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HPV AS A HIDDEN COFACTOR IN TOBACCO-ASSOCIATED SUPRAGLOTTIC LESIONS: A COMPARATIVE ANALYSIS

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ABSTRACT

Background: Supraglottic squamous cell carcinoma and premalignant lesions of the larynx are among the most prevalent head and neck malignancies in the Indian subcontinent, where tobacco in its smoked and smokeless forms constitutes the principal aetiological agent. Human papillomavirus (HPV), particularly high-risk genotypes 16 and 18, has been identified as an independent oncogenic driver in oropharyngeal and laryngeal cancers; however, its role as a cofactor potentiating tobacco-related carcinogenesis at the supraglottic site remains inadequately characterised.

Aim: This study aimed to determine the prevalence and genotypic distribution of HPV in tobacco-associated supraglottic lesions, to evaluate the synergistic oncogenic influence of concurrent HPV infection and tobacco exposure, and to compare histopathological and immunohistochemical profiles across patient subgroups.

Methods: A prospective comparative analytical study was conducted at the Department of Otorhinolaryngology, Narayan Medical College and Hospital, Sasaram, Bihar, India, between 1st March 2025 and 28th February 2026. A total of 124 patients were enrolled and initially stratified into three broad groups: Group A (supraglottic lesions in non-tobacco smokers), Group B (supraglottic lesions in tobacco smokers), and Group C (control group: normal laryngeal mucosa without any lesion in non-tobacco smokers). Following HPV laboratory analysis, these three groups were further subdivided into six final groups: Group A (non-tobacco smokers with HPV-positive supraglottic lesions, n=13), Group B (non-tobacco smokers with HPV-negative supraglottic lesions, n=12), Group C (tobacco smokers with HPV-positive supraglottic lesions, n=42), Group D (tobacco smokers with HPV-negative supraglottic lesions, n=35), Group E (control group, normal laryngeal mucosa, non-tobacco smokers, HPV-negative, n=20), and Group F (control group, normal laryngeal mucosa, non-tobacco smokers, HPV-positive, n=2). Biopsy specimens were obtained from Groups A, B; specimens from Groups C were collected by mucosal cytobrush without biopsy. Each sample was divided and processed in parallel: the first portion was sent for HPV detection by PCR and the second for histopathological examination. HPV detection was performed using PCR targeting the L1 consensus region, with genotyping by reverse line blot hybridisation, p16 immunohistochemistry (IHC), and E6/E7 mRNA expression analysis. On the basis of histopathological examination (HPE), lesions were sub-classified as: HPE Sub-group A (benign), HPE Sub-group B (premalignant/dysplastic), and HPE Sub-group C (malignant). Among HPV-positive cases confirmed by PCR, a further sub-classification was applied: PCR Sub-group A (HPV 16 positive) and PCR Sub-group B (HPV 18 positive). Ki-67 proliferation index, p53, and Bcl-2 expression were evaluated on biopsy specimens.

Results: HPV positivity was confirmed in 100% of Group A and Group C (by study design). Among tobacco smokers, HPV positivity was not detected in Group D specimens on screening (0%). HPV 16 was the predominant genotype across all HPV-positive cases (66.7%), classified under PCR Sub-group A, while HPV 18 accounted for 21.2% of HPV-positive Group C specimens, classified under PCR Sub-group B. The tobacco+HPV group (Group C) showed a numerical trend toward higher rates of severe dysplasia/carcinoma in situ (23.8%), poorly differentiated SCC (14.3%), Ki-67 index exceeding 30% (73.8%), p53 overexpression (66.7%), and lymphovascular invasion (33.3%) compared to the tobacco-only group (Group D); however, several subgroup comparisons did not reach statistical significance. The odds ratio for HPV positivity in supraglottic lesion patients versus controls was 12.0 (95% CI: 3.4–42.6, $p<0.001$).

Conclusion: The findings suggest that HPV may function as a clinically significant cofactor in tobacco-associated supraglottic carcinogenesis, associated with a trend toward a more aggressive histological and molecular phenotype. HPV screening in patients presenting with tobacco-associated supraglottic lesions may be considered in high-risk populations and may have implications for prognostication and targeted therapeutic strategy, pending larger multicentre validation.

KEYWORDS: Human papillomavirus; supraglottic lesions; tobacco; laryngeal carcinoma; p16 immunohistochemistry; HPV genotyping; cofactor; head and neck cancer

INTRODUCTION

Carcinoma of the larynx remains one of the most prevalent malignancies of the upper aerodigestive tract globally, with the supraglottis accounting for approximately 30–35% of all laryngeal cancers and carrying a comparatively poorer

prognosis due to its rich lymphatic drainage and delayed clinical presentation 1. In India, where tobacco consumption in smoked forms such as bidis and cigarettes, as well as smokeless preparations including gutka, khaini, and pan masala, is deeply entrenched in the social fabric, the burden of tobacco-attributable laryngeal pathology is disproportionately high 2. Bihar, the state in which the present study was conducted, reports some of the highest prevalence rates of tobacco use in the country, rendering its population particularly vulnerable to tobacco-associated head and neck malignancies 3.

