Prevalence of HPV is low in oral mucosa

Rashmi Jaiswal[1], TP Chaturvedi[2], Mohan Kumar[3], SK Tripathi[4], Manoj Pandey[1]

Department of Surgical Oncology[1], Faculty of Dental Sciences[2] , Department of Pathology[3], and Department of Forensic Medicine[4] Institute of Medical Sciences, Banaras Hindu University,

Varanasi 221 005, India

Abstract

Aim: The objective of the study was to examine the prevalence of HPV infection and its correlations with p53 and pRb alterations with the presence and absence of HPV DNA in oral mucosa.

Patients and Methods: A total of 178 patients were taken, of which 71 had oral squamous cell carcinoma, 40 had premalignant lesions and 67 had normal healthy mucosa. HPV DNA was detected by PCR. Well established consensus primer set MY 09/11 was used for PCR and p53 and pRb accumulation was studied by using IHC.

Results: The percent positivity of HPV DNA in oral mucosal samples was found to be low (27%). p53 expression was seen in 35%, while pRb expression was seen in 19% oral mucosa. Cancer cases demonstrated strong expression (3+) of p53 and pRb.

Discussion and Conclusion: HPV positivity in all the three groups including cancer, premalignant and normal control was low (26.96%), compared to other parts of India, where the HPV prevalence was found higher (nearly 40-70%). Our study shows higher use of tobacco and alcohol irrespective of HR-HPV presence. Low p53 and pRb expression in HPV positive cases suggests that HPV DNA integrated with host genome, thus blocking the expression of these oncoproteins.

Abbreviations

HPV= Human Papillomavirus, OSCC= Oral Squamous Cell Carcinoma, HNSCC= Head and Neck Squamous Cell Carcinoma, HR-HPV= High risk HPV, PCR= Polymerase Chain Reaction, IHC= Immunohistochemistry

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common solid tumor. Nearly six million new cases are diagnosed and about three million deaths occur every year due to oral squamous cell carcinoma (OSCC), of these 62% cases are from developing countries. Oral cancer is a heterogeneous group of cancers which arises in different parts of the oral cavity, with different predisposing factors, prevalence, and treatment outcomes (Termine *et al.,* 2008; Gillespie *et al.,* 2009; Ferlay *et al.,* 2004). Oral cancer is one of the commonest cancers in India (Koppikar *et al.,* 2005; Kekatpure and Kuriakose, 2010)**.** A study conducted by the National Institute of Public Health in 2011 reported that India accounts for 86% of the world's oral cancer cases. Ninety percent of these cases are due to the use of smokeless tobacco (pan masala, zarda etc.), unlike in the Western countries where smoking is the main reason. Besides tobacco and alcohol, it has also been hypothesized that

there may be a viral involvement in some OSCC. This is based on the evidences for the presence of viral nucleic acids in OSCC tissue (Scully, 2005; Ribeiro *et al.,* 2011; Elango *et al.,* 2011; Balaram *et al.,* 1995). The studies emerging after the development of molecular biology techniques have shown that HPV is one of the risk factor for the oral cancer, besides tobacco and alcohol (Castro and Filho, 2006). However, the prevalence of human papillomavirus (HPV) in the oral cavity and oropharynx has not yet been as well studied as its infection of the genitalia. Human papillomavirus (HPV) are the member of papovaviridae family contains a small (7.2-8.0 kb) circular double standard DNA. It is maintained in host cells as a supercoiled closed molecule or episome and exhibits a pronounced tropism for epithelial cells (Mork *et al.,* 2001; Trottier and Franco, 2006).

Presence of HPV DNA shows pathological changes of the mucosa, particularly dysplasia. The high risk HPVs have been found to affect the epithelial cell by expressing oncoproteins E6 and E7 that alter tumour suppressor pathways after integration into the host genome by degradation of p53 (Nagpal *et al.,* 2002; Ribeiro *et al.,* 2011).
The present study also supports an important role for HPV in a subgroup of head and neck squamous cell carcinoma (HNSCC). PCR method shows the accurate results in HPV detection thus PCR was used in the present study and consensus primer from the L1 region of HPV DNA, MY09/11 was used for detecting prevalence of HPV DNA in oral mucosa.

Material and methods

A total of 178 patients were recruited , of these 71 had OSCC, 40 had oral premalignant lesion and 67 were normal oral mucosa. The cancer cases were recruited from the patients attending the Surgical Oncology outpatient while premalignant and normal controls were recruited from Faculty of Dental Sciences, Sir Sunder Lal Hospital, BHU, Varanasi. The cases were taken from the patients undergoing surgery and biopsies as a part of their treatment. The normal mucosa was obtained from patients undergoing molar extraction surgeries for impacted molars. The study was approved by Institute ethics committee and informed consent was obtained from all the patients and normal controls. After collection of the samples these were snapped frozen in liquid nitrogen and preserved at -80^{o}C until DNA extraction and a portion of the sample was stored in buffered formalin for histology and immunohistochemistry.

DNA extraction

Genomic DNA was isolated from the oral tissues by the modified phenol-chloroform method. The steps followed in DNA isolation were as follows: the homogenized tissue was centrifuged with 1ml of Sucrose-EDTA-Tris buffer at 10,000 rpm for 10 minutes. The supernatant was discarded and 800µl of lysis buffer along with 40µl of SDS was added, followed by proteinase K treatment (10µl). The tubes were allowed to incubate overnight at 37^{o}C. To this equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and incubated at room temperature for 20 minutes. The tubes were centrifuged at 20,000 rpm for 15 minutes and the aqueous phase was collected. 40µl of 3M sodium acetate and double the volume of chilled absolute alcohol was added and allowed to incubate for 1 hour at -20^{o}C. Finally the pellets were washed with 70% alcohol and centrifuged at 5,000 rpm for 5 minutes.

