Original Article

Detection and Genotyping of Human Papilloma Virus-Associated Oral Lichen Planus By In Situ Hybridization Technique

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Summary:

Background: Molecular DNA hybridization has confirmed more than 120 different human papilloma virus (HPV) genotypes. A small group of them have high- risk oncogenic potential. Many studies have described an association of such high risk-HPV genotypes with a variety of oral lesions including squamous cell carcinoma, leukoplakia, and lichen planus. .

Materials and Methods: A total number of 42 tissue specimens, representing 27 patients with oral lichen planus and 15 apparently-healthy oral tissues, were included in this study. The molecular methods for HPV detection and genotyping were performed by in situ hybridization(ISH) using cocktailed- and specific high- risk HPV DNA probes, respectively.

Results: The overall percentage of HPV in the total group of patients with oral lichen planus (OLP) was 33.3 %. Negative HPV DNA- ISH reactions were detected in all tissues of the control group. The overall genotyping results revealed HPV 16- DNA in all HPV- positive oral lichen planus tissues , while none of these OLP tissues showed ISH reactions for HPV 18-DNA or HPV 31/33-DNA.

Conclusions: The significant incidence of such high oncogenic HPV genotype in those patients with oral precancerous lesions could have a relevant importance along its pathogenesis and the multi-steps oral carcinogenesis , HPV-16-associated oral lichen planus that has mostly previliged the site of cheek mucosa represents a herald indicator for spread of such sexually important transmitted infection among Iraqi general population.

Key Words: Oral lichen planus; Human Papilloma Virus; In Situ Hybridization.

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Introduction:

In recent years, the frequency of pre-cancerous lesions and cancers of oral cavity have prompted studies to search for the etiology and pathogenesis of these lesions. It was suggested that chemical carcinogens, radiation energy, chronic irritation and viruses might played an important role in their etiology (1-3).The human papilloma viruses have a specific tropism for squamous epithelial cells and their full productive cycle is only supported in these cells . Update more than 100 different HPV types have been described; of these, nearly 30 can infect the anogenital tract . Some of these HPVs are frequently associated with cancer and are considered high- risk genotypes (for e.g. HPV type 16 and 18) , whereas others give rise to warts and benign lesions and are considered as low- and intermediate- risk genotypes (for e.g. HPV type 6,11,31, 33 and 35) (4).The association of HPV infections in squamous cell pre-cancerous lesions of the uterine cervix has been well established since late 1970s. The role of HPV, in the development of anogenital cancers (including most cervical cancers) has been intensively studied where the oncogenic HPV types are regarded as the most important etiological factors of cervical squamous cell carcinoma(5). These findings had sparked many other scientific studies during the early 1980s which have witnessed the rapid expansion of HPV research from genital tract to cover the other non-genital squamous cell epithelia, thus widening the scope of HPV-associated human tumors to involve the oral cavity, pharynx, larynx, upper airway, and esophagus (6). With the help of the polymerase chain reaction (PCR) and in situ hybridization (ISH), epidemiological and experimental studies have detected and currently indicated for a possible role of human papilloma virus (HPV) infections in the etiopathogenesis of oral squamous cell carcinomas and oral pre-cancer lesions and conditions such as oral leukoplakia and lichen planus. Depending on the sensitivity of the detection method, 40-67% of tissues from oral leukoplakias, 2.5-76% from oral squamous cell carcinomas and 0-87% from lichen planus were described to be infected with HPV 16 or HPV 18 (7). The present study designed to elucidate a possible etiological role for HPV in oral pre-carcinogenesis as well as identifying specific viral genotypes involved in the infection of Iraqi patients with oral lichen planus .

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Materials and Methods:

Patients: Since this study was designed as a retrospective research, the subjects included in this study were represented by their oral tissue samples that were obtained as archival tissue blocks. During the period from December 2006 till March 2007, a collective number of (42) formalin-fixed, paraffin embedded oral tissue blocks enrolled in this study which comprised both patients and control samples. These retrospective paraffin-embedded samples were retrieved from the archives of the period 1991-2006 belonging to Dental Clinics and Departments of Oral Diagnosis and Oral Histopathology /College of Dentistry/ Baghdad University, and Maxillofacial Center / Specialized Surgeries Hospital / Medical City. These blocks included:

A. Oral Lichen Planus (OLP): These included a group of (27) oral lichen planus (OLP) patients who had undergone surgical operation or biopsies .Their data were obtained from the attached histopathological reports including age, gender and provisional clinical diagnosis. Primarily, the diagnosis of these tissue blocks were based on the obtained histopathological records that had accompanied the oral biopsy samples in the hospital files or the histopathological laboratory records that had accompanied each specimen. But following trimming process of these tissue blocks, a confirmatory histopathological re-examination of hematoxyline and eosin (H and E)- stained slides from each obtained tissue blocks was done by further consultant oral histopathologist in the college of Dentistry / Department of oral diagnosis.

