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MOLECULAR DETECTION AND GENOTYPING OF HUMAN PAPILLOMA VIRUS (16/ 18, AND 6/11) IN PATIENTS WITH LARYNGEAL LESIONS USING DNA- IN SITU HYBRIDIZATION

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ABSTRACT

Background: *Laryngeal cancer represents one of the most common head and neck malignancies. Many studies have suggested that human papilloma virus (HPV) might be related to the pathogenesis of many of these malignant and benign head and neck tumors as well as laryngeal lesions. The viral role in laryngeal carcinoma has been studied with conflicting resultsrelated to the population and methodology used.*

Objectives*: This study was designedto determine the percentage as well as genotyping of high-risk and low risk HPV genotypes in archival tissues specimens that range from apparently healthy, through benign tumors growths of vocal cords (nodules and polyps), to laryngeal cancers.*

Study design*: The study is a retrospective one. Molecular detection and genotyping of high risk HPV(16/18)and low risk(6/11) using chromogenic in situ hybridization (CISH) were performed on formalin-fixed ,paraffin embedded (157) laryngeal tissues blocks. After histopathological*

confirmation,they were classified into 45 laryngeal cancer tissue biopsies,35 polyps biopsies,37 nodule biopsies and 40 laryngeal autopsies.

***Results**: The percentage of HPV-16\18 -infected laryngeal cancerous tissues was (53.3%), while the HPV-16\18 -infected laryngeal polyps tissues was (8.6%) and HPV-16\18 -infected laryngeal nodules tissues was (2.7%). The percentage of HPV6\11- ISH signal in infected laryngeal cancer tissues was (24.4%), in benign laryngeal polyps' tissues was (17.2%), and in benign laryngeal nodules tissues was (2.8%).*

***Conclusions**: Our results could pointfor a possible role of HPV, especially the high oncogenic genotypes, in the laryngeal carcinogenesis, wherethese viruses might play this role in the early steps of benign and malignant laryngeal pathogenesis as well as progression of these lesions.*

Key word: Laryngeal lesions, HPV6\11,HPV-16\18, DNA- In Situ Hybridization.

Introduction

Laryngeal cancer is one of the most common head and neck cancers, while it is the second most common malignancy of the upper aero digestive tract that (85% - 95%) of these laryngeal malignancies are squamous cell carcinoma [(1)]. Although tobacco and alcohol use were recognized as two primary risk factors , yet other risk factors were included as well, such as human papilloma virus ,chemical carcinogens , positive family history for malignancy, previous radiotherapy and personal history of head and neck cancers[(2-3)].

HPV is epidemiologically considered as an etiologic factor for laryngeal cancer since it was shown to increase the proliferation of laryngeal epithelial cells [(4)]. HPV DNA has been detected in benign (papillomatosis), indolent (verrucous) carcinoma, and malignant (squamous cell carcinoma). The HPV types associated with laryngeal papillomatosis include low – risk where as HPV16 and 18 are more commonly present in neoplastic lesions (verrucous carcinoma and squamous cell carcinoma).Approximately 25% of laryngeal squamous cell carcinoma(LSCC) harbor HPV infection especially type 16 and type18 [(5)].

Human Papilloma Virus is a member of Papillomaviridae that have a small icosahedral symmetry with circular ds DNA genome. To date more than 200 different types have been described [(6-7)]. Human Papilloma Virus infection of epidermal or mucosal epithelial cells causes

benign and sometime malignant neoplasms [8].HPV usually infects keratinocytes and mucous membranes through direct transmission by skin or mucosal contacts or indirectly by non-sexual transmission via contaminated objects or surface or perinatal transmission [9-10].The association between HPV with genital and uterine cervical neoplasia is well established Since 1970s[11]. HPV-genotypes16,18,31,33,35,39,45,51,52,56,58,59,68,69,73,82 are frequently detected in ano-genital cancers , particularly cancers of the cervix and anus and as such were considered to behigh–oncogenic risk types.The HPV 6,11,40,42,44,54,61,70,71,72,80,and CP6108 were classified as low –risk types that usually detected in genital warts and laryngeal papillomas and the types 26,53 and 66 were considered as probably oncogenic[12-13].

