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Molecular and Virological Study of Nuclear Co-Localization of High Oncogenic Risk Human Papillomavirus 16/18(E6/E7) Genes and Overexpressed P53 Protein in Tissue From Malignant and benign laryngeal tumors

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Abstract :

background: Laryngeal tumors have been the most common malignancies and benign neoplasia of the head and neck tumors . Several studies have demonstrated that infection with human papillomavirus genotypes especial high oncogenic one might be related to the pathogenesis and tumors genesis of the head and neck tumors. Recently, many studies have focused on possible role of HPV oncogenes expression and cell suppressor genes in diagnosing of tumor progression

Objects: To investigate the correlation of nuclear co-localization of high risk oncogenes HPV16/18 E6 and E7 expressions with overexpression tumor suppressor gene p53 protein

Methods: A retrospective study, 157 formalin- fixed, paraffin-embedded (FFPE) including benign and malignant laryngeal tumors tissues blocks were collected. Molecular detection and genotyping of high risk HPV 16 and 18 were conducted by Chromogenic in site hybridization (CISH). Also immunohistochemistry (IHC) tests were perform to investigate the proteins expression of P53 gene as well as high HPV oncogenes E6 and E7 in benign and malignant laryngeal tumors.

Results: The percentage of positive signals of hrHPV16/18 (E6)-IHC of P53 IHC in relation to co-localization of P53 IHC over expression in malignant tumors were (14.3%:2) and benign tumors (polyps and nodules) were (33.3%:1) and (16.7% :1) respectively whereas the percentage of positive signal of hr HPV16 E7 -IHC in relation to over expression P53 IHC in malignant tumors were (50%:1) and (57.1%:8) respectively and in benign tumors (polyps and nodules) were (33.3%:1) and(100%:1) respectively. In results of hr HPV18(E7) – IHC signal and co-localization with 50%:1 and benign tumors (polyps and nodules) were (16.7%:1) for each one. The results of our study show the highest mean value of age was in malignant groups (56.91±17.12), while the lowest mean age was at control groups (27.25±12.15). the mean age of male (43.43±19.91) was more than female (39.68±19.25).

Conclusion: depending of IHC results of both hrHPV16/18 (E6) and (E7) gene expression as well as P53 over expression revealed that high oncogenic genotypes may contribute in pathogenesis and carcinogenesis of early steps of benign and malignant tumors

Key words: HPV16/18, E6 ,E7, P53 , IHC, ISH, Laryngeal tumors , malignant , benign, LSCC.

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Introduction

Squamous cell carcinoma of the larynx is constituted one of important forms of neck and head malignancies . Its comprised for 3% among of all cancers in adults. Recently , the number of people diagnosed with LSCC has risen, menacing a direct danger to human life and health (1,2). Males are more prone to infect with LSCC than females (3). Tobacco smoking and drinking alcohol are the two most significant offers clues. Also the etiology and pathogenesis factors of laryngeal cancer could include viral diseases, types of malnutrition , and local food choices (4). During the last century, the global frequency drifts have assisted a sharp increase in a group of neck and head squamous cell carcinoma (HNSCC) which is clinically associated with high risk HPV instead of typical risk factors (5) . Also in benign (nodules, polyps, papillomatosis), indolent (verrucaus) lesions, and cancerous squamous cell carcinoma, HPV genetic materials have been detected (6). HR-HPV DNA integration into host cells chromosome, predominately for HPV 16/18/31/33/45/53 subtypes , altered epithelial cells DNA by inhibiting tumor suppressor genes p53 and Rb, resulting in increased viral replication and tumor progression (7,8). More than 200 multiple kinds of HPV were detected . Generally, the most high oncogenic HPV genotypes that infect head and neck tumors is HPV16 and HPV18 . HPV16 and HPV18 E6 and E7 proteins expression are enough to immortalize human primary epithelial cells and make pre - malignant HPV-associated squamous intra-epithelial lesions (6). E6 and E7 oncoproteins have been shown to disrupt normal cell growth regulation pathways by inhibiting two well-studied tumor suppressor genes proteins; p53 and retinoblastoma protein (p Rb) (7). HPV16(E6) and HPV18 (E6) proteins bind to p53 and are degraded by ubiquitin path (9). Other biological and transforming functions of the E6 viral protein tend to be independently of p53 (10). It has been found to react with a number of cell proteins , including apoptosis-related proteins like Bak (11) and c-Myc (12), as well as tumor necrosis factor (TNF) (13).

