Do Human Papilloma Viruses Play Any Role in Oral Squamous Cell Carcinoma in North Indians?

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most prevalent malignancy among males in India. While tobacco and alcohol are main aetiological factors, human papilloma virus (HPV) presence has surprisingly increased in head and neck Squamous Cell Carcinoma (HNSCC) in the past two decades but its frequency in OSCCs is still uncertain. We aim to explore the frequency of HPV and its major genotypes in North Indian patients and their association with clinicopathological and histopathological features and p16 expression pattern. Materials and Methods: The study group comprised 250 histologically proven cases of OSCC. HPV was detected by real time PCR in tumor biopsy specimens and confirmed by conventional PCR with PGMY09/PGMY11 primers. Genotyping for high-risk types 16/18 was conducted by type specific PCR. p16 expression was assessed by immunohistochemistry. Results: HPV presence was confirmed in 23/250 (9.2%) OSCC cases, of which 30.4% had HPV 16 infection, 17.4% were positive for HPV 18 and 26.1% had co-infections. HPV presence was significantly associated with male gender (p=0.02) and habit of pan masala chewing (p=0.01). HPV positive cases also had a history of tobacco consumption in 91.3% cases. p16 over expression was observed in 39.1% of HPV positive cases but this was not significantly different from negative cases (p=0.54). Conclusions: The frequency of HPV in OSCC is low in North-India and majority of cases are associated with a tobacco habit. It appears that tobacco shows a confounding effect in HPV positive cases and use of p16 protein as a reliable marker to assess the potential etiological role of HPV in OSCC in our population is not suggested.

Keywords: Oral squamous cell carcinoma - human papilloma virus - p16 - head & neck squamous cell carcinoma.
to sensitivity of the employed technique, the sample size, the state of conservation of the clinical specimens, and epidemiological factors of the studied population.

Apart from having a different epidemiology and aetiology, the HPV-positive HNSCC are different clinicopathologically and showed distinct histopathology. These are usually poorly differentiated, nonkeratinizing and have a basaloïd appearance (Gillison et al., 2000). Furthermore, patients with HPV-positive HNSCC in general tend to be younger at time of diagnosis (Smith et al., 2004) and generally have a better survival and favorable prognosis compared to the HPV-negative patients (Haflkamp et al., 2008; Lassen et al., 2009).

HPV positive oral cancer also represents a distinct molecular phenotype with a unique mechanism of tumorigenesis, independent of the mutagenic effect of tobacco and alcohol. A characteristic over expression of p16, tumor suppressor protein, has been observed in HPV positive HNSCC. Two viral onco-proteins E6 and E7 cause inactivation of p53 and retinoblastoma (Rb) respectively, this event leads further activation of CDKN2A gene and increased expression of p16 has occurred. Thus, p16 over-expression is an indicator of an aberrant expression of viral oncogenes and considered as a surrogate biomarker for HPV presence (Klussmann et al., 2009; Ahmed et al., 2012).

Therefore, the present study aims to explore the frequency of HPV association in OSCC, distribution of its major high risk types and correlation with other risk factors in North Indian population.

Materials and Methods

Study Population: A total of 250 clinically and histologically proven cases of OSCC were included prospectively in the study between Oct 2013 to Jan 2015. Tissue biopsies were collected from department of Surgical Oncology, King George’s Medical University (KGMU) Lucknow after obtaining approval from the Institutional Ethics Committee and written informed consent from the patients.

Sample Collection: Tissue biopsies were collected in 10% buffered formalin at RT for histopathological diagnosis and in 1X phosphate buffer saline (PBS, pH 7.4) and stored at -80°C for molecular analysis. The demographic and clinical details of the patients were recorded on the standard questionnaire.

DNA Extraction: The DNA from biopsy tissues was isolated by commercially available Genomic DNA Mini kit (Invitrogen, USA) as per manufacturer’s instructions. The extracted DNA was eluted in 80μl of the elution buffer (provided in the kit) and kept at -80°C until further use. All the DNA samples were qualitatively confirmed on a 1% agarose gel electrophoresis and the concentration of each DNA sample was quantitated on the standard questionnaire.

