

Immunohistochemical Evaluation of Human Papilloma Virus in Lung Cancer

التقييم المناعي لفيروس الورم الحليمي البشري في سرطان الرئة

Dr. Liwaa H. Mahdi, F.I.B.M.S Pathology , M.B.Ch.B, Assistant professor , Department of Pathology and Forensic Medicine- Collage of Medicine / University of Kufa ,

Dr. Abbas R. Ibraheem Al-Fatlawy, M.B.Ch.B.GP. Cytopathology Al-Hussain Teaching Hospital-Health Center of Karbala

E-mail: Liwaa.Alkulabi@uokufa.edu.iq

الخلاصة

خلفية البحث: سرطان الرئة هو السبب الأكثر شيوعاً للوفاة في كلا الجنسين. وأشد عامل خطر للورم الرئوي هو التدخين وهناك علاقة بين عدوى فيروس الورم الحليمي البشري (HPV) وورم الرئة وقد تم اكتشاف هذا الفيروس في مرضى مصابون بأورام الرئة. ولكن كان هناك جدل قائم في الكشف عن إصابة (HPV) في مريض ورم الرئة الحالي نتيجة الاختلاف في التنوع العرقي والجغرافي وأن هذه الدراسة الحالية للكشف عن نسبة الإصابة بفيروس الورم الحليمي البشري في عينات نسيج سرطان الرئة بواسطة تقنية المناعي النسيجي الكيميائي.

الهدف: صممت هذه الدراسة للكشف عن وجود فيروس الورم الحليمي البشري (HPV) على مرضى السرطان الرئوي من خلال التقنية المناعية والنسجية وعلى عينات مأخوذة عن طريق تقنية الناظور الرئوي والعينات المأخوذة بواسطة جراحة الصدر المفتوح.

المنهجية: تضمنت الدراسة ٤٠ مريضاً ثلاثون منهم ذكورا وعشرة إناث ممن أخذت عيناتهم بتقنية الناظور الرئوي أو عن طريق جراحة الصدر المفتوح صممت هذه الدراسة ، كبحث استرجاعي في قسم الأمراض/كلية الطب/جامعة الكوفة من يناير/ كانون الثاني ٢٠١٤ إلى فبراير/ شباط ٢٠١٥ وشملت العينات ٢٧ (٦٧.٥%) حالة سرطان الرئة الحشفة و٧ (١٧.٥%) حالة سرطان الرئة الغدية و٦ (١٥%) سرطان الخلايا الصغيرة. واعتبرت البثرة (حميدة الجلدية) كسيطرة نوعية في هذه الدراسة. وجمعت جميع هذه الحالات من "مستشفى الصدر التعليمي" وثلاثة مختبرات خاصة في محافظة النجف. و أعمارهم تتراوح بين ٢٧-٩٥ عاماً، وكان المعدل العمري ٦٤.١٥ سنة استخدمت طريقة LSAB+ لتحديد التعبير المناعي النسيجي لبروتين الفيروس (HPV).

النتائج: كانت نسبة الكشف عن فيروس الورم الحليمي البشري والتي تم التحري عنها بطريقة التصبيغ الكيميائي النسيجي المناعي في ٧ سبعة (٢٥.٩%) من أصل سبعة وعشرين حالة سرطان حشفي، وواحد من ٧ حالات من سرطان الغدية (١٤.٣%)، ولكن لم يكشف عن فيروس الورم الحليمي البشري في ٦ ست حالات سرطان الرئة الخلية الصغيرة (٠%)، والكشف عن فيروس الورم الحليمي البشري فيما يتعلق بالتدخين ٧ (٢١.٧٥%) من أصل ٣٢ اثنان وثلاثون حالة، و ١ (١٢.٥%) واحد من أصل ٨ حالات لغير المدخنين. أيضاً كانت نسبة الكشف عن فيروس الورم الحليمي البشري بين الجنسين ٨ ثمانية (٢٦%) حالات الذكور من أصل ثلاثون حالة في حين لم يتم الكشف عن الفيروس جميع حالات الإناث العشرة أما بالنسبة لكشف الفيروس أحلي نسبة إلى العمر فكانت ٤ من أصل ١٣ حالة تتراوح أعمارهم ما بين (٥١-٦٠ سنة) بنسبة (٣٠.٨%) و ٢ من أصل عشر حالات تتراوح أعمارهم ما بين (٦١-٧٠ سنة) بنسبة (٢٠%) و ١ من أصل ثلاث حالات تتراوح أعمارهم ما بين (٤١-٥٠ سنة) بنسبة (٣٣%) و ١ من أصل اثني عشر حالة تتراوح أعمارهم ما بين (٧١-٨٠ سنة) بنسبة (١٢.٥%). أظهرت الدراسة وجود علاقة بين وجود الفيروس ونوع السرطان وحالة التدخين وجنس المريض ولكن دون مغزى ($p > 0.05$).

