Association of Human Papilloma Virus 16 Infection and p53 Polymorphism among Tobacco using Oral Leukoplakia Patients: A Clinicopathologic and Genotypic Study

Seema Sikka, Pranav Sikka[1]

Department of Oral and Maxillofacial Pathology, Sudha Rastogi Dental College, Faridabad, Haryana, India, [1]Department of Pharmacology, L.L.R.M. Medical College, Meerut, Uttar Pradesh, India

Correspondence to:
Dr. Seema Sikka,
Department of Oral and Maxillofacial Pathology, Sudha Rastogi Dental College, Faridabad, Haryana, India.
E-mail: simu50@yahoo.com

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ABSTRACT

Background: Human papillomavirus (HPV) and p53 alterations are speculated to play a role in carcinogenesis. This study was carried out to find out the association of HPV and p53 with precancerous lesions of the oral cavity such as leukoplakia: The objective of this study was to find the association among human papilloma virus (HPV) 16 infections and p53 polymorphism in tobacco using the oral leukoplakia patients.

Methods: A total of 91 oral leukoplakia patients and 100 controls were randomly selected from the out-patient department of a tertiary care dental hospital of North-east India. Blood samples were drawn incisional biopsy was performed from the lesion proper and the tissue was processed for histopathological grading. Cytological smears were taken from the lesional site of leukoplakia patients and buccal mucosa of controls. The rate of HPV infection and p53 polymorphism was detected with the help of polymerase chain reaction, gel electrophoresis and deoxyribonucleic acid sequencing.

Results: The rate of HPV 16 infection was found significantly high in the oral leukoplakia patients. No particular p53 genotype at exon 4 of codon 72 was found to be associated with oral leukoplakia, but "C" allele (proline) at exon 4 of codon 72 was significantly raised in these patients.

Conclusions: Oral leukoplakia, a well-known pre-cancerous lesion, has been shown to be associated with tobacco, but certain other factors like HPV infection and p53 polymorphism may play an important role in its development.

Keywords: Human papilloma virus, oral leukoplakia, p53 polymorphism, tobacco

INTRODUCTION

Oral cancer constitutes 5.5% of all malignancies globally and is the sixth most common cancer world-wide.[1] Oral squamous cell carcinomas (OSCCs) are generally associated with tobacco habits (mainly chewing with/without smoking or alcohol consumption) and usually preceded by premalignant lesions,

most often a persistent leukoplakia.[2] The overall incidence of malignant transformation of oral leukoplakia varies from 8.8% in females versus 5.1% in males with a 30 years follow-up period[3] and similar findings were noted by Silverman in their study for a follow-up period of 7.2 years.[4]

Several etiological factors, particularly tobacco/alcohol consumption and human papilloma virus (HPV) infection, have been shown to be linked with the OSCC.[5] Some studies on HPV, known to be linked with cervical and various other anogenital (i.e. vulvar, penile, perianal and anal) cancers,[6,7] have shown that the prevalence of HPV in OSCC was higher than in normal mucosa; thus, HPV is being regarded as a risk factor and important determinant in oral carcinogenesis.[8-10] Some studies have suggested that HPV exerts its oncogenic property by inhibiting the function of the tumor suppressor proteins p53,[11] but confirming literature is still lacking.

HPV consists of circular double stranded deoxyribonucleic acid (DNA) encased in a small, non-enveloped, icosahedral capsid. The coding strand consists of approximately 10 reading frames, which have been classified as either early (E) or late (L) genes. The transforming property of high risk HPV; however, appears to localize mainly to E6 and E7 proteins.[12-14] The transforming property of E6 protein is attributed to its interaction with the cellular p53 protein. This interaction is mediated by E6 - associated protein (E6-AP) ligase. When E6 binds to p53; it results in ubiquitination of the p53 protein and its subsequent degradation. The level of p53 is decreased in E6 induced immortalized cells and these cells fail to undergo cell cycle arrest following DNA damage.[11] This decrease in the intracellular level of p53 has been shown to increase mutagenesis in human cells *in vitro* as well as results in chromosomal instability.[11,15] A specific set of HPV (including types 16, 18, 31, 33 and 39) is detected in up to 99% of cases of anogenital pre-neoplastic lesions and squamous cell carcinomas. They are termed high-risk HPVs. Their genomes are integrated into the host DNA and are transcriptionally active in both tumor and tumor derived cell lines.[16] Based on this evidence, the International Agency for Research on Cancer (1997) has found HPV 16 and 18 as carcinogenic in humans.[17] Most papilloma viruses have a strong tropism for squamous epithelial cells.[18] The initial infection of epithelial cells occurs in basal and parabasal keratinocytes where viral DNA can be demonstrated by *in situ* hybridization. The initial infection may be cleared by the immune system, may lead to the production of papillomas or result in a latent infection, in which the tissue remains histologically normal. Latent infection of clinically normal appearing mucosa of the upper aerodigestive tract has been well-established. The detection rates of HPV in oral epithelium have varied from 0% to 60%.[12] And the prevalence of HPV in oral cancer patients has been found to be very high in India.[19]

