Human Papilloma Virus Expression in Iraqi Patients with Premalignant and Malignant Laryngeal Lesions: Immunohistochemical Study

التحري عن فايروس الورم الحليمي البشري في آفات حناجر المرضى العراقيين قبل واثناء المرض الخبيث: دراسة مناعية نسيجية كيميائية

Rand Saad Hameed *
Lecturer Dr. Mais Mohammed Salim**
Lecturer Dr. Iftikhar Khdhair Abbas**
Prof. Dr. Liwaa Hussein Mahdi Al-Kilabi**

الخلاصة:
خلفية البحث: فايروس الحليم البشري هو فايروس الحمض النووي، المحب للنسيج الطلائي، غير مغلف، مع جينوم دائري مزدوج الشريط وحوالي 8000 pb، ويبلغ قطرها 55 نانومتر.
الهدف: تسليط الضوء على العلاقة بين افات الحنجرة الحميدة وفايروس الحليم البشري كعامل مسبب في حدوث سرطان الخلايا الحرشفية في الحنجرة.
المنهجية: تم اخذ 50 حالة من حالات سرطان الحنجرة وآفات الحنجرة الحميدة في هذه الدراسة حيث تم حفظ الأنسجة مع الفورمالديهايد وجزءا لا يتجزأ من كتل البارافين. استخدمنا تقنية الفحص المناعي النسيجي الكيميائي (Immunohistochemical) لإثبات العلاقة بين عدوى فيروس الورم الحليمي البشري وافات الحنجرة.
النتائج: بعد اكمال التجارب العملية في المختبر حصلنا على حوالي (47.5 ٪) من فيروس الورم الحليمي البشري إيجابي في سرطان الخلايا الحرشفية وحوالي (20 ٪) من فيروس الورم الحليمي البشري إيجابية في آفات الحنجرة الحميدة.
الاستنتاج:
1. حوالي نصف حالات فايروس الحليم البشري كانت إيجابية وبنسبة 45.5 %.
2. معدل فايروس الحليم البشري للمرضى في العراق قليلة مقارنة مع الحالات في الولايات المتحدة الامريكية حيث بينت بعض الدراسات الى النسبة العالية لفايروس الحليم البشري.
3. تقنية الفحص المناعي النسيجي الكيميائي هي تقنية فعالة للكشف عن فايروس الحليم البشري في سرطان الحنجرة وآفات الحنجرة الحميدة.

التوصيات: الكشف عن فيروس الورم الحليمي البشري في سرطان الحنجرة بأستخدام تقنية PCR وتقنية ISH، واستخدام الحالات في عدد كبير من الدراسات المستقبلية لانتشار فيروس الورم الحليمي البشري بين عامة السكان العراقيين.
الكلمات المفتاحية: فايروس الحليم البشري، سرطان الخلايا الحرشفية، الفحص المناعي النسيجي الكيميائي.

Abstract:
Background: Human Papilloma Virus is a DNA virus, epitheliotropic, non-enveloped, with a double strand circular genome, and about 8,000 pb, measuring 55 nm in diameter.
Aim of study: Highlighting the relationship between the occurrence of premalignant laryngeal lesions and human papillomavirus like a causal factor together with development of laryngeal Squamous Cell Carcinoma.
Methodology: Fifty cases of laryngeal cancer and premalignant laryngeal lesions are incorporated within this study. Tissues were saved within formaldehyde and embedded in paraffin blocks. We used Immunohistochemical technique in order to prove the correlation between HPV infection and those laryngeal lesions.
Results: We obtained about (47, 5 %) HPV positive in laryngeal squamous cell carcinoma and about (20%) HPV positive in premalignant laryngeal lesions
Conclusion:
1. About half of cases were HPV positive (45.5%).
2. The rate of HPV infection in Iraqi patients was low in comparison to United States. Many studies show high rate of HPV detection.
3. Immunohistochemical technique was efficient method for detection of HPV in Laryngeal cancer and its premalignant lesion.