The precipitated DNA pellet was air-dried at room temperature and redissolved in 40µl of Tris-EDTA buffer. The purity and quantity of DNA was assessed spectrophotometrically at 260/280nm. The integrity and quantity of the DNA was assessed semi-quantitatively by using electrophoresis technique.

PCR amplification and HPV detection

The PCR of the extracted DNA samples was carried out using the well established consensus primer set MY09/11 (approximately 450 bp) from L1 region of HPV DNA (table 1). The degenerate primer set used in the present study was taken from the literature (Remmerbech *et al.,* 2004). The L1 consensus primers detect the presence of episomal form of virus predominantly present in the cell and can detect 25 genotypes of HPV in the sample, thus, were used to check the prevalence of HPV DNA. The primers synthesis was done by Imperial Life Sciences (ILS), Gurgaon, Haryana, India. Each PCR experiment was performed with a negative control (sterile water in place of DNA) and a positive control for HPV DNA (known HPV positive sample).

The PCR was carried out in a total volume of 25µl, containing 10X PCR buffer, 0.5mg/ml BSA, 10mMol dNTP each, 100pmol of each primer pool, 2.5U Taq polymerase and 100µg of template DNA. Amplification was performed for 40 cycles with the following parameters: Initial denaturation at 95^0C for 5 minutes; each cycle at 94^0C for 45 seconds, 47.5^0C for 1 minute and 72^0 C for 45 seconds and a final extension at 72^0C for 6 minutes.

Verification of DNA amplification

To verify the amplification electrophoresis was performed with 3% agarose in 1X TAE buffer with the final concentration of 0.5mg/ml EtBr. 5µl of PCR product with gel loading dye (Bromophenol blue and xylene-cyanol) was used for this quantitative analysis. DNA band was visualized under short wavelength ultraviolet transilluminator and photographed. The product length of 450bp from MY09/11 was observed under the UV light.100 bp ladder DNA was used as a marker.

Immunohistochemistry (IHC)

The detection of p53 protein and pRb protein was carried out using mouse monoclonal antibody from Santa Cruz for p53 (Bp-53-12: SC-263) and pRb (Rb IF8:SC-102), a 1:200 dilution was used. Formalin fixed paraffin embedded tissue were used for IHC. 3µm sections were cut and fixed on the slide coated with poly L-lysine. The subsequent steps of the IHC procedures are as follows: deparaffinization of tissue section was performed in xylene and rehydration was done by passing through graded alcohol. The sections were then dipped into citrate buffer (pH=6.0) followed by antigen retrieval in microwave for 20 minutes. This was followed by washing of slides with water and incubating section in 3% hydrogen peroxide for 20 minutes in order to inactivate endogenous peroxidase activity. Then the section were rinsed with Tris buffered saline (TBS) and incubated overnight with primary antibody p53 and pRb at 4^0C. After washing in TBS the sections were incubated with HRP labelled secondary antibody from Biogenex (Super sensitive polymer HRP detection kit-HRP/DAB) for 30 minutes. After washing the specimen, colour development was done by incubating

sections in diaminobenzidine (DAB) solution for 5 minutes. Finally the sections were washed with water and counterstained with hematoxyline and mounted. Negative controls (specimen slides without primary antibody) and positives controls (known positive breast cancer case for p53 and tonsillar carcinoma for pRb) were run in each batch of immunostaining procedure.

Immunoscoring

The Immunoscoring for antibody p53 and pRb was done according to the intensity of staining pattern. Dark brown nuclear staining was considered as positive. Both the antibody p53 and pRb are nuclear antigen thus only nuclear staining was considered. Intensity of staining was assigned as 0 or negative staining when 0-10% cells were stained, 1+, when more than 10 but less than 30% of nuclei were reactive, 2+, when 30-60% of nuclei were stained and 3+ if 60-100% of nuclei were positive (Karimi *et al.,* 2002; Petrescu *et al.,* 2006).

Statistical analysis

Statistical analysis was done with SPSS 16 software. Statistical analysis for comparing the prevalence of HPV, p53 and pRb expression with different clinical condition was done by using Pearson's Chi square test and value of $p<0.05$ was considered significant.

Results

The mean age with carcinoma was 47.8 ±13.2 years, while for the patients with premalignant lesions was 49.45 ± 14.2 years and the mean age of the normal subject was 43.2 ± 15.5 years. The difference in the age between 3 groups was statistically significant ($F=19.5$, $p = 0.000$).

HPV status

A total of 18/71 (25.53%) oral cancer patients, 19/40 (47.5%) premalignant and 11/67 (16.41%) normal controls showed HPV positivity. The total positivity for HPV was (48/178) 26.96% i.e. a low HPV prevalence was seen in the present study. Of the 48 HPV positive cases 34 were male and 14 were females.

A significant association was seen between HPV and tumor stage in cancer group and p53 expression in normal control group showed significant association with HPV (table 2). No significant relation of HPV prevalence was observed with gender, habits, site, and pRb expression in any of the group including cancer, premalignant and normal control.

Expression of p53

Out of 178 patients, 63 cases (35.39%) were p53 positive. Significantly higher p53 positivity was observed in cancer patients as compared to premalignant and normal controls. The strong staining was seen in cancer patients in comparison to premalignant and normal control group (table 3).

p53 expression did not correlate significantly with gender, habits, tumor stage, nodes and site in any of the group, but was significantly correlated with the expression of pRb in all the three groups (table 4).

