Survivin as a Marker of Cervical Intraepithelial Neoplasia and High-Risk Human Papillomavirus and a Predictor of Virus Clearance and Prognosis in Cervical Cancer

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Key Words: Survivin; Apoptosis inhibitor; Human papillomavirus; Cervical intraepithelial neoplasia; CIN; Cervical cancer; Prognosis; Virus clearance; High-risk HPV

Abstract

We analyzed survivin as a marker of cervical intraepithelial neoplasia (CIN) and high-risk human papillomavirus (HR-HPV) and a predictor of HPV clearance and disease outcome in cervical cancer in 302 samples (squamous cell carcinomas [SCCs], 150; CIN lesions, 152) by immunohistochemical staining with survivin antibody and HPV testing using polymerase chain reaction.

HR-HPV types were associated closely with CIN and SCC. There was a significant linear relationship between grade and intensity of survivin expression (P = .0001). Survivin overexpression also was associated strongly with HR-HPV type (P = .0001). Multivariate regression analysis revealed survivin and p16INK4a as equally strong independent predictors of HR-HPV. Deregulated survivin expression did not predict clearance or persistence of HR-HPV after treatment of CIN or survival in cervical cancer in univariate (P = .417) or multivariate analysis. After adjustment for HR-HPV, stage, age, and tumor grade in the Cox regression model, only stage (P = .0001) and age (P = .0001) remained independent prognostic predictors.

Survivin seems to be an early marker of cervical carcinogenesis. Up-regulated survivin expression was an independent predictor of HR-HPV in cervical lesions, most plausibly explained by its normal transcriptional repression by wild-type p53 being eliminated by HR-HPV E6 oncoprotein.

In large epidemiologic studies, the high-risk human papillomavirus (HR-HPV) types are associated with cervical cancer (CC) in almost 100% of cases, in contrast with the low-risk (LR) HPV types that rarely are found in CC and its precursors.1-7 The different oncogenic potential of LR-HPV and HR-HPV seems to be linked, at least in part, with the different functions of 2 viral oncogenes, E6 and E7, and their interactions with 2 cell cycle regulatory proteins, p53 and pRB.2-5,8,9 While the E6 oncoprotein initiates degradation of the p53 tumor suppressor protein, HPV E7 binds to pRB and triggers the release of E2F-like transcription factors, resulting in G1/S transition of the cell cycle.5-10 Through complex mechanisms, these 2 oncoproteins also have an important role in inhibition of apoptosis.2-5,8-14 Inhibition of apoptosis is one of the key features of HPV-induced malignant transformation.2-5,8,10-14 During the past 10 years, marked progress has been made in understanding the regulation of apoptosis, after discovery of a new family of proteins known as inhibitor of apoptosis (IAP).15,16 Until now, 8 members of the human IAP family have been characterized: c-IAP1, c-IAP2, XIAP, NAIP, apollon, ML-IAP/vivin, ILP-2, and survivin.15-17 Since its discovery in 1997,18 survivin has been studied intensely in epithelial and nonepithelial human malignant neoplasms.15,19,20 Survivin is expressed constitutively in most of these malignant neoplasms (and is absent in normal cells), and its expression has been associated with several adverse prognostic signs: shorter overall survival, unfavorable markers of disease progression, increased recurrence rates, and increased resistance to therapy.15,19,20 Some progress also has been made with therapeutic agents targeting the survivin pathway, resulting in increased apoptosis of cancer cells.15,19,20
Until now, few studies have addressed survivin expression in CC and its precursors.21-25 In these studies, various aspects have been analyzed, including expression of survivin in different cervical intraepithelial neoplasia (CIN) grades24 and in different histologic types of CC.21,25 and its relationships with HPV.22,23 None of these studies has analyzed survivin expression as a prognostic marker in CC or its association with any of the viral events, eg, virus persistence or integration, known to be valuable intermediate endpoint markers of CC.1,2,5 As recently discussed,15,19,20 however, expression of survivin in cancer cells can be regulated by several distinct pathways, clearly including also those used by HR-HPV while inhibiting apoptosis and inducing cell transformation.2,5,8,10-14

