and “LCR.” Proportion method was performed to derive the forest plot using MedCalc statistical software version 16.4.3.

**Results:** Among 145 research articles, 33 articles were selected for further analysis, in which 13 articles were related to HPV DNA detection in tissues, 11 articles detected the overexpression of p16<sup>INK4a</sup> and nine articles reported the detection of both HPV DNA and p16<sup>INK4a</sup> expression. Meta-analysis revealed significant heterogeneity ( $P < 0.0001$ ) among the articles. Overall, the study consisted of 3339 patients with OSCC, among which 559 patients were diagnosed with the presence of HPV16 DNA with a random proportion of 20.1% at 95% confidence interval (CI) (13.9–27.1,  $P < 0.0001$ ). Overexpression of p16<sup>INK4a</sup> protein was observed in 709 patients with a random proportion of 25.4% at 95% CI (14.3–38.3,  $P < 0.0001$ ).

**Conclusion:** HPV DNA and expression of p16<sup>INK4a</sup> was suggested as gold standard for the detection of HPV infection in many cases of cancers. Frequency of HPV infection is significantly higher in patients with OSCC as identified through the detection of HPV DNA and p16<sup>INK4a</sup> expression. Even though the association of HPV infection has been established in head and neck cancer, this review could further the establishment of molecular level interaction of HPV in patients with oral cancer.

**Keywords:** Human papillomavirus DNA, meta-analysis, oral squamous cell carcinoma, p16<sup>INK4a</sup>, random effect

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## INTRODUCTION

Oral cancer is the sixth most common form of cancer in the world. The incidence varies because of geographic

location, methods of detection and ethnicity. However, a higher incidence of the cancer is observed in the Southeast Asian region due to excessive use of tobacco. The ratio of

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men to women population prone to oral cancer in India has been reported to be 2:1.<sup>[1]</sup> Apart from the use of tobacco, the prevalence of human papillomavirus (HPV) 16 has been reported in premalignant lesions and cancerous cells, predominantly in the cases of OSCC.<sup>[2,3]</sup> In the Indian scenario, approximately, 45% of cancer cases are diagnosed as oral cancer, among which 20%–50% of the cases are observed to be associated with HPV infection.<sup>[3,4]</sup> Apart from geographic location, the incidence of HPV-induced OSCC also varies with the genotype of virus.

Oral squamous cell carcinoma (OSCC) is one of the most common forms of malignant neoplasms of oral cavity. It affects the epithelial cells of oral and pharyngeal area. The etiological factors of OSCC include tobacco chewing, consumption of alcohol and infections by human papillomavirus (HPV) and hepatitis C virus, among others. In addition, disorders such as actinic cheilitis, erythroplakia, dyskeratosis congenita and sideropenic dysphagia can also progress to OSCC.<sup>[4]</sup>

HPV is a nonenveloped, double-stranded DNA virus of *Papillomaviridae* family. The circular double-stranded DNA is approximately 8000 base pairs in length and has early, late and noncoding genes. The early genes, E1, E2, E3, E4, E5, E6 and E7, are responsible for the control of viral transcription, replication and cellular transformation. L1 and L2 are the late genes involved in encoding proteins required for the formation of the capsid. The replication of virus and the transcription of early genes are regulated by a noncoding long control region (LCR) gene. Based on the genotypic differences, especially in the gene region of E6 and E7, HPV is differentiated into high- and low-risk types.<sup>[5]</sup>

HPV16 has been recognized as an etiological factor for OSCC by the International Agency for Research against Cancer.<sup>[6]</sup> Globally, HPV infection is a sexually transmitted disease; however, it can also be transmitted to the infants through the placenta. Areas of the mouth, tonsils, oropharynx, vagina and vulva are among the primary sites of the infection. In HPV-induced carcinoma, the viral genome gets integrated in the host cell genome leading to a break in E1 and E2 regions. This results in the loss of control for the synthesis of E6 and E7 proteins, which in turn inhibits the functions of tumor suppressor genes including p53 and retinoblastoma protein (pRB). Binding of E7 protein with pRB causes its inactivation, consequently enhancing the expression of p16<sup>INK4a</sup>. The overexpression of p16<sup>INK4a</sup> further prevents the activation of pRB. The detection of overexpressed p16<sup>INK4a</sup> has thus been researched for the early diagnosis of HPV-induced

OSCC along with other molecular typing methods such as immunohistochemistry, *in situ* hybridization and polymerase chain reaction (PCR). On the other hand, the protein encoded by E6 also hinders apoptosis of the cells by interfering with the activity of proteins such as Bak and procaspase, leading to the development of OSCC.<sup>[5,7]</sup>

The following meta-analysis was conducted to address the association of p16<sup>INK4a</sup> and HPV DNA with oral carcinoma. In addition, the study also provides the updated prevalence of HPV-induced OSCC.

