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ASSOCIATION OF HERPESVIRUS INFECTION WITH THE DEVELOPMENT OF GENITAL CANCER

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Clinical observations and epidemiological studies on genital cancer have revealed an association with sexual behavior, thus motivating research into sexually transmitted agents which may be responsible for the neoplasia. In this study, we used the PCR technique to examine the presence of CMV, HSV and EBV viruses in 187 cases of human genital lesions and found that infection with CMV or HSV was associated with cervical cancer. When we stratified according to HPV status this association was found only for HPV-DNA-negative cases. These findings indicate that past infection with CMV or HSV could be interpreted as a surrogate marker of HPV infection. However, these viruses may play an important role themselves in cervical cancer.

Current epidemiological evidence indicates that although certain types of human papillomaviruses (HPVs) are etiologically associated with cervical cancer (Zur Hausen, 1991; Howley, 1991; Vousden, 1993; Demers *et al.*, 1994), other co-factors may exist. The observations that healthy women have a high prevalence of potentially oncogenic HPV types relative to the incidence of cervical cancer, and that HPV-infected cells are not tumorigenic, indicate that additional events may be required for the disease process. Several sexually transmitted agents, including human cytomegalovirus (CMV) and herpes simplex virus (HSV) (Kjaer *et al.*, 1993; De Sanjosé *et al.*, 1994), have also been associated with cervical neoplasia, but the significance of these associations remains to be established. Detection of Epstein-Barr virus (EBV) in genital lesions has been reported, but its role in oncogenesis has not yet been elucidated (Landers *et al.*, 1993).

HSV is known to contain regions in its genome capable of transforming cells *in vitro*, suggesting a possible oncogenic potential *in vivo*. In addition, laboratory findings support an interaction between HSV-2 and HPV16/18 (Hildesheim *et al.*, 1991; Di Paolo *et al.*, 1990), in the development of cervical neoplasms, as was originally proposed by Zur Hausen (1982). Although CMV has been shown to transform a variety of cell lines *in vitro* (Razzaque *et al.*, 1991), it has been difficult to demonstrate a correlation between CMV infection and cervical cancer, due to the high prevalence of CMV infection, as shown by serological tests in many populations. The immediate-early (IE) gene products of CMV can trans-activate other viral or cellular genes (Colberg-Poley *et al.*, 1991; Boldogh *et al.*, 1991), and it has been suggested that concurrent genital infection with CMV and HPV plays a role in the development of cervical cancer (Shen *et al.*, 1993). The action of EBV in genital lesions is not yet clear. It has been proposed that, as in the development of Burkitt's and non-Hodgkin's lymphomas, where abnormal proliferation occurs in EBV-infected lymphoid cells, infected cervical epithelial cells may also show a similar change. This view is supported by detection of EBV in cells obtained from cervical scrapings and by studies showing an association between cervical cancer and EBV infection (Se Thoe *et al.*, 1993; Schmauz *et al.*, 1989).

In an earlier investigation we studied 112 cases of genital lesions for the presence and typing of HPV DNA and for the presence of *ras* gene mutations, in order to examine the hypothesis that HPV co-operates with cellular factors in the carcinogenesis of the cervix, and we found that HPV may co-operate with *ras* oncogene in the progression of the malignancy (Koffa *et al.*, 1994). In an effort to extend these observations and examine the possible co-operation of HPV with other viruses, we examined the presence of CMV, HSV and EBV DNA as well as HPV in 187 cases of genital lesions.

In order to examine possible differences between benign, pre-malignant and malignant lesions, cases of cervicitis and cervical intra-epithelial neoplasia were included, while cases of other types of gynecological cancer (endometrial, ovarian and vulval), were also included in order to study the specificity of viral infections in the process of cervical carcinogenesis. It was found that CMV, HSV and EBV are frequently present in female genital lesions, while CMV and HSV are associated with cervical cancer.