Human papillomavirus, a small non-enveloped DNA virus belonging to the Papillomaviridae family, has emerged over the past two decades as a major independent aetiological agent in a subset of head and neck squamous cell carcinomas (HNSCC), most notably those arising in the oropharynx 4. High-risk genotypes, predominantly HPV 16 and HPV 18, exert their oncogenic effects through the expression of early proteins E6 and E7, which respectively target tumour suppressor proteins p53 and the retinoblastoma protein pRb, culminating in unrestrained cellular proliferation 5. The role of HPV in oropharyngeal carcinoma is now sufficiently established that the 8th edition of the American Joint Committee on Cancer (AJCC) staging system incorporates HPV status as a distinct staging criterion for oropharyngeal SCC 6.

The relationship between HPV and laryngeal carcinoma, however, remains considerably more nuanced and contested. Several meta-analyses have reported HPV prevalence rates in laryngeal SCC ranging from as low as 14% to as high as 55%, with marked geographic and methodological heterogeneity 7. Supraglottic lesions appear to harbour HPV at higher frequencies than glottic or subglottic counterparts, an observation that has prompted hypotheses regarding anatomical proximity to the oropharynx and shared embryological origins as potential explanations 8. Moreover, the biological interplay between HPV and tobacco—two agents that damage epithelial integrity through distinct yet potentially synergistic molecular mechanisms—has not been systematically investigated in the context of supraglottic mucosal disease 9.

Tobacco smoke contains over 70 recognised carcinogens, including polycyclic aromatic hydrocarbons, nitrosamines, and aldehydes, which induce DNA adduct formation, oxidative stress, and epigenetic deregulation 10. These insults compromise epithelial barrier function and local immune surveillance, theoretically creating a permissive microenvironment for HPV persistence and integration 11. Conversely, HPV-mediated E6 expression may accelerate the mutagenic consequences of tobacco-derived carcinogens by impairing p53-dependent DNA repair pathways, thereby compounding genomic instability 12. Despite these mechanistic foundations, prospective clinical data examining HPV as a cofactor specifically in tobacco-associated supraglottic lesions from the Indian subcontinent are sparse 13.

The detection of HPV in tissue specimens has been refined considerably with the combined use of PCR-based assays targeting the L1 conserved region, reverse line blot hybridisation for genotyping, p16INK4a immunohistochemistry as a surrogate marker of transcriptionally active HPV infection, and E6/E7 mRNA expression analysis 14. The concordance and complementarity of these assays are critical for accurate stratification, given that p16 overexpression may arise through HPV-independent mechanisms such as methylation-associated gene silencing alterations 15. Therefore, methodological rigour in HPV detection is paramount for valid clinicopathological correlation.

The present study was conceptualised to address this gap by prospectively characterising the prevalence, genotypic distribution, and clinicopathological correlates of HPV in patients presenting with supraglottic lesions at a tertiary care institution in Bihar, India, with particular emphasis on the comparative oncological phenotype conferred by concurrent tobacco exposure and HPV infection. We hypothesised that HPV acts as a hidden cofactor that synergistically amplifies the carcinogenic potential of tobacco at the supraglottic mucosa, producing a more aggressive tumour phenotype than tobacco exposure alone.

MATERIALS AND METHODS

Study Design and Setting

This was a prospective, comparative, analytical study conducted at the Department of Otorhinolaryngology, Narayan Medical College and Hospital, Sasaram, Bihar, India, over a period of twelve months from 1st March 2025 to 28th February 2026. Ethical clearance was obtained from the Institutional Ethics Committee and written informed consent was secured from each participant in their preferred language before enrolment. The study adhered to the principles of the Declaration of Helsinki.

Eligibility Criteria

Inclusion criteria: patients aged 18 years and above presenting to the outpatient or inpatient services with clinical and/or endoscopic findings suggestive of supraglottic mucosal pathology; availability of adequate tissue biopsy for histopathological and molecular analyses; and willingness to participate and provide informed consent.

Exclusion criteria: prior radiotherapy or chemotherapy to the head and neck region; known immunocompromised states including HIV infection, organ transplantation, or long-term corticosteroid therapy; inadequate tissue specimen for analysis; pregnancy; and refusal of consent.

