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Research Article

Viral Co-Infection of Oncogenic Human Papillomavirus with Epstein–Barr Virus, Human Herpesvirus 8 and Herpes Simplex Virus Type 2 in Malignant Cervical Cancer

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Abstract

Objectives: Cervical cancer (CC) is considered the fourth most common malignancy and the fifth fatal cancer in women, oncogenic *Human papillomavirus (HPV)* are considered a primary cause of development of cervical cancer. It has also been suggested that viral coexistence may also accelerate the progression of cervical lesions to cervical cancer. This study aims to study the coinfection of *Epstein Bar Virus (EBV)*, *Herpesvirus 8 (HHV8)* and *Hepes simplex type 2 (HSV2)* infections in women with cervical cancer with the presence of HPV and their correlations with the clinicopathologic characteristics of patients.

Methods: In this study, 73 samples that tested positive for *Human papillomavirus* in previous study were used for the detection of *EBV, HHV8 and HSV2* in tumor tissue using Polymerase Chain Reaction techniques, and the clinical relevance was analyzed statistically.

Results: Of the 73 samples (48%) were infected with *EBV*, (24.65%) infected with *HHV8* However, none of the cases were infected with *HSV2*. The frequency of co-infections was 22% of cases. No significant association was found between co-infection and other clinicopathologic features.

Conclusion: Therefore, these results represent arguments in favor of the role of *EBV* and *HHV8* among *HPV* positive cases, as potential cofactors in cervical carcinogenesis, which could lead us to develop new therapeutics and preventive vaccines.

Keywords: Human papillomavirus, Epstein Bar, Herpesvirus 8, Hepes simplex type 2, Cervical cancer; PCR

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Worldwide, cervical cancer (CC) is considered the fourth most common malignancy and the fifth fatal cancer in women with around 604,000 new cases and 342.000 new deaths in 2020, respectively.[1,2] In sub-Saharan Africa, CC is the most common cancer in women with about 75.000 new cases and records the highest number of deaths on the continent with more than 50.000 deaths annually.[3]

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The International Agency for Research on Cancer (ICER) reports that CC is the most common form of cancer in Morocco after breast cancer. The report states that every year, 10.4 out of 100,000 age-standardized women suffer from this disease. In addition, 5.8 age-standardized women died from it each year.[4]

Clinical and epidemiological studies have clearly showed the etiologic role of *human papillomaviruses (HPV)* in CC development.[5] Of note, *HPV16 and HPV 18* are considered as the most oncogenic genotypes in the world.[6-7] The oncogenicity of these viruses is mainly mediated by the E6 and E7 oncoproteins which interact with p53 and pRb respectively and affect the cell cycle, apoptosis and cellular adhesion.[7]

Although papillomavirus infection is obligatory for CC progression, it is not sufficient and other genetic and epigenetic events are also involved in CC development and progression. In this field, it was reported that co-infection with viruses such as *herpes simplex virus (HSV1,2), human herpesvirus 8 (HHV-8), Epstein-Barr virus (EBV)* and immunodeficiency virus may promote and accelerate the progression of cervical cancer during the persistence of *HPV* thus it has been shown that *EBV, HSV2 and HHV8* are among the herpes viruses that persistently infect most adults.[8]

Although HSV has not been shown to be a risk factor for cancer, *HPV*, along with *EBV*, is associated with 38% of all infection-related cancers.[9] In fact, a recently published meta-analysis showed that *EBV* and *HPV* co-infection quadrupled the risk of cervical cancer in *EBV*-positive women;[7] similarly, precancerous cervical lesions were twice as common in *EBV*-positive women as in *EBV*-negative women. Therefore, these data may confirm that *EBV* is a potentially active cofactor in cervical cancer development.[7]

EBV is a large double-stranded Deoxyribonucleic Acid (DNA) virus, which was discovered as the first oncogenic virus and responsible for different lymphoid and epithelial malignancies.[10, 11] It has been suggested that *EBV* can transform cells through the interaction of *EBV* receptors with the third component of complement which in turn makes them more susceptible to any viral infection. Of particular interest, *EBV* receptors are mainly expressed in the ecto- and endo-cervix regions which are in favor of the involvement of these viruses as cofactors in CC development.[9, 12]