MATERIAL AND METHODS

Patients and specimens

Tissue specimens were obtained from 187 women with genital lesions, treated at the Department of Obstetrics and Gynecology, Medical School, University of Crete, Heraklion, and the Department of Obstetrics and Gynecology, Medical School, University of Ioannina, Greece. Hematoxylin-eosin-stained sections from all paraffin blocks were reviewed to reconfirm the tumor type and differentiation grade and representative blocks (one per case) were selected for further analysis. The studies on *in situ* cervical cancer comprised all cases with a histological diagnosis of CIN III or *in situ* cancer. The controls were selected from the files of both Departments; they were women who had received routine cervical cytological screening during the period 1990–1994. The Pap smear diagnosis was used to classify subjects as controls (subjects with cervicitis, n = 38) or cases with neoplasia (11 subjects with low-grade CIN, 14 subjects with *in situ* carcinoma of the cervix and 35 subjects with invasive cervical carcinoma, combined for this presentation).

We tested a sample of the controls matched 1:2 to the cases by age group (± 5 years), clinic and appointment date. Informations about marital status, alcohol consumption and cigarette smoking were also available for most of the cases and the controls.

DNA extraction

Five or six 10-μm-thick sections from each formalin-fixed, paraffin-embedded tissue were de-paraffinized by adding 1 ml of xylene, followed by 2 ethanol washes. The samples were then lysed in 400 μl digestion buffer, containing 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, pH 8.0, and 0.1 mg/ml proteinase K (Sigma, St. Louis, MO). Samples were incubated for 24 hr at 37°C, fresh proteinase K was added and the incubation was continued for another 24 hr. The samples

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were then boiled for 8 min and DNA was precipitated with the addition of 20 µl 5 M NaCl and 1 ml ethanol, recovered with centrifugation at 12,000*g* for 15 min at 4°C, washed once with cold 70% ethanol and re-suspended in 20 µl double-distilled water.

Oligonucleotide primers and PCR amplification

The oligonucleotides used for the detection of CMV and EBV DNA have been described (Demler *et al.*, 1988; Rogers *et al.*, 1990). For the detection of HSV DNA, we used a set of general primers (Rogers *et al.*, 1991) that distinguish between type 1 and type 2 by Ava-II cleavage, as well as 2 sets of primers specific for each type, in a multiplex PCR to confirm the typing (Nicoll *et al.*, 1994). For the analysis of HPV status, the results of 112 samples using 4 different sets of primers (Arends *et al.*, 1991) were already published (Koffa *et al.*, 1994), while for all the samples, detection of HPV was also carried out using the general primers GP5 and GP6 (Snijders *et al.*, 1990), which amplify a region of the most conserved L1 ORF and have been successfully applied to clinical samples in several large studies (Van den Brule *et al.*, 1991; Muñoz *et al.*, 1992).

Extracted DNA (1 µl) of each sample was amplified in a reaction solution of 50 µl containing 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 75 mM Tris-HCl, pH 9.0, 0.01% (w/v) Tween, 200 µM of each dNTP, 0.5 µM of each primer and 1.25 U Taq polymerase (Advanced Biotechnologies, Leatherhead, UK). The mixture was heated for 1 min at 95°C, and then subjected to 35 cycles of amplification in the following conditions.

CMV amplification: each cycle consisted of denaturation at 94°C for 50 sec, annealing at 62°C for 30 sec and elongation at 72°C for 50 sec, increasing the elongation time 1 sec per cycle. HSV amplification: using the general primers, each cycle consisted of denaturation at 94°C for 1 min, annealing at 64°C for 40 sec and elongation at 72°C for 50 sec, increasing the elongation time 1 sec per cycle. Using the 2 sets of the specific primers in a multiplex PCR, each cycle consisted of 94°C for 50 sec, 56°C for 40 sec and 72°C for 30 sec. EBV amplification: each cycle consisted of denaturation at 94°C for 1 min, and annealing-elongation at 72°C for 1 min. HPV amplification: each cycle consisted of denaturation at 94°C for 50 sec, annealing at 52°C for 50 sec and elongation at 72°C for 45 sec, increasing the elongation time 1 sec per cycle.

