Cell Cycle Regulators p105, p107, Rb2/p130, E2F4, p21$^{CIP1/WAF1}$, Cyclin A in Predicting Cervical Intraepithelial Neoplasia, High-Risk Human Papillomavirus Infections and Their Outcome in Women Screened in Three New Independent States of the Former Soviet Union

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Abstract

Background: **The growth-controlling functions of the high-risk human papillomaviruses (HPV) depend on their ability to interact with several cellular proteins, including the key regulatory proteins of the cell cycle. We have examined the value of cell cycle regulatory proteins as predictors of the intermediate end point markers in cervical carcinogenesis: (*a*) grade of cervical intraepithelial neoplasia (CIN), (*b*) high-risk HPV type, (*c*) clearance/persistence of high-risk HPV, and (*d*) disease outcome in women participating in a multicenter follow-up study in three New Independent States countries.**

Methods: **Totally, 232 biopsy samples tested high-risk HPV-positive and/or Papanicolaou smear–positive women were immunohistochemically stained for the following cell cycle markers: p105, p107, p130, E2F4, p21$^{CIP1/WAF1/SDI1}$, cyclin A, and Ki-67. In addition, apoptotic index (AI) and mitotic index (MI) were determined in H&E-stained sections. Prospective follow-up data were available to disclose the clinical and virological outcome of the lesions.**

Results: **The expression of Ki-67, p21$^{CIP1/WAF1/SDI1}$, and cyclin A and AI and MI values were markedly increased in high-grade lesions, but only MI was an independent predictor of CIN3 in multivariate analysis. Cyclin A was the only independent predictor of high-risk HPV (odds ratio, 1.09; 95% confidence interval, 1.01-1.18; P = 0.021), exceeding the predictive power of CIN grade and high-grade squamous intraepithelial lesion Papanicolaou smears. None of these markers provided any useful predictive information as to the clinical and virological outcomes during the follow-up. Highly significant correlations (P = 0.0001) were found between AI and MI as well as between MI and cyclin A, Ki-67 and p21$^{CIP1/WAF1/SDI1}$, Ki-67 and cyclin A, and p21$^{CIP1/WAF1/SDI1}$ and cyclin A followed by that between p105 and cyclin A (P = 0.001) and p105 and p130 (P = 0.002).**

Conclusions: **All tested factors related to cell cycle were increased, but only MI and cyclin A was an independent predictor of CIN3 and high-risk HPV carriage, respectively.** (Cancer Epidemiol Biomarkers Prev 2006;15(7):1250–6)

Introduction

The different oncogenic potential of low-risk and high-risk human papillomaviruses (HPV) in inducing cervical cancer and its precursor [cervical intraepithelial neoplasia (CIN)] lesions is attributable to the different interactions of the two viral oncoproteins, E6 and E7, with several cellular proteins of which the most well characterized are the two key regulatory proteins of the cell cycle, p53 and pRb (1-12). The Rb gene family includes three members: p105, p107, and Rb2/p130 (11-16). The Rb/p105 gene is the prototype tumor suppressor gene and is the first of the Rb family members to be identified (12). The Rb/p105 gene maps to the 13q14 chromosome, where deletions and heterozygous mutations are frequent in many human malignancies (12, 17). The second of the Rb family members, p107 gene, maps to a chromosome region 20q11.2 that seems to be rarely altered in human malignancies (12, 18). p107 is most abundantly expressed during the G_1 and S phases and disappears in arrested and differentiated cells (12, 15-17). Rb2/p130 maps to the 16q12.2 chromose and seems to be more restricted in its function in cells that are not in the proliferative phase of the cell cycle (12, 13, 15, 16, 18).

Each of these three pocket proteins elicits G_1-S growth arrest, and their phosphorylation status is regulated in strictly cell cycle–dependent manner (12-18). The growth-suppressive properties of the Rb family members are dependent on their

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ability to regulate the E2F transcription factors (11, 12). In mammalian cells, the E2F family of transcription factors consists of seven members (E2F1-E2F7; refs. 11, 12, 15). In general, E2F1, E2F2, and E2F3 function during the G_1-S progression, whereas E2F4 and E2F5 largely function during quiescence and are involved mainly in repression (11, 12, 15, 19). These three Rb family members show different affinities for the different E2F members in that p105 preferentially binds to E2F1 to E2F3 (but also to E2F4), whereas only p107/E2F4 complexes exist *in vivo*. Rb2/p130 in turn associates with E2F4 and E2F5, switching from E2F5 to E2F4 complex when the cell enters the G_1 phase (11, 12, 15, 16). E2F1 to E2F3 differ from E2F4 to E2F5 in that they have a domain NH_2-terminal to the DNA-binding domain, which bind to cyclin A. E2F4 mutations have been found in high percentage of primary tumors (20). E7 binds to all these pocket proteins and disrupts the interaction of E2Fs, thereby activating E2F-dependent transcription (4, 7, 11-16). The release of E2Fs, which in turn activate transcription of a group of genes encoding key proteins for S-phase progression, like cyclin E or A (21-24).