RECOMMENDATIONS: Detection of HPV in laryngeal cancer using PCR technique (polymerase chain reaction) and ISH technique (Insitu hybridization). And in future study, we will try to use a large number of cases in order to study the prevalence of HPV among Iraqi general population.
Keywords: Human Papilloma Virus**,** Squamous Cell Carcinoma, Immunohistochemistry.

*** B. D. S, Dantist, Dentist trainee, Faculty of Dentistry/University of Kufa.**
E-mail: randasaad2017@gmail.com
**** Department of Pathology and Forensic medicine 2019, Faculty of Medicine/ University of Kufa.**

INTRODUCTION

Tumor of larynx was said to be the next popular neoplasm in connection with the upper part of aero digestive area after oral cavity carcinoma. There are approximately 110,000 to 130,000 new cases diagnosed annually in the world most of these tumors are Squamous cell carcinoma, rating from eighty-five to ninety-five percent of whole tumors of larynx [1, 2].

HPV positivity is strong confirmation of biologically relevant infection. The oncogenic action of this virus is linked with the interaction of E6 and E7 on co-proteins, formed by the early E6 and E7 genes, in that order. These proteins participate in cell proliferation control, managed by tumor suppressor genes p53 and PRB, correspondingly, which are responsible for cell DNA correction and control, accountable for cell transformation and the malignant evolution of these lesions [3, 4, 5, 6, and 7].

AIM OF STUDY

Highlighting the relationship between the occurrence of premalignant laryngeal lesions and human papillomavirus like a causal factor together with development of laryngeal Squamous Cell Carcinoma

METHODOLOGY

The current cross section study was accomplished by the field of pathology and criminal medicine, (College of Medicine in the University of Kufa). All cases incorporated in current study are collected from Al-sadder education Hospital and also from special laboratories at Al-Najaf governorate. The study is collected from Al-sadder Teaching Hospital as well as from private histopathology labs in Al-Najaf governorate.

- Selection of Samples

A. **Study collection:** Fifty cases of laryngeal cancer and premalignant laryngeal lesions are incorporated within this study. Tissues were saved within formaldehyde and embedded in paraffin blocks. The ages are categorized into two age groups, one) <=50(and other) >50). The frequency of males were (33), while for females were (17). The Histological evaluation of the samples was (40) patterns with laryngeal Squamous Cell Carcinoma. and (10) patterns of premalignant laryngeal lesions.
B. **Control:** We used formaldehyde conserved submersed block of nonmalignant specimen (wart) as guidance for assessment of Human papilloma virus in laryngeal tumor tissue.
C. **Positive control section:** The control was treated with reactive component (primary antibody in IHC technique) which is known to express HPV.
D. **Negative control section:** The control unprocessed with immediate element (primary antibody).

- Commend IHC Schedule

1. Break and mounting three to four -micron formal aldehyde-preserved wax–dipped tissues on plus charged slides.
2. Air drying for two hours at fifty-eight C°.
3. Wax removal, dehumidify and rehumidify tissues.
4. Expose tissues to temperature induced recapture of the part of antigen utilizing a proper.
5. Washed with five times of Immunohistochemical cleaning buffer.
6. Placed slides in Poly discoverer Peroxidase hinder for five minute.
7. Washed with three times of Immunohistochemical cleaning buffer.
8. Covered specimen with the Prime Antibody according to industrialization advocated schedule. If anticipating settled antibodies, we propose utilizing our Immune detector Protein Blocker/Antibody Diluent to dilute antibodies.
9. Washed with three times Immunohistochemical cleaning buffer.

10. Covered specimen with Poly discoverer Horseradish peroxidase tag, hatch for forty-five minute.
11. Rinsed with three times of Immunohistochemical cleaning buffer.
12. Prepared Diaminobenzidine by inserting one droplet of Poly discoverer Diaminobenzidine Chromogen per ml of Poly discoverer Diaminobenzidine Buffer and blend.
13. Covered specimen with intended Diaminobenzidine substrate-Chromogen liquid incubated for ten minute.
14. Rinsed with five times of distilled water.
15. Reverser stains and dehydrated.
16. Cover slide with specimen protector [8].

