Evaluation of the possible role of HPV16, CMV and EBV in Cervical Carcinoma progression using In Situ Hybridization technique

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Abstract

Background: Cervical carcinoma was considered as a major problem and life threaten of women; therefore it is worthy to study the association of Epstein–Barr virus and cytomegalovirus co-infection with human papillomavirus type 16 in uterine cervical carcinoma progression. Cervical carcinoma is known closely associated with human papillomavirus.
Objective: To identify whether Epstein–Barr virus and cytomegalovirus play a co-factors role in the cervical carcinogenesis besides human papillomavirus type 16 infection.
Patients and Methods: Current study included paraffin embedded sections from 50 cases of cervical cancer in Baghdad during the period from 2012 till 2014. They were examined for the presence of human papillomavirus type 16, cytomegalovirus and Epstein-Barr virus DNA using in situ hybridization technique.
Results: This study showed that (33, 66%), (22, 44%) and (38, 76%) out of 50 cervical cancer specimens were positive to human papillomavirus type 16, cytomegalovirus and Epstein–Barr virus DNAs signals respectively by using in situ hybridization technique.
Conclusion: The co-operation effects of CMV and EBV onto HPV16 might progress the oncogenesis of cervical carcinoma in female patients.
Key words: Human papillomavirus type 16, cytomegalovirus, epstein-barr virus, in situ hybridization technique, squamous cell carcinoma, adenocarcinoma.
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Received: 23th March 2016
Accepted: 24th July 2016

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Introduction

Human papillomavirus 16 report for 95% of invasive cervical cancers worldwide and it alone is responsible for progressing over than 60% of cervical cancer. Human papillomavirus-16 is a contagious spreads sexually and the infected couple can transmit the disease to the off-springs as well [1]. Cytomegalovirus, also known as HCMV, CMV or (HHV-5) is part of the herpes family of viruses. It is a common virus and spreads through bodily fluids, including saliva, blood, breast milk, semen and urine [2].

Cytomegalovirus is human-to-human transmissible through close bodily contact (coughs and sneezes are also possible routes). Since early 60's that cytomegalovirus was studied for its possible

role in cervical cancer development, but it is still doubtful what the prevalence of cytomegalovirus in cervix is and if CMV can act as a co-factor in cervical carcinogenesis [2].

Epstein-Barr virus (EBV) also called human herpesvirus 4 (HHV-4). The virus was named after Anthony Epstein and Yvonne Barr , as first isolated and described the virus in 1964 from lymphoma samples collected by Denis Burkitt [1] [3]. Epstein–Barr virus was a first tumorigenic human herpes virus that is common in the adult population. It infects >90% of the world's population. The common ways of EBV transmission by saliva, but it has been found in genital secretions, which suggests sexual transmission and led researchers to connect EBV and cervical neoplasia. There have been many reports linking EBV with other sites including breast, lung and gastric carcinomas but with cervical cancer the role of EBV remains controversial, some authors have suggested that EBV could play a role in the carcinogenesis of cervical tumors; other studies do not support this hypothesis [4].

Cervical cancer is the most common form of cancer in women in developing countries as leading cause of cancer-related deaths in the world as a whole [5]. 80% of all cervical cancers is called squamous cell carcinoma followed by 15% to 17% of adenocarcinoma and about 3% to 5% have characteristics of both squamous and adenocarcinoma and are called adenosquamous carcinomas [5].

The purpose of this study was is to identify whether Epstein–Barr virus and cytomegalovirus play a co-factors role in the cervical carcinogenesis besides human papillomavirus type 16 infection.

Patients and Methods

Tissue Samples: The current research was designed as retrospective study. It involved [50] archival formalin fixed paraffin embedded uterine cervix tissue blocks. Samples were collected from (2012) to (2014) from the Teaching Laboratories in Medical City.

They included [28] blocks of squamous cell carcinoma of cervix and [22] blocks adenocarcinoma of cervix. All related histological slides were re-examined and confirmed by the review of freshly prepared haematoxylin and eosin stained slides by certified pathologists and classified according to criteria outlined by the World Health Organization.

