

Expression of Human Papillomavirus DNA and p53 Polymorphisms through Polymerase Chain Reaction in Normal Mucosa and Oral Leukoplakia Individuals with Deleterious Oral Habits

Abstract

Context: Oral leukoplakia (OL) is a well-recognized precancerous lesion with various etiological factors. Most commonly deleterious oral habits such as tobacco smoking and viral etiologies mainly human papillomavirus (HPV) play an important role. p53 polymorphisms mostly homozygous Arginine (Arg) allele has a greater risk of degradation by HPV. Hence, HPV infection and p53 polymorphisms may act as synergistic factors for increased the risk of malignant transformation in oral precancerous lesions. **Aims:** The aim of this study is to evaluate the risk of OL and its malignant transformation due to infection by HPV and p53 polymorphisms in the oral biopsy samples through polymerase chain reaction (PCR). **Subjects and Methods:** A total of 40 individuals were involved— 10 individuals were controls without deleterious habits, 15 were controls with deleterious habits, and 15 were with histologically confirmed OL individuals with deleterious habits. PCR and restriction fragment length polymorphism using smaI enzyme were carried out to evaluate the expression of HPV and p53 polymorphisms. **Statistical Analysis Used:** Chi-square test, Fischer's exact *t*-test, and odds ratio. **Results:** (1) HPV DNA expression was higher in Leukoplakia individuals than controls. (2) p53 genotype with homozygous Arg was more in HPV-infected individuals. **Conclusions:** To conclude HPV infected OL cases were mostly with Arg/Arg type of p53 polymorphism.

Keywords: Human papillomavirus, Leukoplakia, p53, polymerase chain reaction-restriction fragment length polymorphism

Introduction

Oral Leukoplakia (OL) is the most common potentially malignant disorder (PMD) of the oral cavity and has been considered to confer increased risk for the development of oral cancer.^[1] Oral cancer usually involves multiple alterations in the genomic level which progressively gets accumulated during a protracted period, the overall effect of which surpasses the inherent reparative ability of the cell.^[2] The sum total of these alterations are of diagnostic and prognostic relevance which are designated as "Precancerous changes."^[2]

About 70%–90% of PMDs mainly OL s are related to smoking and alcohol use, either alone or in combination.^[3] Several other etiological factors apart from smoking include biological agents such as bacteria, fungi, and chiefly virus. Among all viruses, human papillomavirus (HPV) is the major cancer pathogens in humans which constitute around 23.5%.^[4,5]

HPV is a 55KD, nonenveloped, double-stranded, circular DNA virus that has been implicated in a variety of anogenital and aerodigestive diseases, ranging from common warts to laryngeal papilloma to cervical cancer.^[6] More than 120 different subtypes of HPV have been identified which are divided into 3 major groups as super Group A (Alpha papillomavirus), super Group B (Beta papilloma virus), and the remaining group of HPVs are members of super Group E (Mu and Nu-papilloma viruses).^[5] The alpha papillomavirus group are mucosotropic HPVs which are further divided into two classes based on their risk to humans, that is, High Risk-HPV type (potentially oncogenic) and Low Risk-HPV type (nononcogenic). The E6 and E7 oncoproteins of high-risk HPV have the capacity to mediate carcinomatous transformation of infected keratinocytes by inactivating p53 and retinoblastoma (Rb) tumor suppressor pathways.^[7]

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p53 is a tumor suppressor gene which plays an important role in the maintenance of genomic integrities through induction of cell cycle arrest or apoptosis failing DNA damage. The loss of activity of the wild-type p53 (wtp53) protein can be achieved by two different mechanisms: Either by a mutation of the p53 gene or by binding to the HPV-encoded E6 protein.^[8] A common polymorphism in p53 gene at exon 4 codon 72, encoding for either a proline (CCC/Pro) or arginine (CGC/Arg) amino acid, among which p53Arg homozygous genotype has been implicated to have more susceptibility to HPV-infected cancers.^[8]

This study is to evaluate the risk of tobacco-associated OL and its malignant transformation due to p53 polymorphisms and infection by HPV in the oral biopsy samples through polymerase chain reaction (PCR).

Subjects and Methods

Fifteen consecutive subjects with OL, Healthy individuals with deleterious habits and 10 healthy individuals without deleterious habits visiting the outpatient department of GITAM Dental College, satisfying the inclusion and exclusion criteria were included for the study [Table 1].