- **Tumor marker used**

Anti- HPV, that was a mouse monoclonal antibody, clone BSB-66(SB24), 7ml, Bio SB, extract from cell cultivation floating on the surface which was settled, purified, filter disinfected and diluted in neutralize solution ph. 7.5 and used just as primary antibody for detection of HPV protein [9].

- **Statistical analysis**

To compare the demographic information between patients and control groups, the chi-squared test was used. Correlations among studied parameters were assessed using Pearson's correlation coefficients. Whole counting tests are executed utilizing the statistical package software, a counting bundle, version 20 (SPSS Inc., Illinois, and United States). The level of significance (alpha-level) was set at $p < 0.05$.

RESULTS

Project layout: The microscopic assessment of these specimens with using HPV tumor marker test (positive) was nineteen (47.5%) of samples with laryngeal SCC, while with HPV test (negative) was twenty-one (52.5%) of samples with laryngeal SCC, and with the same test (positive) was two (20%) of cases with premalignant laryngeal lesions, and (negative) was eight (80%) of cases with premalignant laryngeal lesions. Details of subject and their numbers are shown in tables (1), (2), (3).

Table (1): Demographic and Clinical characteristics of studied groups

Variables		Freq.	%
Age (Years)	<= 50	15	30
	> 50	35	70
Gender	Males	33	66
	Females	17	34
Laryngeal squamous cell carcinoma	40		80
Premalignant laryngeal lesions	10		20
Total	**50**		**100%**

Table (1): shows that the majority of age group was at the age categories (>50) BY ABOUT 70%. Regard the gender; males were more than females (66% to 34%) respectively. Furthermore, the diagnosis of studied group was Laryngeal squamous cell carcinoma by about 40%.

Table (2): Immunohistochemistry of studied parameters

Variables		Positive	Negative	Total
HPV	**No.**	**21**	**29**	**50**
	%	**42%**	**58%**	**100%**

Table (2): shows the immunohistochemistry of HPV was 58.

Table (3): Relationship among studied groups according to Immunohistochemistry of HPV infection

Diagnosis		HPV		Total	Chi-square (*p*-value)
		Positive	Negative		
Laryngeal squamous cell carcinoma	**No.**	19	21	40	X^2=2.48 df=1 *p*-value=0.115 (NS)
	%	47.5%	52.5%	100%	
Premalignant laryngeal lesions	**No.**	**2**	**8**	**10**	
	%	20%	80%	100%	
Total	**No.**	**21**	**29**	**50**	
	%	**42%**	**58%**	**100%**	

Table (3) show that most cases were negative for HPV infection among patients suffering from laryngeal squamous cell carcinoma, however, these results were not statistically significant.

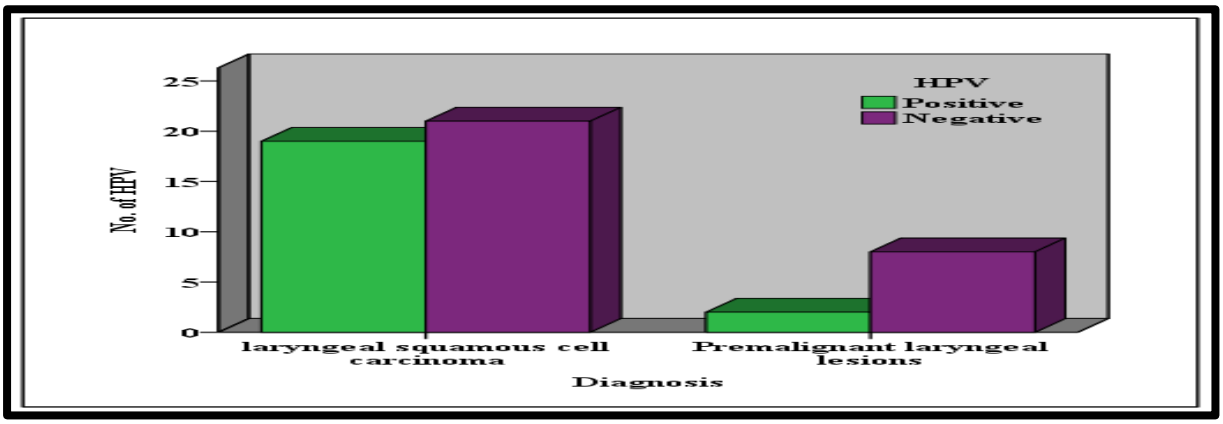

Figure (1): show that most cases were negative for HPV infection among patients suffering from laryngeal squamous cell carcinoma; however, these results were not statistically significant.

