Association of human β-herpesviruses with the development of cervical cancer: bystanders or cofactors

P K S Chan, M Y M Chan, W W H Li, D P C Chan, J L K Cheung, A F Cheng

Abstract

Background/Aim—Human papillomaviruses (HPVs) are important, but not sufficient, for the development of cervical cancer. All three human β-herpesviruses—cytomegalovirus (CMV) and human herpesviruses (HHV) types 6 and 7—have been detected in the cervix. In addition, CMV and HHV-6 can interact with HPVs in vivo. This study examined the possible role of β-herpesviruses in cervical cancer development.

Methods—HPV, CMV, HHV-6, and HHV-7 were detected by the polymerase chain reaction using cervical scrapes taken at colposcopy from 388 women. HPV types were identified using restriction fragment length polymorphisms. Colposcopy guided biopsies were taken from abnormal areas, and the histological findings were regarded as the final diagnoses. The associations between herpesvirus infection and the degree of cervical lesion were analysed with respect to HPV status.

Results—Of the 388 women, 51.8% had a normal cervix, 14.4% had cervical intraepithelial neoplasia grade 1 (CIN1), 8.2% had CIN2, 19.3% had CIN3, and 6.2% had invasive carcinoma. Overall, the positive rates for high, intermediate, and low risk HPVs were 18.8%, 21.4%, and 5.2%, respectively. Fifteen patients harboured HPVs for which the genotype could not be identified. Positive rates for CMV, HHV-6, and HHV-7 were 9.5%, 3.6%, and 3.4%, respectively. HPV positive patients carried a higher risk for high grade lesions (CIN2/3 or carcinoma) (odds ratio (OR), 5.24; 95% confidence interval (CI), 3.19 to 8.62; χ² = 51.79; p < 0.001), whereas those positive for CMV, HHV-6, or HHV-7 did not. Thirteen of 131 patients with high grade lesions had HPV/herpesvirus co-infections, but no association with the cervical lesion was noted. Furthermore, positive rates for herpesviruses among HPV negative, high/intermediate risk HPV negative, and high risk HPV negative subgroups were similarly low and without a significant association.

Conclusions—The ubiquitous nature of herpesviruses may pose difficulty in elucidating their pathogenic role. These results indicate that CMV, HHV-6, and HHV-7 are bystanders rather than cofactors in the oncogenesis of cervical cancer.

Keywords: human papillomavirus; human herpesvirus 6; human herpesvirus 7

Human papillomaviruses (HPVs) are important, but not sufficient to cause cervical cancer.1,2 Recently, the search for cofactors involved in the multistep oncogenic pathway has received much interest. Sexually transmitted agents, particularly viral infections, have been postulated to have a synergistic role in the carcinogenesis of cervical neoplasia.3–4 Apart from herpes simplex virus, which has long been suspected to act as an “initiator” in the development of cervical cancer,3 other herpesviruses are also potential candidates. The immediate–early gene products of cytomegalo virus (CMV) can transactivate other viral and cellular genes,5–7 and it has been suggested that concurrent genital infection with CMV and HPV might increase the risk for cervical cancer.8 A putative oncogene has been found in the human herpesvirus 6 (HHV-6) genome,9–11 and molecular clones of HHV-6 that can transactivate papillomavirus have been reported.12 In addition, the presence of CMV and HHV-6 in the cervix of a considerable proportion of women with normal and abnormal cytologies13–15 gives them an opportunity to interact with genital HPVs.

CMV, HHV-6, and HHV-7 share a high degree of genomic homology and are classified under the same subfamily of the β-herpesvirinae.16 Thus, these herpesviruses might share a similar oncogenic potential. Although all these viruses are ubiquitous and have been detected in the cervix,16–22 epidemiological data regarding their association with genital HPVs and their role in cervical cancer have not reached a consensus. Here, we examined the presence of these β-herpesviruses and HPVs in a series of Chinese women with various cervical lesions to elucidate their role in the development of cervical cancer.