The virus is able to integrate itself into the host genome and use its transcription machinery to express viral proteins from many viral early genes. It has been shown that E6 and E7 of HR-HPV types have an oncogenic potential to disrupt cell cycle check points; after integration, results in elevation of E6 and E7 oncoproteins expression levels, leading to inhibit tumor suppressor proteins p53 and PRB, respectively andinduce cell immortalization through many events[14-15]. So this study aim to investigate the presence of HPV 16/18 and HPV 6/11DNA in benign and malignant laryngeal lesions by using DNA -in situ hybridization technique to elucidate the possible role of these HPV infections in progression of these laryngeal tumors

Material and methods

Patients and tissue samples: The study was designed as retrospective one. A total number of (157) randomly selected formalinfixed, paraffin embedded laryngeal tissue blocks were enrolled in the present study which includes:(45) blocks of laryngeal cancers, (72) blocks of benign laryngeal tumors including (35) laryngeal polyps and (37) laryngeal nodules and forty autopsies laryngeal tissues that had normal tissue appearance on post mortem-histopathological examination was included as an apparently healthy control. The age of these archival tissues ranged from 2 to 80 years.Specimens were collected through the period from September 2014 to January 2016 from the archives of histopathology laboratories of the of Gazi AL- Hariri Teaching Hospital /Baghdad and from Al-Kindy Teaching Hospital/Baghdad,Al-Yarmouk Teaching Hospital , many private histopathology laboratories as well as the archives of Institute of Forensic Medicine in Baghdad during the period from January 2014 to July 2015 while the control group were from September 2015 to January 2016.The diagnosis of these tissue blocks

were based on their accompanied histopathological records. A confirmatory histopathological re-examination of each obtained tissue blocks was done by consultant histopathologist.

Laboratory methods

Four mm thick-tissue sections were prepared and stuck onto positively charged slides. An chromogenic in situ hybridization (ISH) detection system (Zytovision/Germany) was used to target DNA sequences in tissue specimens using a cocktail ofDigoxigenin-labeled long DNAprobes (T-1144-400, ZyoVision GmbH,.Bremerhaven,Germany) for screeningHPV genotypes (6,11,16,18,31,33,35,39,45,51,52,56,58,59,66,68,82). Whereas genotyping of HPV was done by using a specific Digoxigenin-labeled HPV DNA probes(T-1056-400,ZyoVision GmbH,.Bremerhaven,Germany) for the high risk HPV genotypes 16\ 18, and also genotyping for special low-risk HPV genotypes(T-1055-400, ZyoVision GmbH,.Bremerhaven,Germany) for(HPV6\11).Theprocedure of the (CISH) assay adopted by this study was carriedout in accordance with the manufacturer company leaflet(Zytovision/Germany) in the Research Laboratories at CommunicableDiseases Research Unit/ Baghdad Medical College. Positive reactions were performed by replacing the probewith a Digoxigeninhousekeeping gene probe. For the negative control, all reagents were added except the diluted probe. The signals of Chromogenic In Situ Hybridization(ZytoFast Plus Implementation kit AP-Permanent red :T-1151-40) were detected as bright red discoloration when stained with red permanent solution and counter stained with hematoxylin in referring to the HPV screening test and for HPV 6/11 whereas the signals of CISH(ZytoFast Plus Implementation kitHRP-green:T-1073-40) were detected as bright green discoloration and counter stained with nuclear red solution in referring to 16/18 genotypes at the sites of complementary sequences mostly at nuclear signals. Quantification of in situ hybridization signal ofdifferentmolecular markers was evaluated under light microscopy that visualized under (10-40x)and the counting of positive cells was performed at X1000. Insitu hybridization was given intensity and percentage scores,based on intensity of positive signals and number of signals,respectively.The intensity score included no stain and strong intensityof reaction. Positive cells were counted in ten differentfields of 100 cells for each sample and the average of positivecells of the ten fields was determined assigning cases to oneof the three following percentage score categories: Score(1) =1- 25%, Score(2)= 26-50%, Score(3)>50%[16]. Chi-squaretest was used to detect the significances between variables ofour

study. All the statistical analysis was done by SPSS program. P-value was considered significant when< 0.05.