P53 is playing a major role in cell death , cell cycle "checkpoint" monitoring and important for its regulation , DNA damage response , cellular anti-cancer mechanisms and is functionally inactivated in human cancer at a high frequency and senescence . Mutations of P53 gene were founded in over 50% of HNSCC . P53 overexpression in first primary malignancies could be a useful marker for detecting people who are at increased risk of getting secondary primary cancers (9). To the best of our knowledge, the conducted study represents the first in Iraq that highlighting for a possible role of HPV infection in Iraqi patients with laryngeal cancer and to elucidate the relationship exist between HPV infection and laryngeal benign and malignant tumors through evaluate the interaction and expression of high HPV oncogenes (E6,E7) and tumor suppressor gene P53

Material and methods :

Tissue samples:

The hypothesis of study was intended to be a retrospective investigation.(157) specimens of laryngeal formalin fixed , paraffin embedded tissue blokes were collected from patient who exposed to re- sectional or endoscopic laryngeal biopsy that related to (45) laryngeal cancers,(35) benign laryngeal polyps and (37) benign laryngeal nodules and (40) laryngeal autopsies that had normal tissue appearance

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on post mortem histopathalogical examination as an apparently heath control. The ages of laryngeal tissue specimens ranged 2-83 years. They were collected from records of histopathalogical laboratories archives of different hospitals , including: Medical city , AL-Hariri hospital Baghdad ,AL-Yarmok hospital , AL-Kindy teaching hospital , AL-Kadhemiya teaching hospitals well as from any private laboratories . The apparently healthy laryngeal tissues biopsies (without any significant pathological changes) were collected between January 2014 and January 2016, from the archives of Baghdad's Institute of Forensic Medicine were searched. The diagnoses depend on the recoded histopathalogical researches of corresponding patient after confirmatory histopathalogical re-examination of each obtained tissue blokes by histopathologist .

Methodology: Molecular detection and genotyping of human papilloma virus DNA in laryngeal tissue blocks were performed by advanced and recent generation of chromogenic In Situ Hybridization (ISH) (ZytoVigon GmbH, Germany) was used to targeted DNA sequence in tissue samples using a cocktail of Digoxigenin -labeled long DNA probes (T-1144-400 ,ZytoVision GmbH ,Bremerhaven ,Germany) for screening HPV 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 ,ZytoVision GmbH , Bremer haven ,Germany) for the high risk HPV 16/18 genotypes. The process of (ISH)assay embraced by this research was performed in accordance with distributor company booklet . approach contributes were conducted by substituting the probe with a Digoxigenin in house kipping gene probe and for negative control, all reagent kits were placed except for the diluted probe when use ISH detection method (T-1144-400) and (T-1056-400) (ZytoVision, Germany) were used correctly, a deep color signal was generated at specific locations of the hybridization probe in positive result tissue, which can then be anti-stained with hematoxylin.

For measuring the positive cells, tissue samples were assessed through light microscopy at (10x, 40x, and 1000x): (ISH) was assigned an amplitude and a ratio score dependent on intensity. The signal of intensity divided into; no stain and strong stain . Cells with detected sequences (positive) were observed in ten categorical variables of 100 cells for each study, and the mean of positive cells was calculated, assigned situations to another one of 3 percentage score subgroups: score(1) =1-25%, score(2)=26-50 percent, and score (3) >75 percent.

Immunohistochemical method was performed to detect the prevalence P53 overexpression protein in different laryngeal tissue tumors and was done according to the manufacturing company of antibody 80436- Expose mouse and Rabbit specific HRP/ DAB detection Kit(Abcam). The kit was used for defection of anti-p53 Ab [PAb240] ab 26, Anti HPV 16E6 Antibody (CIP5),ANTI-HPV16 (E7)antibody (SPM405) and Anti-HPV18 E7antibody (8E2) ab 100953 in laryngeal tumors were done according to the manufacturing company (Abcam,UK). The strength of nuclear and cytoplasmic staining of P53 positive signal demonstrated as strong stain and no stain while score signal was estimated as under 1% of samples appeared negative, the percent was measured to the nearest 1percent and assigned a score of (0),weak to medium staining (1+:10-75%), or extreme staining (2+: more than 75%) of disperse nuclei. whereas the expression of E6+E7 proteins was assessed using a semi-quantitative method that took into

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account the strength of nuclei and cytoplasm staining as well as the percentile of cells treated (0-4 percent: non expression, 5-25 percent: 1+, 26-50 percent: 2+,>50 percent: 3+ 4+). The negative result were calculated at the absence of stained cell, while the intensity of nuclear and cytoplasmic E6 and E7 protein staining were graded as no staining and as strong staining. Quantification of p53,HPV16+18(E6),HPV16 (E7),HPV16 E7 and HPV18(E7) proteins expression in laryngeal tissue tumors and control were detected under light microscope at 100x , 400x and 1000x. The numbering of positive cells was carried out at1000x Statically analysis; T test , ANOVA test and Chi square tests were applied for all result obtained in this research.

Results

The role of human papilloma virus in laryngeal tumors was tested markers in histopathalogical specimens to risk of malignancy is observing significantly in higher (or lower) tendency for laryngeal tumor tissues from cancer to benign tumor tissues (nodules and polyp) to express a positive markers in comparison to apparently healthy laryngeal tissues. The magnitude of expression of each test marker was measured in two different ways (intensity and at high power field examination constitution).

Based on the relation between age and groups, the study results showed the highest mean value of age was in malignant tumor group(56.91±17.15)while the lowest mean value was showed in A.H. control group(27.25±12.15) . The statically differences among

Table (1) Mean levels of age according to groups and gender were calculated by using student t test and F test.