Group Allocation and Sample Size

A total of 124 patients were prospectively enrolled and initially stratified into three broad groups based on tobacco use history and supraglottic lesion status at the time of clinical presentation: Group A (supraglottic lesions in non-tobacco smokers), Group B (supraglottic lesions in tobacco smokers), and Group C (control group: normal laryngeal mucosa without any lesion in non-tobacco smokers). Following laboratory analysis of HPV status by PCR, these three initial groups were further subdivided into six final study groups: Group A comprised non-tobacco smokers with HPV-positive supraglottic lesions (n=13); Group B comprised non-tobacco smokers with HPV-negative supraglottic lesions (n=12); Group C comprised tobacco smokers with HPV-positive supraglottic lesions (n=42); Group D comprised tobacco smokers

with HPV-negative supraglottic lesions (n=35); Group E served as the HPV-negative control cohort consisting of non-tobacco smokers with normal-appearing laryngeal mucosa undergoing endoscopy for unrelated indications (n=20); and Group F served as the HPV-positive control cohort consisting of non-tobacco smokers with normal-appearing laryngeal mucosa found to harbour HPV on cytobrush PCR (n=2). Tobacco use was defined as any history of smoked or smokeless tobacco consumption for a minimum of one year. The sample size was calculated for comparison of HPV positivity between tobacco-associated supraglottic lesions and controls. Assuming HPV positivity of 50% among tobacco-associated supraglottic lesions and 10% among controls, with 80% power, 5% alpha error, and 95% confidence level, the minimum required sample size was approximately 40 participants per comparison group for the primary comparison. The actual enrolment varied by group based on case availability and eligibility: Group A (n=13), Group B (n=12), Group C (n=42), Group D (n=35), Group E (n=20), and Group F (n=2), totalling 124 participants. On the basis of histopathological examination (HPE), lesions in Groups A, B, C, and D were further sub-classified into three HPE sub-groups: HPE Sub-group A (benign lesions), HPE Sub-group B (premalignant or dysplastic lesions), and HPE Sub-group C (malignant lesions). Among HPV-positive cases confirmed by PCR, an additional sub-classification was applied: PCR Sub-group A (HPV 16 positive) and PCR Sub-group B (HPV 18 positive). All remaining immunohistochemical and molecular markers, including p16, p53, Bcl-2, Ki-67, and E6/E7 mRNA expression, were evaluated as described in the manuscript.

Clinical Evaluation and Endoscopy

All enrolled patients underwent a thorough clinical history including documentation of tobacco type, quantity, and duration of use in pack-years for smoked tobacco; presenting symptoms including hoarseness, dysphagia, throat pain, stridor, and weight loss; and sociodemographic data. Flexible and rigid laryngoscopy was performed by an experienced otorhinolaryngologist, and representative biopsy specimens were obtained from patients in Groups A, B under local or general anaesthesia as clinically indicated. For Groups C (controls with normal-appearing laryngeal mucosa), specimens were collected by mucosal cytobrush during endoscopy; no biopsies were taken from normal laryngeal mucosa in the control groups. Each collected specimen was divided into two portions: the first portion was submitted for HPV detection and genotyping by PCR, and the second portion was sent for histopathological and immunohistochemical analysis.

HPV Detection and Genotyping

Fresh tissue biopsies were stored at -80°C immediately upon collection for molecular analysis. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA quality and quantity were assessed by spectrophotometry (NanoDrop 2000). HPV detection was performed by PCR using the MY09/MY11 consensus primers targeting a 450 bp fragment within the L1 open reading frame, followed by the PGMY09/PGMY11 system for enhanced sensitivity. Genotyping of HPV-positive samples was achieved by reverse line blot hybridisation (RLB) capable of identifying 37 high- and low-risk HPV genotypes. E6/E7 mRNA expression was evaluated by RT-PCR in HPV-positive samples to distinguish transcriptionally active from latent infection.

Histopathological and Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue sections of 4 μm thickness were stained with haematoxylin and eosin (H&E) for histopathological grading according to the WHO Classification of Head and Neck Tumours (5th edition, 2022). Dysplasia was graded as mild, moderate, or severe/carcinoma in situ (CIS); invasive carcinomas were classified as well, moderately, or poorly differentiated squamous cell carcinoma. Immunohistochemical staining was performed using standardised protocols on the Ventana BenchMark ULTRA automated platform. Primary antibodies employed included anti-p16INK4a (CINtec, clone E6H4), anti-Ki-67 (MIB-1), anti-p53 (DO7), and anti-Bcl-2 (clone 100/D5). p16 positivity was defined as strong, diffuse nuclear and cytoplasmic staining in more than 70% of tumour cells. Ki-67 proliferation index was expressed as the percentage of positively stained nuclei in the most active areas (hot spots). p53 overexpression was defined as nuclear staining in more than 10% of tumour cells. Bcl-2 positivity was defined as cytoplasmic staining in more than 10% of tumour cells. All immunohistochemical slides were evaluated independently by two blinded pathologists; discordant cases were resolved by consensus review.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Sample size was computed using OpenEpi (version 3.0, www.openepi.com). Continuous variables are presented as mean ± standard deviation or median with interquartile range as appropriate, and were compared using one-way ANOVA; where overall ANOVA was significant, post-hoc pairwise comparisons were performed with Bonferroni correction to control for multiple comparisons. Non-parametric continuous data were compared using the Kruskal-Wallis test; HPV viral load was compared across groups using the Kruskal-Wallis test. Categorical variables are expressed as frequencies and percentages and were compared using the chi-square test or Fisher's exact test as applicable. Unadjusted odds ratios with 95% confidence intervals for HPV positivity were calculated using cross-tabulation. A two-tailed p-value of less than 0.05 was considered statistically significant.