HPV detection: HPV detection was performed by using the following methodology

Real Time PCR based methodology for HPV detection: Presence of HPV in OSCC samples were detected by Real time PCR (Biorad CFX 96™) using 13 HIGH RISK HPV REAL TIME PCR KIT (Hybridio Limited, China) as per manufacturer’s instructions. Briefly, each PCR reaction contained 17.5μl of PCR master mix, 0.5μl of Taq DNA polymerase and 2μl of DNA. The PCR amplification protocol was as follows- initial denaturation at 95°C for 10min and 45 cycles of - denaturation for 3min at 95°C, annealing for 60sec at 60°C, and extension at 72°C for 20sec. The amplification was followed by a 5sec final extension step at 38°C. Presence or absence of HPV DNA was confirmed by the Ct values obtained for the sample.

Conventional PCR based methodology: Further validation of HPV was done using PGMY09/11 primers as reported by P.E.Gravitt et al, 2000, designed to amplify a 450bp HPV L1 gene fragment. This region is used because it is highly conserved between different HPV types but has sufficient variation for the identification of each one. Cervical cancer cases which were confirmed previously for HPV presence were used as positive control. PCR amplification was carried out in a volume of 20μl containing 50ng/μl of genomic DNA, 4mM of MgCl2 (Invitrogen, USA), 200μM of each dNTPs. Concentration of PGMY09/11 primers was reduced from 10pmol as previously used to 5pmol, also the final concentration of AmpliTaq DNA polymerase (Invitrogen, USA) was reduced from 7.5U/μl to 5U/μl. Amplification was performed in thermal cycler (S100™, Biorad, USA) under the following conditions: initial denaturation at 95°C for 9min, followed by 35 cycles of - 95°C for 1min (denaturation), primer annealing at 55°C for 1min, extension at 72°C for 1min and a final extension at 72°C for 5min. Positive and negative controls were run simultaneously.

HPV genotyping: Samples that were positive for HPV presence were proceeded further for high risk HPV 16 & 18 genotyping. HPV genotyping was done with the previously described type specific primers for HPV 16 and 18 (Sharma et al., 2005). PCR amplification was carried out in a 20μl reaction volume containing 50ng/μl of genomic DNA, 10pmol each of forward and reverse primer, 2mM MgCl2 (Invitrogen, USA), 200μM of each dNTPs, 0.5U/μl of AmpliTaq DNA polymerase (Invitrogen, USA) along with 10X (NH4)2SO4 buffer (Invitrogen, USA). Amplification was performed in thermal cycler (S100™, Biorad, USA) under the following PCR conditions: An initial denaturation at 94°C for 10min, followed by 35 cycles of 94°C for 1min (denaturation), primer annealing at 55°C for 1min, extension at 72°C for 1min and a final extension at 72°C for 5min. Positive and negative controls were run simultaneously.

Expression of p16 by Immunohistochemistry (IHC): Paraffin-embedded tissues were sectioned, 4μm, using a microtome (Leica, Germany), and transferred to tissue bond-coated slides (Biocare, USA). After overnight incubation in a 60°C dry oven, paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded ethanol series 100%, 70% and 50%. Endogenous peroxidase activity was blocked with 3%
hydrogen peroxide in methanol for 30min. Antigen retrieval was done by placing the slides in Tris-EDTA buffer (pH 9.0) in Pascal. These sections after cooling to room temperature (RT) were incubated with p16 primary antibody (Biogenex) at RT for one hour, followed by treatment with polymer based secondary antibody kit with DAB (DAKO, Denmark). Positive reactions were visualized using diaminobenzidine, DAB (1:50). Sections were finally counter-stained with 0.1% hematoxylin. The positive cells expressing the p16 positivity were assessed for cytoplasmic as well as nuclear staining at higher magnification. A tumor was recorded positive if more than 10% of tumor cells showed immunoreactivity (Names et al., 2006).