Age and groups						
	N	Mean	SD	Std. Error	Minimum	Maximum
Control	40	27.25	12.15	2.71	2.00	66.00
Polyps Benign tumors	35	40.57	18.39	3.11	8.00	75.00
Nodules Benign tumors	37	42.49	13.03	2.14	20.00	75.00
Malignant tumors	45	56.91	17.12	2.55	8.00	83.00
p value	0.001***					
Age and gender						
Male	110	43.43	19.91	1.89	4.00	83.00

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Female	47	39.68	19.25	2.80	2.00	74.00
p value	0.276					

group according to age were significant($p<0.05$) , while no significant differences($p>0.05$) were showed between gender and the mean of male(43.43±19.91) was more higher than female(39.68±19.25) as in table(1)

2- HrHPV16/18(E6) /P53-IHC:

Table (2) : Results of HPV16/18(E6) IHC signal Intensity and P53 over expression intensity among studied groups.

Studied groups				P53 intensity		Pearson Chi-Square (P-value)
				NO stain	Strong	
A.H. Control	HPV16/18(E6) intensity	NO stain	N	27	10	P = 0.814 Non Sign. (P>0.05)
			%	93.1%	90.9%	
		Strong	N	2	1	
			%	6.9%	9.1%	
	Total		N	29	11	
			%	100.0%	100.0%	
Nodules Benign tumor tissues	HPV16/18(E6) intensity	NO stain	N	30	6	P = 0.036 Sign. (P<0.05)
			%	100.0%	85.7%	
		Strong	N	0	1	
			%	0.0%	14.3%	
	Total		N	30	7	
			%	100.0%	100.0%	
Polyps Benign tumor tissues	HPV16/18(E6) intensity	NO stain	N	25	7	P = 0.091 Non Sign. (P>0.05)
			%	96.2%	77.8%	
		Strong	N	1	2	
			%	3.8%	22.2%	
	Total		N	26	9	
			%	100.0%	100.0%	
Malignant tumor tissues	HPV16/18(E6) intensity	NO stain	N	21	7	P = 0.128 Non Sign. (P>0.05)
			%	70.0%	46.7%	
		Strong	N	9	8	
			%	30.0%	53.3%	
	Total		N	30	15	
			%	100.0%	100.0%	

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a. HrHPV16/18(E6) /P53-IHC intensities:

The percentage of HPV- infected cells. were evaluated by intensity of early protein HPV16/18(E6) – IHC reaction signals intensities in relation to p53-IHC intensities at a high power fields in laryngeal tumors , tissues under study. In malignant laryngeal tumors . The strong straining signal intensities of hrHPV16/18 (E6) protein expression -IHC in relation to p53expression - IHC intensities were founded in (53.3%:8) , (30%:9) respectively while no staining signal intensities of hrHPV16/18(E6) /P53 were detected in (70%: 21). In polyps laryngeal tumors . The Positive strong staining signal intensities of hrHPV16/18 (E6)/ P53-IHC were observed in (22.2%:2), (3.8%:1) respectively , while no staining signal intensities of hrHPV16/18 (E6)/P53-IHC over expression was detected in (96.2%:25). In nodules laryngeal tumor, the strong signal intensities were observed in (14.3%:1) while no staining signal was observed in (100%: 30) whereas the position strong signal of hrHPV16/18 (E6)/P53-IHC over expression intensities were founded in (9.1%:1) and no staining signal intensities were observed in (93.1%:27) statistically, there are no significant differences among groups except nodules tumors group as well as apparently healthy control ($p<0.05$). as in table (2).

b-HrHPV16/18(E6) /P53-IHC scores

The positive staining signal scores rresults of hrHPV16/18(E6)/P53-IHC over expression in HPV-infected cells of Malignant laryngeal tissues were constitute in (14.3% :12) with score(+) while (100.0%:2) and (35.7%:5) with score(+) and (++) respectively. The negative signal stain of hrHPV16/18(E6)/P53-IHC was(72.4%:21) In polyps laryngeal tumors , t he percentages of positive hrHPV16/18(E6)/p53-IHC showed Low(+) and moderate (++) signal scores were detected in (33.3%:1) and (16.7%:1) respectively. In nodules laryngeal tumors, the percentages of positive signal score hrHPV16/18(E6)/P53-IHC show Low(+) signal scores were detected in (16.7%:l) while negative stain signal scores of HPV 16/18(E6) /P53-IHC were(100%:30). in this group. In apparently healthy control, the percentage of negative signal score was (93.1%:27) while positive signal score was observed in 25% (1) with low score(+) hr HPV16/18(E6)/P53(++).Statistically, no substantial variations exist between the studied groups, with the exception of the polyps laryngeal cancers group and the apparently stable control group (see table) (3).

