Up-Regulation of Plasminogen Activator Inhibitor-2 Is Associated With High-Risk HPV and Grade of Cervical Lesion at Baseline but Does Not Predict Outcomes of High-Risk HPV Infections or Incident CIN

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Abstract

Protease inhibitor serpin-B2 (plasminogen activator inhibitor-2 [PAI-2]) protects pRb from degradation in human papillomavirus (HPV)-18+ HeLa cells. Our objective was to assess whether the pRb-mediated HPV-suppressive effect of PAI-2 in cancer cell lines has implications in the outcome of HPV infections. Cervical biopsy specimens from 225 women were analyzed for PAI-2 expression to assess its value as a predictor of cervical intraepithelial neoplasia (CIN) grade, high-risk (HR) HPV at baseline, outcomes of HR-HPV infections, and the development of incident CIN.

PAI-2 expression increased in parallel with lesion grade. Nuclear PAI-2 expression was significantly related to HR-HPV detection and had a linear relationship with HR-HPV load. PAI-2 expression was of no value in predicting the outcomes of HR-HPV infections. The same was true for PAI-2 as a predictor of surrogate end points (incident CIN 1+, CIN 2+) of progressive disease.

PAI-2 expression is up-regulated on transition from CIN 2 to CIN 3. The HR-HPV suppressive effects of PAI-2 were not related to more favorable outcomes of HR-HPV infections or lower risk of disease progression to CIN.

Practically all cervical carcinomas are caused by high-risk (HR) human papillomavirus (HPV) infections, whereas the low-risk (LR) HPV types are rarely found in cervical carcinoma or its precursor (cervical intraepithelial neoplasia [CIN]) lesions.[1-6] This divergent oncogenic potential of LR and HR HPV is mainly attributable to the differences of the 2 major viral oncoproteins (E6 and E7) to interact with the key regulatory cellular proteins, p53 and pRb.[1,4,7-9] While E6 of HR HPV (but not LR HPV) initiates degradation of the p53 tumor suppressor protein, HPV E7 of HR HPV (but not LR HPV) binds to pRb, resulting in G_1/S transition of the cell cycle.[1,4,7-11] In addition to limiting cell cycle progression through regulation of the E2F family of transcription factors,[7,11] pRb also possesses prosurvival (cytoprotective) activity, directly suppressing apoptosis, independent of growth suppression.[12,13]

These normal activities of pRb are regulated by cellular proteins that interact with pRb.[7,11] One of these pRb-interacting proteins is an intracellular serine protease inhibitor, serpin-B2 (plasminogen activator inhibitor type 2 [PAI-2]).[14] PAI-2 was recently found to bind pRb, colocalizing with pRb in the nucleus and protecting it from proteolytic degradation, leading to up-regulation of pRb levels.[14-17] PAI-2 exists in 2 forms: a nonglycosylated intracellular (42 kDa) form and a glycosylated secreted extracellular (60 kDa) form.[18] PAI-2 inhibits urokinase plasminogen activator very rapidly but inhibits tissue plasminogen activator much more slowly.[15-18] PAI-2 is clearly a multifunctional protein, synthesized by a variety of cell types and promotes, eg, cell survival.[14-16]

The role of PAI-2 in human carcinogenesis has attracted considerable interest.[16,19] Data from several human tumors suggest that of the 2 urokinase inhibitors (PAI-1 and PAI-2),

overexpression of PAI-1 actually promotes tumor progression, whereas high expression of PAI-2 inhibits tumor growth and metastasis. It was suggested that the basis for this apparently paradoxical action of 2 similar serine protease inhibitors lies in the key structural differences controlling their interactions with extracellular matrix components.[16,19-21]

Until now, there are no studies on the expression of PAI-2 in cervical carcinoma or in CIN lesions. However, 2 studies used an HPV-18+ cell line (HeLa) to examine the effects of PAI-2 on the degradation of pRb induced by the viral E7 oncoprotein.[14,22] In their original study disclosing PAI-2 as a pRb-binding protein, Darnell et al[14] demonstrated that PAI-2 expression also protected pRb from E7-mediated degradation, leading to recovery of pRb and inhibition of E7 messenger RNA expression. In a second study, Darnell et al[22] further showed that PAI-2 expression in HeLa cells resulted in restoration of pRb expression and functional silencing of HPV-18 transcription. This, in turn, caused loss of E7 protein expression and restoration of multiple E6- and E7-targeted host proteins, eg, p53, c-Myc, and c-Jun. The authors reasoned that this potent suppressive effect of PAI-2 on pRb-mediated HPV-18 oncogene transcription might have implications in prognosis and in the treatment of HR HPV–associated clinical disease.[22]

To further delineate the role of PAI-2 in HR HPV–associated cervical carcinogenesis, we analyzed (for the first time) a series of cervical biopsy specimens from 225 women included in the Latin American Screening (LAMS) study cohort (n = 12,114) in Brazil and Argentina.[23-26] The study aimed to assess the following: (1) whether the expression of PAI-2 is of any value as predictor of the intermediate end-point markers of cervical carcinogenesis, ie, the grade of CIN and HR-HPV type at baseline; (2) the outcome of these HR-HPV infections; and (3) the development of incident CIN 1 or worse (CIN 1+) and CIN 2+ during the prospective follow-up.[23,24]

Materials and Methods

General Study Design

The ongoing LAMS study is a multicenter screening trial targeting female populations at different risk levels for cervical carcinoma in 2 Latin American countries, Brazil and Argentina.[23] At their baseline visit, a total of 12,114 consecutive women attending the 4 partner clinics (Campinas, Brazil; São Paulo, Brazil; Porto Alegre, Brazil; Buenos Aires, Argentina) were screened for HPV and CIN using 8 different diagnostic tools, as detailed earlier.[23-26] Women testing positive with any of these diagnostic tests were examined by colposcopy (and biopsied) at their second visit. In addition,

a 5% random sample of Papanicolaou smear (Pap)-negative women were recalled for a new Pap test at 12 months, as were 20% of women with negative results on the Hybrid Capture 2 (HC2; Digene, Gaithersburg, MD) test, to assess the rates of incident Pap smear abnormalities and HPV infections, respectively.[23,24] The women with biopsy-confirmed low-grade CIN comprise the prospective cohort (n = 1,011), followed up for a minimum of 24 months. All high-grade lesions were promptly treated and followed up for the same period, using repeated Pap test, colposcopy, and HC2 assay at 12-month intervals.[23-26] For the present analysis of PAI-2, baseline biopsy samples from 225 of the women were available.

