Original Article

# Cytogenetic and micronuclei study of human papillomavirus-related oral squamous cell carcinoma

Pritha Pal[1], Ranjan Raychowdhury[2], Subhasis Basu[3], Prasanta Kumar Gure[4], Suman Das[5], Ajanta Halder[1]

Departments of [1]Genetics, [2]Otolaryngology, [3]Pathology and [5]Oral Maxillo Facial and Surgery, Vivekananda Institute of Medical Sciences, Ramakrishna Mission Seva Pratishthan, [4]Department of Otolaryngology, R. G. Kar Medical College and Hospital, Kolkata, West Bengal, India

## Abstract

**Background:** The association of human papilloma viral infection in oral squamous cell carcinoma is well studied in the Western countries, but its correlation with DNA damage in the form of micronuclei (MN) formation, ceased apoptosis or presence of chromosomal abnormalities has not yet been studied.

**Aim:** The aim of this study is to find any possible correlation between human papillomavirus (HPV) infection and cytogenetic damage in the oral malignant and premalignant population of West Bengal.

**Settings and Design:** A total of 104 malignant and 103 premalignant cases were selected along with 200 controls.

**Methods:** The buccal smear samples were Pap stained for the detection of MN, apoptosis frequency and koilocytes. The buccal swab samples were processed for DNA extraction followed by polymerase chain reaction for the detection of HPV DNA. The peripheral venous blood samples were processed for the detection of any chromosomal abnormality, by the method of human leukocyte culture followed by Giemsa staining.

**Statistical Analysis Used:** Correlation analysis using GraphPad Prism software was used in this study.

**Results:** About 34.6%, 42.3% and 6.73% of malignant and 6.79%, 3.88% and 20.38% of premalignant cases showed the presence of HPV DNA, koilocytes and apoptosis, respectively. The difference between the MN frequencies of premalignant and malignant oral lesions with the control group is significant with respect to various risk factors ($P < 0.05$). One percentage of malignant cases showed the presence of chromosomal break.

**Conclusion:** A considerable percentage of malignant cases showing the presence of koilocytes and viral DNA may indicate the effect of HPV infection leading to the malignancy, which can be correlated with the MN and apoptosis frequency.

**Keywords:** Apoptosis, chromosomal abnormality, human papillomavirus, koilocytes, micronuclei, oral cancer, West Bengal

**Address for correspondence:** Dr. Ajanta Halder, Department of Genetics, Vivekananda Institute of Medical Sciences, Ramakrishna Mission Seva Pratishthan, 99 Sarat Bose Road, Kolkata - 700 026, West Bengal, India.
E-mail: ajantahaldar@yahoo.com

**Received:** 24.04.2018, **Accepted:** 29.09.2018

**Access this article online**

Quick Response Code:

**Website:** www.jomfp.in

**DOI:** 10.4103/jomfp.JOMFP_269_17



**How to cite this article:** Pal P, Raychowdhury R, Basu S, Gure PK, Das S, Halder A, *et al*. Cytogenetic and micronuclei study of human papillomavirus-related oral squamous cell carcinoma. J Oral Maxillofac Pathol 2018;22:335-40.

## INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the 6th most common cancers worldwide[1,2] and 3rd most common cancers in developing world.[3] It is the most common cancer in Southeast Asia (India, Bangladesh, Pakistan and Sri Lanka). Its association with different risk factors such as various addictive habits (intake of betel quid, tobacco, alcohol, etc.), poor oral hygiene and bad oral habits has been already established worldwide. However, in recent years, many research studies suggest that there have been many cases developing this cancer without any history of habits related to these risk factors, which bring out the rise of another potent factor, namely human papillomavirus (HPV) infection.[4] Moreover, the connection of different cancers with cytogenetics or DNA damage is well studied all over the world. The preclinical genomic abnormalities include various parameters such as the presence of micronuclei (MN), apoptosis and the presence of numerical or structural chromosomal anomalies.[5] MN frequency is reportedly high in cases of malignancy than that of premalignancy,[6] apoptosis frequency is conventionally suggested to be low in cases of malignancy, and chromosomal abnormalities are variable.