3- HrHPV16 (E7) /P53-IHC

a. HrHPV16 (E7) /P53-IHC intensities

The positive strong staining signal intensities of hrHPV16(E7)/p53-IHC were observed in (73.3% :11) while no staining signal intensities were found in (60%:18) of malignant laryngeal tumors group. In Polyp laryngeal tumors group, the strong staining signal intensities of hrHPV 16 (E7)/P53-IHC overexpression were observed in (33.3%:3) while no staining signal were founded in (92.3%:24) . In nodules laryngeal tumors, the positive strong staining signal intensities were observed in (14.3%:1) while no staining signal intensities were founded in (100%:30) of this group. Whereas in apparently healthy control group, the positive strong staining signal intensities were observed in (9.1%:1) while no

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staining signal intensities (96.6%:28) 4). of hrHPV16(E7) /p53. Statistically group of malignant laryngeal and group of nodules tumors appeared significant different (P<0.05) as in table (4).

b.HrHPV16 (E7) /P53-IHC scores:

In malignant laryngeal tumors tissues. The positive HPV-infected cells with staining signal scores of hr HPV16 (E7) -IHC in relation to P53 over expression -IHC were detected in (57.1%:8) and (50.1 :1)respectively with moderate signal score (++)hr HPV16 (E7)/P53(+) -IHC and (+)hrHPV16 (E7)/P53(++) –IHC respectively, while the negative signal score were (58.6%:17). In polyps laryngeal tumors, the percentage of positive hrHPV16 (E7)/P5- IHC show moderate (++) staining signal score in (33.3%:1) and low (+) signal score in (33.3%:2) , While negative signal score was recorded in (92.3%:24) . Those with laryngeal nodules tissues that positive moderate signal scores(++) of hrHPV16 (E7)/P53 were constituted (l00%:1) while percentage those with negative signal score for this marker was (92.3%:28) lastly, in apparently healthy control, positive Low (+) scoring of hrHPV16 (E7)/P53(++)were detected in (25%:1) While (96.6%:28) has negative signal score. Statistically all groups to have highly significant differences (p <0.01) except malignant laryngeal tumors group as in table (5).

Table(3):Results of HPV16/18(E6)-IHC signal scores and P53 overexpression IHC scores among studied groups.

Studied groups				P53 scores			Pearson Chi-Square (P-value)
				Negative	+	++	
A.H. Control	HPV16+18 (E6) scores	Negative	N	27	7	3	P = 0.042 Sign. (P<0.05)
			%	93.1%	100.0%	75.0%	
		+	N	0	0	1	
			%	0.0%	0.0%	25.0%	
		++	N	2	0	0	
			%	6.9%	0.0%	0.0%	
	Total		N	29	7	4	
			%	100.0%	100.0%	100.0%	
Nodules Benign tumor tissues	HPV16+18 (E6) scores	Negative	N	30	5	1	P = 0.071 Non Sign. (P>0.05)
			%	100.0%	83.3%	100.0%	
		+	N	0	1	0	
			%	0.0%	16.7%	0.0%	
	Total		N	30	6	1	
			%	100.0%	100.0%	100.0%	
Polyps Benign tumor	HPV16+18 (E6) scores	Negative	N	25	5	2	P = 0.014 Sign. (P<0.05)
			%	96.2%	83.3%	66.7%	
		+	N	1	1	0	

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tissues			%	3.8%	16.7%	0.0%	
		++	N	0	0	1	
			%	0.0%	0.0%	33.3%	
	Total		N	26	6	3	
			%	100.0%	100.0%	100.0%	
Malignant tumor tissues	HPV16+18 (E6) scores	Negative	N	21	7	0	P = 0.113 Non Sign. (P>0.05)
			%	72.4%	50.0%	0.0%	
		+	N	7	5	2	
			%	24.1%	35.7%	100.0%	
		++	N	1	2	0	
			%	3.4%	14.3%	0.0%	
	Total		N	29	14	2	
			%	100.0%	100.0%	100.0%	

Table (4): Results of hrHPV16(E7) – IHC signals intensities and P53 over expression -IHC intensities among the studied groups.

Studied groups				P53 intensity		Pearson Chi-Square (P-value)
				NO stain	Strong	
A.H. Control	HPV16 E7 intensity	NO stain	N	28	10	P = 0.465 Non Sign. (P>0.05)
			%	96.6%	90.9%	
		Strong	N	1	1	
			%	3.4%	9.1%	
	Total		N	29	11	
			%	100.0%	100.0%	
Nodules Benign tumor tissues	HPV16 E7 intensity	NO stain	N	30	6	P = 0.036 Sign. (P<0.05)
			%	100.0%	85.7%	
		Strong	N	0	1	
			%	0.0%	14.3%	
	Total		N	30	7	
			%	100.0%	100.0%	
Polyps Benign tumor tissues	HPV16 E7 intensity	NO stain	N	24	6	P = 0.058 Non Sign. (P>0.05)
			%	92.3%	66.7%	
		Strong	N	2	3	
			%	7.7%	33.3%	
	Total		N	26	9	
			%	100.0%	100.0%	

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Malignant tumor tissues	HPV16 E7 intensity	NO stain	N	18	4	P = 0.035 Sign. (P<0.05)
			%	60.0%	26.7%	
		Strong	N	12	11	
			%	40.0%	73.3%	
	Total		N	30	15	
			%	100.0%	100.0%	

Table (5) :Results of hr HPV16(E7)-IHC signal score and (P53)over expression -IHC signal scores among studied groups.