A common polymorphic site in the wild type p53 gene at codon 72 of exon 4 results in translation to either an arginine residue or a proline residue. The frequency of two alleles varies among ethnic groups that dwell at different latitudes. Polymorphism at p53 codon 72 has been shown to be one of the susceptible factors in HPV associated carcinomas in a Chinese study. It was reported that the p53 containing arginine residue increased the susceptibility of p53 to E6 mediated degradation. Thus, patients who were homozygous for p53 arginine were about 7 times more susceptible to HPV associated carcinogenesis compared with heterozygotes.[20] Despite numerous studies of HPV and oral carcinogenesis, the results are inconclusive.[8,9,21,22] However, in large surveys, normal epithelium has been collected by scraping mucosa and such samples do not include the basal layers, which may contain latent HPVs. In the current study, full-thickness biopsy specimens were taken from patients and controls to avoid such controversy. Further, HPV prevalence in hyperkeratosis or premalignant conditions has been found to be higher than that in malignant disease,[21,23] which may suggest that HPVs are more likely to act as an initiator of epithelial proliferation or play a role in the early stage of oral carcinogenesis. Therefore, objectives of our study included: (1) Detection of HPV 16 in the genomic DNA of oral leukoplakia patients with tobacco habits, (2) study of p53 polymorphism in the genomic DNA of oral leukoplakia patients and (3) to find out the role of HPV infection and p53 polymorphism for the risk of oral leukoplakia.

METHODS

Samples and controls

A total of 91 oral leukoplakia patients and 100 controls with a history of tobacco intake (smoking

and smokeless) and without a habit of alcohol consumption were randomly selected from the outpatient department of a tertiary care dental hospital of North-east India. The presence of tobacco habit for more than 5 years was confirmed from patients and controls. Leukolakia was present on buccal mucosa of all the enrolled patients. Blood samples were drawn and an incisional biopsy was performed from the lesion proper in patients and the tissue was processed for histopathological grading. Cytological smears were taken from the lesional site of leukoplakia patients and buccal mucosa of controls. Cytological samples were immediately frozen and stored at –80°C. The study was approved by the ethical committee of the institution and informed consent was taken from each patient and control.

HPV DNA isolation and detection

Genomic DNA was extracted using the Qiagen kit method. Four leukoplakia cases were excluded from genomic extraction procedures due to systemic illness. The presence of HPV in the oral leukoplakia tissues was detected by polymerase chain reaction (PCR), using primers (MY09 and MY11) from the consensus L1 region. Typing of HPV 16 in the L1 positive samples were carried out using type specific primers (TCAAAAGCCACTGTGTCCTG and CGTGTTCTTGATGATCTGCA) homologous to a region of the E6 gene. The PCR was run for 35 cycles, preceded by an initial denaturation at 95°C for 5 min and followed by 3 min of final extension at 72°C. Each cycle consisted of 1 min denaturation at 95°C, 2 min annealing at 57°C and 1.5 min elongation at 72°C. The reaction mixture for amplification included 1.0 or 2 mM $MgCl_2$, 1 unit Fast Start Taq DNA polymerase (Roche Diagnostics, Germany), 100-200 ng of DNA, 200 µM dNTPs and 2 pmol of HPV primers. A portion of each reaction product was electrophoresed on 2% agarose gel to check for the quality of the desired PCR products. If the amplified products were detected, then, the remaining portions of the samples were digested with 5 unit of MspI for identification of HPV 16. The digested products were analyzed in 2.5% agarose gel to identify the DNA bands (118 bp and 72 bp for HPV 16) [Figure 1]. The resulting gel pattern was visualized under ultraviolet (UV) light after ethidium bromide staining and de-staining in water. DNA from Caski cell lines were used as positive controls for HPV 16 and water, instead of DNA, was used as negative control in each PCR set. For final confirmation of the HPV type, the PCR products were transferred from agarose gel to Gene Screen nylon membrane for Southern hybridization with (^{32}P) labeled HPV type specific probe.