## METHODS

In this case–control prospective study, a stratified sampling method was used to select 407 participants (104 – oral malignant, 103 – oral premalignant and 200 – control) to be interviewed after being informed about the research. Ethical clearance for this study was obtained from the Ethics Committee of Ramakrishna Mission Seva Pratishthan and Vivekananda Institute of Medical Sciences, Kolkata. Nearly 24,550 patients were screened in the Department of ENT, Head and Neck Surgery and Oral and Maxillofacial Surgery of our hospital. Among these, 1070 came out with suspected premalignant and malignant oral lesions. Out of these, 104 patients with histopathologically confirmed cases of oral carcinoma and 103 with premalignant oral lesions and conditions were recruited for this study between June 2013 and October 2017. All cases were newly diagnosed and previously untreated. Clinical characteristics including basic medical data were obtained from medical records. All were resident of different districts of West Bengal. Two hundred controls (cancer free) were recruited simultaneously from the relative of the patients residing in the similar geographic area. Controls were selected among the relatives of the cases who accompanied them and staying in the same localities. Age distribution for the controls was comparable to that of the cases. Cases and controls were matched primarily by frequency of geographic and social origin and secondly by age distribution. They mostly belong to medium-to-low economic classes having similar lifestyle and level of education.

After signing the informed consents, participants were interviewed to collect their demographic data (age, gender and residential history), their daily lifestyle and occupation. Peripheral venous blood, buccal smear and swab samples were collected after obtaining informed consent from all the participants. About 104 malignant, 103 premalignant and 200 control peripheral venous blood samples were processed for detection of the presence of chromosomal abnormalities, by the method of human leukocyte culture followed by Giemsa staining. The buccal smears were taken on slides, Pap stained and examined under microscope for detecting the presence of koilocytes. The corresponding buccal swab samples were dissolved in phosphate-buffered saline solution and DNA was extracted from all the sample solutions following the standard Qiagen protocol, using the Qiagen DNA Mini Kit. Further, polymerase chain reaction (PCR) was performed with all the DNA samples, positive control (HPV 16 plasmid DNA) using the HPV L1 consensus primers [MY11/MY09].

| Primers | Sequence | Amplimer size (approx. bp) |
|---|---|---|
| MY11 | 5'GCCCAAGGACATAACAATGG | - |
| MY09 | 5'CGTCCAAGGGGAAACTGATC | 450 |

### Ethics

Ethical clearance for this study was obtained from the Ethics Committee of Ramakrishna Mission Seva Pratishthan and Vivekananda Institute of Medical Sciences. The study has been independently reviewed and approved by the ethical board as well.

### Statistical analysis

Descriptive analysis was conducted comparing cases with malignant and premalignant oral lesions to the control group in terms of the presence of MN in buccal smear samples. Statistical analysis was done using GraphPad Prism software. All tests were two-sided with a significant level of $P < 0.05$.

## RESULTS

The age–sex distribution and the various risk factor associations are depicted in Table 1. The detailed number of patients and control individuals having/not having various addictions observed in this study is presented in Figure 1.

Almost 42.3% (44/104) of malignant and 3.88% (4/103) of premalignant cases showed the presence of

koilocytes [Figure 2], whereas none of controls showed koilocytes, when observed under the microscope. Nearly 36.4% (36/104) of malignant and 6.79% (7/103) of premalignant cases showed the presence of HPV DNA, whereas none of the control samples showed the presence of the viral DNA. There were few malignant cases (10/104~9.6%) which showed the presence of the viral DNA, in spite of not showing the presence of koilocytes. Twelve malignant nonusers showed the presence of viral DNA. The presence and absence of the viral DNA in malignant and premalignant cases and controls are depicted in Figures 3, respectively.

About 6.73% (7/104) of malignant and 20.38% (21/103) of premalignant cases showed the presence of apoptosis. The range of MN frequency of different cases and controls is depicted in Table 2. The difference between the MN frequencies of premalignant and malignant oral lesions with the control group is significant with respect to various risk factors ($P < 0.05$), as shown in Table 3. The micrographs indicating MN and apoptosis are shown in Figures 4 and 5, respectively. Only one malignant case (~1%) showed the presence of chromosomal abnormality (break). Two metaphase plates one showing normal set of chromosomes and another showing chromosomal break are depicted in Figure 6.