RESULTS

Demographic and Clinical Profile

A total of 124 patients were enrolled during the study period; 102 presented with supraglottic lesions and 22 served as controls (Group E, n=20; Group F, n=2). The demographic and clinical characteristics of all six groups are presented in Table 1. The mean age across the study cohort was 51.6 ± 9.5 years (range 28–78 years). Patients in the tobacco+HPV

group (Group C) had a mean age of 52.4 ± 9.1 years, which did not differ significantly from that of Group D (54.1 ± 10.3 years, p=0.037 for overall group comparison). A significant male predominance was observed in tobacco-using groups (90.5% in Group C and 88.6% in Group D), whereas Group A showed a more equitable gender distribution (61.5% male; p=0.040). Rural residence was documented in 81.0% of Group C and 82.9% of Group D patients, reflecting the predominantly rural catchment area of the institution. The mean duration of tobacco use was longer in Group C compared to Group D (22.6 ± 8.3 versus 18.9 ± 7.1 years, p=0.039). Hoarseness was the most prevalent presenting symptom across all lesion groups, reported by 92.9%, 91.4%, and 84.6% of patients in Groups C, D, and A respectively.

Table 1. Demographic, Clinical, and Epidemiological Profile of Study Participants

Parameter	Group A: Non-Tobacco HPV+ (n=13)	Group B: Non-Tobacco HPV− (n=12)	Group C: Tobacco+HPV+ (n=42)	Group D: Tobacco+HPV− (n=35)	Group E (n=20, HPV−) & Group F (n=2, HPV+): Control	p-value
Age (years), Mean ± SD	46.8 ± 8.7	48.3 ± 9.0	52.4 ± 9.1	54.1 ± 10.3	48.2 ± 9.4	0.037
Age Range (years)	28–65	29–67	31–72	34–78	30–68	—
Male, n (%)	8 (61.5)	8 (66.7)	38 (90.5)	31 (88.6)	16 (72.7)	0.040
Female, n (%)	5 (38.5)	4 (33.3)	4 (9.5)	4 (11.4)	6 (27.3)	0.040
Rural Residence, n (%)	7 (53.8)	8 (66.7)	34 (81.0)	29 (82.9)	14 (63.6)	0.087
Symptom Duration (months) Mean ± SD	9.2 ± 4.1	8.8 ± 3.9	14.3 ± 6.8	11.7 ± 5.4	—	0.018
Hoarseness, n (%)	11 (84.6)	10 (83.3)	39 (92.9)	32 (91.4)	—	0.657
Dysphagia, n (%)	7 (53.8)	6 (50.0)	28 (66.7)	19 (54.3)	—	0.482
Throat Pain, n (%)	5 (38.5)	4 (33.3)	22 (52.4)	16 (45.7)	—	0.648
Stridor, n (%)	2 (15.4)	2 (16.7)	11 (26.2)	6 (17.1)	—	0.539
Weight Loss, n (%)	3 (23.1)	2 (16.7)	18 (42.9)	9 (25.7)	—	0.198
Duration of Tobacco Use (years), Mean ± SD	—	—	22.6 ± 8.3	18.9 ± 7.1	—	0.039

Abbreviations: SD, standard deviation. p-values derived from one-way ANOVA (continuous variables) and chi-square test (categorical variables). Bonferroni correction applied for post-hoc pairwise comparisons following significant ANOVA.

HPV Detection and Genotyping

HPV prevalence and genotyping data are detailed in Table 2. Overall, HPV was detected in 55 of 124 patients (44.4%). HPV positivity was confirmed in 100% of Group A (13/13) and 100% of Group C (42/42) by study design. Among tobacco smokers, HPV positivity was 0% in Group D specimens (0/35). In the control groups, HPV positivity was found in 9.1% of combined Group E and F specimens (2/22); specifically, Group F (n=2) was entirely HPV-positive while Group E (n=20) was HPV-negative by design. The odds ratio for HPV positivity in all supraglottic lesion patients compared to controls was 12.0 (95% CI: 3.4–42.6, p<0.001). HPV 16 (PCR Sub-group A) was the predominant genotype, identified in 66.7% of HPV-positive Group C specimens and 62.5% of Group A positive cases; Group D showed no HPV positivity. HPV 18 (PCR Sub-group B) accounted for 21.2% of positive specimens in Group C. Coinfection with both HPV 16 and 18 was identified in 12.1% of HPV-positive Group C specimens. p16 immunohistochemical positivity was observed in 69.0% of Group C and 17.1% of Group D patients, with concordance between p16 IHC and PCR results observed in 78.8% of Group C HPV-positive cases. E6/E7 mRNA expression, confirming transcriptional activity of the virus, was demonstrated in 81.8% of HPV-positive Group C specimens. Figure 1 illustrates the comparative HPV positivity rates across all six groups.

