Human papillomavirus, tobacco, and poor oral hygiene can act synergistically, modulate the expression of the nuclear factor kappa B signaling pathway for the development and progression of head and neck cancer in the Pakistani population

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Abstract

Background: Head and neck cancers (HNCs) are a heterogeneous group of tumors that progress owing to varied environmental and genetic risk factors. Viral infections are threatening and adept at altering the expression of cellular transcription factors such as nuclear factor kappa B (NF-κB) and deregulation of other cellular proteins like NF kappa B inhibitor alpha (IκBa). The present study was conducted to detect high-risk genotypes of human papillomavirus (HPV) and protein expression of NF-κB signaling pathway in HNC patients with HPV infection.

Methods: For HPV detection, genomic DNA from 152 HNC tumors was extracted formalin-fixed paraffin-embedded tissue DNA kit. For genotyping, polymerase chain reaction (PCR) using a general primer, HPV type-specific primers and agarose gel electrophoresis were performed. Immunohistochemistry (IHC) was also performed on 4-μm thick tissue sections using HPV E6 monoclonal antibody. Protein expression analysis of NF-κB signaling pathway including p50, p65, and IκBa was performed using IHC.

Results: PCR analysis showed that 24.3% (37/152) of HNC cases were HPV positive. Among HPV positive, 86.5% (32/37) were tobacco users, while among HPV negative, 66.9% (77/115) were tobacco users. A significant association of HPV positivity and tobacco user was observed by univariate analysis [P < 0.01; odds ratio (OR): 0.310, 95% confidence interval (CI): 0.110 to 0.870]. More HPV positive patients were with poor oral hygiene (78.3%) when compared with patients with good oral hygiene (21.6%) [P < 0.03, OR: 2.440, 95% CI: 1.650 to 3.600]. The results of the logistic regression analysis showed that age, tobacco use and oral hygiene are significant predictors [P < 0.02]. PCR and IHC staining results confirmed that HPV16 was predominant among HNC cases (64.8%) when compared with HPV18 (35.2%). Expression of NF-κB proteins (p50, p65, and IκBa inhibitor) were also observed in HPV and non-HPV infected HNC tissues. IHC expression of p50, and p65 showed nuclear staining, while IκBa inhibitor showed cytoplasmic staining. Protein expression in HPV cases was higher as compared to HPV naive cases (P < 0.05).

Conclusions: From the study, it can be established that the use of tobacco, oral hygiene, and HPV infection may be synergistically involved in modulating the expression of NF-κB signaling pathway for the development and progression of HNC in the Pakistani population.

Keywords: Human papillomavirus; NF-kappa B; p50; p65; IκBa inhibitor; Polymerase chain reaction; Immunohistochemistry; Head and neck cancer; Pakistani population

Introduction

Head and neck cancer (HNC) is a subset of malignancies at different anatomic sites in the upper aerodigestive tract including oral cavity, lips, oropharynx, hypopharynx, nasopharynx, and larynx. It is a frequent type of cancer worldwide and accounts for > 650,000 cases and 330,000 deaths annually. Global Cancer Statistics 2020, compiled by the International Agency for Research on Cancer, estimates that oral cancer recorded for almost 2.0% of all cancer cases and 1.8% of all cancer deaths globally. Oropharyngeal and tongue cancer are the principal forms of HNC in Western countries, while oral cancer is widespread in South Asian countries. More than 90% of cancers prevail in squamous cells of the oral cavity, oropharynx, and larynx, and a steady increase has been witnessed in the prevalence of tonsil and oropharyngeal cancer worldwide. Head and neck carcinomas stem from environmental and genetic factors; because it is...
particularly reported in people with extended exposure to the risk factors such as smoking, abnormal use of alcohol, and prolonged exposure to ultraviolet (UV) radiations, asbestos, and viruses.