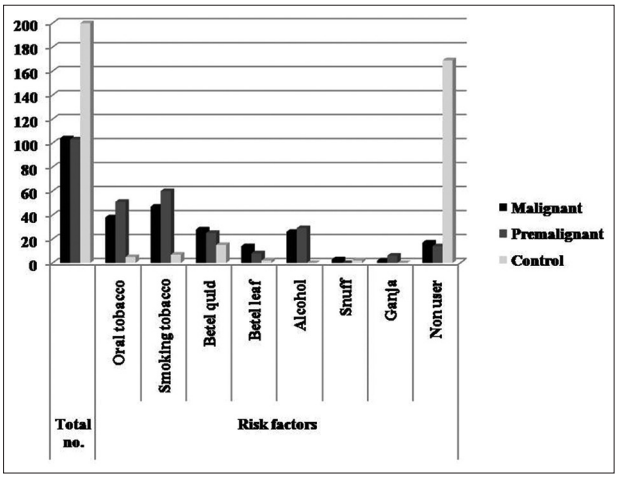


**Figure 1:** The histogram shows the association of various risk factors (including nonuser) and presence of human papillomavirus DNA in malignant and premalignant groups and control individuals

## DISCUSSION

OSCC ranks the 15th most common cancer in males and the 11th most common cancer in females.[7] Its association with different addictions as risk factors is well established worldwide. In developed countries, the use of smoking tobacco is very common, while in developing countries, the chewing practices like intake of paan masala, guthka, zarda, khaini and snuff are more in use,[8,9] along with the use of smoking tobacco such as bidi and cigarette to some extent. However, recent studies stated the prevalence of another risk factor, observed in cancer cases with the history of no addictions or other risk factors. This has been attributed to the human papillomavirus (HPV) in various Western countries,[10] which has resulted in the increase of oropharyngeal carcinoma if compared to oral carcinoma, also among the young agers. The viral prevalence is being tested throughout years with the help of cytological methods for the presence of koilocytes on a primary level, thereby confirming its incidence through molecular methods such as PCR. There is a wide variation of HPV incidence in India, indicating the role of HPV infection in need to get verified more in this developing country. A study from Northern India indicates a low prevalence of HPV and oral carcinoma,[11] which is in contradiction with another study in Southern India, indicating a high prevalence.[12] A study in southern India reported the

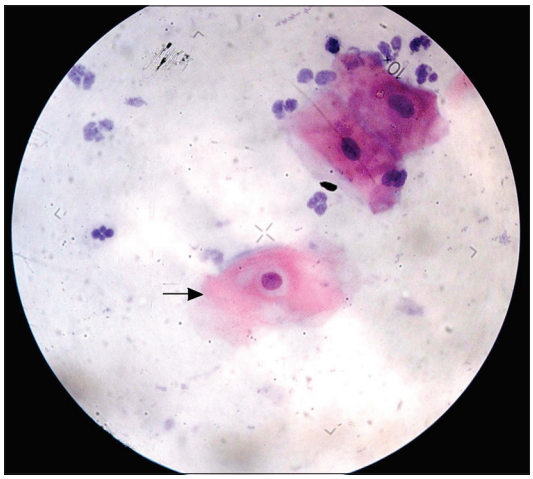

**Figure 2:** Micrograph shows human buccal cells stained by the method of Pap staining and observed at ×100, indicating a koilocyte

**Table 1: The age and sex distribution and various addictions of malignant, premalignant and control individuals are presented here**

| Cases | Sex | | Age | | | | Risk factors* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Male | Female | 20-35 | 36-40 | 41-55 | 56-75 | Oral tobacco | Smoking tobacco | Betel quid | Betel leaf | Alcohol | Snuff | Ganja | Nonuser |
| Malignant | 56 | 48 | 11 | 9 | 36 | 48 | 38 | 47 | 28 | 14 | 26 | 3 | 2 | 17 |
| Premalignant | 71 | 33 | 27 | 14 | 39 | 24 | 51 | 60 | 25 | 8 | 29 | 0 | 6 | 14 |
| Control | 122 | 78 | 37 | 22 | 73 | 68 | 5 | 7 | 15 | 2 | 0 | 2 | 0 | 169 |

