Activation of the ERK/MAP Kinase Pathway in Cervical Intraepithelial Neoplasia Is Related to Grade of the Lesion but Not to High-Risk Human Papillomavirus, Virus Clearance, or Prognosis in Cervical Cancer

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A b s t r a c t

We subjected 302 archival samples (150 squamous cell carcinomas [SCCs] and 152 cervical intraepithelial neoplasia [CIN] lesions) to immunohistochemical staining with extracellular signal–regulated kinase-1 (ERK1) antibody and human papillomavirus (HPV) testing with 3 primer sets. Follow-up data were available for all SCC cases and 67 CIN cases.

High-risk (HR) HPV types were associated with CIN (odds ratio [OR], 19.12; 95% confidence interval [CI], 2.31-157.81) and SCC (OR, 27.25; 95% CI, 3.28-226.09). There was a significant linear relationship between lesion grade and ERK1 staining intensity ($P = .0001$). ERK1 staining was a 100% specific indicator of CIN, with a 100% positive predictive value, but a poor predictor of HR HPV. ERK1 expression did not predict clearance or persistence of HR HPV after CIN treatment. ERK1 staining did not significantly predict survival in cervical cancer in univariate ($P = .915$) or multivariate analysis. After adjustment for HR HPV, ERK1 expression did not predict survival in cervical cancer in univariate ($P = .915$) or multivariate analysis. After adjustment for HR HPV, stage, age, and tumor grade in the Cox regression model, only stage ($P = .0001$) and age ($P = .002$) remained independent prognostic factors.

ERK1 expression seems to be an early marker of cervical carcinogenesis. ERK1 overexpression is not a specific marker of HR-HPV in CIN and cervical cancer, nor does it predict virus clearance after CIN treatment or disease outcome in cervical cancer.

The key etiologic role of human papillomavirus (HPV) in the development of cervical cancer and its precursors has been documented convincingly.1-5 In large epidemiologic studies, high-risk (HR) HPV types have been shown to associate with cervical cancer in almost 100% of cases, in contrast with low-risk HPV types that rarely associate with cervical cancer and its precursors.1,2,5-7 The different oncogenic potential of low-risk HPV and HR HPV seems to be linked, at least in part, with the different functions of 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 from their complex with active hypophosphorylated pRB, resulting in G1/S transition of the cell cycle.8-10

In addition to the strongly transforming proteins E6 and E7, another HPV protein, E5, seems to be weakly oncogenic and has been suggested to potentiate the transforming activity of E7.2-5,11-13 These oncogenic functions of HR HPV E5 are mediated by up-regulation of the epidermal growth factor (EGF) receptor, EGFR,2,5,11-14 known to be expressed in practically all cervical intraepithelial neoplasia (CIN) lesions.15 Whether E5 interacts directly with EGFR remains a matter of dispute, but EGF-dependent and EGF-independent mechanisms have been implicated.14,16 In either case, the key event in E5-induced cell proliferation seems to be an activation of mitogen-activated protein (MAP) kinases (MAPK) in the MAPK signaling pathway.14,16-18

This links HPV E5 protein to the intracellular phosphorylation cascade, initiated by the protein tyrosine kinase growth factor receptors, like EGFR, that on binding with their ligands, activate the oncogene ras on plasma membrane.16,17,19

This
PKC-independent pathway. So far, all of these studies have been conducted in vitro using different cell lines, including cervical cancer cell lines. Interestingly, apart from HR HPV, some low-risk HPV types also seem to be able to activate this ERK/MAPK signaling pathway. HPV-induced activation of this cascade is mediated by the E5 protein, shown to act through at least 2 pathways in human keratinocytes: (1) a phosphokinase-C (PKC)-mediated, EGFR-independent pathway and (2) an EGFR-dependent, PKC-independent pathway. So far, all of these studies have been conducted in vitro using different cell lines, and unlike reports for many other carcinomas, no reports are available on HPV-induced activation of the ERK/MAPK signaling cascade in cervical cancer or its precursor lesions.