Prospective Follow-up

With the aforementioned criteria, women were allocated to the prospective cohort and scheduled to be monitored in the clinic at 6-month intervals for a minimum of 24 months. A total of 1,011 women completed at least 1 follow-up visit, including examination by Pap smear, visual inspection with acetic acid and with Lugol iodine, colposcopy, and biopsy, whenever abnormalities were detected.[24-26] The mean follow-up time at this writing was 21.7 months (SD, 8.09 months; median, 24.2 months; range, 1-54 months).

Outcomes and End Points of Cervical Lesions and HR-HPV Infections

For the present analysis, the data for the 1,011 women were analyzed for the different surrogate end points of progressive disease—progression to CIN 1+ and progression to CIN 2+—and for different outcomes of HR-HPV infections, including incident infections, virus persistence, and HPV clearance. Progression to CIN 1+ was based on detection, in baseline biopsy-negative women, a biopsy-confirmed CIN 1+ lesion in any of the consecutive visits during the follow-up period. Progression to CIN 2+ was defined as any case in which biopsy-confirmed progression from baseline negative, flat HPV with no CIN (N-CIN), or CIN 1 was established by biopsy in the subsequent follow-up visits, as recently detailed.[27]

Times to progression to CIN 1+ and CIN 2+ were calculated from the baseline visit to the respective follow-up visit when the progression event was first detected. Progression rates were calculated by dividing the numbers of progressed cases by woman-months at risk (WMR) and expressed per 1,000 WMR.

Three outcomes of HR-HPV infections were recorded: incident, persistence, and clearance. An incident HR-HPV infection was the appearance of a positive HC2 test (at 1 pg/mL relative light unit [RLU]/cutoff [CO]) among baseline HR-HPV– women at any of the follow-up visits. HR HPV was considered cleared if the HC2 assay was negative at the last follow-up visit. HR-HPV infections were considered persistent in women in whom 2 or more subsequent HC2 assays

were HR-HPV+ and in whom the infection was not cleared at the last follow-up visit. Times to these 3 outcomes were also calculated and expressed as cases/1,000 WMR.

Methods

Because they are detailed in a series of reports,[23-27] the methods used in the LAMS study are described here only as far as pertinent to elaborating the data necessary for the present analysis.

Epidemiologic Questionnaire

All women who gave their consent to participate (n = 12,114) completed a detailed inquiry concerning the risk factors for HPV, CIN, and cervical carcinoma. This structured questionnaire contained questions exploring reproductive history, sexual history, current sexual practices, sexual hygiene, medical history, smoking habits, and contraception.[23,24]

Pap Smears

In the LAMS study, we compared the performance of 3 methods of cervical cytology: conventional Pap and 2 liquid-based cytology techniques (DNA-Citoliq, Digene Brazil, São Paulo; and SurePath, TriPath, Durham, NC).[24] In the present analysis, only the results of the conventional Pap test were used.

Directed Punch Biopsy

Directed punch biopsy specimens (and cone biopsy specimens) were fixed in formalin, embedded in paraffin, and processed into 5-μm-thick H&E-stained sections for light microscopy, following routine procedures. All biopsy specimens were examined within the daily routine in the pathology departments of the partner clinics in the present study and diagnosed by using the commonly agreed-on CIN nomenclature. Pathologists were also asked to report HPV-suggestive morphologic changes in flat lesions with no CIN, ie, N-CIN (flat condyloma).[23-26] The slides from 2 of the centers (Campinas and São Paulo) were subjected to reexamination by 2 authors (M.E. and K.S.). The consensus diagnosis of the pathology panel was considered as the final diagnosis. Interrater reproducibility for CIN 2 and CIN 3 cutoffs in the entire biopsy series reached κ values of 0.951 (95% confidence interval [CI], 0.921-0.981) and 0.967 (95% CI, 0.939-0.995), respectively, and weighted κ values by the intraclass correlation coefficient (ICC) of 0.975 (95% CI, 0.970-0.979) and 0.983 (95% CI, 0.980-0.986), respectively.

Detection of HPV DNA by HC2 Assay

Primary HPV testing was done by using the HC2 assay, using cervical swabs (obtained by a physician) and self-sampling devices (tampons), as described previously.[23,26] The HC2 assay (n = 4,694 tests) was performed by using the automated HC2 test system according to the manufacturer's protocol. The samples were analyzed only for the presence of HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The usual limit of 1 pg/mL of HPV-16 DNA was used as the positive control CO, ie, samples were classified as HR-HPV+ with an RLU/CO of 1.0 pg/mL or more.

Immunohistochemical Detection of PAI-2

A total of 225 slides from the same number of women were available for immunohistochemical analysis of PAI-2. Briefly, 4-μm-thick sections were cut on pretreated glass slides (DAKO, Glostrup, Denmark) specially made for use with the TechMate immunostainer TM 500 automatic immunostainer (BioTek Solutions, Santa Barbara, CA). Sections were deparaffinized using standard methods, after which they were subjected to antigen retrieval by boiling in a microwave oven with 10 mmol/L citrate buffer, pH 6.0. Immunohistochemical analyses were done by using the TechMate TM 500 automatic immunostainer according to the provider's instruction. PAI-2 was detected by using monoclonal PAI-2 antibody (Zymed, South San Francisco, CA), diluted 1:75. Primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat antimouse IgG (No. 6788, dilution 1:250; Abcam, Cambridge, MA). Slides were then processed with universal LSAB-2 single reagents (peroxidase) kit (DakoCytomation, Glostrup, Denmark), and expression of PAI-2 was localized by incubation with diaminobenzidine. Negative control samples were similarly processed by omitting the primary antibody, and biopsy specimens from breast cancer were used as positive control samples.