Figure (1): Relationship among studied groups according to Immunohistochemistry of HPV infection.

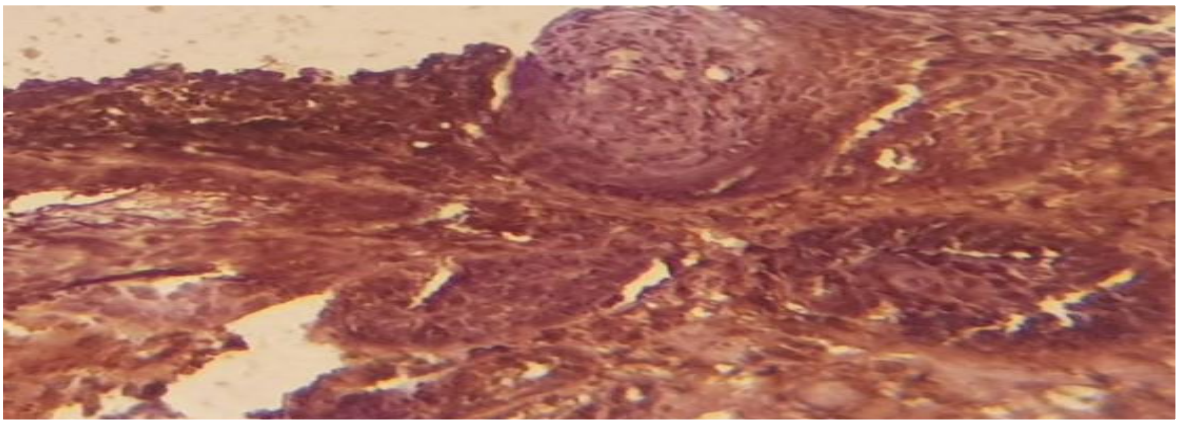

Figure (2): Positive HPV Immunohistochemical of squamous cell carcinoma of larynx shows nuclear expression of viral protein (10 x)

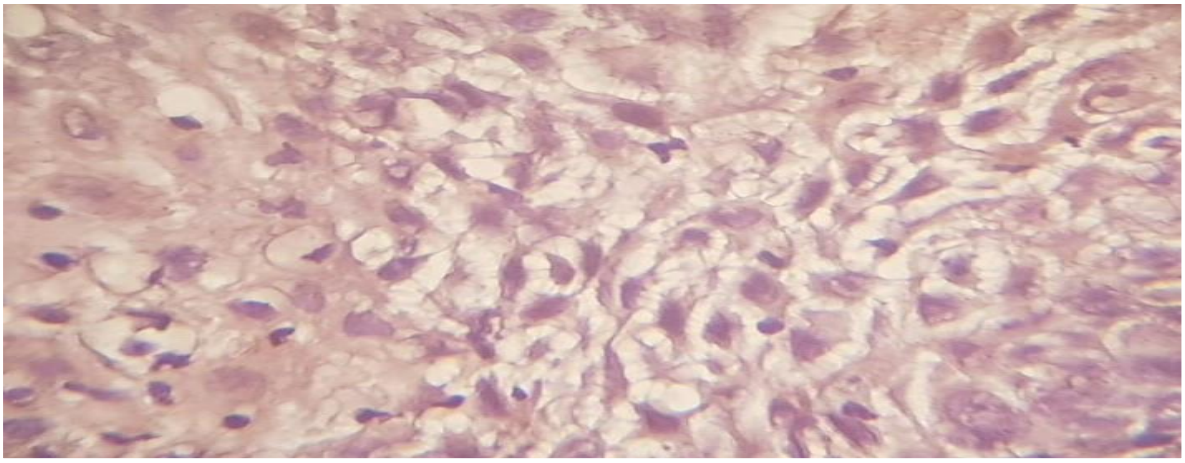

Figure (3): Negative HPV Immunohistochemical of squamous cell carcinoma of larynx shows no nucleus expression of viral protein (10 x)