An informed written consent was obtained before the study. The study protocol was approved by the Ethical committee of the institution.

- Controls: Group 1: (n = 10) Healthy individuals without any deleterious oral habits
- Study group: Group 2: (n = 15) Healthy individuals with deleterious oral habits
- Group 3: (n = 15) Individuals who are clinically and histopathologically diagnosed as cases of OL with deleterious oral habits.

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Healthy individuals with no adverse oral habits and absolutely free of any oral lesions or systemic illness were considered as controls for the study	Individuals with previous treatment of oral epithelial dysplasia were excluded from the study
Untreated individuals with deleterious oral habits and histopathologically confirmed oral epithelial dysplasia alone were considered for the study	Individuals with previous history of any other systemic malignancies or any other systemic disease were excluded from the study
Individuals above the age of 18 years were included in the study	Individuals with any prior neoplasia and cancer metastasis were excluded from the study
	Individuals with any other premalignant lesions (like lichen planus, OSMF) were excluded from the study
	Pregnant and lactating women were excluded from the study

OSMF: Oral submucous fibrosis

Tissue sample collection

A detailed case history was obtained from all individuals before the study. Incisional biopsy samples were collected from the Group 3 individuals. Samples from Group 1 and 2 were collected during routine dental surgical procedures. A part of the tissue was taken for Hematoxylin and Eosin staining to confirm clinical diagnosis of Leukoplakia. Another part of the tissue was placed in TBS buffer and was stored at -20°C for DNA extraction.

HPV DNA isolation and detection

Genomic DNA was extracted using the Qiagen kit method. The presence of HPV in the controls and OL individuals was detected by PCR, using primers (MY09 and MY11) from the consensus L1 region [Table 2].

The PCR was run by an initial denaturation at 94°C for 5 m followed by 35 cycles of 94°C for 1 m, annealing at 51°C for 1 m, extension at 72°C for 1 m followed by final extension at 72°C for 8 m and holding the temperature at 10°C for 1 min. The amplified DNA fragments were observed in 2% agarose gel at 450 base pair [Figure 1].

p53 gene polymorphism at codon 72, exon 4

Extraction of genomic DNA, for p53 detection, was carried out by the same method as done for HPV DNA isolation. A portion of each reaction product was electrophoresed on 2% agarose gel to check for the quality of the desired PCR product at 162 base pairs. After PCR amplification, 2.5 µL PCR products were digested with Sma I enzyme and incubated at 37°C for overnight and then subjected to agarose gel electrophoresis. In CG (Pro/Arg) genotype, one DNA was cut at 135 and 27 and the other was left uncut. In GG (Arg/Arg) genotype, both DNA were left uncut. The gel pattern was visualized under UV light after ethidium bromide staining [Figure 2].

Chi-square test and Fischer’s exact t-test were performed to analyze the results.

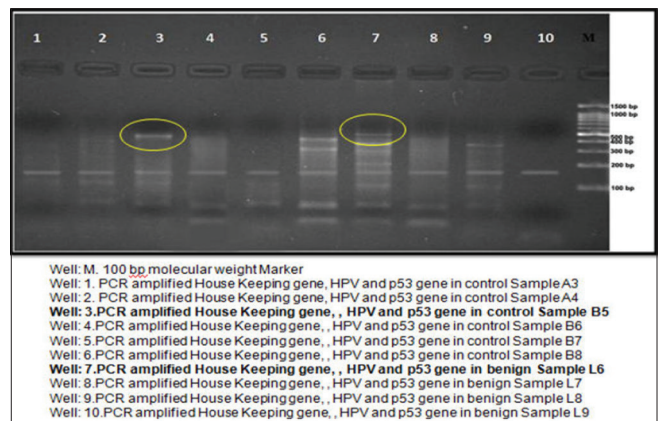


Figure 1: Gel electrophoresis of human papillomavirus and p53 with 100bp ladder (human papillomavirus – 450bp, p53 -162 bp)

