IN SITU HYBRIDIZATION APPROACH FOR DETECTION OF EPSTEIN –BARR VIRUS & HUMAN PAPILLOMAVIRUS TYPE 16 IN A GROUP OF IRAQI WOMEN WITH CERVICAL CARCINOMA [+]

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Abstract:

Aim: **In order to investigate the involvement of (EBV & HPV 16) viruses in the pathogenesis of cervical carcinoma, the presence of the genome of these viruses were investigated in 100 cervical biopsies, histologically classified as squamous cell carcinomas & adenocarcinomas of uterine cervix.** Methods: **detection of DNA signals for both EBV & HPV 16 by non-radioactive In Situ Hybridization technique (ISH).** Results: **A different percentage of positively signals for vital infection was found between the two groups of examined DNA; EBV & HPV 16 infection in cervical carcinoma cases were 52% and 70% respectively; on the contrary in normal cervix tissues a low percentage of viral genomes presence were detected: 44% for EBV and 25% for HPV 16.** Conclusions: The present study shows **there is a compelling body of evidence that cervical carcinoma is related to human papilloma virus infection as a Co-factor and more recently Epstein-Barr virus have been proposed as candidates (other Co-factore) for cervical neoplasia. Our data confirm the involvement of HPV16 & EBV in cervical neoplasia and suggest a possible role of these viruses in cervical cancerogenesis.**

نهج التهجين الموضعي للكشف عن فيروس ابشتاين بار والفيروس الحليمي البشري نوع 16عند مجموعة من النساء العراقيات المصابات بسرطان عنق الرحم

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المستخلص :

الهدف: من اجل التحري عن مدى تداخل فيروس ابيشتاين بار والفيروس الحليمي البشري نوع 16 في التسبب بسرطان عنق الرحم ، وجود الجينوم لكلا الفيروسين بحثت في 100 خزعة من عنق الرحم ، والتي صنفت نسيجياً الى سرطان الخلايا الحرشفية والخلايا الغدية لعنق الرحم ،الطرق: استخدام تقنية التهجين الموضعي الغير مشعة للكشف عن اشارات الحمض النووي (الدنا) لكلا فيروس ابيشتاين بار وفيروس الحليمي البشري نوع 16.

النتائج: وجدت نسب اشارات موجبة مختلفة للأصابة بين المجموعتين الخاضعة لفحص الدنا ، بحيث كانت النسب في حالات سرطان عنق الرحم هي 52 % و 70% لكلا فيروس ابشتاين بار و فيروس الحليمي البشري نوع 16على التوالي وعلى العكس اكتشفت نسب منخفضة للضهور الحيوي لجينوم الفيروسين في أنسجة عنق الرحم الطبيعية : 44٪ لل EBV و 25٪ لل HPV16 .الاستنتاجات: الدراسة الحالية وجدت مجموعة من الأدلة المقنعة على أن سرطان

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عنق الرحم يرتبط بأصابة فيروس الحليمي البشري كعامل مشترك وقد اقترحت مؤخرا فيروس ابشتاين بار كمرشح (عامل مشارك اخر) لنشوء اورام عنق الرحم. والبيانات المتوفرة لدينا تؤكد تورط HPV16 و EBV في اورام عنق الرحم و تشير إلى دور هذه الفيروسات في سرطنة عنق الرحم.

Introduction :

Epstein–Barr virus was a first tumorigenic human herpes virus that is ubiquitous in the adult population. It infects >90% of the world's population [1]. The EBV is transmitted commonly by saliva, but it has been found in genital secretions, which suggests sexual transmission and led researchers to connect EBV and cervical neoplasia [2, 3]. More recently, there have been scattered reports linking EBV with conventional epithelial cancers of other primary sites including breast, lung and gastric carcinomas with regards to cervical cancer the role of EBV remains controversial, some authors have proposed that EBV could play a role in the carcinogenesis of cervical tumors; however, other studies do not support this hypothesis [4, 5, 6].

