Human Papillomaviruses and Cervical Neoplasia in South Carolina

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Abstract

Human papillomaviruses (HPVs), particularly types 16, 18, and 33, have recently been suggested as etiological agents for cervical neoplasia. However, few studies have explored this relationship among low-income, minority women. This case-control study of cervical intraepithelial neoplasia (CIN), detected by Pap smear screening among South Carolina women, investigates the association between HPV positivity and the cytological continuum of CIN. Cervical spatulas and cytobrushes used to collect Pap smears from all women attending health department family planning clinics in three coastal South Carolina counties were saved for subsequent HPV detection and typing. Among this cohort of approximately 6000 cervical samples collected from March through December 1991, those with CIN, atypia, and other cervical abnormalities and women with normal cervical cytology were identified. Women with CIN II or III (n = 28) were 21.9 times more likely to be HPV 16, 18, or 33 positive, while women with CIN I (n = 114) were 11.7 times more likely to be HPV 16/18/33 positive when compared with women having normal cervical cytology (n = 223) and adjusting for potential confounders. Women with atypia (n = 115) were 3.0 times more likely to be HPV 16/18/33 positive. A $\chi^2$ test for trend in increasing HPV 16/18/33 prevalence with increasing severity of cervical lesions was highly significant ($P = 0.0001$). HPV 6 and 11 were not associated with CIN, nor was there a significant trend of increasing prevalence with increasing severity of cervical lesions. Worthy of further research is our finding that the overall prevalence of HPV positivity was low in this relatively high-risk population of low-income, primarily black women.

Introduction

Specific HPVs have been linked to the development of invasive cervical cancer and with the precursor lesions termed cervical intraepithelial neoplasia (1-18). HPV 6 and 11 are causally linked to venereal warts or condylomas and are not thought to be linked to future development of cervical neoplasia, while HPV types 16, 18, 31, and 33 are linked to the development of the more severe lesions (CIN II or CIN III and invasive cervical cancer) (19-20). The presence of HPV 16/18/31/33 alone may not be sufficient to cause cervical cancer. Additional cofactors including cigarette smoking, diet, multiple sex partners, oral contraceptive use, or infection with herpes simplex virus type 2 may also be necessary for the development of cervical cancer (21-25).

Accurate and reliable diagnosis of HPV infection has long been problematic. The currently regarded "gold standard" for HPV DNA typing is the Southern blot method (19), yet due to its cost and labor intensity, few epidemiological studies have used this technique. Conversely, the PCR technique has been shown to be a sensitive and reliable method for HPV typing and may soon replace Southern blot as the gold standard for HPV typing (19). This case-control study used PCR to detect specific HPV types in cervical smear samples.

Although low-income and minority women are at increased risk for cervical neoplasia, few studies have focused on the relationship between HPV positivity and cervical neoplasia in a higher-risk population of this type. This study, based in the coastal region of South Carolina, explored this relationship in a low-income, primarily black population. The research focused on the continuum of CIN in an attempt to temporally link the presence of HPV 16/18/33 to cervical changes prior to the development of cervical cancer. Three specific research questions have been addressed: (a) Are low-income minority women in South Carolina with CIN more likely to have HPV 16/18/33 compared with women from the same population who have normal cervical cytology? (b) Does the prevalence of HPV positivity increase with increasing severity of cervical lesions? and (c) Is the prevalence of HPV positivity greater in this high-risk population than was reported from previous studies in lower-risk populations?

1 The abbreviations used are: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; PCR, polymerase chain reaction; ORF, open reading frame; OR, odds ratio; OC, oral contraceptive; STD, sexually transmitted disease.

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Materials and Methods

Subjects. Between March and December 1991, a cervical cell sample was collected from all women receiving a Pap smear as part of their family planning visit. All women included in this study were patients attending one of 11 clinics in the Trident Health District served by the South Carolina Department of Health and Environmental Control. This population is particularly appropriate for studying the role of HPV in cervical neoplasia because all of the women are sexually active and, thus, at risk for developing cervical cancer. Table 1 shows the demographic profile of clinic clients. Women attending health department clinics are primarily low-income and 60% are black. The mean age of the population included in this study was 23.5 years.

Collection of the Cervical Cell Sample for Cytological Diagnosis and HPV Typing. At each of 11 family planning clinics in the Trident Health District, the nurse practitioner placed all spatulas and cytobrushes used to collect and prepare each Pap smear in a tube containing 5 ml of DNA extraction buffer. The tubes containing the spatulas and cytobrushes used to collect the Pap smear will be referred to as the HPV samples. Genomic DNA was isolated from the cervical cells remaining on the spatulas and used for HPV detection and typing by PCR.