Expression of pRb

Of the total 178 patients, pRb positivity was seen in 33 (18.54%) patients. The pRb expression too was higher in cancer patients compared to premalignant and normal controls. Strong intensity was seen only in cancer patients (table 5).
Similar to results of HPV status and expression of p53, pRb expression also did not showed any significant correlation with gender, habits, tumor stage, nodes and sites in any of the group (table 6).

Discussion

The tobacco consumption and betel quid chewing were known as the major cause for oral carcinomas, however, recently the role of human papilloma virus (HPV) infection is being increasingly suggested by different researchers worldwide (Gillison *et al.,* 2000; Syrjanen, 2005; Campisi *et al.,* 2007; Elango *et al.,* 2011; Gillison *et al.,* 2012). The HPV is a large family of DNA virus and its typing is based on DNA homology besides serotype (zur Hausen, 2009; zur Hausen, 2002). HPV may present in the host cell throughout life without having any signs or symptoms or presenting any clinical or histological evidence of disease, but once it gets integrated into host genome it may cause cancer. HPVs show morphological association with the squamous cell carcinoma and had the ability to immortalize the oral keratinocytes and transforming epithelial cells (Syrjanen, 2005; Shiboski *et al.,* 2005; Soares *et al.,* 2008). However, the individual role of HPVs in oral cancer is difficult to evaluate due to strong confounding influences of tobacco. Evidences suggest that OSCC may develop in the absence of exposure to these agents, or any obvious predisposing genetic defect. The only detection of HPV DNA may not indicate its involvement in head and neck cancer. HPV alone is not a reliable predictor of malignant progression in oral squamous cell carcinoma. Presence of HR-HPV DNA in majority of HNSCC or OSCC may be due to unhealthy life-style or a consequence of genetic instability contributed by the other environmental or host factors.

The incidence of HPV in head and neck cancers has been found lower (Ha *et al.,* 2004; Ritchie *et al.,* 2003; Ribeiro *et al.,* 2011; Elango *et al.,* 2011), whereas, some studies showed higher prevalence of HPV in oral carcinogenesis (Balaram *et al.,* 1995; Bauda *et al.,* 2000; Soares *et al.,* 2008; Khanna *et al.,* 2009). This substantial heterogeneity in detection rates of HPV DNA may be due to several factors including (i) variation in geographical location and the type of population studied (Katiyar *et al.,* 2005) (ii) sub-sites of head and neck cancer (iii) difference in sample size (iv) sensitivity of the HPV testing method (iv) type of primers used (Kreimer *et al.,* 2005).

A low prevalence of HPV was seen in the present study as compared to the studies conducted in other parts of India. The reason for this may be the high prevalence of tobacco chewing habits in this area. Therefore the presence of HPV DNA in buccal mucosa is very high making it the commonest site of cancer in this region.

In the present study, the HPV prevalence was higher in premalignant cases (48%) while, 26% in cancer cases and 16% in normal controls. The rate of HPV detection in different cases as OSCC, pre-malignant and control group varies widely (Ragin *et al.,* 2007, Hansson *et al.,* 2005). Shiboski *et al.*, (2005), showed 20% prevalence rate of HR-HPV in cancer group

(n=38). Zhang *et al.,* (2004), had reported 74% HPV DNA in OSCC patients while 55% HPV DNA in normal controls. Khanna *et al.,* (2009), studied on the population from Varanasi region and showed 64.5% HPV in OSCC, 40% in premalignant cases and 20% in normal oral mucosa by using southern blot technique.

HPV detection rate strongly depends on the sensitivity of the method used. PCR has been considered as high sensitivity assay over the southern blot or FISH. PCR can detect 14-35% of HPV while 25% can be detected by southern blot and 18% by fluorescent i*n-situ* hybridization (FISH) (Remmerbech *et al.,* 2004; Coutlee *et al.,* 2002*).* Miller *et al.,* (1996), reported high frequency of HPV DNA by using PCR (37%) than by using southern blot technique (27%) and even low rate by using ISH (25%) ($p \leq 0.005$). PCR is a rapid, highly sensitive approach with great effectiveness and could be used as a valuable tool for detecting low copy number of HPV DNA in oral samples. Since oral samples have much lower viral load than the cervical samples, thus PCR (real detection of 10 copies of viral DNA in 1 million cells), is most widely used for HPV DNA detection with higher accuracy. However, reports showed that PCR still produces varied results with prevalence rates of HPV prevalence from 0% to 100%. The reason may be the inherent differences in the population studied or the choice of the primer used in the PCR which may affect the range of HPV detection or depends on the viral DNA sequence targeted. The most commonly used primer in the HPV testing in OSCC tissue involves the use of consensus primer sets within highly conserved regions in the L1 genes. The L1 regions primers have the ability to detect a wide spectrum of HPV types, but during virus integration into the host genome. MY09/MY11 primer from L1 region can detect wide range genotypes of HPV in the sample (nearly 25 sub-types). The amplified region generated by these primers is of approximately of 450bp. The only disadvantage of these consensus primers is that these cannot detect HPV type 35. To overcome this problem the use of nested PCR has been recommended (Remmerbech *et al.,* 2004). Ribeiro *et al.,* (2011), reported 7.8% HPV-positive cases while, Herrero *et al.,* 2003, found 9.6% of HPV DNA by using L1 consensus primers in their study. Nagpal *et al.,* (2002), reported 33.6%, HPV positivity by using L1 consensus primer. Gillison *et al.,* (2000), detected 22% HPV DNA by using L1 consensus primers.