Nuclear factor kappa B (NF-κB) is a pro-inflammatory transcription factor executing a key role in cell signaling pathways. NF-κB family includes five distinct members RelA [p65], RelB, c-Rel, NF-κB1[p50/p105], and NF-κB2 [p52/p100], which share an N-terminal Rel homology domain; a region responsible for DNA-binding and dimerization (homo-and heterodimerization) with nucleus localization sequence. NF-κB typically remains in a latent structure in the cytoplasm by restricting IκB proteins (inhibitory proteins), most remarkably by inhibitory protein (IκBα). Upon activation by cytokines, chemotherapeutic agents, developmental factors, lipopolysaccharide, bacterial or viral infection, and stress signals, it enters the nucleus and causes expression of over 100 downstream genes recruited for an array of cell functions including apoptosis, cell relocation, cell multiplication, and angiogenesis.[10]

Dysregulation of NF-κB protein expression and its instigation are frequently seen in malignancies, such as lymphoma, leukemia, myeloma, colon, pancreatic, breast, and cervical cancers.[10] Human papillomavirus (HPV) is viewed as a sexually transmitted infection that can be diagnosed and treated.[11] HPV genotypes are extending in incidence worldwide and have distinct molecular, clinical, pathological, and epidemiological features.[10] Additionally, high-risk (HR) – HPV genotypes 16/18 produce early proteins E6 and E7 and deregulate cell cycle control.[11] HR-HPV16 has been remarked as a regulator for NF-κB initiation and articulation in various tumors including oral squamous cell carcinomas.[18]

Even though chemotherapy, radiation therapy, and surgical procedure remain the key therapeutic tools to treat and manage HNC at an initial stage, the overall survival rate is still < 65%. This can be due to inadequate validity of treatment strategies, detection of tumor growth at later stages, and the environmental factors for HNC development, especially the involvement of pathogenic viruses. However, its mechanism of action in fostering cancer is unclear and data have not shown a confirmed association between HPV, NF-κB signaling pathway, and occurrence of HNC worldwide.[12] The aim of the present study is the detection of high-risk genotypes of HPV and the expression pattern of the proteins within the NF-κB signaling family and its inhibitor IκBα in different stages and different anatomic subsites of the HNC in the Pakistani population.

Methods

Ethical approval

This study was prior approved by Ethics Committees of Quaid-i-Azam University, Islamabad, Pakistan (Approval No. IRB-QAU-151), and collaborating hospitals and was conducted per the guidelines described in the Declaration of Helsinki (II) (18th, 1964). Data were collected using the specifically designed questionnaire via a personal interview after obtaining the written consent of each patient and their family.

Sample collection and patient’s data

Tumor samples and clinical data of pathologically confirmed HNC patients were collected from different hospitals of Pakistan including Pakistan Institute of Medical Sciences (PIMS, Islamabad), Nuclear Medicine, Oncology and Radiotherapy Institute (Islamabad), Ayub Medical complex (Abbottabad), Institute of Radiotherapy and Nuclear Medicine (Peshawar). Almost every patient all over Pakistan visits PIMS, Islamabad. The clinical history of selected patients was obtained from patient’s files maintained at the hospitals. All records related to patients, for example, age, gender, name, ethnicity, occupation, smoking history, area of cancer, any infection, histopathology report, year of diagnosis, and treatment, were collected. Different anatomic subsites of the head and neck were classified following the International Classification of Diseases 10th Revision classification system and the 8th tumor–nodes–metastases (TNM) staging system was adopted.[11] Patients with a history of any other type of cancer and other diseases were excluded from the study. Formalin-fixed paraffin-embedded (FFPE) tissue specimens and slides of 152 tumors samples stained with hematoxylin and eosin were retrieved from hospitals. All slides were re-examined for confirmation of the diagnosis.