Methods

STUDY POPULATION

A total of 388 Chinese women referred to the colposcopy clinic at Queen Elizabeth Hospital, Hong Kong, for the management of abnormal cervical cytologies were recruited with written informed consent. In Hong Kong, cervical cancer ranks fourth for new cancers and seventh for deaths from cancer in women. The median age at diagnosis is 56 years with an age standardised rate of 12.7/100 000.18
CLASSIFICATION OF CERVICAL LESIONS

All recruited women were examined by colposcopy with biopsies taken from abnormal areas and followed by local surgical treatment if necessary. All histological assessments were performed by an experienced pathologist. The classification of cervical lesions was based on historical findings according to the World Health Organisation’s classification. When there were discrepancies between the histological findings of colposcopic biopsies and excised tissues, the worst results were regarded as the final diagnoses. Women with normal colposcopy/biopsy had follow up cytology performed at three to six month intervals. For the purpose of our study, women with normal colposcopy/biopsy who had normal cytology results throughout the subsequent 12 month follow up period were classified as normal. The virology results were unknown to clinicians and the pathologist throughout the entire follow up. The study protocol was approved by the local institution ethics committee.

VIRAL DNA DETECTION

A cervical scrape sample was obtained from each woman at the time of colposcopy. Total DNA was extracted by a previously described method. The quality of the DNA was assessed by the polymerase chain reaction (PCR) targeting the 358 bp fragment of the human β-globin gene. The presence of CMV, HHV-6, and HHV-7 DNA was detected by nested PCR. The primer sets used have been shown to be specific and do not cross amplify other herpesviruses (table 1). Briefly, 5 µl of the extracted DNA was amplified in a 50 µl reaction mixture containing PCR buffer (10 mM Tris/HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl2), 200 µM of each dNTP, 1 unit of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), and 0.25 µM of each primer. An initial denaturation at 94°C for four minutes was followed by 30 cycles of one minute each for denaturation (94°C), annealing (55°C), and extension (72°C), with an eight minute additional extension step at 72°C after the last cycle. An aliquot of 2 µl of the first round PCR product was amplified in a second reaction (55°C, and 8.2°C) had biopsy confirmed CIN2, 75 (19.3%) had biopsy confirmed CIN3, and 24 (6.2%) had invasive cervical carcinoma (two patients with invasive adenocarcinoma, others had invasive squamous cell carcinoma).

For the detection of HPV DNA, a single round PCR based on the degenerate primers MY09 and MY11 was used (table 1). These primers are capable of amplifying at least 40 genital HPV types. The amplification conditions were the same, except that the reaction was run for 40 cycles. HPV types were further characterised by restriction fragment length polymorphism using endonucleases RsaI and DdeI, as described previously.

The detection limit of PCR was estimated by limiting dilution using plasmids containing the target sequences of CMV, HHV-6, HHV-7, and HPV types 6, 11, 16, and 18, respectively. The nested PCRs for CMV, HHV-6, and HHV-7 had a sensitivity equivalent to five to 10 genome copies of template, whereas the detection limit for HPV PCR was 100 genome copies.

To avoid possible cross contamination, all PCR reactions were carried out under stringent conditions following the recommendations of Kwok and Higuchi. A negative control was included after each fifth sample. In addition, all positive samples were repeated in a separate PCR run and were all reproducible.

STATISTICAL ANALYSIS

The age distribution of different patient groups was compared by independent samples t test. Associations between viral DNA positivity and the degree of cervical lesion were assessed by calculating the odds ratios (OR) and 95% confidence intervals (CI). The χ2 test or Fisher’s exact test was used as appropriate to compare categorical variables. Two tailed p values of < 0.05 were regarded as significant.

