Turk J Med Sci
31 (2001) 509-515

Mehmet YALTIRIK[1]
Ayla ÖZVEREN[2]
Canan ALATLI[2]
Nevin BÜYÜKAKYÜZ[1]

Detection of Human Papilloma Virus in Benign, Malignant and Pre-Cancerous Lesions of Oral Mucosa By in Situ Hybridization

Received: August 03, 2000

[1]Department of Oral Surgery, Faculty of Dentistry, Istanbul University
[2]Department of Tumor Pathology and Oncological Cytology, Institute of Oncology, Istanbul University, İstanbul - TURKEY

Abstract: HPV is an epitheliotropic virus, predominantly associated with human skin and mucosal lesions. In many studies, the presence of HPV types 6/11, 16/18 and 31/33/51 have been demonstrated in oral lesions.

The aim of this study is to investigate the presence of specific types of HPV in oral benign and precancerous epithelial lesions and oral carcinomas, and the etiological significance of HPV in oral epithelial malignancies.

In the present study, twenty oral lesions, of which seven were papilloma, seven squamous cell carcinoma, four leukoplakia and two lichen planus, were tested for different types of HPV. In addition, these patients were questioned regarding their alcohol consumption, smoking habits and presence of oral prothesis.

Two of the seven specimens from patients with squamous cell carcinoma were positive for 16/18 and 31/33/51HPV types. In four of the seven papilloma, specimens HPV types 6/11 and 31/33/51 were positive. Three of the six precancerous lesions were positive for all types of HPV tested.

In conclusion, it can be stated that the presence of HPV in oral lesions may have a part in the development of malignancy.

Key Words: HPV, In situ hybridization, oral lesions

Introduction

In recent years, the frequency of pre-cancerous lesions and cancers in the oral cavity has prompted studies as to the aetiology and pathogenesis of these lesions. It is suggested that chemical carcinogens, radiation energy, chronic irritation and viruses play an important part in their aetiology (1-4).

It is well known that viruses generally infect the anogenital region. Infections of the larynx, conjunctiva and oral cavity have also been reported. (5-10). Kashima et al. (4), Zeuss et al. (10), Watts et al. (11), Woods et al. (12), and Miller et al. (13) suggested that specific types of human papilloma virus (HPV) exhibited various distributions in the lesions of the oral cavity. In their studies, they demonstrated that HPV 6/11 was present in benign cases, while HPV 16/18 and HPV 31/33/51 were present in intraepithelial neoplasms and cancers.

HPV infection is generally seen in the keratinocytes of the epidermis. Initially, viral particles pass from an eroded region of the epithelium into the cells in the basal layer (17, 18). After penetrating into the cells of the basal layer, the virus stimulates the synthesis of regulatory proteins for viral DNA replication. Early viral genes stimulate cellular division in basal cells. As a result of this stimulation, excess cellular division reveals itself as hyperplasia in the upper layers of the epithelium. The cells on the more superficial layers undergo nuclear degeneration and perinuclear cytoplasmic vacuolation, called koilocytosis. As the basal cell differentiates and proceeds towards the upper layers, viral DNA replication is stimulated, synchronized and stabilized by cellular DNA replication. As the infected keratinocytes are shed, the virus infects other areas (16-20).

Well known etiological factors in the development of oral leukoplakia and cancer are smoking, irritation of oral restorations, and poor oral health. Alcohol is not considered as a direct etiological agent in oral cancer, but is a factor which decreases the immunological resistance of the patient, providing suitable conditions for other carcinogenic factors (3,15-20).

The aim of this study is to investigate the presence of specific types of HPV in cytological material and tissue sections of oral lesions, since HPV is thought to have a synergistic effect in the pathogenesis of benign and

precancerous lesions and cancers of the oral cavity, together with other etiological factors such as micro traumas and chemical agents.

Materials and Methods

In this study, a total of 20 cases were investigated, 7 of which were diagnosed as squamous cell carcinoma, 7 as papillomatous lesion, and 6 as lichen planus or leukoplakia as a result of clinical and histopathological studies. All cases were investigated for location, age, gender, smoking habits and alcohol consumption. In the histopathological examination of squamous cell carcinoma, differentiation grade, presence koilocytosis and keratosis were evaluated (Table 2). In the papilloma and precancerous lesion groups only koilocytosis and keratosis were graded as well as the presence of HPV types (Table 3).