B. Control group: A collective group of (15) tissue specimens with normal histological appearance (i .e without any significant pathological changes), taken from oral mucosal tissues(cheek and / or lip) during surgical operations for oral lesions and from those around sound surgically extracted tooth for orthodontic purposes, were properly subjected to fixation as well as paraffin embedding and used for this research work as an age- and sex- matched healthy control group.

Methodology: Tissue Sectioning and Slide Preparation: At the histopathological department of Teaching laboratories / Medical City, the obtained formalin-fixed paraffin-embedded blocks were subjected to cut as serial thin sections of (4 µm) thickness and were sticked on charge slides In order to prevent carry-over DNA contaminations from one tissue sample to another, only one disposable cutting knife, which was specified for each tissue block, was used and then each section was sticked on a single charged slide. The 1st and 2nd tissue slides were specified for hematoxyline and eosin staining whereas many subsequent 4 µm thickness-paraffinized tissue sections were specified for the following procedures of in situ hybridization. As positive and negative HPV controls, it was feasible to include such tissue-containing charged slides in each experiment by using cervical tissue blocks, proved by PCR to have both cocktailed and high risk-oncogenic HPV genotypes, as a positive controls, as well as negative control from those apparently healthy cervical tissues, that were also proved by PCR technique to be negative for HPV.

Molecular Detection and Genotyping of HPV DNA: Molecular detection and genotyping of HPV DNA in those oral tissue blocks were performed by a recent generation of in situ hybridization (ISH), using a cocktailed- as well as specific- biotinylated DNA probes for high oncogenic-risk HPV genotypes 16, 18, and 31/33, respectively. In Situ Hybridization for Detection Cocktailed (Generic) – HPV Genotypines: In this study, the instructions of manufacturing company (Dako corporation Co./ Denmark) for performing all processes of in situ hybridization for detection and genotyping HPV were followed and according to the details in (8). In Situ Hybridization of HPV Genotype 16 , 18 , 31/ 33 Biotinylated DNA Probes:The same procedures for in situ hybridization for detection of cocktailed probes of HPV (Dako cytomation Code No. K0601) were followed, except for the step of the temperature for stringent washing of these steps was changed to 58°C for 20-40 minutes Assessment of the Results: Within 2 hours, the obtained results were assessed by examining the processed slides under light microscope; a deposition of a soluble blue purple product at the sites of hybridization of the probes to their targets is a positive indicator for the presence of the questioned group of HPV. Staining Interpretation: Positive signals, corresponding to areas of hybridization, will appear as blue or blue-purple regions within individual cells of tissue. Overdevelopment of the substrate-chromogen may result in black signals. For hybridization involving DNA targets, counterstaining with nuclear counter stains may make the interpretation of positive signals difficult if too much counter stain is used. The use of hematoxylin is not recommended, since the blue color of the positive reaction is easily obscured by the blue counter stain. A light counter stain of the tissue section using Nuclear Fast Red is recommended if additional morphological details are desired.

Statistical Analysis: The suitable statistical methods (9) were used in order to analyze and assess the results including the followings:

1- Descriptive statistics:

A- Statistical tables including observed frequencies with their percentages.

B- Graphical presentation by (bar & pie - charts).

2- Inferential statistics:

These were used to accept or reject the statistical hypotheses, they include the followings:

A) Binomial test (z-test).

B) Chi-square $\chi 2$.

C) Student (t-test).

Correlation is considerd significant when $P < 0.05$.

The Results:

The age of the patients who were affected by oral lichen planus ranged between 20-65 years where as age of those in the healthy control group ranged between 25-58 years . The mean age of 27 patients with oral lichen planus was 42.56 ±12.74 years where as the mean age of 15 cases in the healthy control group was 39.40 ± 12.46 years. Statistical analysis shows non-significant difference (p>0.05). The age group of patients mostly affected with oral lichen planus is 20-40 years (51.9 %) followed by the age stratum 41-60 years (40.7 %), and the lastly affected age stratum was those more than 60 years(7.4%). Statistical analysis shows significant difference (p<0.05)(figure 1).

