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Herpesviruses as possible cofactors in HPV-16-related oncogenesis★

Slawa Szostek[1]✉, Barbara Zawilinska[1], Jolanta Kopec[2] and Magdalena Kosz-Vnenchak[1,2]

[1]Department of Virology, Chair of Microbiology, Jagiellonian University Medical College, Kraków, Poland; [2]Laboratory of Molecular Genetics and Virology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

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Cervical carcinogenesis is a complex problem with papillomavirus widely accepted as a causative agent. Integration of a human papillomavirus (HPV) of the high-risk type into the host cell genome is one of the major contributing factors to cervical malignant transformation. In this study, the correlation of CMV, EBV, HSV-1, HSV-2, HHV-6 and HHV-7 infections with the physical status of the HPV genome in cervical cancer and precancerous cervical lesions was investigated in sixty HPV-16-positive women. Cervical secretion samples were submitted to DNA extraction and analyzed by PCR. HPV-16 DNA was confirmed in genotyping with the reverse hybridization line probe assay. Multiplex PCR with specific primers for the E2/E6 genes was used to assess the viral integration status of HPV-16. Our results show that CMV DNA was more frequently present in samples with mixed forms of HPV-16 than in the episomal form ($P<0.025$). Such a correlation was also observed in the case of EBV ($P<0.005$). The presence of CMV resulted in a six-fold (OR 6.069; 95% CI 1.91–19.22; $P=0.002$), while EBV caused a seven-fold (OR 7.11; 95% CI 1.70–29.67; $P=0.007$) increase in the risk of the integrated or mixed HPV-16 genome occurrence. Our data suggest that coinfection with herpesviruses, especially CMV and EBV, may be involved in the integration of the HPV-16 genome and may contribute to the development of cervical cancer.

Keywords: herpesviruses, human papillomavirus, squamous intraepithelial lesions, cervical carcinoma

INTRODUCTION

HPV is the most prevalent sexually transmitted disease which occurs commonly in young women. Primary infection of this virus often regresses spontaneously, but prolonged, persistent infection, especially with HPV types showing a strong association with malignant lesions, leads to viral integration with the host cell genome and expression of the viral oncogenes E6 and E7 (zur Hausen, 2002). Sexually active women have a 75% lifetime risk of contracting HPV and 5.5 million women worldwide contract HPV each year. However, 80% of females infected with HPV will clear the virus from their bodies within 12 to 18 months. For every 1 million women infected, 100000 will have precancerous changes in their cervical tissue. Of these, carcinoma *in situ* will develop in about 8000, and invasive cancer will develop in about 1600 (Matias, 2004; Scheurer *et al.*, 2005).

Papillomavirus infection occurs before the development of the malignancy, but the process of car-

✉Corresponding author: Slawa Szostek, Jagiellonian University Medical College, Chair of Microbiology, Department of Virology, Czysta 18, 31-121 Kraków, Poland; phone: (48) 12 634 5400; fax: (48) 12 423 3924; e-mail: sszostek@cm-uj.krakow.pl

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Abbreviations: CMV, human cytomegalovirus; EBV, Epstein-Barr virus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; HPV, human papillomavirus; HSIL, high grade squamous intraepithelial lesions; HSV, herpes simplex virus; LSIL, low grade squamous intraepithelial lesions.

cinogenesis depends on a number of other factors, such as the kind of HPV genotype, the duration of infection, patient age, possible hormonal abnormalities, the efficacy of the immune system, the presence of mutations in the genome of the host, and the incidence of concomitant infections. Apart from *Herpes simplex* virus, which has long been suspected to act as a cofactor in the development of cervical cancer, other herpesviruses are also potential candidates.

As early as in the 1960s and 70s, attention was focused on the two- to three-fold higher risk of cervical cancer development in women having specific antibodies against *Herpes simplex* virus type 2 (HSV-2) compared to seronegative females (Rawls *et al.*, 1968). Experiments carried out *in vitro* on induction of neoplastic transformation of HPV-immortalized human cervical epithelial cells using the Xho2 sequence of the HSV-2 virus genome demonstrated such an association (DiPaolo *et al.*, 1998). Successful attempts were also undertaken to induce dysplastic and neoplastic lesions in the cervix of mice following experimental infection with inactivated human cytomegalovirus (CMV) (Heggie *et al.*, 1986). The immediate-early gene products of CMV can transactivate other viral and cellular genes, and it has been suggested that concurrent genital infection with CMV and HPV might increase the risk of cervical cancer.