Screening of p53 gene polymorphism at codon 72, exon 4

Extraction of genomic DNA, for p53 detection, was carried out by the same method (above mentioned) except the reaction mixture (10 µl) for amplification included 1.8 µl $MgCl_2$ and dNTPs, 0.1 µl Taq DNA polymerase (Roche Diagnostics, Germany), 1 µl of DNA and 0.2 µl of p53 primers each and 6.7 µl H_2O. The forward primer used was 5' TTTTCACCCATCTACAGTCCCCCTTG3' and the reverse primer was 5' TAGGAGCTGCTGGTGCAGGGGCCCCG3'. A portion of each reaction product was electrophoresed on 2% agarose gel to check for the quality of the desired PCR product (162 base pairs). After PCR amplification, 10 ul products were digested with 5 units of Sma I in a total 15 µl volume and incubated at 37°C for 4 h and then subjected to agarose gel electrophoresis. In CC (Pro/Pro) genotype (+/+) both DNA were cut at 135 and 27 base pairs. In CG (Pro/Arg) genotype, one DNA was cut at 135 and 27 and the other was left uncut. In GG (Arg/

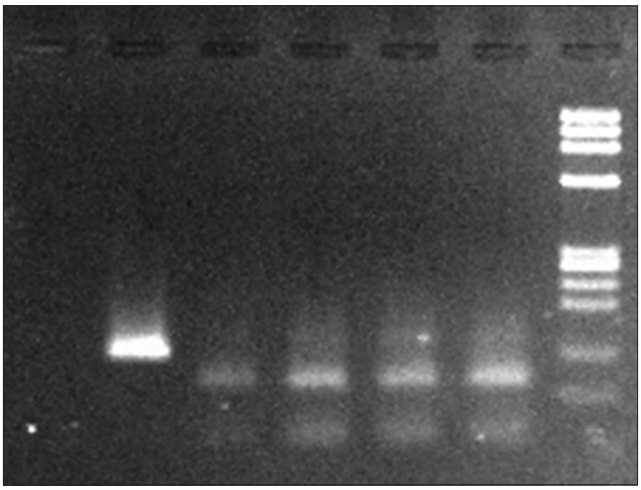

Figure 1: Agarose gel electrophoresis of human papilloma virus 16 polymerase chain reaction products (digested and undigested)

Arg) genotype, both DNA were left uncut. The resulting gel pattern was visualized under UV light after ethidium bromide staining and destaining in water. 10% of the amplicon was subjected to sequencing using exon specific sequencing primers. Sequencing was performed with a Dye Deoxy Terminator Cycle Sequencing Kit (ABI-Perkin Elmer, Weiterstadt, Germany) in an ABI-Prism 3,100 Avant Genetic Analyser (ABI-Perkin Elmer). Exons 4-9 were completely sequenced in all cases [Figure 2]. The re-sequencing results matched exactly with the genotype data from PCR restriction fragment length polymorphism method. Chi-square test was employed to analyze the results.

RESULTS AND OBSERVATIONS

Out of 91 oral leukoplakia patients and 100 controls from North-east India, most of the patients and healthy controls belonged to the low-income group (family income <100 US$ per month) and had similar nutritional status. None of the patients and controls was exposed to specific occupational or environmental carcinogens; so, environmental effects, other than tobacco use, were similar. Distribution of age, sex and tobacco habits in patients and controls are shown in [Table 1]. No significant difference was noted in number of smokers in oral leukoplakia and control groups statistically. Numbers of smokeless tobacco users were significantly less in oral leukoplakia group compared to controls (P < 0.0001). But, the numbers of mixed habitual were similar in both groups. Mean smoking and smokeless tobacco doses were not significantly different in oral leukoplakia patients and controls. However, the mean age of the controls was similar with that of oral leukoplakia patients. 45% of oral leukoplakia samples were HPV infected as compared to 23% in controls. HPV infection was associated with increased risk of oral leukoplakia (OR = 2.8, 95% CI = 1.2-6.5) [Table 2]. Four leukoplakia cases were excluded from the study due to the systemic illness.

No significant association between codon 72 polymorphism and oral leukoplakia was observed [Table 3] but, allele "C" (Proline) at this locus was significantly raised in leukoplakia patients (P = 0.04, OR = 1.57, 95% CI = 1.03-2.39) [Table 3].