Endonuclease cleavage

CMV-EBV confirmation: 10- to 20-µl aliquots of the amplification products were digested for 3 hr at 37°C with 30 U of RsaI or HaeIII restriction endonucleases for CMV and with 30 U of HindIII for EBV DNA.

HSV typing: after PCR amplification using the general primers, 10- to 20-µl aliquots of the PCR products were digested for 3 hr at 37°C with 30 U of AvaII. This endonuclease recognizes 2 sites on the sequence of HSV-1 amplification product and one site on the sequence of HSV-2 amplification product. Digestion products were electrophoresed through a 2% agarose gel. Gels were stained with ethidium bromide and photographed on a UV-light transilluminator. Enzymes were supplied by New England Biolabs (Beverly, MA) and the conditions followed for digestion were those recommended by the supplier.

Nucleotide sequence analysis

The HSV PCR product of one sample, using the general primers, remained uncut after AvaII and BglI digestion, a restriction site of which is located near AvaII. This 478-bp PCR product was electrophoresed in a 2% agarose gel, purified and subjected to direct sequencing, using the Sequenase Kit, Version 2.0 (USB, Cleveland, OH), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed on a Macintosh computer using StatWorks (version 1.2) software and Microsoft Excel 4.0. Fisher's exact test was used for 2 × 2 tables and the Chi-squared test for significance.

RESULTS

The histological classification of the 187 examined samples revealed 38 cases of cervicitis, 11 cervical intra-epithelial neoplasias I-II (CIN I-II), 14 *in situ* carcinomas of the cervix, 35 invasive carcinomas of the cervix, 18 normal-benign endometrial lesions, 50 endometrial adenocarcinomas, 10 ovarian benign lesions, 8 ovarian carcinomas, and 3 vulval carcinomas.

Out of the 187 samples, 59 were found positive for HPV, 28 for CMV (Fig. 1), 15 for HSV (Figs. 2, 3), while only 3 for EBV (Fig. 4) DNA. Out of the 15 cases positive for HSV-DNA, only 3 were positive for HSV-2: 2 cases of SCC and 1 case of normal endometrium (Table I).

FIGURE 1 – Detection of CMV DNA. PCR products were electrophoresed through a 2% agarose gel. Lane M, molecular-weight marker pUC18/HaeIII; lanes 1, 3, 4, 6, positive samples; lanes 2, 5, 7, 8, 9, negative samples.

FIGURE 2 – Detection and typing of HSV DNA using the set of the specific primers. The PCR products (110 bp for HSV-1 and 93 bp for HSV-2) were electrophoresed through a 3% agarose gel. Lane M, molecular-weight marker pUC18/HaeIII; lanes 1, 5, negative samples; lanes 2, 4, positive samples for HSV-1; lanes 3, 6, positive samples for HSV-2.

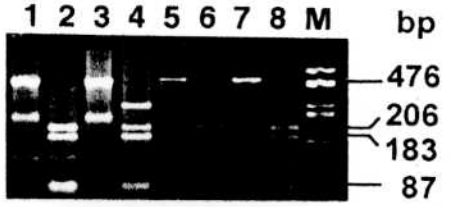

FIGURE 3 – Typing of HSV amplification products using the general primers. After AvaII digestion, which recognizes 2 sites in HSV-1 and one site in HSV-2 PCR products, the samples were electrophoresed through a 2% agarose gel. Odd lanes, uncut PCR products; even lanes, HSV-1 samples (providing 206-, 183- and 87-bp fragments); lane M, molecular-weight marker pUC18/HaeIII.

One of the HSV PCR products using the general primers remained uncut after AvaII and BglI digestions used for the typing of the virus. To determine the type, the 2 sets of specific primers were used in a multiplex PCR reaction to reveal the HSV-1 type. The initial PCR product was sequenced and revealed 4 nucleotide substitutions, which however did not alter the amino-acid sequence (Fig. 5).