Studied groups				P53 scores			Pearson Chi-Square (P-value)
				Negative	+	++	
A.H. Control	HPV16 E7 scores	Negative	N	28	7	3	P = 0.048 Sign. (P<0.05)
			%	96.6%	100.0%	75.0%	
		+	N	0	0	1	
			%	0.0%	0.0%	25.0%	
		++	N	1	0	0	
			%	3.4%	0.0%	0.0%	
	Total		N	29	7	4	
			%	100.0%	100.0%	100.0%	
Nodules Benign tumor tissues	HPV16 E7 scores	Negative	N	30	6	0	P = 0.00 Highly Sign. (P<0.01)
			%	100.0%	100.0%	0.0%	
		++	N	0	0	1	
			%	0.0%	0.0%	100.0%	
	Total		N	30	6	1	
			%	100.0%	100.0%	100.0%	
Polyps Benign tumor tissues	HPV16 E7 scores	Negative	N	24	4	2	P = 0.006 Highly Sign. (P<0.01)
			%	92.3%	66.7%	66.7%	
		+	N	2	2	0	
			%	7.7%	33.3%	0.0%	
		++	N	0	0	1	
			%	0.0%	0.0%	33.3%	
	Total		N	26	6	3	
			%	100.0%	100.0%	100.0%	
Malignant tumor tissues	HPV16 E7 scores	Negative	N	17	5	0	P = 0.151 Non Sign.
			%	58.6%	35.7%	0.0%	
		+	N	4	1	1	

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			%	13.8%	7.1%	50.0%	(P>0.05)
		++	N	8	8	1	
			%	27.6%	57.1%	50.0%	
	Total		N	29	14	2	
			%	100.0%	100.0%	100.0%	

3- HrHPV18 (E7) /P53-IHC:

a. HrHPV18 (E7) /P53-IHC intensities

In malignant laryngeal tumors group, the percentages of hrHPV- infected cells with strong intensities of hrHPV18(E7)/P53 overexpression-IHC in (40%:6) while no stain signal intensities for this markers were founded in (63.3%: 19). In laryngeal polyps tumors, the percentage of strong staining intensities of hrHPV 18(E7)/P53-IHC was observed in (22.%:2) .While in nodules tissues, the percentages of strong signal intensities were observed in (28.6%:2), while no staining signal of hrHPV 18(E7)/P53-IHC was observed in(100%:30). Lastly in apparently healthy control, the strong signal intensities hrHPV18(E7)/p53-IHC were detected in (9.1%:1) and no staining signal intensities were deleted in (96.6%:2) . statistically, there is highly significant differences with laryngeal nodules tumors group ($p<001$) as in table (6).

b.HrHPV18 (E7) /P53-IHC intensities

Also in present results of positive hrHPV18(E7)/p53overexpression-IHC signal staining with moderate(++) scores in malignant laryngeal tumors were detected in (50%:1) and (++)hrHPV18(E7)/p53(+)-IHC was (14.3:1) respectively while, the percentages of malignant tissues that showed low(+) signal score for hrHPV18(E7)/p53-IHC tests were (21.4%) and those with low(+) hrHPV18(7)/P53 (++) were (50%:1) respectively while laryngeal polyps with moderate (++) score signal of hrHPV18(E7)/P53(+) was expressed in (16.7%:1),while others with low(+) score hrHPV18(E7)/P53(++) . The percentage of negative signal was (88.5%:23) .In the nodules laryngeal tumors, the percentage of hrHPV18(E7)/P53-IHC with moderate(++) signal scores (16.7%:1) while with Low (+) signal scores showed(100%:1). Negative results for these markers were founded in(100%:30). In apparently healthy control, the percentages of moderate(+2) scoring of hrHPV 18(7)/P53 -IHC constituted (25%:1), while negative results for these markers constituted (96.6 : 28) as in table (7) .

Table (6) :Results of hrHPV18(E7)–IHC signal intensities and P53 over expression IHC signal intensities among studied groups.

Studied groups				P53 intensity		Pearson Chi-Square (P-value)
				NO stain	Strong	
A.H.	HPV18 E7	NO stain	N	28	10	P = 0.465

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Control	intensity		%	96.6%	90.9%	Non sign. (P>0.05)
		Strong	N	1	1	
			%	3.4%	9.1%	
	Total		N	29	11	
			%	100.0%	100.0%	
Nodules Benign tumor tissues	HPV18 E7 intensity	NO stain	N	30	5	P = 0.003 Highly Sign. (P<0.01)
			%	100.0%	71.4%	
		Strong	N	0	2	
			%	0.0%	28.6%	
	Total		N	30	7	
			%	100.0%	100.0%	
Polyps Benign tumor tissues	HPV18 E7 intensity	NO stain	N	23	7	P = 0.431 Non Sign. (P>0.05)
			%	88.5%	77.8%	
		Strong	N	3	2	
			%	11.5%	22.2%	
	Total		N	26	9	
			%	100.0%	100.0%	
Malignant tumor tissues	HPV18 E7 intensity	NO stain	N	19	9	P = 0.828 Non Sign. (P>0.05)
			%	63.3%	60.0%	
		Strong	N	11	6	
			%	36.7%	40.0%	
	Total		N	30	15	
			%	100.0%	100.0%	

Tables (7): Results of hrHPV18 (E7)-IHC signal scores and P53 over expression-IHC signal scores among studied groups.