Data analysis: The diagnostic criteria used for the detection of HPV considered only those OSCC cases that were positive by at least two methods described above (Real time PCR, PCR, 16 PCR & 18 PCR). The real time PCR data was analyzed using Bio-Rad CFX Manager software v3.0. The results are presented in mean±SD and percentages. The Chi-square test was used to compare the dichotomous/categorical variables and unpaired t-test was used to compare the continuous variables. The univariate binary logistic regression was carried out to find the strength of associations. The odds ratio with its confidence interval was calculated. The p-value<0.05 was considered significant. Kaplan-Meier survival curve was made and survival time was compared by using Log rank test. All the analysis was carried out by using SPSS 16.0 version (Chicago, Inc., USA).

Results

The study encompasses 250 histologically proven cases of OSCC. Out of these HPV presence was confirmed in 23 (9.2%) cases (Figure 1A&B) according to diagnostic criteria predefined in data analysis. Table 1 summarizes the clinical, histopathological, demographic and behavioral characteristics of patients in HPV positive and negative cases.

The mean age of HPV positive patients was 47.17 while HPV negative cases had mean age 47.69, but this difference was statistically insignificant. HPV presence associated significantly with male gender [p=0.02*, OR (95%CI) =0.34 (0.13-0.83)]. Buccal Mucosa was the most frequent site (52.2%) in patients. Most of HPV positive cases were well differentiated SCC (60.9%), eight cases were moderately differentiated or keratinized while only 4.3% cases showed a basaloid morphology (Figure 2A). HPV positivity did not find to be associated with age, marital status, domicile, sub-site, stage, tumor grade, nodal status and outcome.

In HPV positive cases 91.3% had taken tobacco in any form while only 8.7% patients had no history of any risk factor. History of multiple risk factors was present in 13.00% cases. HPV positivity significantly associated with habit of pan masala chewing [p=0.01*, OR (95%CI) = 0.32 (0.13-0.79)].

HPV subtypes in OSCC: Out of 23 HPV positive cases 30.4% cases had HPV 16 infection while 17.4% were positive for HPV 18. Six cases (26.1%) co-expressed...
Table 1. Association of HPV with Clinicopathological, Histopathological, Demographic and Behavioral Characteristics in OSCC Patients