*Some of the malignant and premalignant cases and control individuals have more than one addiction

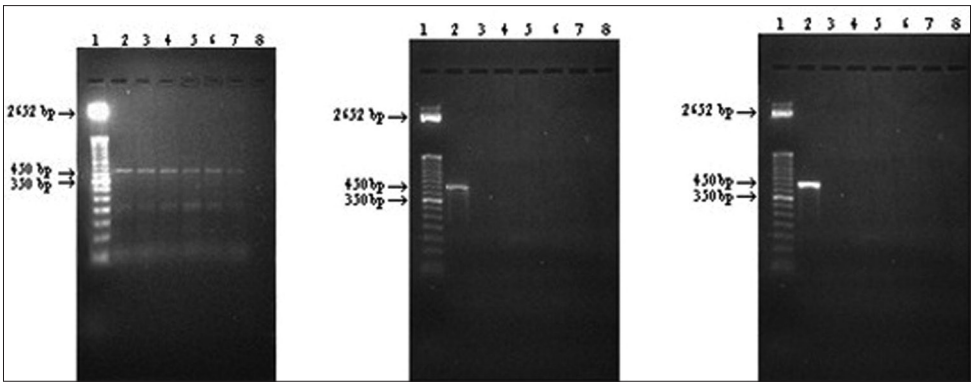


**Figure 3:** Agarose gel electrophoresis showing the presence or absence of human papillomavirus DNA in malignant, premalignant and control samples (lane 3–7), +ve control (lane 2), –ve control (lane 8)

**Table 2: The range of micronuclei frequency among malignant, premalignant and control groups is presented here**

| Cases | MN frequency (%) | | |
|---|---|---|---|
| | 0.1-0.5 | 0.6-1.0 | 1.1-1.5 |
| Malignant | 9 | 81 | 14 |
| Premalignant | 82 | 21 | 0 |
| Control | 2 | 0 | 0 |

MN: Micronuclei

**Table 3: Significant difference of the micronuclei frequency observed in the human buccal cells (indicating the *P* values) between the cases and controls**

| Frequency | Cases | |
|---|---|---|
| | Premalignant and malignant (range) | Control (range) |
| MN frequency (%) | 0.1-1.5 | 0-0.1 |
| *P* | <0.05* | |

*Denotes that the difference of mean values of micronuclei frequency with respect to cases and control groups is significant. MN: Micronuclei

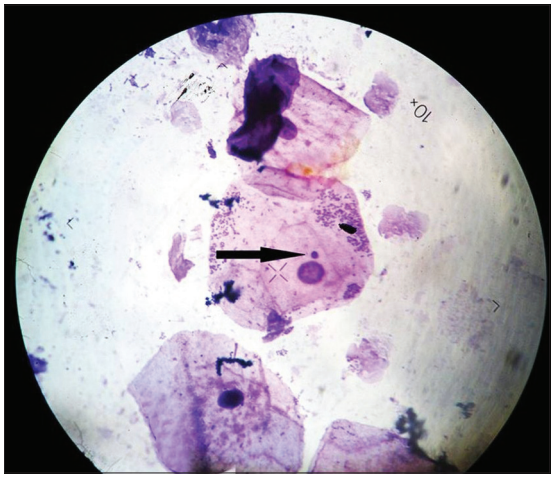

**Figure 4:** Micrograph shows human buccal cells stained by the method of Pap staining and observed at ×100. The solid black arrow indicates micronuclei in the cell

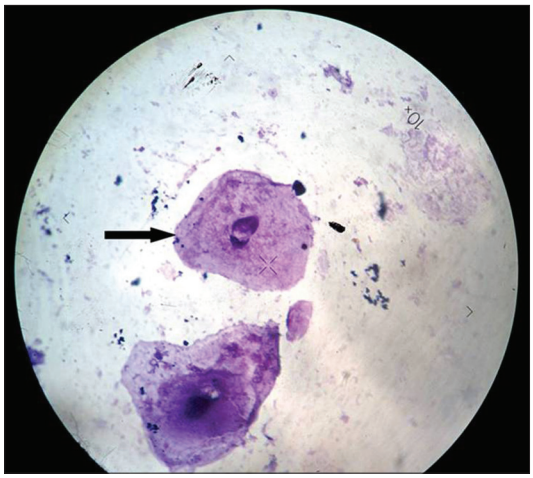

**Figure 5:** Micrograph shows human buccal cells stained by the method of Pap staining and observed at ×100. The solid black arrow indicates an apoptotic cell