In our study, a Patho Gene DNA Probe Assay Kit (Enzo Diagnostic Inc.) was used and the presence of HPV 6/11, 16/18 and 31/35,51 types were investigated by the method of in situ hybridization. Three different HPV probe reagents, HPV6/11 tissues control slide and HPV16 probe control slide were provided. Before the

Table 1. Findings of localization, histopathological diagnosis, age, gender, smoking habits and alcohol consumption in all cases.

Localization	Diagnosis	Age	Gender	Smoking	Alcohol
Lingual mucosa	Squamous cell carcinoma	51	Male	+	-
Lingual mucosa	Squamous cell carcinoma	38	Female	+	+
Lingual mucosa	Squamous cell carcinoma	31	Female	-	-
Lingual mucosa	Squamous cell carcinoma	58	Female	-	-
Lingual mucosa	Squamous cell carcinoma	65	Male	+	+
Lower lip	Squamous cell carcinoma	63	Male	-	-
Upper lip	Squamous cell carcinoma	72	Female	-	-
Cheek mucosa	Papillom	72	Female	+	+
Cheek mucosa	Papillom	34	Male	+	+
Cheek mucosa	Papillom	32	Male	+	-
Cheek mucosa	Papillom	33	Male	-	-
Lower lip	Papillom	15	Female	-	-
Palate	Papillom	72	Female	-	-
Palate	Papillom	55	Female	-	-
Lower lip	Leukoplakia	38	Male	-	-
Lower lip	Leukoplakia	35	Female	-	+
Lower lip	Leukoplakia	50	Male	+	+
Cheek mucosa	Leukoplakia	33	Male	+	+
Cheek mucosa	Lichen planus	42	Male	+	+
Cheek mucosa	Lichen planus	32	Male	+	+

Table 2. Differentiation grade, presence of koilocytosis and presence of HPV types in squamous cell carcinoma specimens.

Diagnosis	Localization	Grade	Paraffin section				Imprint slide		
				HPV			HPV		
			Koilocytosis	6/11	16/18	31/33/51	6/11	16/18	31/33/51
Squamous cell carcinoma	Lower lip	I	Moderate	-	-	-	-	-	-
Squamous cell carcinoma	Upper lip	III	Mild	-	-	-	-	-	-
Squamous cell carcinoma	Lingual mucosa	III	Mild	-	-	-	-	-	-
Squamous cell carcinoma	Lingual mucosa	II	Mild	-	-	-	-	-	-
Squamous cell carcinoma	Lingual mucosa	II	Mild	-	-	-	-	-	-
Squamous cell carcinoma	Lingual mucosa	II	Mild	-	-	-	-	+	+
Squamous cell carcinoma	Lingual mucosa	II	Mild	-	-	+	-	-	+

Table 3. Koilocytosis, keratosis and HPV positivity in papilloma and precancerous lesion groups.

Diagnosis	Localization	Paraffin section					Imprint slide		
				HPV			HPV		
		Koilocytosis	Keratosis	6/11	16/18	31/33/51	6/11	16/18	31/33/51
Papillom	Palatinum of mouth	Moderate	Orthokeratosis	+	-	-	-	-	-
Papillom	Palatinum of mouth	Severe	Parakeratosis	-	-	-	-	-	-
Papillom	Lower lip	Mild	Hyperkeratosis	-	-	-	-	-	-
Papillom	Cheek mucosa	Severe	Hyperkeratosis	-	-	-	-	-	-
Papillom	Cheek mucosa	Mild	Hyperkeratosis	-	-	+	-	-	-
Papillom	Cheek mucosa	Mild	Hyperkeratosis	+	-	-	-	-	-
Papillom	Cheek mucosa	Moderate	Hyperkeratosis	+	-	+	-	-	-
Leukoplakia	Cheek mucosa	Mild	Hyperkeratosis	-	-	+	-	-	+
Leukoplakia	Lower lip	Moderate	Hyperkeratosis	+	+	-	-	-	-
Leukoplakia	Lower lip	Mild	Parakeratosis	-	+	-	-	-	-
Leukoplakia	Lower lip	Mild	Hyperkeratosis	-	-	-	-	-	-
Lichen planus	Cheek mucosa	Mild	Hyperkeratosis	-	-	-	-	-	-
Lichen planus	Cheek mucosa	Moderate	Parakeratosis	-	-	-	-	-	-