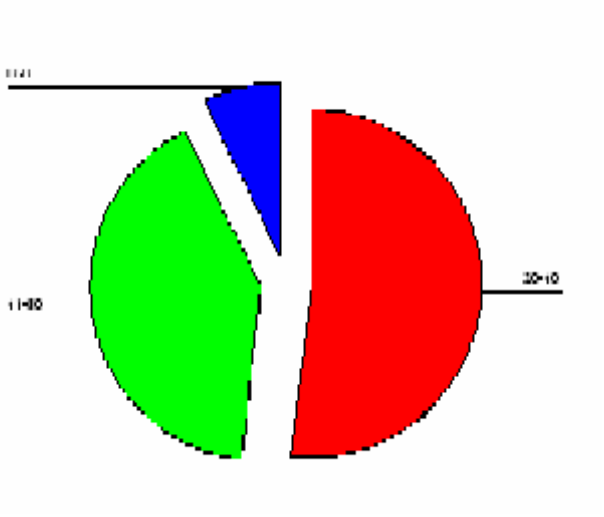

Figure 1. Distribution of patients with oral lichen planus according to their age stratification (years).

Only ten of oral lichen planus patients (37%) were males whereas the rest 17 cases (constituted 63%) were females.Statistical analysis shows highly significant difference (p<0.01)(Table 1).

Table 1: Percentage of oral lichen planus according to the sex of patients.

Study Groups	No. of Tested Cases	Gender Of Patients *			
		Male		Female	
		No.	%	No.	%
Oral Lichen Planus	27	10	37.0	17	63.0
Healthy Control	15	5	33.3	10	66.7

*Statistically the difference is highly significant (P<0.01).

The majority (81.5%) of oral lichen planus were affecting, and so were feasible to be collected from, cheeck mucosa while the rest(18. 5%) of OLP tissues were obtained from lower lip of patients.The statistical analysis shows that the difference between these two sites is highly significant(P<0.01) (Table 2). Table 3 shows the percentage of generic-HPV DNA detection in the group of oral lichen planus tissues compared to their control group of normal oral tissues included in this ISH experiment that using biotinylated wide spectrum-HPV probes which are prepared to hybridize to human papilloma viruses of HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-45, HPV-51, and HPV-52 (*Code No. Y1404*) (*Dako Cytomation., Denmark*).

Table 2: Percentage of oral lichen planus according to their sampling sites.

Study Groups	No. of Tested Cases	Anatomical Location *			
		Cheeck Mucosa		Lower Lip	
		No.	%	No.	%
Oral Lichen Planus	27	22	81.5	5	18.5
Healthy Control	15	12	80.0	3	20.0

*Statistically the difference is highly significant (P<0.01).

Table 3: Percentage of cocktailed HPV DNA Detection in oral lichen planus.

Study Groups	No. of Tested Cases	Cocktailed HPV DNA Detection Via In Situ Hybridization*			
		Positive		Negative	
		No.	%	No.	%
Oral Lichen Planus	27	9	33.3	18	66.7
Healthy Control	15	0	0	15	100

*Statistical analysis shows significant difference (p<0.05).

The results were interpreted as directed by that manufacturing company. Positive hybridizatoion signals were detected in form of dark-blue or blue-purple granules located in positive cells while absence of such hybridizatoion signals defined negative cells (Figure 2 and 3). Nine out of 27 OLP tissue blocks revealed positive hybridizatoion signals representing 33.3% of the total group of oral lichen planus. No HPV DNA was detected in all the examined tissues of the healthy control group. The statistical analysis shows significant difference (p<0.05).

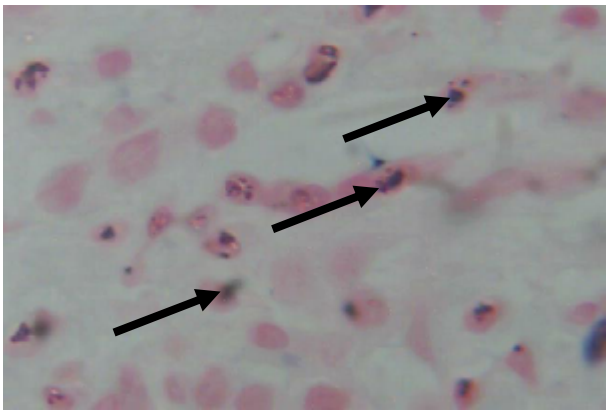

Figure 2: Positive in situ hybridization reaction showing HPV 16- DNA within nuclei of the cells of tissue blocks from patient with oral lichen planus. BCIP/NBT-chromogen stained & counter stained by nuclear fast red (X1000).