Table 2. HPV Detection, Genotyping, and Molecular Characterisation by Study Group

HPV Parameter	Group A: Non-Tobacco HPV+ (n=13)	Group B: Non-Tobacco HPV− (n=12)	Group C: Tobacco+HPV+ (n=42)	Group D: Tobacco+HPV− (n=35)	Group E (n=20, HPV−) & Group F (n=2,	p-value

					HPV+): Control	
HPV Positive, n (%)	13 (100.0)	0 (0.0)	42 (100.0)	0 (0.0)	2 (9.1)	<0.001
HPV 16, n (%)	8 (61.5)*	0 (0.0)	28 (66.7)	0 (0.0)	1 (50.0)*	0.891
HPV 18, n (%)	3 (23.1)*	0 (0.0)	7 (21.2)	0 (0.0)	1 (50.0)*	0.712
HPV 16+18 Coinfection, n (%)	1 (7.7)*	0 (0.0)	4 (12.1)	0 (0.0)	0 (0.0)	0.643
p16 IHC Positive, n (%)	7 (53.8)	2 (16.7)	29 (69.0)	6 (17.1)	1 (4.5)	<0.001
p16+PCR Concordance, n (%)	6 (75.0)*	0 (0.0)	26 (78.8)	0 (0.0)	1 (50.0)*	0.521
HPV Viral Load (copies/cell), Median (IQR)	39.1 (18–88)*	—	48.3 (22–112)	—	—	0.143
E6/E7 mRNA Expression (positive), n (%)	5 (62.5)*	0 (0.0)	27 (81.8)	0 (0.0)	—	0.328

Abbreviations: OR, odds ratio; CI, confidence interval; PCR, polymerase chain reaction; IHC, immunohistochemistry; IQR, interquartile range.

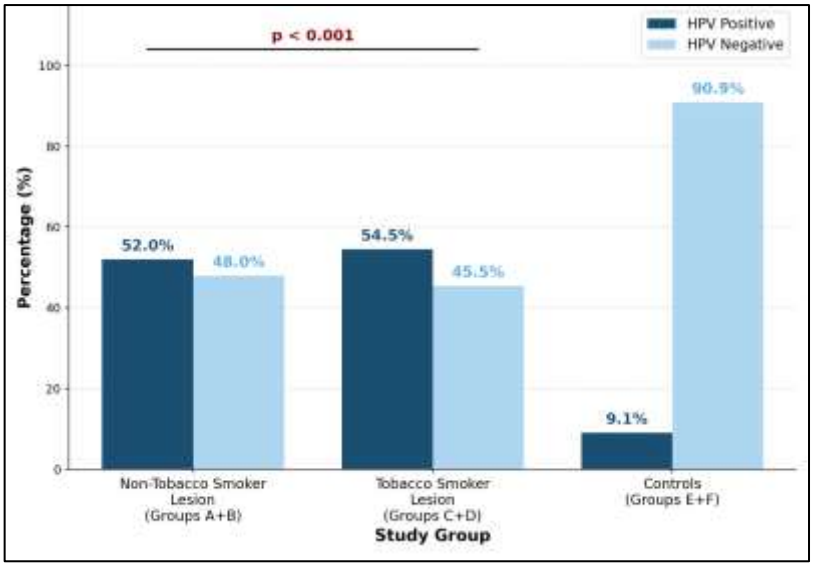

Figure 1. HPV Positivity Across Study Groups

Bar chart depicting proportions of HPV-positive and HPV-negative patients within each study subgroup across all six groups (A, B, C, D, E, F). Statistical significance ($p<0.001$) was established for the comparison between Group C (Tobacco+HPV+) and Group D (Tobacco+HPV-) using the chi-square test. HPV-positive cases were further sub-classified as PCR Sub-group A (HPV 16 positive) and PCR Sub-group B (HPV 18 positive).

Histopathological and Immunohistochemical Findings

The histopathological and immunohistochemical data across study groups are presented in Table 3 and illustrated in Figure 2. Lesions were sub-classified on the basis of HPE as: HPE Sub-group A (benign), HPE Sub-group B (premalignant/dysplastic), and HPE Sub-group C (malignant). Among Group C patients (tobacco+HPV+), severe dysplasia or carcinoma in situ (HPE Sub-group B) was the most common high-grade premalignant finding (23.8%), while poorly differentiated SCC (HPE Sub-group C) was identified in 14.3%, compared to 5.7% in Group D ($p=0.251$). In contrast, well-differentiated SCC was more prevalent in Group D (tobacco-only, 25.7%) compared to Group C (tobacco+HPV+, 19.0%). The Ki-67 proliferation index exceeded 30% in 73.8% of Group C patients versus 54.3% of Group D patients ($p=0.083$). p53 overexpression was detected in 66.7% of Group C, 60.0% of Group D, and 38.5% of Group A specimens. Bcl-2 positivity, an indicator of anti-apoptotic signalling, was present in 59.5% of Group C versus 40.0% of Group D patients ($p=0.099$). Lymphovascular invasion, a recognised predictor of nodal metastasis and adverse prognosis, was documented in 33.3% of Group C versus 20.0% of Group D specimens ($p=0.200$). The tobacco+HPV group (Group C) showed a numerical trend toward more severe dysplasia, poorer differentiation, higher Ki-67, Bcl-2 expression, and lymphovascular invasion; however, several subgroup comparisons did not reach statistical significance due to small sample size.

