HPV detection in stained cytological cervical specimens and correlation with cytology and histology

M. KOFFA1,2, H. SIMIAKAKI3, M. ERGAZAKI1,2, M. PAPAEFTHIMIOU3, K. KARAKATSANI1, E. DIAKOMANOLIS4 and D.A. SPANDIDOS1,2

1Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens; 2Laboratory of Virology, Medical School, University of Crete, Heraklion; 3Department of Clinical Cytology, ‘Alexandra’ Hospital, Athens; 4Department of Colposcopy, ‘Alexandra’ Hospital, Athens, Greece

Received August 14, 1995; Accepted September 4, 1995

Abstract. It has been suggested that detection of high risk human papillomavirus (HPV) in low grade cervical intraepithelial neoplasia contributes to transition to high grade lesions and cancer. Currently, the PAP smear is the primary screening tool to identify women with cervical disease, specifically cervical intraepithelial neoplasia (CIN). In the present study we examined the utility of HPV detection and typing by PCR, in women with cytological and/or histological evidence of low grade squamous lesion (LGSL), using stained PAP cervical smears. HPV infection was confirmed in 21 out of the 31 (68%) specimens examined.

Introduction

The significant role of human papillomavirus (HPV) in cervical and anogenital neoplasia is well known (1-3). HPV types 16, 18, 31, 33 and 35 have been detected in association with up to 80% of high grade lesions (CIN II and III) and with up to 90% of invasive cervical cancers (4,5). These types are also found in low grade lesions (CIN I and wart virus change only) and even in women with negative cervical biopsies (6,7). The detection of these viral types in an individual patient might be predictive of the progression to a higher-grade lesion (8,9).

The management of patients with persistent borderline or wart virus change, or mild dyskaryosis is not clear as there is no cytopathological criterion to distinguish between patients who will have a low- or high-grade lesion on subsequent biopsy (10,11). Moreover, about 50% of lesions diagnosed as CIN I will regress spontaneously, and a too early intervention may result in the unnecessary treatment of women who are at little or no risk of developing carcinoma. There are however, patients with CIN I in whom the disease will persist or progress to carcinoma. HPV testing may usefully augment cytology by helping to decide whether a patient with a mild abnormality needs immediate referral for colposcopy (9) providing a quality assurance in cervical cytopathology (12).

Although the detection of HPV infections using colposcopy, histology and cytology has been claimed, these techniques are not able to identify the virus itself, but detect the (sub-) clinical manifestation of the HPV infection (13). Moreover, the formation of koilocytes, the main cytological and histological criterion for HPV infection, and HPV-specific colposcopie pattern, can also be caused by agents other than HPV, while koilocytes can be absent in HPV infections (14). Thus the sensitivity and specificity of each of these techniques in HPV detection are rather low and controversial (15). Furthermore, they fail to discriminate between the different types of HPV (16).

With the use of the polymerase chain reaction (PCR) technique, viral DNA has been detected in cervical smears from women with no history of cervical lesions attending routine screening clinics and in substantial proportions of women with CIN or cervical carcinomas (17-20). PCR is known to be more sensitive than in situ hybridization, Southern blotting, or filter hybridization in the detection of HPV DNA (21,22). The PCR technique is thought to increase the sensitivity of HPV DNA screening tests and give a more accurate frequency of infections as well as better estimate of disease progression.

In the present study we evaluated the potential contribution of HPV analysis in the prediction of low-grade CIN progression, as well as the correlation between HPV detection and typing by PCR with cytological and histological diagnosis. 31 stained cytological specimens from PAP smears with a cytological and/or histological diagnosis of low grade squamous lesion (LGSL) were examined and 21 were found positive for HPV DNA.

Materials and methods

Patients and specimens. Specimens were obtained from 50 women with cytological and/or histological evidence of low grade squamous lesions (LGSL: HPV and/or CIN I), treated at the Department of Cytology, ‘Alexandra’ Hospital, Athens, Greece. The cervical smears used were already fixed and stained to obtain the cytological diagnosis.
DNA extraction. For each case, one slide with stained cervical smear was used. The slides were soaked for 48 h in xylene followed by ethanol wash, to remove the coverslip. The cells were then scraped into a 1.5 ml Eppendorf, with the addition of 400 μl digestion buffer containing 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0 and lysed with the addition of 0.1 mg/ml proteinase K (Sigma, St. Louis, MO). Samples were incubated for 24 h at 37°C. Fresh proteinase K was added and the incubation was continued for another 24 h. The samples were then extracted once with phenol/chloroform and once with chloroform. DNA was precipitated with the addition of 20 μl 5 M NaCl and 1 ml ethanol, recovered with centrifugation at 13000 rpm for 15 min at 4°C, washed once with cold 70% ethanol and resuspended in 20 μl double distilled water.

Oligonucleotide primers and PCR amplification. All specimens were examined for the presence of amplifiable DNA using a set of primers for a fragment of β-globin gene (23). For the distinction of HPV types a multiplex PCR was employed, using simultaneously four pairs of primers (for HPV types 11, 16, 18 and 33), with each virus type giving different length of amplified DNA (24). Two μl of the extracted DNA of each sample was amplified in a reaction solution of 50 μl containing 20 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 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). The mixture was heated for 1 min at 95°C, and then subjected to 35 cycles of amplification.