Histopathologically, the HPV infected samples included 10 cases of mild dysplasia, 21 cases of moderate dysplasia and 6 cases of severe dysplasia. The correlation between HPV infection and the degree of dysplasia was found to be non-significant (P = 0.28) [Table 4]. About 65% of the 37 HPV infected oral leukoplakia patients were found to be smokers, 5% were smokeless tobacco chewers and 30% had mixed habits. 35% of the 23 controls were smokers, 39% were smokeless tobacco chewers and 26% had mixed habits [Table 5].

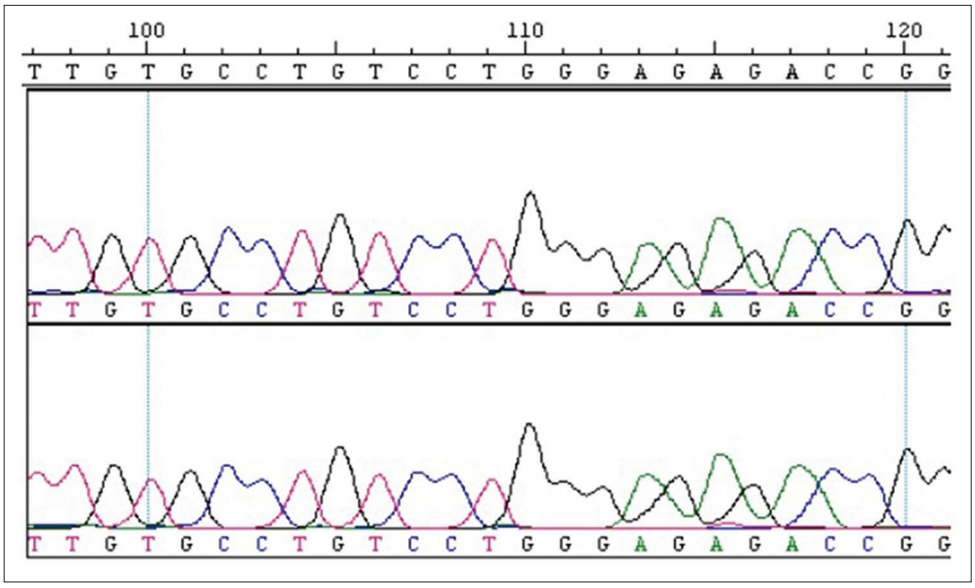

Figure 2: Electrophorogram showing a segment of deoxyribonucleic acid sequence of a polymerase chain reaction product for p53 (exon 4) gene

Table 1: Demography and tobacco exposure of oral leukoplakia patients and controls

Subjects and tobacco habits	Controls (*n*=100) (%)	Leukoplakia (*n*=91) (%)	*P* value
Sex			
Male	87 (87)	82 (90)	<0.001
Female	13 (13)	9 (10)	-
Age (years)			
Mean±SD	49±11	48±10	>0.05
Smoking habit only			
Smokers	45 (45)	54 (59)	-
Lifetime smoking range (PY)	1-50	2-90	-
Mean smoking dose±SD (PY)	18±10	25±13	>0.05
Smokeless tobacco habit only			
Smokeless tobacco users	37 (37)	9 (10)	<0.0001
Lifetime smokeless tobacco using range (CY)	1-480	3-700	-
Mean smokeless tobacco dose±SD (CY)	87±59	126±52	>0.05
Mixed tobacco habitual			
Smoking as well as Smokeless tobacco using habit	18 (18)	28 (31)	-
Lifetime smoking range (PY)	1-32	2-75	-
Mean smoking dose±SD (PY)	12±9	29±13	-
Lifetime smokeless tobacco using range (CY)	4-120	6-225	-
Mean smokeless tobacco dose±SD (CY)	35±19	70±53	-

SD=Standard deviation, CY=chewing-year (i.e., taking smokeless tobacco once in a day for 1 year=1 CY), PY=pack-years (one packet per day for 1 year=1 PY [1 pack=20 cigarettes or 40 bidies]). All patients and controls had tobacco exposure

Table 2: HPV infection status in leukoplakia patients and controls

HPV infection	Controls *n*=100 (%)	Leukoplakia *n*=87 (%)	Risk OR (95% CI)
HHPV (infected)	23 (23)	37 (43)	2.8 (1.2-6.5)
HPV (non-infected)	77 (77)	50 (57)	