After the Pap smear and the HPV sample were collected and the Pap smear request form was completed, these were sent to the Cytology Section of the South Carolina Department of Health and Environmental Control Bureau of Laboratories in Columbia. At this laboratory, a cytological diagnosis was made of the Pap smear, and these results were provided to study researchers. A unique study number was assigned to each HPV sample, and the samples were taken to Dr. Pinisi’s laboratory for DNA extraction and HPV analysis. In the HPV analysis laboratory samples were identified solely by their study number.

Disease misclassification was limited by having the same cytology laboratory interpret all Pap smears in the cohort. This limits the potential for the introduction of error when different criteria for cytological diagnosis from different laboratories are applied. Furthermore, the same pathologist reviewed all abnormal smears as well as a 10% sample of all normal smears; therefore, the same pathologist’s abnormal cytological criteria were applied to all smears in this cohort.

A total of 5995 Pap smears and cervical samples were collected. Within this cohort of family planning patients receiving Pap smears, 3141 (52.4%) had normal cytology, 44 were found to have either CIN II or CIN III (0.7%), 179 had CIN I (3.0%), 1211 had cervical atypia only (20.2%), and 1420 (23.7%) had cervical abnormalities not included in the previously listed mutually exclusive categories; these include those with inflammatory cellular changes only and those with cytological evidence of trichomonas, herpes, HPV cellular changes (koilocytosis), or chlamydia.

Detection of Human Papillomavirus DNA Types in Cervical Smear Samples. The PCR technique used for HPV detection and typing was that described by Pao et al. (26) with some modifications. This method is based on the amplification of a segment of the E6 ORF and is, therefore, highly specific for individual HPV types. Primers were designed to amplify a segment of DNA of different size in each HPV type (26), so that identification of specific HPV types can be based on the size of the amplification products obtained, resolved by electrophoresis on a 2% agarose gel, and stained with ethidium bromide.

Positive control reactions used plasmid DNA templates containing the entire genome of HPV types 6b, 11, 16, 18, and 33. Negative controls (set up last, using the same batch of reagents used for the samples) contained calf thymus DNA as a template. In addition, an amplification reaction was carried out each time with a genomic DNA sample that had previously proved positive for an HPV, as an additional positive control. Each reaction mix contained, in addition to the HPV-specific primers, a pair of primers designed to amplify a segment of the β-lactamase gene (present in all plasmids used in the laboratory), to exclude contamination of cervical DNA samples by plasmid DNA from the laboratory. Finally, in a separate tube, an amplification reaction was carried out in parallel, for each sample, using exactly the same amount of template DNA used in the HPV amplification reaction, primers specific for the β-globin gene, and the same batch of reagents used in the HPV analysis reactions, to ensure that adequate amounts of genomic DNA were present in each reaction and that no contaminants able to inhibit PCR amplification were present in the samples. To avoid cross-contamination of human genomic DNA samples, all reagents were aliquoted in single-use aliquots, and DNA templates were handled using aerosol-resistant pipet tips or positive displacement pipets (Rainin).

HPV typing was performed on a randomly selected subset of samples: 68% of those with CIN (n = 151); 10% of those with atypia only (n = 121); 10% of those with inflammation or infection only (n = 155); and 8% of those with normal cervical cytology (n = 242).

HPV typing results were inconclusive for 7.3% of the 669 samples for which typing was performed. The majority (47 of 49) were inconclusive because the β-globin gene could not be amplified in the sample, which is perhaps indicative of not having recovered sufficient DNA to determine HPV presence. In only two samples was β-lactamase identified, which indicates a contamination at some point in the procedure. These samples also were labeled as inconclusive and were not included in this study.

HPV positivity was determined as either positive or negative for HPV 6/11 and, again, for HPV 16/18/33. HPV typing of all study subjects was performed and assessed...
by one reviewer. Only those samples that unequivocally produced a definite band of the expected size for a given HPV subtype were termed positive; no category including questionable HPV positivity was created.

