The relationship between c-FLIP expression and human papillomavirus E2 gene disruption in cervical carcinogenesis☆

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Abstract

Objective. Human papillomavirus (HPV) is the essential causative factor in cervical carcinogenesis, and apoptosis inhibition is one of the key features of HPV-induced malignant transformation. This study is to investigate the possible cause–effect association between high-risk HPV and cellular FLICE-like inhibitory protein (c-FLIP), an important apoptosis regulator, during cervical carcinogenesis.

Methods. A series of 80 archival samples, including 20 squamous cervical carcinomas (SCC) 54 cervical intraepithelial neoplasia (CIN) lesions and 6 normal cervical tissues, were subjected for c-FLIP immunohistochemical staining and HPV HC-II analysis. Typing HPV-16 infection was analyzed by the polymerase chain reaction (PCR), and its status was assessed with the integrity and disruption of the HPV-16 E2 gene, which was amplified in three overlapping fragments.

Results. The types of HR-HPV infection and E2 disruption were associated closely with cervical lesion severity. There was a significant relationship between lesion grade and c-FLIP expression level. c-FLIP overexpression was also closely associated with HR-HPV infection and its integration status. Multivariate regression analysis revealed c-FLIP as a strong independent predictor for CIN, with 100% PPV, and showed 90.9% PPV in detecting HR-HPV, and remained a significance factor to rule out which case has no HR-HPV integration, with a 94.7% sensitivity and a 90.0% NPV.

Conclusions. The present data approved that c-FLIP overexpression is related significantly to the presence of HR-HPV infection and its integration status during progression of cervical squamous cell cancer and confirmed the role of c-FLIP as an early marker of cervical carcinogenesis.

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Keywords: Cellular FLICE-like inhibitory protein; Human papillomavirus; E2 gene; Cervical carcinogenesis

Introduction

Invasive cervical carcinoma (CC) is a multi-step process, developing through stages designated cervical intraepithelial neoplasia (CIN), low-grade and high-grade, in order of increasing severity. These lesions, which are often associated with human papillomavirus (HPV) infection, represent a paradigm for in vivo human carcinogenesis. Over 100 types of HPVs have now been described and been categorized into low-risk, intermediate-risk and high-risk type, based on their association with clinical disease. HPV-16 is the predominant high-risk type detected in both CC and CIN. High-risk HPVs (HR-HPV) are thought to cause the carcinogenesis of CC through binding and inactivation of tumor suppressors, p53 [1] and retinoblastoma protein (Rb) [2], by the viral-transforming protein E6 and E7 respectively. HPV-transforming protein E2 is involved in viral genome replication and transcriptional control. Binding of E2 to sites near the constitutive early promoter of HR-HPV can negatively regulate expression of E6 and E7, leading to cell cycle arrest and the efficient immortalization of primary human keratinocytes [3]. In pre-neoplastic cells, the HPV genome has been

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found as an extra-chromosomal viral element. However, integration of HR-HPV into the host genome is frequent in CC [4,5]. When the viral DNA integrates, disruption of E2 expression has been found in several studies and viral integration has been proposed as an activation mechanism for progression from high-grade CIN to invasive CC. Sequential decrease of viral E2 protein was found to be associated with the transformation of cervical intraepithelial lesions, being highest in CIN1 and lowest in CIN3 [6], and this E2 protein was found inversely related to the viral DNA copy number [7]. Disruption of E2 expression is proposed to be associated with poor prognosis in CC [8]. However, detailed events that occur during HPV integration are still under investigation.

Apoptosis is a tightly regulated form of cell death that is implicated in the defense against pathogens and cancer. Inhibition of apoptosis is one of the key features of HPV-induced malignant transformation [9]. Apoptotic cell death can be triggered by various stimuli, including engagement and activation of death receptors belonging to the TNF receptor (TNFR) superfamily [10]. An important regulator of this pathway is cellular FLICE-like inhibitory protein (c-FLIP) [11]. Two splice variants, c-FLIPS and c-FLIPL, were expressed in mammalian cells. c-FLIPS consists of two death effector domain (DED) motifs, and c-FLIPL additionally contains a catalytically inactive caspase domain. Since c-FLIP has shown to act as dominant negative inhibitors of FADD, a crucial mediator of death receptor signaling, it was suggested to inhibit death-receptor-mediated apoptosis by displacing DED-containing caspase-8 from the death-inducing signaling complex (DISC). High expression of c-FLIP was previously detected in many human malignancies and its expression has been crucially related to favor tumor growth and immune escape of tumors [12,13]. Some progress also has been made with therapeutic agents targeting the c-FLIP pathway, resulting in increased apoptosis of cancer cells [14,15]. To our knowledge, little is known about c-FLIP expression with respect to human cervical tumorigenesis in vivo, or its association with any of the viral events, e.g., virus persistence or integration, known to be valuable intermediate endpoint markers of CC and cervical carcinogenic progression associated with HPV.