Human papillomavirus type 16 (HPV16) is a cancer related virus that causes dysplastic tissues to grow on the infected area, and it's a causative agent of cervical cancer with over than 60%, HPV 16 is a contagious spreads mostly through sexual contacts and the infected couple can transmit the disease to the Off-springs as well [7].Sometimes HPV 16 has no harmful effect on the human body & it remains dormant in the body but it can be transmitted to others as a result of sexual contacts and cause diseases [8, 9]. Cervical cancer continues to cause significant morbidity and mortality over the past few decades. After breast cancer, cervical cancer is the second most common type of cancer in women worldwide [10] High-risk human papillomaviruses are the causative agent of cervical cancer .It is arising through disruption of the p53 pathways and the product of the retinoblastoma gene by the human papillomavirus oncoproteins E6 and E7 [11,12].

Materials and Methods :

A: Patients and tissue samples:

We analyzed 50 cases of invasive cervical carcinoma treated between (2008) to April (2012) and 50 cases of non-malignant cervical tissues treated between January 2011 to December 2011 at department of Histopathology –Teaching Laboratories that belongs to the Medical City Teaching Hospital, in Baghdad. The mean age of the patients with invasive cervical carcinoma was 48 ± 12 years (range, 25 to 82 years).While the mean age of the females in the control group was 46 ± 10 years (range, 27–71 years).

All slides were reviewed, and the clinico-pathological data including histological type, grade of carcinoma and age at initial diagnosis were collected. The histological type of invasive cervical carcinoma was squamous cell carcinoma in 42 (84%) cases, adenocarcinoma in 8 (16%) cases and adenosquamous carcinoma not detected. Tumor grade was determined by differentiation categories: moderately differentiated carcinomas were diagnosed in (28/50) (56%) of the cases, while each of well differentiated & poorly differentiated carcinomas were made (11/50) (22%) of the cases.

B: Study Design:

This study was designed as a two retrospective populations. It involved 100 selected & well preserved formalin fixed, paraffin embedded cervical tissue blocks distributed as the following: **The**

first population, included 50 blocks of cervical carcinomas tissues as a case of study & 50 blocks of non-malignant cervical tissues as a control of study to determine the EBV infection. **The second population** consisted of 30 blocks of cervical carcinoma tissues as a case of study and 20 blocks of non-malignant cervical tissues as a control of study, all the blocks in the second population selected randomly from the first population to determine HPV16 infection then the degree of Co-infection between EBV & HPV16 was evaluated in study groups.

C: In Situ Hybridization Techniques for EBV & HPV 16 detection:

We used a biotinylated probe for DNA of EBV & HPV 16. Tissue fragments were placed on positively charged slides and baked in a vertical position at 65˚C in an oven overnight. Afterward, the slides were routinely deparaffinizated. The slides were placed on the boiling citric buffer solution heated in a specific jar on hotplate until it was boiled, or kept at 98˚C, then slides placed in the solution for 15 minutes. Enzymatic digestion was performed with proteinase K (1X) for 15minutes at 37˚C , the slides were dehydrated in series concentrations of alcohol, and dried at room temperature. Then slides were incubated with suitable diluted probe solution overnight at 37˚C. (The probe for EBV & HPV 16 was denaturized in oven at 95˚C for 8-10 minutes). Visualization of the reaction was possible through labeling the probe with biotin and detection of the hybridized probe by addition of an enzyme-conjugated streptavidin alkaline phosphatase (streptavidin-AP), [13] and then incubated for 20 minutes at 37˚C. Upon addition of the single component BCIP/NBT solutions (suitable substrate) then slides were incubated at room temperature (20-25˚C) for about 40 minutes until the color development was optimal.

Finally, the slides were counterstained with eosin and mounted. Positive controls consisted of a case of nasopharynx lymphoepithelioma-like carcinoma previously knows to be EBV positive and cervical carcinoma positive for HPV 16.