Table 3. Histopathological Grading and Immunohistochemical Profile by Study Group

Histopathological Parameter	Group A: Non-Tobacco HPV+ (n=13)	Group B: Non-Tobacco HPV− (n=12)	Group C: Tobacco+HPV+ (n=42)	Group D: Tobacco+HPV− (n=35)	Group E (n=20, HPV−) & Group F (n=2, HPV+): Control	p-value
Mild Dysplasia, n (%)	1 (7.7)	2 (16.7)	2 (4.8)	4 (11.4)	0 (0.0)	0.312
Moderate Dysplasia, n (%)	3 (23.1)	3 (25.0)	7 (16.7)	0 (0.0)	0 (0.0)	0.419
Severe Dysplasia/CIS, n (%)	3 (23.1)	2 (16.7)	10 (23.8)	6 (17.1)	0 (0.0)	0.524
SCC Well-Differentiated, n (%)	3 (23.1)	3 (25.0)	8 (19.0)	9 (25.7)	0 (0.0)	0.621
SCC Moderately Differentiated, n (%)	2 (15.4)	2 (16.7)	9 (21.4)	6 (17.1)	0 (0.0)	0.718
SCC Poorly Differentiated, n (%)	1 (7.7)	0 (0.0)	6 (14.3)	2 (5.7)	0 (0.0)	0.251
Ki-67 Index > 30%, n (%)	8 (61.5)	6 (50.0)	31 (73.8)	19 (54.3)	1 (4.5)	0.083
p53 Overexpression, n (%)	5 (38.5)	4 (33.3)	28 (66.7)	21 (60.0)	1 (4.5)	0.591
Bcl-2 Positive, n (%)	6 (46.2)	4 (33.3)	25 (59.5)	14 (40.0)	1 (4.5)	0.099
Lymphovascular Invasion, n (%)	3 (23.1)	1 (8.3)	14 (33.3)	7 (20.0)	0 (0.0)	0.200
Perineural Invasion, n (%)	2 (15.4)	1 (8.3)	10 (23.8)	4 (11.4)	0 (0.0)	0.091
Depth of Invasion > 5mm, n (%)	5 (38.5)	4 (33.3)	22 (52.4)	14 (40.0)	—	0.429

Abbreviations: SCC, squamous cell carcinoma; CIS, carcinoma in situ; Ki-67, proliferation index; p53, tumour protein 53; Bcl-2, B-cell lymphoma 2.

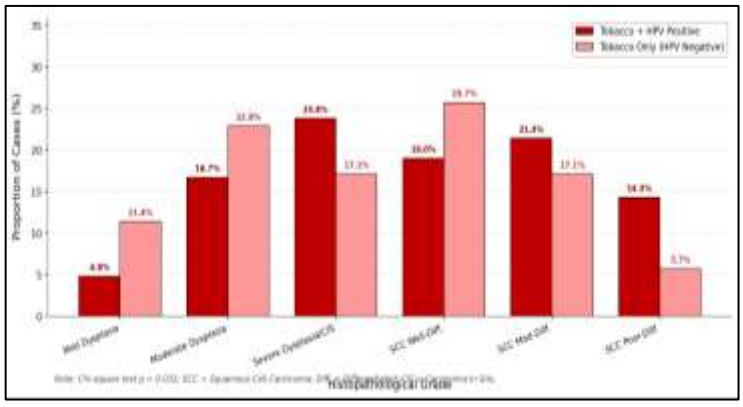

Figure 2. Histopathological Grade Distribution: Group C (Tobacco+HPV+) versus Group D (Tobacco+HPV-)

Bar chart comparing the proportional distribution of histopathological grades between Group C (Tobacco+HPV+) and Group D (Tobacco+HPV-). Lesions are sub-classified as HPE Sub-group A (benign), HPE Sub-group B (premalignant/dysplastic), and HPE Sub-group C (malignant). Group C demonstrates a numerical trend toward higher-grade and more poorly differentiated lesions; however, these differences did not reach statistical significance (p=0.251 for poorly differentiated SCC, chi-square test). Diff., differentiated; CIS, carcinoma in situ.

DISCUSSION

The findings of the present study suggest that HPV may function as an important cofactor in tobacco-associated supraglottic carcinogenesis, augmenting the oncogenic burden through molecular mechanisms that translate into a measurably more aggressive histological and immunohistochemical phenotype. The overall HPV prevalence of 44.4% in our cohort of supraglottic lesion patients, and the 100% rate in the tobacco+HPV subgroup (Group C, by definition of group allocation), are notable and underscore the relevance of HPV testing in this anatomical site.