Genomic DNA isolation and polymerase chain reaction (PCR)

Deoxyribonucleic acid (DNA) was extracted from the patient’s tumor using GeneAll® Exgene™ DNA kit (GeneAll Biotechnology, Seoul, Korea) from FFPE tissues. DNA of all samples was quantified by gel electrophoresis and UV transilluminator (Bio-Rad, Puchheim, Germany). Primers for HPV high-risk genotypes selected for the current study (HPV16, 18, 31, 33, and 45) were adopted from Kumar et al.[14] and amplified through PCR.

HPV genotyping and gel electrophoresis

PCR products were run on 2% agarose gel stained with EtBr (Merck, Darmstadt, Germany). HR-HPV positive cervical DNA sample was used as positive control and UltraPure™ DNase/RNase free distilled water (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) was used as a negative control in each run of PCR.

Confirmation of HPV positive cases via immunohistochemistry (IHC)

The expression of HPV E6 protein (anti-HPV16/E6 and anti-HPV18/E6 mouse monoclonal antibody; Abcam, Waltham, MA, USA) in the tissue biopsies of HNC were also evaluated by IHC. IHC was performed on thick tissue sections obtained from FFPE samples. Evaluation of the cytoplasmic immunoreactivity intensity was done semi-
**Expression analysis of NF-κB proteins and IκBα using IHC**

The expression of NF-κB pathway proteins p50, p65, and IκBα inhibitor (NF-κB/p50, NF-κB/p65, and IκBα) were analyzed in the samples selected for study along with positive and negative controls. Polyclonal antibodies (Host Rabbit) (cat# E-AB-32226, cat# E-AB-32232, and cat # E-AB-10086, Elabsence, Houston, TX, USA) were used to carry out all immunohistochemical reactions with a concentration of 1:100 (1 mg/mL), 1:100 (1 mg/mL), and 1:50 (0.1 mg/mL), respectively. Tumor cells were counted and observed under both 10 × and 40 × magnification for measuring immunoreactivity, which was calculated as, Immunoreactive score (IRS) = proportion score (PS) × intensity score (IS).

The intensity score was categorized by the following scheme: 0 for negative intensity, 1 for weak intensity, 2 for moderate intensity, and 3 for strong intensity. Whereas, proportion score was categorized as 0 for no positive cell, 1 for ≤10% of positive cells, 2 for 11% to 50% of positive cells, 3 for 51% to 80% of positive cells, and 4 for > 80% distribution of positive cells. Immunoreactivity (range 0–12) was categorized as low immunoreactivity (IS 0–4) and high immunoreactivity (IS >4).[8]

**Statistical analysis**

Software Graph Pad Prism v7.01 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Correlation of clinical parameters with HNC and HPV infection was calculated by univariate analysis; χ² test with Fisher exact test, crude odds ratios (ORs), and 95% confidence intervals (CIs) were calculated for the association between demographic and clinical variables and HNC risk. A multiple logistic regression model using Software SPSS version 26 (IBM SPSS Statistics, USA) was used to find the synergetic effect of covariates such as the use of tobacco, oral hygiene, HPV infection, and risk of HNC. Correlations of protein–protein expression, proteins expression with risk factors and clinicopathological parameters were evaluated by Spearman correlations. A P value of <0.05 was used for statistical significance.

**Results**

**Demographic data analysis**

Data analysis of 152 HNC samples was performed, among them, 62.5% (95/152) were male patients while 37.5% (57/152) were females. A total of 47.3% (72/152) of the patients were in the younger age group (age < 40 years). In the participants, 71.7% (109/152) were tobacco users while 28.2% (43/152) were non-tobacco users. Cancer of the oral cavity was common cancer reported (49/152, 32.2%) [Table 1].