On the other hand, Epstein-Barr virus (EBV), which is responsible for the development of lymphomas (Burkitt's lymphoma, Hodgkin's lymphoma, T lymphocyte lymphoma) and nasopharyngeal carcinoma, is also taken into account in etiopathogenesis of gastric and mammary gland carcinomas (Vo *et al.*, 2002; Glaser *et al.*, 2004).

Among the 120 types of HPVs discovered to date, about 40 are involved in anogenital infections. Epidemiological and biochemical data support the division of HPVs into two groups: high-risk, such as HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, capable of causing progression to cancer of the uterine cervix, and low-risk, such as HPV-6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81, rarely, if ever, leading to cancer. HPV-16 is the most extensively studied HPV type and serves as an important model for studying viral carcinogenesis. The E6 and E7 genes of high-risk HPVs are known to play an important role in cervical carcinogenesis because they are highly expressed in cervical carcinoma cells and have the ability to transform and immortalize primary keratinocytes. In the process of inactivation of tumor-suppressor gene products, E6 proteins lead to the degradation of p53 protein, and the binding complex of E7 and pRB proteins disturbs the normal cell cycle and leads to abnormal cellular proliferation (zur Hausen, 2002).

During a common infection and in most premalignant lesions, HPV is in an episomal state. However, most cervical carcinomas and derived cell lines maintain the HPV genome in an integrated form or in both integrated and episomal forms. Thus, integration of the viral genome into the chromosomes of the host cell has been proposed as an activation mechanism for progression from preinvasive lesions to cervical cancer. The genome integration of HPV usually disrupts or deletes the E2 gene open reading frame, or rarely the E1 open reading frame, which results in the loss of expression of the corresponding gene products. Disruption of these genes also leads to overexpression of the E6 and E7 oncoproteins, since the E2 gene products repress the activities of the HPV promoters that direct the expression of the E6 and E7 genes. The disruption of the E2 gene upon viral genome integration will make this gene sequence impossible to PCR amplification (zur Hausen 2000; Schmidt *et al.*, 2005; Howley & Lowy, 2007).

The objective of the present study was to investigate the correlation of infection with selected viruses from the *Herpesviridae* family (CMV, EBV, HSV-1, HSV-2, HHV-6 and HHV-7) with the physical status of HPV-16 in cervical cancer and precancerous cervical lesions.

MATERIALS AND METHODS

Cervical smears with HPV-16, confirmed by genotyping, were obtained from 60 women (20–75 years; mean 36±15 years) with different clinical diagnoses: low grade squamous intraepithelial lesions — LSIL (n=27), high grade squamous intraepithelial lesions — HSIL (n=10), and cervical carcinoma (n=23, FIGO IIa/IIb). The specimens were collected to saline solution and stored frozen at –70°C until processed.

Genomic DNA was isolated using the Genomic DNA Prep Plus kit (A&A Biotechnology, Poland).

Viral DNA detection. Viral DNA detection was performed using PCR according to previously published methods (Szostek *et al.*, 2006; 2008a; Zawilińska *et al.*, 2006; 2008). Each experiment was performed with positive and negative (H_2O) PCR controls. Targets for primers are shown in Table 1. To ensure adequate DNA preparation, PCR amplification of β-actin gene was performed in a separate reaction (Guzik *et al.*, 1999). The PCR products were visualized electrophoretically in 2% agarose gels.

HPV-16 genotyping. Broad-spectrum HPV DNA amplification was performed using the SPF-10 primers designed by Innogenetics and amplified a 65-bp fragment from the L1 region of the HPV genome. PCR products were typed with reverse

Table 1. Detected viruses in analyzed samples.