Cyclin A overexpression is a frequent phenomenon in low-grade and high-grade CIN lesions (8, 24). Other cell cycle regulators include a class of molecules called cyclin-dependent kinase (CDK) inhibitors, of which there are two functional groups: the KIP/CIP (p21, p27, and p57) and the INK4 (p15, p16, p18, and p19) groups (3, 11, 12, 21). In this study, we were interested in $p21^{CIP1/WAF1/SDI1}$, which is the transcriptional target of p53 and a critical determinant of the G_1 arrest as a response to DNA damage (6, 11, 12, 21, 22). $p21^{CIP1/WAF1/SDI1}$ inhibits a broad range of CDKs, including both CDK4 and CDK2, and with its two mutually competitive binding sites can also bind and inhibit proliferating cell nuclear antigen (6).[12] High-risk HPV E7 is capable of blocking this ability of $p21^{CIP1/WAF1/SDI1}$ to inhibit CDK and proliferating cell nuclear antigen–dependent DNA replication through direct binding to the COOH terminus of $p21^{CIP1/WAF1/SDI1}$, which is necessary for HPV DNA replication and G_1-S transition (6, 25).[12]

We recently conducted a cohort study testing 3,187 women for optional screening tools in three New Independent States of the Former Soviet Union, and ~900 of these women were followed-up to assess the natural history of their HPV infections (the New Independent States cohort study; refs. 26, 27). We also analyzed the temporal relationships between acquisition, persistence, and clearance of high-risk HPV and the clinical outcome of the lesions (28-31).

In the present study, we have analyzed the expression of several cell cycle proteins in baseline biopsy samples (n = 155) to estimate their usefulness as predictors of the intermediate end point markers in cervical carcinogenesis. The following proteins were included: the three pocket proteins (p105, p107, and p130), the transcriptional factor E2F4, cell cycle regulator (cyclin A, promoting G_1-S and G_2-M transition), and a CDK inhibitor ($p21^{CIP1/WAF1/SDI1}$ responsible for G_1-S arrest). Cell proliferation was monitored by analyzing the expression of Ki-67 proliferation marker expressed at the G_1, S, G_2, and M phases of the cell cycle (32) as well as using apoptotic index (AI) and mitotic index (MI). The rationale for selecting these particular proteins is that E7 of the oncogenic HPV types is capable of interacting with all of them (1-3).

[12] M. Branca, C. Giorgi, M. Ciotti, et al. Upregulation of proliferating cell nuclear antigen (PCNA) is closely associate with high-risk human papillomavirus (HPV) and progression of cervical intraepithelial neoplasia (CIN), but does not predict disease outcome in cervical cancer. Int J Gynecol Cancer 2005, submitted for publication.

Materials and Methods

Materials

Study Design. The samples for this study were obtained from women participating in a cross-sectional/cohort study in three New Independent States of the Former Soviet Union, enrolled by six outpatient clinics in Moscow, Novgorod (Russia), Minsk (Belarus), and Riga (Latvia) between 1998 and 2002 as has been described earlier (26-28, 30, 31). Three target populations of women with different risk for HPV infections were enrolled: (*a*) women participating in cervical cancer screening, (*b*) those attending gynecologic outpatient clinics with different indications, and (*c*) patients examined at standard clinics. All women had Papanicolaou (Pap) smear and HPV testing was done with Hybrid Capture II (HCII; Digene, Silver Spring, MD). Women with cytologic abnormality consistent with HPV-CIN (ASC or above) and those testing positive with HCII were referred to colposcopy and biopsy confirmation. Punch biopsies were fixed in formalin and processed for H&E-stained sections for light microscopic examination following the routine procedures. A total of 497 biopsy samples were taken at the first control visit (baseline biopsies). All biopsies were reexamined. The concordance (intraclass correlation coefficient, weighted κ) between the two raters was 0.758 [95% confidence interval (95% CI), 0.684-0.815]. Cervical lesions were graded using the CIN nomenclature, extended by the lowest category (HPV-NCIN), denoting lesions with HPV morphology but devoid of CIN (1, 26, 27). Due to the national regulations preventing the delivery of tissue blocks abroad, altogether 232 biopsies (baseline and FU) were available for this immunohistochemical analysis, including 155 of the baseline biopsies in following categories: 41 cases of metaplasia, 42 cases of HPV-NCIN, 35 cases of CIN1, 16 cases of CIN2, 13 cases of CIN3, and 8 cases of invasive squamous cell carcinoma.