### Evaluation of the Immunohistochemical Staining for PAI-2

For logistical reasons, slides for immunohistochemical staining were available from 225 women. In normal and metaplastic squamous epithelium, expression of PAI-2 was weak, predominantly with cytoplasmic (and scattered nuclear) staining confined to superficial layer cells ❚Image 1❚. In CIN lesions and cervical carcinoma, cytoplasmic and nuclear PAI-2 expression was markedly increased ❚Image 2❚, ❚Image 3❚, and ❚Image 4❚. In original grading of the cytoplasmic PAI-2 staining, semiquantitative scoring into 4 categories was used, as follows: 0, no expression of PAI-2; 1, weak staining (equivalent to normal squamous epithelium); 2, moderately increased staining (intermediate and parabasal cells stained); and 3, strongly increased staining (all layers PAI-2+). In statistical analysis, the staining results were also treated as dichotomous categorical variables (negative-weak vs moderate-strong) or by using 3-tiered grading as negative-weak, moderate, and strong. Nuclear staining of PAI-2 was graded only as a dichotomous variable: present or absent.
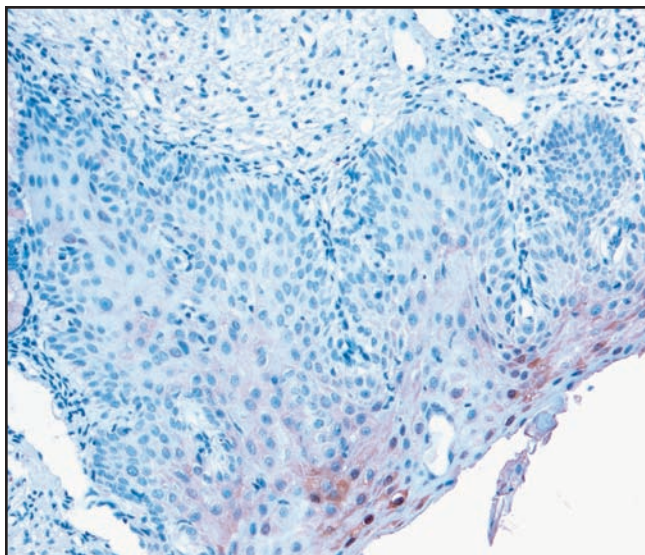
Image 1 Normal cervical epithelium undergoing physiologic squamous cell metaplasia. Plasminogen activator inhibitor-2 (PAI-2) expression is equivalent to that in normal squamous epithelium. PAI-2 expression is weak in intensity, predominantly cytoplasmic, and confined to the cells in the upper (and scattered intermediate) layers of the metaplastic epithelium. A few cells with nuclear PAI-2 expression are found close to the epithelial surface (immunohistochemical analysis for PAI-2, ×100).
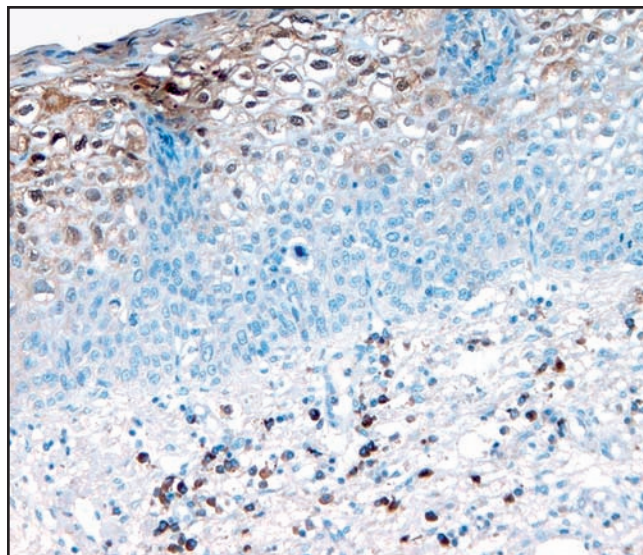
Image 2 A low-grade cervical intraepithelial neoplasia 1 lesion with characteristic features of human papillomavirus infection (koilocytes) from intermediate layers upward. As compared with the metaplastic epithelium, both cytoplasmic and particularly nuclear expression of plasminogen activator inhibitor-2 (PAI-2) is increased in intensity, and positive staining is also present in lower levels of the epithelium. Yet, there is a major difference as compared with the high-grade lesions (immunohistochemical analysis for PAI-2, ×100).
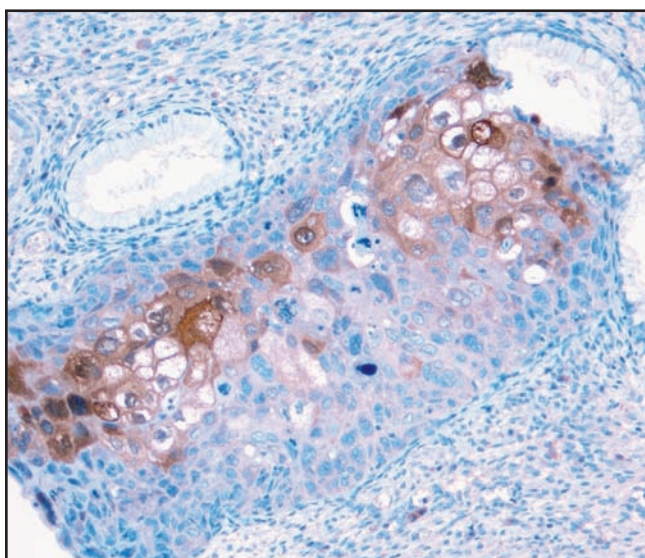
Image 3 A high-grade cervical intraepithelial neoplasia 3 lesion penetrating into the underlying glandular openings. Plasminogen activator inhibitor-2 (PAI-2)+ cells are found throughout the full thickness of the epithelium, indicating marked up-regulation. The staining intensity is variable, with some cells showing intense cytoplasmic and/or nuclear expression, while in the rest of the cells, PAI-2 expression is less intense (immunohistochemical analysis for PAI-2, ×200).
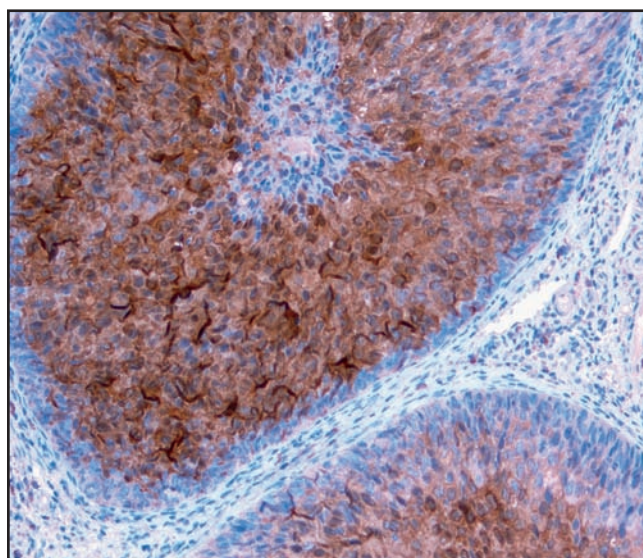
Image 4 An invasive squamous cell carcinoma with intense expression of plasminogen activator inhibitor-2 (PAI-2). Positive immunostaining is detected in practically all cancer cells, being an indicator of a marked overexpression of PAI-2, even more diffuse than in the cervical intraepithelial neoplasia grade 3 lesion shown in Image 3 (immunohistochemical analysis for PAI-2, ×100).