fixation of the biopsy specimens, they were imprinted on six separate slides covered by Poly-L-Lysine. After being fixed at least for 15 minutes in 96% alcohol, the imprint slides were stained by the Papanicolaou (Pap) technique, and those that were rich in cells were marked. Three slides of each case were chosen for the application of in situ hybridization. These slides were destained by 1% HCL/ethyl alcohol solution for 15 minutes. Then, they were dehydrated in a sequential alcohol series.

The biopsy specimens were fixed in 10% buffer-formalin solution for 4-8 hours and after routine tissue processing they were embedded in paraffin blocks. In order to determine the 6/11, 16/18, and 31/33,51 types of HPV, three separate sections of 4-6 microns were prepared from each case and were placed on slides with specific pits. Deparaffinization processes were carried out on both the biopsy specimen slides and the tissue control slide of HPV 6/11.

Then, Proteinase K working solution was obtained by diluting the Proteinase K stock solution ten-fold with wash buffer. Biopsy specimen and tissue control slides were placed on the 37ºC heating block, 0.4 ml Proteinase K working solution was added and the slides were incubated at 37ºC for 15 minutes. The imprint slides were incubated in the same solution at 37ºC for 4-5 minutes. Then, the biopsy specimens were incubated in Quench Reagent (buffered NaCl in EDTA 3% hydrogen peroxide) for 10 minutes at 37ºC. Later, 0,06% H_2O_2 was dropped on the imprint slides instead of Quench Reagent. Then they were incubated at room temperature for 30 minutes.

Afterwards, all slides were dehydrated. After these procedures were complete, one drop of each solution containing HPV 6/11 DNA probe, HPV 16/18 DNA probe and HPV 31/33/51 DNA probe, labelled by biotin, were dropped on the tissue sections. DNA denaturation and hybridization were obtained by incubating them first for 8-10 minutes at 95ºC and then for 20-30 minutes at 37ºC.

The imprint slides were incubated for 5 minutes at 95ºC for denaturation and 16 hours at 37ºC for hybridization.

Later, post-hybridization reagent was dropped on all the slides to fix the hybridizations formed, and the slides were stored at 37ºC for 10 minutes. In order to determine the target nucleotide, detection reagent (streptovidin-biotinlated horseradish peroxidase in HCL) was dropped on the slides and they were incubated at 37ºC for 10 minutes. Then, all the slides were incubated with chromogen-substrate (3 amino 9 ethylcarbazole) solution at room temperature for 10 minutes.

The slides were stained with Meyer's Haematoxylin for counter staining and then they were covered by cover glasses, using an aqueous mounting medium.

The stained slides were examined under a light microscope, and the presence of HPV was determined to be a positive reaction by the presence of pink-red or brick red staining in the nuclei of epithelial cells.

Results

Location, age, gender, smoking and alcohol consumption of the 20 cases are shown in Table 1. Clinically, in five of seven squamous cell carcinoma cases the lesions were located in the lingual mucosa. The tumours were located in the lower and upper lip in the other two cases respectively. In four of seven papilloma cases the lesions were located in the cheek mucosa. The lesions were located in the palate in the other two cases, and in one case it was located in the lower lip. Three of the four leukoplakia cases were located in the lower lip and one in the cheek mucosa. Two of the lichen planus cases were located in the cheek mucosa.

In the paraffin sections of the seven cases diagnosed as squamous cell carcinoma, one case was evaluated as Grade I, four cases were Grade II and two cases Grade III. Six cases revealed mild koilocytosis and one case moderate koilocytosis in the surface epithelium. Diskeratosis was observed in one case, orthokeratosis in three cases, parakeratosis in two and hyperkeratosis in one case. In the sections of squamous cell carcinoma cases stained for HPV, only one case was positive for type 31/33/51. The imprint slide of the same case was also positive for HPV 31/33/51 (Figure2). In the imprint slide of another case positive staining HPV for 16/18 was observed (Figure 1).

