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Role of Polymorphism of p53 Gene Codon 72 in HPV-associated oral lichen planus in south Indian population: A PCR-based study

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Abstract--Background: High-risk Human Papillomavirus infections, and polymorphism of the p53 gene, have been implicated in oral potentially malignant disorders. The present study determines whether the polymorphism at codon 72 of the p53 gene and the presence of HPV-16/18 strains are associated with OLP patients. Materials and methods: The biopsy specimens were obtained from 250 OLP individuals. The samples were processed for HPV-DNA analysis and polymorphism of the p53 gene using PCR. Data were analyzed by applying the Chi-square test. Results: There were 103 positive HPV sample specimens with 34 males and 69 females which were shown to be statistically significant ($p < 0.001$). Arg/Arg, Pro/Pro, and Arg/Pro were found in 19.4%, 31.1%, and 50% of HPV-positive cases of OLP, respectively, compared to 17%, 47%, and 36% of HPV-negative samples, which were found to be statistically significant between the two groups. The Arginine allele (44%) was more prevalent in HPV-associated OLP than in HPV-negative OLP cases (35%) which was found to be statistically significant between the groups. Conclusion: The results of the current study relate to the premalignant potential of OLP based on the occurrence of codon 72 of the p53 gene polymorphism in HPV-associated OLP patients in terms of genotype and allele.

Keywords--arginine, human papillomavirus, oral lichen planus, p53 polymorphism, proline.

Introduction

The World Health Organization (WHO) has considered oral lichen planus (OLP) as an oral potentially malignant disorder (OPMD) [1,2]. It is one of the inflammatory conditions associated with a markedly elevated risk for Oral Squamous Cell Carcinoma (OSCC). OLP is reportedly more common in India (2.6%), followed by Sweden (1.9%), Turkey (1.15%), Japan (0.5%), and Malaysia (0.3%). According to various studies, the rate of malignant transformation of OLP ranged from 0.04% to 1.74%. It is estimated that OSCC will evolve from OLP at a rate of approximately 1% every five years [3]. The majority of OPMD-related OSCCs are caused by genetic changes. While not all OPMDs have the potential to develop into OSCC, they form a group of disorders that display a few unique morphological traits, some of which have a higher propensity to develop into a malignancy. The propensity of OLP for malignancy has long been debatable. The lack of broadly adopted diagnostic criteria for OLP has been identified as the primary impediment in understanding the malignant changes of OLP [2].

Squamous epithelial cells in the oral cavity are infected by the Human Papillomavirus (HPV) E6 and E7 oncoproteins, which are crucial for oncogenesis in cervical cancer, driven by high-risk strains of HPV-16/18 [4,5]. Patients with both wild-type and p53 mutations were shown to have high-risk HPV infection [6]. The E6 and E7 viral proteins expressed by the two high-risk HPV-16/18 strains interact in unison in the degeneration of keratinocytes and are essential for maintaining the oncogenic phenotype. While the E7 protein integrates to the cellular tumour suppressor protein Rb and deactivates it, the E6 forms a complex with the p53 protein and controls its proteolytic disintegration by ubiquitin pathways, which interferes functionally with the management of the cell cycle [7,8]. It is well-documented that more than half of all malignancies are caused by mutations in the p53 gene, a paramount gatekeeper gene in managing the replication process, apoptosis, and Deoxyribonucleic Acid (DNA) transcriptional regulation. The over-expression of p53 gene was found in OPMDs as opposed to OSCC [2]. Inflammatory processes and cytokine pathways trigger DNA damage, induce cellular proliferation, suppress apoptosis, and fundamentally alter the proteins of oral epithelium, creating a milieu that is similar to malignancy in OLP and facilitating its malignant evolution [1].

According to the reports on molecular changes employing microsatellite biomarkers, the p53 tumour suppressor gene is inactivated in oral malignancies and oral lesions resulting in changes in gene expression. Oral malignancies and OPMD have also shown the inhibitory effect of p53 gene through mutations, over-expression, deletions, and adherence to oncoproteins. Over 25% to 70% of oral cancers have p53 mutations, which make up the most often mutated gene [9]. Proline (Pro) or Arginine (Arg) amino acids, which are encoded by the frequent polymorphism in the p53 gene at codon 72, have been associated with a higher risk of developing specific malignancies, with the Arg/Arg genotype being more common in cervical cancer and a preponderance of the Pro/Pro genotype in lung cancer [4,10]. There is a debate concerning the mechanisms enabling the over-expression of p53 in OLP. According to numerous authors, p53 over-expression is a type of cellular reaction to the hyper-proliferative condition frequently observed

in OLP. The normal mechanism of activation of the p53 system prioritizes molecular action in OLP to facilitate the repair of DNA [11].

