The Presence of Human Papillomavirus-16/-18 E6, p53, and Bcl-2 Protein in Cervicovaginal Smears from Patients with Invasive Cervical Cancer

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Abstract

Cervical cancer is the second leading cause of death from cancer in women worldwide, and recent epidemiological studies have strongly implicated the sexually transmitted human papillomavirus (HPV) as a causative agent. The ability of high-risk HPVs to contribute to malignant progression seems to depend on expression of the viral E6 and E7 oncoproteins. The E6 oncoprotein forms a complex with the cellular tumor suppressor protein p53, leading to degradation of p53 via ubiquitin-dependent proteolysis. Thus, E6 expression results in the loss of p53 function in cells, including stimulation of apoptosis and inhibition of the expression of the antiapoptotic protein bcl-2. Recently, we found increased bcl-2 expression in cervical carcinoma cell lines containing mutated or E6-inactivated p53 (X. Liang, S. Mungal, A. Ayscue, J. D. Meissner, P. Wodnicki, G. Gordon, S. Lockett, and B. Herman. J. Cell. Biochem., 57: 509–520, 1995). Based on these findings, we examined Papanicolaou smears from 94 women with varying degrees of cervical carcinoma for the presence or absence of p53, HPV-16/-18 E6, and bcl-2 proteins using immunofluorescence microscopy. Our findings indicate that there is a statistically significant, inverse association between the presence of p53 and invasive cervical disease [odds ratio (OR), 0.3; 95% confidence interval (CI), 0.1–0.7]. Moreover, the odds of being diagnosed with an invasive stage of cervical cancer were 3.7 times higher (95% CI, 1.6–8.8) for women positive for the E6 protein and 17 times higher (95% CI, 5.5–58.3) for women positive for the bcl-2 protein compared with women negative for E6 or bcl-2. Women with invasive cervical cancer were also 4.59 times more likely to test positive for the presence of more than one marker (95% CI, 1.8–11.8). χ2 analysis demonstrated a strong association between the presence of E6 and bcl-2 (P < 0.001) as well as between the presence of E6 or bcl-2 and diagnosis (P = 0.015 and < 0.001, respectively). In the multivariate analysis, the presence of bcl-2 (OR, 18.8; 95% CI, 5.5–67.8) and age at diagnosis (≥50 years: OR, 7.8; 95% CI, 2.5–24.5) showed significant association with invasive cervical disease. These findings indicate that: (a) the presence of the bcl-2 protein is strongly associated with the development of invasive cervical disease; (b) the pattern of the presence of high-risk HPV-E6, p53, and bcl-2 proteins may be useful for identifying women at increased risk for the development of invasive cervical cancer; and (c) a defect in apoptosis may partially underlie the development of cervical cancer.

Introduction

Cancer of the uterine cervix is the second most common female cancer, representing approximately 15% of all neoplasms. It is the most common cancer among women in India, accounting for 26% of female cancers and resulting in about 90,000 Indian women developing the disease annually (1). It has also been estimated that at least 15,000 cases are diagnosed each year in the United States, resulting in about 4600 deaths (2). With an international overall survival rate of only 40% (3), cancer of the uterine cervix continues to remain a key international health problem.

Epidemiological studies have strongly implicated the sexually transmitted HPV2 as a causative agent (4, 5). Thus far, approximately 100 different genotypes have been identified (6), and those types associated with genital lesions have been classified as either "low-risk" or "high-risk" based on their association with benign or malignant lesions (7). The low-risk HPVs, such as HPV-6 and -11, are generally associated with benign warts and condylomas. The high-risk HPVs, such as HPV-16 and -18, have been found in approximately 84% of cervical carcinomas (8) and are associated with cervical dys-
Apoptosis and Cervical Cancer

plasia and CIN. HPV-16 is the most prevalent high-risk genotype and is present in about 50% of all genital lesions (9).