**Information on Risk Factors for CIN: The Pap Smear Request Form.** A Pap smear request form accompanied each Pap smear and HPV sample collected. This form included information on additional risk factors for cervical neoplasia. The nurse practitioner collecting each Pap smear completed this form by reviewing the patient’s chart and/or directly asking the patient for the information requested on the form. The information obtained from this form included the patient’s age; race; parity; gravidity; number of induced abortions; current status as pregnant, postpartum, nursing, or postabortion; current oral contraceptive or intrauterine device use; current smoking status; history of any sexually transmitted disease, cervicitis, cryosurgery, conization, radiation therapy, or chemotherapy; and history of high-risk sexual behavior (i.e., early age at first sexual intercourse of multiple sex partners). To assess the patient’s risk profile, the nurses were requested to check all categories that applied. In addition, nurses were asked to record any previous abnormal Pap smears and the dates of these smears.

**Statistical Analysis.** The odds ratio and its 95% confidence interval were used to estimate the association between HPV exposure and the disease continuum. Multiple logistic regression was used to adjust for potential confounding variables in deriving the maximal likelihood estimates of combined odds ratios and 95% confidence intervals. Known risk factors for cervical neoplasia (27–28) which were also found to be associated with HPV or CIN were included in the logistic regression models as confounders. The following confounders were included in the final logistic regression models estimating the adjusted odds ratio for HPV positivity and the CIN continuum: age; race; current smoking and OC use; high-risk sexual behavior; and history of any STD. Age was dichotomized around age 25 because the average age of patients in this study was 23 and because being younger than 25 was correlated with HPV positivity in earlier unpublished work.

**Results**

**Risk Factors for HPV Positivity.** Table 2 shows the crude odds ratios and their 95% confidence intervals for the relationship between HPV positivity (either HPV 6/11 or HPV 16/18/33 positivity) and selected risk factors for CIN. HPV positivity was significantly associated only with being younger than 25 years of age (OR = 2.0). HPV positivity was not significantly associated with race, current cigarette smoking status, current oral contraceptive use, high-risk sexual behavior, having a history of any STD, or having ever been pregnant.

**Risk Factors for CIN.** Table 3 shows the crude odds ratios for the relationship between CIN (I–III) and the same selected risk factors explored in Table 2. Women diagnosed with CIN are compared with women with normal cervical cytology. In summary, the following risk factors were correlated with CIN: age less than 25 years (OR = 1.6), current OC use (OR = 1.5), and having a history of any STD (OR = 2.2).

**HPV Positivity across the CIN Continuum.** Tables 4 and 5 show the distribution of HPV positivity among subjects across the CIN continuum. HPV positivity was defined in two ways: (a) presence of HPV 16/18/33 (Table 4) and (b) presence of HPV 6/11 (Table 5).

From the literature, we anticipated an increased prevalence of HPV positivity with increasing severity of cervical lesions. We further anticipated a stronger relationship between cervical abnormalities and HPV 16/18/33 than with HPV 6/11.

From Table 4, the prevalence of HPV positivity significantly increased with increasing severity of cervical lesions; after adjusting for confounders, the χ² test for trend was 40.5 (P = 0.0001). After adjusting for the confounders (age, race, smoking status, high-risk sexual behavior, and OC use), women with CIN II/III were 21.9 times more likely to be HPV 16/18/33-positive, and women with CIN I were 11.7 times more likely to be HPV 16/18/33-positive, when compared with women having normal cervical cytology. Although not statistically significant at the P = 0.05 level, women with atypia (adjusted OR = 3.0) and those with inflammation or infections only (adjusted OR = 2.6) were more likely to be HPV 16/18/33-positive when compared with women with normal cervical cytology.

In contrast with the strong association observed for HPV 16/18/33 and the CIN continuum, HPV 6 and 11 were not associated with the CIN continuum. No trend of increasing HPV 6/11 prevalence with increasing severity of cervical lesions was observed. The prevalence of HPV 6/11 positivity varied little across the CIN continuum; 2.6% of those with CIN I were positive, compared with 3.5% of those with atypia and 1.8% of those with normal cervical cytology. The low prevalence of HPV 6/11 detection in this population clearly limits the power of this analysis, as illustrated by the very wide confidence intervals.
Human Papillomaviruses and Cervical Neoplasia

