Human Papillomavirus-related Serological Markers of Invasive Cervical Carcinoma in Brazil

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Abstract

Masked sera from 194 cases and 217 controls participating in a case-control study of cervical cancer in Brazil were examined for antibodies to human papillomavirus (HPV) E6 and E7 by radioimmunoprecipitation assay. Radiolabeled full-length E6 and E7 proteins expressed by in vitro transcription and translation in rabbit reticulocyte lysate were used as antigens. The antibody prevalences in cases and controls were: 54.1% versus 6% for E6; 30.4% versus 4.6% for E7; 63.4% versus 10.1% for either E6 or E7; and 21.1% versus 0.5% for both E6 and E7. The corresponding odds ratios were 35 (95% confidence interval (CI), 15–83), 10 (95% CI, 4–25), 28 (95% CI, 13–61) and 87 (95% CI, 10–736). The most marked contrast between cases and controls was observed for sera with high antibody titers (cpm > 6000) with an odds ratio of 239 (95% CI, 29–1946) for E6 or E7. Seroreactivity in cases was partially type specific; women who had HPV-16 DNA in the genital tract had higher antibody prevalence rates than those who were negative for HPV DNA. Reactivity to the E6 protein was associated with the stage of disease; the antibody prevalence was 62.7% in cases with stages II–IV and 31.0% in cases with stage I (P < 0.005). HPV-16 serology and HPV polymerase chain reaction were compared as markers for invasive cervical cancer. The sensitivity and specificity estimates were: 63.4% and 89.9% for HPV-16 serology; 53.8% and 94.9% for HPV-16 DNA; 84.4% and 82.7% for any HPV DNA; and 90.7% and 75.5% for a combination of HPV-16 serology and any HPV DNA. The specificity for high E6 or E7 titers was 99.5%. The HPV-16 E6 and E7 serological assays confirm the association of HPV's with cervical cancer previously established by HPV DNA assays. The serological assays may be useful as an adjunct to pathological diagnosis and potentially of value for clinical management of HPV-associated cervical cancers.

Introduction

The predominant role of HPV's in the etiology of invasive cervical carcinoma has been recognized by epidemiological (1) as well as experimental data (2). Nearly all of the epidemiological studies have relied on the detection of HPV DNA in the genital tract as a marker of HPV exposure in their comparisons of case and control women. In most recent studies, 70–90% of women with cervical cancer, but only 5–17% of control women, have been reported to have HPV DNA in the genital tract (1). HPV-16 has been found to be the predominant virus in cervical cancer in most parts of the world.

The prevalence of HPV DNA in the genital tract is indicative of either current virus infection or virus-associated abnormality. In contrast, serological markers may be expected to reflect the entire past history of HPV exposures of the subject and thus provide additional tools for the investigation of cervical cancer. Serological studies of HPV infections have sought to define markers of HPV infection and HPV-associated neoplasia. A wide variety of antigens (bacterially expressed or in vitro translated proteins, synthetic peptides, viral capsids, etc.) have been used for this purpose. For most antigens, the specificity or reproducibility of the observed reactivities have been difficult to establish (3). However, one observation has been consistently confirmed in numerous studies. Antibodies to HPV-16 E7, and to a lesser extent, to HPV-16 E6 are found more frequently in women with HPV-associated cervical cancer than in control women (4–10).

The main objectives of the present study were to assess the relationship of HPV's with cervical cancer in Brazil using HPV-specific serological markers and in addition, to compare serology with the detection of HPV DNA in the genital tract as a marker for invasive cervical cancer. The serological markers were also examined for their correlation with the clinical stage of disease and their HPV type specificity. We prepared E6 and E7 proteins of HPV-16 by in vitro...
transcription and translation, a technique which conserves conformational epitopes and allows for the production of full-length proteins. Antibodies were measured by radioimmunoprecipitation assay of 125I-labeled proteins. All of the case and control sera collected in an epidemiological study of HPV and invasive cervical cancer in Brazil were tested in a blinded fashion.