Prompted by the recent data on p16INK4a as a useful marker of HR HPV types and CIN, we analyzed a series of cervical carcinomas and CIN lesions to assess whether ERK1 (as a marker of an activated ERK/MAPK signaling cascade) might be of any use in predicting the grade of CIN, the HPV type, clearance of the virus after eradication of CIN, and the prognosis in cases of cervical cancer. Expression of ERK1 was studied using immunohistochemical analysis in CIN lesions treated by conization and followed up by serial polymeerase chain reaction (PCR) for HPV clearance, and survival data in cervical cancer were related to ERK1 expression in the surgical samples.

Materials and Methods

The material of this study comprises the retrospective component of the HPV-Pathogen National Institute of Health project and was collected from the files of the Pathology Departments of 2 Italian hospitals (S. Orsola Malpighi Hospital, Bologna, and Maggiore Hospital, University of Trieste). Altogether, this prospective biopsy material comprises samples from 302 patients with an invasive cervical squamous cell carcinoma (SCC) or CIN diagnosed and treated in these hospitals between June 1986 and March 2002. Of the 302 cases, 114 CIN and 38 SCC cases were from Bologna, and 38 CIN lesions and 112 SCCs were available from Trieste. The mean age of patients with CIN was 35.5 years (range, 18-79 years), and that of patients with SCC was 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, 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 cases from Bologna had been followed up at 6-month intervals after cone treatment (mean, 10.5 months; range, 2.4-27.6 months) and subjected to repeated colposcopy, Papanicolaou 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. The clinical International Federation of Gynecology and Obstetrics (FIGO) stage of the disease was known for 125 patients with SCC.

Biopsy and Surgical Samples

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. On histologic examination, the lesions were graded using nomenclature and categorized as CIN1, CIN2, or CIN3. The histologic diagnosis of SCC was confirmed in all cases, and 2 adenocarcinomas originally present were excluded from this series.

Immunohistochemical Analysis for ERK1

Immunohistochemical staining for ERK1 MAPK expression was completed using 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 concentration, 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 nonspecific binding sites with goat-rabbit serum. Sections were incubated with the primary antibody, polyclonal rabbit ERK1 antibody (No. 7947, dilution 1:50; Abcam, Cambridge, England), in a humidified chamber for 1 hour at room temperature. This polyclonal (IgG) antibody was raised in rabbits against a synthetic peptide mapping within subdomain XI of rat ERK1. This antibody reacts with ERK1 (p44) and, to a lesser extent, ERK2 (p42) MAPKs.

The 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 ERK1 protein was localized by incubation with diaminobenzidine. As a final step, the slides were stained.
with light hematoxylin counterstaining. Negative control samples 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 evaluated by using a light microscope (Leitz Diaplan, Leitz Wetzlar, Heidelberg, Germany) equipped with a digital camera (Leica DG300, Leica, Heidelberg, Germany). In normal squamous epithelium, weak positive (cytoplasmic) staining for ERK1 was detected, confined to the upper parabasal and lower intermediate cell layers. In original grading of the immunohistochemical staining, semiquantitative scoring into 3 categories was used: 1, slight staining, equivalent to that of normal squamous epithelium; 2, moderately increased staining, in which the numbers of positively stained cells (cytoplasmic and/or nuclear) are clearly increased; and 3, intense staining, with almost all cells diffusely staining throughout the lesion (intense nuclear or cytoplasmic staining).

**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, as described in the following sections.

**DNA Extraction**

Paraffin-embedded tissue sections (5-µm sections not weighing more than 25 mg) were treated with xylene to remove paraffin and digested with ATL buffer 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, Hilden, Germany).

**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'-GGCGGCACCACCATGTACCCT-3'; antisense, 5'-AGGGGCCGGACTGTCATACT-3'). The PCR mix contained 200-µmol/L concentrations of each deoxynucleoside
A low-power view of high-grade cervical intraepithelial neoplasia 3, showing several mitotic figures and apoptotic cells. Positive immunohistochemical staining for extracellular signal–regulated kinase-1 (ERK1) is easily detectable in the nuclei of the neoplastic cells in all layers. In addition, distinct positive cytoplasmic staining also is present, which is most intense in the cells in the lower half of the lesion (ERK1, original magnification ×100).