HSV-2 is a sexually transmitted infectious agent reported as a potential cofactor in CC development. Several independent studies propose that HSV-2 infections are correlated with a higher incidence of cervical cancer but with consistent identification of *HPV* DNA.[13] HSV infections result in unexpected cellular DNA synthesis and chromosomal amplifications. The mutational status of HSV-2 genome indicates that this virus is not a typical DNA tumor virus. It is assumed that persistent or abortive infections induce permanent genetic alterations that interfere with the differentiation of the cervical epithelium and subsequently induce abnormal proliferation, suggesting that HSV-2 may be a cofactor in some, but not all, cases of CC.[13]

HHV-8, also called *Kaposi's sarcoma-associated herpes virus (KSHV)*, was first described in 1994 and has been linked to several malignancies in the human population such as KS, primary effusion lymphomas (PEL) and multicentric Castleman's disease (MCD).[14, 15] *HHV*-8 is commonly transmitted during oral, vaginal and anal sex, the same route of *HPV* and *HHV*-8 transmission, suggesting that it could be involved in CC development and/or progression as a potential cofactor.[15]

In Morocco, great interest was given to *HPV* detection and genotyping in CC cases but data on co-infection with other viruses that could play a crucial role in CC development and/or progression are scarce. In this regards, the present study was planned to investigate the co-infection status of CC cases with *HPV and EBV, HSV2 and HHV8*, in Moroccan women with CC.

Methods

Patients and samples

DNAs from 73 *HPV* positive cervical cancer samples were available from our laboratory DNA bank. These DNAs were obtained from patients recruited at Ibn Rochd University Hospital Center in Casablanca, Morocco.

Socio-demographic and clinicopathologic characteristics of recruited patients is reported in Table 1. Accordingly, reported data showed that most recruited patients were in

Table 1. Clinico-pathological characteristics of the cervical cancer patients (n=73).

Characteristics	Effective	Percentage (%)
Age at diagnosis		
27-41	12	16.44
42-61	41	56.16
>61	20	27.40
Histological type		
SCC	40	54.79
AC	33	45.21
Clinical stage		
I	14	19.18
II	53	72.60
III	6	8.22
IV	0	0.00
Menopause		
Yes	51	69.86
No	22	30.14
Childbirth delivery		
None	7	9.59
Vaginal birth	58	79.45
Caesarean section	8	10.96
Marital status		
Single	14	19.18
Married	59	80.82

the age range of 42–61 years (56.16%) with a mean age of 54 years. Histological analysis subtyped specimens to squamous cell carcinoma (SCC), reported in 54.79% of cases and adenocarcinoma (AC) in 45.21% of cases. According to the classification of the International Federation of Gynecology and Obstetrics (FIGO), most patients were diagnosed at an early stage II (71.60%) and I (19.17%). Regarding reproductive factors, most women were postmenopausal (69.86%), patients gave birth vaginally (79.45%) and most of the recruited cases were married (80.82%).

The study protocol was approved by the Ethics Committee for Biomedical Research of the Faculty of Medicine and Pharmacy of Casablanca, Morocco (3/2018 on 30.04.2018).

Assessment of the Quality and Quantity of Collected DNAs

Before analyses, *HPV* positive DNAs collection from cervical cancer cases was firstly quantified using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). To assess the quality of the DNAs, all extracted DNAs were subjected to a PCR test to detect the *β-globin* gene using specific primers (Table 2), as a housekeeping gene.[16]

Viral DNA Amplification

The presence of *EBV*, HSV-2 and *HHV*-8 viral sequences in DNA extracted from frozen cervical cancer specimens was determined by PCR amplification using specific primer sets (Table 2). For HSV-2 and *HHV*-8 a single PCR round was used, however, amplification of *EBV* DNA was performed using a nested PCR with two specific primers' pairs.

PCR amplification was carried out in a total volume of 25 µl containing 1.5 mM $MgCl_2$, 100 µM each dNTP, 0.2 µM forward and reverse primers, 100 ng genomic DNA and 0.25 U Taq DNA polymerase in 1x PCR buffer. The amplification mixtures were first denatured at 94°C for 7 min. Then, 35 cycles of PCR were performed with denaturation at 94°C for 1 min, primer annealing for 1 min at corresponding Tm and primer extension for 1 min at 72°C. At the end of the last cycle, the mixtures were incubated at 72°C for 10 min.

For every reaction, a negative control in which DNA template was omitted from the amplification mixture was included. To evaluate the outcome of each PCR, all PCR products were analyzed by electrophoresis on 2% agarose gel at 70 V for 1.5 h, stained with ethidium bromide and visualized under UV light.