The term "malignization" refers to conditions in which the function of p53 is compromised by mutations or other inactivating mechanisms, which may be a crucial step in the course of a malignant transformation. The form of cell response, which may be observed in some instances of OLP malignancy, is an effort to protect the epithelial framework in order to prevent the development of ulcerations. In this situation, the cells are damaged by T-lymphocytes of the associating inflammatory infiltration. They are then potentially exposed to mutagenic stimuli such as O₂ and Ni free radicals, and cyclooxygenase-2 pathway. Later these cells show an upsurge in their proliferation rate rather than undergoing apoptosis. These processes cause the build-up of oncogenic molecular events resulting in the emergence of cancer. Nonetheless, OLP has a low risk of malignant transformation [11].

According to a meta-analysis, Asia, especially India, has a greater rate of HPV-associated OLP lesions [8]. The prevalence of HPV-16 and HPV-18 strains was found to be 42% and 47% respectively in South Indian Population. However, the data on the subpopulation in the Western part of the country had 15% of HPV-16 infection with no evidence of HPV-18 strain. Furthermore, there is no detailed information on the prevalence of p53 codon 72 polymorphism in the Indian population [5]. In addition, the results based on the molecular analysis of OLP in this arena are quite scarce. Consequently, the present study has been conducted to determine whether the polymorphism at codon 72 of the p53 gene and the presence of HPV-16/18 are associated with OLP patients from the South Indian population.

Materials and Methods

A clinico-histopathological investigation that was approved by the Institutional Ethics Committee was carried out in accordance with the STROBE criteria for observational studies. Data were collected from 250 individuals who came to the Department of Oral Medicine and Radiology and were clinically diagnosed with reticular and erosive OLP between September 2018 and December 2021. The study was carried out after receiving written informed consent, in accordance with the Declaration of Helsinki. Patients with a history of systemic diseases, who were smokers/drinkers, who had immune-mediated hypersensitivity reactions to tooth restorations, or who were taking any drugs, such as oral hypoglycemics or angiotensin-converting enzyme inhibitors, were excluded from the study. Specimens with appropriate tissue measuring at least 5x5 mm were acquired from the 250 cases and stored in a biopsy vial containing DNA stabilizer solution.

Each sample was separated into two parts: one is for histopathologic evaluation to validate the clinical diagnosis and the other is for characterization and typing of HPV-DNA as well as for detection of polymorphism. Cases were subsequently removed from the study if they did not fulfill both the clinical and histological criteria for OLP as defined by the Van der Meij and Van der Waal criteria. Following the evaluation of Haematoxylin and Eosin-stained sections by two expert oral pathologists, a consensual histological diagnosis was achieved in all

patients. The section of biopsy sample that was processed for HPV-DNA analysis was transported to a deep freezer (-80° C) immediately and preserved until the specimens were retrieved for further procedures.

HPV-DNA Extraction

The Cetyltrimethylammonium bromide (CTAB) technique was used to extract genomic DNA from the tissue sample. To assess the quality of the DNA, PCR was performed using GAPDH primers (Product Size: 496bp; GAPDH F: TTCTGGGGACTGGCTTTCC; GAPDH R: AAAGTGGTCGTTGAGGGCAA). Bands were recognized in all the 250 specimens. To eliminate contamination, a no template control is included in the last lane. To visualize the bands, the isolated DNA was loaded onto a 1.2% Agarose gel electrophoresis.

Quantification of Isolated DNA

The quantity of extracted DNA was measured using a UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). A 50-times dilution of the stock is made by mixing 1 µl DNA with 49 µl sterile distilled water. To ensure the purity of the DNA preparations, the A260/A280 ratio was determined.