Results

Age Distribution: The archival specimens collected in this study were related to laryngeal cancers patients whose ages were ranged from 8 -80 years and the mean age of those laryngeal cancers patients was (56.91 ± 17.122) years. The mean age of patients with laryngeal nodules was (42.49± 13.027) years ranged whom age from 20 - 75 years, while the mean age of patients with laryngeal polyps was (40.57 ± 18.393) years and ranged from 8 - 75 years and the mean age of apparently healthy individuals was (27.25 ± 17.148) years and was ranged from 2 - 66 years. The most commonly affected age stratum of laryngeal cancer group was 61-80 years constituting (48.6%). In comparison with benign groups(polyps and nodules),the most affected age stratum (20-40) yearsconstituted (40%)and(48.6%), respectively.There are significant differences (P<0.01) among study groups according to age distribution (Table 1 and2).

Sex distribution: the percentage of the males with laryngeal cancers was higher (86.7%) than the percentage of their laryngeal cancer female counter parts (13.3%).Regarding the patients whom suffering from benign laryngeal tumors for nodules and polyps , the percentage of males was also higher (73%) and (65.7%), respectively, than the percentage of female counter parts with laryngeal nodules and laryngeal polyps (27%) and (34.3 %) respectively. While the percentage of males for an apparently healthycontrol was higher (77.5%) than the percentage of females (22.5%) .The male / female ratios of the patients with laryngeal cancer and laryngeal benign tumors (nodules and polyps) were 6.5and (2.7and 1.9), respectively ,while the male/ female ratio of apparently healthy control was 3.4 .The statistical analysis showed significant difference (P> 0.05) among the studied groups (Table 3).

Molecular detection

1-Screening of HPV DNA-ISH: The present results of positive -ISH signal of scoring and intensity for screening HPV-DNA was (33.3%) ,(75.6%) respectively, in laryngeal cancer was higher than that found in benign tumors (polyps) (17.1%),(37.1%), respectively and (nodules)(10.8%),(27%),respectively. Significant differences were found among groups (**Table 4)andfigure(1)**.

2.HPV16/18 DNA-ISH: The positive -ISH signal of scoring and intensity for HR-HPV-DNA detection was (53%) ,(66.7%) respectively, in laryngeal cancer was higher than that found in patients with laryngeal benign tumors (polyps)(8.6%),(17.1%),respectively and (nodules)(2.7%),(5.4%),respectively. Significant differences were found among groups (T**able 5)(figure(1).**

3-HPV6/11 DNA-ISH: The present results of positive -ISH signal of scoring and intensity for LR-HPV-DNA were (24.4%) ,(28.9%), respectively, in laryngeal cancers was higher than that found in benign tumors (polyps) (17.1%),(22.9%), respectively and (nodules)(10.8%),(18.9%),respectively. Significant differences were found among groups(**Table 6).**

Discussion

Laryngeal cancer is the most frequent malignant tumor among head and neck cancers which is associated with several environmental and endogenous risk factors [(17)].HPV has been detected in benign, pre-malignant and malignant lesions of the larynx [(18)] where the infection with high-risk HPV types (HR-HPV) has been etiologically linked toa subset of these lesions suggesting a different carcinogenetic and tumorigenetic pathways [(2-3)].

The results of age match with the results of many other studies; Hafkampet al,(2003) and Wei etal., (2012) found that laryngeal cancer was common in patients with a mean age of 57years and an age range of 27-84 years[(19-20)] . Also numerous researches (Forshadpour etal.,2011;Mooren etal.,2014 ; Vietia etal.,2014;Hernandez etal.,2014) are in agreement with the results of current study that laryngeal cancer increased with the development of age [(21-24)]. On the contrary, Ogaetal., (2016) results disagree with our results and with the above mentioned researches they found the mean age of Nigerian patients with head and neck cancers including laryngeal cancer was 43.3 years [(25)].