**Materials and Methods**

**Study Subjects.** The study design was described in detail in a previous report (11). In brief, between June 1990 and June 1991, women with a diagnosis of invasive cervical cancer and women selected as controls were recruited from seven hospitals in São Paulo city. The cases were women between 25 and 79 years of age whose diagnosis was confirmed by histopathology and who had no previous treatment for the disease. Controls were enrolled from five general hospitals from which the cases were recruited. Controls were frequency matched to cases in 5-year age groups. Women who were admitted for treatment of a gynecological condition or who had a hysterectomy or conization were ineligible as controls. Women with a psychiatric illness were ineligible as cases or controls. Evidence of a gynecological or cytological abnormality detected on examination after recruitment was not a criterion for exclusion.

All study participants had a pelvic examination performed by a gynecologist. At that time, exfoliated cells from the ecto- and endocervix were collected for virological analyses. The HPV diagnosis was made, as described, by PCR, using a combination of general and type-specific primers (12). Briefly, the specimens were screened first for the presence of any HPV using general primers 5 and 6 which permit the detection of a broad spectrum of genital HPVs at the subpicogram level (13). The general primer-PCR-positive samples were subjected to HPV type specific PCR using a mixture of HPV-6, HPV-16, HPV-33, HPV-11-, HPV-18-, and HPV-31-specific primers. Nucleotide sequences of primers and probes used, as well as conditions, have been published (14). To analyze the quality of target DNA for PCR purposes, samples were also analyzed by PCR using human β-globin gene-specific primers. Only β-globin-positive samples were included in the analysis.

Serum specimens were collected at the time of the gynecological examination and stored at −20°C.

Two hundred six women with invasive cervical cancer and 238 control women were eligible; 199 and 225 agreed to participate. Reasons for nonparticipation were refusal (3 cases, 11 controls), death (3 cases), and inability to locate (1 case, 2 controls). Five cases and eight controls had no serum collected. The number of participants included in the analyses were 194 cases and 217 controls. The 194 cases were categorized in clinical stages following the Federation Internationale des Gynaecologistes et Obstétristes classification scheme as stage I (n = 42), stage II (n = 64), and stages III–IV (n = 70). Eighteen cases had no stage information.

**Detection of Antibodies to HPV 16 E6 and E7 Proteins by TT-RIPA.** Antibodies were detected by radioimmunoprecipitation of full-length HPV-16 E6 and E7 proteins prepared by in vitro transcription and translation as described previously (15). The specificity of the test has been established using rabbit antiserum to HPV-16 E6 and E7 proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of precipitated proteins (15). Briefly, pGem-1 plasmids containing the E6 or E7 open reading frame of HPV-16 (16, 17) (gifts of Dr. Peter Howley) were linearized downstream of the T7 promoter with appropriate restriction enzymes. E6 and E7 mRNA were transcribed with T7 RNA polymerase in the presence of cap analogue (5‘7-meGpppG) (Stratagene, La Jolla, CA). One μg of RNA was used to program a micrococcal-nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of 40 μCi of [35]S-methionine (Amersham, Arlington Heights, IL). Five μl of serum and 5 μl of rabbit reticulocyte lysate in 200 μl of RIPA buffer (10 mM Tris-HCl, pH 8-140 mM NaCl-2.5 mg/100 ml NaN3-0.1% Nonidet P-40) were incubated for 3 h at 4°C. Protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO) were added, and the reaction was further incubated for 12 h at 4°C. The beads were washed twice each with 500 μl of RIPA buffer, TSA solution (RIPA buffer without Nonidet P-40), and 10 mM Tris-HCl, pH 6.8. The beads were then resuspended in 100 μl of 60 mM Tris-HCl (pH 6.8), 2 g/100 ml sodium dodecyl sulfate, and 100 μl dithiothreitol, boiled for 5 min, and quickly chilled on ice. The supernatant was transferred to a scintillation vial containing 10 ml of Aquasol-2 (Du Pont New England Nuclear, Boston, MA), and cpm were measured in a liquid scintillation counter.