Single polymerase chain reaction (PCR) assay

A single PCR assay was employed to determine the target HPV-16 and HPV-18 DNA. PGMY09/11 primers were used to detect HPV (500 bp HPV-16 and 200 bp HPV-18). The following primers for the E6 region were used in the PCR:

- HPV16F: 5'GTCAAAAGCCACTGTGTCCT3'
- HPV16R: 5'CCATCCATTACATCCCGTAC3'
- HPV18F: 5'CCGAGCACGACAGGAACGCT3'
- HPV18R: 5'TCGTTTTCTTCCTCTGAGTCGCTT3'

Each PCR reaction mixture contained 2 µL of 10 times PCR buffer with MgCl₂ (1.5mM), 2 µL of dNTP mix (2.5mM), 2 µL of oligonucleotide primer F (10picomoles/µL), 2µL of oligonucleotide primer R (10picomoles/µL), 10.70 µL of H₂O, 1 µL of template DNA (50ng/µL), 0.30 µL of Taq-polymerase (5 U) making the total amount of each reaction to 20.0 µL. The following were the thermocycling conditions: 3 minutes of initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 50 seconds, 40 seconds of annealing at 50°C, 1.30 minutes of extension at 72°C, and 40 seconds of final extension at 72°C.

Analysis of p53 polymorphism

Genomic DNA from each sample was amplified using an allele-specific PCR to study the variant. To amplify the Arg and Pro alleles, two sets of primers (forward [F] and reverse [R]) were employed as follows:

- Arg F: TCC CCC TTG CCG TCC CAA
- Arg R: CTG GTG CAG GGG CCA CGC
- Pro F: GCC AGA GGT TGC TCC CCC

- Pro R: CGT GCA AGT CAC AGA CTT

The PCR products were 177 base pairs (bp) for the Pro allele and 135 bp for the Arg allele based on which set of primers could attach. The PCR reaction was carried out in a final reaction volume of 20 μ l, containing 10 μ l of 2x Emerald Master Mix, 1 μ l of Forward and Reverse Primers (10 pmol/ μ l), and DNA. Initial denaturation at 94°C for 3 minutes was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 68°C (for Arg primer) & 60°C (for Pro primer) for 30 seconds, and elongation at 72°C for 30 seconds. Finally, samples were incubated for 5 minutes at 72°C. The resultant PCR product was identified using 2% agarose gel electrophoresis.

Statistical analysis

SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, USA), was used to perform the statistical analyses. Following PCR amplification, the genotype distribution and allelic frequencies were evaluated using the Chi-square test of association at a 5% significance level to determine the risk of the observed polymorphisms being related to positive HPV-DNA.

Results

The age of the patients providing samples ranged in age from 15 to 75 years with 48% (n=120) and 52% (n=130) males and females respectively. The p53 codon 72 polymorphisms were studied in a study sample of OLP with and without HPV-DNA. A single Arginine or Proline base pair substitution resulted in three different genotypes: homozygote for Arg (Arg/Arg), homozygote for Pro (Pro/Pro), and heterozygote (Pro/Arg). A total of 250 OLP samples were examined for PCR detection of HPV infection, with 103 sample specimens being positive for HPV-16 and HPV-18, and 147 cases being negative for all types of HPV. There were 34 males and 69 females with HPV-related OLP lesions. The gender distribution of OLP cases with and without HPV was shown to be statistically significant ($p < 0.001$). According to the results in Figure 1, the Arg/Arg homozygous genotype was observed to be less common in the entire study sample than Pro/Pro and Arg/Pro.

The frequencies of the three p53 genotypes were found to be statistically highly significant in the sample population. Arg/Arg, Pro/Pro, and Arg/Pro were found in 19.4%, 31.1%, and 50% of HPV-positive cases of OLP, respectively, compared to 17%, 47%, and 36% of HPV-negative samples of OLP (Table 1). In HPV-positive patients, the frequency of Arg/Arg and Ar/Pro genotypes was found to be higher than in HPV-negative cases. In HPV-negative group, the Pro/Pro genotype was shown to be more common than in HPV-positive cases. Figure 2 shows that the Proline allele was observed to be more common in the study sample than the Arginine allele. However, the Arginine allele (44%) was more prevalent in HPV-positive OLP than in HPV-negative OLP cases (35%). As shown in Table 1, the Proline allele (65%) is more prevalent in HPV-negative OLP than in HPV-positive OLP patients (56%). The difference in allele distribution between the two groups was determined to be statistically significant ($p < 0.001$).