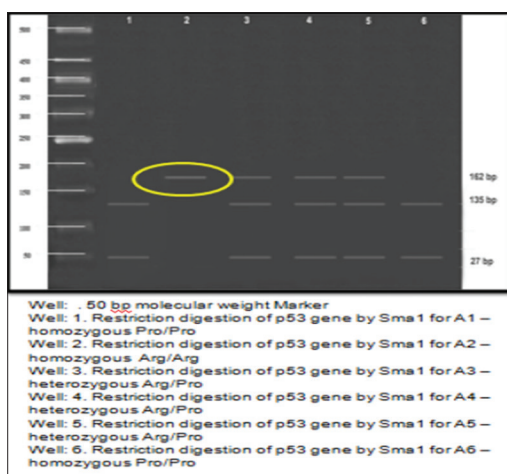


Figure 2: restriction fragment length polymorphism - p53 polymorphisms digested by Sma1 enzyme (Arginine/Arginine – 162bp, Arginine/Proline – 162,135,27bp, Proline/Proline -135, 27bp)

Results

A total of 40 individuals were included in the study. The demographic data, the status of HPV expression, and type of p53 polymorphisms of all the individuals involved in the study are included in Table 3.

Human papillomavirus analysis

HPV was detected in 1 out of 25 individuals of Group 2 and 3 out of 15 individuals of Group 3. In the present study, HPV was seen mostly associated with sites exposed to microtrauma i.e buccal mucosa in Leukoplakia individuals, but these associations were statistically nonsignificant. Among 3 HPV infected OL patients, 1 was smokeless tobacco chewer and 2 were smoke tobacco chewers. There was no significant association between HPV infection and risk of OL (odds ratio = 6, 95% confidence interval = 0.56–63.9, $P > 0.13$). The correlation between HPV infection and the degree of dysplasia was also found to be nonsignificant ($P = 0.28$).

p53 polymorphisms analysis

No significant association between codon 72 polymorphism and OL was observed but, there was a significant correlation between the presence of Arg/Arg type of p53 polymorphism and HPV-positive cases of Leukoplakia ($P = 0.05$).

Discussion

In India, it has been reported that 30%–80% malignancies of oral cavity arise from premalignant lesions such as Leukoplakia. The major risk factor for the development of premalignant lesions and malignant lesions is tobacco consumption. Tobacco is being in use as both smoke and smokeless forms (khaini and betel quid).^[9] Tobacco-specific nitrosamines are the strong carcinogens associated with tobacco smoke.^[10] Apart from deleterious habits, accumulative evidence indicates that individual susceptibility to precancer also depends on genetic predisposition and viral infections mainly HPV.^[11]

Table 2: Primers used for polymerase chain reaction amplification

Primer name	Sequence from 5'-3'
Forward Primer HPV	TGTCAAAAACCGTTGTGTCC
Reverse Primer HPV	TCTGAGTCGCTTAATTGCTC
Forward Primer p53	TTTTACCCATCTACAGTCCCCCTTG
Reverse Primer p53	TAGGAGCTGCTGGTGCAGGGGCCCC
Forward Primer B-actin	CAAGGCCAACCGCGAGAAGATG
Reverse Primer B-actin	GTCCAGGGCGACGTAGCACAGC

Table 3: Demographic data of all the three groups

Demographic data	Group 1	Group 2	Group 3
Age			
Mean	40	50.466	46.733
SD	15.151	14.050	8.353
Gender			
Males	6 (10)	12 (15)	12 (15)
Females	4 (10)	3 (15)	3 (15)
Site of lesion			
Buccal mucosa	-	-	9 (15)
Palate	-	-	3 (15)
Others	-	-	3 (15)
Tobacco habits			
Smoke tobacco	-	10 (15)	9 (15)
Smokeless tobacco	-	5 (15)	6 (15)
Grade of dysplasia			
Mild	-	-	2 (15)
Moderate	-	-	9 (15)
Severe	-	-	4 (15)
HPV status			
Absent	10 (10)	14 (15)	12 (15)
Present	0 (10)	1 (15)	3 (15)
p53 polymorphisms			
Arg/Arg	2 (10)	4 (15)	3 (15)
Arg/Pro	5 (10)	7 (15)	7 (15)
Pro/Pro	3 (10)	4 (15)	5 (15)
HPV and p53 polymorphisms			
Arg/Arg + HPV	-	0 (15)	2 (15)
Arg/Pro + HPV	-	1 (15)	1 (15)
Pro/Pro + HPV	-	0 (15)	0 (15)