D: Evaluation of DNA ISH Signals for EBV & HPV16:

The positive cells presented (bluish black discoloration) staining at the site of complementary sequences as nuclear as well as cytoplasmic signals. The viral signal patterns were classified as punctate, diffuse, or mixed. A punctate pattern, that is, distinct dot-like signals or scattered tiny particles in the nucleus, was considered to indicate integrated viral genome, and a diffuse pattern, that is, large globular homogeneously dense staining in the nucleus & cytoplasm, was considered to represent episomal viral genome, while the mixed patterns were considered to indicate integrated & episomal viral genome. The intensity scale included negative corresponding to no detectable ISH reaction and positive graded as low, Moderate, and high intensity of reaction [14].

Results & Discussions :

1. Detection of EBV infection (First Population):

The presence of ISH signals for EBV DNA were monitored in (52%) of cervical carcinoma cases. It was higher than that found in control group which made (44%).

The prominent intensity of replication was (36%) of cervical carcinoma cases, showing high intensity, while (20%) of the control group showing low intensity. As shown in figure (1-A).

Furthermore, patterns of replication showed that (20%) of cervical carcinoma cases were mixed patterns (which involved integrated as well as episomal EBV DNA), while (32%) of

the control group showed diffused (episomal) EBV DNA that may reflect asymptomatic non progressive cervical intraepithelial lesions [14]. As shown in figure (1-A).

Significant difference was found between ISH EBV DNA signals in comparing signal intensities and patterns of replication with study groups as shown in table (1):

Table(1): Frequency distribution of positive EBV DNA signals intensity & patterns of replication between study groups

Study Groups	Total EBV ISH-ve results	Positivity according to Intensity of replication			Positivity according to Patterns of replication			Total EBV ISH+ve results
		High	Moderate	Low	Diffused	Punctate	Mixed	
Case 50	**24 /50** (48%)	**18** (36%)	**5** (10%)	**3** (6%)	**9** (18%)	**7** (14%)	**10** (20%)	**26 /50** (52 %)
Control 50	**28 /50** (56%)	**7** (14%)	**5** (10%)	**10** (20%)	**16** (32%)	**4** (8%)	**2** (4%)	**22 /50** (44 %)
Total 100	**52** %	**25** 52.1%	**10** 20.8%	**13** 27.1%	**25** 52.1%	**11** 22.9%	**12** 25%	**48** %
		P-value:0.030 , p<0.05, Significant			**P-value:0.038 , p<0.05, Significant**			

This finding is similar to what has been reported previously by [15, 16]. However Malin & Larissa *et al* [17,18]; concluded that EBV was present in high amounts in the genital tract, indicating that a sexual route of transmission and relation to genital disease is possible, and suggested further studies using real-time PCR as a useful in determining the role of different herpes viruses in human disease.

Furthermore, for most of the tumor viruses the viral genome persists in an integrated or episomal state. The EBV-DNA tumor viruses' infection is latent, the viral genome persists in some form in the tumor tissue, and a subset of viral genes is expressed in the tissue so the EBV may act as a Co-factor especially in the cervical malignancy and the incidence may found in certain geographic areas [19, 20].

2. Detection of HPV16 infection (Second Population):

The presence of ISH signals for HPV16 DNA in the cervical carcinoma cases were made (70%) which was higher than that found in control group which made (25%).

The prominent intensity of replication was (33.3%) for cervical carcinoma as a high intensity, while (15%) of the control group showing low intensity. As shown in figure (1-B).

The most prominent patterns of replication was found to be as diffused (episomal DNA) pattern which made (43.3%) for case & (25%) for control group that may reflect asymptomatic non progressive cervical intraepithelial lesions [14]. As shown in figure (1-B).