## MATERIALS AND METHODS

### Sources of data

Literature search was performed using databases such as PubMed (maintained by the United States National Library of Medicine) to collect the related studies published from 1995 to 2016. The keywords used for the search included “HPV infection,” “Oral squamous cell carcinoma” p16<sup>INK4a</sup>, “HPV DNA,” early genes “E6” and “E7,” late gene “L1,” “L2” and noncoding control “LCR.”

### Selection criteria

English articles involving studies on fresh and frozen or formalin-fixed, paraffin-embedded (FFPE) tissue samples and articles retrieved after a particular search were included in the analysis. Articles having studies on cell lines, biofluids, head and neck cancer, polymorphism, methylation, healthy control and microRNA were excluded from the analysis. Review and meta-analysis articles, along with those in other languages than English, were also excluded from the analysis.

### Statistical analysis

Proportion method was performed, and forest plot was derived to carry out meta-analysis by using MedCalc software version 16.4.3 (Ostend, Belgium). The proportion of HPV DNA and p16<sup>INK4a</sup> in OSCC cases was analyzed at 95% confidence interval (CI). Random effects model (DerSimonian and Laird method) was used in the analysis due to the presence of significant heterogeneity.

## RESULTS

The study included 145 research articles, out of which 112 articles were excluded. The excluded articles include the articles, which were in other languages ( $n = 6$ ), either review articles or meta-analysis ( $n = 16$ ), related to cell line study ( $n = 14$ ), biofluids ( $n = 6$ ), head and neck cancer ( $n = 2$ ) and articles related to polymorphism, methylation, healthy control or microRNA ( $n = 68$ ). Among the 33 research articles included in the analysis, 9 articles

conducted the study with the help of fresh tissue sample whereas 24 articles conducted the study with the help of frozen or FFPE tissue samples.

Of the 33 research articles, 28 articles were found to be related with the study of HPV DNA in OSCC cases. There was significant heterogeneity ( $P < 0.0001$ ) in the studies ( $I^2 = 94.68\%$  with 93.27–95.79 CI at 95%). These studies included 3083 patients, among which 559 patients were reported to be positive for HPV DNA. Proportion of positive cases for HPV16 was analyzed in the present meta-analysis. The average proportion of positive cases was found to be 18.11%. However, the studies done by Tania *et al.* (2015) and Chen *et al.* did not find any positive case with 0.00–2.91 and 0.00–8.81 CI at 95%, respectively. On the other hand, Nemes *et al.* (2016) found a high proportion of 81.82% with 64.54–93.02 CI at 95%. However, Rietbergen *et al.* (2014) included the highest number of patients in the study [Figure 1].

Among the 33 articles, twenty research articles were related with p16<sup>INK4a</sup>. There was significant heterogeneity ( $P < 0.0001$ ) in the selected studies ( $I^2 = 98.07\%$  with 97.64–98.42 CI at 95%). These studies included 2578 patients, among which 709 patients were reported to be positive for HPV DNA. Proportion of positive cases for p16<sup>INK4a</sup> was determined by following the random effect analysis. A study conducted by Singh *et al.* had a low proportion of 3.6% with 1.65–6.72 CI at 95% whereas a study by Gröbe *et al.* had a high proportion of 89.64% with 84.86–93.32 CI at 95%. Out of the twenty research articles, 13 were found to have proportion between 0% and 25% [Figure 2]. However, nine research articles were

found to be associated with the study of both HPV DNA and p16 expression [Figure 3].