Table 1
Frequencies of polymorphism of p53 gene codon 72 with respect to gender, genotype and allele in the study sample

Variables	Patients	OLP with HPV N(%)	OLP without HPV N(%)	Chi-square test	p-value
Gender	Male	34 (33)	86 (58.6)	15.77	<0.0001**
	Female	69 (67)	61 (41.4)		
Genotype	Arg/Arg	20(19.4)	25(17)	6.61	0.04*
	Pro/Pro	32 (31.1)	69(47)		
	Arg/Pro	51(50)	53(36)		
Allele	Arg	91(44)	103(35)	4.26	0.04*
	Pro	115(56)	191(65)		

*Significant; ** Highly significant

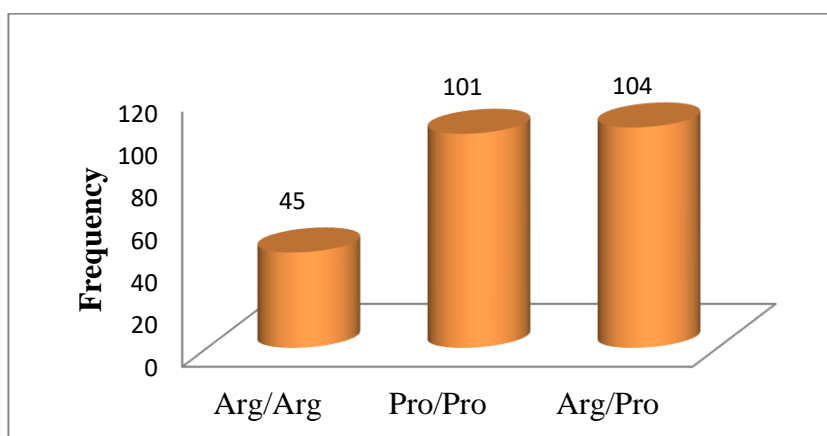


Figure 1. Frequency of distribution of the three p53 genotypes in the study sample

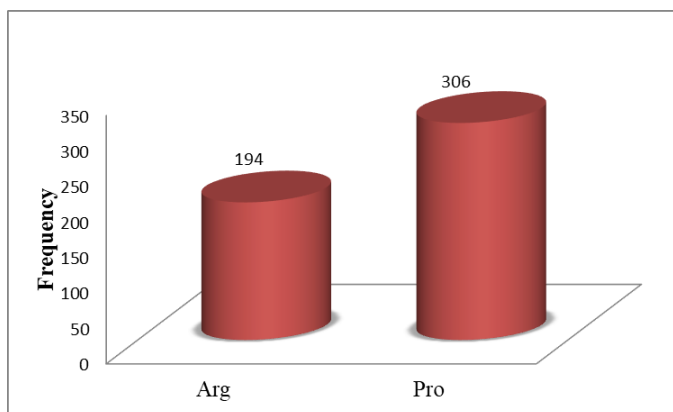


Figure 2. Frequency of distribution of Arginine and Proline allele in the study sample

Discussion

The results of the study showed a statistically significant relationship between gender and HPV-positive/negative groups. Females (67%) had an increased prevalence of OLP-associated HPV samples. It is noteworthy that in the present study of OLP samples without a history of using tobacco or alcohol, a statistically significant correlation between genotype, allele, and HPV infection was established. This was consistent with the earlier study that described OLP as a mucocutaneous disorder typified by nonspecific inflammation that resulted in extensive basal epithelial layer degradation [12]. It is imperative to explore the premalignant nature of OLP not only in the histopathological sample but also in the molecular analysis of the two alleles of p53 polymorphism. Keratinocyte degeneration in OLP is caused by apoptosis [13]. According to findings by Pim et al. [14], Proline has a lower propensity to cause apoptosis than Arginine. The reduced apoptosis occurring in presence of Proline polymorphism, enables exaggerated proliferation and malignant transformation of OLP [13,15].

The p53 codon 72 Arg/Pro polymorphism has received a lot of attention recently. One of the most often mutated genes in malignancies is the tumour protein p53 gene, which is found on chromosome 17p13. It has been suggested that this gene plays a key determining role in cancer. The significance of single-nucleotide polymorphisms (SNP) in p53 and their associated risk of various malignancies have been extensively explored since mutations do not increase the non-familial risk of developing cancer in the general population. They have been subjected to comprehensive evaluation as risk modulators for various cancers and other disorders [12]. It is now understood that the p53 tumour suppressor gene regulates the cell replication process, DNA repair, and apoptosis to maintain genomic integrity [16,17].