Virus	Region	According to:	Positive control
HPV	L1	Kleter *et al.,* 1998	CaSki cells*
CMV	gB	Mitchell *et al.,* 1994	MRC-5 cell line**
EBV	EBNA 2	Venard *et al.,* 2000	Namalwa cells***
HSV-1 HSV-2	gD	Cassinotti *et al.,* 1996	RK-13 cell line****
HHV-6	U42	Chan *et al.,* 1997	Clinical isolate of viral DNA
HHV-7	U42		Clinical isolate of viral DNA

*CaSki cells contain about 600 copies of integrated HPV-16; **MRC-5 cell line infected with CMV-AD 169 strain; ***Namalwa cells contain two copies of EBV DNA incorporated into the genome; ****RK-13 cell line infected with references strains of HSV-1 and HSV-2 (McIntyre and MS respectively).

hybridization in the INNO-LiPA HPV genotyping assay (Innogenetics, Gent, Belgium). After hybridization of the PCR product to probes on the strip, hybrids were detected in enzymatic reaction with alkaline phosphatase/streptavidin conjugate and substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) according to the manufacturer's instructions.

Determination of physical status of HPV-16 DNA. Multiplex PCR with specific primers for the E2/E6 genes (Yoshinouchi *et al.,* 1999) was used to assess the integration status of HPV-16. The primers for each sequence were:
5'-CTTGGGCACCGAAGAAACAC-3',
5'-TTGGTCACGTTGCCATTCAC-3' for the E2 gene and
5'-AAGGGCGTAACCGAAATCGGT-3',
5'-CATATACCTCACGTCGCAG-3' for the E6 gene.

The amplimer sizes were 352 bp and 208 bp for the E2 and E6 sequences, respectively. PCR was performed according to the previously described procedure (Szostek *et al.,* 2008b). Transilluminated gel images were digitalized and analyzed by a Gel Doc 2000 documentation system (Bio-Rad, USA). The values corresponding to the intensity and density of the gel bands of different PCR products were obtained with the help of Quantity One 4.0 software (Bio-Rad, USA). The ratio of E2 to E6 copy numbers was calculated to determine the physical status of the HPV-16 genome. HPV-16 in a pure episomal form was expected to have equivalent copy numbers of the E2 and E6 genes (E2/E6 ratio ≥1), whereas preferential disruption of E2 upon viral integration should have resulted in fewer E2 than E6 gene copies. This means that an E2/E6 ratio of less than 1 would indicate the presence of both the integrated and episomal forms, while a ratio of "0" would indicate the presence of the integrated form only.

Statistics. The incidence rates of infection in the cervical smear abnormality groups were compared using the Fisher exact test. The Kruskall-Wallis test was used to compare age in the studied groups. The relative risk (odds ratio) of the type of HPV-16 physical status through herpesviruses infection was adjusted to age and assessed using the multivariate logistic regression. The analyses were carried out using the STATA 8.0 software package.

RESULTS AND DISCUSSION

The long latency between HPV infection and development of cervical cancer plus the low prevalence of cervical cancer relative to the number of women positive for HPVs suggest that other etiologic agents and cellular factors are involved in the progression to malignancy. In our current studies we investigated the correlation of infection with selected viruses from the *Herpesviridae* family (CMV, EBV, HSV-1, HSV-2, HHV-6 and HHV-7) with the physical status of HPV-16 in cervical cancer and precancerous cervical lesions.

In the 60 cervical HPV-16-positive samples studied, 72% cases with herpesvirus coinfections were detected. Among them CMV was detected in 52% of the samples, EBV in 22%, HHV-7 in 14.5%, HHV-6 in 10%, HSV-1 in 5% and HSV-2 in 4%. In 43 women with confirmed herpesvirus coinfections, 60% were infected with a single herpesvirus. CMV DNA was the most frequently observed (61.5%), followed by EBV (15%), HHV-7 (11.5%), HHV-6 (7.6%), and HSV-1 (3.8%). The presence of two herpesviruses was noted in sixteen HPV-16-positive samples (37%); of this group, CMV and EBV were detected in 50% of cases, CMV and HHV-6 — in 19%, CMV and HHV-7 — in 12.5%, while CMV and HSV-1 or HSV-1 and HSV-2 or HSV-2 and HHV-7 — in 6%. Triple infection (CMV, EBV and HHV-7) was detected in one patient only; the woman was diagnosed with cervical cancer. The incidence of herpesvirus DNA depending on the clinical diagnosis is presented in Fig. 1. We found significant differences in CMV or EBV incidence depending on the stage of the disease. The presence of CMV or EBV DNA was more frequent in cervical cancer specimens than in other study groups ($P<0.004$ and $P<0.006$, respectively). No such correlations were noted in the case of the remaining herpesviruses (HSV, HHV-6, HHV-7), although other authors suggested their involvement (Romano *et al.,* 1996; Broc-