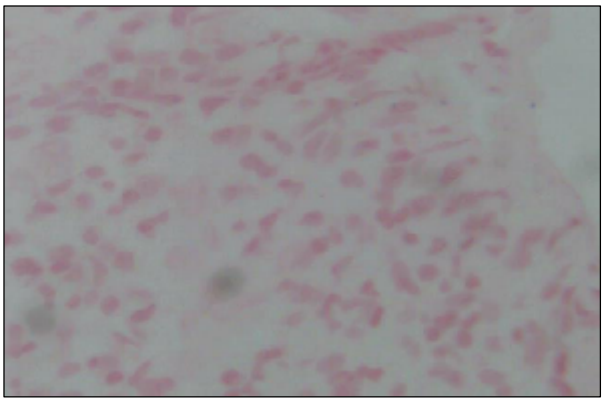

Figure 3: oral lichen planus epithelium showing negative ISH reaction for cocktailed HPV-DNA; BCIP/NBT stained and counter stained by nuclear fast red (X400).

Three different high-risk HPV genotypes were examined by ISH in tissues from positive-oral lichen planus of the present study using their respective(specific) DNA probes . The genotype HPV16 was detected in all (100%) of HPV-positive oral lichen planus tissue blocks. However, neither HPV 18 nor HPV 31/33 was detected in any positive-OLP tissue specimens on performing the following two consecutive ISH runs for HPV 18 and HPV 31/33, respectively .The bulk (77.8%) of HPV DNA-positive tissue specimens with oral lichen planus was detected in the age stratum (41-60) years which statistically showed very highly significant differences among other age strata (Figure 4).
HPV DNA was detected in two males out of 10 (20%) and in seven out of 17(43.5%) females tissue specimens with oral lichen planus . The chi-square analysis of percentge of detection of generic HPV-DNA in oral lichen planus tissues according to patients gender showed non-significant differences (figure 5).

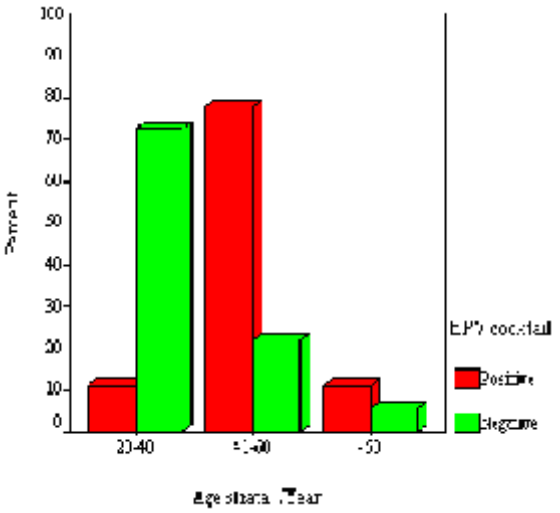

Figure 4: The chi-square evaluation of percentge of detection of generic HPV- DNA in oral lichen planus tissues according to age strata of patients.

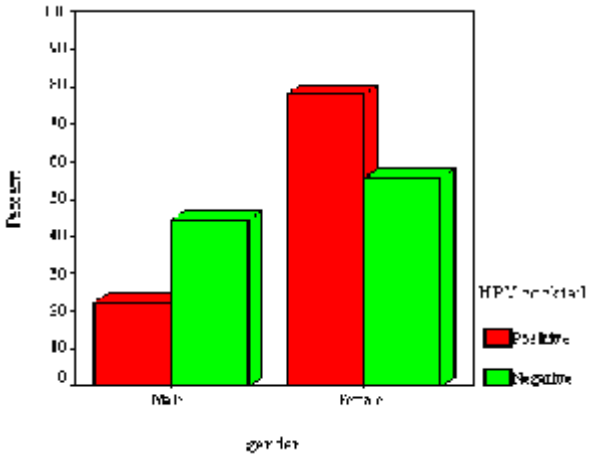

Figure 5: The chi-square analysis of percentge of detection of generic HPV-DNA in oral lichen planus tissues according to patients gender.