In the present study, we analyzed c-FLIP expression in different grades of cervical dysplasia and CC with respect to tumor progression by immunohistochemistry method and characterized the disruption of HPV-16 E2 gene in those lesions using the overlapping primer-based polymerase chain reaction (PCR) to explore the possible cause–effect association between HR-HPV integration and c-FLIP expression during cervical tumorigenesis.

Materials and methods

Tissue specimens

Tissue specimens were obtained via biopsy of suspicious cervical lesions, conization, and hysterectomies at the Department of Gynecology and Obstetrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology in China, from September 2000 to October 2002. All specimens 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 histological examination, lesions were graded according to WHO criteria and categorized as CIN1, CIN2, or CIN3 and CC. The histological diagnosis of CC was confirmed in all cases, and the clinical International Federation of Gynecology and Obstetrics (FIGO) disease stage was available for them. Patients diagnosed with adenocarcinoma were excluded. A total of 74 lesions, including 14 CIN1, 18 CIN2, 22 CIN3 and 20 invasive CC (range from stage Ia to Ila) were enrolled in this study. As a source of non-neoplastic tissue, normal cervical sections were also obtained from 6 samples of hysterectomy performed for other reasons.

Immunohistochemical analysis

Immunohistochemical staining for expression of the c-FLIP 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) in a microwave oven (85 °C–95 °C, 3 times for 5 min each), followed by blocking the nonspecific binding sites with goat serum. Sections were incubated with the primary antibody, polyclonal rabbit FLIP antibody (No. RB-9250-P0, dilution 1:100; Lab Vision, CA, USA), in a humidified chamber at 4 °C overnight. This polyclonal antibody has been raised in rabbits against a synthetic peptide derived from C-terminus of human FLIP (including c-FLIPS and c-FLIPL). This antibody reacts with human and mouse FLIP, and other species have not been tested. Primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat anti-rabbit IgG at room temperature for 30 min. Slides then were processed with the universal SP histostain™-plus kit (ZYMED, Carlsbad, CA, USA), and expression of c-FLIP was localized by incubation with 3,3′-diaminobenzidine tetrahydrochloride substrate. 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 colon carcinoma were used as positive control samples.

Evaluation of immunohistochemical staining

Immunohistochemical staining was examined using a light microscope (Olympus, Japan) equipped with a digital camera (Nikon, Japan). c-FLIP was detected as predominantly cytoplasmic staining in positive specimens. Slides were evaluated by two independent pathologists without clinicopathological knowledge. In original grading of immunohistochemical staining, 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) were clearly increased; and 3, intense staining, with almost all cells staining strongly. In original grading of immunohistochemical staining, 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) were clearly increased; and 3, intense staining, with almost all cells staining strongly. In statistical analysis, the staining results were used as dichotomous categorical variables combining the aforementioned categories as negative–weak (0–1) and moderate–intense (2–3; strong) or using the 4-tier categorization.

DNA extraction

Paraffin-embedded tissue samples (5-μm sections not weighing more than 25 mg) were treated with xylene to remove paraffin and digested with tissue lysis buffer (10 mmol/l Tris–Cl, pH 8.0, 1 mmol/l EDTA, 0.1 mol/l NaCl) and protease K (10 mg/ml, re-added every 24 h) for 72 h at 56 °C in a water bath, and DNA was extracted according to the manufacturer’s instructions (QIAamp DNA mini kit, QIAGEN, Hilden, Germany).

High-risk HPV’s testing and typing

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 a 498-bp β-actin fragment (sense: 5′-AGCCATGTAACATTGCTATCC-3′; antisense: 5′-TGGCGGTAAGGTCTTGGC-3′). HPV testing was performed by the hybrid capture II (HC-Il, Digene) microplate test [16]. Then, the positive samples were amplified for typing HPV-16 using primer mix as
described [17] located within the E7 region of the HPV-16 genome (315-bp product, sense: 5’-AGAAGACGCTGATCAT-3’; antisense: 5’-TTATGTTTCTGAGAAGAGA-3’).