The prevalence of CMV and HSV viruses was found markedly higher in cases of cervical lesions compared with the other genital lesions, with the exception of ovarian tumors, where only a small number of cases was examined.

CMV and HSV infection was also more common in carcinomas of the cervix (34.3% and 22.9% respectively) as compared with cases of cervicitis (5.3% and 2.6%, $p = 0.017$ and 0.001 respectively). Presence of CMV DNA was also higher among intra-epithelial neoplasms I-II (36.4%) and *in situ* carcinomas of the cervix (35.7%) compared with cases of cervicitis ($p = 0.018$ and 0.011 respectively); the same trend was shown for HSV, but was not statistically significant. However, no differences in the incidence of CMV and HSV infection were observed between intra-epithelial neoplasms and carcinomas of the cervix, suggesting a possible involvement of the 2 viruses in the early stages of carcinogenesis of the cervix.

EBV DNA was detected only in one case each of CIN II, SCC and endometrial adeno-acanthoma. Such a low level of detection is in accordance with previous observations (Landers *et al.*, 1993; Wong *et al.*, 1993).

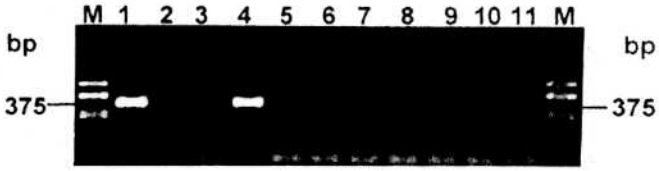

FIGURE 4 – Detection of EBV DNA. PCR products were electrophoresed through a 2% agarose gel. Lane M, molecular-weight marker pUC18/HaeIII; lanes 1, 4, positive samples; lanes 2, 3, 5–11, negative samples.

Combination of CMV and HSV infection was detected in only 4 cases (3 in invasive carcinomas of the cervix and 1 in CIN II).

Analysis of marital status, alcohol consumption and cigarette smoking history did not reveal any statistically significant association with risk of cervical cancer. On the assumption that HPV infection plays a significant role in cervical cancer, we examined the prevalence of CMV, HSV and EBV infection according to the HPV status, showing no significant differences between HPV-negative and HPV-positive samples (Table II). However, comparison among HPV-negative women showed significantly higher prevalence of CMV infection in invasive carcinomas (33.3%, $p = 0.009$) and *in situ* carcinomas of the cervix (33.3%, $p = 0.017$) than in cervicitis (0%). The prevalence of HSV was also higher in cases of invasive carcinomas (33.3%, $p = 0.037$) than in cases of cervicitis (4.3%). No differences in the rate of CMV, HSV or EBV infections were found between cervical intra-epithelial neoplasias, *in situ* carcinomas and invasive carcinomas of the cervix among HPV-negative women.

In contrast, comparison among HPV-positive women showed no statistically significant differences in the prevalence of CMV and HSV infection in cases of cervicitis, neoplasm and carcinoma of the cervix, although CMV was still more prevalent in cases of CIN and carcinoma than in controls. The presence of CMV and HSV DNA was not correlated to any increasing risk, and it was of no separate etiological significance, possibly due to the higher oncogenic potential of HPV virus as compared with that of CMV and HSV. Thus, only among HPV-negative women were these 2 infections significantly associated with risk of cervical cancer.