β-globin: Each cycle included a denaturation step at 94°C for 50 sec, an annealing step at 56°C for 40 sec and an elongation step at 72°C for 40 sec, increasing the elongation time 1 sec per cycle.

HPV: Each cycle included a denaturation step at 94°C for 50 sec, an annealing step at 56°C for 50 sec and an elongation step at 72°C for 50 sec, increasing the elongation time 1 sec per cycle. To establish type specificity of primer-directed amplification, each set of primers was tested with template plasmid DNA of the five HPV types 6b, 11, 16, 18 and 33. PCR products were analyzed on a 2% agarose gel and photographed on a UV light transilluminator.

Results

The presence of amplifiable DNA, using primers for a fragment of β-globin gene, was confirmed in 31 out of the 50 stained smears examined. The remaining 19 cytological preparations either contained only very small number of cells, too few to be successfully scraped off the slide, or chemicals used for the fixation and staining for cytology remained after extraction, inhibiting the PCR reaction.

Detection and typing of HPV DNA was performed in the 31 specimens with a cytological and/or histological diagnosis of low grade squamous lesion (LGSL). In 21 (68%) cases the presence of HPV DNA was confirmed, while 10 cases were found negative for HPV DNA.

The typing of the virus revealed the higher prevalence of HPV16, found in 11 cases (36%). HPV11 was found in 4 cases (13%), HPV18 in 3 cases (10%), HPV33 in 1 case (3.2%), while in 2 cases (6.5%) HPV16 and HPV11 were simultaneously detected (Fig. 1). The primers used for the multiplex PCR were within the E6 gene, providing amplification whether the HPV genome was episomal or integrated. Moreover, the length of the PCR products (315-143 bp) was relatively short and could be amplified from the very small yields of low-molecular-weight DNA produced from such preparations.

After 3 years of follow-up, persistence of the disease was found in 4 cases (13%) with high-risk HPV: 2 cases with HPV-16 (6.5%) and two cases (6.5%) with HPV-18. Progression of the disease was found in only one case with HPV-11 (3.2%), in which no histological diagnosis was available. In 26 cases (84%) (9 cases with HPV-16, 3 cases with HPV-11, 2 cases with both HPV-16 and HPV-11, one case with HPV-18, one case with HPV-33 and 10 cases with no detectable HPV DNA of the above types) with a negative cytological diagnosis, regression of the disease was detected (Table I, Fig. 2).

Discussion

It has been suggested that HPV testing may usefully augment cytology by helping to decide whether a patient with a mild abnormality needs immediate referral (25), while testing for high-risk HPV types appears to have more clinical utility (12). The sensitivity of the PCR method provides a more accurate frequency of infections.

Formalin-fixed paraffin-embedded tissues, fresh tissues and exfoliated cervical cells recovered from cervical scrapes have been used for HPV DNA detection by PCR (26-28). However, fixed and stained cervical smears already used for cytology, which provide the possibility of retrospective studies with follow-up of the patients as well, have never been used. It is also possible to compare the results of the PCR analysis to cytology within the same sample, while confirmation as well as typing of the virus can provide further support for the treatment selection. Moreover, the ability to use archival cervical smears provides material for the investigation of cervical neoplasia and has important implications even in forensic medicine. The only disadvantage of this technique is that it obviously destroys the cytology specimen.
Table I. HPV typing in stained cervical smears with low grade CIN and status of the disease after a 3-year follow-up of the patients.

<table>
<thead>
<tr>
<th>HPV type</th>
<th>No of patients</th>
<th>Regression</th>
<th>Persistence</th>
<th>Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>HPV-11</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HPV-18</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>HPV-33</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV-16+HPV-11</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None of the above</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>26 (84%)</td>
<td>4 (13%)</td>
<td>1 (6.5%)</td>
</tr>
</tbody>
</table>

Due to the high sensitivity of the PCR technique, relatively low amounts of target DNA are required, which is important in the case of stained cytological material as used in this study. Moreover, the employment of the ‘hot start’ PCR technique provides more specific and efficient amplification from low target copy numbers (29).

In the current study HPV detection was confirmed in 21 out of the 31 cases (68%) examined with a cytological and/or histological diagnosis of HPV and/or CIN I. In ten cases HPV DNA was not detected, probably due to the presence of other type of HPV than 11, 16, 18 and 33, or to low viral copy number. This rate of detection is in accordance with previous studies (30,31) detecting HPV DNA in 64%-75% of CIN I with cytological evidence of HPV infection, pointing to the questionable value of koilocytosis as specific marker of HPV infection, suggesting that HPV analysis is needed to prevent overclassification and overtreatment. Moreover, prevalence of high-risk HPV types (55%) found in this study has also been reported (25).

The present study emphasizes the necessity of confirmation of HPV presence in cytological specimens by a more sensitive and specific molecular technique such as the PCR. The persistence or progression to high grade lesions in patients with mild cytological abnormalities can be predicted by molecular detection of HPV in some cases, particularly when combined with cytological analysis. However, the magnitude of this prediction is dependent on the population of patients studied and the clinical role of this approach therefore remains to be defined.

We intend to extend this pilot study and continue long-term follow-up to evaluate whether this additional test can assist in predicting which women are at greater risk of eventually developing cervical cancer.

References