Analysis of HPV-16 E2 gene integrity

The E2 gene was amplified in three overlapping fragments, each of which is smaller in size than the internal control amplimer, using the following primer pairs [5]: amplimer a (475-bp product): a1, 5’-ACCTGACCTCCATCAGATCT-3’; a2, 5’-ACTGACCTCCATCAGATCT-3’, amplimer b (477-bp product): b1, 5’-TTGTGAAGAAGCATCAGTAACT-3’; b2, 5’-TAAAGATTAGATCATCACCCT-3’, amplimer c (276-bp product): c1, 5’-TGAATGATACACTACACCATCACCATA-3’; c2, 5’-GGATGCAGTATCAAGATTTGTT-3’. These primers were validated using DNA extracted from CaSki and SiHa cells, which contain intact and disrupted E2 genes respectively [18,19].

An aliquot of 5 μl of DNA was used for each PCR reaction, which contained, in a total volume of 50 μl, 200 μM/l of each deoxynucleoside triphosphate, 1.5 mM/L of magnesium chloride, 1× PCR buffer, 40 pmol of sense and antisense primers, 1 U of Taq DNA polymerase (Fermentas, Burlington, Canada). The PCR conditions were as follows: 95 °C for 5 min then 40 cycles of 95 °C for 1 min, 58 °C for 2 min, and 72 °C for 1.5 min, followed by 72 °C for 10 min and a final resting temperature of 4 °C. The reference HPV-16 clone (obtained from Dr. E-M deVilliers, Heidelberg, Germany) and DNA extracted from CaSki cells were amplified as positive controls. The omission of template DNA served as a negative control. The amplified products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Statistical analyses

Statistical analyses were performed using the SPSS software packages (V12.0). Frequency tables were analyzed using the Fisher Exact Test (two-tailed) and/or the Chi-squared test, with Pearson correlation and/or likelihood ratio used to assess the significance of correlations between categorical variables. 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 (OR) and 95% confidence intervals (95% CI). Performance indicators of c-FLIP as a marker of CIN, HR-HPV infection or HR-HPV’ status 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 x SE). In all tests, P values less than 0.05 were considered statistically significant.

Results

HR-HPV infection and HPV-16 E2 gene disruption

HR-HPV infection was determined by HC-II. 24 Cases were HR-HPV non-infected and 56 cases were infected. In the latter cases, 40 cases were HPV-16 E7-positive (Fig. 1A).

Among all CIN lesions, 70.4% were HR-HPV-positive, contrasted with none of those without CIN. HR-HPV infection was even more common in SCC cases, in which 90% positive infection rate was observed. HR-HPV detection was associated with SCC at an OR of 4.0 (95% CI, 2.06–7.75), with any grade of CIN at an OR of 1.375 (95% CI, 1.14–1.655), and with CIN3 at an OR of 2.5 (95% CI, 1.53–4.09).

The overlapping primer pairs were validated initially using DNA extracted from CaSki and SiHa cells: all three fragments could be amplified from SiHa cells but only one (amplimer c) could be amplified from SiHa cells, as predicted from the published data [19]. The different fragments of HPV-16 E2 gene were amplified in all HPV-16-positive cases. A failure of amplification of one or more amplimer from these lesions indicated HPV-16 E2 disruption (Fig. 1B). We found intact E2 in 21 of 40 HPV-16-positive cases (group A), and the remaining 19 cases were HPV-16 E2 disrupted (group B). The ratio of E2 disruption in different HPV-16-positive lesions was 0, 12.5%, 42.9% and 85.7% for CIN1, CIN2, CIN3 and CC, respectively. There exists a significant tendency for increased E2 disruption with increasing lesion severity (P trend < 0.05; Table 1).

Expression of c-FLIP in cervical lesions

c-FLIP expression was detected as predominantly cytoplasmic staining in positive specimens, as shown in Fig. 2. Table 2 shows the expression of c-FLIP related to the grade of the lesion in all specimens. Epithelium in normal cervix and CIN1 mainly stained negatively of c-FLIP, with the rates of 66.7% and 57.1% respectively. The most remaining cases of these groups only showed weakly c-FLIP-positive cells scattered among the
normal basal or parabasal layer and dysplastic cells. 9 of 18 CIN2 lesions (50%), 16 of 22 CIN3 lesions (72.7%) and 17 of 20 CC lesions (85%) displayed strong signals (moderate–intense) of c-FLIP.