HPV: Human papillomavirus; SD: Standard deviation; Arg: Arginine; Pro: Proline

The present study was based on study of the expression of HPV and p53 polymorphisms in individuals with a mean age of 45 years in all the 3 groups considered. This study reported that OL was more frequent in buccal mucosa followed by the palate. Mitra *et al.*^[10] and Sarnath *et al.*^[11] gave the reasons for the varying sites of occurrence of Leukoplakia which include continuous contact of irritants, variations in the degree of keratinization and permeability of the oral mucosa, contribute to the modulating effects of tobacco toxins.^[12] According to the present study mostly males with smoke tobacco habits were more susceptible for OL which can be due to the synergistic effect of elevated temperature in the oral cavity of males thereby making

the epithelium more susceptible to the genotoxic effect of tobacco products.^[13]

Mucosotropic or epitheliotropic viruses mostly HPVs are capable of causing lesions in both oral mucosa as well as in cervical mucosa because of a similar type of epithelium. Hence, it is important to investigate the relationship between HPV and its contribution to premalignancy.^[14]

According to Terai *et al.*^[15] and Califano *et al.*^[6] the prevalence of HPV infection shows extensive variation in healthy mucosa ranging from 12% to 81.1%, but the prevalence of HPV in the present study were— Group B-6.7% and Group C-20%, respectively. This low prevalence of HPV in the oral cavity without any lesions was due to the salivary immune response which includes IgA, cystatins, and proteolytic enzymes that protect the oral mucosa.^[3] Other studies by Giovannelli *et al.*,^[16] Gichki *et al.*,^[17] Miller *et al.*^[18] and Sikka *et al.*^[19] the prevalence of HPV ranges from 17 to 40.8% in OPMD, which was in accordance with the present study. Martinez *et al.*^[20] reported that increased frequency of HPV in OPMDs than in the oral carcinomas was due to the “hit and run” theory, according to which viral genome does not need to be present to maintain cell transformation once genetic damage has been inflicted at an early stage.^[20] The site prevalence of HPV according to Mravak-Stipetic *et al.*^[21] and the present study was mostly in areas more exposed to micro-trauma.

Apart from the viral basis of etiology, carcinogenesis also has a genetic basis of occurrence. The link between viral contribution and genetic factors is due to the effect of viral proteins on tumor suppressor genes—a major step in viral carcinogenesis.

Most frequently effected tumor suppressor gene by the viral proteins is p53 which is located at locus 17p13.1, the name was due to its molecular mass: Which was in the 53 KD fractions of cell proteins. A p53 has many polymorphisms due to exonic and intronic sequence variations. Most commonly occurring polymorphisms is at exon 72 which include homozygous Arg allele, homozygous Pro allele and heterozygous Pro and Arg alleles. According to Mousami *et al.* Arg/Arg polymorphism of p53 codon has more risk of OL. The frequencies of p53 polymorphisms in this study were in correlation with findings of Sikka *et al.*^[19] Mitra *et al.*^[10] reported that individuals with an Arg form of p53 were less efficient in processing tobacco-associated DNA damage signals and hence were more susceptible to develop OL.

An interesting observation in the present study was a significant correlation between frequency of expression of HPV and frequency of the homozygous Arg type of p53 polymorphism, thereby indicating a higher risk of Arg/Arg type of p53 allele to HPV-mediated degradation which could be an important risk factor for malignant transformation of HPV infected OPMDs. Hou *et al.*,^[22]

Storey *et al.*^[23] and Nagpal *et al.*^[24] reported similar findings regarding HPV and p53 polymorphisms.

To summarize, although a majority of premalignant lesions occur in individuals with deleterious habits, only some of these lesions are transforming into malignancy. Hence, identification of these high-risk premalignant lesions with increased susceptibility to malignancy and their early detection may help in downstaging of oral cancer and can have a better prognosis. Thus, detection of HPV and type of p53 polymorphism can be highly effective in this scenario as cancer predictive biomarkers in view of reducing global head and neck cancer burden.

Conclusion

So to conclude, this study focuses on the role of HPV in the premalignant lesion and also its role in the degradation of p53. Although the sample size in the present study was small, there was a significant result correlating the presence of HPV and Arg/Arg genotype of p53 in individuals with Leukoplakia. However, larger sample size involving wider cross-section of the population should be included to achieve an affirmative result. This study may provide a stepping stone for the future research in which HPV and p53 polymorphisms may be considered as important markers for suggesting the malignant transformation which further can play a major role in treatment modalities and prognosis.

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Conflicts of interest

There are no conflicts of interest.

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