Atypia

CIN I

Infection or inflammation

Normal cervical cytology

Normal cervical cytology

Human Papillomaviruses and Cervical Neoplasia

Atypia

CIN I

Infection or inflammation

Normal cervical cytology

Table 4 Odds ratios for HPV 16/18/33 positivity and the CIN continuum

<table>
<thead>
<tr>
<th>Cervical cytology</th>
<th>n</th>
<th>HPV 16/18/33-positive</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN II/III</td>
<td>28</td>
<td>35.7%</td>
<td>19.5 (6.4-59.9)</td>
<td>21.9 (6.4-74.5)</td>
</tr>
<tr>
<td>CIN I</td>
<td>114</td>
<td>24.5%</td>
<td>11.7 (4.7-29.1)</td>
<td>11.7 (4.3-32.0)</td>
</tr>
<tr>
<td>Atypia</td>
<td>115</td>
<td>6.1%</td>
<td>2.3 (0.8-7.1)</td>
<td>3.0 (0.9-9.8)</td>
</tr>
<tr>
<td>Infection or inflammation</td>
<td>140</td>
<td>10.7%</td>
<td>2.4 (0.8-6.9)</td>
<td>2.6 (0.8-8.4)</td>
</tr>
<tr>
<td>Normal cervical cytology</td>
<td>223</td>
<td>2.7%</td>
<td>1.0 REF</td>
<td>1.0 REF</td>
</tr>
</tbody>
</table>

χ² test for trend

50.6, P = 0.0001

4 REF, referent group.

Discussion

Our results are consistent with those observed in other studies exploring the relationship between HPV positivity and the CIN continuum in different populations (12-16, 23, 29-31). Among low-income, primarily black women in South Carolina, those with CIN II/III were 20 times more likely to be HPV 16/18/33-positive and those with CIN I were 12 times more likely to be HPV 16/18/33-positive when compared with those with normal cervical cytology and adjusting for confounders. A significant trend toward increasing HPV prevalence with increasing severity of cervical lesions was noted for HPV types 16/18/33 only. HPV 6/11 positivity was not significantly associated with the CIN continuum.

In a study comparable with this investigation, which used Southern blot and not PCR to detect HPV, Negriini et al. (23) found that among low-income, primarily black (77%) women residing in the Washington, DC area, 13.3% of 15 women with CIN II/III were HPV 16/18-positive compared with 6.1% of 165 women with CIN I and 1.4% of 281 with normal cytology. The crude odds ratio for HPV 16/18 positivity was 10.4 for women with CIN II/III and 7.3 for women with CIN I, when compared with women with normal cervical cytology. Van Den Brule et al. (29) used PCR to determine HPV positivity and found that 21.5% of 177 Dutch gynecology outpatients with histories of CIN were HPV 16/18/31/33-positive compared with 3.5% of 1346 women with normal cervical cytology. The crude odds ratios for the relationship between HPV 16/18/31/33 and these two groups of women were 7.6. These results were quite comparable to ours in both the magnitude of the odds ratio and the prevalence of HPV positivity. Bauer et al. (30) used consensus primers to detect HPV by PCR among UC-Berkeley student health patients and found that 92% of 12 women with atypia or dysplasia were HPV-positive and 31% of 421 women with normal cytology were HPV-positive; the crude odds ratio was 24.6.

Interestingly, the prevalence of HPV positivity (either HPV 16/18/33 or HPV 6/11) found when we used PCR in this population of young, low-income, sexually active family planning patients was lower than the published reports noted above (23, 30-31). Hallam et al. (31) estimated that the prevalence of HPV positivity among family planning patients in England was 53% when PCR was used to detect HPV 16/18/31. The PCR-based HPV 16/18/31/33 prevalence rates noted by Van Den Brule (29) among Dutch women with normal cytology (3.5%), however, were comparable to our results (4% HPV 6/11/16/18/33-positive in women with normal cervical cytology).

Possible explanations for our finding a lower HPV prevalence in this South Carolina population include: (a) Our method of HPV typing used primers targeted to a different region of the HPV genome (the E6 ORF) while other researchers (specifically Bauer et al.) used primers from another region (the L1 ORF). When using our PCR technique, we specifically detected only HPV types 6, 11, 16, 18, and 33. Conversely, those using consensus primers targeted to L1 detect a range of at least 17 different HPV types. Thus the difference in HPV positivity may be due to our system, which does not detect all of the HPV types others have included when conducting HPV typing. (b) Our study of clients attending state health department family planning clinics includes a population very different from that explored by others. Bauer et al. (30) assessed HPV positivity in a California college population, whereas our South Carolina population included women of a lower socioeconomic status, lower educational attainment, and different rural versus urban distribution. These differences may explain the discrepancy in HPV prevalence.