Statistical Methods

Statistical analyses were performed using the SPSS for Windows, version 16.0.2.1 (SPSS, Chicago, IL) and Stata/SE 10.1 (Stata, College Station, TX) software packages. Frequency tables for categorical variables were analyzed by using the χ^2 test, with the likelihood ratio or Fisher exact test to assess the significance of the correlation. Differences in the means of continuous variables were analyzed by using nonparametric tests (Mann-Whitney) or analysis of variance.

Correlations between cytoplasmic and nuclear PAI-2 staining were calculated by using bivariate correlation (Spearman R test). To calculate the reproducibility of the CIN 2 and CIN 3 cutoffs between the original diagnoses (LAMS) and the reviewers' ratings (M.E. and K.S.), the nonweighted (Cohen) κ and weighted κ were used. The latter was calculated by using the ICC, with parallel 2-way random model and consistency settings and the test for interrater agreement with the unique raters and default weight settings. Logistic regression models using a stepwise backward or forward approach and default likelihood ratio enter and removal statistics were used to analyze the power of PAI-2 as a predictor of the viral and disease end points, calculating crude odds ratios (ORs) with 95% CIs.

Performance indicators (sensitivity, specificity, positive predictive value [PPV], negative predictive value [NPV], and their 95% CIs) of PAI-2 in predicting baseline high-grade CIN or HR HPV, as well as the longitudinal predictive values for incident CIN and the 3 viral outcomes, were all calculated using STATA/SE software and the algorithm introduced by Seed and Tobias,[28] which also calculates the area under receiver operating characteristic curve (AUC). In all tests, values of P of less than .05 were considered statistically significant.

Results

In bivariate correlation analysis, nuclear and cytoplasmic expression of PAI-2 was significantly correlated ($P = .0001$; Spearman $R = 0.282$). The paired-samples test (Wilcoxon; P

= .059) corroborates this finding. Using dichotomized values for both, the regular κ was 0.280 (95% CI, 0.267-0.293; $P = .0001$) and the weighted κ (ICC) somewhat higher, at 0.442 (95% CI, 0.271-0.573; $P = .0001$), indicating fair "reproducibility" between nuclear and cytoplasmic PAI-2 expression.

Expression of PAI-2 in baseline cervical biopsy specimens was related to the lesion grade as shown in ▮Table 1▮. There was a significant linear trend of increasing up-regulation of cytoplasmic PAI-2 expression ($P = .004$) in parallel with increasing grade of CIN and somewhat less significant for nuclear PAI-2 expression ($P = .028$ for linear trend). Major up-regulation of cytoplasmic and nuclear PAI-2 takes place on transition from CIN 2 to CIN 3.

When dichotomized (moderate-strong vs negative-weak), up-regulated cytoplasmic PAI-2 expression predicted CIN 3+ with an OR of 2.90 (95% CI, 1.25-6.69; $P = .008$) and CIN 2+ with an OR of 2.01 (95% CI, 1.05-3.84; $P = .029$). Positive nuclear PAI-2 expression predicted CIN 3+ with an OR of 3.70 (95% CI, 1.37-9.97; $P = .004$) and CIN 2+ with an OR of 2.05 (95% CI, 1.02-4.11; $P = .036$). No useful data were provided by the calculation of the performance indicators (sensitivity, specificity, PPV, NPV, and AUC).

▮Table 2▮ shows the association of cytoplasmic and nuclear expression of PAI-2 with the detection of HR HPV and its semiquantitative (HC2 assay) viral load, as well as performance indicators calculated for dichotomized PAI-2 values. With 3-tiered grading, cytoplasmic PAI-2 was up-regulated more often in HR-HPV+ cases than in HR-HPV– lesions ($P = .044$ for linear trend). Viral load seemed to increase in parallel with the increasing up-regulation of PAI-2 ($P = .010$; Kruskal-Wallis test). Cytoplasmic PAI-2 was not a particularly good marker of HR HPV, with an AUC of 0.558.

Nuclear expression of PAI-2 seemed to be significantly ($P = .032$) related to HR-HPV detection (OR, 2.05; 95% CI, 1.06-3.93). The mean viral load of HR HPV was almost twice as high in HR-HPV+ cases as in HR-HPV– lesions ($P = .006$; Mann-Whitney test). Nuclear PAI-2 showed somewhat better

▮Table 1▮
Expression of Plasminogen Activator Inhibitor-2 as Related to Lesion Grade[*]

	Normal (N-CIN)	CIN 1	CIN 2	CIN 3	Squamous Cell Carcinoma
Cytoplasmic expression[†]					
Negative-slight up-regulation	42 (45)	29 (45)	9 (41)	8 (22)	0 (0)
Moderate up-regulation	30 (32)	21 (33)	7 (32)	9 (25)	1 (100)
Marked up-regulation	22 (23)	14 (22)	6 (27)	19 (53)	0 (0)
Nuclear expression[‡]					
Absent	34 (36)	24 (38)	8 (36)	5 (14)	0 (0)
Present	60 (64)	40 (63)	14 (64)	31 (86)	1 (100)

CIN, cervical intraepithelial neoplasia; N-CIN, flat human papillomavirus with no CIN.
[*] Data are given as number (percentage).
[†] $P = .043$ (Fisher exact test); $P = .004$ for linear trend.
[‡] $P = .068$ (Fisher exact test); $P = .028$ for linear trend.