Studied groups				P53 scores			Pearson Chi-Square (P-value)
				Negative	+	++	
A.H. Control	HPV18 E7 scores	Negative	N	28	7	3	P = 0.048 Sign. (P<0.05)
			%	96.6%	100.0%	75.0%	
		+	N	1	0	0	
			%	3.4%	0.0%	0.0%	
		++	N	0	0	1	
			%	0.0%	0.0%	25.0%	
	Total		N	29	7	4	
			%	100.0%	100.0%	100.0%	

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Nodules Benign tumor tissues	HPV18 E7 scores	Negative	N	30	5	0	P = 0.00 Highly Sign. (P<0.01)
			%	100.0%	83.3%	0.0%	
		+	N	0	0	1	
			%	0.0%	0.0%	100.0%	
		++	N	0	1	0	
			%	0.0%	16.7%	0.0%	
	Total		N	30	6	1	
			%	100.0%	100.0%	100.0%	
Polyps Benign tumor tissues	HPV18 E7 scores	Negative	N	23	5	2	P = 0.139 Non Sign. (P>0.05)
			%	88.5%	83.3%	66.7%	
		+	N	3	0	1	
			%	11.5%	0.0%	33.3%	
		++	N	0	1	0	
			%	0.0%	16.7%	0.0%	
	Total		N	26	6	3	
			%	100.0%	100.0%	100.0%	
Malignant tumor tissues	HPV18 E7 scores	Negative	N	19	9	0	P = 0.458 Non Sign. (P>0.05)
			%	65.5%	64.3%	0.0%	
		+	N	6	3	1	
			%	20.7%	21.4%	50.0%	
		++	N	4	2	1	
			%	13.8%	14.3%	50.0%	
	Total		N	29	14	2	
			%	100.0%	100.0%	100.0%	

Discussion:

Laryngeal squamous cell carcinoma (LSCC) is the most common malignant neoplasm of upper respiratory tract in adults (10). Despite smoking and alcohol consumption are considered as a carcinogenic factors, many researchers theorized that multiple viral infection could act synergistically to increase hyperplasia and the pre-malignant mutation or to expedite their progression to cancer (11,12). On a worldwide scale, HPV16 has importantly been the most widely prevailing genotypes (82% of positive cases) ; and HPV18 genotype is considered the second then other sporadic genotypes as HPV31, HPV33, HPV35 come the third in several malignant and benign head and neck neoplasia including laryngeal one (5,8,13) . The association between HPV infection and the presence of many malignant and benign tumors of upper respiratory tract has been reported (14,15,16). Malignant transformation of infected HPV cells can occurs only when high oncogenic risk HPV integrated in to host cell genome result in dysfunction of two main pathway of cell growth cycle : retinoblastoma protein (p RB) mediated by P 16 INK4A and P53 (17,18). High oncogenic HPV E6 and E7 interact with two

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important tumor suppressor genes p53 and p Rb result in activation of them , causing genetic instability and shifting cell toward a more malignant phenotype and more facilitate viral replication (19,20). P53 have a complex biology and one of most popular tumor suppressor genes exposed to mutations and the loss of heterozygosity of chromosome 17P with various point mutation are shown to exist in over 50% of head and neck tumors including laryngeal tumors (21,22,23). In further studies , a rather frequent coexistence of HPV DNA and p53 mutation was founded and gain all sites and histological grads were involved (24). In Iraq , and up to our knowledge . This is the first study that performed CISH technique for detect the nuclear co- localization of high oncogenic risk human papillomavirus 16/18(E6/E7) genes and overexpressed p53 protein in archived histopathological specimens (FFPE) with malignant and benign (polyps and nodules) tumors.

1-Distribution of age and gender in patients with laryngeal malignant and benign tumors:

In current study , as shown in table (1), the patients' ages from 8-83 year and the average age of those with malignant tumors were (56.91±17.12) years with highly significance differences from that of benign tumors (polyps and nodules) with mean age of forties. While the percentage of the males with laryngeal tumors were higher 110(43.43 %) than the percentage of their laryngeal tumors female counterparts 47(39.68%) The results of ages are compatible with the results of many several studies as in (25) who founded the affected age by laryngeal carcinoma was 8-84 years . Also the present study agreed with kim etal., (2019) who founded increased laryngeal cancer with mean age (63.29± 9.7) years was much higher than those without laryngeal cancer during follow up period of 5322 subjects were newly diagnosed with laryngeal cancer(26) . In benign tumors, Singhal etal., (2009) founded that benign tumors of the larynx was common in patients with an age range 21-30 years(27) .Herein, these results were compatible with our results. Moreover, the results of our study are closely moving with the results of Rutt etal.,(2010) who founded that the studied 99 cases of laryngeal benign tumors have mostly presented in patients aged between 25-29 stratum with percentage (62.6%) . Muniraju and Vidya, (2017) founded that laryngeal lesions was common in affected stratum between 31-40 years(29) .The study differs with other studies as with Chinthapeta etal .,(2017) study that showed the highest incidence of hoarseness was observed between the groups of 41 – 50 years (30) and differs with Sadek etal.,(2019) study that revealed the average age of all studied patients was 41.7 years with a range from 10 to 75 years(31). The similarities and differences of age distribution in patients with difference types of laryngeal tumors . These studies supported the development of laryngeal cancer with ages. This could be showed by prolonged chance of exposing to smoking ,alcohol consumption as well as environmental carcinogens like chemicals, radiation and viruses, which were regarded as significant factors that promoting within laryngeal cancers progression (32). Furthermore, the noted deficiency in the immune response at these ages, owing to the cancerous decline in host defense, could contributed to the production of cellular DNA alteration , which could be considered another important impact of development of such malignancies (33). While benign tumors occurs, probably, due to prolonged use of the voice ,role of viruses chemicals ,gastro esophageal reflux disease (GERD) can causes heart burn as well as increases the chance of esophagus cancer (34). As far as genetic syndromes are concerned, individuals who have syndromes induced by inherited defects (mutations) in specific genes have a really

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serious chance of throat cancer, like laryngeal cancers like Fanconi anemia. When young subjects with this condition are older, they could exposed to a high risk of getting cancer of a throat and mouth, which encourages neoplasia (35).While the exact explanation for differences in incidence increment of malignant and benign tumors between male and female are unknown. However , the increased incidence in male then female could be due to the major risk elements of cigarette and alcohol consuming that are more commonly in men ,also may be due to the frequent exposure to inhalational irritants at workplaces, homes as well as malnutrition. Some of work occupation of men that differ from women occupation may constitute a risk factor for incidence of laryngeal malignant and benign tumors such as prolong and intense displays to wood dust, fume of paint, and chemical materials that used in metal industries , oil derivatives , plastics, and industries of textures which can also raises the dangerous of laryngeal and hypopharyngeal tumors (36). Asbestos is a natural fiber that once was widely used it as an insulation material agent in a variety of items. Asbestos exposure is a significant cause of mesothelioma as well as lung cancer. A potential correlation between asbestos exposure and laryngeal cancer has also been discovered in the some experiments (37).

2-Detection of hrHPV16/18(E6)-IHC/ P53-IHC:

From the results of present study, the prevalence of HPV16 as well as 18 were higher in malignant tumors than in benign tumors (polyps and nodules) , it has revealed that p53 overexpression and HPV 16/18(E6/E7) were significantly higher in laryngeal malignant tumors tissues than in either laryngeal benign tumors(polyps and nodules) or apparently normal laryngeal tissues reflecting prevalence of HPV16 and HPV18 with high potential of viral integrated into genome of laryngeal tissue cells with opportunities of increased viral load because of active episomal and integrated viral replication ,also may reflect reactivation of past infection , while low percentage of hr HPV16/18(E6/E7)/P53 expression may revealed persist infection with episomal replication in benign tumors or may these laryngeal tumors infected with other low risk genotypes of HPV, therefore have low level damage or not in their DNA to induce transcriptional up regulation of p53 gene (38).

Despite the importance of many factors , the variability of many results reviewed below as the size of study sample , tumor types , techniques of methods and condition of test that use in detection of HPV (E6 and/or E7) as well as p53 that have a role in varying the percentage of p53 expression(39).

According to replication events of HPV, episomal hrHPV genome integrated to laryngeal cell genome with high expression of E6 and E7 and down regulation or degradation of p53 genes products that in agreement with present that showed elevated percentage of E6 and E 7 of HPV16 and lease in HPV18 with low or absent level of p53 in some case and overexpression p53 in other cases (40).

The devotion between score (+1) and (+2) of HPV16/18 (E6) , HPV16 (E7) , HPV18 (E7) as well as p53 may belong to the intratypic variants of HPV16(E6) and/or HPV18(E6) because transcription and translation of HPV genome is differ among genotypes and complex therefore ,to explain the important of elevated level of hrHPV16/18(E6/E7) in associated with p53 overexpression , we must understanding two critical types of p53 mutations . Autosomal p53 gene mutation has been reported in over 80% of head

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and neck malignancy(41). There were two important mutations of p53 gene were detected namely null and non-null mutation. In theory , the researcher must be distinguish among wild type p53(P53-low-IHC), null mutant (P53-negative-IHC) and non-null mutant (overexpression) in tumors. When non- null p53 mutant occurred lead to increased p53 stability and its products accumulate chiefly in nucleus of neoplastic cells ,therefore , it can detected by immunohistochemistry (IHC) meanwhile null mutation leading to unstable truncated p53 that not detected by IHC(42,43) . whole wild type p53 expression can discovered in decreased levels by IHC (44).Some researchers founded that wild-type (low p53-IHC) was a good progenitor predictor because of very short life (30 minute) while other has been reported the opposite that wild type is a poorly prognostic predictor based on the level of wild type p53 and null mutants in low category(45).