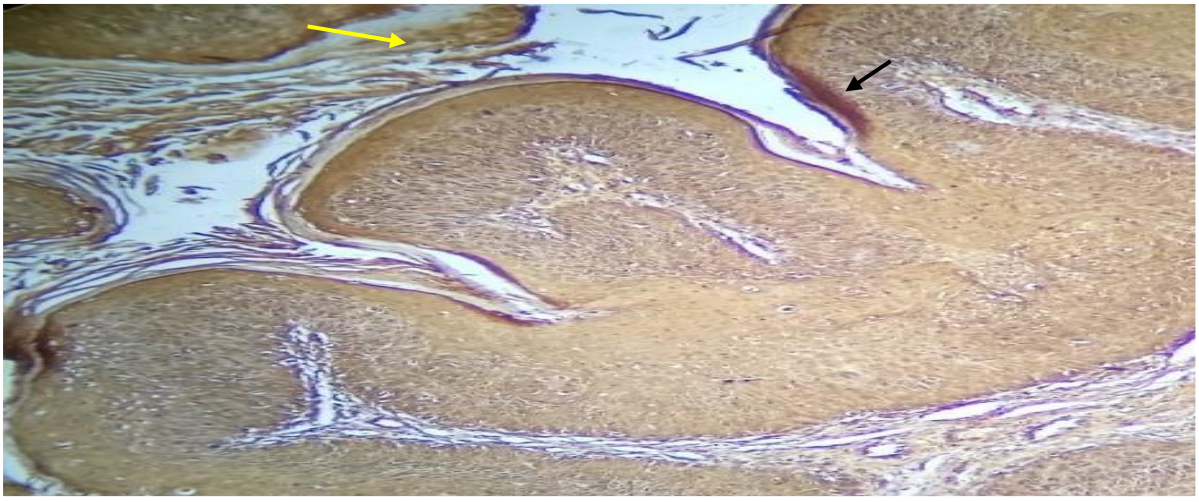

Figure (4): Positive HPV Immunohistochemical of wart shows nuclear expression of viral protein (positive control)

DISCUSSION

Argumentation: The present study acts the fundamental and remarkable move completed at Iraq for determining the function of Human papilloma virus in the development of laryngeal carcinoma.

Distribution of Laryngeal Cancer Study Relating to Gender

In the current study, the majority of age group about 70% was at age categories (<50) male was more than female (66% to 34%), this is because the most recent study showed that the genetic polymorphism of genes such as xenobiotic metabolizing enzyme (genotypes of cytochrome p-450 and glutathione s-transferees GST) has shown to increase the risk of laryngeal cancer. (Study in Brazil) [79] (Jahnke V, Matthias C, Fryer A, Strange R). Males are more affected than females with frequency (66%), these data agree with other assessment carried out in India by (D. K. Sharma) because the males are more than females due to many factors like occupational factors with frequency of laryngeal tumor were about (96.6%). HPV in patients with laryngeal squamous cell carcinoma (52 .5%) out of all cases, while in premalignant lesions revealed (80%).

HPV Immunohistochemical Study

The quality is confirmed through assessment by positive detector employed for Human papilloma virus evaluation. The favorable dominance that utilized nonmalignant lesion (verruca) that recognized to show Human papilloma virus. The marking system was supposed by different modification utilized for accounting HPV spotting result; all the specimens are examined for plus result of nucleus and cytoplasm.