In benign lesions, Singhal etal., (2009) found that benign tumors of the larynx were common in patients with an age range 21-30 years.Herein, these results were compatible with our results [(26)]. Moreover, the results of our study are closely moving with the results of Sharma and Sohal, (2013)who found that laryngeal benign tumors have mostly presented in patients aged between 21-30 years[(27)]. Also numerous researches as in the studies of (Hegdeetal.,(2005); Omland., etal.,(2014); Filho etal.,2013) [(28-30)] are in agreement with the results of current study in that the

age stratum of patients with benign tumors ranged from 20-40 years.The study differs with other studies: Avilaetal.,(2014) and Nassaret al.,(2010) studies that found the affected age stratum with benign laryngeal lesion was 41-50 years and42-68 years , respectively, and with an average age of 47.7 years and 50±8.2 years , respectively[31,32]. The results of age distribution in patients with differences laryngeal lesions studies supported the association of laryngeal cancer development with age.This could be explained by the prolonged chance of exposure to environmental carcinogens such as chemicals, radiation and viruses, which were regarded as important promoting factors in the development of laryngeal cancers [33]. In addition, the observed impairment in the immune system in such agesmight lead to the accumulation of cellular DNA mutations that could be regarded as an additional significant factor in the development of such malignancies [34].Benign lesion occurs, probably, due to prolonged use of the voice ,role of viruses chemicals ,gastro esophageal reflux disease and increase the chance of cancer of the esophagus that may be increases the risk of aero-digestive tract cancers as laryngeal and hypopharyngeal cancers[35]. Genetic syndromes as people with syndromes caused by inherited defects (mutations) in certain genes have a very high risk of throat cancer, including cancer of the larynx such as Fanconi anemia [36, 37].

Regarding **sex distribution,** our results match with most other studies, like Nassearetal.,(2010) in that the prevalence of laryngeal cancer in male (98%) was more than in female(2%)(32).Also Allegra etal.,(2012) demonstrate that male incidence rates are consistently 19.8 times higher (95.25%) than those in female (4.8%)[38]. However,the opposite was revealed where high female rates than males were observed .This increase in women may be related to increase the use of tobacco and alcohol consumption by womenas well as nutritional and hormonal factors that could have a role in increasing laryngeal cancers[39-41].

For patients suffering from **benign laryngeal nodules and polyps,** the percentage of males was also higher (73%and 65.7%, respectively) than the percentage of female counterparts (27% and 34.3%, respectively). The male / female ratios of those patients were 2.7and 1.9, respectively. According to many studies,male incidence rate with benign laryngeal tumor is higher than female rate as in the studies ofSinghaletel.,(2009);Durayetal.,(2011);Avila etal.,(2014);Omlandetal.,(2014) [26,42,43,29]who found the percentage of male with benign laryngeal lesions was higher than female percentage (73.8%/26.2%), (70.5%/29.5%), (97%/3%), (69.49%/30.5%) respectively. The current study differ from Filhoetal (2013) study who showed

that the incidence rate of male: female distribution differed in relation to polypoid lesions characteristics of benign laryngeal lesions demonstrate that the male /female ratio in angiomatous polyps was 1.86 with male percentage 65.08% and female percentage 34.92% while in gelatinous polyps the percentage of female was higher(66.67%) than male percentage (33.33%) and male: female ratio was 0.49[30].The exact explanation for this incidence increment and gender differences are unknown. However ,the frank predominance male: female ratio was suggested to be due to the main risk factors of smoking and alcohol uses that are more common in men. Some of work occupation of men that differ from women occupation may constitute a risk factor for laryngeal lesions incidence such as long and intense exposures to wood dust, paint fumes, and certain chemicals used in the metal working, petroleum, plastics, and textile industries which can also increase the risk of laryngeal and hypopharyngeal cancers. Some studies have also found a possible link between asbestos exposure and laryngeal cancer[44, 45].

Molecular detection of HPVDNA in laryngeal lesions

The presence results of positive -ISH signal of scoring and intensity for screening HPV-DNA was (33.3%) in laryngeal cancer was significantly higher than that found in benign tumors: in polyps (17.1%) and in nodules (10.8%).

The study of de Oliveira et al., (2006) and Morshed etal., (2008) [46,17]results were similar with the current study; they foundthe prevalence of total HPV-DNA in patients with laryngeal cancer were (37%)and (35%)respectively by using PCR. Moreover, the current results was similar to the results of Halec and his associates, (2013) who found that the total HPV positive by using wide spectrum of HPV-DNA was (35%) in patients with laryngeal cancers by using PCR [47].Vieta and his coworkers(2014) detect the prevalence of total HPV-DNA in larynx cancer was 32.39% using PCR which was closely related to our results[23].