Statistical Analysis

All analyzes were performed with the statistical package SPSS by chi-square test and Fisher's exact test, for a possible association with clinico-pathological data and prevalence of *EBV, HHV-8 and HSV-2 viruses*, p values <0 .05 were considered statistically significant.

Results

Our first concern in this work was to check the quality of the DNA samples. Amplification of *β-globin* gene was successful in all DNA specimens highlighting a 256bp fragment in all cases, suggesting that all samples were adequate for further analyses.

In this study, *EBV* DNA was detected in 47.95% (35/73) and *HHV*-8 in 24.66% of samples (18/73). Of interest, among the 73 samples tested, 16 showed co-infection with both *EBV* and *HHV*-8 viruses (22%). However, all samples were HSV-2 free (Fig. 1).

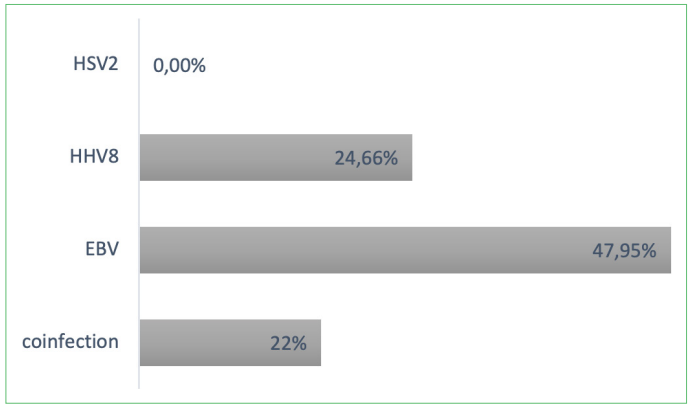

Figure 1. Percentage of *EBV* and *HHV-8* positive cases in *HPV* positive cervical cancer samples.

Table 2. The Primer Sequences Used for PCR Tests[17, 18]

Oligonucleotide	Sequences of primers		Tm	PCR product sizes
β-globin	*PC04*	5'-CAA CTT CAT CCA CGT TCA CC-3'	54°C	256 bp
	GH20	5'-GAA GAG CCA AGG ACA GGT AC-3'		
EBV	*EBV1-F*	5'-GCG GGT GGA GGG AAA GG-3'		
	EBV1-R	5'-GTC AGC CAA GGG ACG CG-3'	58°C	245 pb
	EBV2-F	5'-AGG CTG CCC ACC CTG AGG AT-3'		
	EBV2-R	5'-GCC ACC TGG CAG CCC TAA AG-3'	66°C	
HSV2	*HSV2-F*	5'-TAT GCC TAT CCC CGG TTG GA-3'		
	HSV2-R	5'-CGT GCC ATC CGA ATA AAC GTG-3'	55°C	715 bp
HHV8	*KS-1*	5'-CCG AGG ACG AAA TGG AAG TG-3'		
	KS-2	5'-GGT GAT GTT CTG AGT ACA TAG CGG-3'	55°C	233 bp

Distribution of *EBV* and/or *HHV*-8 positive cases according to clinico-pathological data is reported in Table 3. Statistical analysis showed that *EBV* infection is significantly associated with patients age (p=0.001), histological type (p=0.001) and marital status. Indeed, 68.29% of *EBV* positive cases are aged between 42 and 61 years old and most cases harboring *EBV* DNA were diagnosed with SCC (77.50%). Regarding the marital status, *EBV* positive cases was reported in 59.32% of married cases and only 21.43% of single ones.

HHV-8 virus was reported in 18 *HPV* positive DNAs. Statistical analysis showed that *HHV*-8 is also significantly associated with patients age (p=0.033) and histological type (p=0.026). Like *EBV*, most *HHV*-8 positive cases prevail among cases diagnosed with squamous cell carcinoma (30.00%) as compared to adenocarcinoma cases (18.18%). However, *HHV*-8 positive cases were mostly detected among young people aged between 27 and 41 years old. Of note, no significant association was found between co-infection and clinico-pathological features.