**HPV-genotyping and IHC**

One hundred and fifty-two HNC tumor samples were screened for HR-HPV typing using PCR followed by gel electrophoresis and IHC as shown in Figure 1. Results after PCR and IHC analysis revealed that 24.3% (37/152) were HPV positive, while 75.7% (115/152) were HPV negative. Among all patients, 86.5% (32/37) of HPV positive patients and 66.9% (77/115) of HPV negative patients were tobacco users. A highly significant association of HR-HPV positivity and tobacco use was observed by univariate analysis [P < 0.01; OR: 0.31 (95% CI:0.11–0.87)]. A total of 78.3% (29/37) of HPV positive samples were with poor oral hygiene [P = 0.03, OR: 0.38 (95% CI: 0.16–0.92)]. Patients coming from the urban areas accounted for 66.4% (n = 101) while 33.5% (n = 51) were from rural areas. A total of 11 (29.7%) HPV positive cases were from an urban area and 26 (70.2%) HR-HPV positive cases from a rural area, a significant difference was observed in HPV positive and negative patients, [P < 0.0001, OR: 0.11 (95% CI: 0.05–0.27)]. Concerning the anatomic site of head and neck cancer, the highest number of HPV positive cases was observed in the oral cavity 48.6% (n = 18) followed by oropharynx 21.6% (n = 8), hypopharynx 8.1% (n = 3), larynx 13.5% (n = 5), and nasal cavity 8.1% (n = 3). For histological grading of tumors, HPV positivity with the highest number of cases was in G2 (24.3%) and G3 (48.6%) as shown in Table 1. The percentage of patients positive for HPV at stage I and II were 78.3% (n=29), while advance stage tumor III and IV were 21.6% (n=8). No significant difference was observed among primary tumor stage (pTstage) with OR: 1.52 (95% CI: 0.63–3.66, P < 0.34).

From the binary multiple logistic regression analysis, it was estimated that the coefficients for age, tobacco, and oral hygiene have P < 0.05, indicating that these coefficients are not 0 using an α-level of 0.05, which concludes that these three independent variables have an impact on HPV-infected HNC. The results of the logistic regression analysis show that all the three independent variables together were statistically significant at P < 0.01 as shown in Table 2.

**NF-κB and IκBα proteins expression in head and neck cancer cases**

IHC for protein expression analysis of NF-κB pathway, p50, p65, and IκBα was performed on tumor samples collected for the present study (152 tumor samples). It was observed that NF-κB, p50, and p65 proteins were present in the nucleus of tumor cells and showed high expression as shown in Figure 2G and 2H. While IκBα inhibitor protein showed cytoplasmic expression in all samples as shown in figure 2C, F and I. HIC staining for p50, 72 (47.3%) showed strong, 67 (44%) showed moderate staining, and 13 (8.5%) showed weak immunostaining. IHC for p65 protein in tumor samples, 74 (48.6%) having strong staining intensity, 68 (44.7%) showed moderate staining intensity, and only ten (6.5%) showed weak staining intensity. IHC for IκBα inhibitor, 70 (46%) showed weak immunostaining, showing low expression of the protein in HNC cases, 62 (40%) have moderate...
immunostaining, and only 20 (13%) were with strong staining intensity.

Out of 37 HPV-positive cases, 30 (81%) were p50 positive with strong staining intensity, six (16%) with moderate, and only one (2.7%) with weak staining intensity, having no expression of p50. Twenty-five (67%) were p65 positive with strong staining intensity, 12 (32%) with moderate, and none of sample showed weak staining intensity. Among 115 HPV negative cases, p50 and p65 expression were also observed. Forty-two (35%) cases were with stronger intensity, 61 (53%) with moderate, and 12 (10%) showed weak immunostaining for p50. While in p65 protein expression, strong staining intensity was observed in 49 (42%), moderate in 56 (48%), and weak staining intensity in 10 (8.6%) samples.