Prompted by the recent data suggesting p16INK4a as a specific biomarker of HR-HPV types and CIN,9,10,26 we analyzed a series of cervical carcinomas and CIN lesions to assess whether survivin expression might be of any use in predicting the following: (1) the grade of CIN, (2) HR-HPV type, (3) clearance of the virus after eradication of CIN, and (4) the prognosis of CC. Survivin expression was studied using immunohistochemical analysis in CIN lesions treated by conization and monitored by serial polymerase chain reaction (PCR) for HPV clearance, and survival data for CC were related to survivin expression in surgical samples.

Materials and Methods

This study comprises the retrospective component of the HPV-Pathogen ISS project,27 and materials were collected from the files of the pathology departments of 2 Italian hospitals (Azienda Ospedaliera S. Orsola Malpighi, Bologna, and Ospedale Maggiore, University of Trieste, Trieste). The prospective biopsy material was from 302 patients with invasive cervical squamous cell carcinoma (SCC) or CIN diagnosed and treated in these 2 hospitals between September 1986 and October 2002. Of these 302 cases, 114 CIN and 38 SCC cases were provided by Bologna, and 38 CIN lesions and 112 SCCs were from Trieste. The mean age of all patients with CIN was 35.5 years (range, 18-79 years) and that of patients with SCC, 59.2 years (range, 27-89 years; P = .0001).

Available Data

For all cases from Bologna, HPV status was determined by PCR, as reported in separate recent studies,28-30 whereas the samples from Trieste were examined for HPV status during the present study. Complete follow-up data were available for all 150 SCC cases, with a mean follow-up of 51.7 months (range, 1-218 months). Furthermore, all CIN patients from Bologna had been followed-up at 6-month intervals after cone treatment (for a mean of 10.5 months; range, 2.4-27.6 months) and subjected to repeated colposcopy, Papnicolaou smear, and biopsy (if residual disease was suspected). A minimum of 2 serial PCR analyses were available from 67 cases and recently reported as a part of a larger study on HPV clearance.30 The clinical International Federation of Gynecology and Obstetrics (FIGO) disease stage was available for 125 cases of SCC.

Biopsy and Surgical Samples

The colposcopic biopsy specimens and surgical samples were fixed in 10% buffered formalin, embedded in paraffin, and processed for 5-µm-thick paraffin sections stained with H&E for routine diagnosis. All slides were reexamined to confirm the diagnosis. During histologic examination, lesions were graded using CIN nomenclature and categorized as CIN 1, CIN 2, or CIN 3. The histologic diagnosis of SCC was confirmed in all cases, and 2 adenocarcinomas originally present were excluded from this series.

Immunohistochemical Analysis for Survivin

Immunohistochemical staining for expression of the IAP, survivin, was completed by standard procedures. In brief, the 5-µm paraffin sections cut on poly-L-lysine–coated microscopy slides were deparaffinized and rehydrated in graded alcohols. The sections were heated in citrate buffer (0.01 mol/L, pH 6.0; DAKO Target Retrieval Solution, DAKO, Carpinteria, CA) in a microwave oven (85°C-95°C, 3 times for 5 minutes each), followed by blocking the nonspecific binding sites with goat-rabbit serum. Sections were incubated with the primary antibody, polyclonal rabbit survivin antibody (No. 0469, dilution 1:100; Abcam, Cambridge, England), in a humidified chamber for 1 hour at room temperature. This polyclonal (IgG) antibody has been raised in rabbits against a full-length recombinant human survivin. This antibody reacts with human survivin; other species have not been tested. Primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat antirabbit IgG (No. 6720, dilution 1:200; Abcam). Slides then were processed with the universal LSAB-2 single reagents (peroxidase) kit (DakoCytomation, Glostrup, Denmark), and expression of survivin was localized by incubation with diaminobenzidine. As a final step, the slides were stained with light hematoxylin counterstaining. Negative control slides were processed similarly by omitting the primary antibody, and biopsy specimens from breast cancer were used as positive control samples.