| Characteristics                  | HPV Positive (n=23) | HPV Negative (n=227) | OR     | 95% CI   | p-value1 |
|----------------------------------|---------------------|----------------------|--------|----------|----------|
| Age in years                     |                     |                      |        |          |          |
| <30                              | 2                   | 7                    | 3.1    | 2.57     | 0.39-16.86 | 0.32     |
| 30-40                            | 5                   | 73                   | 32.2   | 0.616    | 0.15-2.43  | 0.49     |
| 41-50                            | 7                   | 69                   | 30.4   | 0.91     | 0.25-3.32  | 0.89     |
| 51-60                            | 5                   | 42                   | 18.5   | 1.07     | 0.26-4.29  | 0.92     |
| >60                              | 4                   | 36                   | 15.9   | 1        | Ref.      |          |
| Gender                           |                     |                      |        |          |          |
| Male                             | 14                  | 186                  | 81.9   | 0.34     | 0.13-0.83  | 0.02*    |
| Female                           | 9                   | 41                   | 18.1   | 1        | Ref.      |          |
| Marital status                   |                     |                      |        |          |          |
| Married                          | 23                  | 223                  | 98.2   | NA       | NA       |          |
| Unmarried                        | 0                   | 4                    | 1.8    | 1        | Ref.      |          |
| Residence                        |                     |                      |        |          |          |
| Rural                            | 16                  | 159                  | 70     | 0.97     | 0.38-2.48  | 0.96     |
| Urban                            | 7                   | 68                   | 30     | 1        | Ref.      |          |
| Subsite                          |                     |                      |        |          |          |
| BM                               | 12                  | 115                  | 50.7   | 0.99     | 0.35-2.77  | 0.08     |
| FM                               | 0                   | 4                    | 1.8    | -        | -        |          |
| LA                               | 4                   | 40                   | 17.6   | 0.95     | 0.25-3.58  | 0.94     |
| Palate/Upper alveolus            | 0                   | 6                    | 2.6    | -        | -        |          |
| RMT                              | 1                   | 5                    | 2.2    | 1        | 0.18-19.06 | 0.58     |
| Tongue                           | 6                   | 57                   | 25.1   | 1        | Ref.      |          |
| Site                             |                     |                      |        |          |          |
| Left                             | 16                  | 116                  | 51.1   | 2.18     | 0.86-5.51  | 0.09     |
| Right                            | 7                   | 111                  | 48.9   | 1        | Ref.      |          |
| Stage                            |                     |                      |        |          |          |
| I                                | 1                   | 21                   | 9.3    | 0.47     | 0.05-3.88  | 0.48     |
| II                               | 4                   | 45                   | 19.8   | 0.88     | 0.26-2.93  | 0.84     |
| III                              | 7                   | 51                   | 22.5   | 1.37     | 0.50-3.74  | 0.53     |
| IV                               | 11                  | 110                  | 48.5   | 1        | Ref.      |          |
| Smoking                          |                     |                      |        |          |          |
| Yes                              | 12                  | 110                  | 48.5   | 1.16     | 0.49-2.73  | 0.73     |
| No                               | 11                  | 117                  | 51.5   | 1        | Ref.      |          |
| Pan massala                      |                     |                      |        |          |          |
| Yes                              | 8                   | 141                  | 62.1   | 0.32     | 0.13-0.79  | 0.01*    |
| No                               | 15                  | 86                   | 37.9   | 1        | Ref.      |          |
| Tobacco                          |                     |                      |        |          |          |
| Yes                              | 19                  | 171                  | 75.3   | 1.55     | 0.50-4.76  | 0.43     |
| No                               | 4                   | 56                   | 24.7   | 1        | Ref.      |          |
| Alcohol                          |                     |                      |        |          |          |
| Yes                              | 7                   | 66                   | 29.1   | 1.06     | 0.42-2.71  | 0.89     |
| No                               | 16                  | 161                  | 70.9   | 1        | Ref.      |          |
| Multiple habit                   |                     |                      |        |          |          |
DNA of both HPV 16 and 18 subtypes and 26.1% cases were negative for both 16 and 18 or supposed to had HPV subtypes other than 16/18 (Table 2, Figure 1C & D).

**Correlation of HPV 16 and 18 with clinicopathological variables**

Table 2. HPV Subtypes in OSCC Patients

| HPV Result       | No. of Patient | %   |
|------------------|----------------|-----|
| HPV positive cases | 23             | 9.2 |
| HPV negative cases | 227            | 90.8|
| HPV Types        |                |     |
| HPV16 only       | 7              | 30.4|
| HPV18 only       | 4              | 17.4|
| HPV16 and 18 co-infection | 6      | 26.1|
| Negative for HPV 16/18 or suspected subtypes other than 16/18 | 6 | 26.1 |

Abbreviation: HPV- Human Papilloma Virus

Association of HPV type 16 & 18 with all clinical, demographic & behavioral profile of patients were evaluated but it was found to be shown no association with any of these (data not shown).

**Correlation of HPV with Survival of Patients**
Factors involved in our study population. and betel nut chewing habits were the major etiological which was present, but the co-infection of 16/18. Tobacco (17.47%). In one fourth of the cases it is not single subtype HPV was associated in 9.2% of OSCC cases with HPV (70.6%) was reported (Kulkarni et al., 2011). Our exception in southern India where higher prevalence of 2005; Alok et al., 2006; Chaudhary et al., 2013) with an HPV associated OSCC between 15-37.9% (Priya et al., 2008). In one study 5.9% positivity was reported by Mark W. Lingen., 2013, worldwide level varies from 0-30%.In North America Indian population.