Results

The 388 recruited Chinese women were aged between 16 and 88 years (mean, 40.4; SD, 11.4). Two hundred and one patients (51.8%) had a normal cervix, 56 (14.4%) had biopsy confirmed cervical intraepithelial neoplasia grade 1 (CIN1), 32 (8.2%) had biopsy confirmed CIN2, 75 (19.3%) had biopsy confirmed CIN3, and 24 (6.2%) had invasive cervical carcinoma (two patients with invasive adenocarcinoma, others had invasive squamous cell carcinoma).

Table 1 Primers used for the polymerase chain reaction

<table>
<thead>
<tr>
<th>Target DNA</th>
<th>Primer</th>
<th>Amplicon (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV morphological transforming region II</td>
<td>Outer CMTR1: 5'-CTG TCG GTG ATG GTG TCT TT-TC-3'; CMTR2: 5'-CCC GAC AGG CCG AAA AGA AA-3'</td>
<td>230</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Inner CMTR3: 5'-CTC CTG GTG ATG ATC GTG TTC TT-3'; CMTR4: 5'-CTG ACC TAC CAA CGT AGT ATG TTT-3'</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>HHV-6 major capsid protein encoding region</td>
<td>Outer H6-6: 5'-AAG CTT GCA CAA TGCC CAA AAA ACA G-3'; H6-7: 5'-CTC GAG TAT ATA CGG GAC ACC CCT AAT C-3'</td>
<td>223</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Inner NH-6: 5'-CTC ATT TTG CAG GCA GGA TCT GTT-3'; NH-7: 5'-TGT TAG GAT ATA CCG ATG TG TGG CT-3'</td>
<td>130</td>
<td>24</td>
</tr>
<tr>
<td>HHV-7 U10 region</td>
<td>Outer P1: 5'-TAT CCC AGG TGT TTT CAT ATG TCA-3'; P2: 5'-GCC TCG GAG TAG CAG TAT CGG GTT-3'</td>
<td>186</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Inner P3: 5'-AGA TGA TAG ACA GAG GTT TG-3'</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>HPV L1 ORF</td>
<td>MY09: 5'-CTG CCM ARR GGA WAC TAG TAA TG-3'; MY11: 5'-GCM CAG GGW CAT AXA AAT GG-3'</td>
<td>450</td>
<td>26</td>
</tr>
</tbody>
</table>

CMV, cytomegalovirus; HHV, human herpesvirus; HPV, human papillomavirus; ORF, open reading frame.
Overall, 191 of the 388 cervical samples (49.2%) were positive for HPV DNA. All the remaining 197 HPV negative samples were positive in the β-globin PCR, indicating that an adequate preparation had been obtained. Seventy three (38.2%) of the 191 HPV positive women harboured high risk HPV types (HPV types 16/18), 83 (43.5%) harboured intermediate risk types (HPV types 31, 33, 35, 52, 58), 20 (10.5%) carried low risk types (HPV types 6/11), and the remaining 15 (7.9%) had HPVs in which the genotypes could not be identified based on their restriction patterns. Figure 1 shows the restriction patterns of representative HPV types. Twenty patients had coinfection with two types of HPVs, and they were classified according the HPV type that was associated with a higher risk. The positive rates for CMV, HHV-6, and HHV-7 were 9.5%, 3.6%, and 3.4%, respectively. Members of the HPV positive group were significantly younger than those without HPV infection (mean age, 39.2 ± 41.5 years; p < 0.05 by t test). In contrast, women positive for CMV, HHV-6, or HHV-7 had no significant difference in age distribution (fig 2).

Table 2 shows the distribution of viral DNA positive samples among various degrees of cervical lesion. When the viral DNA positive rates between patients with normal/low grade cervical lesions (a normal cervix or CIN1) and those with high grade cervical lesions (CIN2/3 or carcinoma) were compared, the presence of HPVs carried an increased risk for high grade cervical lesions (OR, 5.24; 95% CI, 3.19 to 8.62; χ² = 51.79; p < 0.001). In contrast, the presence of CMV, HHV-6, or HHV-7 did not significantly increase the risk for high grade cervical lesions (CMV: OR, 0.81; 95% CI, 0.36 to 1.79; χ² = 0.3; p = 0.585. HHV-6: OR, 1.09; 95% CI, 0.31 to 3.66; p = 1.0 by Fisher’s exact test).