prevalence being 40.4%,[13] while another study from southern India shows HPV prevalence of 80%–90%.[14] A study from West Bengal indicates HPV positivity in head-and-neck squamous cell carcinoma tumors being 69%.[15] However, another study from Southern India has stated no role of HPV in oral carcinogenesis.[16] This is also contradicted by another study of West Bengal.[17] In India, HPV DNA has been detected less frequently in tumor specimens from individuals having habits of predisposing risk factors than the nonusers.[18] This is also consistent with another in Kerala which says that negative history of tobacco usage has shown a trend toward HPV positivity in OSCC patients.[19] This study also suggests that the combined effect of oral tobacco and HPV is also a potent factor in oral carcinogenesis, especially in case of infection by high-risk strains. Another study also reported high incidence of HPV infection in tobacco chewers.[20] Numerous studies have suggested additive effect of tobacco and alcohol consumption and HPV.[21,22] However, a study from North India suggests an inclination toward tobacco habit in the oral carcinoma.[11] While HPV-16 and HPV-18 have been found as a significant risk factor for oral cancerous lesions in Western studies,

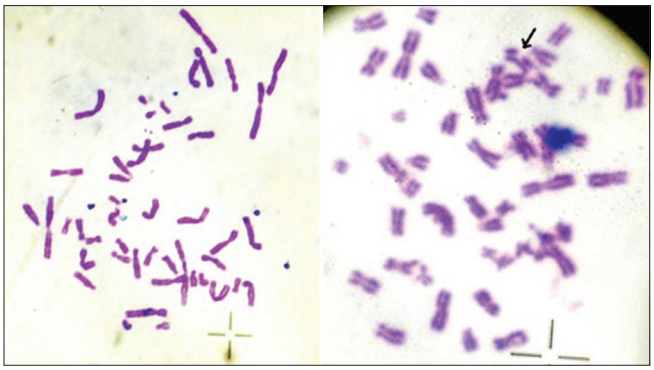

**Figure 6:** Micrographs show metaphase plates; one showing normal set of human chromosomes and the other showing the presence of chromosomal break

in India, this role needs to be verified more, especially in case of premalignant oral lesions such as leukoplakia.[23]

Like all other carcinoma, oral cancer also results from various mutations and chromosomal abnormalities in genes controlling the cell cycle and DNA repair.[24] In addition to the potential for metastasis, this can also be characterized by the loss of the ability of cells to evolve to death when genetic damage occurs. The induction by carcinogens of micronucleated cells, both *in vivo* and *in vitro*, is a sign of the genotoxic effect of such substances. Hence, various cytogenetic tests including the micronucleus test in addition to tests of various degenerative alterations indicative of apoptosis (karyorrhexis, pyknosis and condensed chromatin), which examine the presence of MN, apoptosis and any chromosomal anomaly under microscope, give a promising method to detect the occurrences of cytogenetic alterations in the oral epithelium due to this carcinoma.[4] However, many phytochemicals including essential plant oils have shown their benefits in various cancer cell lines.[25] The authors observed MN frequencies in exfoliated buccal cells in normal mucosa, precancerous lesions and squamous cell carcinoma of Indian participants and concluded that the MN frequency may be a marker of epithelial carcinogenic progression.[26] Here, we have focused on the MN formation, apoptosis frequency and detection of chromosomal abnormalities and observed in various cases of premalignant and malignant stages of OSCC and their association with various risk factors in participants of West Bengal, which will help us to analyze the extent of carcinogenic invasion and compare these with the control groups. The prevalence of HPV in oral cancer has been well documented in several studies from different geographical regions of the world. This incidence has been studied earlier in various Western countries stating the infection of HPV as an independent risk factor leading to this malignancy in participants not having any kind of history of patent risk factors such as intake of tobacco (chewed or smoked), alcohol and betel quid.[10] The microscopic indication of koilocytes can serve as a valuable biomarker for the detection of HPV incidence,[27] further leading to this malignancy in the cases obtained from West Bengal. Hence, in order to find the effect of HPV infection in this zone, we have carried out this study which indicates that although our numbers are small, it would appear that a considerable percentage of the presence of koilocytes states the prevalence of HPV working as a factor in this carcinogenesis.