The possible mechanisms by which these oncogenic HPVs act seem to involve interactions between the HPV early proteins E6 and E7 and cellular proteins involved in growth control and apoptosis. The E7 protein of HPV-16/18 binds the retinoblas-
toma p105 protein (10), whereas the E6 protein of HPV-16/18 complexes with the p53 gene product (11, 12). Studies on HPV-infected cervical carcinoma cell lines from our laboratories demonstrated that E6 complexes with p53 in the cytoplasm of HPV-16/18-infected cells, which may prevent p53 from exerting its activities in the nucleus of cells (13). Complexation of p53 with high-risk HPV-E6 in vitro results in the rapid degradation of p53 through a ubiquitin-dependent proteolysis (14). Thus inactivation of p53, whether by abnormal localization or degradation of p53, results in the loss of p53-mediated functions, among which are stimulation of apoptosis (programmed cell death) and suppression of bcl-2 expression, a M, 26,000 cellular protein that inhibits apoptosis (15). Recent evidence suggests that viral infection can up-regulate bcl-2 expression (16, 17), and analysis of cervical carcinoma cell lines containing inactive p53 in our laboratory has recently shown that these cells contain increased bcl-2 protein levels (18). Thus, it is possible that the relative levels of expression and interactions of p53, HPV-16/18 E6, and bcl-2 could play a role in the development of cervical carcinogenesis. Therefore, analysis of these proteins in exfoliated cells from patients with varying grades of cervical disease may provide clues toward elucidating the roles of these proteins in cervical cancer, with potential implications for early diagnosis and prevention.

Materials and Methods

Cervical Smears. All subjects were seen at the clinics of the Regional Cancer Centre and the S. A. T. Hospital for Women and Children. Patients were selected randomly from two patient populations. Smears from women with invasive cervical cancer were selected from patients referred to the Regional Cancer Centre after a provisional diagnosis of invasive cancer was made by the referring gynecologist. The diagnosis was subsequently confirmed histologically after biopsy. Smears from normal women and from women with inflammatory and precancerous conditions were obtained from patients attending the gynecology outpatient clinics of the S.A.T. Hospital for Women and Children who presented for various gynecological complaints. All such patients undergo routine Papanicolaou smears.

The disease status of each patient was assessed by a cytopathologist, who categorized cell smears as either normal, LSILs (CIN I), HSILs (CIN II and III), or invasive cancer. A total of 94 cervical smears was evaluated in this study. Of the 94 patients, 52 had invasive squamous cell carcinoma; 6 had HSILs (CIN II and III); 10 had LSILs (CIN I); and 26 had either normal or slightly inflammatory smears. Cervicovaginal cells were collected with an Ayre’s spatula and placed in tissue culture medium. Cells were centrifuged at slow speed to remove mucus and other debris and then suspended in 0.05% cysteine-containing buffer. Cell monolayers were then prepared on poly-
tyrosine-coated slides, fixed in cold acetone, and stored at −20°C until analysis.

Immunofluorescence Microscopy. The cell smears were analyzed for the presence of p53, HPV-16/18 E6, and bcl-2 oncoproteins using indirect immunofluorescence microscopy as described previously (13, 18). Briefly, cells were incubated with either anti-p53 antibody (polyclonal antibody 1801; On-
cogene Science, Lake Placid, NY; 1:10 dilution), anti-HPV-16/ -18 E6 antibody (Oncogene Science; 1:10 dilution), or a ham-
tser monoclonal anti-bcl-2 antibody (kindly provided by David Hockenbery, Fred Hutchinson Cancer Research Center, Seattle, WA; 1:20 dilution). The hamster 6C8 antihuman bcl-2 mono-
clonal antibody, which was used in the immunofluorescence analysis, has been characterized previously and has been shown to be specific for the detection of the bcl-2 protein (18). Rabbit antihuman bcl-2 polyclonal antibody (PharMingen, San Diego, CA), produced against a synthetic cysteine-containing peptide (amino acids 41–54) of the human 239-amino acid bcl-2 protein, was also used for immunofluorescence experiments. The specificity and sensitivity of this antibody has also been documented previously (18). Results using these two antibodies were consistent. The specificity and sensitivity of the anti-HPV-16/18 E6 and p53 antibodies have been documented in literature ob-
tainable from the company from which the antibodies were purchased. Primary antibody binding sites were visualized with either FITC-conjugated, sheep antimouse IgG (Organon Teknika Corp., West Chester, PA; 1:60 dilution) or tetrameth-
ylrhodamine isothiocyanate-conjugated, goat antihamster IgG (Jackson Immunoresearch Laboratories, West Grove, PA; 1:50 dilution). Slides were mounted in Vectashield (Vector Labora-
tories, Burlingame, CA), and cells were imaged using an epi-
fluorescence microscope coupled to a low-level camera and a digital image analysis system as described previously (19).