Table 2: Prevalence of HPV, CMV, HHV-6, and HHV-7 DNA in the cervix of 388 women according to the degree of cervical lesion

<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>No. tested</th>
<th>High risk HPV</th>
<th>Intermediate risk HPV</th>
<th>Low risk HPV</th>
<th>CMV</th>
<th>HHV-6</th>
<th>HHV-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/inflamed</td>
<td>201</td>
<td>10 (5.0)</td>
<td>24 (11.9)</td>
<td>14 (7.0)</td>
<td>17 (8.5)</td>
<td>7 (3.5)</td>
<td>7 (3.5)</td>
</tr>
<tr>
<td>CIN1</td>
<td>56</td>
<td>16 (28.6)</td>
<td>19 (33.9)</td>
<td>2 (3.6)</td>
<td>9 (16.1)</td>
<td>2 (3.6)</td>
<td>2 (3.6)</td>
</tr>
<tr>
<td>CIN2</td>
<td>32</td>
<td>6 (18.8)</td>
<td>8 (25.0)</td>
<td>0</td>
<td>3 (9.4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CIN3</td>
<td>75</td>
<td>28 (37.3)</td>
<td>24 (32.0)</td>
<td>4 (5.3)</td>
<td>8 (10.7)</td>
<td>3 (4.0)</td>
<td>3 (4.0)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>24</td>
<td>13 (54.2)</td>
<td>8 (33.3)</td>
<td>0</td>
<td>0</td>
<td>2 (8.3)</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>Total</td>
<td>388</td>
<td>73 (18.8)</td>
<td>83 (21.4)</td>
<td>20 (5.2)</td>
<td>37 (9.5)</td>
<td>14 (3.6)</td>
<td>13 (3.4)</td>
</tr>
</tbody>
</table>

† 15 women had HPVs for which the genotype could not be identified.
CIN, cervical intraepithelial neoplasia grade; CMV, cytomegalovirus; HHV, human herpesvirus; HPV, human papillomavirus.
Table 3 Prevalence of coinfection with HPV and herpesviruses in the cervix of 388 women according to the degree of cervical lesion

<table>
<thead>
<tr>
<th>Cervical lesion</th>
<th>No. (%) of women with coinfection of HPV and the indicated virus</th>
<th>CMV</th>
<th>HHV-6</th>
<th>HHV-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/infamed</td>
<td>201</td>
<td>8 (4.0)</td>
<td>2 (1.0)</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>CIN1</td>
<td>56</td>
<td>6 (10.7)†</td>
<td>2 (3.6)‡</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>CIN2</td>
<td>32</td>
<td>1 (3.1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CIN3</td>
<td>75</td>
<td>7 (9.3)‡</td>
<td>2 (2.7)</td>
<td>3 (4.0)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>Total</td>
<td>388</td>
<td>22 (5.7)</td>
<td>6 (1.5)</td>
<td>7 (1.8)</td>
</tr>
</tbody>
</table>

†One woman coinfected with HHV-6.
‡One woman coinfected with HHV-7.

PATIENTS NEGATIVE FOR HIGH/INTERMEDIATE RISK HPV TYPES
Among the 232 patients who were negative for high or intermediate risk HPVs, the positive rates for CMV, HHV-6, and HHV-7 were 8.6%, 3.9%, and 3.0%, respectively. Of these 232 patients, 44 had high grade lesions (CIN2/3 or carcinoma), with four (9%), three (6.8%), and none positive for CMV, HHV-6, and HHV-7, respectively. No significant association between herpesvirus infection and high grade cervical lesion was observed (CMV: OR, 1.08; 95% CI, 0.29 to 3.69; p = 1.0 by Fisher's exact test). HHV-6: OR, 2.22; 95% CI, 0.42 to 10.57; p = 0.377 by Fisher's exact test. HHV-7: OR, 0.00; 95% CI, 0.00 to 3.37; p = 0.352 by Fisher's exact test.