Higher power detail of another cervical intraepithelial neoplasia grade 3 (CIN3) lesion. The immunohistochemical expression of extracellular signal–regulated kinase-1 (ERK1) is practically absent in this lesion, in which no cytoplasmic immunostaining can be detected. A few cells with nuclear staining are scattered close to the epithelial surface. For CIN3, this was an unusual ERK1 pattern (ERK1, original magnification ×400).

Detail of an invasive squamous cell carcinoma. All invasive foci are decorated with intense, diffuse staining for extracellular signal–regulated kinase-1 (ERK1), which is detectable against the ERK1-negative, blue stromal background. Immunohistochemical staining of this intensity represents grade 3 in our grading. In this case, the immunostaining is confined to the cytoplasm, with no positively stained nuclei easily recognizable (ERK1, original magnification ×250).

Another case of invasive cancer, also associated with strong overexpression of extracellular signal–regulated kinase-1 (ERK1). Compared with the lesion in Image 5, the immunohistochemical staining pattern is different in that intense cytoplasmic and strong nuclear staining for ERK1 are clearly discernible. Fewer than half of the nuclei stained only blue, showing absent ERK1 expression (ERK1, original magnification ×400).
triphosphate (dNTP), a 1.5-mmol/L concentration of magnesium chloride, 1x PCR buffer, 40 pmol of sense and antisense primers, and 1.25 U of AmpliTaq Gold (Applied BioSystems, Branchburg, NJ). The PCR conditions were as follows: 94°C for 10 minutes for 1 cycle; 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 25 cycles; and 72°C for 7 minutes.

The samples then were amplified for the presence of HPV using different sets of degenerated primers as described separately for MY09/MY11,35 GP5+/GP6+,36 and biotinylated short PCR-fragment primers (SPF) primer mix located within the L1 region of the HPV genome.37 The PCR conditions for MY09/MY11 were as follows: 94°C for 10 minutes for 1 cycle; 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 30 seconds for 40 cycles; followed by an extension step at 72°C for 7 minutes. The PCR mix contained a 200-µmol/L concentration of each dNTP, 40 pmol of each primer, 1x PCR buffer, and 1.25 U of AmpliTaq Gold. For the GP5+/GP6+ primers, the following conditions were used: 94°C for 10 minutes for 1 cycle; 95°C for 30 seconds, 44°C for 60 seconds, and 72°C for 90 seconds for 40 cycles; and a final extension step at 72°C for 7 minutes. Amplification with SPF primer mix was carried out as follows: 94°C for 10 minutes for 1 cycle; 94°C for 30 seconds, 52°C for 45 seconds, and 72°C for 45 seconds for 40 cycles; and a final extension step at 72°C for 7 minutes. Positive and negative control samples were included in each amplification.

None of the samples was positive exclusively with the primer set MY09/MY11, whereas GP5+/GP6+ and 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 SPF and GP5+/GP6+ primers and the other 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.

**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 bromochloroindolyl 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.37

**Statistical Analyses**

Statistical analyses were performed using the SPSS and STATA software packages (SPSS for Windows, version 11.5, SPSS, Chicago, IL; and STATA/SE 8.2, STATA, College Station, TX). Frequency tables were analyzed by using the $\chi^2$ test, with Pearson correlation and/or likelihood ratio used to assess the significance of the correlation between the categorical variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using the exact method. Differences in the means of continuous variables were analyzed by 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 ORs (and 95% CI). Performance indicators of the ERK1 marker for detecting CIN or HR HPV were calculated by using the conventional contingency tables to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with the 95% CI based on the F distribution ($\pm 1.96 \times SE$). 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 enter and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log survival plots. In all tests, a $P$ value of less than .05 was considered statistically significant.