الاستنتاج: وجود فيروس الورم الحليمي البشري في الأنسجة السرطانية الحشفية وخاصة الذكور والمدخنين بنسبة أعلى من باقي سرطانات الرئة الأخرى ولكن دون مغزى ولذلك فإن نوع السرطان وجنس المريض والتدخين وعمره له تأثير كبير في وجود فيروس الورم الحليمي البشري في سرطان الرئة. وأن فيروس الورم الحليمي البشري قد يلعب دوراً هاماً كعامل خطر الإصابة بسرطان الرئة.

التوصيات: إدراج هذه الدراسة المناعية النسيجية الكيميائية للكشف عن فيروس HPV مع المعلومات المرضية الأخرى سوف تعطي نتائج عملية بشكل أكثر دقة وينصح مزيد من الدراسة مع عدد كبير من حالات سرطان الرئة ومع تقنيات أخرى مثل تفاعل سلسلة البلمرة المتعددة (PCR) وتقانة التهجين الموضعي (ISH). للكشف عن فيروس الورم الحليمي البشري في حساسية عالية وبصورة أكثر دقة.

Abstract:

Background: Lung cancer is the common repeated cause of death in both gender. The highest risk factor for lung tumor is the smoking and the relationship of human papilloma virus (HPV) infection and lung tumor has been reported. HPV can be detected in patient suffering from lung tumors. But there was conflict in the detection of (HPV) infection in patients with lung tumor as a result of racial and geographical diversiform, this current study to detect the rate of HPV infection in lung cancer tissue sample by immunohistochemical technique.

Aim of study: This study was designed to detect the presence of HPV in lung cancer patients by immunohistochemistry in specimens taken as lobectomy, tru cut biopsy or bronchoscopic biopsy.

Material and Methods: A total forty 40 lung cancer tissue samples of thirty 30 male and ten 10 female patients, who had undergone lobectomy or tru cut bronchoscopic biopsy from their lungs, where involved in this study, that performed in the Department of Pathology, Faculty of Medicine, a University of Kufa from January 2014 to February 2015, twenty seven patients 27 were squamous cell carcinoma (67.5%) and seven 7 patient were adenocarcinoma (17.5%) And six 6 patients were small cell carcinoma (15%). wart (benign skin lesion) was considered as control involve in this study. All these cases were collected from Al-Seder Teaching Hospital and

three private laboratories in AL-Najaf governorate. Their ages were ranging from 37-95 years, with mean 64.15 year and standard deviation ± 11.45 , thirty two 32 patient were smoker, twenty four 24 were male, while eight 8 were female smoker, while the remaining eight 8 of cases not smoking, six 6 were male, and two 2 were female cases. Labeled Streptavidin-Biotin (LSAB+) method for Immunohistochemical detection of HPV was employed. The statistical analysis was officiated using the SPSS version 20.0 statistical software program. The Differences of HPV prevalence among gender, smoking status, age groups and histological cell types, were calculated using the Pearson's Chi-square test. and correlation- regression test at P value <0.05 and ≥ 0.3 respectively.

Results: The immunohistochemistry detection of HPV was reported in seven 7(25.9%) out of twenty seven 27 cases of squamous cell carcinoma, and one 1 case (14.3%) out of seven 7 cases of adenocarcinoma, But there was no detection of HPV in six 6 cases of small cell lung cancers (0%).

Conclusions: HPV expressed in lung cancer tissue, mainly in squamous cell carcinoma specially in male smoker patient but with no significant

Type of cancer and smoking and gender and age may have significant effect on expression of HPV in lung cancer.

HPV play significant role as risk factor in lung cancer.

Recommendations:

1- Incorporation of HPV immunohistochemical study with other pathological parameters will give more accurate prediction of clinical outcome.

2- Further study with large number of lung cancer cases and with other techniques like PCR and ISH are recommended to detect the HPV in high sensitivity and specificity.

Keyword : Lung cancer, Expression, HPV.