Human Papilloma Virus in the Obtainable Laryngeal Lesion

The present project outcomes revealed that nineteen cases of laryngeal cancer show Human papilloma virus. Immunohistochemical spotting of nucleus that appears dark brown color in their microscopically staining part because of HPV integration with DNA genome of the host cell and its replication in nuclei, 19 (47,5%) cases are with laryngeal squamous cell carcinoma, 2(20%) cases are with premalignant laryngeal lesions. As a result, laryngeal cancer shows 47.5% positively for HPV because the nature of microenvironment of laryngeal tissue has epithelial junction area between squamous and columnar epithelium which is the favorable site for HPV infection in addition to its tropism to the epithelial cells **(10)** with ($p=0.115$). All patients in our study are non-smoking and non-alcohol. This study agrees with another study carried in Poland when HPV which was detected in 36 patients (27.5%) out of 130 tissue samples and no significance correlation, on the other hand, the study does not agree with this study because heavy smoking and alcoholic patients were included by Kamal Morshed **(11).** There was a large study carried in large hospitals on 674 Chinese patients (smokers and drinkers), it showed that HPV was not the common cause of laryngeal squamous cell carcinoma, and agreed with our study in that HPV was more in non-smokers ($p<0.05$) and non-drinkers people ($p<0.05$) **(12).**

CONCLUSION

Immunohistochemical technique was efficient method for detection of HPV Laryngeal cancer and its premalignant lesion, the rate of HPV in Iraqi patients was low in comparison to United States, many studies show high rate of HPV detection and about half of cases were HPV positive.

RECOMMENDATIONS:

1. Detection of HPV in laryngeal cancer using PCR technique (polymerase chain reaction) and ISH technique (In situ hybridization)
2. In future study, we will try to use a large number of cases in order to study the prevalence of HPV among Iraqi general population.

REFRENCES:

1. Jamal A,Siegel R,Ward E,Murray T,Xu J,Thun MJ.Cnncer statistics.2007. *CA Cancer J Clin*.2007; 57:43-66.
2. Barnes L, Tse LY, Hunt JL. Barnes L, Eveson J, Reichart P, Sidransky D, et al. Tumours of the hypo pharynx, larynx and trachea: introduction. World Health Organization Classification of Tumours: Pathology and Genetics of Head and Neck Tumours. Lyon, France: *IARC Press*; 2005.
3. J.A. Langendijk, A. Psyrri, The prognostic significance of p16 overexpression in *or pharyngeal squamous cell carcinoma: implications for treatment strategies and future clinical studies*, Ann. On col.21 10 2010 1931–1934, Epub2010/ 08/19.
4. Duray A, Descamps G, Arafa M, Decaestecker C, Emmeline M, Sirtaine N, et al. High incidence of high-risk HPV in benign and malignant lesions of the larynx. *Into J Oncol* 2011; 39:51–9.

5. International Agency for Research on Cancer IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Human Papillomaviruses, vol. 90, World Health Organization, Lyon, 2005.
6. J.A. Langendijk, A. Psyrri, The prognostic significance of p16 overexpression in oropharyngeal squamous cell carcinoma: implications for treatment strategies and future clinical studies, Ann. On col.21 10 2010 1931–1934, Epub2010/ 08/19.
7. Duray A, Descamps G, Arafa M, Decaestecker C, Emmeline M, Sirtaine N, et al. High incidence of high-risk HPV in benign and malignant lesions of the larynx. *Into J Oncol* 2011; 39:51–9.
8. Bio SB Mouse / Rabbit Poly Detector DAB HRP Brown Detection system.
9. Bio SB inset IHC of HPV on a FFPELSIL of the cervix tissue.
10. Wei-Chih chen, Hui-Ching Chuang, et. al. Clinical impact of human papillomavirus in laryngeal squamous cell carcinoma: a retrospective study.2017; 5.
11. Kamal Morshed. Association between Human Papillomavirus Infection and Laryngeal Squamous Cell Carcinoma. Journal of Medical Virology 2010; 82:1017–1023.
12. Yanan Xu, Suru Liu, Hongliang Yi, Jiadong Wang, Pin Dong, Xiaoyan Li, Shankai Yin. Human Papillomavirus Infection in 674 Chinese Patients with Laryngeal Squamous Cell Carcinoma.2014; 9(12): e115914.