In response to DNA damage, this gene activates its downstream target (p21WAF1) to halt the cell cycle and aid in cell repair. However, p53 can cause apoptosis by stimulating the gene transcription like PUMA, Bax, and Fas if indeed the cellular damage is severe and further beyond regeneration [15]. Several studies have also proven that p53 can induce apoptosis in a transcriptionally independent manner [14]. Evidence suggests that apoptosis resulting from the production of the p53 protein is the primary cause of OLP [11,15]. Oral epithelial dysplasia and OSCC have both been associated with p53 gene dysfunction or mutant p53 [17]. The Proline-rich region of p53 is one of the components of the protein that is assumed to be necessary for its potential to trigger apoptosis. Interestingly, there is a frequent polymorphism in this domain at position 72, which either encodes for Proline or an Arginine residue.

According to the pattern of this polymorphism, a series of recent investigations have identified potentially significant biochemical variations in the activity of the p53 gene. Initial studies have prompted interest in this context that suggested the polymorphism could influence the vulnerability of the p53 gene to HPV E6-induced disintegration and that the Arg72 protein was more subjected to degeneration than the Pro72 form [14]. The findings of the present study was concurrent with the finding that Arginine allele was more prevalent in HPV-associated OLP group compared to that of the HPV-negative OLP sample.

Furthermore, a statistically significant association was found between the polymorphism of the p53 gene codon 72 in HPV-associated OLP and HPV-negative OLP group. Epidemiological studies have also described that variations in polymorphism may influence the susceptibility to certain malignancies, whether or not they are HPV-related [14].

Tabatabaei et al., [13] reported that the heterozygous genotype (Arg/Pro) had the highest frequency of 71.4%, followed by the homozygous genotype Arg/Arg and Pro/Pro with a frequency of 21.4% and 7.1% respectively in the OLP sample. Further, in another similar study conducted by Yanatatsaneeji et al. in OLP cases, the Pro/Pro genotype had been identified as one of the genomic risk factors that could cause OLP and was the most prevalent [15]. However, the results of the present investigation in HPV-associated OLP sample demonstrated that the frequency of Arg/Pro was the most prevalent genotype followed by Pro/Pro and Arg/Arg form. The findings of the present study were retrieved by comparing the HPV-associated OLP cases with HPV-negative OLP samples. On the contrary, it is significant to highlight the study conducted by Storey et al., [18] who compared HPV-positive cervical malignancies and HPV-negative control group who were healthy and normal.

He claimed that a distinct p53 allele polymorphism renders women genetically extremely vulnerable to contracting cervical cancer if exposed to the high-risk HPV-16/18 strains. He further demonstrated that p53 is increasingly susceptible to HPV E6-mediated proteolytic degradation in-vivo. It was further documented that the Caucasian women carrying a greater frequency of p53 Arg homozygous alleles have an approximately seven-fold increased likelihood of contracting HPV-induced cervical cancer compared to those carrying Pro/Arg heterozygous genotype. The results of this investigation concur with those of Chuery et al., [4] who found that the Arg allele, Arg/Arg, and Arg/Pro genotypes were considerably more prominent in HPV-positive samples than in HPV-negative samples. The prevalence of the Pro/Pro genotype in the present study was, however, more common in the HPV-negative group than in the positive group, which was consistent with the earlier study [4]. It should also be noted that a study by Makni et al. revealed significant inter-laboratory variations in the proportions of Arg/Arg, Arg/Pro, and Pro/Pro genotypes [19].

P53 is a tumor-suppressor gene that prevents the development of cancer by repairing DNA damage and causes disruption of the cell cycle through the induction of apoptosis [5]. The concurrently aging process could also favour malignant transformation though. p53 inactivation is a frequent occurrence in OSCC which were influenced by factors such as molecular changes occurring in the p53 pathway, and the presence of the HPV virus [20]. The insight into the relationship between p53 polymorphisms and predisposing factors of carcinogenesis has recently attracted a lot of attention. In this gene, 13 polymorphisms have been identified. The one that is most commonly explored is a single nucleotide polymorphism (SNP) that causes Proline to substitute Arginine in the trans-activating site at codon 72 in exon 4. For this SNP, the G allele encodes an Arginine where a Proline would normally be found at position 72 of the protein. This results in a minor allele homozygote known as a Pro/Pro genotype, a major allele homozygote referred to as an Arg/Arg genotype, or a

heterozygote form also known as an Arg/Pro [13,21]. Pro72 and Arg72 are two polymorphism variations that affect the structure and functionality of the p53 protein [22].