Currently, there is no unanimity on the most appropriate method for the detection of HPV in HNSCC. Therefore it is still required to develop more sensitive primers for the detection of a wide range of HPV. p53 functions as tumor suppressor protein by arresting the cell-cycle and by triggering apoptosis. p53 overexpression has been investigated independently in different malignancies for their potential value as a prognostic marker. p53 produces a nuclear phospholipids which control cells to enter into S phase in cell-cycle and in normal conditions also stops growth and apoptosis in injured cells (Karimi et *al.,* 2002). p53 comes into action when DNA is damaged and arrest the cell-cycle so as to allow the repair. In case if repair fails, p53 directs the cell to apoptosis. Hence p53 mutations/inactivation lead to uncontrolled proliferation of cells (Kinra *et al.,* 2005; Harris, 1990). Mutation of p53 tumors suppressor gene is very common event in SCC of head and neck cancer. HPVs also represent to be another potential prognostic factor for SCC of HNC. This oncogenic potential of HPV may be due to the ability of HPV oncoprotein E6 that promotes the degradation of p53 by binding

with it. Since p53 has multiple cellular roles it can also be possible that E6 may affect apoptotic function.

pRb is also an important cell-cycle regulatory protein, which also shows an important relationship with HPV. HPV oncoprotein E7 binds pRb and degrades it by allowing the transcription of E2F-dependent genes. Phosphorylation of pRb by cyclin D-CDK4-16 complex in the mid to late G1 phase of the cell cycle release E2F from pRb and subsequently, leads to the progression of cell cycle (Jayasurya *et al.,* 2005).

In our study p53 expression was seen lower in HPV positive cases. Similarly low pRb expression was seen in HPV positive cases in all the three groups. Low p53 and pRb expression in HPV-positive cases suggests, that the HPV DNA has been integrated with host genome, thus blocking these genes, and inactivating G1-S cell-cycle checkpoint. These findings support the earlier studies which state that HPV blocks p53 and pRb by expressing their oncogenes. Elango *et al.,* (2011), reported low expression of p53 (18%). Also the absence of pRb expression in their study suggests pRb degradation and formation of a complex between the two proteins, E7 and pRb in HPV mediated carcinogenesis. Nemes *et al.,* (2006), reported low p53 expression in HR-HPV positive cases. Similarly, low pRb expression was also observed in HPV-positive cases in their report. Soares *et al.,* (2008), had reported significant higher expression of pRb (81%) in HPV-positive cases, thus showing significant relationship between pRb and HPV tumors ($p=0.044$) whereas no significant association has been observed in the present study between HPV positivity and pRb, but a significant association was observed between p53 and pRb expression in the present study. The significance between p53 and pRb expression suggests activation of both the pathways in the same patients.

The p53 and pRb are the two important, interconnected molecular pathways which are frequently found in various human cancers. The p53 and pRb may emerge as the most promising molecular prognostic marker in HPV associated oral carcinogenesis.

Conclusions

The present study show low overall prevalence of HPV in OSSC higher prevalence was found in premalignant patients compared to normal and cancer cases. Higher prevalence has been found in buccal mucosa, and male patients. Present study failed to demonstrate any significant relationship between HPV and habits of alcohol and tobacco thus, suggesting that HPV may act as facilitator or bystander of tobacco and alcohol to cause the oral squamous cell carcinoma or vice versa. Our study failed to define the type of HPV most prevalent in oral mucosa thus we suggests that the patients coming with the oral squamous cell carcinoma must undergo HPV testing irrespective of presence or absence of chewing or alcohol habits.

Further studies are needed to define the exact relation of HPV, habits and oral squamous cell carcinoma.

References

[1]. Termine N, Panzarella V, Falaschini S, Russo A, Matranga D, Muzio LL, *et al.* HPV in oral squamous cell carcinoma *versus* head and neck squamous cell carcinoma biopsies: A meta-analysis, 1988–2007. Ann Oncol. 2008;19:1681–1690.

[2]. Gillespie MB, Rubinchik S, Hoel B, Sutkowski N. Human papillomavirus and oropharyngeal cancer: What you need to know in 2009. Curr Treat Options Oncol. 2009;10(5-6): 296-307.

[3]. Ferlay J, Bray F, Pisani P, Parkin DM. International Agency for Research on Cancer (IARC). GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. Cancer Base No. 5, version 2.0. Lyon, France: IARC Press; 2004.

[4]. Koppikar P, deVilliers EM, Mulherkar R. Identification of human papillomaviruses in tumors of the oral cavity in an Indian community. Int J Cancer. 2005;113:946-950.

[5]. Kekatpure V, Kuriakose MA. Oral cancer in India: Learning from different populations. Cancer Prevention. 2010. http://www.nypcancerprevention.com/issue/14/cancer_prevention/feature/india.shtml. Issue 14. 2010.

[6]. Scully C. Oral cancer: The evidence for transmission. Br Dent J. 2005;199(4): 203-7.

[7]. Castro TPPG, Filho IB. Prevalence of human papillomavirus (HPV) in oral cavity and oropharynx. Rev Bras Otorinolaringol. 2006;72(2):272-82.

[8]. Mork J, Lie AK, Glattre E, Hallmans G, Jellum E, Koskela P, *et al.* Human papillomavirus infection as a risk factor for squamous cell carcinoma of the head and neck. N Engl J Med. 2001;344:1125-31.

[9]. Trottier H, Franco EL. The epidemiology of genital human papillomavirus infection. Vaccine. 2006;24:S1/4–S1/15.