The overall prevalence of HPV associated OSCC at worldwide level varies from 0-30%.In North America 5.9% positivity was reported by Mark W. Lingen., 2013, in Canada 4% by Jarry Machado et al., 2010. 27.5% in China by Li-Li Gan et al., 2014 and only 3% in Bangladesh by Mahmuda Akhter et al., 2013.

Most Indian studies have reported prevalence of HPV associated OSCC between 15-37.9% (Priya et al., 2005; Alok et al., 2006; Chaudhary et al., 2013) with an exception in southern India where higher prevalence of HPV (70.6%) was reported (Kulkarni et al., 2011). Our study is in concordance with previous studies and shows that HPV was associated in 9.2% of OSCC cases with HPV 16 showing higher (30.4%) prevalence than HPV 18 (17.47%). In one fourth of the cases it is not single subtype which was present, but the co-infection of 16/18. Tobacco and betel nut chewing habits were the major etiological factors involved in our study population.

In contrast to oropharyngeal cancer, oral cancer and other HNSCC occasionally harbor HPV (Dayyani et al., 2010; Machado et al., 2010). Its presence is strongly associated with oropharynx, most notably in tonsils and base of tongue (El-Mofy, 2003; Gillison et al., 2004). This might be due to the fact that these tumors may constitute an etiologically different subgroup within head and neck tumors. HPV might play a role in progression of premalignant lesions to advanced cancer form (Miller et al., 2001) but evidences in this direction are not sufficient.

Low prevalence of HPV in oral cavity carcinoma was reported by Jerry Machado et al., 2010. Luca Scapoli et al., 2008 also found a very low prevalence of HPV in oral cavity (2%) and demonstrated no significant correlation of HPV in OSCC.

Association of HPV with other factors is also controversial worldwide, for instance Luciano Marques-Silva et al., 2012 reported no association of HPV positivity with age. To the contrary Abdul Samad Gichki et al., 2012 reported higher HPV incidence between 20-59 years of age. Jerry Machado et al., 2010 found no significant association between HPV presence and smoking, alcohol status, tumor differentiation, stage and survival. Our study is in concordance with previous reports of no significant association of HPV positivity with age, marital status, demographic profile, tumor stage, grade and site of tumor. HPV in our case series was significantly associated with male gender (p=0.02) this is might be due to the fact that HNSCC is more common in males compared to females because of the presence of traditional risk factors here.

Earlier studies report that majority of HPV related carcinomas of the oropharynx are nonkeratinizing squamous cell carcinoma (NKSCC) with a characteristic basaloid cellular morphology and these tumors were found to be more responsive to treatment with a favorable patient outcome and good prognosis. But HPV positive OSCC, unlike oropharynx, do not exhibit distinct morphology and have poor prognosis (El-Mofy et al., 2014). Our study is in agreement with studies where HPV positive cases did not demonstrate basaloid morphology (p=0.10) and we also found no significant differences in survival outcome (p=0.62) between HPV positive and negative cases.

It is assumed that carcinogenic potential of HPV increases with viral integration to host genome (Spence et al., 2005). Smoking induces DNA damage which may favor the integration of HPV to human genome at these sites (Luo et al., 2013) thus enhancing the oncogenic potential of virus. In our study majority of HPV positive cases (91.3%) had one or multiple of the tobacco related habits like tobacco and pan masala chewing/smoking. We have observed significant relationship of HPV with age. To the contrary Abdul Samad Gichki et al., 2012 reported higher HPV incidence between 20-59 years of age. Jerry Machado et al., 2010 found no significant association between HPV presence and smoking, alcohol status, tumor differentiation, stage and survival. Our study is in concordance with previous reports of no significant association of HPV positivity with age, marital status, demographic profile, tumor stage, grade and site of tumor. HPV in our case series was significantly associated with male gender (p=0.02) this is might be due to the fact that HNSCC is more common in males compared to females because of the presence of traditional risk factors here.

HPV positive patients was better (16.5) compared to HPV negative patients (12.9) but the difference was not significant (p=0.54). (Figure 3A, B & C). The survival status of patients in the study is shown in Table 3.