**Statistical Analysis.** The distributions of cpm in cases and controls were compared using the Mann-Whitney test (18). In addition, the sera were categorized as antibody positive or negative, using as cutoff values the mean plus three SDs of the control sera after the outliers in the control sera were excluded (6). Frequencies of antibody-positive sera in different groups were compared by the χ2 test (18). To estimate the association between a positive serological test and cervical cancer, ORs and 95% CIs were calculated as approximations of relative risks using unconditional logistic regression analysis (19). ORs were estimated after adjustment for age, as well as for other variables (socioeconomic status, number of Papanicolaou smear examinations, parity, number of sexual partners, age at first intercourse, and duration of oral contraceptive use) which have been previously found to be potential confounders of the association between HPV and cervical cancer (20). The correlation between E6 and E7 antibodies was expressed as a Pearson correlation coefficient. A test for trend was performed where necessary using the method of Mantel (18). The sensitivity and specificity of the serological and DNA assays for invasive cervical cancer were calculated using the pathological diagnosis as the gold standard.

**Results**

**Antibodies to HPV-16 E6 and E7 Proteins in Cases and Controls.** The distributions of cpm values among cases and controls for HPV-16 E6 and E7 are shown in Fig. 1, A and B. The cpm values of case sera against E6 and E7 were significantly higher than those of control sera (P = 0.0001 for both E6 and E7). High cpm values (>6000) were found almost exclusively in case sera, in 33.5% of the cases and none of the controls for E6, and in 23.7% of the cases and 0.5% of the controls for E7 (P < 0.0001 for both E6 and E7). The cutoff cpm values for categorizing sera as antibody positive were calculated to be 2218 for E6 and 2461 for E7. Table 1 shows the antibody prevalence in cases and controls to E6, E7, and various combinations of E6 and E7, and the corresponding ORs for cervical cancer in relation to a positive serological test. The antibody prevalence was sig-
Correlation between Antibodies to HPV-16 E6 and E7.

Among cases, antibody prevalences to E6 and E7 were high but they showed no relationship with any of the above risk factors. Among controls, antibody prevalences to both proteins were low. Antibodies to E7, but not to E6, decreased with age ($P < 0.005$; Table 2). Antibody prevalence to the E7 protein increased with the number of sexual partners but the trend was not statistically significant.

No clear relationship was observed between antibody prevalence in the controls and age at first intercourse, use of oral contraceptives, smoking, or parity (data not shown).

Relationship between Antibodies to HPV-16 E6 and E7 and HPV DNA Diagnoses. It was of interest to know if antibodies to HPV-16 E6 and E7 proteins were specific for infection with HPV-16. Therefore, distributions of cpm values and antibody prevalence were examined by three HPV diagnostic categories: HPV-16 positive; other HPV positive; and HPV negative (Fig. 2, A and B). For E6, the cpm values of the HPV-16-positive cases were significantly higher than that of other HPV-positive ($P = 0.001$) as well as HPV-negative cases ($P = 0.004$). For E7, the cpm values of HPV-16-positive cases were significantly higher than that of HPV-negative cases ($P = 0.025$) but not of other HPV-positive cases ($P = 0.19$). Table 2 shows the prevalences of antibodies to E6 and E7 in case sera by HPV diagnosis. Analysis of frequencies revealed significant differences between the diagnostic categories in reactivity to E7, E6 and E7, and E6 or E7 ($P < 0.01$ for all comparisons), but not in reactivity to E6 ($P = 0.103$). The greatest difference between the diagnostic categories was observed for sera with a high titer of antibody to E6 ($P < 0.0001$). For each protein and combination of proteins, the highest seroreactivity was observed among HPV-16-positive cases, and the reactivity of other HPV-positive and HPV-negative cases were not significantly different.

Among controls, antibodies to either E6 or E7 were observed in 1 of 10 subjects who were HPV-16 positive, in 3 of 22 subjects who were positive for other HPV, and in 15 of 160 subjects who were HPV negative. The antibody frequencies between diagnostic categories among controls were not significantly different.