Expression of Plasminogen Activator Inhibitor-2 as Related to Detection of High-Risk HPV and Its Viral Load*

	HPV+ (n = 112)	HPV– (n = 59)	Mean Viral Load (95% CI)†
Cytoplasmic expression‡			
Negative-slight up-regulation	42 (37.5)	29 (49)	1.78 (1.05-2.51); n = 71
Moderate up-regulation	32 (28.6)	19 (32)	2.63 (1.63-3.62); n = 51
Marked up-regulation	38 (33.9)	11 (19)	3.67 (2.76-4.58); n = 49
Nuclear expression§			
Absent	31 (27.7)	26 (44)	1.61 (0.77-2.44); n = 57
Present	81 (72.3)	33 (56)	3.06 (2.44-3.67); n = 114

ANOVA, analysis of variance; CI, confidence interval; CO, cutoff; HPV, human papillomavirus.
* Data are given as number (percentage).
† Semiquantitative viral load determined by the relative light units/CO ratio in the Hybrid Capture 2 assay, log-transformed.
‡ For HPV+/HPV–, $P = .091$ (χ^2, log rank); $P = .044$ for linear trend. For viral load, $P = .008$, ANOVA; $P = .010$, Kruskal-Wallis.
§ For HPV+/HPV–, $P = .032$ (χ^2, log rank); $P = .031$ for linear trend. For viral load, $P = .007$, ANOVA; $P = .006$, Mann-Whitney.

performance in detecting HR HPV, but the AUC of 0.582 is not very impressive.

Cytoplasmic and nuclear PAI-2 expression as a predictor of incident, clearance, and persistent HR-HPV infection is summarized in ∎**Table 3**∎. PAI-2 expression is not a useful predictor of these 3 viral outcomes, as calculated by the sensitivity-specificity balance (AUC), which remained near the 0.5 limit. The only exception was the AUC of 0.718 for incident infections, but only 1 incident event was observed in this series. As to the times to clearance and time to incident HR HPV (not calculable), there were no differences related to PAI-2 expression. As to the nuclear expression (present or absent) of PAI-2, almost the same applies. Using longitudinal performance indicators, nuclear PAI-2 showed 90.9% sensitivity, 37.8% specificity, 26.3% PPV, and 94.4% NPV as predicting HR HPV persistence (AUC, 0.643; 95% CI, 0.529-0.758; data not shown).

∎**Table 4**∎ gives the data on PAI-2 as a predictor of the 2 surrogate end points of progressive disease (incident CIN 1+,

CIN 2+). Cytoplasmic PAI-2 expression (3-tiered grading) was practically identical in the baseline biopsy specimens that subsequently progressed to incident CIN 1+, with no statistical difference. Longitudinal performance indicators do not provide any useful values, with an AUC of 0.478. Exactly the same is true with cytoplasmic PAI-2 as a predictor of incident CIN 2+ (AUC, 0.511). However, the NPV exceeded 90%, implying that negative-weak PAI-2 precludes progression to CIN 2+ with that level of accuracy (95% CI, 76.9%-98.2%).

When similar data were calculated for nuclear PAI-2 expression, still no significant associations were found with progression to CIN 1+ or CIN 2+. It seems that progression is less probable in cases with nuclear PAI-2 expression as compared with PAI-2– lesions at baseline. Owing to this difference in favor of PAI-2– cases, also the longitudinal indicators give AUC values below 0.5. When calculated the other way around, negative nuclear PAI-2 predicts incident CIN 1+ with an AUC of 0.568 (95% CI, 0.439-0.696) and incident CIN 2+ with an AUC of 0.622 (95% CI, 0.443-0.801). Positive

Expression of Plasminogen Activator Inhibitor-2 as a Predictor of Different Viral Outcomes*

	End Point					
	Incident HR-HPV		HR-HPV Cleared		HR-HPV Persistence	
	Yes (n = 1)	No (n = 55)	Yes (n = 14)	No (n = 42)	Yes (n = 11)	No (n = 45)
Cytoplasmic expression†						
Negative-slight up-regulation	0 (0)	24 (44)	5 (36)	19 (45)	2 (18)	22 (49)
Moderate up-regulation	0 (0)	19 (35)	6 (43)	13 (31)	5 (46)	14 (31)
Marked up-regulation	1 (100)	12 (22)	3 (21)	10 (24)	4 (36)	9 (20)
Nuclear expression‡						
Absent	0 (0)	18 (33)	5 (36)	13 (31)	1 (9)	17 (38)
Present	1 (100)	37 (67)	9 (64)	29 (69)	10 (91)	28 (62)

HR-HPV, high-risk human papillomavirus.
* Data are given as number (percentage).
† For incident HR-HPV, $P = .233$ (Fisher exact test); $P = .233$ for linear trend. For HR-HPV cleared, $P = .740$ (Fisher exact test); $P = .848$ for linear trend. For HR-HPV persistence, $P = .166$ (Fisher exact test); $P = .090$ for linear trend.
‡ For incident, cleared, and persistent HR-HPV, $P = 1.000$, $P = .751$, and $P = .084$ (Fisher exact test), respectively.

nuclear PAI-2 has 93.9% NPV for incident CIN 2+. Times to progression to CIN 1+ and CIN 2+ were identical in different categories of PAI-2 expression, with no significant differences (data not shown).

Discussion

Some of the key functions of PAI-2 seem to be mediated through pRb, which has a pivotal role in the regulation of cell proliferation and sensitivity to apoptosis.[11,15] It was recently shown that calpain cleavage of pRb promotes pRb loss by proteasome degradation, eg, during tumor necrosis factor α–induced apoptosis.[15] However, the pRb-binding protein PAI-2 protects pRb from this calpain cleavage, leading to up-regulation of pRb and enhanced cell survival. These data clearly confirm PAI-2 as an important cell survival factor that counteracts pRb repression by proapoptotic signal transduction.[15] In addition, there are other well-known mechanisms of pRb cleavage, most notably the binding with the E7 oncoprotein of HR-HPV types.[1-4,7-9] In this respect, of utmost interest are the recent observations linking PAI-2 with oncogenic HPV, reported in 2 experimental studies on HPV-18+ HeLa cells.[14,22] PAI-2 expression was shown to exert a potent suppressive effect on HPV-18 oncogene transcription, mediated by pRb. If applicable in clinical lesions, these findings could have important implications in prognosis and even treatment of HPV lesions.[14,22]

Until now, however, there have been no published studies assessing the potential prognostic and predictive value of PAI-2 in cervical carcinoma or its precursor lesions, despite the fact that specific markers predicting disease outcome in cervical carcinoma are urgently needed.[29,30] Because one of the key mechanisms leading to a progressive phenotype of CIN lesions is the inhibition of normal pRb functions by E7 oncoproteins,[1,2,7-9] it seemed feasible to assess whether these pRb-mediated HPV-suppressive effects of PAI-2 in HPV-18+ HeLa cell line could be directly translated to a clinical setting, ie, related to more favorable outcomes of HR-HPV infections or shown as a lower risk of disease progression measured by surrogate end points (CIN 1+/CIN 2+).