Evaluation of Immunohistochemical Staining

Immunohistochemical staining was examined using a light microscope (Leitz Diaplan, Leitz Wetzlar, Heidelberg, Germany) equipped with a digital camera (Leica DG300, Heidelberg, Germany). In normal squamous or columnar cervical epithelium, no positive staining for survivin was detected. In original grading of immunohistochemical staining,
A semiquantitative scoring into 4 categories was used: 0, negative; 1, weak staining, scattered in single cells or a diffuse, weak reaction in the squamous epithelium; 2, moderately increased staining, in which positively stained cells (cytoplasmic and/or nuclear) were clearly increased; and 3, intense staining, with almost all cells staining diffusely throughout the lesion (intense nuclear or cytoplasmic staining). In statistical analysis, the staining results were used as dichotomous categorical variables combining the aforementioned categories as negative-weak and moderate-intense (0-1 and 2-3) or using the 4-tier categorization.

Image 1 Normal squamous epithelium of the cervix stained for survivin antibody. Only single cells in the parabasal layer demonstrate nuclear staining for survivin in immunohistochemical analysis (survivin, original magnification ×100).

Image 2 Normal transformation zone demonstrating the process of immature squamous metaplasia. Also the metaplastic squamous epithelium remains negative for survivin expression in immunohistochemical analysis (survivin, original magnification ×100).

Image 3 A low-grade cervical intraepithelial neoplasia lesion showing characteristic morphologic features of human papillomavirus infection (koilocytes) in the upper layers of the epithelium by immunohistochemical analysis. Notice weakly survivin-positive cells scattered among the dysplastic cells in the lower third and some within the koilocyte cell layers (survivin, original magnification ×100).

Image 4 A high-grade cervical intraepithelial neoplasia lesion (CIN 3; on the left) and its junction with the normal squamous epithelium (on the right). Notice strong immunohistochemical staining for survivin in all layers of the CIN 3 lesion and the lack of staining in the normal epithelium, except for some scattered parabasal cells (survivin, original magnification ×40).
HPV Testing

The 114 CIN and 38 SCC cases from Bologna already had been tested for HPV for other purposes using PCR, as reported in separate studies. In the present study, the 150 paraffin-embedded sections (112 SCC and 38 CIN) from Trieste were subjected to HPV testing (see “Polymerase Chain Reaction”).

DNA Extraction

Paraffin-embedded tissue samples (5-µm sections not weighting more than 25 mg) were treated with xylene to remove paraffin and digested with ATL tissue lysis buffer (QIAGEN, Hilden, Germany) and Proteinase K overnight at 56°C in a thermomixer, and DNA was extracted according to the manufacturer’s instructions (QIAamp DNA mini kit, QIAGEN).

Polymerase Chain Reaction

To verify the extraction and the quality of DNA from the paraffin-embedded tissue samples, 5 µL of each sample was amplified with a primer set recognizing the β-actin gene (sense: 5’-GGCGGCACCACCATGACCTC-3’; antisense: 5’-AGGGGCCGGACTGCACTACT-3’). The PCR mix contained 200 µmol/L of each deoxynucleoside triphosphate, 1.5 mmol/L of magnesium chloride, 1× PCR buffer, 40 pmol of sense and antisense primers, 1.25 U of AmpliTaq Gold. For the GP5+/GP6+ primers, the following conditions were used: 94°C for 10 minutes, 1 cycle; 95°C, 30 seconds, 44°C, 60 seconds, and 72°C, 90 seconds, for 40 cycles; and with the final extension step at 72°C for 7 minutes. Amplification was electrophoresed on a 2% agarose gel and visualized under UV light.