[10]. Nagpal JK, Patnaik S, Das BR. Prevalence of high-risk human papilloma virus types and its association with p53 codon 72 polymorphism in tobacco addicted oral squamous cell carcinoma (OSCC) patients of eastern India. Int J Cancer. 2002;97(5):649-53.

[11]. Ribeiro KB, Levi JE, Pawlita M, Koifman S, Matos E, Eluf-Neto J, *et al.* Low human papillomavirus prevalence in head and neck cancer: Results from two large case-control studies in high incidence regions. Int J Epidemiol. 2011;40(2):489-502.

[12]. Remmerbech TW, Brinckmann UG, Hemprich A, Chekol M, Kühndel K, Liebert UG. PCR detection of Human papillomavirus of the mucosa: Comparison between MY09/11 and GP5+/6+ primer sets. J Clin Virol. 2004;30(4):302-8.

[13]. Karimi S, Bahadori M, Mohammadi F, Afsharieh AM, Khodadad K. Immunohistochemical detection of p53, bcl-2 and retinoblastoma proteins in primary mediastinal large B cell lymphoma. Tanaffos. 2002;1(4): 27-35.

[14]. Petrescu A, Mârzan L, Codreanu O, Niculescu L. Immunohistochemical detection of p53 protein as a prognostic indicator in prostate carcinoma. Rom J Morphol Embryol. 2006;47(2):143-6.

[15]. Gillison ML, Koch WM, Capone RB, Spafford M, Westa WH, Wu L, *et al*. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92:709-20.

[16]. Syrjänen S. Human papillomavirus (HPV) in head and neck cancer. J Clin Virol. 2005;32 (Suppl 1):S59-66.

[17]. Campisi G, Panzarella V, Giuliani M, Lajolo C, Di Fede O, Falaschini S, *et al.* Human Papillomavirus: Its identity and controversial role in oral oncogenesis, premalingant and malignant lesions. Int J Oncol. 2007;30(4): 813-23.

[18]. Elango KJ, Suresh A, Erode EM, Subhadradevi L, Ravindran HK, Iyer SK, *et al.* Role of human papilloma virus in oral tongue squamous cell carcinoma. Asian Pac J Cancer Prev. 2011;12(4):889-896.

[19]. Gillison ML, Broutian T, Pickard RKL, Tong Z-y, Xiao W, Kahle L, *et al.* Prevalence of oral HPV infection in the United States, 2009-2010. J Am Med Assoc (JAMA). 2012;307(7):693-703.

[20]. zur Hausen H. Papillomaviruses in the causation of human cancers-a brief historical account. Virology. 2009;2: 260–5.

[21]. zur Hausen H. Papillomaviruses and cancer: From basic studies to clinical application. Nat Rev Cancer. 2001;2:342-350.

[22]. Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: Increasing trends in the U.S. population ages 20-44 years. Cancer. 2005;103(9):1843-9.

[23]. Soares RC, Oliveira MC, Souza LB, Costa AL, Pinto LP. Detection of HPV DNA and immunohistochemical expression of cell cycle proteins in oral carcinoma in population of Brazilian patients. J Appl Oral Sci. 2008;16(5): 340-344.

[24]. Ha PK, Califano JA. The role of human papillomavirus in oral carcinogenesis. Crit Rev Oral Biol Med. 2004;15(4):188-96.

[25]. Ritchie JM, Smith EM, Summersgill KF, Hoffman HT, Wang DH, Klussmann JP, *et al.* Human papillomavirus infection as a prognostic factor in the carcinoma of the oral cavity and oropharynx. Int J Cancer. 2003;104:336-44.

[26]. Balaram P, Nalinakumari KR, Abraham E, Balan A, Hareendran NK, Bernard HU, *et al.* Human papillomaviruses in 91 oral cancers from Indian betel quid chewers-high prevalence and multiplicity of infections. Int J Cancer. 1995;61(4): 450-454.

[27]. Bauda M, Gorgoulis VG, Kastrinakis NG, Giannoudis A, Tsoli E, Danassi-Afentaki D, *et al.* "High -risk" HPV types are frequently detected in potentially malignant and malignant oral lesions, but not in normal mucosa. Mod Pathol. 2000;13:644-53.

[28]. Khanna R, Rao GRK, Tiwary SK, Rai A, Khanna S, Khanna AK. Detection of human papilloma virus 16 and 18 DNA sequences by southern blot hybridization in oral leukoplakia and squamous cell carcinoma. Indian J Surg. 2009;71: 69-72.

[29]. Katiyar S, Hedau S, Jain N, Kar P, Khuroo MS, Mohanta J, *et al.* p53 gene mutation and human papillomavirus (HPV) infection in esophageal carcinoma from three different endemic geographic regions of India. Cancer Lett. 2005;218(1):69-79.

[30]. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: A systematic review. Cancer Epidemiol Biomarkers. 2005;14(2):467-75.

[31]. Ragin CCK, Modugno F, Gollin SM. The epidemiology and risk factor of head and neck cancer: A focus on HPV. J Dent Res. 2007;86(2):104-14.

[32]. Hansson BG, Rosenquist K, Antonsson A, Wennerberg J, Schildt EB, Bladström A, *et al.* Strong association between infection with human papillomavirus and oral and oropharyngeal

squamous cell carcinoma: A population-based case-control study in southern Sweden. Acta Otolaryngologica. 2005;125(12):1337-44.

[33]. Zhang Z-y, Sdek P, Cao J, Chen W. Human papillomavirus type 16 and 18 DNA in oral squamous cell carcinoma and normal mucosa. Int J Oral Maxillofac Surg. 2004;33: 71-74.