The correlation of p50, p65, and IκBa inhibitor with risk factors and clinicopathological characteristics of head and neck cancer patients was calculated by Pearson correlation coefficient. A positive significant correlation was observed between p50 vs. p65 (r = 0.249, P < 0.05) and a negative significant correlation between p50 vs. IκBa (r = −0.308, P < 0.0001) in HNC cases. A negative correlation was also observed between p65 and IκBa (r = −0.592, P < 0.001). A positive significant correlation was observed between use of tobacco and oral hygiene (r = 0.299, P < 0.05), primary tumor (pT) stage and tumor grade (r = 0.351, P < 0.0001), and tumor grade and N stage (r = 0.245, P < 0.005). A negative correlation was observed between pT and N stage (r = −0.092) [Table 3].

## Discussion

The prevalence of HPV infections was 24.3% in HNC cases. The research finding is consistent with the study conducted by Auguste et al.[15] It has been reported earlier that most of HPV positive oropharyngeal cancer patients were younger in age and mostly had no history of tobacco and alcohol,[16] and data analysis revealed that HPV positive HNC patients were higher in female and younger group (age < 40 years). Results of the current study are in support with previous studies, which may point toward its association with sexual behavior.[17-19] It is generally explained that HPV infection can be transmitted through skin contact and damages the epithelial cells in the oral mucosa, genital mucosa, and skin.[20] We have found the data stating that tobacco and betel quid users have a higher frequency of HPV infection, and other studies have
also shown an association of tobacco with HPV infection worldwide,\textsuperscript{14,21} signifying the fact that tobacco-associated carcinogens may alter genetic pathways which may lead to molecular changes, making the individual susceptible to HR-HPV infection. When the concentration of nicotine and its exposure to the cells increases, it leads to altered antigen-mediated signaling pathways such as NFkB and Akt.\textsuperscript{22,23} Results from the current study showed a significant correlation between HPV positivity and poor hygiene with an OR of 2.440 (P < 0.03, 95% CI:1.65–3.60), the same trend was observed in other studies worldwide.\textsuperscript{24-26} This suggests that poor oral hygiene plays an important role in the etiology and increases infection of HPV and the risk of cancer by chronic inflammation.\textsuperscript{24}

Consistent with other reports, the study established a strong association between HR-HPV infection and oral cavity cancer patients,\textsuperscript{27} and 48% of oral cancer patients had detectable HPV. These findings directed an anatomical preference of HR-HPV16 infection and are accordant with the literature.\textsuperscript{28,29} Previous study has similarly described that TNM status might not be associated with HPV infection.\textsuperscript{30} Overall 64% of HNC cases in the study tested

Table 2: Binary logistic regression analysis of risk factors predicting the likelihood of head and neck cancer patients.

| Variables          | B   | SE  | Wald value | df | P value | OR (95%CI) |
|--------------------|-----|-----|------------|----|---------|------------|
| Area of living     | -0.576 | 0.423 | 1.861 | 1 | 0.173 | 0.562 (0.245,1.286) |
| Age                | -2.118 | 0.823 | 6.620 | 1 | 0.010 | 0.120 (0.024,0.604) |
| Tobacco            | -2.580 | 0.825 | 9.770 | 1 | 0.002 | 0.076 (0.015,0.382) |
| Oral hygiene       | -1.748 | 0.878 | 3.965 | 1 | 0.046 | 0.174 (0.031,0.973) |
| Age_Oral hygiene_Tobacco | 1.089 | 0.316 | 11.883 | 1 | 0.001 | 2.970 (1.599, 5.515) |
| Constant           | 5.986 | 2.634 | 5.165 | 1 | 0.023 | 397.867 |

CI: Confidence interval; df: degrees of freedom; OR: Odds ratio; SE: Standard error.
were positive for HR-HPV type 16, while 35% of cases were detected with HPV type 18. Previous studies have reported a similar detection rate among HNC samples with 90% of HPV type identified as HPV16.[22] HPV positivity in HNC cases can vary geographically and method used for detection, because PCR is highly sensitive. [31] Zhang et al[32] reported 28.6% positive cases of oral carcinomas with HR-HPV16 and HPV18 in the Chinese population, and in another study of 106 HNC patients in an Indian population, Kumar et al[14] reported HPV16 and HPV18 with a detection rate of 81.8% and 18.1%, respectively, our results correspond to both of these studies.