For other studies, the percentages of total HPV-DNA ranged 0% to over 80%:Fakhry etal(2008) found total HPV-DNA -ISH in 40% of patients with head and neck squamous cell carcinomas , while the Maxwell etal.,(2010) found total HPV-DNA-ISH in (84.3%) with a similar types of such tumors[48,16] .The differences in the prevalence rates of total HPV-DNA among the studies may belong to study sample size and the technique that selected to detection of HPV-DNA. On other hand, the high percentage may reflect an active reproduction rate of the viruses in laryngeal epithelial tissues that revealed active infection or reactivated of past infection with probable viral

genome insertion into cellular DNA, while the low percentage may represent persistent infections [(49)].

Genotyping of HPV16\18-DNA-CISH in laryngeal lesions

Our data for positive -ISH signals of HR-HPV 16/18-DNA detection (53%) in laryngeal cancers was higher than that found in patients with laryngeal benign tumors: in polyps (8.6%) and in nodules (2.7%)with significant differences were found among groups.

Since HR-HPVs DNA have been shown to possess oncogenic potential therefore, the association between HR-HPV infection and laryngeal lesions (benign or \and malignant) has been suggested and investigated previously. Many researchers have detected and genotyped of HR-HPV-DNA in laryngeal tumors, but their results were highly controversial. The range of these studies was between (0%) to over (66%). Our result ranks the middle among these studies. Unfortunately,for a shortage of researches that use HPV16/18 probe in detection and genotyping of HR-HPVDNA were noticed,therefore this study has compare the current results with those in other sites of head and neck cancers as an anatomical sites closely related to the larynx, as in Jitani etal.,(2015)[(50)] in their CISH study for analysis of HPV16\18 DNA prevalence rate in oral cavity SCC patients was 44.4% in male whereas 55.6% in female which were in consistent with our study, while Masterson etal.,(2016)[(51)] molecular analysis for detection of HPV-DNA in head and neck cancer found the prevalence of HPV16 DNA was 45.2%. These studies are relatively agreed with our study. The present results are lower than many studies the study of Dury etal.,(2011) who demonstrated that high incidence of high-risk HPV-DNA was (77%) and the HPV16-DNA also was (77%) in patients with benign and malignant lesions of larynx[(42)]; in Kreimer etal.,(2005) systemic review study found the distribution of HR-HPV genotypes16 and 18 in laryngeal SCC worldwide were 69.2% and 17%.Respectively[(52)]; in Lin etal., (2013) study, the prevalence of the total HR HPV-DNA was 56% while HR-HPV16\18-DNA was 68% in oropharyngeal cancer [(53)].

On other hand, the obtained results of our current study are much higher than those results of many studies that showed lower prevalence of high risk HPV-DNA as in Mohammed Ali,(2009) ISH-study for detection and genotyping of human papilloma virus-associated oral lichen planus who found that the most prevalence type was HPV16-DNA with high rate (100%) while no HPV18-DNAprevelance (0%) in the same tissues[(54)].

Also many studies in different geographic origins showed variation in results reported as in the study of Liu etal.,(2010) who found the prevalence HPV16\18-DNA was 4/84(4.8%),and the detection of HPV16 and HPV18 (DNA) separately were 29/84(34.5%) and 6/84(7.1%)respectively by using E6 and E7 amplified –RT -PCR test [55]. The same disagreement results was reported by Chernock etal.,(2013) who found HPV-DNA in 65%while HR-HPV16-DNAwas 30% in tissues with SCC of the larynx[56]. However, a lower percentage was noticed in Hernandez etal.,(2014) study who found the percentage of HPV16-DNA was (6.1%), and their lower percentage of HPV18-DNA in patients with invasive laryngeal cancers in United States was in agreement with the current results[24].