There was a direct relationship between the increasing grade of lesion and the intensity of c-FLIP staining in which the frequency of intense c-FLIP expression increased from 0% to 45.5% in specimens without CIN and those interpreted as CIN3 (P < 0.05). With the 2-tier category of staining (negative–weak vs. moderate–intense), the latter was significantly associated with high-grade lesion (CIN3 or CC). When CC cases were excluded, this association was still exhibited (P < 0.05).

Expression of c-FLIP related to HR-HPV infection and HPV-16 integration

The relationship between the c-FLIP expression and the presence or absence of HR-HPV in cervical lesions was assessed. As shown in Table 3. Moderate and intense levels of c-FLIP expression were associated with HR-HPV at an OR of 1.705 (95% CI, 1.282–2.267; P < 0.05). There was a significant

Table 1 HR-HPV infection and grade of cervical lesions

<table>
<thead>
<tr>
<th>Lesion grade</th>
<th>Cases</th>
<th>HR-HPV infection</th>
<th>HPV-16 E2 disruption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>CIN1</td>
<td>14</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>CIN2</td>
<td>18</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>CIN3</td>
<td>22</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>SCC</td>
<td>20</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>56</td>
<td>24</td>
</tr>
</tbody>
</table>

HR-HPV, high risk human papillomavirus; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.

Fig. 2. Expression of c-FLIP in different cervical tissues detected immunohistochemically. (A) A normal cervical squamous epithelium stained with c-FLIP antibody showing no positive staining (20×). (B) A low-grade cervical intraepithelial neoplasia (CIN1) lesion with the characteristic morphological features of HPV infection (koliocytes) in the upper layers of the epithelium, displayed weak positive staining localized in the lower one-third cells (blue arrow) and scattered in some of the intermediate layer cells (10×). (C) A high-grade cervical intraepithelial neoplasia lesion (CIN2–3) showing strong immunohistochemical staining in the dysplastic cells (10×). (D) Another CIN3 lesion with intense immunostaining (40×). (E) Invasive squamous cell carcinoma showing intense diffuse staining in tiny nests of tumor cells clearly detectable against the negative blue stromal background (20×). (F) A poorly differentiated invasive squamous cell carcinoma. Cancer cells expressing intense immunohistochemical staining, distributing equally throughout the lesion (40×).
correlation between the different grades of c-FLIP expression and the HR-HPV infection status in the lesions, while moderate and intense expression levels were more frequent in HR-HPV-positive lesions than those in HR-HPV-negative lesions, of which 58.3% were completely c-FLIP-negative (14/24). In the HR-HPV-positive cases, 71.4% showed moderate–intense c-FLIP, as contrasted to 16.7% of the HR-HPV-negative cases ($P < 0.05$).

We then excluded 16 cases with HR-HPV infection other than type 16 (group C), and investigated whether the expression of c-FLIP had an association with HPV-16 E2 disruption in the remaining 64 cases including HR-HPV-negative and HPV-16-positive specimens. Contrary to HPV-negative cases, moderate–intense level of c-FLIP expression in the 40 cases infected with HPV-16 was significantly higher. Of those HPV-16 infected, cases with E2 disruption (group B) tended to have a higher grade of dysplasia, and c-FLIP was significantly more expressed in this group than in cases without E2 disruption (group A) ($P < 0.05$). There is a more significant trend that increased c-FLIP expression correlated with loss of intact E2.

As a final step, we calculated the performance indicators (sensitivity, specificity, PPV, and NPV) for c-FLIP staining as a marker of CIN, HR-HPV infection and viral integration, the latter was typified by the status of HPV-16 E2 disruption. As shown in Table 4, moderate–intense c-FLIP expression was a 100.0% specific indicator of CIN, with a 100.0% PPV, because none of the specimens without CIN showed increased c-FLIP expression. Negative staining, however, did not rule out CIN, because the NPV was less than 20%. As anticipated from the data in Table 3, c-FLIP expression predicted HR-HPV infection with a 77.8% specificity and a 90.9% PPV. But for HR-HPV infection status, negative c-FLIP expression was more usable to discern the case which had no HR-HPV infection, because of a 94.7% sensitivity and a 90.0% NPV. High c-FLIP expression, however, was not so efficient to indicate HR-HPV infection, and the specificity is only 42.9%, with a 60.0% PPV.