The histological diagnosis of *in situ* and invasive cervical carcinomas was SCC in 41 cases, AC in 6 cases and SCC + AC in 2 cases. According to the degree of differentiation of the 49 *in situ* and invasive cervical carcinomas, there were 14.3% (n = 7) grade I, 42.9% (n = 21) grade II, 10.2% (n = 5) grade

TABLE I – DETECTION OF HPV, CMV, HSV AND EBV VIRUSES IN LESIONS OF THE FEMALE REPRODUCTIVE TRACT

Type of lesion	Number of patients	HPV-positive (%)	p (χ^2)	CMV-positive (%)	p (Fisher's test)	HSV-positive (%)	p (Fisher's test)	EBV-positive (%)
Cervicitis	38	15 (39.5)		2 (5.3)		1 (2.6)		0
Cervical intraepithelial neoplasia (CIN) I–II	11	7 (63.6)		4 (36.4)	0.018	2 (18.2)		1 (9.1)
In situ carcinoma of the cervix	14	5 (35.7)		5 (35.7)	0.011	1 (7.1)		0
Carcinoma of the cervix	35	23 (65.7)	0.021	12 (34.3)	0.017	8 (22.9)	0.001	1 (2.9)
Endometrial normal-benign lesions	18	0		0		1 (5.6)		0
Endometrial adenocarcinomas	50	8 (16.0)		3 (6.0)		1 (2.0)		1 (2.0)
Ovarian benign lesions	10	0		0		0		0
Ovarian carcinomas	8	0		2 (25.0)		1 (12.5)		0
Vulval carcinomas	3	1		0		0		0
Total	187	59		28		15		3

p values are referred to comparison of cervicitis controls with each case group: CIN I–II, *in situ* carcinoma and carcinoma of the cervix.

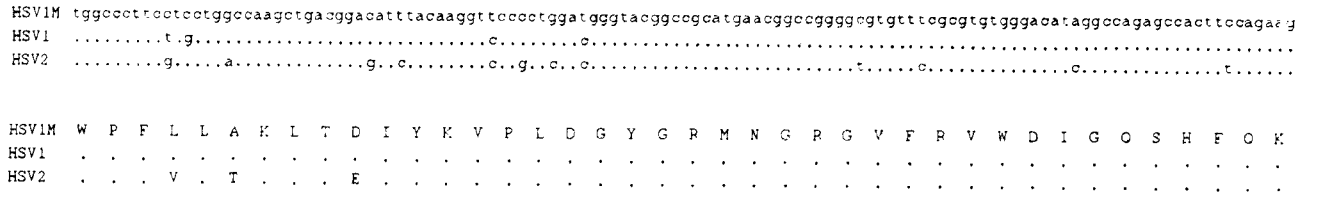

FIGURE 5 – Nucleotide and amino-acid comparison between the 120-bp isolated sequence (HSV1M) and HSV-type-1 and type-2 regions of the polymerase gene (5959-6078 bp) (Quinn and McGeoch, 1985). The 4 substitutions found in HSV1M did not alter the amino-acid sequence.

TABLE II – PREVALENCE OF CMV, HSV AND EBV INFECTION IN HPV-DNA-NEGATIVE AND -POSITIVE CERVICAL LESIONS

	Number of patients	CMV (%)	HSV (%)	EBV (%)
HPV-negative lesions				
Cervicitis	23	0	1 (4.3)	0
CIN I–II	4	1 (25.0)	1 (25.0)	0
In situ carcinoma	9	3 (33.3) $p = 0.017$[1]	1 (11.1)	0
Invasive carcinoma	12	4 (33.3) $p = 0.009$[1]	4 (33.3) $p = 0.037$[1]	1 (8.3)
HPV-positive lesions				
Cervicitis	15	2 (13.3)	0	0
CIN I–II	7	3 (42.9)	1 (14.3)	1 (14.3)
In situ carcinoma	5	2 (40.0)	0	0
Invasive carcinoma	23	8 (34.8)	4 (17.4)	0

[1]Each case group (*in situ* carcinoma and invasive cervical carcinoma) is compared with cervicitis controls.–*p* values are referred to Fisher's exact test.