**Results**

Table 11 shows the expression of ERK1 related to grade of the lesion in cone (for CIN) or surgical (for SCC) specimens. There was a direct relationship between the increasing grade of the lesion and the intensity of the ERK1 staining, in that the frequency of strong ERK1 expression increased from 0% (0/10) in biopsy specimens without CIN to 73.2% in those with SCC ($P = .0001$). By using the 2-tier category of staining (normal or increased), increased ERK1 expression was associated with a high-grade lesion (CIN3 or cancer) at an OR of 15.09 (95% CI, 7.42-30.68). When SCC cases were excluded, this association had an OR of 8.90 (95% CI, 4.05-19.54) ($P = .0001$).

Of all CIN lesions, 70.5% were positive for HR HPV, contrasted with only 11.1% of those without CIN (data not shown). HR HPV types were even more common in SCC cases, 77.6%; the remaining 22.4% were HPV-negative (4.9%) or HPV-positive but the virus type could not be
ERK1 (40.2%) and in those with normal expression, the death rate was practically identical in cases with increased ERK1 expression (40.0%) \((P = .633)\). In Kaplan-Meier analysis, the survival curves crossed at several points, resulting in \(P = .915\) (log-rank test) \(\text{Figure 1}\). HR HPV showed a slight positive effect on survival in that 64.0% of HR HPV–positive and 43.8% of HR HPV–negative women were alive (OR, 1.46; 95% CI, 0.964-2.21; \(P = .044\)). In Kaplan-Meier analysis, this difference was significant, however \((P = .036); \) log-rank test). As usual, the FIGO stage was a powerful predictor of survival in the Kaplan-Meier analysis \((P = .0001); \) log-rank test).

In multivariate survival analysis (Cox regression), ERK1 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 \((P = .0001)\) and age \((P = .002)\) proved to be independent predictors of patient survival (OR, 1.04; 95% CI, 1.020-1.076). When FIGO stage 1 was used as the reference, the OR for stage 2 was 3.04 (95% CI, 0.96-9.65); for stage 3, the OR was 7.06 (95% CI, 2.37-20.97); and for stage 4, the OR was 32.90 (95% CI, 9.76-110.90) for dying of the disease.

\(\text{Table 1}\) shows the expression of ERK1 related to the presence or absence of HR HPV in cervical lesions. In the whole series, increased ERK1 expression was associated with HR HPV \((OR, 1.44; 95\% \text{ CI, 0.700-2.98); } P = .208\). There was no significant association between ERK1 expression and the HR HPV type in the lesion, although strong expression was more frequent \((53.0\%) \text{ in HR HPV–positive than in HR HPV–negative lesions (38}\% \text{ [29/76])}. When the 2-tier category of staining \((increased \text{ or normal}) \text{ was used, the percentages for HR HPV–positive cases were 68.8\% and 31.2\%, and those for HR HPV–negative cases were 60.4\% and 39.6\%, for increased and normal ERK1, respectively \((P = .208)\). When only CIN lesions were counted, the association of increased ERK1 expression with HR HPV was not significant \((P = .542)\).

We then calculated the performance indicators \((sensitivity, specificity, PPV, NPV\)) for ERK1 staining as a marker of CIN and HR HPV. As shown in \(\text{Table 3}\), increased ERK1 staining is a 100% specific indicator of CIN, with a 100% PPV, because none of the biopsy specimens without CIN showed increased ERK1 expression. Negative staining, however, does not rule out CIN, because the NPV was only 20.0%. As anticipated from the data in Table 2, ERK1 expression was a poor predictor of HR HPV.