Discussion

According to a recent task force on searching for prognostic factors in CC, there is an urgent need for more specific markers capable of predicting the disease and its 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. One of the key mechanisms leading to the progressive phenotype in CIN lesions is the inhibition of apoptosis by HR-HPV oncoproteins [20]. Thus, it was of interest to assess whether some apoptosis inhibitory factors, such as FLIP, would be of any value in predicting CIN progression and CC.

FLIP, a potent intracellular inhibitor of apoptotic cell death mediated by death receptor signaling, was originally discovered as a family of viral inhibitors of death-receptor-mediated cell death found in several herpesviruses and its cellular homologs were subsequently identified [11]. Since its discovery, FLIP has attracted considerable attention in many human malignant neoplasms [21,22]. Surprisingly involvement of c-FLIP in CC and its precursors remains to be defined, despite accumulated evidence suggesting several potentially important functions for this novel member of the death receptor pathway. Accordingly, FLIP 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 [14,15]. In many respects, CC would be an ideal target to analyze these properties of FLIP, not the least because its precursors are well defined and its viral cause well established. Until now, practically all of these potential links between FLIP and HPV have been completely unexplored. In the present study, by identifying the status of HR-HPV infection and HPV-16 integration, we demonstrated the in vivo up-regulation of c-FLIP in correlation with the malignant transformation of cervical epithelium.

Here, we provide data concerning the in situ expression of c-FLIP in human CIN and CC, and its potential relevance to tumor development. We analyze 80 normal, benign and malignant human cervical epithelial specimens by immunohistochemical assay and confirm that c-FLIP is predominantly up-regulated in high-grade CINs and malignant lesions of the cervix, namely CIN2, CIN3, and CC. FLIP expression was detected in only two of six normal cervix, and at low levels. In contrast, expression was observed in 77.8% (14 of 18) of CIN2, 86.4% (19 of 22) of CIN3, and 95% (19 of 20) of CC. We were able to establish an almost linear relationship between the grades of CIN and the intensity of FLIP expression (Table 2). The proportion of negative expression was inversely related to the grade of dysplasia.
to CIN grades, whereas high expression (moderate to intense) increased in parallel with CIN grades ($P<0.05$). Therefore, it clearly appears that FLIP expression is more frequent in malignant cervical lesions, suggesting that it may, in certain cases, be implicated in the pathogenesis of CC, possibly by offering distinct advantages to the tumor cells. Recent evidence has shown by transfection that overexpression of FLIP in melanoma cells significantly renders their resistance to the pro-apoptotic effect of recombiant FasL and TRAIL, this result has suggested that defective Fas signaling owing to inhibition by FLIP may be implicated in tumorigenesis [23]. Indeed, in murine tumor models, inhibition of Fas signaling by overexpression of FLIP confers an advantage to tumors within an immunocompetent setting [24,25]. Escaping death-receptor-mediated cell death may, therefore, enable cervical malignant cells to evade elimination by the immune system.

In the present data, high FLIP expression was already evidenced in 14.3% of CIN1 lesions (2/14) and increased in parallel with lesion grade. In fact, FLIP overexpression proved to be a 100.0% specific marker of CIN, because it was never found in specimens without CIN (Table 4). Moreover the PPV for predicting CIN was 100.0%, when we calculated the specificity and PPV in predicting CIN2 and CIN3, FLIP expression (negative–weak vs. moderate–intense) predicted CIN3 with a specificity of 90.0% and a PPV of 91.1% (data not shown). Therefore, FLIP overexpression in cervical lesions can be considered as an early marker of cervical carcinogenesis.

Recent study has shown that p16INK4a seems to be a specific biomarker of cells harboring HR-HPV infection [20,26], our data are similar to this interpretation. It is interesting that FLIP expression is related significantly ($P<0.05$) to HR-HPV infection (Table 3). In lesions with HR-HPV, only 28.6% had negative or weak expression, contrasted with 77.7% of the lesions without HR-HPV (14/18). It seems intriguing to combine these 2 markers (and possibly others) to calculate the performance indicators for such a combination and the practical implications of these data remain to be established.