## CONCLUSION

In this study, the significant difference of MN frequency between the cases and control groups with respect to various risk factors indicates the already established fact of using this parameter as an important biomarker in estimating the cancer progression. The usual pattern of reduced apoptosis frequency in premalignant and malignant cases in comparison to the controls also supports the previous studies in this field. Moreover, a considerable percentage of malignant cases showing the presence of HPV DNA indicate a possible correlation between the HPV infection and the development of oral malignancy in this population too, as already established in the Western countries. However, its independent role is not yet depicted since association with other risk factors are taken into consideration, and a very small percentage of nonusers have shown the malignancy with an impact of the viral infection. Hence, a higher sample size is definitive for bringing out this role. Furthermore, a very small percentage of malignant cases showing the presence of chromosomal abnormality (chromosomal break) cannot exert the fact of this cytogenetic damage created as a result of this viral infection. This observation cannot imply a possible relation between these factors, rather just implementing the creation of DNA damage as a result of any other risk factor in association with the HPV infection, since this malignant case also showed the presence of viral DNA. This correlational study also focuses on the possible link between the viral infection and the caused DNA damage in the form of MN, ceased apoptosis, chromosomal damage, etc., A considerable number of malignant cases showing both the factors into play may indicate a possible impact of the viral infection on the DNA of the affected patients as an outcome of MN formation and further cancer progression with the usual trend of stoppage of the programmed cell death or may be the created environment of the viral factor playing a positive role in the interplay of different risk factors finally leading to the DNA damage and malignancy. A very small percentage of malignant

cases showing the presence of chromosomal abnormality in the form of chromosomal break cannot confirm the correlational link between this viral infection and this cytogenetic damage, yet assessing the damage created by any other risk factor in association with HPV infection, since this malignant case also showed the presence of the viral DNA. To bring out such correlation, a much higher sample size with extensive statistical analysis is required.

## Acknowledgment

We are grateful to Swami Nityakamananda, Secretary of Ramakrishna Mission Seva Pratishthan, to kindly allow us to conduct the study in this institution. We are also thankful to Dr. Sanchita Roy and Dr. Chinmoy Panda for their extensive help and guidance. We are indebted to DST Inspire Fellowship, New Delhi, for giving the financial assistance.

## Financial support and sponsorship

This study was financially supported by DST Inspire Fellowship, New Delhi.

## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Kadashetti V, Chaudhary M, Patil S, Gawande M, Shivakumar KM, Patil S, *et al*. Analysis of various risk factors affecting potentially malignant disorders and oral cancer patients of central India. J Cancer Res Ther 2015;11:280-6.
2. Coelho KR. Challenges of the oral cancer burden in India. J Cancer Epidemiol 2012;2012:1-17.
3. Fazeli Z, Pourhoseingholi MA, Pourhoseingholi A, Vahedi M, Zali MR. Mortality of oral cavity cancer in Iran. Asian Pac J Cancer Prev 2011;12:2763-6.
4. Rakesh S, Janardhanan M, Vinodkumar RB, Vidya M. Association of human papilloma virus with oral squamous cell carcinoma – A brief review. Oral Maxillofac Pathol J 2010;1:63-6.
5. Kingsley K, O'Malley S, Ditmyer M, Chino M. Analysis of oral cancer epidemiology in the US reveals state-specific trends: Implications for oral cancer prevention. BMC Public Health 2008;8:87.
6. Dórea LT, Meireles JR, Lessa JP, Oliveira MC, de Bragança Pereira CA, Polpo de Campos A, *et al*. Chromosomal damage and apoptosis in exfoliated buccal cells from individuals with oral cancer. Int J Dent 2012;2012:457054.
7. Alhazzazi TY, Alghamdi FT. Head and neck cancer in Saudi Arabia: A Systematic review. Asian Pac J Cancer Prev 2016;17:4043-8.
8. Rodriguez T, Altieri A, Chatenoud L, Gallus S, Bosetti C, Negri E, *et al*. Risk factors for oral and pharyngeal cancer in young adults. Oral Oncol 2004;40:207-13.
9. Juntanong N, Siewchaisakul P, Bradshaw P, Vatanasapt P, Chen SL, Yen AM, *et al*. Prevalence and factors associated with oral pre-malignant lesions in Northeast Thailand. Asian Pac J Cancer Prev 2016;17:4175-9.
10. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, *et al*. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol 2013;31:4550-9.
11. Singh V, Husain N, Akhtar N, Kumar V, Tewari S, Mishra S, *et al*. Do human papilloma viruses play any role in oral squamous cell carcinoma in North Indians? Asian Pac J Cancer Prev 2015;16:7077-84.
12. 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;12:889-96.
13. Bijina BR, Ahmed J, Shenoy N, Ongole R, Shenoy S, Baliga S, *et al*. Detection of human papilloma virus in potentially malignant and malignant lesions of the oral cavity and a study of associated risk factors. South Asian J Cancer 2016;5:179-81.
14. Kulkarni SS, Kulkarni SS, Vastrad PP, Kulkarni BB, Markande AR, Kadakol GS, *et al*. Prevalence and distribution of high risk human papillomavirus (HPV) types 16 and 18 in carcinoma of cervix, saliva of patients with oral squamous cell carcinoma and in the general population in Karnataka, India. Asian Pac J Cancer Prev 2011;12:645-8.
15. Mitra S, Banerjee S, Misra C, Singh RK, Roy A, Sengupta A, *et al*. Interplay between human papilloma virus infection and p53 gene alterations in head and neck squamous cell carcinoma of an Indian patient population. J Clin Pathol 2007;60:1040-7.
16. Laprise C, Madathil SA, Allison P, Abraham P, Raghavendran A, Shahul HP, *et al*. No role for human papillomavirus infection in oral cancers in a region in southern India. Int J Cancer 2016;138:912-7.
17. Pal P, RaychowdhuryR, Halder A. Oral carcinoma, HPV Infection, arsenic exposure – Their correlation in West Bengal, India. Otolaryngol Open Access 2016;6:266.
18. Gupta S, Gupta S. Role of human papillomavirus in oral squamous cell carcinoma and oral potentially malignant disorders: A review of the literature. Indian J Dent 2015;6:91-8.
19. Kane S, Patil VM, Noronha V, Joshi A, Dhumal S, D'Cruz A, *et al*. Predictivity of human papillomavirus positivity in advanced oral cancer. Indian J Cancer 2015;52:403-5.
20. 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;61:450-4.
21. Schwartz SM, Daling JR, Doody DR, Wipf GC, Carter JJ, Madeleine MM, *et al*. Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. J Natl Cancer Inst 1998;90:1626-36.
22. Smith EM, Ritchie JM, Summersgill KF, Hoffman HT, Wang DH, Haugen TH, *et al*. Human papillomavirus in oral exfoliated cells and risk of head and neck cancer. J Natl Cancer Inst 2004;96:449-55.
23. Bhargava A, Shakeel M, Srivastava AN, Raza TS, Rizvi S, Varshney P, *et al*. Role of human papilloma virus in oral leukoplakia. Indian J Cancer 2016;53:206-9.
24. Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmueller S, *et al*. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: The HUMN project perspective on current status and knowledge gaps. Mutat Res 2008;659:93-108.
25. Aras A, Iqbal MJ, Naqvi SK, Gercek YC, Boztas K, Gasparri ML, *et al*. Anticancer activity of essential oils: Targeting of protein networks in cancer cells. Asian Pac J Cancer Prev 2014;15:8047-50.
26. Stich HF, Curtis JR, Parida BB. Application of the micronucleus test to exfoliated cells of high cancer risk groups: Tobacco chewers. Int J Cancer 1982;30:553-9.
27. Khovidhunkit SO, Buajeeb W, Sanguansin S, Poomsawat S, Weerapradist W. Detection of human papillomavirus in oral squamous cell carcinoma, leukoplakia and lichen planus in Thai patients. Asian Pac J Cancer Prev 2008;9:771-5.