Materials and methods

DNA probe hybridization detection system in situ kit a complete hybridization and immunodetection system/High Sensitivity (Maxim Biotech Inc. USA): IH-60001 (IHD-0050), was used to target the DNA sequences of HPV16, CMV and EBV in tissue specimens.

Positive control: was prepared from tissues which had been with the target marker and test previously. They included HPV16 associated uterine cervical carcinoma, nasopharyngeal carcinoma for EBV and CMV associated breast carcinoma tissue samples.

Negative control: all reagents were added except the diluted probe for the negative control.

Positive control probe: was prepared from a test tissue section and processed in identical manner to the test section but by replacing the probe with housekeeping gene probe.

ISH procedure for HPV16, CMV and EBV DNAs detection: All process was done based upon the manufacturer's instructions (Maxim DNA probe hybridization and detection system in situ kit Cat No.IH-60001 (IHD-0050) \ USA). Biotinylated DNA probes of HPV16, CMV and EBV were used. Tissue section were adhered onto positively charged slides and baked vertically overnight in an oven at 65˚C. Afterward, deparaffinizated the

slides. Then the slides immersed in boiling citric buffer solution for 15 minutes heated in a specific jar on hotplate at 98˚C. Proteinase K (1X) for 15minutes at 37˚C for enzymatic digestion, followed by dehydration in alcohol, then dried at 37˚C. Slides incubation with diluted probe solution overnight at 37˚C. Addition of an enzyme-conjugated (Streptavidin-AP) for detection of the hybridized probe, the slides were incubated for 20 minutes at 37˚C. Then addition of the BCIP/NBT solutions to the slides and incubated at (20-25˚C) for about 40 minutes until the colour development. Lastly, all slides were counterstained with eosin and mounted**.**

DNA ISH Signals Evaluation for HPV16, CMV and EBV

Bluish black discoloration was the signal that represented the positive cells after binding at the site of complementary sequences of interest in nucleus (integrated genome) or in cytoplasm (episomal genome). The viral signal patterns of replication were classified as punctate (linear), diffuse, or mixed. The punctate pattern as distinct dot-like signals or scattered tiny particles in the nucleus primarily was considered to represent linear viral genome and maybe indicate an integrated viral genome as in HPV16 and EBV carcinogenesis or only linear genome as in CMV infection, and a diffuse pattern, that is, large dense globular homogeneously staining in the nucleus or cytoplasm, represent episomal viral nucleic acid, while the mixed patterns were supposed to represented integrated or linear and episomal viral genome [6, 7, 8, 9], according to the author opinion the viral intensity of replication scales as negative result to no noticeable ISH signals and positive graded as low (past infection), Moderate (chronic or latent infection), and high intensity of reaction (recent infection or continuous viral replication).

Statistical analysis

Chi-square was used to detect the significance differences among the variables of our study. by using SPSS ver. 18.0.

The significance differences among variables (p-value) in any test was:-

S=Significant difference ($P<0.05$), NS = Non Significant difference ($P>0.05$), HS=Highly Significant difference ($p>0.0001$).

Results

The results showed that the most common histological types of cervical carcinoma were squamous cell cervical carcinoma (56%), followed by the adenocarcinoma (44%), and all results interpretation as shown below in table (1), table (2), figure 1 and figure 2.

The HPV16 DNA ISH signals had been detected in 33 (66%) patients. While, the rest of cases were negative as 17 (34%) patients. Moderate intensity of HPV16 DNA replication was the most prominent mode of replication as (36%) in cervical carcinoma tissues, followed by (12%) showed low intensity. Furthermore, diffused (episomal HPV16 DNA) pattern of replication was the most prominent patterns to HPV16 DNA in cervical carcinoma which made (46%). Followed by (6%) as mixed patterns of HPV16 DNA replication (which involved integrated as well as episomal HPV 16 DNA).

Table (1): ISH – DNA Signals of EBV, HPV 16 and CMV according to the Cervical carcinoma types.