In the paraffin sections of the seven cases diagnosed as papilloma, three cases revealed mild koilocytosis, two cases moderate koilocytosis and two cases severe koilocytosis in the surface epithelium. Orthokeratosis was observed in one case, parakeratosis in one case and hyperkeratosis in five cases. In the section stained for HPV, one case was positive for type 31/33/51 and three cases were positive for HPV type 6/11 (Figure 3). In none of the papilloma cases were the imprint slides positive for any types of HPV.

In the paraffin sections of the five cases diagnosed as leukoplakia, one case revealed moderate koilocytosis and two cases mild koilocytosis in the surface epithelium. Parakeratosis was observed in one case and hyperkeratosis in three cases. In the sections stained for HPV, two cases were positive for type 16/18 (Figure 4), one case was positive for type 6/11 and one case was positive for type 31/33/51. The imprint slide of the same case was also positive for HPV type 31/33/51. The imprint slides of other cases did not reveal any positive staining.

In the paraffin section of the two cases diagnosed as Lichen planus, one case revealed mild koiloyctosis and one case moderate koilocytosis in the surface epithelium. Neither the paraffin sections nor the imprint slides showed any positive staining for any type of HPV.

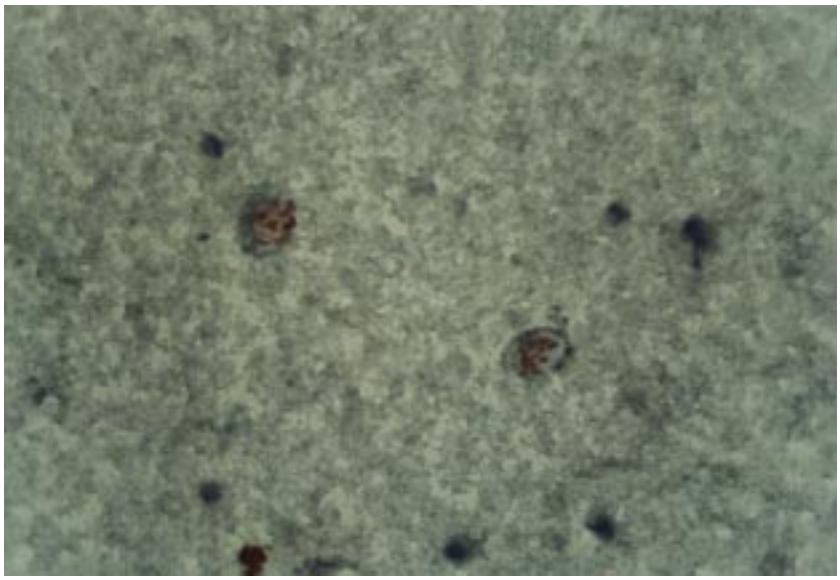

Figure 1. Positivity to HPV 16/18 types observed by the ISH method in the imprint preparation of a case diagnosed as squamous cell carcinoma (X400).