The samples then were amplified for the presence of HPV using different sets of degenerated primers as described separately for MY09/MY11, GP5+/GP6+ and biotinylated short PCR-fragment (SPF) primer mix located within the L1 region of the HPV genome. The PCR conditions for the MY09/MY11 were as follows: 94°C for 10 minutes, 1 cycle; 94°C, 30 seconds, 55°C, 45 seconds, and 72°C, 30 seconds for 40 cycles; followed by an extension step at 72°C for 7 minutes. The PCR mix contained 200 µmol/L of each deoxynucleoside triphosphate, 40 pmol of each primer, 2 mmol/L of magnesium chloride, 1× PCR buffer, and 1.25 U of AmpliTaq Gold. For the GP5+/GP6+ primers, the following conditions were used: 94°C for 10 minutes, 1 cycle; 95°C, 30 seconds, 44°C, 60 seconds, and 72°C, 90 seconds, for 40 cycles; and with the final extension step at 72°C for 7 minutes. Amplification was electrophoresed on a 2% agarose gel and visualized under UV light.

None of the samples were exclusively positive with the primer set MY09/MY11, whereas GP5+/GP6+ and the SPF primer mix alone gave positive results in 12 and 34 cases, respectively. Of the remaining cases, 42 samples were positive for HPV DNA when amplified with the SPF and GP5+/GP6+ primers and another 44 when using the triple set of primers (MY09/MY11, SPF mix, and GP5+/GP6+). The amplified products were electrophoresed on a 2% agarose gel and visualized under UV light.

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HPV Typing

HPV typing was done using the reverse-hybridization assay. The denatured, biotinylated, amplified product (10 µL) was hybridized with specific oligonucleotide probes, which are immobilized as parallel lines on membrane strips (InnoLiPA, Innogenetics, Ghent, Belgium). After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with bromochloroindoyl phosphate/nitroblue tetrazolium chromogen yields a purple precipitate that can be interpreted visually. Based on the position of the visualized line, it is possible to determine the HPV genotype.33

Statistical Analyses

Statistical analyses were performed using the SPSS and STATA software packages (SPSS for Windows, version 11.5, SPSS, Chicago, IL; STATA/SE 8.2, STATA, College Station, TX). Frequency tables were analyzed using the \( \chi^2 \) test, with Pearson correlation and/or likelihood ratio used to assess the significance of correlations between categorical variables. Differences in the means of continuous variables were analyzed using nonparametric tests (Mann-Whitney) or analysis of variance. Logistic regression models using a stepwise backward approach and the likelihood ratio statistic for removal testing were used to analyze the power of different covariates as predictors of the outcome variables (CIN, HR-HPV), calculating crude odds ratios (ORs) and 95% confidence intervals (CIs).

Performance indicators of survivin as a marker of CIN or HR-HPV were calculated using conventional contingency tables to calculate sensitivity, specificity, and positive (PPV) and negative predictive value (NPV), with 95% CI based on the F distribution (± 1.96 × SE). Univariate survival (life table) analysis for the outcome measure (HPV clearance, HPV persistence, cancer survival) was based on the Kaplan-Meier method. Multivariate survival analysis was run by using the Cox proportional hazards model in a backward stepwise manner with the log-likelihood ratio significance test and using the default values for inclusion and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log survival plots. In all tests, values less than .05 were considered statistically significant.

Results

Table I shows the expression of survivin related to the grade of the lesion in cone (for CIN) or surgical (for CC) specimens. Survivin expression was not detected in normal or metaplastic squamous epithelium. There was a direct relationship between the increasing grade of lesion and the intensity of survivin staining in that the frequency of intense survivin expression increased from 0% to 58% in biopsy specimens without CIN and those interpreted as CIN 3 (P = .0001). With the 2-tier category of staining (negative-weak vs moderate-intense), the latter was associated with high-grade lesion (CIN 3 or cancer) at an OR of 13.07 (95% CI, 6.49-26.29). When cancer cases were excluded, this association still had an OR of 13.07 (95% CI, 5.67-30.12; P = .0001).