**DISCUSSION**

The comprehensive meta-analysis of research articles in the present study included 3339 patients diagnosed with OSCC. Among them, 559 patients were positively diagnosed with the presence of HPV16 DNA with a random proportion of 20.1% at 95% CI (13.9–27.1,  $P < 0.0001$ ) and 709 patients were found to have the overexpression of p16<sup>INK4a</sup> protein with a random proportion of 25.4% at 95% CI (14.3–38.3,  $P < 0.0001$ ). The meta-analysis was performed to determine the prevalence of HPV DNA and p16 in OSCC.

Major etiological factors for the development of oral lesions and OSCC include alcohol and tobacco consumption along with the presence of HPV infection in the diagnosed individual. The lesions may develop because of a single factor or due to synergistic effect of multiple factors. Singh *et al.*<sup>[8]</sup> reported the prevalence of HPV infection in patients with OSCC involved in the consumption of tobacco and betel leaves. They also revealed that the concordance of HPV-induced OSCC is significantly more in men than in women ( $P = 0.02$ ). Similarly, another Indian study by Balaram *et al.*<sup>[9]</sup> reported the concordance of HPV16 and 18 in 42% and 47% of the patients, respectively, suggesting viral infection as an vital etiological factor along with the consumption of betel leaves. The use of betel levels, as hypothesized, may lead to the synergistic mutagenic effect in the development of OSCC. A study conducted by Gan *et al.*<sup>[10]</sup> in Chinese population reported the prevalence of combined infection in the development

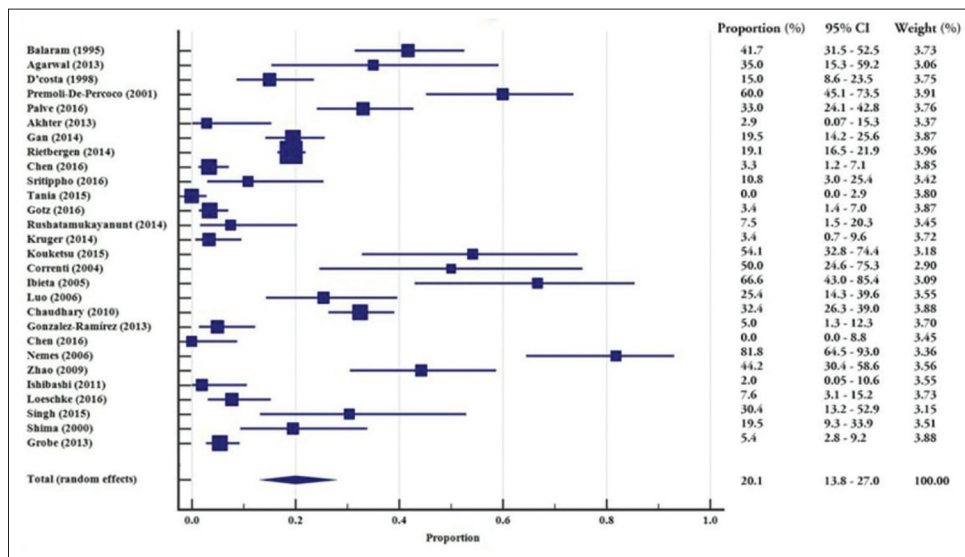


Figure 1: Forest plot of human papillomavirus 16 DNA in oral squamous cell carcinoma samples