Correlation of HPV with p16 protein expression

p16 expression was evaluated in all HPV positive cases and equal number of HPV negative cases (n=23) that were selected randomly. Diffuse nuclear staining with some cytoplasmic positivity for p16 protein was seen in 39.13% HPV positive patients and 30.43% HPV negative patients (Table 1, Figure 2B). p16 over expression was not associated with the presence of HPV ( p=0.54).

Table 3. Association of Survival of Patients with HPV Presence and Its Subtypes.

| HPV Status | Median Survival in Month |
|------------|--------------------------|
| HPVα       | 16.5                     |
| Negative   | 12.9                     |
| Total cases| 15.6                     |
| HPV16b     | 14                       |
| Positive   | 12                       |
| HPV18c     | 13                       |
| Positive   | 14                       |
| Negative   |                         |

According to Log rank test median survival of HPV positive patients was better (16.5) compared to HPV negative patients (12.9) but the difference was not significant (p=0.54). (Figure 3A, B & C). The survival status of patients in the study is shown in Table 3.

Discussion

HPV has been identified as a prime suspect in the etiology of HNSCC due to their morphological similarities with genital epithelia and their ability to transform and immortalize oral keratinocytes (Termine et al., 2008). Speculation on the role of HPV in the etio-pathogenesis of oral carcinoma has been voiced worldwide. We have explored the frequency and major genotypes of HPV in 250 histologically confirmed cases of OSCC in North-Indian population.

The overall prevalence of HPV associated OSCC at worldwide level varies from 0-30%.In North America 5.9% positivity was reported by Mark W. Lingen., 2013, in Canada 4% by Jarry Machado et al., 2010, 27.5% in China by Li-Li Gan et al., 2014 and only 3% in Bangladesh by Mahmuda Akhter et al., 2013.

The limitation of the present study was that we were unable to evaluate the oncogenic expressions of HPV E6/E7 in our case series due to scarce sample amount. Furthermore, due to lack of relevant information regarding behavioral history of patients, the route of transmission of virus is not recognized here. The FDA approved
hybrid capture method was not implemented in our cases. However we have utilized both in-house conventional PCR assay and a kit based real time PCR analysis to confirm HPV presence. Further sub typing for 16 and 18 also show independent positive results. Hence we feel confident about the presence of HPV in our cases. Despite of some limitations our data is contributing needful information regarding HPV presence in OSCC of North Indian population that will be useful for the future treatment implications.

HPV positive cancers usually show over-expression of p16, while the loss of the p16 expression by deletion, hypermethylation or mutation is common in tobacco related cancers. Therefore, p16 up regulation is an indication of expression of viral oncogenes and we can expect the presence of HPV (Tran et al., 2007; Vidal et al., 2008; Klussmann et al., 2009; Ahmed et al., 2012), but its relevance for the site of HNSCC other than oropharynx is ambiguous. In our cases presence of HPV was not significantly associated with p16 expression and all p16 positive cases had history of tobacco consumption. It is possible that tobacco related oncogenic pathway co-existed with HPV related events in our cases. Hence, p16 expression was not found to be a reliable marker for HPV presence in our population. Our findings are in concordance with Pradit Rushatamukayanan et al., 2014 who also could not relate p16 expression for HPV presence. Our findings are contributing needful information regarding HPV presence in OSCC.

In conclusion, our findings illustrate that 9.2% OSCC cases harbor HPV in North Indian population which is slightly lower than that observed in previous Indian studies and we report tobacco as a major risk factor in both HPV negative as well as positive cases. Therefore the independent role of HPV in the causation of oral cancer is difficult to evaluate in our case series due to the strong confounding influence of tobacco. We also find that p16 expression is not a reliable marker in the oral cavity to assess the potential etiologic role of HPV. Further studies on larger and well defined population are needed to elucidate the role of HPV induced oral oncogenesis and co-carcinogenesis pathways need to be explored.

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