Of all CIN lesions, 70.5% were HR-HPV–positive, contrasted with only 11% of those without CIN (1/9) (data not shown). HR-HPV types were even more common in SCC cases, 77.6%, with the remainder, 22.4%, negative (4.9%) or HPV-positive but the virus type could not be determined (17.5%). HR-HPV detection was associated with SCC at an OR of 27.25 (95% CI, 3.28-226.09), with any grade of CIN at an OR of 19.12 (95% CI, 2.31-157.81), and with CIN 3 lesions at an OR of 29.33 (95% CI, 3.47-247.85).

Table 2 shows the expression of survivin related to the presence or absence of HR-HPV in cervical lesions. Moderate and intense levels of survivin expression were associated with HR-HPV at an OR of 4.41 (95% CI, 2.42-8.02; P = .0001). There was a significant association between the different grades of survivin expression and the HR-HPV type in the lesions, while moderate and intense expression levels were more frequent in HR-HPV–positive than in HR-HPV–negative lesions, of which 16% were completely survivin-negative (12/74). Of the HR-HPV–positive cases, 84.6% showed moderate to intense survivin, as contrasted to 55% of the HR-HPV–negative cases (41/74; P = .0001).

Table 1

Survivin Expression and Grade of Cervical Lesions*

<table>
<thead>
<tr>
<th>Lesion Grade</th>
<th>Negative</th>
<th>Weak</th>
<th>Moderate</th>
<th>Intense</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN Negative (n = 9)</td>
<td>9 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1 (n = 20)</td>
<td>8 (40)</td>
<td>5 (25)</td>
<td>5 (25)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>2 (n = 21)</td>
<td>4 (19)</td>
<td>8 (38)</td>
<td>6 (29)</td>
<td>3 (14)</td>
</tr>
<tr>
<td>3 (n = 93)</td>
<td>2 (22)</td>
<td>11 (12)</td>
<td>26 (28)</td>
<td>54 (58)</td>
</tr>
<tr>
<td>SCC (n = 143)</td>
<td>1 (0.7)</td>
<td>19 (13.3)</td>
<td>48 (33.6)</td>
<td>75 (52.4)</td>
</tr>
<tr>
<td>Total (n = 286)</td>
<td>24 (8.4)</td>
<td>43 (15.0)</td>
<td>85 (29.7)</td>
<td>134 (46.9)</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.

Table 2

Survivin Expression Related to High-Risk HPV Types in the Lesions*

<table>
<thead>
<tr>
<th>High-risk HPV</th>
<th>Negative</th>
<th>Weak</th>
<th>Moderate</th>
<th>Intense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present (n = 201)</td>
<td>10 (5.0)</td>
<td>21 (10.4)</td>
<td>66 (32.8)</td>
<td>104 (51.7)</td>
</tr>
<tr>
<td>Absent (n = 74)</td>
<td>12 (16)</td>
<td>21 (28)</td>
<td>18 (24)</td>
<td>23 (31)</td>
</tr>
<tr>
<td>Total (n = 275)</td>
<td>22 (8.0)</td>
<td>42 (15.3)</td>
<td>84 (30.5)</td>
<td>127 (46.2)</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus.

* Data are given as number (percentage). Cases were included that were HPV-negative. Pearson P = .0001; \( \chi^2 \) test.
We then calculated the performance indicators (sensitivity, specificity, PPV, and NPV) for survivin staining as a marker of CIN and HR-HPV. As shown in Table 3, moderate or intense survivin expression is a 100.0% specific indicator of CIN, with a 100.0% PPV, because none of the biopsy specimens without CIN showed increased survivin expression. Negative staining, however, does not rule out CIN, because the NPV was less than 20%. As anticipated from the data in Table 2, survivin expression predicted HR-HPV with an 84.5% sensitivity and an 80.5% PPV.

Of the HPV-positive, CIN-treated women, the HR-HPV infection cleared in 41 (61%) of 67 during 705 woman months at risk, giving a monthly clearance rate of 5.8%, ie, 58/1,000 woman months at risk. Of the cases with moderate or intense expression of survivin, the infection cleared in 63% (32/51), compared with 57% (8/14) of cases with negative or weak survivin expression (P = .762). The corresponding percentages for virus persistence were 16% (8/51) and 7% (1/14), respectively (P = .670). In univariate (Kaplan-Meier) survival analysis, survivin expression (0-1 vs 2-3) was not a significant predictor of virus clearance (P = .947; log-rank) or virus persistence (P = .702; log-rank) after treatment of CIN.