The HPV DNA was detected in 9 out of 22 (40.45%) oral lichen planus tissues in the group of cheek mucosa group while none of the oral lichen planus tissues from lower lip group had showed any ISH reactions for cocktailed/ generic HPV DNA. (table 4).

Table 4: The percentge of generic HPV- DNA according to anatomical sites of oral lichen planus .

Anatomical Sites Of Oral Lichen Planus	No. of Tested Cases	Cocktailed HPV DNA Detection Via In Situ Hybridization			
		Positive		Negative	
		No.	%	No.	%
Cheek Mucosa	22	9	40.45	13	59.55
Lower Lip	5	0	0	5	100
Total	27	9	33.3	18	66.7

The chi-square analysis of percentge of detection of generic HPV-DNA in oral lichen planus tissues according to their sampling sites showed non-significant difference (p>0.05) (Figure 6).

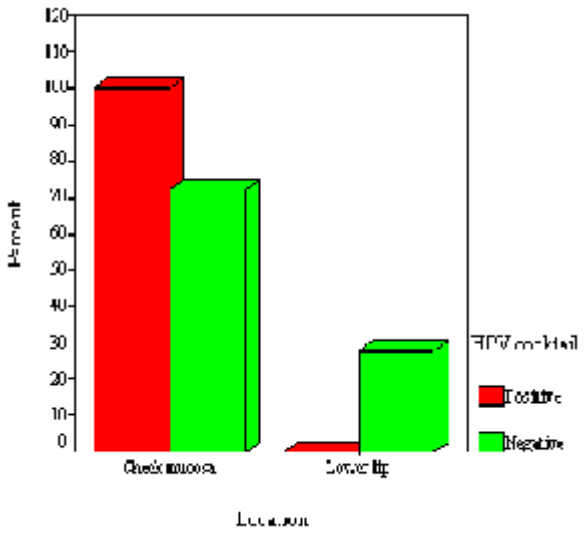

Figure 6: The chi-square analysis of percentge of detection of generic HPV-DNA in oral lichen planus tissues according to their sampling sites.

Disscusion:

The concept of a two-step process of cancer development in the oral mucosa i.e., the initial presence of a precursor (pre-malignant) lesion that subsequently developing into cancer, is well established (7).The WHO Experts Meeting in 1997 has subdividing oral pre-cancer into pre-cancerous lesions, such as leukoplakia and erythroplakia, and pre-cancerous conditions, such as submucous fibrosis and lichen planus. Internationally, Oral lichen planus which is a chronic inflammatory oral mucosal disorder, affects approximately 1-2% of those general adult population attending oral pathology and oral medicine clinics (10, 11). The human papilloma virus (HPV) infection has attracted a great attention because of the oncogenic potential of certain strains, in particular HPV-16, - 18, -31, and -33 that ultimately have been signed out to be the most likely cause-candidates in the course of cervical cancer(12). Also studies have discovered that human keratinocytes expressing E6 and E7 genes of HPV 16 became immortal and similar findings were noticed in the oral epithelial cells(13, 14). Since then the researchers, in their majority of studies on head and neck lesions, have focused on such high oncogenic potential-HPV types. They have been detected in 20-30% of oral squamous cell carcinoma (15). The other types—such as HPV- 6, -11, and -33 have not been identified in many oral malignancies(16). Recent testing for HPV DNA relies mainly on three commonly used techniques; hybrid capture, polymerase chain reaction (PCR), and in situ hybridization(ISH).These molecular techniques have a higher sensitivity and specificity than cytopathological and histopathological techniques in diagnosing HPV infection (17). Since nucleic acid-based probe test is the most advanced form of biochemical diagnostic methodology available today, where the amount of target molecules detected by means of this in situ procedure is impossible to match by other physical or chemical means(18); for this, the present research work has utilized in situ hybridization using both cocktail and specific biotinylated HPV DNA-probes to detect and genotype HPV in Oral lichen planus. Epidemiological and experimental studies have indicated a possible role of HPV in the etiopathogenesis of oral premalignant lesions and tumors(19-20).However, in Iraq, there is neither such seroepidemiological study of HPV among healthy population nor experimental study of role of HPV in oral premalignancy and oral tumorigenesis.