III, undifferentiated 2% (n = 1) and for 15 cases the differentiation grade was not available. Clinical assessment of the 35 invasive cervical carcinomas showed that 14.3% (n = 5) were stage Ia, 54.3% (n = 19) stage Ib, 5.7% (n = 2) stage II, 2.9% (n = 1) stage IIIb, 2.9% (n = 1) stage IV, while for 7 cases the stage was not available. Separate analysis of the cases revealed no significant associations between viral infection and histological type, degree of differentiation or stage of the tumor. There were no clear trends with age, although HSV infection was more frequent in women under 40 ($p = 0.063$).

DISCUSSION

It has been suggested that synergism between multiple infections may be a cause of cervical cancer (Zur Hausen, 1982), and this has received epidemiological support from some studies (Hildesheim *et al.*, 1991; Dillner *et al.*, 1994; Jha *et al.*, 1993; Schmauz *et al.*, 1989). The increased risk of cervical cancer with seropositivity against multiple infections has been explained in several ways. It was suggested that an increased number of infections posits a measure of sexual activity, and thus higher possibility of HPV infection. It is possible that long-standing infections will promote malignancy through chronic irritation.

In this study, we found that CMV and HSV appears to be significantly associated with cervical cancer. However, among HPV-DNA-positive women, no other infection conveyed an increased risk for cervical cancer, although CMV infection was more common than in HPV-DNA-negative women. In contrast, among HPV-DNA-negative women, only those with CMV or HSV infection were at a significantly increased risk of carcinoma of the cervix.

HSV detection appeared to differ qualitatively from that of CMV. HSV had a weaker association with cervical cancer, but its presence was more common in HPV-negative than in HPV-positive cases, although this inverse association may not be significant. More definite conclusions on the association between cervical cancer and sexually transmitted agents among HPV-negative women would require a study of much larger populations. HSV-2 DNA was detected in only 2 out of the 35 cases with cervical cancer (5.8%), as compared with HSV-1 detected in 6 cases (17.1%). Type-1 genital infection has been used as an indicator of orogenital acquisition, however this may not be valid in populations in which type 1 constitutes a large proportion of genital disease (Guinan *et al.*, 1985). The frequency of type-1 genital infection varies greatly in different studies (Kimura *et al.*, 1991; Lycke, 1991; Jha *et al.*, 1993; Whitley, 1994). It has been reported that factors affecting the frequency of type-1 genital infection include the geographic area, primary compared with recurrent infection, gender and population sample. Our results rely on the validity of PCR to detect past infection, due to the higher sensitivity and specificity of the method as compared with current serological tests. Our aim was to detect past exposure, not only the actual clinical disease. A number of reports on the comparison of Southern blot, ViraPap and PCR indicated the superiority of PCR (Schiffman *et al.*, 1991; Guerrero *et al.*, 1992). Studies comparing HPV and other factors using different methods (*e.g.*, DNA hybridization, ViraPap, or serological studies) assessed different exposures (current or past), and the observed interaction might not reflect the biological process (Guerrero *et al.*, 1992).

The intratypic polymorphism of HSV genome found in one of the samples examined, further supports the employment of sequence analysis in tracing transmission of HSV in certain regions and in determining whether infection is a result of re-activation of a latent virus or is due to re-infection with an exogenous virus (Sakaoka *et al.*, 1994).

Our findings could be interpreted as indicating that past infections with CMV or HSV are surrogate markers of HPV. The suggestion of HPV interaction with CMV and HSV in cervical carcinogenesis (Shen *et al.*, 1993; Hildesheim *et al.*, 1991) is not supported. However, it cannot be excluded that genital infections with other sexually transmitted agents, such as CMV and HSV viruses, may play a separate etiological role in cervical cancer. In favor of this is the strong association found in our study between cervical cancer and infection with CMV or HSV among HPV-DNA-negative women. Different carcinogenic

factors, cellular or not, acting independently or synergistically, are involved at different steps of the carcinogenesis cascade. Care should therefore be taken not to limit future studies on genital oncogenesis to only one of these factors, but rather to extend them towards considering the complex interactions as well as different pathways arising from the multistep model.

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