Relationship between E6 and E7 Antibodies and Clinical Stages of Cervical Cancer. The correlation between seroreactivity of cervical cancer patients to HPV-16 E6 and E7 protein and clinical stage of disease was examined. Fig. 3, A and B show the distributions of cpm values of case sera for E6 and E7 by clinical stages (stage I, stage II, and stages III–IV). The cpm values of E6 in more advanced stages were significantly higher than that in stage I ($P = 0.01$ for stage I versus stage II; $P = 0.005$ for stage I versus stages III–IV), but there was no significant difference in the distributions of cpm values between stage II and stages III–IV ($P = 0.61$). In contrast, the distributions of cpm values of E7 did not show any significant difference between clinical stages. Table 4 shows the prevalence of antibodies to HPV-16 E6 and E7.
Table 2  Prevalence of antibodies to HPV-16 E6 and E7 among cases and controls by age and number of sexual partners

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Cases n = 194</th>
<th>Controls n = 217</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>E6 (+)*</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>12</td>
<td>66.7</td>
</tr>
<tr>
<td>35-44</td>
<td>43</td>
<td>46.5</td>
</tr>
<tr>
<td>45-54</td>
<td>60</td>
<td>55.0</td>
</tr>
<tr>
<td>55-64</td>
<td>46</td>
<td>54.3</td>
</tr>
<tr>
<td>65+</td>
<td>33</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Number of sexual partners</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>14.3</td>
</tr>
<tr>
<td>1</td>
<td>103</td>
<td>50.5</td>
</tr>
<tr>
<td>2-3</td>
<td>61</td>
<td>59.0</td>
</tr>
<tr>
<td>4+</td>
<td>30</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>$P$ (χ² for trend)</td>
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</tbody>
</table>

Table 3  Antibody prevalence (%) in case sera by HPV DNA diagnoses

<table>
<thead>
<tr>
<th></th>
<th>HPV-16 n = 96</th>
<th>Other HPVb n = 57</th>
<th>No HPVc n = 29</th>
<th>P</th>
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<tbody>
<tr>
<td>E6</td>
<td>61.5</td>
<td>49.1</td>
<td>41.4</td>
<td>0.103</td>
</tr>
<tr>
<td>E7</td>
<td>42.7</td>
<td>21.1</td>
<td>13.8</td>
<td>0.002</td>
</tr>
<tr>
<td>E6 or E7</td>
<td>72.9</td>
<td>59.6</td>
<td>41.4</td>
<td>0.006</td>
</tr>
<tr>
<td>E6 and E7</td>
<td>31.3</td>
<td>10.5</td>
<td>13.8</td>
<td>0.006</td>
</tr>
<tr>
<td>E6 cpm &gt; 6000</td>
<td>51.0</td>
<td>19.3</td>
<td>10.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E7 cpm &gt; 6000</td>
<td>35.4</td>
<td>12.3</td>
<td>13.8</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*12 cases that were β-globulin negative were excluded from this analysis.

bOther HPV, HPV-18 (n = 16); HPV-31 (n = 2); HPV-33 (n = 2); HPV-31/33 (n = 2); and HPV unknown (n = 35).

Fig. 2.  A, distribution of cpm values for seroreactivity to E6 among case sera by HPV DNA diagnosis. Horizontal line, cutoff value (2218) for positivity. B, distribution of cpm values for seroreactivity to E7 among case sera by HPV DNA diagnosis. Horizontal line, cutoff value (2461) for positivity.

and of high titers of antibodies to E6 and E7 in case sera by clinical stage of disease. Prevalence of antibodies to E6 in more advanced stages was significantly higher than that in stage I (stage I, 31.0%; stage II, 62.5%; and stages III–IV, 62.9%; χ² for trend = 9.06; P < 0.005), but it did not differ between stage II and stages III–IV (P = 0.996). High cpm values to E6 were found more frequently in advanced stages of disease (stage I, 19.0%; stage II, 35.9%; and stages III–IV, 42.9%; χ² for trend = 6.17; P < 0.02). For E7, neither the prevalence of antibodies nor the magnitude of cpm values showed any association with clinical stages.

The above analyses were repeated for the 88 cases which had HPV-16 DNA in the genital tract. The results were similar to those obtained with all 176 cases and showed that antibodies to E6, but not to E7, increased in the advanced stage of disease (data not shown).