Statistical Analysis. Patients were divided into two groups, as having either invasive disease of the cervix (cervical cancer) or noninvasive disease of the cervix (normal and CIN I–III), and ORs were calculated as estimates of the relative risk to test for any significant association between age at diagnosis, age at first live birth, parity, expression versus absence of proteins (p53, HPV-E6, and bcl-2), total number of positive markers per smear (smears were scored as being positive for one of the three markers versus positive for two of the three markers versus positive for all three markers), and invasive disease. Ninety-five % CIs were calculated according to the method of Sclesselman (20). In addition, 2χ2 analysis was performed to test whether the proportion of cases positive for p53, HPV, and bcl-2 increased with increasing severity of cervical disease.

Logistic regression was used to estimate risks, which were adjusted for multiple risk factors using SAS statistical software (SAS Institute, Inc., Cary, NC). All risk factors were dichoto-
mized. All variables that were significant in the bivariate analy-
ysis were included in the first multivariate model. Any inter-
acting terms that were significant at P < 0.5 were included in the first multivariate model as well. The final model, reported here, excluded risk factors and interaction terms that were not statistically significant (P > 0.05) in the preliminary model. CIs for the adjusted ORs were calculated using the estimated log-
istic coefficient and the corresponding SE.

Results

The mean age at diagnosis of all women was 50 (range, 26–75; SD, 10.5) years. Age at diagnosis was associated with disease status; women who were diagnosed with invasive cervical cancer were older. Parity and age at first birth did not vary significantly among the four groups (normal, CIN I, CIN II and III, and invasive cancer), although the mean number of children and age at first birth were higher for women with invasive cervical cancer than for the other three groups.

We examined HPV expression in subjects with invasive cancer using in situ hybridization of biopsy specimens. Of the 52 subjects with invasive cancer, 41 were positive, and 11 were
Expression of p53, HPV-16/-18 E6, and bcl-2 proteins in cervicovaginal epithelial cells. A. positive staining for p53 protein in normal cervicovaginal cells. B. absence of staining for p53 protein in malignant cervicovaginal epithelial cells. C. positive staining for HPV-16/-18 E6 protein in malignant cervicovaginal epithelial cells. D. absence of staining for HPV-16/-18 E6 protein in normal cervicovaginal epithelial cells. E. positive staining for bcl-2 protein in malignant cervicovaginal epithelial cells. F. absence of staining for bcl-2 protein in normal cervicovaginal epithelial cells. ×25. Insets in A, C, and E. positive p53, E6, and bcl-2 staining, respectively, at higher magnification. ×100. Normal p53 staining was concentrated in the nucleus (A, inset, arrows), whereas E6 and bcl-2 staining were found in the cytoplasm; E6 appeared as diffuse clumps of staining in the cytoplasm of cells (C, inset, arrows), whereas bcl-2 staining was punctate in nature (E, inset, arrows).

negative for HPV-16. None of the samples were found to be positive for HPV-6, -11, or -18. None of the 11 samples negative for HPV-16 expressed the E6 protein. Of the 41 HPV-positive samples, 8 were negative for E6. Of the 10 patients with CIN I, 2 were positive for HPV-16 and E6, whereas 1 was positive for HPV-11. Of the 6 samples with CIN II or III, 4 were positive for HPV-16, and 3 of these 4 were positive for E6. We were unable to do testing in the normal and inflammatory samples because of the lack of biopsy specimens.

The expression of p53, HPV-16/-18 E6, and bcl-2 proteins showed characteristic cytological localization. In normal cells, p53 staining was found predominantly in the nucleus, with less staining in the cytoplasm (Fig. 1A, note brighter, centrally stained nuclei of cells). No HPV-E6 staining and little bcl-2
staining were observed in normal cells (Fig. 1, D and F). In cells from patients with cervical disease, when present, p53, E6, and bcl-2 staining was restricted to the cytoplasm of cells (Fig. 1, A–C). Significant variations in the expression of the three proteins in relation to the cytopathology of the smear was also evident. p53 was found to be expressed predominantly in normal or low-grade disease (i.e., CIN I; Table 1). When present in abnormal cells, p53 was localized to the cytoplasm, in a distribution similar that of E6. In contrast to p53, the expression of E6 and bcl-2 was predominantly found to occur in advanced disease. E6 and bcl-2 expression was found predominantly in cells of higher-grade lesions (CIN II and III) and invasive disease. E6 and bcl-2 expression was found predominantly to occur in advanced cervical disease. In contrast to our findings regarding p53 staining, increased bcl-2 protein would be associated with invasive cervical disease (Table 3; OR, 4.2; 95% CI, 1.8–11.8).