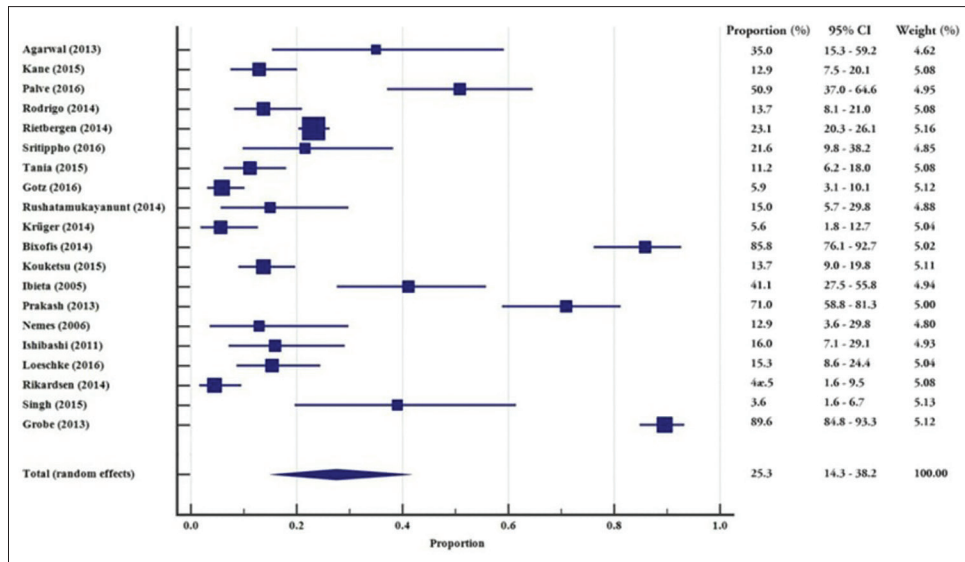


Figure 2: Forest plot of p16<sup>INK4a</sup> immunohistochemistry in oral squamous cell carcinoma samples

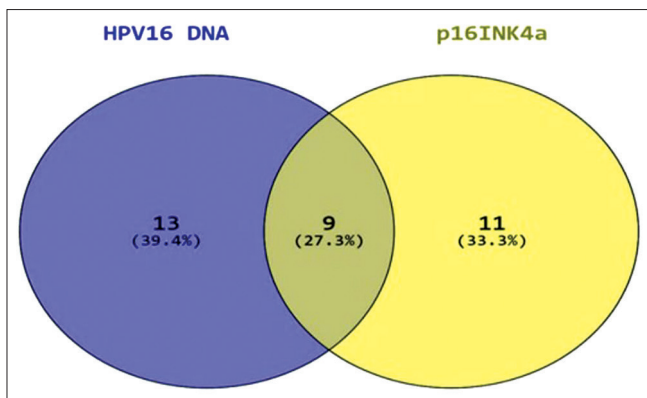


Figure 3: Representation of study distribution using Venn diagram

of cancerous lesion in the oral region, due to the presence of HPV16/18 along with traditional factors (tobacco and alcohol). They found that patients with preexisting HPV infection and history of tobacco and alcohol consumption have a high risk of developing cancer in the oral region. The odds ratio of the factors was found to be 13.3 with 95% CI of 3.1–56.8. However, González-Ramírez *et al.*<sup>[11]</sup> conducted a study on eighty Mexican patients diagnosed with OSCC and found that high-risk-HPV was a controlling factor in the occurrence of oral cancer at a young age, i.e., <45 years even without the traditional risk factors of alcohol and tobacco consumption. Similar conclusion was obtained by another study by Ibieta *et al.*<sup>[12]</sup> in which no significant difference was observed in the HPV-positive and HPV-negative controls, with respect to smoking and drinking as the cofactors. A study on 124 patients with oral cancer by Kane *et al.*<sup>[13]</sup> also demonstrated the prevalence of HPV-induced OSCC in 31.3% of patients without the history of tobacco use. In addition, HPV infection being one of the sexually transmitted diseases, significant

multiplicative interaction ( $P < 0.001$ ) was found between HPV16/18 and age at the first intercourse in a study by Chen *et al.*<sup>[14]</sup> for the development of OSCC.

Molecular and immune-staining techniques have been used in the detection of HPV infection in patients with OSCC. The presence of the DNA is usually detected by performing DNA extraction and PCR from frozen or FFPE tissue samples. However, the genotyping of the virus is detected by the use of specific primers in quantitative PCR (qPCR), southern hybridization or by *in situ* hybridization.<sup>[15]</sup> Chaudhary *et al.*<sup>[16]</sup> applied hybrid capture-II assay and PCR for the detection of HPV DNA in 222 patients with OSCC. The comparison of the methods was done based on their specificity and sensitivity. They concluded that the hybrid capture-II assay was more sensitive in the diagnosis of HPV-induced OSCC when compared with PCR. The detection of HPV DNA is usually done to detect the regions of L1, E6 and/or E7, as these are the genes present in the genome of the virus primarily involved in the process leading to the development of cancerous cells. Chen *et al.*<sup>[17]</sup> and Palve *et al.*<sup>[18]</sup> detected the presence of E6 transcript by SYBR Green-based qPCR assay. On the other hand, the transcripts E6/E7 were detected by consensus PCR in the study conducted by Shima *et al.*<sup>[19]</sup>