These differences are a reflection of high prevalence of HPV in their general population since oro-sexual transmission and multipartners and are not common in our society and thus may constitute a probable cause for HPV infection in laryngeal lesions could related of the transmission of carcinogenic agents such as the HR-oncogenic HPV types mixed with saliva pool in the floor of mouth through oropharynx, and constantly these sites leading to constant carcinogen exposure. In addition,the thinner layer of stratified squamous epithelium at larynx provides less protection against these carcinogenic agents. Moreover, the episomal- HPV genome is frequently integrated in to host cell genome and an important and exciting consequences of this pattern of integration is that only E6 and E7 gene remain unaffected . Continued expression of these viral genes in tumor tissues can play a role maintenance of the malignant phenotype [50]. In an indirect way this could be related to the patient's genetic makeup. certain host genetic factors, such as polymorphisms or variations in specific human leukocyte antigens (HLA) class II have been implicated in the natural history of HPV infection; some increase the risk of HPV persistence by several folds, whereas others are associated with a reduced risk of persistent HPV infections [57,58]. The host genetic makeup may play an important role in susceptibility to HPV infection and HPV intratypic variants in different geographical regions may also determine the association with the risk of head and neck cancer and laryngeal cancer [59,60]. In addition to the host and pathogen genetic variation, the difference of the detection methods employed in the studies,environmental factors, sample size, the quality & sensitivity of the techniques used in these studies might also account for data discrepancies [61]. In view of these facts, the present data suggest that HPV infection in the studied laryngeal tissues might play a role in the laryngeal pathogenesis of our series of studied cases.

Genotyping of HPV6\11 in laryngeal lesions:

The present results of positive -ISH signal for LR-HPV-DNA was (24.4%) in laryngeal cancers was higher than that found in benign tumors: (polyps) (17.1%) and (nodules) (10.8%)with significant differences were found among groups. The current results are lower than the results of many studies: in Mooren etal.,(2013) study , the prevalence rate of HPV6 was (63%) and for HPV11 was (33%) in patients with head and neck papilloma and laryngeal dysplasia; inLupu and sarafoleanu ,(2014) study detection studies of HPV6 and HPV11 in patients with recurrent respiratory papillomatosis were 31% and 17%, respectively[22,62].Moreover,Aboguniun etal.,(2014) demonstrated that the prevalence rates of LR-HPV DNA in head and neck lesions was (34.6%). Our results were higher than the results of Dury etal.,(2011) who found that the percentage of low risk was 6% in patients with benign lesions of larynx[63,42]. Other study in US society found that the prevalence of LR-HPV-DNA in patients with laryngeal tumors was HPV6 (1.4%)while HPV11was(0.7%).These differences among studies might be an indirect evidence of the progressive nature of laryngeal keratosis comes from observations on the period of latency of the HPV infection. The period of latency is very important to estimate the first diagnosis and the development of carcinoma.Recurrent benign papillomatous growths are initially arising most often in the vocal cords, with subsequent spread to other areas of the respiratory tract, beginning from latency to pre-malignant and development of cancer. Clinically, papillomas tend to arise from the junction of squamous and respiratory (ciliated) epithelium,an area of iatrogenically induced squamous metaplasia. HPV DNA detection in laryngeal mucosa could be performed in all patients with prolonged exposure to tobacco and alcohol[64,65].

Conclusion

HPV16\18 infections might be the most frequent genotypes in Iraqi population with different laryngeal lesions as well as carcinoma, these findings may indicate that HPV16\18 could played an important role in their carcinogenesis The presence of positive -ISH signal of screening HPV-DNA,HPV16\18, HPV6\11 in laryngeal cancersin asignificantly higher percentages than those found in benign tumors (polyps and nodules); these may point for an active reproduction rates of these viruses in epithelial tissues of the larynx and in turn represent an active infection or reactivated of past infection with a probable viral genomic insertion into the cellular DNA.

Table (1): Distribution of study groups according to their mean and range of their age (years)

Studied groups	N	Mean Age / Year	Standard Deviation	Standard Error	Range	
					Min	M
A.H. Control	40	27.25	17.148	2.711	2	66
Laryngeal nodules	37	42.49	13.027	2.142	20	75
Laryngeal polyps	35	40.57	18.393	3.109	8	75
Laryngeal cancers	45	56.91	17.122	2.552	8	80
Total	15					

Table (2): Distribution of age strata according to the histopathological diagnosis of studied groups.