As a final step, we tested the value of survivin expression as a predictor of disease outcome in patients with CC. Of the 150 patients with SCC, 91 (60.7%) were alive and 59 died during a mean follow-up of 52 months. Survival was slightly better in cases with increased survivin expression (60.2% [74/123]) than in cases with negative or weak expression (55% [11/20]; P = .0001). In Kaplan-Meier analysis, the survival curves crossed at 70 months of follow-up, resulting in a P value of .417 (log-rank test). HR-HPV showed a slight positive effect on survival in that 64.0% (71/111) of HR-HPV–positive and 44.0% (14/32) of HR-HPV–negative women were alive (P = .044; OR, 1.46; 95% CI, 0.964-2.21). In Kaplan-Meier analysis, this difference was significant (P = .0306; log-rank). As usual, the FIGO stage was a powerful predictor of survival in Kaplan-Meier analysis (P = .0001; log-rank).

In multivariate survival (Cox regression) analysis, survivin expression did not prove to be a significant independent prognostic factor but was removed from the model when adjusted for age, HR-HPV status, tumor grade, and FIGO stage. In the final Cox model, only the FIGO stage and age proved to be independent predictors of patient survival (both P = .0001). When FIGO stage 1 was used as the reference, the OR in stage 2 was 2.70 (95% CI, 0.85-8.60); in stage 3, 7.57 (95% CI, 2.53-22.59); and in stage 4, 63.22 (95% CI, 16.47-242.76) for dying of the disease. The mean age of women who were alive was 54.2 years compared with 66.7 years for those who died (P = .0001).

### Discussion

In the prediction of HPV-associated cervical disease (cancer and CIN), several issues are important. According to a recent task force on prognostic factors in CC, there is an urgent need for more specific markers capable of predicting the disease outcome in individual patients. In the prediction of CIN, the role of persistent HR-HPV as a cause of treatment failure has achieved increasing attention in the recent literature. Thus, monitoring this risk of disease recurrence after cone treatment using a suitable marker would be of considerable clinical value.

Two such molecules have been explored as potential markers of HR-HPV and CIN: p16INK4a and extracellular signal–related kinase-1 (ERK1). As an indicator of aberrant function of the p16INK4a/cyclin D/Rb pathway due to interference by the E7 oncoprotein, p16INK4a has been implicated as a specific marker of CIN and HR-HPV in several studies. In ERK1, in turn, is linked with another HPV-transforming protein, E5, whose effects are mediated through the activation of potent transcription factors (c-fos, myc, Ets1, Ets2, Elk-1, c-jun) by the ERK/mitogen-activated protein kinase (MAPK) signaling pathway. These divergent mechanisms also reflect the different associations of p16INK4a and ERK1 with HR-HPV and CIN.

One of the key mechanisms leading to the progressive phenotype in CIN lesions is the inhibition of apoptosis by HR-HPV oncoproteins. Thus, it was of interest to assess whether the members of the IAP family, such as survivin, would be of any value in predicting CIN progression or disease outcome in CC, which was done in the present study. Since its discovery in 1997, survivin has attracted considerable attention in many human malignant neoplasms (reviewed...
by Li\textsuperscript{15} and Altieri\textsuperscript{19,20}). Surprisingly few studies have addressed this IAP member in CC and its precursors,\textsuperscript{21-25} despite accumulated evidence suggesting several potentially important functions for this novel member of the IAP family.\textsuperscript{15,19,20} Accordingly, several lines of evidence currently imply that the inhibition of apoptosis by survivin in vivo and in vitro might be more selective compared with cytoprotection (apoptosis inhibition) by the other IAP members.\textsuperscript{19,20} Furthermore, survivin seems to be a pivotal cancer gene, not only because of its sharp expression in malignant lesions and absence in normal tissues, but also because of the potential exploitation of this pathway in cancer diagnosis and therapy.\textsuperscript{15,19,20,38} In many respects, CC would be an ideal target to analyze these properties of survivin, not the least because its precursors are well defined and its viral cause well established. Until now, practically all of these potential links between survivin and HPV have been completely unexplored.\textsuperscript{21-25}