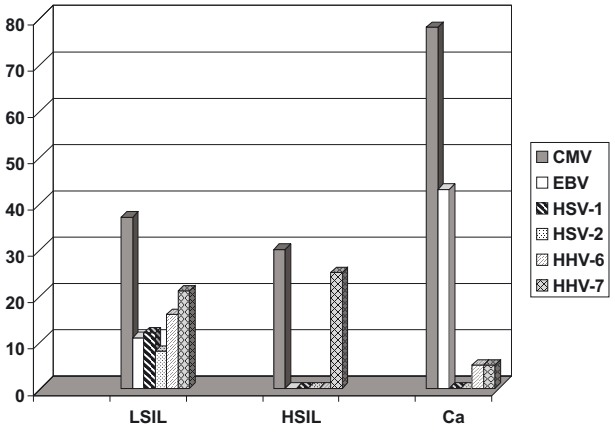

Figure 1. Frequency of DNA of herpesviruses in HPV-16-positive women with different clinical diagnoses.
It can be observed that CMV and EBV DNA was more frequent in cervical cancer (Ca) specimens than in LSIL group ($P<0.004$) and HSIL group ($P<0.006$).

colo *et al.*, 2008). Although recent reports describing investigations carried out in Italy do not confirm a direct correlation between CMV and the process of cervical oncogenesis, they indicate a possible cooperation of cytomegalovirus and another herpesvirus, HHV-6, in development of intraepithelial cervical lesions (Broccolo *et al.*, 2008). The mechanism of cellular transformation with an involvement of CMV might be associated with expression of early viral proteins IE1 and IE2, which were proven to be mutagenic in an animal model (Shen *et al.*, 1997). The advocates of the *„hit and run"* theory believe cytomegalovirus to be a transformation-initiating factor and they explain problems with direct detection of this infection in carcinoma cells by elimination of viral DNA in the course of oncogenesis

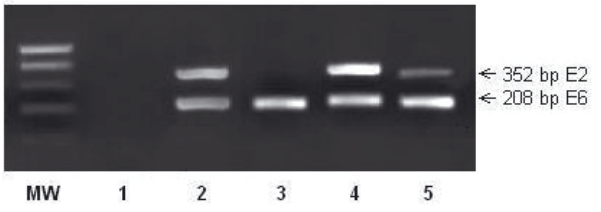

Figure 2. Representative gel showing PCR products for E2/E6 HPV-16 genes obtained using multiplex PCR.
Samples were collected from women with different states of intraepithelial cervical lesions. MW, molecular size marker 100–500 bp; lane 1, negative control (H_2O); lane 2, two PCR products with similar density which represent episomal form of HPV-16, sample from patient with LSIL; lane 3, PCR product for E6 HPV-16 only which is indicative of integrated form, sample from patient with cervical carcinoma; lane 4, more intensive PCR product for E2 HPV-16 than for E6 gene which is accepted as an episomal form, sample from patient with LSIL; lane 5, PCR product for E2 HPV-16 with lower density than PCR product for E6 HPV-16, pattern characteristic for mixed (episomal and integrated) form, sample from patient with HSIL.

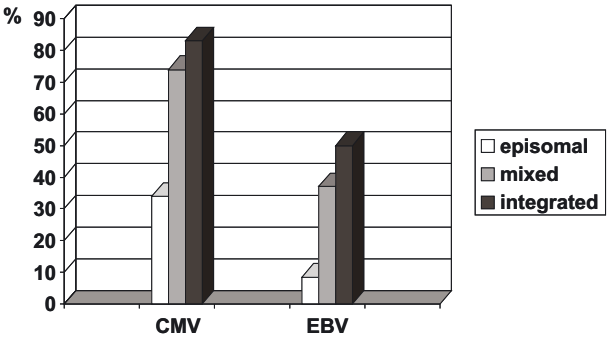

Figure 3. Frequency of distribution of CMV and EBV DNA in patients with different HPV-16 DNA physical states.
CMV and EBV DNA were more frequently present in samples with the integrated and mixed forms of HPV-16 ($P<0.005$).