[34]. Coutlee F, Gravitt P, Kornegay J, Hankins C, Richardson H, Lapointe N, *et al.* The Canadian women's HIV study group, Franco E. Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. J Clin Microbiol. 2002;40(3): 902–907.

[35]. Miller CS, White DK, Lexington KY. Human papillomavirus expression in oral mucosa, premalignant conditions and squamous cell carcinoma: A retrospective review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1996;82:57-68.

[36]. Herrero R, Castellsagué X, Pawlita M, Lissowska J, Kee F, Balaram P, *et al.* Human papillomavirus and oral cancer: The International Agency for Research on Cancer multicentre study. J Natl Cancer Inst. 2003;95(23):1772-83.

[37]. Kinra P, Turlapati SPV, Mehta A, Rai R. Study of p53 and bcl-2 oncoproteins in ulcerative colitis with dysplasia. Med J Armed Forces India (MJAFI). 2005;61(2):125-129.

[38]. Harris AL. Mutant p53 the commonest genetic abnormality in human cancer. J Pathol. 1990;162:5-6.

[39]. Jayasurya R, Sathyan KM, Lakshminarayanan K, Abraham T, Nalinakumari KR, Abraham EK, *et al.* Phenotypic alteration in Rb pathway have more prognostic influence than p53 pathway proteins in oral carcinoma. Mod Pathol. 2005;18(8):1056-66.

[40]. Nemes JA, Deli L, Nemes Z, Marton IJ. Expression of $p16^{INK4A}$, p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma. Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;102:344-352.

Authors' contributions

RJ: Literature search, Design of the HPV studies and interpretation of their results, preparation of manuscript and drafting the manuscript

TPC: Helped in normal sample collection, preparation of manuscript

MK: Concept and design of the pathological part of manuscript, pathological data interpretation

MP: Concept and design, preparation of final manuscript, data analysis and interpretation.

Funding

None

Acknowledgement

None

Ethical Considerations

Authors declare that the present study was approved by the Institute Ethics Committee.

Conflict of Interest

Authors declare that there are no conflicts of interests

Table 1: Sequence of primer, and its product size

Primers	Nucleotide Sequence	Size
MY09/11	MY09-5´CGTCCMARRGGAWACTGATC-3´ MY11-5´-GCMCAGGGWCTATAAYAATGG-3´	**450bp**

Where M= A/C, R=A/G, W=A/T, Y=C/T (Remmerbech *et al.,* 2004)

Table 2: Relation of HPV status with other clinico-pathological features using Chi square test with (p=0.05) as level of significance

Variables	Cancer Cases			Premalignant Cases			Normal Controls		
	HPV Positive	HPV Negative	P Value	HPV Positive	HPV Negative	P Value	HPV Positive	HPV Negative	P Value
Gender									
Male	13/18 (72.2%)	44/53 (83%)	0.320	14/19 (73.6%)	18/21 (85.7%)	0.342	7/11 (63.6%)	25/56 (44.6%)	0.249
Female	5/18 (27.7%)	9/53 (16.9%)		5/19 (26.3%)	3/21 (14.2%)		4/11 (36.36 %)	31/56 (55.3%)	
Habits									
Tobacco	9/18 (50%)	24/53 (45.2%)	0.686	4/19 (21%)	5/21(23.8%)	0.112	8/11 (72.7%)	8/56 (15.%)	0.360
Tobacco + Smoking	5/18 (27.7%)	12/53 (22.6%)		6/19 (31.5%)	5/21 (23.8%)		1/11 (9%)	1/56 (1.7%)	
Tobacco + Alcohol	3/18 (16.6%)	8/53 (15%)		2/19 (10.5%)	4/21 (19%)		0/11 (0%)	0/56 (0%)	
Tobacco + Smoking + Alcohol	0/18 (0%)	6/53 (11.3%)		0/19 (0)	4/21 (19%)		0/11 (0%)	0/56 (0%)	
None	1/18 (5%)	3/53 (5.6%)		7/19 (36.8%)	3/21 (14.2%)		2/11 (18.18 %)	47/56 (83.9%)	
Tumor Size									
T1-2	7/18 (38.8%)	9/53 (16.98%)	0.05*	-	-	-	-	-	-
T3-4	11/18 (61%)	44/53 (83%)		-	-		-	-	
Nodes									
N0	9/18 (50%)	18/53 (33.9%)	0.289	-	-	-	-	-	-
N1-3	9/18 (50%)	35/53 (66%)		-	-		-	-	
Site									
Buccal Mucosa	13/18 (72.2%)	33/53 (62.2%)	0.509	18/19 (94.7%)	19/21 (90.4%)	0.231	9/11 (81.8%)	46/56 (82.1%)	0.895
Others	5/18 (27.7%)	20/53 (37.7%)		1/19 (5.2%)	2/21 (9.5%)		2/11 (18.1%)	10/56 (17.8%)	
p53									
Positive	6/18	27/53		5/19	8/21		4/11	13/56	

	(33%)	(50.9%)	0.596	(26.3%)	(38%)	0.625	(36.3%)	(23.2%)	0.021*
Negative	12/18 (66.6%)	26/53 (49%)		14/19 (73.6%)	13/21 (61.9%)		7/11 (63.6%)	43/56 (76.7%)	
pRb									
Positives	4/18 (22.2%)	15/53 (28.3%)	0.423	5/19 (26.3%)	2/21 (9.5%)	0.163	1/11 (9%)	6/56 (10.7%)	0.493
Negative	14/18 (77.7%)	38/53 (71.6%)		14/19 (73.6%)	19/21 (90.4%)		10/11 (90.9%)	50/56 (89.2%)	