Table 1

<table>
<thead>
<tr>
<th>% present</th>
<th>Expression</th>
<th>Normal</th>
<th>CIN I</th>
<th>CIN II/III</th>
<th>Invasive cancer</th>
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<tbody>
<tr>
<td>n</td>
<td>(n = 26)</td>
<td>(n = 10)</td>
<td>(n = 6)</td>
<td>(n = 52)</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>54</td>
<td>60</td>
<td>33</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>HPV-16/18 E6</td>
<td>27</td>
<td>20</td>
<td>50</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>At least 1 present</td>
<td>65</td>
<td>60</td>
<td>83</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>1 present</td>
<td>54</td>
<td>40</td>
<td>33</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>2 present</td>
<td>12</td>
<td>10</td>
<td>50</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>3 present</td>
<td>10</td>
<td></td>
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Table 2

<table>
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<tr>
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<th>Invasive cases</th>
<th>Normal cases</th>
<th>OR* (95% CI)</th>
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</thead>
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<tr>
<td>p53 expression vs. absence (referent)</td>
<td>13</td>
<td>22</td>
<td>0.3 (0.1–0.7)</td>
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<tr>
<td>HPV-16/18 E6 expression vs. absence (referent)</td>
<td>31</td>
<td>12</td>
<td>3.7 (1.6–8.8)</td>
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<tr>
<td>bcl-2 expression vs. absence (referent)</td>
<td>34</td>
<td>4</td>
<td>17.5 (5.5–58.3)</td>
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<td>Age at diagnosis, &gt;50 yr vs. ≤50 yr (referent)</td>
<td>33</td>
<td>8</td>
<td>7.4 (2.8–19.2)</td>
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<tr>
<td>Parity, &gt;3 vs. ≤3 (referent)</td>
<td>28</td>
<td>12</td>
<td>2.9 (1.2–6.9)</td>
</tr>
<tr>
<td>Age at first live birth, &gt;19 vs. ≤19 yr (referent)</td>
<td>37</td>
<td>22</td>
<td>2.2 (0.9–5.3)</td>
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<tr>
<td>No. of markers present, &gt;1 vs. ≤1 (referent)</td>
<td>27</td>
<td>8</td>
<td>4.6 (1.8–11.8)</td>
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Table 3

<table>
<thead>
<tr>
<th>Marker 1</th>
<th>Marker 2</th>
<th>Cases</th>
<th>Controls</th>
<th>OR* (95% CI)</th>
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<tbody>
<tr>
<td>p53</td>
<td>HPV-16/18 E6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>Absent (referent)</td>
<td>17</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td>Present</td>
<td>Absent</td>
<td>4</td>
<td>15</td>
<td>0.3 (0.07–1.12)</td>
</tr>
<tr>
<td>Present</td>
<td>Present</td>
<td>22</td>
<td>5</td>
<td>4.2 (1.2–15.7)</td>
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<tr>
<td>Present</td>
<td>Present</td>
<td>9</td>
<td>7</td>
<td>1.4 (0.4–5.5)</td>
</tr>
<tr>
<td>bcl-2</td>
<td>HPV-16/18 E6</td>
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<td></td>
<td></td>
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<tr>
<td>Absent</td>
<td>Absent (referent)</td>
<td>11</td>
<td>17</td>
<td>1.0</td>
</tr>
<tr>
<td>Present</td>
<td>Absent</td>
<td>7</td>
<td>21</td>
<td>0.7 (0.2–2.5)</td>
</tr>
<tr>
<td>Present</td>
<td>Present</td>
<td>28</td>
<td>3</td>
<td>17.2 (3.7–80.4)</td>
</tr>
<tr>
<td>Present</td>
<td>Present</td>
<td>6</td>
<td>1</td>
<td>10.8 (1.0–120.5)</td>
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<tr>
<td>Bcl-2</td>
<td>HPV-16/18 E6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>Absent (referent)</td>
<td>12</td>
<td>29</td>
<td>1.0</td>
</tr>
<tr>
<td>Present</td>
<td>Absent</td>
<td>9</td>
<td>17</td>
<td>17.1 (1.3–170.0)</td>
</tr>
<tr>
<td>Present</td>
<td>Present</td>
<td>6</td>
<td>9</td>
<td>1.5 (0.4–5.9)</td>
</tr>
<tr>
<td>Present</td>
<td>Present</td>
<td>25</td>
<td>3</td>
<td>22.4 (5.1–98.9)</td>
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</tbody>
</table>

a OR adjusted for age at diagnosis; the referent group is indicated.