The associative statistical analysis is presented in Table 4, an increased risk of cervical cancer and *EBV* infection with *HPV* was found in patients with a histological type of SCC (odds ratio (OR) = 3.58; 95% confidence interval (95% CI) = [1.03; 12.4], who are postmenopausal (OR=1.15; 95% CI = [0.423; 3.15]) and married women (OR=4.35; 95% CI = [1.10;

Table 4. Potential risk factors (Histological type, Menopause, Marital status) of EBV, HHV8 and its correlation among HPV-positive cervical cancer patients

Factors	Odds ratio value	95% Confidence interval
EBV among HPV-positive		
Histological type	3.58	[1.03; 12.4]
Menopause (yes/no)	1.15	[0.423; 3.15]
Marital status	4.35	[1.10; 17.2]
HHV8 among HPV-positive		
Histological type	0.245	[0.0670; 0.895]
Menopause (yes/no)	1.16	[0.358; 3.78]
Marital status	1.11	[0.270; 4.58]
EBV/HHV8 co-infection among HPV-positive		
Histological type	0.346	[0.0859; 1.39]
Menopause (yes/no)	1.36	[0.330; 5.58]
Marital status	1.10	[0.211; 5.74]

Odds ratio value < 1: Not significant; Odds ratio value> 1: significant

Table 3. Association between viral infection and clinico-pathological characteristics.

Characteristics	N	EBV status		p	HHV-8 status		p	Co-infection		p
		P	%		P	%		P	%	
Ag Age at diagnosis										
27-41	12	3	25.00	0.001	4	33.33	0.033	2	26.67	0.645
42-61	41	28	68.29		11	26.83		8	19.51	
>61	20	4	20.00		3	15.00		2	10.00	
Histological type (WHO)										
SCC	40	31	77.50	1.000	12	30.00	0.026	10	25.00	0.192
AC	33	4	12.12		6	18.18		2	6.06	
Clinical stage (FIGO)										
I	14	5	35.71	0.922	2	14.29	0.652	3	21.43	0.784
II	53	25	47.17		14	26.42		7	13.21	
III	6	5	83.33		2	33.33		2	33.33	
IV	0	0	-		0	-		0	-	
Menopause										
Yes	51	25	49.02	0.780	13	25.49	1.000	9	17.65	0.780
No	22	10	45.45		5	22.73		3	13.64	
Childbirth delivery										
None	7	2	28.57	0.559	2	28.57	0.541	1	14.29	0.993
Vaginal birth	58	29	50.00		14	24.14		9	15.52	
Caesarean section	8	4	50.00		2	25.00		2	25.00	
Marital status										
Single	14	3	21.43	0.037	2	14.29	1.000	3	21.43	0.462
Married	59	35	59.32		16	27.12		9	15.25	

P: positive cases; %: percentage.

17.2]). with regard to the potential risk factors (Histological type, Menopause, Marital status) of *HHV*8 in patients with *HPV* positive cervical cancer, the infection was found in postmenopausal patients (OR= 1.16; 95% CI = [0.358; 3.78]) and married women (OR=1.11; 95% CI = [0.270; 4.58]). And finally for the co-infection of the *EBV* and *HHV*8 , we also found an infection with two parameters respectively, postmenopausal women and married women (odds ratio (OR) = 1.36; 95% confidence interval (95% CI %) = [0.330; 5.58] and (odds ratio (OR) = 1.10; 95% confidence interval (95% CI) = [0.211; 5.74]).