Comparison of Antibodies to HPV-16 E6 and E7 with HPV DNA Diagnosis as Markers of Cervical Cancer. HPV DNA diagnoses of genital tract specimens were available for the study subjects. Therefore, it was possible to compare HPV-16 E6 and E7 antibodies in the serum with HPV-16 DNA or any HPV DNA in the genital tract as markers for invasive cervical cancer. Both serology and HPV DNA diagnosis showed a significant association between a positive test and invasive cervical cancer (OR = 28 for antibodies to E6 or E7, 71 for HPV-16 DNA, and 33 for any HPV DNA) (11). Assuming that all cervical cancer cases are associated with HPV, the assays were evaluated as diagnostic tests for invasive cancer (Table 5). The HPV-16 serological assay detected more cases than HPV-16 DNA diagnosis but fewer than the generic HPV DNA diagnosis. The combination of serology and DNA diagnosis resulted in the detection of HPV-associated cervical cancer in more cases (91%) than HPV DNA diagnosis alone (84%). HPV-16 DNA assay had the highest specificity (94.9%), followed by HPV-16 serology (89.9%), and any HPV DNA assay (82.7%). HPV-16 serology for high antibody titers to E6 or E7 had a sensitivity of 40.7% and a specificity of 99.5%.
The E6 and E7 proteins of HPVs are responsible for the transforming activities of oncogenic HPVs, and they are expressed consistently in cells of HPV-associated cancers (16, 17, 21–26). They are also expressed in the course of transient cytopathic HPV infections which do not lead to malignancy. Previous investigations have shown that antibodies to E6 and E7 proteins are found in small proportions of normal controls and cervical intraepithelial neoplasia grade III cases, and that the prevalences and titers of these antibodies increase in women who have invasive cervical carcinoma (4–8, 10, 27, 28).

The major aims of our study were to assess the relationship of HPVs with cervical cancer using serological markers and to evaluate antibodies to E6 and E7 proteins as markers for invasive cervical cancer. We tested all of the sera collected in a case-control study of invasive cervical cancer in Brazil for reactivity to in vitro translated HPV-16 E6 and E7 proteins. We chose E6 and E7 proteins of HPV-16 because HPV-16 was the predominant type in cervical cancers in the Brazil study and accounted for 54% of the cases (11). The serum reactivities of case and control sera were analyzed in two ways: (a) by a comparison of the distributions of the cpm values; and (b) by a comparison of the antibody prevalence rates calculated on the basis of appropriate cutoff values. The results of these two analyses were consistent with one another and showed marked differences between cases and controls. The ORs for the association of cervical cancer seroreactivity to E6, E7, either E6 or E7, and both E6 and E7 ranged from 10 to 87. The differences between cases and controls were most impressive where the frequencies of sera with high cpm values were compared; 40.7% of case sera, as compared to 0.5% of control sera, had high titers to either protein, giving an OR of 239 (95% CI, 29–1946). These findings support and confirm the association of HPV with invasive cervical cancer that was previously established by case-control studies using HPV DNA diagnosis.

Higher prevalences of E6 and E7 antibodies in invasive cervical cancer cases as compared to controls have been observed in studies which used a variety of E6 and E7 immunological reagents. However, as compared to other reagents, in vitro translated proteins showed a greater ability to distinguish between cases and controls. The difference between reagents was best illustrated by tests with the E6 protein. In our previous study with E6 peptides, the antibody prevalences in cases and controls were 16 and 4%, respectively (6). In a recent report, reactivity to bacterially expressed E6 proteins was observed in 33% of the sera from cervical cancer patients and 3% of control sera (10). In comparison, the reactivity to E6 in TT-RIPA was 54% among cases (62% among HPV-16 DNA-positive cases) and 6% among controls in the present study, and 56% among cases of HPV-16-associated cervical cancers and 1.7% among controls in Colombia and Spain (15). The very likely reason for the greater reactivity of sera in TT-RIPA is the potential of the assay to measure antibodies to conformational epitopes. The high prevalence of antibody to E6 protein indicates that the protein is expressed and is seen by the immune system of cancer patients. The immunogenicity of the HPV-16 E6 protein in cancer patients has implications for vaccine development and suggests that current
efforts to develop E7-based vaccines should be extended to include the E6 protein.