(* = Significance at <0.05)

Table 3: Result of IHC for p53 protein in cancer, premalignant and normal control

	Grade of p53 stain		
	Grade 1	Grade 2	Grade 3
OSCC (n=71)	11 (15%)	8 (11%)	14(19%)
Premalignant (n=40)	9 (22%)	4 (10%)	0 (0%)
Normal (n=67)	12 (17%)	5 (7%)	0 (0%)

Table 4: Relation of p53 expression with other clinico-pathological features using Chi square test with (p=0.05) as level of significance

Variables	**Cancer Cases**			**Premalignant Cases**			**Normal Controls**		
	p53 Positive	**p53 Negative**	**P Value**	**p53 Positive**	**p53 Negative**	**P Value**	**p53 Positive**	**p53 Negative**	**P Value**
Gender									
Male	28/33 (84.8%)	29/38 (76.3%)	0.355	11/13 (84.6%)	21/27 (77.7%)	0.744	8/17 (47%)	23/50 (46%)	0.787
Female	5/33 (15.1%)	9/38 (23.6%)		2/13 (15.3%)	6/27 (22.2%)		9/17 (52.9%)	27/50 (54%)	
Habits									
Tobacco	13/33 (39.3%)	25/38 (65.7%)	0.286	2/13 (15.3%)	7/27 (25.9%)	0.747	2/17 (11.7%)	6/50 (12%)	0.864
Tobacco + Smoking	6/33 (18.1%)	5/38 (13.1%)		5/13 (38.4%)	6/27 (22.2%)		0/17 (0%)	1/50 (2%)	
Tobacco + Alcohol	4/33 (12.1%)	4/38 (10.5%)		2/13 (15.3%)	2/27 (7.4%)		0/17 (0%)	0/50 (0%)	
Tobacco + Smoking + Alcohol	8/33 (24.2%)	4/38 (10.5%)		2/13 (15.3%)	4/27 (14.8%)		0/17 (0%)	0/50 (0%)	
None	2/33 (6%)	0/38 (0%)		2/13 (15.3%)	8/27 (29.6%)		15/17 (88.2%)	43/50 (86%)	
Tumor Size									
T1-2	7 /33 (21.21%)	9/38 (23.68%)	0.377	-	-	-	-	-	-
T3-4	26/33 (78.78%)	29/38 (76.31%)		-	-		-	-	
Nodes									

N0	13/33 (39.39%)	15/38 (39.47%)	0.745	-	-	-	-	-	-
N1-3	20/33 (60.6%)	23/83 (60.52%)		-	-		-	-	
Site									
Buccal Mucosa	24/33 (72.7%)	22/38 (57.8%)	0.951	12/13 (92.3%)	25/27 (92.5%)	0.83	15/17 (88.2%)	39/50 (78%)	0.895
Others	9/33 (27.2%)	16/38 (42.1%)		1/13 (7.6%)	2/27 (7.4%)		2/17 (11.7%)	11/50 (22%)	
pRb									
Positives	19/33 (57.5%)	0/38 (0%)	0.000**	6/13 (46.1%)	1/27 (3.7%)	0.001**	5/17 (29.4%)	2/50 (4%)	0.04*
Negatives	14/33 (42.4%)	38/38 (100%)		7/13 (53.8%)	26/27 (96.2%)		12/17 (70.5%)	48/50 (96%)	

(* = Significance at <0.05, ** = Significance at <0.005)

Table 5: Result of IHC for pRb protein in cancer, premalignant and normal control

	Grade of pRb stain		
	Grade 1	Grade 2	Grade 3
OSCC (n=71)	5 (7%)	6 (8%)	8 (11%)
Premalignant (n=40)	7(17%)	0 (0%)	0 (0%)
Normal (n=67)	4 (5%)	3 (4%)	0 (0%)

Table 6: Relation of pRb expression with other clinico-pathological features using Chi square test with (p=0.05) as level of significance

Variables	**Cancer Cases**			**Premalignant Cases**			**Normal Controls**		
	pRb Positive	**pRb Negative**	**P Value**	**pRb Positive**	**pRb Negative**	**P Value**	**pRb Positive**	**pRb Negative**	**P Value**
Gender									
Male	14/19 (73.6%)	43/52 (82.6%)	0.398	4/7 (57.1%)	28/33 (84.8%)	0.096	4/7 (57.1%)	28/60 (46.6%)	0.600
Female	5/19 (26.3%)	9/52 (17.3%)		3/7 (42.8%)	5/33 (15.1%)		3/7 (42.8%)	32/60 (53.3%)	
Habits									
Tobacco	7/19 (36.8%)	26/52 (50%)	0.633	2/7 (28.5%)	7/33 (21.2%)	0.904	1/7 (14.2%)	6/60 (10%)	0.926
Tobacco + Smoking	6/19 (31.5%)	11/52 (21.1%)		2/7 (28.5%)	9/33 (27.7%)		0/7 (0%)	1/60 (1.6%)	
Tobacco + Alcohol	3/19 (15.7%)	8/52 (15.3%)		1/7 (14.2%)	5/33 (15.1%)		0/7 (0%)	0/60 (0%)	
Tobacco + Smoking + Alcohol	1/19 (5.2%)	5/52 (9.6%)		0/7 (0%)	4/33 (12.1%)		0/7 (0%)	0/60 (0%)	
None	2/19 (10.5%)	2/52 (3.8%)		2/7 (28.5%)	8/33 (24.2%)		6/7 (85.7%)	53/60 (88.3%)	
Tumor Size									
T1-2	2/19 (10.52%)	14/52 (26.92%)	0.19	-	-	-	-	-	-
T3-4	17/19 (89.47%)	38/52 (73.07%)		-	-		-	-	
Nodes									
N0	5/19 (26.31%)	23/52 (44.23%)		-	-		-	-	