INTRODUCTION:

Lung carcinoma is the main reason of the mortalities for people of both genders worldwide^(1,2,3). This increasing in mortality rate is mainly because most of patients with carcinoma of lung are detected at preceding stage of progression. Developed early detection of cancer, which was referred as premalignant lesions, surely will assist to reducing the mortality rate. It is, as well, a big problem, taking in to account the fact that the total lung is a possible place of malignancy, and carcinoma has multiple various possible pathways to progress⁽⁴⁾. Lung cancer develops in two separate compartments - the central (transporting air) and the peripheral (respiratory) part of the lung. There are four histological types of cancer, including small cell cancer and heterogeneous group of non-small cell cancer, which includes squamous cell carcinoma, adenocarcinoma including a non-invasive type of bronchioloalveolar carcinoma, large cell cancer and many others, much less frequent subtypes. Prolonged duration exposure of the epithelium lining the lungs to various carcinogenic substances, including most of all cigarette smoking, lead to a numerous genetic changes of the cells make it in various problems. These multistage sets of changes result in variable morphological consequences with tumor progression and proliferation^(5,6). Consequently, lung tumor perhaps progress in the major bronchus, tiny bronchioles as well as alveoli. Squamous cell carcinoma is most often placed centrally whereas adenocarcinoma and large cell cancer are typically encountered in the peripheral part of the lung^(5,6).

Human papilloma virus (HPV) involved within pathogenesis in some human being carcinomas like carcinomas of uterine, cervix, urinary bladder and oropharynx⁽⁷⁾. Depending on the existing information, HPV are generally correlated through the progression of cervical tumors in addition. HPV of type 16 as well as 18 kinds recurrently associated with severe inside the layer of cells that forms the surface lesions in addition to invasive malignant neoplasm. First evidence of probability association of HPV within lung squamous cell carcinoma were demanding through Syrjäne, depend on histological information⁽⁸⁾. After that, HPV DNA was revealed within different separated suitcases of lung carcinoma when numerous biopsies have been examined^(9,10).

Aim of the Study:

This study was designed to detect the presence of HPV in lung cancer patients by immunohistochemistry in specimens taken as lobectomy ,tru cut biopsy or bronchoscopic biopsy.

Material and Methods

Forty cases with lung tumor are incorporated within this revise. These patient had thoracotomy in addition to tru cut biopsy or bronchoscopic biopsy. Forty 40 samples lung cancer patient blocks had been fixed with formalin and embedded with paraffin wax these cases were collected from laboratory of Histopathology in Alsader Teaching Hospital in Al-Najaf and from three private laboratories in this governorate. The ages of patients ranges from 37 to 95 years with mean 64.15years with standard deviation ± 11.45 . The ages are categorized to age groups were from 31-40 years one1(2.5%) male case, and from 41-50 years two 2 (6.7%) male cases, and one 1 (10%)female case,from51-60 years were ten10(33.3%) male case and three 3(30%) female cases, from 61-70 were seven 7 (23.3%)male and three 3 30% female case. Were from71-80years ten 10 (33.3%) male cases and two (2) 30.0%female cases while >80 years one 1 (10. %) female case. Total numbers of male Patients thirty (30) 75% and female patients were ten 10(75 %.)The number of patient who are smoked thirty two 32(80%),twenty five 25 (83.3%) were male and seven7(70%) were female. while eight 8(20%) were nonsmoker Five 5 (16.7%)were male and three 3 (30.0%) were female. Ethical approval was confirmed by review of freshly prepared hematoxylin and eosin-stained slides and classified according to criteria by World Health Organization (WHO).The histological diagnosis of these tissue blocks was twenty seven 27 cases of squamous cell carcinoma .and seven 7 cases of adenocarcinoma, and six 6 cases were small cell carcinoma..formalin fixed embedded block of benign skin lesion (wart) as control for diagnosis of HPV in lung cancer tissue were used.