We assessed whether survivin expression might be of use in predicting any of the several outcome measures: grade of CIN, HR-HPV type, clearance of the virus, and prognosis of CC. Survivin expression was totally absent in the 9 lesions rediagnosed as not being CIN (Table 1). This absence of survivin expression in normal epithelium is consonant with the data established in several other human malignant neoplasms,\textsuperscript{15,19,20} including CC.\textsuperscript{21,25} There are 2 reports, however, in which survivin expression also was detected in nonneoplastic cervical epithelium.\textsuperscript{23,24} In one of these, weak survivin expression was confined to the basal cell layer,\textsuperscript{24} whereas in the other study,\textsuperscript{23} moderately strong survivin expression in suprabasal cells was present in 70% of cases with normal epithelium. The former is more consistent with our observations, whereas the latter is strictly contradictory and difficult to explain in the light of the currently accumulated data on survivin expression in human tissues.\textsuperscript{15,18-20}

We were able to establish an almost linear relationship between the grade of CIN and the intensity of survivin expression (Table 1). The proportion of negative expression was inversely related to CIN grade, whereas moderate and intense expression increased in parallel with CIN grade ($P = .0001$). This observation is similar to that reported in one of the previous studies,\textsuperscript{24} but again, strictly contradictory to findings of the other study,\textsuperscript{23} in which survivin expression declined from CIN 1 through cancer. Yet another study analyzing survivin transcripts by reverse transcriptase–PCR failed to demonstrate a linear relationship to CIN grade.\textsuperscript{22}

We also analyzed the relationship of survivin expression to histologic differentiation of CC and to FIGO stage but failed to establish any correlation ($P = .204$ and $P = .142$, respectively). There is some indication that survivin expression in oral SCC is related to aggressive, high-grade lesions,\textsuperscript{39} but it was considered as an early marker of oral carcinogenesis in another study.\textsuperscript{40} The latter interpretation is consonant with the present data, in which moderate or intense survivin expression was evidenced already in 35% of CIN 1 lesions (7/20) and increased in parallel with lesion grade. In fact, survivin overexpression proved to be a 100.0% specific marker of CIN, because it never was found in biopsy specimens without CIN (Table 3). Also, the PPV for predicting CIN was 100.0%. In 1 previous study, specificity and PPV in predicting CIN 2 and 3 were calculated for survivin transcripts,\textsuperscript{22} 52.9% and 65.2%, respectively. When we did the same, survivin expression (negative-weak vs moderate-intense) predicted CIN 3 with a specificity of 68% and a PPV of 83.3% (data not shown).

It recently has been shown that p16\textsuperscript{INK4a} seems to be a specific biomarker of cells harboring HR-HPV infection,\textsuperscript{9,10,26} whereas ERK1 as a marker of the ERK/MAPK cascade (activated by HPV E5 protein) did not show any such specificity for oncogenic HPV types.\textsuperscript{37} One recent study reported the most intense survivin expression in cervical lesions with morphologic signs of HPV, but no type-specific data are given.\textsuperscript{23} It is interesting that the present data indicate that survivin expression is related significantly ($P = .0001$) to HR-HPV types (Table 2). In lesions with HR-HPV, only 15.4% had negative or weak expression, contrasted with 45% of the lesions without oncogenic HPV (33/74).