Significant difference was found between ISH HPV 16 DNA signals in comparing signal intensities and patterns of replication with study groups as shown in table (2):

Table(2): Frequency distribution of positive HPV16 DNA signals intensity & patterns of replication between study groups

Study Groups	Total HPV16 ISH-ve results	Positivity according to Intensity of replication			Positivity according to Patterns of replication			Total HPV16 ISH+ve results
		High	Moderate	Low	Diffused	Punctate	Mixed	
Case 30	**9** (30%)	**10** (33.3%)	**6** (20%)	**5** (16.7%)	**13** (43.3%)	**3** (10%)	**5** (16.7%)	**21** (70%)
Control 20	**15** (75%)	**2** (10%)	**0** (0%)	**3** (15%)	**5** (25%)	**0** (0%)	**0** (0%)	**5** (25%)
Total: 50	**24** %	**12** 46%	**6** 24%	**8** 30%	**18** 69.2%	**3** 11.5%	**5** 19.2%	**26** %
		P-value:0.008 , p<0.05, Significant			**P-value:0.009 , p<0.05, Significant**			

Our results came compatible with Jung *et al*., 2010, reported that high risk of HPV 16 was detected in (69.5%) by using ISH assay [14].However, that observation was insufficient to show whether the virus has got a role in primary cervical carcinoma or it is only due to an ascending infection. This may need further studies to be proved. In an indirect way this could be related to the patient's genetic factors [21].The host genetic factors may play an important role in susceptibility to HPV16 infection in different geographical regions particularly MHC class II [22].In addition to the host and pathogen genetic variation, the difference of the detection methods employed in the studies might also account for data interpretations.

Human papillomavirus 16 (HPV16) genome integration into the host genome is thought to be one of the causes of cancer progression and explain there's a persistence HPV infection. However, there is controversy concerning the physical status of HPV16 in premalignant cervical lesions [23, 24].

For this reason, a hypothesis was made in the present study for the ISH signals of HPV DNA, the detection of HPV16 in both normal and abnormal cervical tissues may reflect that HPV16 was a sexual transmitted agent by a male vector and continuous predisposition (high dose) of HPV16 into cervical tissues & viral genome integration (persistence infection) may be help in the progression into carcinoma, but the high percentage of episomal viral DNA in cervical carcinoma & control group indicate that HPV16 sharing in carcinogenic process as a co-factor besides other factors like a genetic mutation , external environmental factors, immunological disorders or even EBV infection to induce cervical carcinoma as shown in table (2).

3. The EBV & HPV 16 Co-infection (Second Population):

For cases of the study group , both EBV & HPV16 DNA Co-infection were detected in (12/30) of the cases as (24%) while in the control group were detected in (2/20) as (4%) as shown in table (3): Significant difference was found between EBV & HPV16 Co-infection signals detected by ISH in comparing with study groups.

Table(3): Co-infection between EBV & HPV16 in cervical carcinoma

Study Groups	ISH Total results for co-infection of EBV & HPV 16				Total of Study Groups
	EBV	HPV16	EBV & HPV16	Negative	
Case	8 (16 %)	9 (18 %)	12 (24 %)	1 (2 %)	30 60%
Control	8 (16 %)	3 (6 %)	2 (4 %)	7 (14 %)	20 40%
Total	16 32%	12 24%	14 28%	8 16%	50 100%
P-value	**P-value:0.004 , p<0.05, Significant**				

The pervious results were compatible with Adi Prayitno, [25]; he reported 89% of cervical cancers were infected with HPV16 while 68% were infected with EBV & HPV16.

Moreover, Aromseree *et al* [26]; demonstrated the EBV-HPV co-infections, especially with HR-HPV16 which was significantly increased in cervical carcinoma groups. Interestingly, this co-infection was not associated with HPV integration but raised the possibility that EBV could be a possible cofactor and induce HPV16 episomal form to contribute to cervical cancer development and this will support our study findings with highly significantly compatible.

For more reasonable interpretations to rule out the HPV16 as main and as causative agents for Iraqi cervical carcinoma, it is possibly due to the practice of circumcision in Muslim population, furthermore, it is clearly stated that the risk of cervical cancer for a given women is predictable by the sexual behavior of her husband as much as for her own sexual behavior, and it has been confirmed that male circumcision protects the wives of husbands from cervical cancer. This clearly shows that male sexual behavior is a central determinant of the incidence of cervical cancer [27].