On the other hand , other different studies have showed the clinical importance of non-null mutation (p53 over-expression) in patient with laryngeal malignant tumors and it consider a prognostic indicator , however it is still controversial (46). Null mutation of P53 (p53-negative-IHC) on the other hand, are associated with early, metastatic disease, and a poor prognosis, and they truly represent an independent indicator of survival rate in cancers such as ovarian cancer (47,48). However according splicing occurrences, there are a few explanations ,different expressions of E6 and E7 are processed by a single primary transcript that is separately spliced in the E6 gene full length (ft)transcript, which may result in two internally truncated E6 protein variants (E*I) and *II) from fused to short non E-6 sequences (E6* III or E6 *IV). E6 *I transcripts produce the truncated E6*I protein and the fl E7 protein, which have been used to distinguish between carcinogenic and non-carcinogenic HPV subtypes (49,50) .Another reason for elevate results of HPV16/18(E6) is that the E6 protein should stimulate the protein telomerase in different laryngeal tissue cells, leading to an increased life-span of the infected cells throughout interferon sensing by disrupting INF regulate factor 3 and mediate pro-apoptotic protein degradation (BAK and BAX), though the precise mechanism by which this occurs is still unknown (51) .

Another explanation that hr E6 proteins have PDZ-binding motive at C-terminus ,the primary function of PDZ- proteins (ex: Dlg ,Secribed ,MAGI 1,2,3) . In response to cell biological activities , include the control of cell growth, cell polarity, and cell adhesion. The predicted increase in E6 levels may also be due to E6 protein interacting with many other proteins including such SRC kinase and the pro-apoptotic BAK, improving its proliferative and apoptotic effects (52,53). Despite hrHPV E6can bind directly to p53 gene and block its transcription. E6 can affect p53 by preventing its acetylation and hence block stabilization of p53 protein through binding of E6 to histone acetyltransferases p300 and CBP (CREB- binding) which would normally acetylate p53 and hence activate it. Another possible explanation is that p53 isoform bind to hr E6 protein leading to p53 degradation and block apoptosis (54,55,56) .

2-Detection of hrHPV16/18(E7)-IHC/ P53-IHC:

While elevated level of HPV16 E7 and HPV18 E7 in laryngeal malignant tumors higher than in benign tumors may be belong to increase transcription and translation of E7 transcript from many sources as from the full length of E6/E7m RNA, or from spliced E6 *I transcripts (species , B,E,I) and from E6 *II m RNA(C,F,J) might play a role in HPV types carcinogenicity (57) .

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Another reason for the enhanced E7 protein level could be belong to the important role of E7 protein whenever the attachment of histone deacetylases (HDACs) interaction was shown to be essential for episomal preservation of the S phase system and is thus considered a critical event for effective viral replication in suprabasal cells (58). On other hand, the increase level of mutant p53 expression and decrease copies of wild type p53 in malignant laryngeal tissues as a part of the malignant process . At the same time may indicate that benign tumors cells and normal tissues have low level damage or not in their DNA to induce transcriptional up-regulation of p53(59). It may well be down to lack of wild type p53 function or an inability to trigger p53 correctly, compromising the ability to regulate cellular proliferation and development (60). It was previously established that mutant p53 protein would form a tetramer with wild type p53 and operate as a dominant negative to suppress physiological functions of p53, likely by causing an inactive configuration of DNA binding domain or even other associated components and limiting the ability to trans-activate/ repress target genes(61). however it is well accepted now that loss off heterozygosity of wild type p53 is very important for the defective p53 activity and this could be through deletion of wild type p53 or replacement by mutant one (62). Both condition could be associated with HPV infection when viral integrated with cell genome . P53 binding to E protein causing escape of the cell form cell check point surveillance ,subsequently leading to genome stability and cell immortalization(7).

The major part of p53 mutations are missense mutations that result in message residue changes, with the majority of them happening in the DNA binding domain. Over-expression of non-functional variants of p53 function is common when both p53 alleles are mutated (63). Exon 5-8 missense mutations are often responsible for such over-expression (41). However, a reduction in transcriptional activity can result in a decrease in Mdm2 as a result of mutant p53 stabilization, resulting in an increase in number of non-functional/gain-of-function mutant p53 protein (64).

It could be concluded that the p53 negative of laryngeal malignant cases in the present study which are constituted 72.4% ,58.6% and 65.5% respectively for (HPV16/18(E6)/P53 ,HPV16E7/P53 and HPV18E7 of total laryngeal malignant tumors and in a part from techniques failure might results from biallelic deletion of Tp53 gene very low mutant or wild-type p53 , a nonsense mutation or a truncated p53 protein in its N-terminal portion which would not be detected by monoclonal antibody of P53 test. Another explanation , P53 may be randomly in a small proportion of cells or the detected p53 may be normal one but it is somehow abnormal stabilized or increased in amount(65,66).

Conclusion : : depending of ISH/IHC results of both hr HPV 16/18 (E6) and (E7) as well as P53 over expression revealed that high oncogenic genotypes may contribute in pathogenesis and carcinogenesis of early steps of benign and malignant tumors.

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