Immunohistochemistry: The immunohistochemistry staining technique used within the modern review were Labeled Streptavidin Biotin (LSAB+) procedure which were functional designed for HPV's discoloration.The formalin fixed and paraffin embedded tissuesubjected to microtome section to (4 μ m) sections in thickness, and then labeled on charged slides. Then all sections were dried out by set the slides in a 60 °C oven for 60 minutes.After thatdewaxation by immersion of slides in xylene baths 5 min each. ,thenrehydrated through repeated dilutions of alcohol,in two time alteration of fresh complete 100% ethanol for 3 min. andin two time alteration of 90% ethanol for 3min.then in 80% ethanol for 3min.and put in phosphate buffer saline PBS clean bath for further rehydration (30 min. at room temperature).Clean the slide by means of deionized(H₂O)Water Purifying in addition to transfer them in a microwave ovenin citrate buffer two time 5 minutes. Then get rid of extra solution by means of a quick movement and with awareness. Clean all slide just about the section. Put 100 μ l Anti-Human Papilloma(HPV) which is monoclonal antibody monoclonal mouse anti(HPV)protein,Dakoprimary antibody solution, coat the tissue slice. Incubate for at least 60 minutes. At 37 °C in humidify hall. Then put in 100 μ l Biotinylated link Antibody (K0679)to all slides, coating the tissue slice .Incubate in a humidity hall for at nearly all 30 minutes at room temperature. Then Put in to all slides 100 μ l Streptavidin / peroxidase (K0679) , coat the section.incubate in humid chamber for at least 20 minutes at room temperature.Then

put adequate drops of DAB+ (K0679). incubate 5-10 minutes. after that until preferred dye response is realistic, following check by the microscope. Then put in sufficient Lillie's modification (S3309) on the way to cover slide then put slide inside a bath of Lillie's modification(Mayer's hematoxylin) Wash the slides in water gently for 5 minutes. Skin wart used as control were all steps done for immunohistochemistry by using two slides one treated with primary antibody used as positive control ,the second not incubated with primary antibody were used as negative control.

Scoring system:

The standard factor for positive immunoreactions is dark brown spontaneous in the nucleus for HPV.each cancer was given a score consistent with the intensity of the nuclear or cytoplasm staining

- no staining=0
- weak staining=1
- moderate staining=2
- strong staining=3

And the degree of stained cells

- 0%=0
- 1–10%=1
- 11–50%=2
- 51–80%=3
- 81–100%=4

The ending immunoreactive score determined by multiplying the intensity and extent of positivity scores of stained cells with the minimum score of 0 and a maximum score of 12^(11,12,13).

Statistical analysis

Statistical study was achieved by means of the SPSS 20.0 statistical software program. Variation of HPV revealing among gender, smoking status, age groups, histological cell types, were calculated using the Pearson's Chi-square test.

RESULTS:

In the studied group HPV immunoreexpression was reported in eight(8) out of 40 cases of lung cancer represented (20.0%) within type of tissue and thirty two 32 cases were negative represented (80.0%) within type of tissue as shown in table 1. the immunodetection rate looks positively well correlated to smoker patients.

The immunoreexpression of HPV in related to age groups of presented cases were seen in table (2) that shows the difference among these age groups was statically no significant. ($p>0.05$)..However, the immunodetection rate look not correlated with age group.

Table (1) Clinicopathological features of lung SQC and AC and SCC.

	Number of subjects (%)			
	SQC	ADC	SCC	P-value*
Total	27 (100)	7 (100)	6 (100)	
Gender				0.029
Male	23 (85.2%)	5 (71.4%)	2 (33.3%)	
Female	4(14.8%)	2(28.6%)	4(66.7%)	
Age (years)				0.92
≤60	13 (48.5%)	2(28.5%)	2 (33.3%)	
≤70	5 (18.5%)	3(42,5%)	2 (33.3%)	
>71	9 (33%)	2(28.5%)	2(33.3%)	
Smoking				0.249
Non smoker	4(14.8%)	3(42.85%)	1 (16.6%)	
Smoker	23(85.2%)	4 (57.15%)	5 (83.4%)	

SQC= squamous cell carcinoma; AC=adenocarcinoma,SCC=small cell carcinoma .

*P-values for difference between SQCs and ACs and SCC were obtained from Chi square test.

This table shows the clinico-pathological features of patients regarding to gender and age groups with smoking habit.there is significant association between the gender and types of lung cancer.

Table(2)Relationships between HPV imunohistochemical staining and clinicopathologicparameter of patients with lung tumors.

Parameters	HPV-	HPV+	P Value
Age			
≥60years	12	3	0.73
<60years	20	5	
Gender			
male	22	8	0.68
female	10	0	
smoking			
smoker	25	7	0.553
non smoker	7	1	
Tumor type			
SQC	20	7	0.3
ADC	6	1	
SCC	6	0	

Note :negative HPV immunostaining and positive HPV immunostaining were detected by immunohistochemistry.

Abbreviation :SQC squamous cell carcinoma,ACCadenocarcinoma,SCC small cell carcinoma

This table shows the relationship between the HPV immunohistochemical staining and clinicopathological parameters of patients suffer from variable types of lung cancer. there is no significant relationship between the HPV immunostain and types of lung cancer.