ISH – DNA / HPV16							
Tumor Types			**Positive -ISH**		**Negative - ISH**		***P* - Value**
Cases	NO.	%	NO.	%	NO.	%	0.03 Significant
SCC	28	56%	22	44%	6	12%	
AD	22	44%	11	22%	11	22%	
Total	50	100%	33	66%	17	34%	
ISH – DNA / CMV							
Tumor Types			**Positive -ISH**		**Negative - ISH**		**P - Value**
Cases	NO.	%	NO.	%	NO.	%	0.02 Significant
SCC	28	56%	16	32%	12	24%	
AD	22	44%	6	12%	16	32%	
Total	50	100%	22	44%	28	56%	
ISH – DNA / EBV							
Tumor Types			**Positive -ISH**		**Negative - ISH**		**P - Value**
Cases	NO.	%	NO.	%	NO.	%	0.02 Significant
SCC	28	56%	18	36%	10	20%	
AD	22	44%	20	40%	2	4%	
Total	50	100%	38	76%	12	24%	

The presence of ISH CMV DNA signals in cervical carcinoma tissues had been detected in 22 (44%) patients, while the remaining of specimens were negative as 28 (56%) patients. The most common intensity of replication to CMV DNA was (12%) in cervical carcinoma as low intensity, while (10%) showed moderate intensity. The prominent patterns of replication of CMV DNA were found to be as diffused (episomal) pattern which made (16%). Followed by (6%) as punctate patterns of replication (which involved linear CMV DNA).

The EBV DNA of ISH signals were monitored in 38 (76%) of cervical carcinoma cases, while 12 (24%) were negative cases. The most prominent intensity of replication to EBV DNA was (26%) showing low intensity, while (24%) showing moderate intensity. Furthermore, patterns of replication of EBV DNA showed that (28%) of cervical carcinoma cases were diffused patterns (episomal), while (20%) showed mixed Patterns of replication (which involved integrated or linear as well as episomal EBV DNA).

The co-existence of DNA ISH signals for HPV16, CMV and EBV in cervical carcinoma tissues altogether reflects a possible role of these viruses in the carcinogenesis of cervical carcinoma as shown in table (2). HPV16 DNA signals in cervical carcinoma cases were detected in the moderate intensity of replication, and this may reflect moderate reproduction rate of the virus in cervical carcinoma or viral latency or old infection with continuous proliferation and noticeable episomal viral DNA. The CMV and EBV DNA signals in cervical carcinoma cases were detected in the low to moderate intensity of replication, and this may reflect a low to moderate reproduction rate of the virus or viral latency or maybe old infection in cervical carcinoma with continuous proliferation in addition to noticeable episomal and linear CMV or integrated EBV genomes.

Table (2): HPV16, CMV and EBV Co-infection in Cervical carcinoma tissues.

Virus (es)	No.	%
HPV16 infection	1	2%
CMV infection	1	2%
EBV infection	16	32%
HPV16, CMV and EBV co-infection	11	22%
HPV16 and EBV co-infection	11	22%
HPV16 and CMV co-infection	10	20%
CMV and EBV co-infection	0	0%
Total	50	100%