HPV=Human papilloma virus, OR=Odds ratio, CI=Confidence interval. Four leukoplakia cases excluded due to systemic illness

Table 3: Distribution of genotypes at p53 exon 4 (codon 72) among leukoplakia patients and controls

Genotypes at p53 exon 4 (codon 72)	Control *n*=94 (%)	Leukoplakia *n*=86 (%)	*P* value	OR (95% CI)
Arg/Arg				
GG	21 (22)	14 (16)	-	-
Pro/Arg				
CG	51 (54)	38 (44)	0.943	1.12 (0.50-2.48)
Pro/Pro				
CC	22 (24)	34 (40)	0.087	2.32 (0.98-5.50)
Alleles at p53 exon 4				
Arg				
G	93 (49)	66 (38)	-	1.57 (1.03-2.39)
Pro				
C	95 (51)	106 (62)	0.044	-

Six genotypes could not be determined in controls, one genotype in Leukoplakia. Arg and Pro are amino acids at polymorphic site, GG, CC, CG are genotypes at polymorphic site. OR=Odds ratio, CI=Confidence interval

Table 4: Histopathological findings in HPV infected leukoplakia samples

Degree of dysplasia	HPV infected *n* (%) 37 (43)	HPV non-infected *n* (%) 50 (57)	*P* value
Mild dysplasia	10 (27)	15 (30)	0.28
Moderate dysplasia	21 (57)	26 (52)	
Severe dysplasia	6 (16)	09 (18)	

Four hyperplastic-infected leukoplakia samples were not considered for risk analysis. HPV=Human papilloma virus

DISCUSSION

In our study with oral leukoplakia patients, 59% were smokers, 10% were smokeless tobacco users and 31% were mixed habitual [Table 1]. Numerous studies show that the evidence for tobacco smoking etiology for oral leukoplakia is quite strong. Not only 70-90% of oral leukoplakia patients have such a habit, but 78% of lesions either completely

Table 5: Tobacco habits in HPV infected samples

Tobacco habits	HPV infected controls (*n*=23), (%)	HPV infected leukoplakia (*n*=37), (%)	*P* value
Smokers only	8 (35)	24 (65)	0.07
Smokeless tobacco users only	9 (39)	2 (5)	
Mixed habituals	6 (26)	11 (30)	

HPV=Human papilloma virus

disappear or regress within 12 months of smoking cessation.[24]

In our study, only 10% of the oral leukoplakia patients were females [Table 1]. The reason for the less number of females might be because probably lesser females visited for their dental problems to the dental college. The number of smokeless tobacco users among oral leukoplakia was very less (n = 10), out of which majority (n = 7) were females. Since females are mostly tobacco chewers so females as well as tobacco chewers were present less number in the oral leukoplakia samples. Similarly, less number of females was taken for the controls to match the oral leukoplakia samples since in the controls, 87% were males and 13% were females. Among the controls also, the majority (11 of 13) of smokeless tobacco users were females. Oral leukoplakia has always been found to be more prevalent in males except in regional populations in which women use tobacco products like men.[25] In United States, 74% of the affected persons were males in 1935 compared to 69% in 1988.[26] This decrease is a welcome change as oral leukoplakia in males has a much higher risk of dysplasia or malignant change than similar lesions in females.[27] The gender distribution varies ranging from a strong male pre-dominance in different parts of India to almost 1:1 in the western world. A study conducted in Ernakulum district of Kerala, India showed that the annual age adjusted incidence was 2.1 per 1000 among men and 1.3 per 1000 among women. The highest incidence of oral leukoplakia (6 per 1000) was in men who both chewed and smoked.[28]

Out of 37 of our HPV infected samples, 65% were smokers, 30% mixed habituals and 5% smokeless tobacco users [Table 5]. In a study by Dai *et al*., chewers (71.4%) were more likely to be positive for HPV DNA than smokers (32.1%).[29] Kozomara *et al*. reported higher incidences of HPV infection among smokers in their study of OSCC patients ($P < 0.05$).[30] So, both smokers and chewers could be equally infected by HPV. These results suggest that rate of HPV infection was not significantly associated with the habit present.