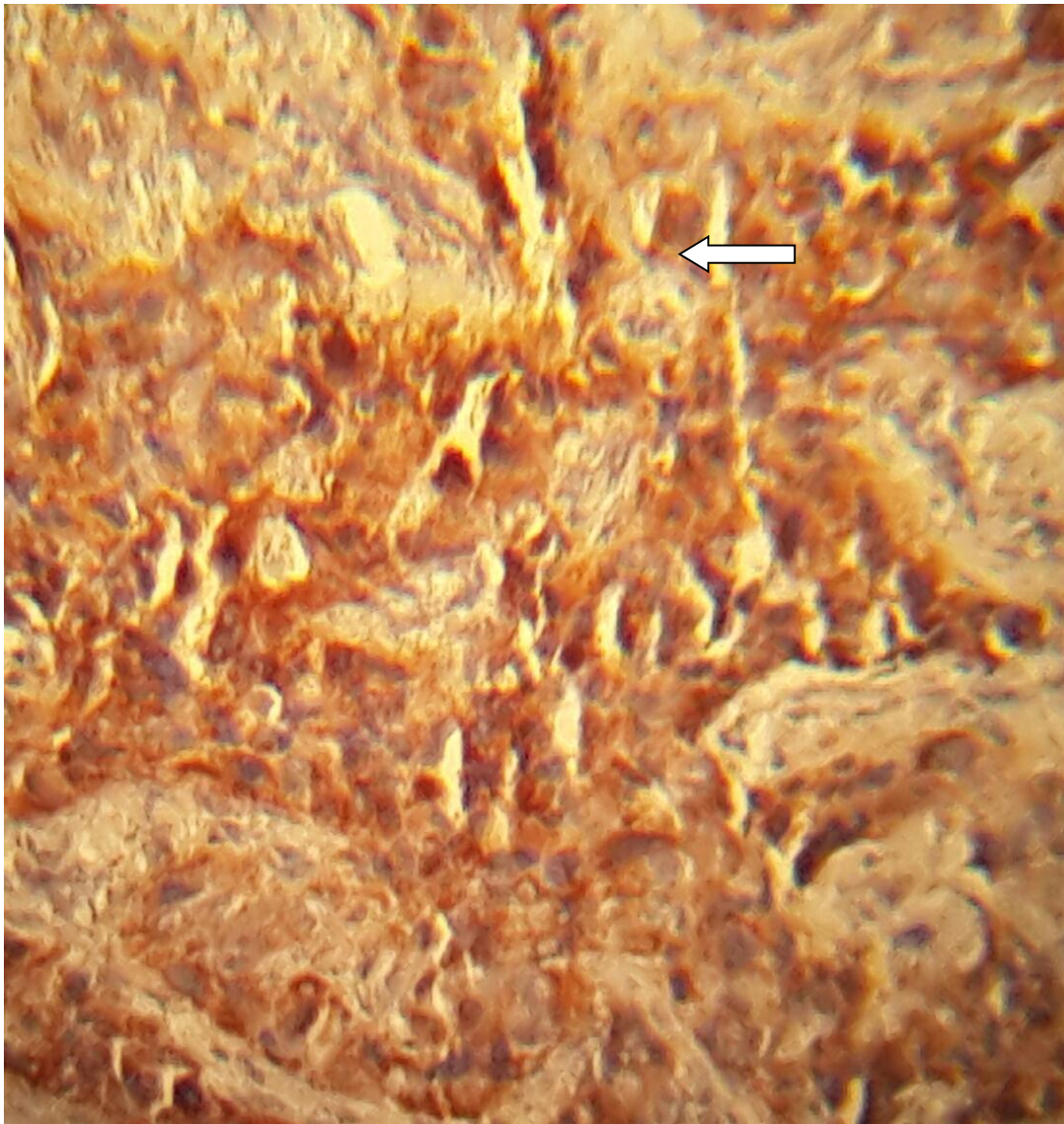


Figure 1. HPV immunostain positive in squamous cell carcinoma of lung shows both cytoplasmic and nuclear viral protein expression (Arrow), X40