Age Groups / Year		Studied groups				Pearson Chi-Square (P-value)
		Apparently Healthy Control	Laryngeal Nodules	laryngeal Polyps	Laryngeal Cancers	
< 20	N	13	0	5	2	P = 0.00 Highly Sign. (P<0.01)
	%	32.5%	0.0%	14.3%	4.4%	
20 – 40	N	19	18	14	6	
	%	47.5%	48.6%	40.0%	13.3%	
41 - 60	N	7	14	11	17	
	%	17.5%	37.8%	31.4%	37.8%	
61 – 80	N	1	5	5	20	
	%	2.5%	13.5%	14.3%	44.4%	
Total	N	40	37	35	45	
	%	100.0%	100.0%	100.0%	100.0%	

***Highly significant difference (P<0.01) by using Pearson Chi-Square test.**

Table(3):Distribution of study group according to their gender.

Gender			Studied groups				Pearson Chi-Square (P-value)
			Apparently healthy control	Laryngeal nodules	Laryngeal polyps	Laryngeal Cancers	
	Male	N	31	27	23	39	P = 0.163 Non Sign. (P>0.05)
		%	77.5%	73.0%	65.7%	86.7%	
	Female	N	9	10	12	6	
		%	22.5%	27.0%	34.3%	13.3%	
Total		N	40	37	35	45	
		%	100.0%	100.0%	100.0%	100.0%	

*** Non-Significant differences using Pearson Chi- square test at 0.05 level**

Table(4):The Screening HPV-DNA-CISH signal results of the studied groups according to their scores.

HPV Screening Scores		Studied groups				Pearson Chi-Square (P-value)
		Apparently healthy control	Laryngeal nodules	Laryngeal polyps	Laryngeal Cancers	
Negative	N	32	27	22	11	P = 0.00 Highly Sign. (P<0.01)
	%	80.0%	73.0%	62.9%	24.4%	
+	N	2	4	6	13	
	%	5.0%	10.8%	17.1%	28.9%	
++	N	1	4	5	15	
	%	2.5%	10.8%	14.3%	33.3%	
+++	N	5	2	2	6	
	%	12.5%	5.4%	5.7%	13.3%	
Total	N	40	37	35	45	
	%	100.0%	100.0%	100.0%	100.0%	

***Highly significant differences (P<0.01).**

Table(5):The Screening HPV-DNA-CISH signal results of the studied groups according to their intensities.

HPV Screening Intensity		Studied groups				Pearson Chi.Square (P-value)
		Apparently healthy control	Laryngeal nodules	Laryngeal polyps	Laryngeal Cancers	
NO Stain	N	32	27	22	11	P = 0.00 Highly Sign. (P<0.01)
	%	80.0%	73.0%	62.9%	24.4%	
Strong Stain	N	8	10	13	34	
	%	20.0%	27.0%	37.1%	75.6%	
Total	N	40	37	35	45	
	%	100.0%	100.0%	100.0%	100.0%	

***Highly significant differences (P<0.01)**

Table (6): The HPV16/18 DNA-CISH signal results of the studied groups according to their scores.

HPV16/18 scores		Studied groups				Pearson Chi-Square (P-value)
		Apparently healthy Control	Laryngeal Nodules	Laryngeal Polyp	Laryngeal cancers	
Negative	N	37	35	29	15	P = 0.00 Highly Sign. (P<0.01)
	%	92.5%	94.6%	82.9%	33.3%	
+	N	1	1	3	6	
	%	2.5%	2.7%	8.6%	13.3%	
+++	N	2	1	3	24	
	%	5.0%	2.7%	8.6%	53.3%	
Total	N	40	37	35	45	
	%	100.0%	100.0%	100.0%	100.0%	

***Highly significant differences (P<0.01)**

Table (7):HPV16/18DNA-CISH signal results of the studied groups according to their intensities

HPV16 /18 intensity		Studied groups				Pearson Chi-Square (P-value)
		Apparently healthy Control	Laryngeal nodules	Laryngeal polyps	Laryngeal Cancers	
NO stain	N	37	35	29	15	P = 0.00 Highly Sign. (P<0.01)
	%	92.5%	94.6%	82.9%	33.3%	
Strong	N	3	2	6	30	
	%	7.5%	5.4%	17.1%	66.7%	
Total	N	40	37	35	45	
	%	100.0%	100.0%	100.0%	100.0%	

***Highly significant differences (P<0.01)**

Table(8):The HPV6/11-DNA-CISH signal results of the studied groupsaccording to their scores.