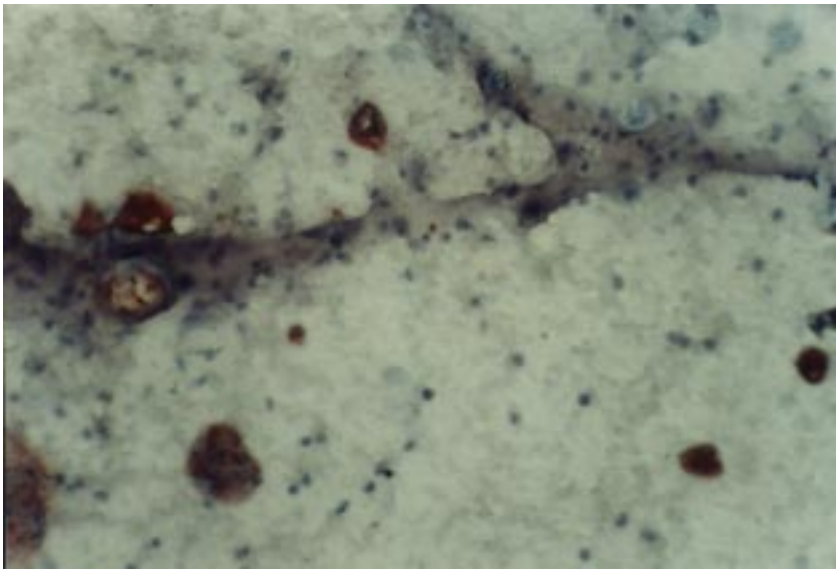

Figure 2. Positivity to HPV 31/33/51 types observed by the ISH method in the imprint preparation of a case diagnosed as squamous cell carcinoma (X400).

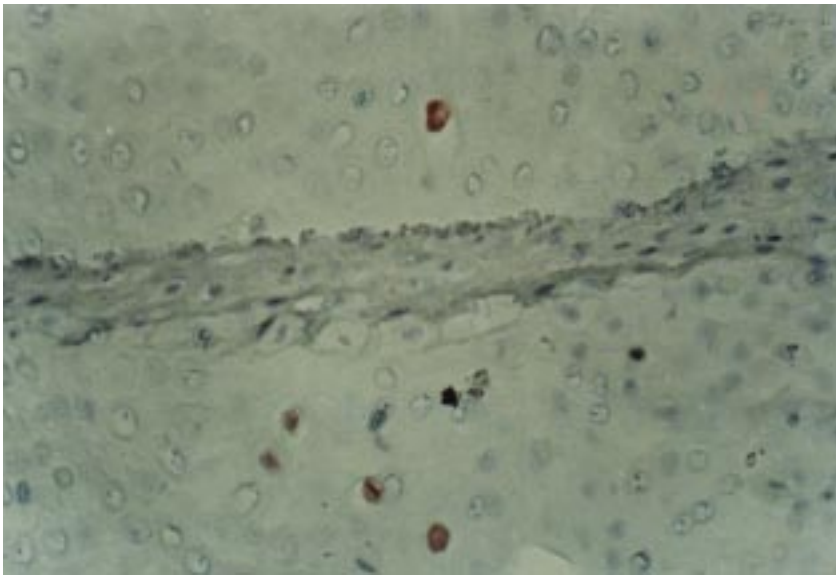

Figure 3. Positivity to HPV 6/11 types observed by the ISH method in the paraffin section preparation of a case diagnosed as papilloma (X250).

Discussion

Human papilloma viruses are a group of heterogenous viruses. More than 65 genotypes have been established for them to date. In the experimental studies carried out on animals, it has been demonstrated that these viruses play a part in oncogenesis as co-factors (21).

The fact that the presence of HPV has been established in 90 percent of cervical cancers has suggested the a etiologic role played in the development of benign and malignant lesions of the oral mucosa, which has the same histologic structure as the mucosa of the genital region (12).

DNA of HPV 16, which is held responsible for the a etiology of cervical carcinomas, has been demonstrated to play a part in carcinogenesis, as it is integrated in the genome of the host cell. However, in studies carried out to explain the a etiological role of HPV in cancers of the head and neck region, it has been demonstrated that DNA of HPV 16 has been present both in an episomal form, and as integrated into the genome. On the basis of this finding, it has been suggested that HPV may be latent for a long time in the episomal form in the oral mucosa, and that it may be responsible for the initiation and development of a tumoral growth as a result of a

multicarcinogenic interaction together with some other carcinogens and co-carcinogens (11, 22).

In the studies carried out in order to investigate chronic use of cigarette/tobacco and alcohol together with the presence of HPV, it has been reported that the cases of epidermoid carcinoma which are found to be HPV positive, together with the use of cigarettes and alcohol, have formed the largest group (70.6%) (22).