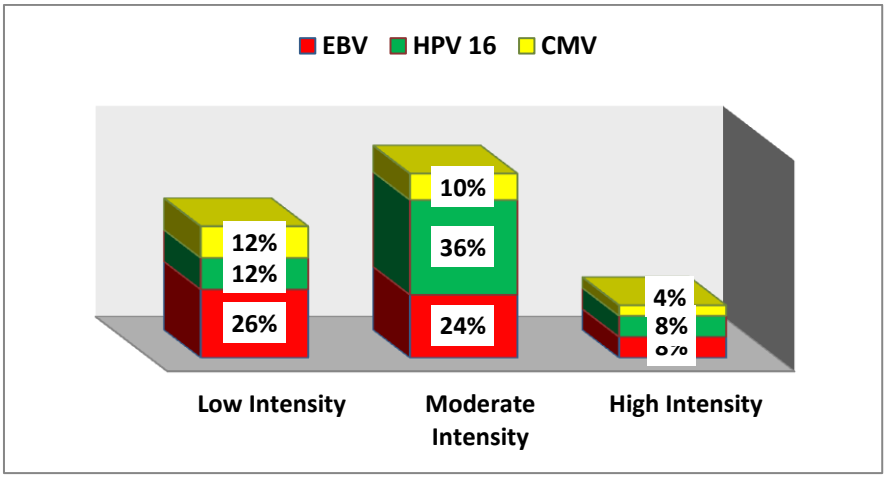

Figure (1): Viral intensity of replication in cervical carcinoma tissues**.**

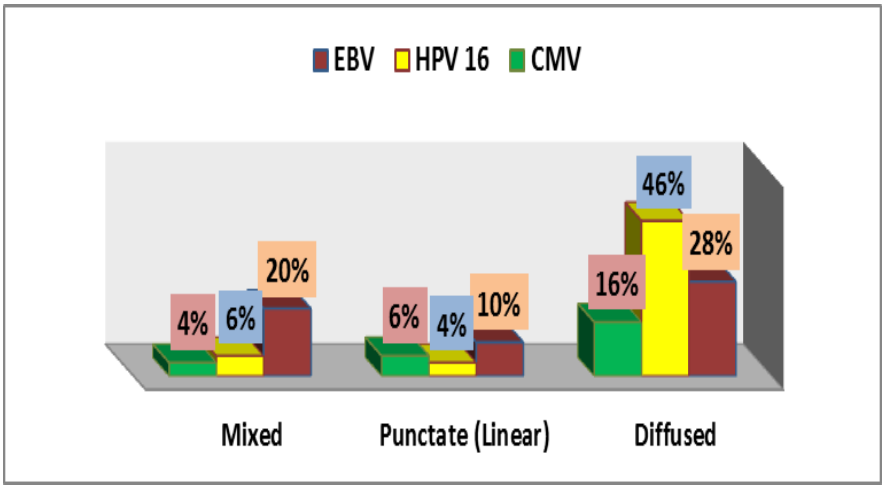

Figure (2): Viral patterns of replication in cervical carcinoma tissues.