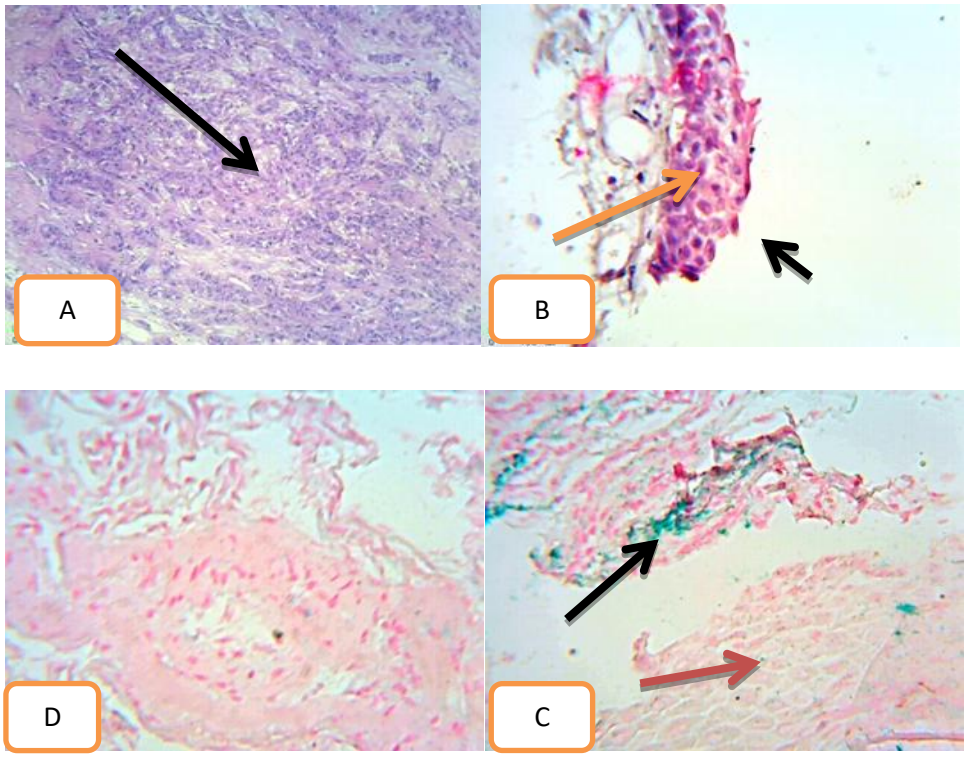
HPV6 / 11 Scores			Studied groups				Pearson Chi-Square (P-value)
			A.H. Control	Nodules	Polyps	Malignant Patients	
	Negative	N	39	30	27	32	P = 0.015 Sign. (P<0.05)
		%	97.5%	81.1%	77.1%	71.1%	
	+	N	1	3	2	2	
		%	2.5%	8.1%	5.7%	4.4%	
	++	N	0	4	6	11	
		%	0.0%	10.8%	17.1%	24.4%	
Total		N	40	37	35	45	
		%	100.0%	100.0%	100.0%	100.0%	

Significant differences (P<0.05)

Table (9): Distribution of signal intensities of positive HPVDNA 6/11-CISH among studied groups.

HPV6 / 11 intensity			Studied groups				Pearson Chi-Square (P-value)
			Apparently healthy control	Laryngeal nodules	Laryngeal polyps	Laryngeal cancers	
	NO stain	N	39	30	27	32	P = 0.015 Sign. (P<0.05)
		%	97.5%	81.1%	77.1%	71.1%	
	Strong	N	1	7	8	13	
		%	2.5%	18.9%	22.9%	28.9%	
Total		N	40	37	35	45	
		%	100.0%	100.0%	100.0%	100.0%	

Significant differences using Pearson Chi- square test at 0.05 level



Figure(1):Microphotographs of total HPV-DNA and HR-HPV 16/18 DNA CISH-signals :A- Normal laryngeal tissue(x100).B-Laryngeal cancer, the tissue where two signal pattern for total HPV are noticed (punctuated orange arrow , diffused black arrow,x400) at complementarity sequence sites. C-Green signal of HPV16/18 in laryngeal cancer tissue (punctuated red signal (diffused black signal (x400). D- normal laryngeal cancer (x400)

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