Since the oral cavity is an easily accessible environment for many carcinogens and co-carcinogens, and because the lesions in the oral cavity are asymptomatic in the early stages of development, it has been found crucial to take biopsies in order to follow-up and diagnose at an early stage any possible malignant change in oral benign and precancerous lesions. It has been emphasized, in many studies, that the in situ hybridization (ISH) method is as reliable as the polymerase chain reaction (PCR) method, and that, at the same time, it has been advantageous in the follow-up of HPV positivity in lesions due to the more practical aspect of the method (23, 24).

We have determined the presence of HPV 6/11 in 3 cases and HPV 31/33/51 in one case out of 7 papilloma cases that were included in our study. In other studies on this subject, it has been reported that positivity for HPV 6/11 has been high in papilloma cases (5, 8, 9, 25).

We have determined the positivity of HPV 6/11 and 16/18 in one, HPV 16/18 in another, and HPV 31/33/51 in still another case out of 6 cases that were diagnosed as having lichen planus or leukoplakia. Our findings are in agreement with the literature (9, 20, 26).

Moreover, the fact that HPV 16/18 positivity, which is determined mostly in cancer cases, appears also in leukoplakia cases suggests that these lesions should be more carefully followed up. In our study, the presence of HPV 16/18 was established in 1 out of 7 squamous cell carcinoma cases, and positivity of HPV 31/33/51 in one case. In this case, a few cells in the imprint slide were positive for HPV31/33/51, whereas no positivity was observed in the tissue section. It is indeed difficult to classify this finding; however, we may conclude that HPV positive cells may have been very superficially located so that they were imprinted, and the rest of the tissue revealed no positivity for HPV. With the exception of this squamous cell carcinoma case, it can be stated that biopsy specimens showed more positivity for HPV in general than imprint slides, which included for fewer cells.

These data, when compared with the findings in the literature as to HPV types, are consistent with the results obtained by Abdalsayed (5), Tsuchiya et al. (8) and Young and Min (9) but do not agree with these of Shayer and Greer (24) and Miller et al. (13).

Consequently, we determined the presence of various subtypes of HPV DNA in 9 out of a total of 20 cases. The negative results in 11 cases are probably related to the absence of HPV DNA in the biopsies, or its presence in the cells of a different region of tissue, or the presence of a different type of HPV other than the type we used.

Acknowledgments

This study was supported by Istanbul University Research Fund, Project Number: 589/171193, and was prepared as a PhD thesis with the subject 'Human Papilloma Virus Expression with Insitu Hybridisation in Benign Precancerous and Mailing Lesions of the Oral Mucosa', in 1996, under the supervision of Assoc. Prof. Dr. Nevin Büyükakyüz, in Istanbul University's Faculty of Dentistry, Department of Oral Surgery.

Correspondence author:

Mehmet YALTIRIK

Başbakanlık Toplu Konutları

III. Etap A 602/66

34660 Halkalı - İSTANBUL

References

1. Chang F, Syrjanen S, Kellokoski J, Syrjanen K: Human papillomavirus (HPV) infections and their associations with oral disease. J Oral Pathol Med 20: 305-317, 1991.
2. Hanson BG, Forslund O, Bjerre B, Lindholm K, Nordenfelt E: Human papilloma virus types in routine cytological screening and at colposcopic examinations. Eur J Obstet Gynecol Reprod Biol 52: 49-55, 1993.
3. Scully C: Oral Cancer: New insights into Pathogenesis. Dental Update 95-100, 1993.

4. Kashima HK, Kutcher M, Kessis T, Levin LS, De Villiers EM Shan K: Human papillomavirus in squamous cell carcinoma leukoplakia, lichen planus, and clinically normal epithelium of the oral cavity. Ann Otol Rhinol Laryngol 99: 55- 61, 1990.

5. Abdelsayed R A. Study of human papillomavirus in oral epithelial dysplasia and epidermoid carcinoma in the absence of tobacco and alcohol use. Oral Surg Oral Med Oral Pathol 71: 730-732, 1991.

6. De Villiers EM: Heterogeneity of the human papilomavirus group. J Virol 63: 4898-903, 1989.

7. Kellokoski JK, Syrjönen SM, Syrjönen KJ, Yliskoski M: Oral mucosal changes in women with genital HPV infection. J Oral Pathol. Med 19: 142-148, 1990.