Discussion

Cervical cancer is the fourth most common malignancy worldwide and is one of the leading causes of death in women with an incidence of 570,000 new cases and 311,000 deaths.[19] developing countries, it is the most common cancer and the third leading cause of cancer death. [20] Indeed most cervical tumors (95%) arise from epithelial cells and include SCC, adenocarcinoma and AC, 99.7% of which are caused by high-risk (HR) *HPV*, such as *HPV*16 or *HPV*18,[21] but this infection remains insufficient because the latency period between HR-*HPV* infection and progression cervical cancer suggests the involvement of other etiological agents in the progression of malignancy. In this regard, the detection of other viruses such as *EBV*, HSV2 and *HHV*8 may increase the risk and promote the development of cancer.[22] It is clear that one of the most intriguing research problems is the possible synergistic effects of *HPV* and other viruses in promoting cervical carcinogenesis and its progression ,Therefore in the present study, we sought to assess the prevalence of co-infections in CC tissues of the three viruses belonging to the Herpes family (*EBV*, *HHV*8 and HSV2) among the samples with an *HPV*-positive result 73/80 (91.3%), By analyzing the presence of herpesviruses, we highlighted the presence of DNA sequences belonging to the *EBV* genome in 47.95% of cases (35/73), (24.66%) for *HHV*8, on the other hand no case tested positive for HSV2. Regarding the involvement of *EBV*, our results are in agreement with several studies which also reported positive results for the coexistence of *EBV* and *HPV*. In Thailand Aromseree et al were able to detect the *EBV* genome in (32%),[23] Similarly, a syrein study reported coexistence of *EBV* among 34% of *HPV* positive samples.[24] Joharinia et al showed that the frequency of *EBV* in cervical samples was 12.7% (13 samples out of 101).[22] On the other hand, the prevalence of *EBV* found in our work is clearly higher than the rate reported in a previous study in a Bulgarian population by Staykova et al which showed that only 9.6% of all samples were positive for *EBV* and *HPV* simultaneously.[25] The significant reduction in sequence prevalence of the virus in cervical cancer compared to our study could be due to a change in exposure to this virus and/or the disappearance of its host. This is probably related to the improvement of living conditions as well as the increase in the socio-economic level of the Bulgarian population. According to a meta-analysis based on 25 publications, a transformation of cervical cells via the complement receptor C3d which is widely expressed in the cervix, making the cervical cells more sensitive to various oncogenic stimuli which clearly explains our results.[26] Indeed *EBV*/*HPV* co-infection in CC varies from 12.7% to 81.8%[27,28] while *EBV* was frequently associated with *HPV*16 and *HPV*18[29] increasing the risk of integration of *HPV*16 into cervical cells, thereby increasing the genomic instability of infected cervical cells.[28,30] In addition, *HPV*+/*EBV*+ cervical cancer shows increased methylation of RB1 and E-cadherin (CDH1) gene promoters compared to *HPV*+/*EBV*– tumors.[31] clinic-pathological and the presence of *EBV* we found no correlation between this virus and the following classic prognosis parameters, histological type, FIGO, Childbirth Childbirth and menopause, On the other hand a significant correlation is established with age and marital status ($p<0.05$). Relatively There are few studies on the role of co-infection between *HPV* and *HHV*-8 in cervical cancer. In our study, the prevalence of *HHV*-8 in *HPV* positive cervical lesions was detected in 24.65%, our results join an Iranian study which detected the *HHV*-8 genome in 22.9%.[32] However, a Chinese study showed that the frequency of *HHV*-8 was similar in the *HPV*-positive and *HPV*-negative groups (9.4% vs. 8.0%).[33] Therefore, based on all these studies, we can hypothesize that *HHV*8 may play a role in uterine cancer progression by causing chronic inflammation. It has been shown that *HHV*-8 co-infection in SiHa cell lines may increase expression levels of several inflammatory factors including *interleukin 6 (IL-6), chemokine (CXC motif) ligand 1 (CXCL1), chemokine (CXC motif) ligand 1 (CXCL1), chemokine (CXC motif) CC) ligand 5 (CCL5), interleukin 8 (IL-8), macrophage migration inhibitory factor (MIF) and plasminogen activator inhibitor 1 (PAI-1)*,[34,35] With regard to Statistical analyses, a significant difference was found respectively with age groups ($p=0.033$). And the histological type ($p=0.026$) which agrees with the statistical analyzes of another similar study which found a significant result with age ($p=0.015$), while no significance was found for other parameters. These results are reasonable because it is possible that reactivated *HHV*-8 is more common in women with cervical cancer due to aging. associated immunosuppression.[36,37]

In contrast, no cases of HSV2 were detected in *HPV*-positive cervical lesions, as well as our results. Another Iranian study also showed no HSV2 genomic findings in cervical cancer tissue using PCR testing,[38] while Staykova and colleagues

were able to detect infection with this virus in 30.8% of lesions infected with *HPV*.[25] In addition, for the first time, our study found a coinfection between *EBV* and *HHV*8 in the same samples infected with *HPV* (16.44%) and with therefore This infection could accelerate the progression of the cancer.

Taken together, our results suggest that *EBV* and *HHV*8 may be potential active cofactors rather than mere indicators of cervical cancer pathogenesis and progression in the presence of *HPV*. Given the successful prevention of cervical cancer by *HPV* vaccination and upcoming vaccines against both viruses, further molecular and translational/clinical studies on *EBV* and *HHV*8 are needed to improve still their prevention efforts, especially in affected Morocco and other developing countries at the highest rates.

Disclosures

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