It has been noted that p53 polymorphism with homozygous Arg/Arg genotype is more subject to degradation by HPV E6 protein than either the Arg/Pro heterozygote or the Pro/Pro homozygote genotype and may be associated with a poor prognosis [18,23]. The Pro/Arg polymorphism might affect the outcome of HPV infection because p53 is indispensable for host response against malignancies. No conclusive relationship between either the presence or lack of a specific polymorphism of p53 in tumours that are not HPV-associated has been established yet. Contrarily, our findings suggest that the p53 polymorphism at codon 72 may offer a significant risk factor in HPV-associated OLP cases due to the preponderance of Arg allele, Arg/Pro, and Arg/Arg genotype in the HPV-positive group [7].

In oral malignancies with HPV positivity, Nagpal et al. [5] revealed a remarkable decrease in the frequency of the Pro/Pro genotype. This suggests that individuals with the Arg/Arg genotype may be more prone to HPV infection and oral malignancies. Tandle et al., [10] on the contrary, reported that there was neither increased frequency of the Pro/Pro nor Arg/Arg genotypes in oral malignancy as compared to healthy control. In terms of geographical variance, it is documented that the people living near the equator appear to have a greater frequency of Pro alleles compared to populations inhabiting the nations farther North. There are also considerable racial and ethnic differences in the genotype pattern of the p53 codon 72 polymorphism [4,6]. Fakhrijou et al. found that the rate of p53 expression in OLP was higher than in normal counterparts in the Iranian population. He further suggested that p53 might be utilized as a biomarker and that p53 polymorphism could be used to predict poor prognostic outcomes [24].

Nevertheless, the study results from India by Katiyar et al. suggest that the p53 codon 72, Arginine polymorphism might not appear to represent a significant risk factor for the development of oral, cervical, and breast cancers in the Indian population [7]. On the other hand, codon 72 polymorphism was assumed to work independently of one another, and their expression levels were also believed to influence the risk of developing cancer. The findings of Addala et al., suggest that the p53 codon 72 polymorphism might act as a genetic risk factor for OSCC. The p53 protein modulates cell genomic stability in a pleiotropic manner. Additionally, the p53 Arg72 protein molecule might be associated with an elevated risk of malignancies in the South Indian Population [25].

To the best of our knowledge, the data from the current study are the first to imply that the Arg allele, Arg/Arg, and Arg/Pro genotypes were related to a higher likelihood of positive HPV-DNA expression in OLP cases in the South Indian population. The results of extensive research on the relationship of polymorphism to high-grade lesions or cervical cancer vary depending on the geographical location of the study [4]. Given that the function of the p53 polymorphism at codon 72 may vary depending on the study population, further extensive research in different populations to explore the relationship of p53 polymorphism to high-grade oral lesions should be established. A thorough review of the literature

indicates that impaired p53 gene function has been linked to the occurrence and progression of epithelial dysplasia and OSCC. Henceforth, detecting p53 polymorphism may aid in the detection of high-risk lesions such as OLP that have the potential for malignant transformation [26]. Since this was a cross-sectional study without a long-term follow-up, it was not possible to determine whether the association between Arg/Arg, Arg/Pro genotypes, and positive HPV-DNA expression is sustained over time or associated with an elevated risk of developing neoplastic lesions. The use of molecular biology to diagnose oral precancerous lesions and cancer may significantly improve the early detection of molecular alterations that are inconspicuous under the microscope since such polymorphism could represent a genetic predisposing factor.

Conclusion

In the study sample, it was shown that females had a greater preponderance of HPV infections. The Arg/Arg and Ar/Pro genotypes were more frequently found in OLP cases that tested positive for HPV. In HPV-negative patients, a greater prevalence of the Pro/Pro genotype was seen. The Arginine allele was more common in OLP with HPV-positive cases, whereas the Proline allele was quite common throughout the entire sample of OLP. Based on the relevance of the occurrence of codon 72 of the p53 gene polymorphism in the HPV-associated OLP samples in terms of genotypes and alleles, the results of the current study relate to the premalignant potential of OLP.

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