p16<sup>INK4a</sup> has long been considered as a biomarker in the identification of head and neck cancer along with cancer in dysplastic cervical epithelia.<sup>[20,21]</sup> The integration of viral genome in the host and the splitting of its genome in between the E1 and E2 regions lead to the loss of control in the expression of E7. This protein in turn deactivates pRB protein, leading to the overexpression of p16<sup>INK4a</sup>. Its detection, therefore, confirms the inhibition of host



cell apoptosis, leading to the conversion of normal cells into cancerous cells.<sup>[51]</sup> The integration also leads to the production of mRNA of the p16 protein, which can also be used in the detection of cancer.<sup>[22]</sup> In addition, inhibition of pRB and overexpression of p16<sup>INK4a</sup> protein also, to some extent, confirm the role of HPV and therefore make it an essential etiological factor in the development of cancer.

qPCR has been used for the detection of oncogenic mRNA (mRNA of E6 and E7) and DNA.<sup>[23,24]</sup> Several studies have been performed to correlate the expression of mRNA with the prevalence of HPV infection in cancer cases. A study conducted by Olthof *et al.*<sup>[25]</sup> in HPV genome was found to integrate itself in between the human genes such as Fanconi Anemia Complementation Group C and Histone Deacetylase 2, which play a role in head and neck cancer. In addition, RNA *in situ* hybridization was found to be more sensitive than DNA hybridization.<sup>[26]</sup> Thus, mRNA profiling can be performed along with the detection of DNA for early and confirmatory detection of HPV infection.<sup>[18,26]</sup> However, its use as a biomarker in the diagnosis of OSCC is contradictory due to its instability as compared to DNA and protein. RNA can also be silenced by host defense mechanism and hence may not lead to the development of cancer. Therefore, p16<sup>INK4a</sup> can be assessed for confirming the role of HPV in cancer as overexpression of p16<sup>INK4a</sup> is due to the activity of proteins produced from HPV mRNA.<sup>[27]</sup>

Sritippho *et al.* (2015)<sup>[28]</sup> reported the expression of p16 in 21.6% of the patients with OSCC. They also reported a significant association ( $P < 0.05$ ) between HPV DNA and p16 with odds ratio of 20 at 95% CI (1.9–211.8). It was observed that detection of p16 overexpression demonstrated 40% sensitivity and 79.3% specificity, hence p16 can be used in the detection of HPV infection rather than as a marker for HPV-induced OSCC identification. Its role as a biomarker in detecting OSCC has been supported by a study reported by Agarwal *et al.* (2013). They found that 22.5% of patients with OSCC were positive for HPV16. Among these patients, 85.17% of the cells were found to be positive for p16. Prakash *et al.*<sup>[29]</sup> also reported the overexpression of p16 in several cases (71%) of patients with OSCC. Similar study conducted by Gröbe *et al.*<sup>[30]</sup> concluded that p16 overexpression can be considered as a useful diagnostic biomarker for the detection of HPV infection. It has been also found in some studies that the HPV DNA and p16<sup>INK4a</sup> overexpression is localized in the region of cancerous lesion and is observed to decrease beyond the focal point. The immunohistochemical staining is often used to detect the expression of protein, which is further evaluated based on intensity, localization,

distribution and proportion of the stain in the tumor cells. The tissue samples exhibiting more than 70% p16<sup>INK4a</sup> expression in the tumor cells, with diffuse staining in cytoplasmic and nuclear region, are considered as tumor of the laryngeal region.<sup>[31]</sup>

## CONCLUSION

The above meta-analysis of HPV infection, identified through the presence of HPV16 DNA and p16<sup>INK4a</sup> expression, implies that the frequency of HPV infection is significantly higher in patients with OSCC. Association of HPV infection as a causative factor in cervical and in a subset of oropharyngeal squamous cell carcinomas is highly established; however, there is a paucity in the published data to reveal the association as of HPV infection as causative factor in OSCC. This review may open up further research to establish the molecular level interaction of HPV in patients with OSCC, and early identification of disease status may serve in future therapeutic implications to reduce the disease burden for the betterment of patient care.

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## Conflicts of interest

There are no conflicts of interest.

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