N1-3	14/19 (73.68%)	29/52 (55.76%)	0.221	-	-	-	-	-	-
Site									
Buccal Mucosa	14/19 (73.6%)	32/52 (61.5%)	0.696	7/7 (100%)	30/33 (90.9%)	0.709	4/7 (57.1%)	51/60 (85%)	0.133
Others	5/19 (26.3%)	20/52 (38.4%)		0/7 (0%)	3/33 (9%)		3/7 (42.8%)	9/60 (15%)	

Legends for table

Table 1: Sequence of primer, and its product size

Table 2: Relation of HPV status with other clinico-pathological features using Chi square test with (p=0.05) as level of significance

Table 3: Result of IHC for p53 protein in cancer, premalignant and normal controls.

Table 4: Relation of p53 expression with other clinico-pathological features using Chi square test with (p=0.05) as level of significance

Table 5: Result of IHC for pRb protein in cancer, premalignant and normal control

Table 6: Relation of pRb expression with other clinico-pathological feature using Chi square test with (p=0.05) as level of significance

Legends for figures

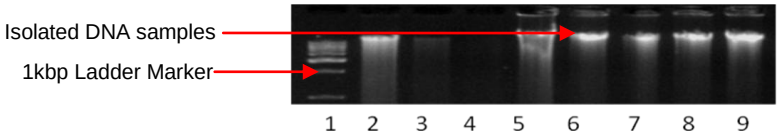
Figure 1: Isolated DNA samples from oral mucosa under UV light

Figure 2: Amplified PCR product of HPV DNA in electrophoresis gel

Figure 3: Photomicrograph showing positive expression of p53 staining in oral mucosa

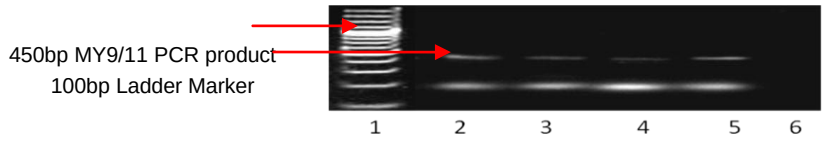
Figure 4: Photomicrograph showing positive expression of pRb staining in oral mucosa

FIGURES



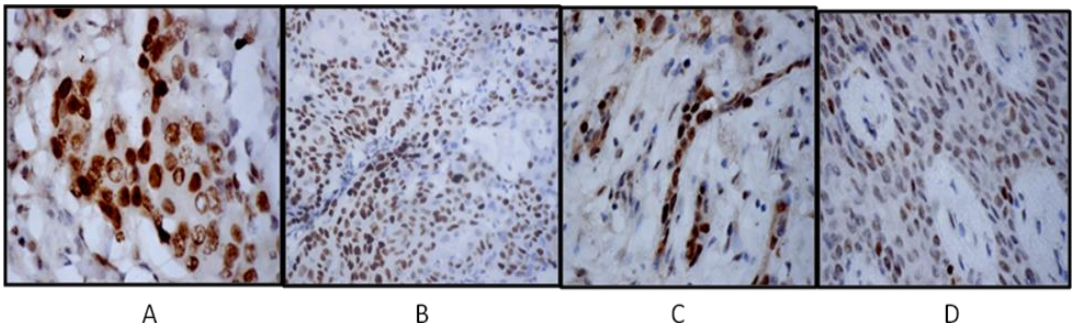
Lane 1= 1kbp ladder marker, Lane 2-9= DNA samples

Figure 1: Isolated DNA samples from oral mucosa under UV light



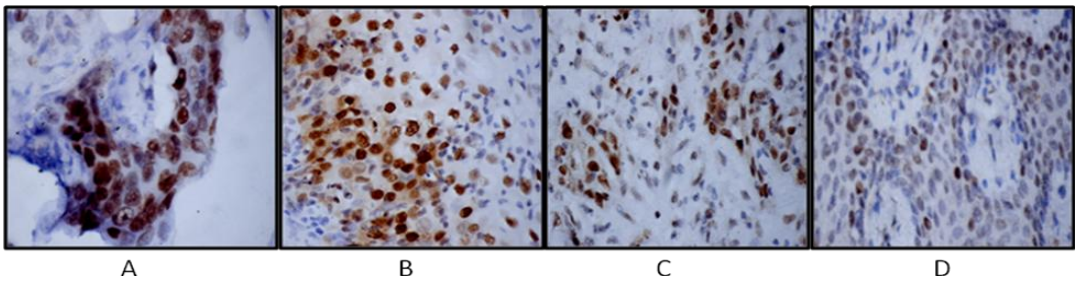
Lane 1=100bp ladder marker, Lane 2-6= PCR product

Figure 2: Amplified PCR product of HPV DNA in electrophoresis gel



A= Positive control (Breast carcinoma) for p53 expression, B= Positive p53 expression in cancer case, C= Positive p53 expression in premalignant lesion, D= Positive p53 expression in normal control.

Figure 3: Photomicrograph showing positive expression of p53 staining in oral mucosa



A= Positive control for pRb expression (Tonsillar carcinoma), B= Positive pRb expression in cancer case, C= Positive pRb expression in premalignant lesion, D= Positive pRb expression in normal control.

Figure 4: Photomicrograph showing positive expression of pRb staining in oral mucosa