According to our working formulation, if cytoprotective[15-17,19,20] in clinical HPV lesions, PAI-2 should increase its expression on cleavage of pRb by HR-HPV E7 oncoproteins, and, accordingly, clear-cut up-regulation should be detectable in HR-HPV+ CIN lesions. Furthermore, if sustained enough, this HPV-suppressive effect might be evidenced by less aggressive outcomes (ie, clearance) of these HR-HPV infections and, even more significantly, as a lower risk of disease progression associated with PAI-2 up-regulation.

To explore the first part of this concept, we related PAI-2 expression to the lesion grade of the baseline biopsy specimens

❚Table 4❚
Expression of Plasminogen Activator Inhibitor-2 as a Predictor of the Two Surrogate End Points of Disease Progression*

	End Point			
	Incident CIN 1+	Incident CIN 1+	Incident CIN 2+	Incident CIN 2+
	Yes (n = 18)	No (n = 81)	Yes (n = 9)	No (n = 90)
Cytoplasmic expression†				
Negative-slight up-regulation	7 (39)	28 (35)	3 (33)	32 (36)
Moderate up-regulation	7 (39)	32 (40)	3 (33)	36 (40)
Marked up-regulation	4 (22)	21 (26)	3 (33)	22 (24)
Nuclear expression‡				
Absent	8 (44)	25 (31)	5 (56)	28 (31)
Present	10 (56)	56 (69)	4 (44)	62 (69)

CIN, cervical intraepithelial neoplasia.
* Data are given as number (percentage).
† For incident CIN 1+, $P = .946$ (Fisher exact test); $P = .738$ for linear trend. For incident CIN 2+, $P = .826$ (Fisher exact test); $P = .822$ for linear trend.
‡ For incident CIN 1+, $P = .277$ (χ^2, log rank). For incident CIN 2+, $P = .155$ (Fisher exact test).

(Table 1). Cytoplasmic and nuclear PAI-2 expression seemed to be related to the grade of the cervical lesion. While about 45% of normal (N-CIN) and CIN 1 lesions were negative or demonstrated weak expression, more than 50% of CIN 3 cases depicted marked up-regulation of cytoplasmic PAI-2 (OR, 2.90; 95% CI, 1.26-6.69), which gives a significant trend ($P = .004$). A similar, albeit less significant ($P = .024$), trend was seen for nuclear PAI-2 expression, with positive expression showing a sharp increase (>20%) on transition from CIN 2 to CIN 3 lesions. As stated, there are no previous studies on PAI-2 expression in CIN lesions to enable direct comparisons. However, the nearest parallel is a study in which precursor lesions of another HPV-related cancer, head and neck squamous cell carcinoma (HNSCC),[1-4] were studied for expression of PAI-2 using a similar immunohistochemical approach.[31] The authors report PAI-2 expression in normal epithelium and dysplastic lesions, with a clear decline in SCC lesions. They did not provide grade-specific data for dysplasia, however. In principle, these data are in alignment with the present observations, in which a minority of normal epithelium showed intense expression of PAI-2 (Table 1) and the only SCC lesion in the series presented with moderate up-regulation.

These observations are also consonant with PAI-2 data on HeLa cells,[14,22] considering the known natural history of CIN lesions.[1-3] It is well established that a substantial proportion of HR-HPV infections can be transient, with no or only minor (N-CIN) epithelial changes in the biopsy specimen. Such transient infections could explain why some (23%) of the normal/N-CIN lesions also showed intense up-regulation of PAI-2, which could be a response to pRb degradation by HR-HPV E7 oncoprotein.[22] On the other hand,

the vast majority (>75%) of these low-grade lesions showed no PAI-2 or only weak-moderate up-regulation (Table 1). It is tempting to speculate that those particular low-grade lesions with intense overexpression of PAI-2 could represent transient HR-HPV infections destined to clear spontaneously, orchestrated by the effective HPV-suppressive effects of PAI-2, with restored pRb expression, silencing of HPV-18 transcription, loss of E7 protein expression, and restoration of the key E6- and E7-targeted host proteins (p53, c-Myc, c-Jun).[22] This should be relatively easy to control using immunohistochemical analysis for multiple markers.

On the other hand, the sharp increase of PAI-2 expression on transition from CIN 2 to CIN 3 could coincide with the selection of a clone destined to progressive disease, with integrated HR HPV, degradation of pRb, and compensatory up-regulation of cytoplasmic and nuclear PAI-2. Evidently in such cases, PAI-2 expression fails to suppress the HR HPV–driven process of progression toward invasive disease. If, however, the data of Hasina et al[31] on HNSCC implicating down-regulation of PAI-2 expression (and silencing of its gene transcription) among invasive SCC are translated to these CIN lesions, one could hypothesize that the CIN 3 lesions with only moderate (25%) and particularly the lesions with no or weak PAI-2 expression (22%) should represent the true high-risk lesions for progression. Unfortunately, only 1 SCC was included in our series, precluding reliable conclusions whether this concept holds true. Some indirect evidence is provided by the present data, suggesting that the development of incident CIN seems to be more common among cases with low or absent PAI-2 at baseline, as discussed later.

Another part of our working hypothesis would imply that overexpression of PAI-2 bears a close association with detection of HR HPV in the biopsy specimens, if considered to be up-regulated as a response to pRb degradation by HR-HPV E7.[22] Indeed, cytoplasmic PAI-2 is more frequently negative or weak in lesions testing HR-HPV– and more often shows intense expression in HR-HPV+ lesions (Table 2). Nuclear PAI-2 expression is even more closely correlated with HR-HPV+ lesions (OR, 2.05; 95% CI, 1.06-3.93). Both cytoplasmic and nuclear PAI-2 expression were linearly related to the semiquantitative (HC2) viral load of HR HPV ($P = .008$ and $P = .007$, respectively). This association is not consistent enough, however, to make immunohistochemical assessment of PAI-2 a useful predictor of HR HPV, as recently shown with some other markers (eg, p16[INK4A], survivin, and hTERT).[32] With the sensitivity-specificity balance (AUC) not exceeding 0.6, one cannot consider PAI-2 to be of any use as a predictor.