This discriminative power between the HR-HPV and LR-HPV types seems similar for survivin and p16\textsuperscript{INK4a} with almost identical (84.5%) sensitivity and (80.5%) PPV (Table 3), as well as ORs around 4.5.\textsuperscript{26} When entered in a multivariate regression model, survivin and p16\textsuperscript{INK4a} were equally strong independent predictors of HR-HPV (OR, 3.23; 95% CI, 1.72-6.09; OR, 3.45 [95% CI, 1.83-6.52], respectively). The practical implications of these data remain to be established. It seems intriguing to combine these 2 markers (and possibly others) to calculate the performance indicators for such a combination, which will be done as soon as the analysis of all 13 markers programmed for this study\textsuperscript{27} have been completed.

During the past few years, persistent HR-HPV infections have achieved increasing attention as a cause of significantly increased risk of failure of treatment for CIN.\textsuperscript{30,35,36} Monitoring this risk of disease recurrence after cone treatment using a suitable marker would be of considerable clinical value, and we were interested to see whether survivin expression might be of any such predictive value for HR-HPV persistence or clearance. In univariate survival analysis, the survivin expression pattern did not provide any useful information as a predictor of these 2 viral events in the cervix following the treatment of CIN. Together with previous data,\textsuperscript{26,37} this implies that at least these 3 biomarkers (survivin, ERK1, and p16\textsuperscript{INK4a}) cannot substitute for HPV testing in monitoring the risk of disease recurrence after cone treatment.\textsuperscript{30,35,36}

As recently concluded by the Prognostic Factor Committee of the European Society of Gynaecological Oncology,\textsuperscript{34} there is an urgent need for specific prognostic biomarkers in cervical
carcinoma. In searching for such potential prognostic markers, Branca et al.\textsuperscript{26,37} recently showed that p16\textsuperscript{INK4a} and ERK1 expression were of no value as predictors of disease outcome in CC. Unfortunately, the same proved to be the case with survivin analyzed by univariate and multivariate techniques in the present study. When entered in a Cox multivariate model, survivin expression was removed from the model, and of all the entered variables (HR-HPV, tumor grade, survivin, FIGO stage, age), only the last 2 remained significant independent prognostic factors at the $P = .0001$ level. Thus, survivin expression analyzed by immunohistochemical analysis does not seem to have independent predictive value for disease outcome in CC. Similar data have been reported in urothelial carcinoma\textsuperscript{41} and in lung cancer\textsuperscript{42}; survivin was not related to disease prognosis. As recently reviewed,\textsuperscript{15,19,20} however, there is ample evidence to suggest that survivin expression in several human tumors is an ominous prognostic sign. Additional studies clearly are needed to establish the prognostic significance of survivin in CC based on a larger cohort and longer follow-up than available in the present study.

Taken together, our results indicate that survivin expression is an early marker of cervical carcinogenesis, being expressed with increasing intensity from low-grade CIN onward. It is interesting that survivin overexpression is related significantly to the presence of HR-HPV types in CIN and CC, closely resembling p16\textsuperscript{INK4a} \textsuperscript{9,10,26} Review of the data from the studies on the molecular basis of survivin overexpression in human cancer reveals that there is something that might provide a plausible explanation for such a molecular link between HR-HPV and deregulated survivin.\textsuperscript{19} Among the several mechanisms responsible for deregulated survivin, one is the transcriptional repression of survivin by wild-type p53.

Indeed, 3 groups have independently reported that survivin is one of the genes that is repressed transcriptionally by wild-type p53.\textsuperscript{43-45} It also was shown that transcriptional repression of survivin contributed to p53-dependent apoptosis,\textsuperscript{44,45} while on the other hand, survivin expression antagonized p53-induced cell death.\textsuperscript{45} It is self-evident that the well-established function of HR-HPV (but not LR-HPV) E6 oncoprotein to mediate the degradation of p53 through binding to it would provide a plausible explanation of why up-regulation of survivin is associated so intimately with HR-HPV types in cervical lesions. Because it is not confined exclusively to lesions with HR-HPV types, however, survivin deregulation in cervical carcinogenesis probably has (several?) other triggering mechanisms, as currently established in other settings.\textsuperscript{15,19,20} This is a practically unexplored field for basic HPV researchers.

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