8. Tsuchiya H, Tomita Y, Shirasawa H, Tanzawa H, Sato K, Simizu B: Detection of human papilloma-virus in head and neck tumours with DNA hybridization and immunohistochemical analysis. Oral Surg Oral Med Oral Pathol 71: 721-725, 1991.

9. Young SK, Min KW: In situ DNA hybridisation analysis of oral papillomas, leukoplakias, and carcinomas for human papillomavirus. Oral Surg Oral Med Oral Pathol 71: 726-729, 1991.

10. Zeuss MS, Miller CS, White DK: In situ hybridisation analysis of human papillomavirus DNA in oral mucosal lesions Oral Surg Oral Med Oral Pathol 71: 714-720, 1991.

11. Watts SL, Brewer EE, Fry TL: Human papillomavirus DNA types in squamous cell carcinomas of the head and neck. Oral Surg Oral Med Oral Pathol 71: 701-707, 1991.

12. Woods KV, Shillitoe EJ, Spitz MR, Schantz SP, Adle-Starthz K: Analysis of human papillomavirus DNA in oral squamous cell carcinomas. J Oral Pathol Med 22: 101-108, 1993.

13. Miller CS, Zeuss MS, White DK: Detection of HPV DNA in oral carcinoma using polymerises chain reaction together with in situ hybridisation. Oral Surg Oral Med Oral Pathol 77: 480-486, 1994.

14. Gopolakrishnun R, Weghort CM, Lehmun TA: Mutated and wild type p53 expression and HPV integration in proliferate verrucose leukoplakia and oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Radiol Oncol 471-477, 1997.

15. Smotkin D: Virology of human papillomavirus. Clin Obstet Gynecol 1989: 32: 117-124, (1989).

16. Villa LL, Brentani RR: Human papillomavirus Up-Date. Int J Cancer 48: 163-166, 1991.

17. Park M, Vande Woude GF: Principles of molecular cell biology of cancer: Oncogenes. In: Devita VT, Hellmann S, Rosenberg SA. Cancer Principles & Practice of Oncology. JB Lippincott Company, Philadelphia, 1989; pp: 45-48.

18. Vande Woude S, Vande Woude GF: Principles of molecular cell biology of cancer: General aspect of gene regulation. In: Devita VF, Helman S, Rosenberg SA. Cancer Principles & Practice of Oncology. JB Lippincott Company, Philadelphia, 1989; pp: 31-44.

19. Beutner KR: Human papillomavirus infection. J Am Acad Dermatol 20: 114-123, 1989.

20. Zur Hausen H: Papillomaviruses and p53. Nature 393: 217, 1998.

21. Migliorati CA, Jones AC, Baughman PA: Use of exfoliative cytology in the diagnosis of oral hairy leukoplakia. Oral Surg Oral Med Oral Pathol 76: 704-710, 1993.

22. Chang K-W, Chang C-S, Lai K-S, Chou M-J, Choo K-B: High prevalence of human papillomavirus infection and possible association with Betel Quid chewing and smoking in oral epidermoid carcinomas in Taiwan. J Med Virol 28: 57-61, 1989.

23. Fukushima K, Ogura H, Watanabe S, Yabe Y, Masuda Y: Human papillomavirus type 16DNA detected by the polymerise chain reaction in non-cancer tissues of the head and neck. Eur Arch Otol Rhinol Laryngol 251: 109-112, 1994.

24. Shroyer KR, Greer RO: Detection of human papillomavirus DNA by in situ DNA hybridisation and polymerise chain reaction in premalignnant and malignant oral lesions. Oral Surg Oral Med Oral Pathol 71: 708-731, 1991.

25. Syrjanen SM, Sryrjanen KJ, Lamberg MA, Finland K: Detection of human papillomavirus DNA in oral mucosal lesions using in situ DNA-hybridisation applied on paraffin sections. Oral Surg Oral Med Oral Pathol 62: 660-667, 1986.

26. Gassenmaier A, Hornstein OP: Presence of human papillomavirus DNA in benign and precancerous oral leukoplakias and squamous cell carcinomas. Dermatologica 176: 224-233, 1988.