There was a significant decreasing trend in the proportion of cases positive for p53 with increased severity of cervical disease (Table 1; χ² = 7.7; P < 0.01). We also observed a significant trend in the proportion of cases positive for HPV-E6 (Table 1; χ² = 9.4; P < 0.005) and bcl-2 (Table 1; χ² = 34.6; P < 0.001) with increasing severity of cervical disease.

We have demonstrated previously that the HPV-16/18E6 protein can complex with the p53 protein in the cytoplasm of HPV-infected cervical carcinoma cells (13). This complexation is thought to target p53 for rapid degradation via ubiquination (14). If true, one might expect to see decreased expression of p53 in HPV-16/18 E6-expressing cells. Therefore, we examined the presence of p53 and HPV-E6 proteins in cervicovaginal epithelial cells in noninvasive cases versus invasive cancer cases and calculated the OR adjusted for age at diagnosis (Table 3). As expected, the largest percentage of p53-positive cells was found to be normal or inflammatory or to have low-grade disease. As disease severity increased, the percentage of cells demonstrating p53 staining decreased, whereas the percentage of HPV-E6-positive samples increased. The largest percentage of samples that were positive for E6 and negative for p53 staining was found in invasive cancer (Table 3; OR, 4.2; 95% CI, 1.2–15.7).

p53 has also been shown to suppress the expression of bcl-2 (15); if p53 activity is inactivated due to either enhanced degradation or localization of the p53 protein to the cytoplasm via interaction with HPV-E6 (13), then the suppression of bcl-2 expression might be diminished. To test this hypothesis, we also examined the relationship between the presence of p53 and bcl-2 proteins in cervicovaginal epithelial cells in noninvasive (controls) versus cases (invasive cancer) and calculated OR for age at diagnosis (Table 3). Our prediction would be that an inverse correlation between the presence of p53 and bcl-2 proteins should exist, and that decreased p53 protein but increased bcl-2 protein would be associated with invasive cervical disease. In contrast to our findings regarding p53 staining, bcl-2 staining was associated almost exclusively with invasive cervical disease (Table 3; OR, 17.2; 95% CI, 3.7–80.4), and the highest percentage of bcl-2-expressing cells was found to be p53 negative. A higher risk (Table 3; OR, 22.4; 95% CI, 5.1–98.9) for the presence of invasive cancer as well as a strong association between the presence of E6 and bcl-2 proteins were...
observed ($\chi^2, P = 0.001$). There was also a strong association between the presence of the E6 or bcl-2 protein and cytopathological diagnosis ($P = 0.015$ and 0.001, respectively). In the multivariate analysis, the expression of bcl-2 (OR, 18.8; 95% CI, 5.5–67.8) and age at diagnosis ($\geq$50, OR, 7.8; 95% CI, 2.5–24.5) showed significant associations with invasive cervical disease (Table 4).

### Discussion

One of the most intensively studied issues in gynecological pathology is the factors involved in the progression of precancerous processes of the uterine cervix to invasive cancer. This question is of vital importance, because it addresses the biological characteristics that typify neoplasia, the cellular features that can be recognized as signifying high risk, and the possibility that the use of such information will simplify the management of women with abnormal Papanicolaou smears. Cellular and molecular characteristics of the pathobiology of cervical cancer and its precursor lesions need to be identified and evaluated in the context of diagnostic cytopathology. This approach will aid in the identification of those lesions at high risk for progression to invasion, will provide potential targets for intervention, and in addition, will provide surrogate end point biomarkers for chemopreventive approaches (21).

Previously published data suggest that expression of high-risk HPV-E6 results in the loss of p53 due to enhanced degradation. Transfection of human mammary epithelial cells with HPV-16 led to sharply reduced levels of the p53 protein (22), whereas E6 expression was associated with a decrease in p53-mediated transcriptional repression (23), transactivation (24), and disruption of p53-mediated G1 arrest. To determine whether high-risk HPV-E6 expression results in the loss of p53 in vivo, we examined cervicovaginal smears from patients with invasive cervical cancer for the presence or absence of p53 and HPV-16/18 E6. We observed an inverse relationship between the presence of E6 and p53 proteins. Our findings also demonstrated a statistically significant inverse association between the presence of p53 and invasive cervical cancer. Moreover, the odds of being diagnosed with invasive cervical cancer were almost four times higher for women positive for E6. Thus, the presence of high-risk HPV-E6 and the absence of p53 seem to be associated with an increased risk of development of high-grade cervical disease.