Our study showed that 43% of the oral leukoplakia samples were HPV infected compared to 23% in controls (OR: 95% CI = 2.8 [1.2-6.5]) [Table 2]. The incidence of HPV infected head and neck tumors varies in the scientific literature between 0% and 100%. Terai *et al*. pointed out that the rate depends on a number of factors including the source of the specimen, the detection method and the number of subjects and their ages. They found HPV in 30 out of their 37 specimens.[31] A study by Kozomara *et al*. shows a significantly higher infection in the group of smokers.[30] HPV infection rate was found to be 40.8% in premalignant lesions and 0% in control subjects in a study by Nielsen *et al*.[32] Bouda *et al*. found HPV in 30 of 34 oral leukoplakia patients.[10] It is, nevertheless, very significant that HPV type 16, a type demonstrated in oral leukoplakias and carcinomas, has been shown to induce dysplasia in squamous epithelium in an otherwise sterile *in vitro* environment.[33] HPV prevalence in hyperkeratosis and premalignant conditions has been found to be higher than that in malignant disease, which may suggest that HPVs are more likely to act as an initiator of epithelial proliferation or play a role in the early stage of oral carcinogenesis.[34] The detection rate of HPV-16 DNA in epithelial dysplasia (31/51, 61%) was found to be higher than that in normal mucosa (16/44, 36%) and in OSCC (30/86, 35%) in a study by Sugiyama *et al*.[35] Although a subset of normal population may carry HPV infections, each study could not demonstrate the presence of HPV in normal specimens. This wide range of HPV infection can be explained by different detection methods, varying amount of infection in various locations as well as different ethnic populations in patients. The presence of more HPV in premalignant lesions of the oral cavity as compared to normal mucosa in our study is strongly suggestive of HPV and host genome interaction.

In our study, p53 genotype did not modulate the risk of oral leukoplakia [Table 3]. Since GG (Arg/Arg) genotype was found in 16%, CG (Pro/Arg) in 44% and Pro/Pro in 40% of the oral leukoplakia patients similar to control samples [Table 3]. But, a study by Mitra *et al*. showed

that GG (Arg/Arg) genotype was at greater risk for developing oral leukoplakia.[19] One possible reason for this difference in results could be different sample sizes in these two studies. We can speculate that the processing of the signals generated from the tobacco associated DNA damage is differentially regulated by p53 alleles for the development of oral leukoplakia. So, study with more samples may be needed to get an association between p53 polymorphism and risk of oral leukoplakia. But, some studies failed to observe any risk of GG (Arg/Arg) genotype at codon 72 of p53 for oral cancer and HNSCC,[29] indicating a possible role of GG (Arg/Arg) variant of p53 codon in early stages of oral cancer development only. It was speculated that processing of the signals generated from the tobacco associated DNA damage is differentially regulated depending on specific p53 variant present and the development stage of the incipient tumor.[36] In our study, the oral leukoplakia patients showed a significantly higher "C" (proline) allele frequency (62%) at codon 72 of p53 as compared to controls.(P = 0.044) [Table 3]. Studies by Nagpal *et al*. found a striking reduction in Pro/Pro allele frequency in HPV positive cases, indicating Arg/Arg genotype to be more susceptible to HPV infection and oral carcinogenesis.[37] Also, a study by Storey *et al*. (1998) showed that arginine form of p53 was significantly more susceptible than the proline form to E6 mediated degradation.[38] Summersgill *et al*. found no association between p53 codon 72 polymorphism and HPV infection or between the p53 polymorphism and the risk of oral cancer.[39]

Saini *et al*. in their study, found HPV was found to be significantly associated with OSCC ($P < 0.001$) when compared to controls without any association between codon 72 polymorphism and oral cancer.[40] According to a study by Wang *et al*., combined risk genotypes of p53-related genes may modify risk of HPV16-associated oral cancer, especially in young patients, never-smokers and patients with oropharyngeal cancer.[41]

The following conclusions were drawn from our study:

- It was observed that HPV 16 infection increases the risk of oral leukoplakia.
- p53 genotype of codon 72 did not modulate the risk of oral leukoplakia, but proline allele was more prevalent in oral leukoplakia.

Thus, the process of carcinogenesis involves a complex conglomerate of tobacco habits, HPV infection and p53 polymorphism. The relative role of each factor as a causative factor for oral cancer and oral leukoplakia is debatable and is under investigation. The current research focuses on the role of HPV and genetic factors (p53) as causative agents in the development of oral leukoplakia, to combat cancer at an early stage.

CONCLUSIONS

Oral leukoplakia is a well known precancerous lesion. It is known to be associated with tobacco. But recently HPV and p53 have also been implicated in its pathogenesis. More research needs to be carried out to explore their role in the causation of leukoplakia.

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