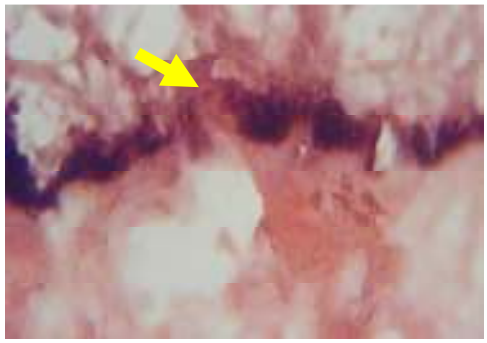

Figure: SCC, high intensity, diffused.

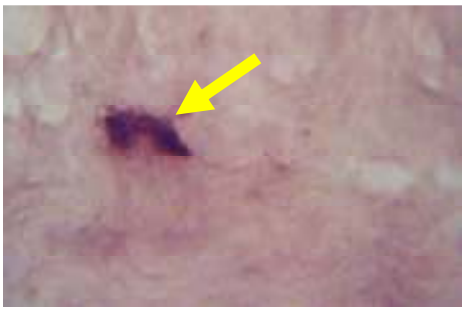

Figure: AD, high intensity, diffused.

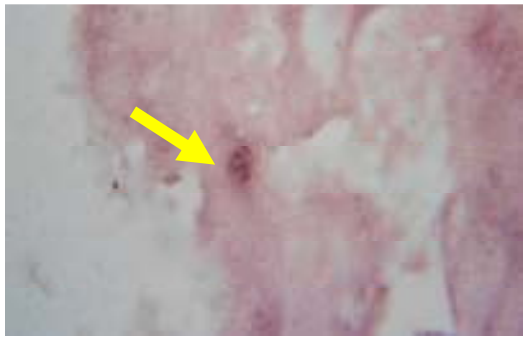

Figure: SCC, low intensity, punctate.

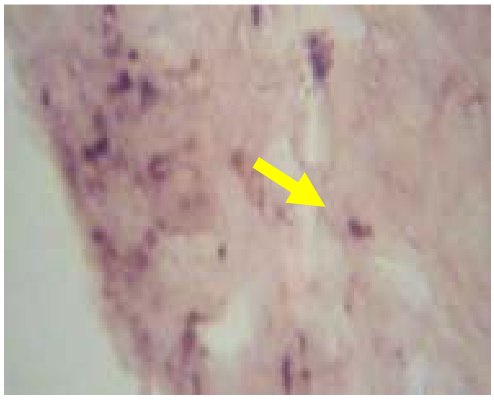

Figure: cervix control tissue, low intensity, mixed.

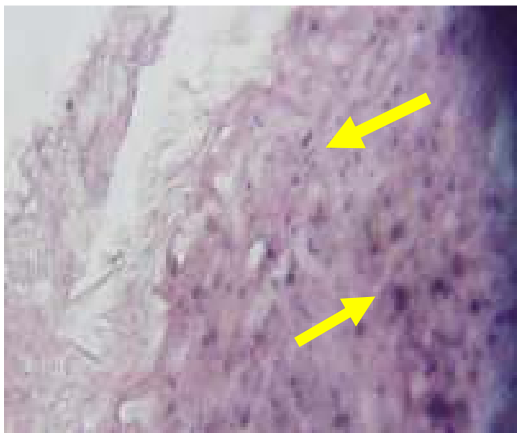

Figure: SCC, moderate intensity, mixed.

Figure 1-A: EBV ISH DNA signals under 1000X, in squamous cell carcinomas (SCC) & adenocarcinomas (AD)

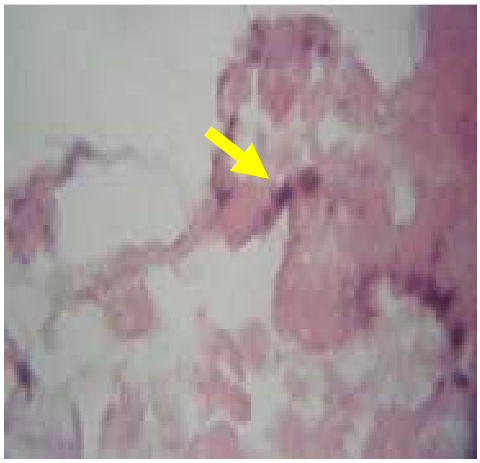

Figure: AD, low intensity, diffused.