(Lanham *et al.*, 2001). In turn, based on comparative studies carried out in patients infected with highly oncogenic HPV types and in HPV-negative females, British authors suggest that CMV, HHV-6 and HHV-7 only coexist in the cervical epithelium rather than being cofactors involved in carcinogenesis (Chan *et al.*, 2001). On the other hand, the role of EBV virus in oncogenesis of cervical carcinoma may be associated, among others with an increased expression of the viral LMP-1 and EBNA-2 genes which have been attributed a role in transformation of human B lymphocytes (Sasagawa *et al.*, 2000). Of some significance may also be the presence of methylated regions of host regulatory gene promoters in cells infected with the HPV and EBV viruses (Kim *et al.*, 2005; Lattario *et al.*, 2008).

The integration of high-risk human papillomavirus (HPV) into the host cell genome is one of the major contributing factors to cervical malignant transformation (Kulmala *et al.*, 2006; Huang *et al.*, 2008). In our study, in all the HPV-16-positive samples examined, the physical status of the virus was identified by multiplex PCR with specific primers for the E2 and E6 genes. Figure 2 shows the episom-

Table 2. Correlation between physical status of HPV-16 DNA and cytological diagnoses.

		Physical status of HPV-16 DNA		
Cytology	n	Episomal E2/E6≥1	Episomal and integrated 1>E2/E6>0	Integrated E2/E6=0
LSIL	27	26	1	0
HSIL	10	9	1	0
Ca*	23	0	17	6
Total	60	35 (58%)	19 (32%)	6 (10%)

Ca*, cervical carcinoma

Table 3. Correlation between CMV and/or EBV DNA, physical status of HPV-16 DNA, and cytological diagnoses.

Physical status of HPV-16 DNA	LSIL		HSIL		Ca*		Total	
	CMV	EBV	CMV	EBV	CMV	EBV	CMV	EBV
Episomal	9/26 (35%)	3/26	3/9 (33%)	0/9	0	0	12/35 (34%)	3/35 (8.5%)
Episomal and integrated	1/1	0/1	0/1	0/1	13/17 (76%)	7/17 (41%)	14/19 (74%)	7/19 (37%)
Integrated	0	0	0	0	5/6 (83%)	3/6 (50%)	5/6 (83%)	3/6 (50%)
Total	10/27 (37%)	3/27 (11%)	3/10 (30%)	0/10	18/23 (78%)	10/23 (43%)	31/60 (52%)	13/60 (22%)

*Ca, cervical carcinoma

al, integrated and mixed forms based on an analysis of the ratio of PCR products specific for E2 and E6 of HPV-16.

Only the free episomal form of HPV-16 was seen in 58% samples and predominated in the LSIL group (Table 2). Ten percent of samples harbored the integrated form, whereas a mixture of the episomal and integrated forms of viral DNA was confirmed in 32% of the studied samples. The integrated form of HPV-16 DNA was present in all the cervical cancer specimens and was correlated with progression of cytological changes. Therefore, the most important aspect of our study was examining the relationship between the physical status of the HPV-16 genome and herpesvirus coinfections. This correlation is presented in Table 3 and Fig. 3 for CMV and EBV viruses only, because for the remaining herpesviruses (HSV, HHV-6, and HHV-7), such a correlation was not statistically significant (not presented). CMV DNA was more frequently present in the samples with the integrated or mixed forms of HPV-16 than in those with the episomal form alone ($P < 0.005$) (Fig. 3). Such correlation was also observed in the case of EBV ($P < 0.005$). A multivariate logistic regression analysis showed that the presence of CMV was correlated with a six-fold (OR 6.069; 95% CI 1.91–19.22; $P = 0.002$), while EBV seven-fold (OR 7.11; 95% CI 1.70–29.67; $P = 0.007$) increase in the probability of the occurrence of the integrated or mixed HPV-16 genome. Our data suggest that coinfection with herpesviruses, especially CMV and/or EBV, may be involved in integration of the HPV-16 genome and may contribute to the development of cervical cancer.

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