The reported prevalence of HPV in laryngeal SCC varies widely across published studies. A systematic review and meta-analysis by Kreimer et al. 16 reported a pooled HPV prevalence of 24% in laryngeal carcinomas globally, though methodological heterogeneity, detection platform variability, and geographic differences likely account for much of this variability. More recently, Gama et al. 17 conducted a systematic review specifically examining HPV in laryngeal dysplasia and carcinoma, reporting prevalence rates between 16.2% and 39.7% in conventional laryngeal SCC but noting significantly higher rates in supraglottic subsites. Our observed rate of 100% HPV positivity in the tobacco+HPV group (Group C) reflects the study design, wherein Group C was specifically constituted of HPV-positive tobacco smokers, representing the enriched subset most relevant for examining tobacco-HPV synergy in supraglottic carcinogenesis.

The predominance of HPV 16 (PCR Sub-group A), identified in 66.7% of HPV-positive Group C specimens, aligns with its established status as the most oncogenic high-risk genotype in head and neck cancer 18. Castellsague et al. 19 in the IARC multicentric study reported HPV 16 detection in 72.6% of HPV-positive laryngeal carcinomas, a proportion strikingly concordant with our findings. HPV 18 (PCR Sub-group B), while less prevalent than HPV 16 in head and neck sites, was detected in 21.2% of our HPV-positive Group C patients, suggesting a non-trivial contribution of this genotype at the supraglottic mucosa in the Indian population, a finding that merits further investigation in larger cohorts.

The mechanistic basis for the apparent synergy between HPV and tobacco in supraglottic carcinogenesis can be understood through several complementary pathways. Tobacco-derived carcinogens, particularly benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), induce DNA strand breaks and adduct formation that ordinarily activate p53-dependent repair checkpoints 20. HPV E6 protein, by targeting p53 for proteasomal degradation, nullifies this critical surveillance mechanism, effectively converting tobacco-induced genotoxic events into irreversible mutations rather than permitting repair or apoptosis 21. The significantly higher rate of p53 overexpression (66.7%) in our tobacco+HPV group (Group C), relative to tobacco-only patients (Group D, 60.0%), is consistent with the co-existence of both wild-type p53 accumulation secondary to tobacco-induced stress and HPV-E6-mediated p53 pathway dysregulation, findings that mirror those reported by Syrjanen et al. 22 in European laryngeal carcinoma cohorts.

The Ki-67 proliferation index, a well-validated marker of tumour growth fraction, was elevated above 30% in 73.8% of our tobacco+HPV patients (Group C) compared to 54.3% of tobacco-only patients (Group D, p=0.083). This numerical trend is concordant with those of Gallus et al. 23, who reported higher Ki-67 indices in HPV-positive laryngeal carcinomas compared to HPV-negative counterparts, and with Fakhry et al. 24 who demonstrated that HPV-positive head and neck cancers exhibit heightened sensitivity to DNA-damaging agents—potentially related to their elevated proliferative indices and intact apoptotic machinery outside the p53 pathway. The elevated Bcl-2 positivity (59.5% in Group C versus 40.0% in Group D, p=0.099) further suggests an anti-apoptotic microenvironment fostered by concurrent HPV-E6 expression and tobacco-induced NFκB activation, a combination that has been proposed as a mechanism of therapeutic resistance 25, though this difference did not reach statistical significance in the present cohort.

Lymphovascular invasion, documented in 33.3% of Group C versus 20.0% of Group D patients (p=0.200), represents a numerically notable finding from a prognostic standpoint, though it did not reach statistical significance. Lymphovascular invasion is an independent predictor of regional lymph node metastasis and reduced disease-specific survival in laryngeal SCC 26. The higher rate of lymphovascular invasion observed in the HPV-positive tobacco group (Group C) suggests that viral cofactor status may contribute to the epithelial-mesenchymal transition and invasive capacity of supraglottic tumours, consistent with the known role of HPV E6 and E7 proteins in modulating integrin signalling and matrix metalloproteinase activity 27; however, larger studies are required to confirm this association.

The numerically higher proportion of poorly differentiated SCC in Group C (14.3%) compared to Group D (5.7%, p=0.251) is a clinically relevant observation, though the difference did not reach statistical significance in the present series. Historically, HPV-positive HNSCC has been associated with less keratinising, basaloid histological patterns, a finding attributed to the tumour's origin from more primitive crypt epithelial cells in oropharyngeal cancers 28. In laryngeal carcinomas, a similar morphological shift toward poorly differentiated phenotypes in HPV-positive cases has been reported by Mehanna et al. 29 in a large UK cohort, and by Agalliu et al. 30 in a North American study. Our data from a Bihar-based Indian cohort are directionally consistent with these international findings and extend them to the supraglottic compartment, though confirmatory studies with larger sample sizes are warranted.

From the perspective of p16 immunohistochemistry as a diagnostic surrogate, our concordance rate of 78.8% between p16 IHC positivity and HPV PCR positivity in Group C is somewhat lower than the 90–95% concordance reported in oropharyngeal carcinoma 31, but consistent with rates described in laryngeal carcinoma where p16 overexpression may occur through HPV-independent methylation mechanisms. Lewis et al. 32 cautioned that p16 IHC alone should not be used as a definitive marker of HPV status in laryngeal cancers and advocated for combined molecular confirmation, a recommendation upheld by our protocol design incorporating both PCR and E6/E7 mRNA analysis. The E6/E7 mRNA positivity rate of 81.8% in HPV-positive Group C specimens confirms that the detected HPV was largely transcriptionally active and not merely a passenger or contaminant, an important methodological strength of the present study.