The data presented herein demonstrate that HPV DNA can be found and localized in epithelial cells of OLP, representing 33%. Up to best knowledge, there is also no similar molecular study on HPV DNA in OLP in Iraq. The present data support the earlier observation and also comparable with the Danish study of Nielsen et al in 1996(21) who detected HPV DNA in 40% of OLP and with the three Italian studies of Giovannelli et al, 2002(23), Campisi et al, in 2004(22) and Cianfriglia et al in 2006(20) who detected HPV DNA in 19.7%, 20% and 27.1% of OLP, respectively. However, in a Turkish study done by Yaltirik et al,2001(3), no HPV 6/11, HPV 16/18, HPV 31/33 were found in

OLP although these tissues showed koilocytosis and hyperkeratosis or parakeratosis which are pathologically indicative of HPV infection.These are related to the small number (only 2 cases) enrolled and to the presence of other types of HPV not included among the range of ISH used. None of the present normal oral tissues in control group studied herein was found to contain HPV DNA. The present result is in accordance with elsewhere studies, such as the Danish study of Nielsen et al in 1996(21), who were unable to demonstrate HPV in any of the 20 normal control tissues even by using four different sophisticated laboratory techniques, and also with that done by Bouda et al (2000)(24). However, in many other recent studies, HPV DNA was detected in 0.4%, 6%, 20% , and in up to 40%-60% of individuals with healthy tissues (20, 22, 25, 26). These wide variations in detection rate of HPV DNA in individuals without any obvious oral lesion could be related to the study population, type of sample collected, method of DNA detection (hybridization versus PCR), and the primers and specific probes utilized. In addition, and as in many cases, they could be related to difference in patients age, pathology, and specific risk factors from one study to another(20). Although many strains of HPV have been associated with oral pre- cancers and cancers(19), the association of HPV-31,-33-,and -35 have not been as strong as that for HPV-16/18(7). In this study, the strain most often associated with OLP is HPV-16 while none of these tissues showed ISH-reactions for either HPV-18 or -31/33 genotypes.This result is consistent with Al-Bakkal et al ,1999(27) who found 16 times expression of HPV-16 type in oral pre-cancers and cancers than benign lesions. However, and unexpectedly, HPV-18 was the most prevalent genotype detected in OLP by(23).These findings could indicate a different distribution pattern of HPV types, probably reflecting the different geographical as well as genetic origions of sampling. In Iraq, HPV-18 and -31/33 seem to play a trivial or no role in OLP pathogenesis. It is also possible that such oral lesions could be caused by another (rare, novel or even new) HPV types(28), which are out of the range of probes used in the present study and as such are not detected. It is likely that the use of combination of other HPV probes would not only increase the rate of HPV detection but might also detect other distinct HPV types. There seems to be a distinct predilection for HPV-associated oral pre-cancer lesions to occur on the buccal mucosa(7). The present results are compatible with these results, yet the difference in the present study is not statistically significant.The male gender predilection for HPV-associated oral carcinomas has not yet been demonstrated in HPV-associated oral pre-cancer lesions(7).In this study, HPV-associated oral pre-cancer lesions were detected in 43.5% of female patients while such lesions were found in only 20% of male counterparts .These results are in consistent with(20) who also found that HPV-positive oral pre-cancers tend to occur in female patients(22%; 2 out of 9) than male counterparts (16%; 1 out of 6). The HPV-associated oral pre-cancer patients are in contrast to their oral carcinoma patients counterparts elsewhere in that they have not been associated with their younger age (7).In the present study, highly significant percentage of HPV positivity was detected in the age group 41-60 years. In this respect, only older age afflected by OLP was found to be a significant risk determinant for the presence of HPV DNA(23). Conclusively, the absence of HPV in normal samples, along with the detection of such respective percentage of HPV in these pre cancerous condition suggests an association of this virus with oral carcinogenesis and indicates for an early role more than late one for involvement of HPV in oral premalignant development in the oral cancer progression (20). The studies of HPV infection are still in the early phase, and aspects such as HPV load and persistence in infected cells of the oral mucosa need to be analyzed in more detail. Further investigations with a higher numbers of cases in combination with analysis of the viral gene expression are necessary aiming to find out the prognostic value of the HPV infection for this facultative premalignant disease.

Conclusions:

1.HPV-16-associated oral lichen planus has mostly previliged the site of cheek mucosa of the patients in the age stratum 41-60 years.

2. Despite a proposed low prevalence of HPV infection in our country,the significant incidence of HPV in those patients with oral precancerous lesions could represents a herald indicator for spread of such sexually important transmitted infection among Iraqi general population.

3. The detection of such high oncogenic genotype, namely HPV-16 in that oral pre-cancerous condition could have a relevant importance in its pathogenesis and, along many other risk factors, in the process of oral carcinogenesis.

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