PATIENTS NEGATIVE FOR ALL HPV TYPES
Among the 197 patients who were negative for HPV DNA, 14 (7.1%) were positive for CMV alone, seven (3.6%) were positive for HHV-6 alone, six (3.0%) were positive for HHV-7 alone, and one (0.5%) was coinfectected with CMV and HHV-6. Most of these HPV negative but herpesvirus positive patients had a normal cervix or low grade cervical lesions; only two patients had CIN2 (both infected with CMV), two patients had CIN3 (one infected with CMV, the other infected with HHV-6), and two other patients had invasive carcinoma (both were infected with HHV-6).

Discussion
The involvement of cocarcinogens in HPV related oncogenesis is supported by observations that the UV part of sunlight is necessary in the development of epidermodysplasia verruciformis, x rays are necessary in laryngeal papillomatosis, and carcinogens containing bracken fern are necessary in oesophageal papillomatosis of cattle. As for cervical cancer in humans, nicotine, oral contraceptives, dietary variables, and coinfections with other sexually transmitted agents have all been suggested as potential cocarcinogens, but conclusive evidence has yet to be produced.

In our study, the quality of the extracted DNA preparation was assessed by PCR targeting the β-globin gene. Although the amplified β-globin sequence is approximately 100 bp shorter than the target of HPV PCR, given that the original samples were cervical scrapes, it is likely that the β-globin positive results indicated an adequate preparation for HPV PCR.

Our results show that infection with HPV, as expected, was associated with an increased risk for high grade cervical lesion. However, all the three β-herpesviruses examined were present at low frequencies and did not carry a significant increase in risk for high grade cervical lesions. Among the 24 patients with invasive carcinoma, only two were infected with HHV-6, one with HHV-7, and none with CMV. Although both HHV-6 positive patients
who had invasive carcinoma were negative for HPV; the overall picture does not suggest a causal association. Because a marginal effect of herpesviruses could be masked by the presence of high/intermediate risk HPV-type infections, we analysed the positive rates of herpesviruses among the high risk HPV negative, high/intermediate risk HPV negative, and HPV negative and herpesvirus/herpesvirus coinfected subgroups with respect to the degree of cervical lesion, but still found no positive evidence.

It has been shown in vitro that CMV can enhance the transforming ability of bovine papillomavirus, and HHV-6 can enhance the expression of HPV oncoproteins E6 and E7. If these mechanisms are relevant in the development of cervical cancer, the ability of these herpesviruses to persist in cervical epithelial cells might be crucial. However, the available epidemiological data are conflicting. Reports of detecting CMV in cervical cancer, using similar highly sensitive PCRs, have not reached a consensus. Han et al reported the presence of CMV in 67% of Taiwanese patients with cervical cancer. In contrast, Thompson et al found that only 4% of Australian women with cervical cancer were positive for CMV.

Findings on HHV-6 are also inconclusive. Yadav et al detected HHV-6 sequences in 10 of 26 cervical carcinoma tissues by in situ hybridisation. On the other hand, Romano et al reported that only one of 85 women with abnormal cervical cytology was positive for HHV-6, and in that series none of the six patients with invasive carcinoma had HHV-6 detected by PCR. As for HHV-7, although it has been detected in the uterine cervix, investigations on its association with cervical cancer are lacking.

Attempts to elucidate the pathogenic role of herpesviruses in human diseases are often complicated by the fact that they are ubiquitous. Our results, in contrast to those of others, suggest that CMV, HHV-6, and HHV-7 are bystanders rather than cofactors in the development of cervical cancer. Nonetheless, the approach that we used cannot localise the development of cervical cancer. Nonethe-


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