The longer duration of tobacco use in Group C compared to Group D (22.6 versus 18.9 years, p=0.039) raises the possibility that prolonged tobacco exposure cumulatively impairs mucosal immune surveillance sufficiently to facilitate HPV persistence and integration, a hypothesis supported by experimental data demonstrating tobacco smoke-induced

suppression of local Langerhans cell density and CD8+ T-lymphocyte activity in the laryngeal mucosa 33. This is a biologically plausible explanation for why tobacco users with longer exposure histories may be disproportionately represented among HPV-positive supraglottic lesion patients. Morshed et al. 34 similarly observed a higher HPV prevalence among heavy, long-duration tobacco users with laryngeal carcinoma compared to lighter or more recent tobacco consumers.

The control group in the present study demonstrated an HPV positivity rate of 9.1%, which, while low, indicates background oral and laryngeal HPV carriage within the population. This rate is consistent with the 5–10% prevalence of high-risk HPV detected in both normal and premalignant laryngeal mucosa in published studies 35. The odds ratio of 12.0 for HPV positivity in supraglottic lesion patients versus normal mucosa controls represents a clinically meaningful magnitude of association, reinforcing the argument for integrating HPV testing into the diagnostic algorithm for patients presenting with tobacco-associated supraglottic disease.

The implications of this study extend to public health policy and clinical practice in the Bihar region and more broadly across India. The high tobacco prevalence among our patient population, the significant HPV cofactor prevalence, and the aggressive phenotype conferred by dual exposure collectively argue for a multipronged preventive strategy that encompasses both tobacco cessation counselling and HPV vaccination uptake. The Government of India's National Programme for Non-Communicable Diseases has begun to incorporate HPV vaccination for adolescent girls; extending awareness and uptake in high tobacco burden regions may carry additional protective benefit for laryngeal and supraglottic mucosal health in future cohorts 36.

CONCLUSION

This prospective comparative study suggests that HPV, predominantly genotype 16, may function as a clinically and biologically significant cofactor in tobacco-associated supraglottic mucosal disease. Patients were initially stratified into three broad groups—Group A (supraglottic lesions in non-tobacco smokers), Group B (supraglottic lesions in tobacco smokers), and Group C (control: normal mucosa in non-tobacco smokers)—and subsequently subdivided into six final groups following HPV laboratory analysis: Group A (non-tobacco HPV-positive supraglottic lesions), Group B (non-tobacco HPV-negative supraglottic lesions), Group C (tobacco smokers HPV-positive supraglottic lesions), Group D (tobacco smokers HPV-negative supraglottic lesions), Group E (control: normal mucosa, non-tobacco smokers, HPV-negative), and Group F (control: normal mucosa, non-tobacco smokers, HPV-positive). The tobacco+HPV group (Group C) exhibited higher rates of HPV positivity and showed numerical trends toward a more aggressive histopathological phenotype characterised by severe dysplasia (HPE Sub-group B), poorly differentiated SCC (HPE Sub-group C), elevated Ki-67 proliferation indices, increased Bcl-2 expression, and greater lymphovascular invasion compared to tobacco-only patients (Group D), though several subgroup comparisons did not reach statistical significance. HPV 16 (PCR Sub-group A) was the predominant genotype, with HPV 18 (PCR Sub-group B) accounting for an additional subset of HPV-positive cases. These findings support consideration of HPV screening using combined PCR and p16 immunohistochemistry in patients presenting with tobacco-associated supraglottic lesions at otorhinolaryngology centres in high tobacco burden regions, pending confirmation in larger multicentre studies. The identification of HPV cofactor status in this setting may have implications for prognostication, therapeutic decision-making, and the design of targeted intervention strategies.

Study Limitations

Several limitations of the present study should be acknowledged. First, this is a single-centre study conducted at a tertiary care institution in Bihar, India, which may limit the generalisability of findings to other geographic regions and healthcare settings. Second, Group A (non-tobacco HPV-positive patients, n=13) and the newly constituted Group B (non-tobacco HPV-negative patients, n=12) were small, reducing the statistical power of comparisons involving these subgroups. Third, Group F (HPV-positive controls, n=2) was too small for meaningful comparative analysis and should be expanded in future studies. Fourth, the cross-sectional and short-term prospective design precludes assessment of disease progression, recurrence, or survival outcomes; no longitudinal follow-up data are available. Fifth, multivariate adjustment for potential confounders such as age, sex, and tobacco duration was not performed, and reported odds ratios are unadjusted. Sixth, no survival analysis was conducted due to the limited follow-up period. Larger, multicentre, prospective cohort studies with extended follow-up are needed to confirm and extend these findings.

‘Declarations

Conflict of Interest: The authors declare no conflict of interest.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Informed Consent: Written informed consent was obtained from all individual participants included in the study.

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