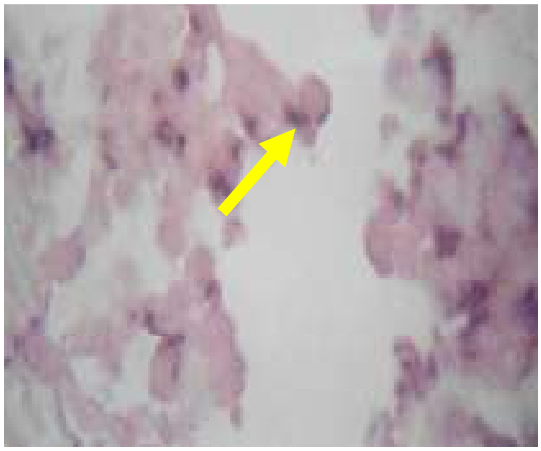

Figure: SCC, moderate intensity, diffused.

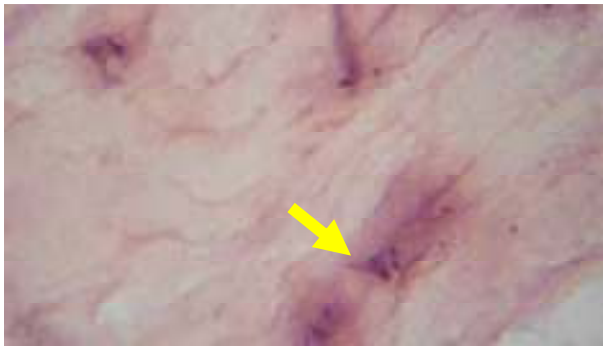

Figure: SCC, high intensity, punctate

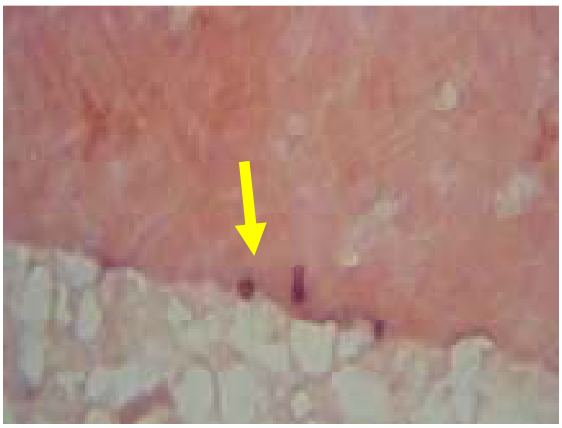

Figure: SCC, low intensity, diffused. Punctate

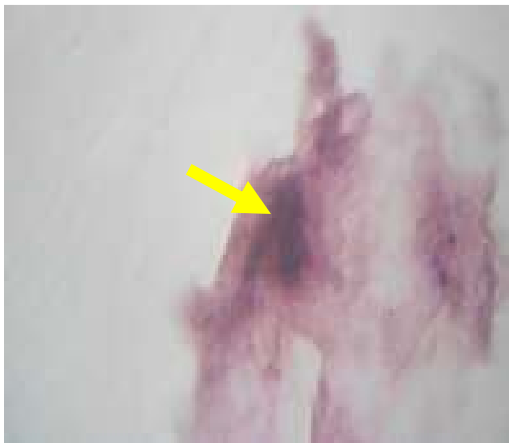

Figure: cervix control tissue, low intensity, diffused.

Figure 1-B: HPV 16 ISH DNA signals under 1000X, in squamous cell carcinomas (SCC) & adenocarcinomas (AD)

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