Among controls, the antibody prevalence to E6 and E7 was low and the positive sera had low titers. These reactivities are very likely the result of past infections with HPV-16 or related viruses and should therefore be more frequently found in women who are at increased risk for genital HPV infections. Antibody prevalence to E7 increased with the number of sexual partners but the trend was not statistically significant. The low antibody prevalence may have reduced the ability to detect associations. The decrease in E7 antibody prevalence by increase in age might suggest that antibody titers decline with age. Since our controls were hospitalized patients, it is possible that the findings do not apply to the general population. It will be possible to evaluate the specificity of the low levels of E6 and E7 antibodies by examining if these specimens are also reactive with HPV-16 capsids. A serological assay with HPV-16 capsids, which has been described recently (29), appears to be a marker for past infections.

There was a degree of type specificity in the E6 and E7 antibody response when it was examined by comparisons of the response in cases in three diagnostic categories: those who had HPV-16 DNA in the genital tract; those who had other HPV DNA; and those who had no HPV DNA in the genital tract. The type specificity of the E7 response was evident both in a comparison of antibody prevalence rates (P = 0.002) and the prevalence of sera with high titers (P = 0.002). With respect to E6, significant differences in antibody prevalence rates in the three diagnostic categories were found only in sera with high titers (P < 0.0001). It is possible that sera with high titers to E6 and E7 in case women who were diagnosed as HPV negative (for example, E7 values in Fig. 2B) may represent a degree of type specificity in the E6 and E7 antibody responses for invasive cancer suggests that the assays may be useful in epidemiological studies examining the role of HPVs in other cancer, such as vulvar, penile, and oral carcinomas. The serological tests should also be evaluated as prognostic indicators and for monitoring of response to treatment.

Antibodies to E6 appeared to be a marker for an advanced stage of disease. The prevalence as well as titers of antibodies to E6 increased significantly in the higher grades of disease. These results suggest that antibodies to HPV-16 E6 might be a marker of invasiveness or tumor mass. It is possible that E6 antibody assays would be useful as a prognostic marker and for monitoring response to treatment in women with invasive cancer, but this remains to be evaluated in prospective studies. Antibodies to E7 were not related to disease stage in our study.

For epidemiological and clinical studies of HPV-associated invasive cervical cancer, a test with a high degree of sensitivity and specificity for HPV and invasive cancer may have some utility as an adjunct to pathology for the diagnosis of cancer, and as a means for determining the proportion of cancers associated with HPV. In our study, we had the opportunity to compare HPV serology and HPV PCR for their correlation with invasive cervical cancer. HPV-16 serology was more sensitive than the HPV-16 DNA assay, probably because serology, but not the DNA diagnosis, identifies infection with closely related viruses. The 54% of cases that were positive by HPV-16 DNA PCR probably represent all, or nearly all, of the cancers that are HPV-16 associated. As compared to PCR using a generic probe, HPV-16 serology was less sensitive, probably because serology cannot identify infections with distantly related oncogenic viruses. The most sensitive measurement was a combination of DNA diagnosis and HPV-16 serology. The sensitivity may increase if serological assays using E6 and E7 proteins of other oncogenic HPVs were used. The specificity of the various assays ranged from 95% for HPV-16 DNA to 76% for the combination of any HPV DNA and HPV-16 serology. The presence of HPV DNA in a small proportion of controls is not unexpected and probably represents women with active or chronic HPV infection. The low prevalence of antibody in controls most likely results from past infection with HPV-16 or related viruses.

Serology for HPV-transforming proteins, in conjunction with HPV DNA diagnosis, appears to be a promising strategy to identify HPV-associated invasive cancers. Further studies should clarify the type specificity of the antibody responses and establish more precisely the relative performance of serology and DNA diagnosis for the detection of invasive cervical cancer. The 99.5% specificity of high titers of E6 or E7 antibody responses for invasive cancer suggests that the assays may be useful in epidemiological studies examining the role of HPVs in other cancer, such as vulvar, penile, and oral carcinomas. The serological tests should also be evaluated as prognostic indicators and for monitoring of response to treatment.

References


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