Approximately 15–20% of cervicovaginal smears from all categories of cervical disease were found to contain both p53 and E6 proteins. This may seem counterintuitive, in that E6 expression leads to p53 degradation. The polyclonal antibody used in the present study (polyclonal antibody 1801) detects both the wild and mutant types of p53; therefore, it is not possible to distinguish whether we are detecting mutant or wild-type p53. However, p53 mutation is an extremely rare event in cervical cancer, and thus, it is unlikely that we are detecting mutant p53. In the small percentage of smears that contain both proteins, it may be that these cells came from the transition zone between normal and tumor tissue and thus represent early stages of transformation. The presumed low levels of these proteins would not be fully transforming. Unfortunately, it is not possible using indirect immunofluorescence to determine levels of expression quantitatively. In addition, collection of exfoliated cells with an Ayre’s spatula does not allow direct comparison of normal versus dysplastic and tumor cells within a single individual or assessment of the relative contributions of normal versus diseased cells in each sample. For example, it is possible that exfoliated cells collected from invasive cancer patients may contain a larger tumor: normal cell ratio than women with LSILs given these patients’ larger tumor burden. This would have the effect of overestimating the OR for each marker.

Very recent experiments have suggested that E6 may function in inhibiting apoptosis in certain cells (14, 17, 25, 26). Apoptosis plays a critical role in tissue homeostasis by counterbalancing mitosis. It is an essential component of many physiological processes, including embryonic development, clonal selection in thymocytes (27, 28), and protection against disease (29). Apoptosis selectively eliminates unnecessary or damaged cells, which, if allowed to proliferate, could lead to malformation, autoimmune reactions, or neoplasia (30). p53 has been shown to be required for apoptosis induced by adenovirus E1A (31), ionizing radiation (32), and etoposide (33). It has been suggested that p53-induced apoptosis following DNA damage may be a defense mechanism of the organism by which cells with mutations are deleted before they can proliferate. Thus, loss of p53 activity due to high-risk HPV-E6 expression may impair the apoptotic response in virally infected cells.

Evidence is beginning to emerge that p53 regulates other proteins involved in controlling apoptosis. bcl-2 was originally identified as an oncogene due to its involvement in B-cell lymphomas (34). In these tumors, bcl-2 is translocated from its normal 18q21 chromosomal site to the 14q32 locus, fusing it to the immunoglobulin heavy chain gene and resulting in overexpression of the bcl-2 mRNA and protein (35, 36). Subsequently, it was discovered that bcl-2 was able to block programmed cell death induced by a wide variety of death-inducing stimuli. P53 has been shown to regulate bcl-2 expression in breast cancer cells (37) and M1 murine leukemia cells (38). In mice deficient in p53, increased bcl-2 expression was found in certain tissues (39). Recently, data has been published indicating that bcl-2 transfection of keratinocytes blocks their differentiation and extends their viability but (unlike high-risk E6 and E7) does not immortalize normal human keratinocytes (38). Interestingly, however, it has been shown that bcl-2 completely blocks p53-induced apoptosis (16). Thus, both E6 and bcl-2 interfere with the function of p53. However, neither E6, which immortalizes keratinocytes at a low efficiency, nor increased bcl-2 expression, in and of itself, is sufficient to immortalize keratinocytes. Recent findings from our laboratory have demonstrated that there is an inverse relationship between the levels of p53 and bcl-2 in cervical carcinoma cells (18). In total, these findings suggest that the relative level of p53 may modulate the expression of the apoptotic regulator bcl-2, and that loss of p53 expression in combination with increased bcl-2 expression may lead to transformation of cervical epithelial cells.

To test this hypothesis, we examined cervicovaginal smears for any relationships that might exist between the presence or absence of p53 and bcl-2 as well as E6 and bcl-2, because E6 expression lowers or abolishes p53 expression. As was the case with p53 and E6, we found an inverse relationship.