About 75.5% of cervical lesion cases were HR-HPV infected and 54.1% of those cases were type 16 HPV infected. Epidemiological study has shown that most of the cervical lesions can be attributed to HPV infection, and that low-grade lesions can progress to high-grade with the cancer-associated high-risk type of HPV [27], among which HPV-16 is predominantly detected. HPV-16 E2 is involved in the transcriptional and replication control of HPV and it has been predicted that down-regulation of E2 facilitates further replication of HPV. Several studies, using different methods, have proven that, in cervical carcinomas associated with HR-HPV, the E2 gene is always inactivated when integration of the viral genome into the cellular genome, leading to disruption of the E2 gene [8,28,29]. In such conditions, autonomous viral DNA replication is abolished, and expression of the E6 and E7 oncoproteins is up-regulated. In our study, we found greater loss of HPV-16 E2 gene expression in CIN3 than that in CIN1 and CIN2 (Table 1), suggesting that higher HPV-16 integration occurs as neoplastic transformation progresses, which is consistent with the report of Hopman et al. [30]. We also found that, in CC cases with HPV-16 infection, the ratio of integration is 85.7% (12/14, Table 1), which is significantly higher than that in CIN lesions. This might suggest that the loss of HPV-16 E2 function during viral integration has been an activation mechanism for progression from high-grade CINs to invasive CC.

A significant proportion of CIN1 and CIN2 lesions regress spontaneously. And it takes years for preinvasive CC to progress into invasive cancer. Women who were infected with HPV-16 have a higher rate of progression of CIN [27]. However, HPVs by themselves are insufficient for malignant transformation and additional cellular changes must be involved [31]. We found the augmentation of c-FLIP expression in conjunction with repression in intact E2 expression in HPV-16-infected cervical lesions. And it was also found that high c-FLIP expression more frequently is present in E2-disrupted cervical lesions (group B, Table 3), compared with that in full HPV-16 E2 expressed ones (group A, Table 3). Loss of HPV-16 E2 in conjunction with the increase of c-FLIP expression suggested the overexpression of c-FLIP mainly when HPV integration occurred. The influence of c-FLIP expression on integration of HR-HPV has not been reported. The regulatory mechanism controlling c-FLIP expression and that of HPV genes associated with carcinogenesis remains obscure. Recently it was reported that, in addition to antiproliferative properties and cell cycle arrest, HPV-16 E2 could also induce apoptosis [32,33]. Remarkably, E2 activates apoptosis mainly through the extrinsic pathway of apoptosis [33], via activation of caspase 8, which can be negatively regulated by c-FLIP [34]. Therefore, there is a possibility that the products of the HPV-16 E2 genes may interact with and alter c-FLIP expression and HPV-16 integration occurrence may have predominant role in promoting c-FLIP expression. Here we propose loss of HPV-16 E2 in conjunction with the increase of c-FLIP expression (group B) suggesting a close circuit comprising HPV E2 and c-FLIP, where HPV E2 regulates the expression and function of c-FLIP.

### Table 4

**Performance indicators of c-FLIP as a marker of CIN and HR-HPV infection**

<table>
<thead>
<tr>
<th>Outcome variable</th>
<th>c-FLIP performance* (95% CI)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CINb</td>
<td>50.0 (36.7–63.3)</td>
<td>100 (100.0–100.0)</td>
<td>100 (100.0–100.0)</td>
<td>18.2 (7.0–36.0)</td>
<td></td>
</tr>
<tr>
<td>HR-HPV</td>
<td>71.4 (59.6–83.2)</td>
<td>77.8 (52.0–94.0)</td>
<td>90.9 (82.4–99.4)</td>
<td>46.7 (28.0–66.0)</td>
<td></td>
</tr>
<tr>
<td>Integrationc</td>
<td>94.7 (74.0–100.0)</td>
<td>42.9 (34.0–78.0)</td>
<td>60.0 (41.0–77.0)</td>
<td>90.0 (55.0–100.0)</td>
<td></td>
</tr>
</tbody>
</table>

* a c-FLIP staining (negative–weak vs. moderate–intense).
* b Any grade of CIN (squamous cell carcinoma cases excluded).
* c The integration status of HPV-16 infection.
which controls tumorigenesis. It might be possible that HPV-16 oncogenes E6 and E7 do not directly influence the effect of c-FLIP regulation of cells, since it was reported that the induction of apoptosis appears to be an autonomous function of E2 because E2-induced apoptosis is detected in many HPV-negative carcinoma cell lines [33]. On the other hand, nevertheless, mechanisms that activate c-FLIP, or the activation itself, may facilitate HPV integration.

Taken together, our results indicate that c-FLIP expression is an early marker of cervical carcinogenesis, being expressed with increasing intensity from low-grade CIN onward. It is interesting that c-FLIP overexpression is related significantly to the presence of HR-HPV infection and its integration status in CIN and CC, which is an unexplored field for basic HPV researchers and deserves to be demonstrated by extensive molecular biological studies.

References