Of HPV-positive women treated CIN, 41 \((61\%)\) of 67 experienced clearing of the HR HPV infection during 705 women-months at risk, giving a monthly clearance rate of 5.8% \((ie, 58/1,000 \text{ women-months at risk})\). Of the cases with increased ERK1 expression, 54.0% experienced clearing of the infection, compared with 83.3% of those with normal ERK1 expression \((P = .059)\). The corresponding percentages for virus persistence were 16.0% for cases with increased ERK1 expression and 8.3% for cases with normal ERK1 expression \((P = .441)\). In univariate (Kaplan-Meier) survival analysis, increased ERK1 staining was not a significant predictor of virus clearance \((P = .439); \) log-rank test) or virus persistence \((P = .645); \) log-rank test) \(\text{Figure 1}\). Thus, the ERK1 expression pattern \((normal \text{ or increased}) \text{ did not predict clearance or persistence of HR HPV types in the cervix after treatment for CIN.)

As the final step, we analyzed the value of ERK1 staining as a predictor of disease outcome in patients with cervical cancer. Of 150 patients with SCC, 91 \((60.7\%)\) were alive, and 59 \((39.3\%)\) died during a mean follow-up of 51.7 months. The mean survival time for patients who eventually died was 25.3 months \((range, 0.1-177.6 \text{ months})\). The death rate was practically identical in cases with increased ERK1 \((40.2\%)\) and in those with normal expression (40.0%) \((P = .633)\). In Kaplan-Meier analysis, the survival curves crossed at several points, resulting in \(P = .915\) (log-rank test) \(\text{Figure 1}\). HR HPV showed a slight positive effect on survival in that 64.0% of HR HPV–positive and 43.8% of HR HPV–negative women were alive (OR, 1.46; 95% CI, 0.964-2.21; \(P = .044\)). In Kaplan-Meier analysis, this difference was significant, however \((P = .036); \) log-rank test). As usual, the FIGO stage was a powerful predictor of survival in the Kaplan-Meier analysis \((P = .0001); \) log-rank test).

In multivariate survival analysis (Cox regression), ERK1 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 \((P = .0001)\) and age \((P = .002)\) proved to be independent predictors of patient survival (OR, 1.04; 95% CI, 1.020-1.076). When FIGO stage 1 was used as the reference, the OR for stage 2 was 3.04 (95% CI, 0.96-9.65); for stage 3, the OR was 7.06 (95% CI, 2.37-20.97); and for stage 4, the OR was 32.90 (95% CI, 9.76-110.90) for dying of the disease.
Discussion

Predicting disease outcome is a major challenge in modern medicine. Concerning the prediction of HPV-associated cervical disease, several issues are important.1,2,5 These are related to the management of women with diagnosed CIN and those with cervical cancer. In the former, 2 issues are closely linked with disease outcome: (1) curative (radical) excision of CIN by cone, laser, or large loop excision of transformation zone (also known as LLETZ), and (2) clearance or persistence of HR HPV after treatment.2,5,34 Apart from the well-recognized risk of recurrence associated with the margin involvement in the cone, the role of persistent HR HPV as a cause of treatment failure has achieved increasing attention in recent literature.34,38,39 The prognosis of cervical cancer, on the other hand, is determined by several predictors (clinical and molecular), and, according to a recent task force on prognostic factors in cervical cancer, there is an urgent need for more specific markers capable of predicting the disease outcome in individual patients.40

Table 3
Performance Indicators of ERK1 as a Marker of CIN and High-Risk HPV Type*

<table>
<thead>
<tr>
<th>Outcome Variable</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN‡</td>
<td>70.3 (62.9-77.7)</td>
<td>100.0 (100.0-100.0)</td>
<td>100.0 (100.0-100.0)</td>
<td>20.0 (13.5-26.5)</td>
</tr>
<tr>
<td>High-risk HPV</td>
<td>81.0 (78.8-83.3)</td>
<td>26.3 (21.1-31.5)</td>
<td>74.3 (69.2-79.5)</td>
<td>34.4 (28.8-40.0)</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; ERK, extracellular signal–regulated kinase; HPV, human papillomavirus; NPV, negative predictive value; PPV, positive predictive value.