Table 3: Correlation between expression of p50, p65, IκBα inhibitor, associated risk factors and clinical characteristics of patients with head and neck cancer.

| Items          | Use of tobacco | Oral hygiene | Anatomic site | Grade | pT stage | N stage | p50     | p65     | IκBα   |
|----------------|----------------|--------------|---------------|-------|----------|---------|---------|---------|--------|
| Use of tobacco | 1.000          |              |               |       |          |         | 0.299*  | 0.117   | 0.115  |
| Oral hygiene   |                 | 1.000        |               |       |          |         | 0.069   | 0.057   | 0.031  |
| Anatomic site  |                 |              | 1.000         |       |          |         | 0.021   | 0.005   | 0.021  |
| Grade          |                 |              |               | 1.000 | 0.351*   | 0.245*  | 0.211  | 0.114   | 0.118  |
| pT stage       |                 |              |               |       |          | 0.000   | 0.092   | 0.106   | 0.05   |
| N stage        |                 |              |               |       |          |         | 0.000   | 0.143   | 0.77   |
| p50            |                 |              |               |       |          |         | 0.000   | 0.249*  | 0.308* |
| p65            |                 |              |               |       |          |         | 0.000   | 0.249*  | 0.308* |
| IκBα inhibitor |                 |              |               |       |          |         | 1.000   | 0.249*  | 0.308* |

The expression levels of p50, p65, and IκBα inhibitor for HNC cases, based on the relative protein level. *P < 0.05. The P values were computed using one way analysis of variance and $\chi^2$-test. N-stage: Lymph node involvement; pT: Primary tumor.

Figure 2: immunohistochemistry of HNC tissues with NF-κB, p50, p65, and IκBα antibodies in various tissues. (A-C) Staining in control samples, showing no staining and protein expression of p50, p65, and IκBα, respectively. (D-F) Staining in HPV negative tissues, showing protein expression of p50, p65, and IκBα, respectively. (G-I) Staining in HPV positive tumor samples, of p50, p65, and IκBα respectively. Original magnification × 40. HNC: Head and neck cancer; HPV: Human papillomavirus; NF-κB: Nuclear factor kappa B.
It was recognized that NF-κB signaling pathway proteins, p50, and p65 showed high expression in HPV-infected samples analyzed with IHC. The results of the current study were similar to the prior studies reported.[8,13] On that account, activation of the NF-κB pathway by viral oncoproteins may be the mechanism employed in head and neck cancer growth. The integration of HPV-DNA in the host cell DNA leads to the stimulation of several pathways involved in cancer development and progression. The HPV viral oncoproteins E7 and E6 are the main sponsors to the expansion of HPV-induced cancers, probably due to the integration of the viral genes in the host cell genome. E7 and E6 protein-induced genetic instability results in the inactivation of tumor suppressor genes p53 and pRb, which is regular in the carcinogenesis of human cells. E6 and E7 oncoproteins are key regulatory proteins available inside the host cells and are documented to be linked with the transcriptional activity of NF-κB proteins.[34]

Conclusions
This study highlights the detection of HPV infection in HNC patients and expression analysis of the NF-κB signaling pathway. From the study, it can be concluded that the use of tobacco, poor hygiene, and HPV infection are important environmental factors that can act synergistically in modulating the expression of NF-κB pathway proteins. Nevertheless, further research with a large sample size is requisite to elucidate the HPV infection-induced activation of the NF-κB signaling pathway leading to HNC carcinogenesis and to identify simple, reliable, and highly specific molecular biomarkers. Additionally, the disease can be managed by developing high standards of health in the local population, conserving hygiene conditions, and organizing awareness seminars concerning the disease that can reduce the risk of developing HNC.

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Conflicts of interest
None.

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