DISCUSSION:

in the current study the results have exposed that eight 8 out of forty 40 cases of lung carcinoma are express HPV immuohistochemical nuclear and cytoplasmic staining which is appear brown color in their histological staining section .7 (25.9%) cases with squamous cell carcinoma out of 27 (74.1%) (7out of27)of cases with squamous cell carcinoma, (17.5%) of all cases, and 1 (14.3%)case were with adenocarcinoma out 7(1out of seven) (85.7%) cases of adenocarcinoma (2.5%) of allcases, whereas zero (0%) cases out of six 6(0out of 6) cases were with small cell carcinoma. As a result, lung cancer show 20% positively for HPV with ($p=0.327$). but with no significance. The finding rates of HPV in lung carcinoma tumors are topic to large differences. In a new assessment of 85 revision documentation.about2500samples

demonstrate a finding rate unreliable beginning (0 - 100%).(Syrjanen *et al*) ⁽¹⁴⁾ This study agree with other study presented by(Castillo *et al*) ⁽¹⁵⁾ performed in three America Latin countries Mexico ,Colombia, Peru 36 cases were subjected to study 22 male and 14 female ,14 cases with SQCa,13 with ADC, and 9 cases with SCC. The HPV revealed mainly in SQCa but not agree with expression of HPV in ADC were revealed null and in contrast SCC expressed HPV. The immunohistochemical analysis of the outcome shows that HPV expression in 7 (21.9%) out of 32 cases smoker of lung cancer and 1(12.5%) out of 8 cases nonsmoker of lung cancer. In our study all smokers and nonsmoker of the obtainable lung cancer were no significantly correlated to the of HPV(p value =0.535). There was no correlation between the patients' smoking history and the detection of HPV but the rate of HPV infection higher in smoker than nonsmoker patients. Current study nearly agree with (Kato *et al*) ⁽¹⁶⁾ .in Japan were from 42 cases of NSCLC smoker HPV positive were about 16.7. But our study not agree with study by (Ya-Wen Chen *et al*) ⁽¹⁷⁾ were HPV detected more in nonsmoker than smoker patient HPV immunoexpression revealed that 8(26.7%) (8 out 30) male of presented samples with lung cancer while not any of female cases were exposed HPV. The difference among gender group were statically not significant (p value=0.68) our finding nearly as study performed by (Yang Fei *et al*) ⁽¹⁸⁾ were revealed that HPV expressed in male higher than female with p value >0.05 ,also our study not agree with study by (Cheng YW *et al*) were HPV expressed more in female gender than male gender ⁽¹⁷⁾ .The immunoexpression of HPV in related to age groups of presented cases revealed no HPV immunoexpression in age group (31-40 year), and revealed 1(33.3%) (1 out 3) cases in age group (41-50 year), and 4(30.8%) (4 out of 13) cases in age group (51-60 year), and 2(20%) (2 out of ten 10) cases in age group (61-70 year), and revealed that 1(8.3%) (1 out of 12) cases in age group (71-80 year), and revealed that zero 0 (0%) (0 out of one 1) case in age group (>80 year). The difference among these ages groups were statically not significant (value=0.73.). However, the immunohistochemistry detection. rate look higher with age group (51-60 year). this study correlated with study by (Cheng YW *et al*) ⁽¹⁷⁾ were revealed that HPV16E6 immunostaining more expressed in age >65 years than age ≤65 years with p value =0.690 and immunostaining of HPV18E6 expressed in age ≤65 years higher than age >65 years with p value=0.404.

CONCLUSION

The prevalence of HPV among the lung cancer cases examined has been found 20%, mainly in squamous cell carcinomas.

The small cell carcinoma tissues did not show any expression of HPV in immunohistochemical technique.

The prevalence of HPV in Al-Najaf governorate is still not known because of limited studies, in comparison to western Europe and Asia and American Latin where many studies report high rate of detection of HPV.

The using of immunohistochemical technique are well correlated method for detection of HPV in lung carcinoma.

The expression of HPV in lung carcinoma have focused on squamous cell carcinoma (25.9%) from 27 cases of squamous cell carcinoma, while adenocarcinoma 1(14.3%) out of 7 cases of adenocarcinoma.

The detection rate varied from 0% to 100%, depending on the technique used and the sample size.

RECOMMENDATIONS:

1. Genetic DNA analysis of HPV gene in lung cancer that could be detect genotypes by ISH and PCR technique to accurate occurrence of oncogenic HPV genotypes and development of lung cancer.
2. Larger study including all types of lung cancer for further investigation HPV over expression and decisive the relation connecting genotypes and progression of lung carcinoma.
3. Further studies with large number of cases are needed to study the actual prevalence of HPV infection among Iraqi general population
4. Type of sample in detection of HPV DNA more précised in fresh frozen samples than the paraffin embedded blocks.

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