* Values in parentheses are the 95% confidence intervals.
† ERK1 staining (grades 2 and 3/grade 1) only.
‡ Any grade of CIN (squamous cell carcinoma cases excluded).
As an indicator of aberrant function of the p16INK4a/cyclin D/Rb pathway due to interference by the E7 oncoprotein of HR HPV, p16INK4a has been implicated as a specific marker of CIN and HR HPV. In addition to the well-known strong oncopgenes, E6 and E7, E5 also seems to be oncogenic and capable of potentiating the transforming activity of E7. The known molecular mechanisms of E5-induced cell proliferation are completely different from those of E6 and E7, and ERK2 overexpression in the early phases of prostate, colon, and bladder carcinogenesis, with progressive loss of ERK-positive cells when poorly differentiated. In our material, there was no difference in ERK1 expression in the 3 histologic grades of tumor differentiation, however (P = .857). When these findings are considered together, ERK1 as an indicator of an activated ERK/MAPK signaling pathway seems to be an early marker in a wide range of epithelial human tumors, including cervical carcinoma and its precursors. In our series, ERK1 overexpression proved to be a 100% specific marker of CIN, and was never found in biopsy specimens without CIN (Table 3).

As recently concluded by the Prognostic Factor Committee of the European Society of Gynaecological Oncology, there is an urgent need for specific prognostic biomarkers in cervical carcinoma. We recently showed that p16INK4a expression was of no value as a predictor of disease outcome in cervical cancer. Similarly, in the present analysis, there was no indication that ERK1 has any prognostic value in cervical cancer, even in univariate analysis (Figure 2). When entered in the Cox multivariate model, ERK1 was removed from the model, and of all entered variables (HR

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HPV, tumor grade, ERK1, FIGO stage, age), only the last 2 remained significant independent prognostic factors ($P = .0001$ and $P = .002$ for FIGO stage and age, respectively).

The results of immunohistochemical staining for ERK1 were different from those of $\text{p16}^{\text{INK4a}}$ observed in our recent study,\textsuperscript{11} in that ERK1 expression bears no association with the presence of HR HPV in cervical lesions. Although both $\text{p16}^{\text{INK4a}}$ and ERK1 are linked with 2 oncogenic HPV proteins, E7 and E5, respectively, the differences observed in the present study are feasibly explained by the divergent molecular pathways, through which these 2 transforming proteins stimulate cell proliferation. Whereas E7 does interfere with the $\text{p16}^{\text{INK4a}}$/cyclin D/Rb pathway,\textsuperscript{9,10,41} E5 mediates overexpression and activation of the ERK/MAPK signaling cascade.\textsuperscript{13,14,16,17,28} The major difference between the 2 mechanisms is that E7 seems to be specific in its actions, whereas this is not the case with E5 functions. Thus, $\text{p16}^{\text{INK4a}}$ is a highly specific marker of cells expressing HR HPV, in contrast with ERK1, which did not show any specificity to HR HPV in the present study. Clearly, more data are needed on the possible interactions between E5 and E7 and how the former potentiates the transforming capacity of the latter, as suggested some time ago.\textsuperscript{11,12}

Two plausible explanations for this lack of specific association between HR HPV and ERK1 overexpression can be offered: (1) Apart from E5, there are multiple other mechanisms that mediate their effects through activation of ERK/MAPK pathways. (2) ERK/MAPK stimulation is not restricted to E5 of the oncogenic HPV types. The former is an established fact, because a wide variety of extracellular signals use ERK/MAPK signaling pathways in the activation of nuclear transcription factors.\textsuperscript{21,22,24,25} There also is recent evidence implying that E5 of the nononcogenic HPV types can activate the ERK/MAPK pathway, as is shown to be the case for 1 cutaneous HPV and 1 mucosal low-risk HPV—HPV2a,\textsuperscript{18} and HPV6b,\textsuperscript{29} respectively.

Taken together, our results suggest that ERK1 expression seems to be an early marker of cervical carcinogenesis, as suggested for other human epithelial neoplasia,\textsuperscript{20} being expressed with increasing intensity starting from the low-grade CIN. It is important to note, however, that ERK1 overexpression is not a specific marker of HR HPV in CIN and cervical cancer, and it does not predict disease outcome in the latter.

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