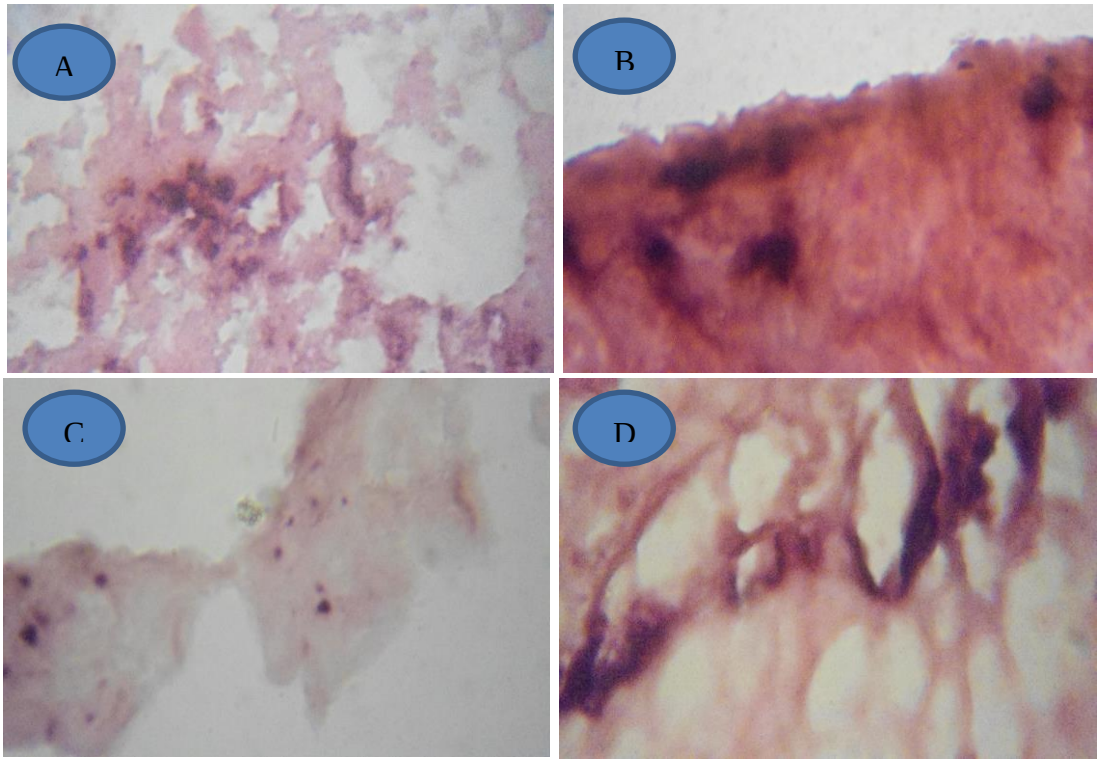

Figure (3): DNA ISH signals in cervical carcinoma tissues.(A) HPV16, SSC, high intensity, diffused (X1000). (B) EBV, AD, low intensity-diffused (X1000).(C) EBV, SCC, high intensity, punctate (X1000). (D) CMV, SSC, high intensity, diffused (X1000).

Discussion

The current results agree with Jung et al., 2010, noticed that HR-HPV 16 was detected in (69.5%) by using ISH assay [6].However, that results was insufficient to show whether the virus has got a role in primary cervical carcinoma or it is only due to an ascending infection[3]. The (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 [3]. So that, a hypothesis was made in the present study for the ISH signals of HPV16 DNA, the detection of HPV16 in an abnormal cervical tissues may reflect that HPV16 was a sexual transmitted agent by a male vector (Seminal Fluid) and viral genome integration (persistence infection) may be help in the progression into carcinoma. Furthermore, the high percentage of episomal viral DNA in cervical carcinoma indicate that HPV16 act as a co-factor besides other factors like a genetic mutation , external environmental factors, immunological disorders or even EBV or CMV infection to induce cervical carcinoma. The pervious results were agreed with Adi Prayitno, [3]; reported 89% of cervical cancers were infected with HPV16 while 68% were infected with EBV and HPV16 co-infection. Other proposed that high EBV load in the genital tract, indicating that a sexual route of transmission and relation to genital disease is possible. In addition to, many studies using real-time PCR as a useful in determining the role of different herpes viruses in human disease. Aromseree *et al* [4]; demonstrated the EBV-HPV co-infections, particularly with HR-HPV16 was significantly increased in cervical carcinoma patients. Surprisingly, EBV-HPV16 co-infection was not related to HPV16

integration but raised the possibility that EBV could be a possible cofactor and stimulate HPV16 episomal form to initiate to cervical cancer and this will support our findings with highly significantly compatible. According to the CMV role in cervical cancer, Since early 60's CMV was studied for its possible role in cervical carcinogenesis, but it is still doubtful CMV can act as a co-factor in cervical cancer[2]. This study revealed that the frequency of Cytomegalovirus infection in the cervix was 26% in all cervical samples and 56% in HPV positive women. Hence, despite results showed that Cytomegalovirus shedding in cervical samples is frequent more studies should be performed to clarify if Cytomegalovirus infection is an opportunistic infection in HPV-infected cases, or if it contributes for cervical immunosuppression that will favour HPV-associated carcinogenesis [2].

In conclusions, The current study reveals an evidence that cervical carcinoma is related to high risk human papilloma virus 16 infection as a Co-factor not as a main causative agent and more recently Cytomegalovirus and Epstein-Barr virus have been proposed as candidates (other Co-factors) for cervical neoplasia progression. Our conclusion advises to do extra-investigations to confirm the reality role of HPV16, CMV and EBV co-existence in cervical carcinoma and the interaction with tumor suppressor genes or other cellular proteins, which must be studied with a large sample size in Iraqi population.

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