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**Table 4** Logistic regression analysis of variables associated with invasive cervical cancer

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, $&gt;$50 vs. $\leq$50 yr (referent)</td>
<td>7.8</td>
<td>2.5-24.5</td>
</tr>
<tr>
<td>bcl-2 expression vs. absence (referent)</td>
<td>18.8</td>
<td>5.2-67.8</td>
</tr>
<tr>
<td>Constant</td>
<td>0.2</td>
<td>0.1-0.5</td>
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*$\chi^2 = 9.85; df = 8; P = 0.0270$.
between the presence of p53 and bcl-2 proteins. This inverse correlation between p53 and bcl-2 proteins may partly explain the process of tumor progression in the uterine cervix. Because it has been documented that p53 inhibits bcl-2 expression (37), increased bcl-2 expression in the absence of (or expression of inactivated) p53 may provide transformed cells with a selective advantage for survival, leading to oncogenesis and tumor progression. Overproduction of the bcl-2 protein has also been shown to abrogate p53-induced apoptosis, suggesting that if sufficiently high levels of bcl-2 protein are produced, even if wild-type p53 is present, it may no longer be able to induce apoptosis (39). Thus, the level of bcl-2 protein expression seems to be a key role in the regulation of cervical epithelial cell growth.

A strong association of expression between E6 and bcl-2 protein was observed, as well as an association between the presence of the E6 or bcl-2 protein and diagnosis. Thus, the loss of p53 and increased amounts of high-risk HPV-E6 and bcl-2 proteins seem to be associated with high-grade cervical disease. The absence of p53 in high-grade cervical precancer and invasive cancer and the concomitant expression of the E6 protein suggests that the inactivation of the former may be due to complex formation with E6. This may indicate that in HPV-mediated carcinogenesis, expression of the E6 protein seems to be a critical event. This is also reflected in the finding that the odds of being diagnosed with high-grade cervical disease was almost 5 times greater in patients positive for E6. We also found a statistically significant inverse association between the presence of p53 and advanced cervical disease. Moreover, the odds of being diagnosed with an invasive stage of cervical cancer were almost 18 times higher for women positive for bcl-2 protein compared with women negative for bcl-2. However, in a multivariate analysis, only the presence of bcl-2 and age at diagnosis showed significant associations with advanced cervical disease.

χ² analyses were performed to test whether the proportion of cases positive for p53, HPV, and bcl-2 increased with increasing severity of cervical disease, with particular emphasis on differences between normal and invasive cervical cancer. There was a significant inverse trend in the proportion of cases positive for p53 with increased severity of cervical disease. We also observed a significant trend in the proportion of cases positive for HPV-E6 and bcl-2 with increasing severity of cervical disease. One concern about this analysis is the fact that our patient population consisted of only a small number of women with dysplasia, relative to those free of disease or those with invasive cervical cancer. Due to the low numbers of women with dysplastic lesions, our study does not have the power to address issues related to the multistage disease process (although it is suggestive) and must await more comprehensive studies of large numbers of women with dysplastic lesions. These studies are currently underway. To determine whether this small number of dysplastic samples affected our findings, we reanalyzed our data excluding the LSIL and HSIL cases. We obtained essentially the same results as those when these samples were included, suggesting that our initial findings are valid.

The alterations in expression of high-risk HPV-transforming protein E6, the tumor suppressor protein p53, and the antiapoptotic protein bcl-2, which we have found to occur invasive cervical cancer, are of considerable significance, both from a diagnostic viewpoint and in the elucidation of cellular events occurring during cervical carcinogenesis. The major significant finding of this study is the presence of the bcl-2 protein in invasive cancer. This increased presence of the bcl-2 protein does not seem to be due to chromosomal translocation, as is the case in B-cell lymphoma (13). High levels of the bcl-2 protein have also been seen in the absence of this characteristic translocation in neuroblastoma, cancer of the prostate, colon, lung, nasopharynx, and breast, and leukemia (40–42). The finding in this study that the odds of being diagnosed with advanced cervical disease were increased 19-fold in women whose cervicovaginal smears contained the bcl-2 protein strongly suggests that bcl-2 plays a significant role in the development of cervical cancer and that alterations in apoptosis may in part underlie the mechanism of development of cervical cancer.

References


The presence of human papillomavirus-16/-18 E6, p53, and Bcl-2 protein in cervicovaginal smears from patients with invasive cervical cancer.

M R Pillai, S Halabi, A McKalip, et al.