These results are not completely generalizable to previously reported studies of invasive cervical cancer (17-18) since we limited our investigation to cytological CIN findings. However, if one assumes that CIN and invasive cervical cancer are part of the same disease

Table 5 Odds ratios for HPV 6/11 positivity and the CIN continuum

<table>
<thead>
<tr>
<th>Cervical cytology</th>
<th>n</th>
<th>HPV 6/11-positive</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN II/III</td>
<td>28</td>
<td>0.0%</td>
<td>1.8 (0.5-7.5)</td>
<td>2.9 (0.5-18.3)</td>
</tr>
<tr>
<td>CIN I</td>
<td>114</td>
<td>2.6%</td>
<td>2.0 (1.5-8.6)</td>
<td>4.7 (0.8-26.6)</td>
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<td>115</td>
<td>3.5%</td>
<td>2.0 (1.5-8.6)</td>
<td>4.7 (0.8-26.6)</td>
</tr>
<tr>
<td>Infection or inflammation</td>
<td>140</td>
<td>2.1%</td>
<td>1.2 (1.3-5.3)</td>
<td>2.5 (0.4-15.5)</td>
</tr>
<tr>
<td>Normal cervical cytology</td>
<td>223</td>
<td>1.8%</td>
<td>1.0 REF</td>
<td>1.0 REF</td>
</tr>
</tbody>
</table>

χ² test for trend

1.24, P = 0.871

4 REF, referent group.
continuum, studying the relationship between preinvasive cervical neoplasia and HPV offers an important methodological advantage. Because case-control studies cannot establish temporality (that HPV precedes invasive disease), focusing on preinvasive neoplasia helps to ensure that this prevalence measure of HPV presence does indeed precede invasive disease.

Unfortunately, we did not have ready access to histological cervical biopsy findings which could verify the exact CIN grade of cases identified cytologically as having CIN. However, this misclassification of disease status affects only the case groups and results in misclassification of the exact grades within CIN. The result of this misclassification would be, most probably, a biasing of the odds ratios for specific CIN levels and HPV toward the null. However, the magnitude of each odds ratio by CIN level (Table 4) clearly indicates a strong association between HPV 16/18/33 and CIN despite this potential methodological advantage. Because case-control studies cannot establish temporality (that HPV precedes invasive disease). We anticipate developing a prospective cohort study to follow that this prevalence measure of HPV presence does indeed precede invasive disease.

Given the inability of case-control studies to establish that HPV positivity precedes CIN development, we anticipate developing a prospective cohort study to follow, by HPV positivity, women with no CIN to subsequent development of CIN. We anticipate also evaluating the role of other cofactors (e.g., age, cigarette smoking status, contraceptive use) in CIN development. In a feasibility assessment, we found that 65% of women included in this case-control analysis have subsequently returned to these same clinics for annual or repeat Pap smears. Thus, such a follow-up study is feasible in this population.

The following measures were taken to reduce potential biases. Women who had been previously treated for CIN were not eligible. Controls had normal cervical cytology. Since the HPV DNA type was unknown at the time of study enrollment there was no way to selectively enroll a higher percentage of cases with virus than without, and thus overt selection bias was not a problem in this study.

Pap smear request form data are subject to misclassification because in all cases information was not directly asked of each subject. Nurses who completed the request form either reviewed the chart to obtain risk factor information or presumably directly asked the patients for this information if the information did not appear in the chart. We have no way of knowing how this information was obtained for a given patient. We do not anticipate, however, that misclassification of information gained from the request form would be differentially misclassified since neither the HPV type nor the cervical cytology was known when this information was provided on the request form. Furthermore, information provided on the request form appears to be consistent with that obtained in personal interviews from a small study piloting the use of an interviewer-administered questionnaire in the homes of these same family planning clients. The prevalence of current smoking (25%), current OC use (70%), and having even been pregnant (63.6%) were comparable with the Pap smear request data.

South Carolina leads the nation in cervical cancer incidence (32) and mortality rates (33). Our finding of a relatively low prevalence of HPV, which is thought to be etiologically linked with cervical neoplasia, warrants further investigation into the reasons for this observed lower HPV prevalence in a population (low-income, sexually active, and minority) which is at increased risk for cervical neoplasia.

In the future, we will explore possible reasons for the lower HPV prevalence in this higher-risk population by reassessing the HPV positivity in the same sample using consensus primers from the L1 ORF of HPVs to determine whether our lower HPV positivity rates are a function of the high specificity of our PCR analysis with E-6 primers or are a characteristic of this high-risk population. It will be of interest to assess whether other "oncogenic HPV types," which eluded detection by the method used in this study, are particularly prevalent in this geographical area and can contribute to a high incidence of cervical cancer in this population.

Acknowledgments

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References

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