However, this association of PAI-2 with HR HPV, albeit not perfect, leaves room for discussion about the implicated links to HeLa cell data[22] and HNSCC data.[31] Being constitutively expressed in morphologically normal cervical epithelium only at a very low level (Image 1), any up-regulation

in normal/N-CIN lesions could be ascribed to detection of HR HPV in these lesions. In fact, more than 50% of these normal/N-CIN lesions were HR-HPV+ (flat HPV lesions with no CIN), and HPV positivity increased in parallel with increasing CIN grade (data not shown). Thus, this association of PAI-2 with HR HPV would also neatly explain why PAI-2 up-regulation was more common in high-grade lesions, as discussed earlier. This is clearly shown in the Mantel-Haenszel test for confounding, which indicated that the association of PAI-2 and HR HPV (Table 2) was not confounded by the histologic grade (ie, the association remained constant across the histologic spectrum), whereas the association of PAI-2 with histologic grade (Table 1) was confounded by HR-HPV detection (ie, PAI-2 overexpression in CIN 3 was markedly more common among HR-HPV+ lesions than in HR-HPV– lesions; data not shown). These data clearly substantiate the presented concept that the marked up-regulation of PAI-2 on transition from CIN 2 to CIN 3 is, indeed, due to its association with HR HPV, not to CIN 3 as such.

To provide evidence for the third and fourth elements of our working hypothesis, we assessed whether PAI-2 expression has any favorable impact on the outcome of HR-HPV infections. It can be speculated that if the HPV-suppressive effect of PAI-2 demonstrated in HeLa cells would bear any clinical relevance as suggested,[22] it might confer less aggressive outcomes (ie, clearance) to these HR-HPV infections and, if sustained enough, should be also associated with a lower risk of disease progression to CIN 1+ and CIN 2+ end points. To our disappointment, we were unable to provide any confirmatory data to support either of these concepts. Thus, incident infections, virus clearance, or HR-HPV persistence did not show any direct association with cytoplasmic or nuclear PAI-2 expression (Table 3). Similarly, the longitudinal predictive indicators (sensitivity, specificity, PPV, and NPV) for dichotomized PAI-2 expression did not provide any useful results in predicting the 3 outcomes of HR-HPV infections. Unfortunately, the same was true with the association of PAI-2 in baseline biopsy specimens and the disease progression to CIN 1+ and CIN 2+ surrogates during the follow-up (Table 4). There was no difference in PAI-2 expression patterns among progressive and nonprogressive lesions, and longitudinal predictive indicators were of no value in discriminating the incident CIN 1+ and CIN 2+ cases from cases that did not progress.

Taken together, the present data indicate that PAI-2 expression in cervical biopsy specimens is closely related to histologic grade of the lesion, with most marked up-regulation on transition from CIN 2 to CIN 3. This seems to be attributable to the close link between PAI-2 and HR HPV, shown to be consistent across the histologic spectrum and resulting in enrichment of PAI-2 overexpression among HR-HPV+ CIN 3 lesions. However, we were unable to provide confirmatory data

to support the concept of Darnell et al,[22] suggesting that the HPV-suppressive effects of PAI-2 demonstrated in HPV-18+ HeLa cells might have implications in prognosis and treatment of HPV lesions. In our prospective setting, PAI-2 expression in baseline biopsy specimens did not have any impact on the outcome of HR-HPV infections or on disease progression to CIN 1+ and CIN 2+ surrogate end points during follow-up.

This failure to find a favorable prognostic impact of PAI-2 does not necessarily invalidate the original concept of pRb-mediated HPV-suppressive effects of PAI-2, however. It is clear that pRb as one of the key regulatory proteins is under control of a complex system of molecules, PAI-2 being only one of those. Until now, only a few proteolytic mechanisms targeting pRb degradation have been identified.[15] Apart from the degradation by the HPV E7 oncoprotein,[1,2,7-9] pRb may be cleaved and inactivated in response to death receptor signals, through the action of caspases.[33] These, in turn, are inhibited by a group of molecules known as apoptosis inhibitors (IAPs), including survivin.[34] Other recently disclosed proteins participating in the regulation of pRb turnover are calpains, exerting cleavage of pRb before proteasome degradation.[15] It is interesting that the cytoprotective effect of PAI-2 was shown to be mediated by protection of pRb from this calpain cleavage.[15]

In cervical lesions, a delicate balance must exist between the dual functions of pRb: (1) limiting cell cycle progression through regulation of the E2F family of transcription factors (to be abrogated by HPV E7)[7,11] and (2) its prosurvival (cytoprotective) activities, stimulated, eg, by PAI-2 and all IAPs.[12,13] To illustrate this complexity, one of these IAPs (survivin) also was recently shown to be intimately linked with HR HPV, most plausibly explained by the fact that the normal transcriptional repression of survivin by wild-type p53 is eliminated by the HR-HPV E6 oncoprotein.[34]

At this point, we should emphasize that PAI-2 expression is also a function of lack of pRb, ie, in the absence of any measurement of pRb in the cells, it is not possible to determine if the detected expression of PAI-2 is high enough to bring the level of pRb back to normal or close to normal, so as to reverse the effects of its absence. Taken with the observed strong relation between PAI-2 expression and lesion grade, as well as viral load, we could speculate that PAI-2 is perhaps a function of pRb absence more than its capacity to bring back pRb. Unfortunately, we were unable to include the assessment of pRb among the markers in the present series. On the basis of the preceding findings, we think it highly unlikely that 1 single marker analyzed in 1 (baseline) sample only could accurately predict the subsequent long-term outcomes of HR-HPV infection and/or disease progression. In this respect, it will be of interest to assess whether PAI-2 has any prognostic role in cervical cancer, similar to findings in several other human tumors.[16,19]

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References

1. zur Hausen H. Papillomaviruses in the causation of human cancers: a brief historical account. *Virology.* 2009;384:260-265.

2. Syrjänen KJ. Biology of human papillomavirus (HPV) infections and their role in squamous cell carcinogenesis. *Med Biol.* 1987;65:21-39.

3. Syrjänen K, Syrjänen S. *Papillomavirus Infections in Human Pathology.* New York, NY: Wiley; 2000.

4. Syrjänen S, Syrjänen K. The history of papillomavirus research. *Cent Eur J Public Health.* 2008;16(suppl):S7-S13.

5. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med.* 2003;348:518-527.

6. Cliffort GM, Gallus S, Herrero R, et al. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: pooled analysis. *Lancet.* 2005;366:991-998.

7. Syrjänen SM, Syrjänen KJ. New concepts on the role of human papillomavirus in cell cycle regulation. *Ann Med.* 1999;31:175-187.

8. von Knebel Doeberitz M. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *Eur J Cancer.* 2002;38:2229-2242.

9. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus–induced oncogenesis. *J Virol.* 2004;78:11451-11460.

10. Branca M, Ciotti M, Santini D, et al. p16[INK4A] expression is related to grade of CIN and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. *Int J Gynecol Pathol.* 2004;23:354-365.

11. Santopietro R, Shabalova IP, Petrovichev N, et al. Cell cycle regulators p105, p107, Rb2/p130, E2F4, p21[CIP1/WAF1], cyclin-A in predicting CIN, high-risk human papillomavirus infections and their outcome in women screened in three new independent states of the former Soviet Union. *Cancer Epidemiol Biomarker Prev.* 2006;15:1250-1256.

12. Du W, Pogoriler J. Retinoblastoma family genes. *Oncogene*. 2006;25:5190-5200.

13. Chau BN, Wang JY. Coordinated regulation of life and death by RB. *Nat Rev Cancer*. 2003;3:130-138.

14. Darnell GA, Antalis TM, Johnstone RW, et al. Inhibition of retinoblastoma protein degradation by interaction with the serpin plasminogen activator inhibitor 2 via a novel consensus motif. *Mol Cell Biol*. 2003;23:6520-6532.

15. Tonnetti L, Netzel-Arnett S, Darnell GA, et al. SerpinB2 protection of retinoblastoma protein from calpain enhances tumor cell survival. *Cancer Res*. 2008;68:5648-5657.

16. Croucher DR, Saunders DN, Lobov S, et al. Revisiting the biological roles of PAI2 (SERPINB2) in cancer. *Nat Rev Cancer*. 2008;8:535-545.

17. Myöhänen H, Vaheri A. Regulation and interactions in the activation of cell-associated plasminogen. *Cell Mol Life Sci*. 2004;61:2840-2858.

18. Belin D. Biology and facultative secretion of plasminogen activator inhibitor-2. *Thromb Haemost*. 1992;70:144-147.

19. Duffy MJ. The urokinase plasminogen activator system: role in malignancy. *Curr Pharm Des*. 2004;10:39-49.

20. Croucher DR, Saunders DN, Stillfried GE, et al. A structural basis for differential cell signalling by PAI-1 and PAI-2 in breast cancer cells. *Biochem J*. 2007;408:203-210.

21. Croucher D, Saunders DN, Ranson M. The urokinase/PAI-2 complex: a new high affinity ligand for the endocytosis receptor low density lipoprotein receptor–related protein. *J Biol Chem*. 2006;281:10206-10213.

22. Darnell GA, Antalis TM, Rose BR, et al. Silencing of integrated human papillomavirus type 18 oncogene transcription in cells expressing serpinB2. *J Virol*. 2005;79:4246-4256.

23. Syrjänen K, Naud P, Derchain SM, et al. Comparing Pap smear cytology, aided visual inspection, screening colposcopy, cervicography and HPV testing as optional screening tools in Latin America: study design and baseline data of the LAMS Study. *Anticancer Res*. 2005;25:3469-3480.

24. Longatto-Filho A, Maeda MYS, Eržen M, et al. Conventional Pap smear and liquid-based cytology as screening tools in low-resource settings of Latin America: experience of the Latin American Screening Study. *Acta Cytol*. 2005;49:500-506.

25. Sarian L, Derchain SM, Naud P, et al. Evaluation of visual inspection with acetic acid (VIA), Lugol's iodine (VILI), cervical cytology and HPV testing as cervical screening tools in Latin America: this report refers to partial results from the LAMS (Latin AMerican Screening) study. *J Med Screen*. 2005;12:142-149.

26. Longatto-Filho A, Eržen M, Branca M, et al. Human papillomavirus (HPV) testing as an optional screening tool in low-resource settings of Latin America: experience from the LAMS Study. *Int J Gynecol Cancer*. 2006;16:955-962.

27. Syrjänen K, Shabalova I, Naud P, et al. Persistent high-risk human papillomavirus infections and other end-point markers of progressive cervical disease among women prospectively followed up in the New Independent States of the Former Soviet Union and the Latin American Screening study cohorts. *Int J Gynecol Cancer*. 2009;19:934-942.

28. Seed PT, Tobias A. Summary statistics for diagnostic tests. *Stata Tech Bull*. 2001;59:9-12.

29. Syrjänen KJ. Summary statement on prognostic factors in carcinoma of the uterine cervix. *CME J Gynec Oncol*. 2003;8:298-301.

30. von Knebel-Döberitz M, Syrjänen K. Molecular markers: how to apply in practice. *Gynecol Oncol*. 2006;103:18-20.

31. Hasina R, Hulett K, Bicciato S, et al. Plasminogen activator inhibitor-2: a molecular biomarker for head and neck cancer progression. *Cancer Res*. 2003;63:555-559.

32. Branca M, Ciotti M, Giorgi C, et al. Predicting high-risk human papillomavirus (HPV) infection, progression of cervical intraepithelial neoplasia (CIN) and prognosis of cervical cancer with a panel of 13 biomarkers tested in multivariate modelling. *Int J Gynecol Pathol*. 2008;27:265-273.

33. Tan X, Wang JY. The caspase-Rb connection in cell death. *Trends Cell Biol*. 1998;8:116-120.

34. Branca M, Giorgi C, Santini D, et al; for the HPV-Pathogen ISS Study Group. Survivin as a marker of cervical intraepithelial neoplasia and high-risk human papillomavirus and a predictor of virus clearance and prognosis in cervical cancer. *Am J Clin Pathol*. 2005;124:113-121.