Follow-up and Viral Outcomes. All women who presented with low-grade lesions (HPV-NCIN or HPV-CIN1) were subjected to prospective follow-up, whereas high-grade lesions were promptly treated as detailed before (28-31). Follow-up at 6-month intervals included examination by colposcopy, Papanicolaou (Pap) smear, and punch biopsy (in case of suspected progression). Cytologic samples for HPV DNA testing with HCII were collected at each follow-up visit. Altogether, follow-up data were available from a total of 887 women (29-31).

Methods

Detection of HPV DNA. HPV testing of scrapings taken with the HCII sampling kit was done with the HCII test according to manufacturer's instructions The HCII test was done using the probe panel B, which detects the high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (26).

Real-time PCR for HPV Physical Status. A novel real-time PCR method developed by our group was used to analyze the physical state of HPV16-positive samples (33, 34).

Immunohistochemistry for Cell Cycle Markers. Immunohistochemical staining for cell cycle markers was completed following standard procedures. In brief, the 5-μm paraffin sections cut on poly-L-lysine-coated microscopy slides were first deparaffinized and rehydrated in graded alcohols. Antigen retrieval was done in 1 mmol/L EDTA (pH 8.0) by heating sections in either a pressure cooker or a microwave oven according to the antibody used followed by blocking the nonspecific binding sites with goat/rabbit serum. Sections were incubated with the primary antibody in a humidified chamber for 1 hour at room temperature. The antibodies and the dilutions used are listed in Table 1. Slides were then processed with ULTRAVISION/AP method (Lab Vision Corp.,

Table 1. Characterization of the antibodies and the assays used

Antibody	Manufacturer	Antigen retrieval	Dilution
pRb/p105	Biogenex Menarini (Italy)	1 mmol/L EDTA (pH 8.0) by heating in a microwave	1:200
pRb/p107	DBA (Italy)	1 mmol/L EDTA (pH 8.0) by heating in a pressure cooker	1:500
pRb/p130	Prodotti Gianni (Italy)	1 mmol/L EDTA (pH 8.0) by heating in a microwave	1:100
E2F4	Bio-Optica (Italy)	1 mmol/L EDTA (pH 8.0) by heating in a microwave	1:100
Cyclin A	DBA, Italy (Novocastra)	1 mmol/L EDTA (pH 8.0) by heating in a microwave	1:100
$p21^{CIP1/WAF1/SDI1}$	Inalco Division (Calbiochem)	1 mmol/L EDTA (pH 8.0) by heating in a microwave	1:100
Ki-67	DakoCytomation (Denmark)	1 mmol/L EDTA (pH 8.0) by heating in a microwave	1:50

Fremont, CA) used to visualize the immunostaining. As a final step, the slides were stained with a light hematoxylin counterstaining. Negative controls were similarly processed by omitting the primary antibody, and biopsies from breast cancer were used as positive controls.

Evaluation of the Immunohistochemical Staining. Immunostaining for all antibodies was quantified by counting the cells exhibiting positive staining with a given antibody in 10 randomly selected high-power fields (×40; within the site of the most severe lesion in the biopsy), and the results were expressed as percentages of all epithelial cells in those areas (minimum of 2,000 cells). Intraobserver and interobserver reproducibility of the counts were in the order of 95%. In statistical analysis, the staining results were used as continuous (scale) variables in the nonparametric tests.

MI and AI. Mitotic figures were counted from H&E-stained sections using a dual-headed microscope by two observers with an objective magnification ×40 (field diameter 490 μm) as described previously (35). The volume-corrected MI method was used, which expresses the number of mitotic figures per square millimeter of the neoplastic (CIN or cervical cancer) epithelium in the section. Apoptotic cells were counted using the same method in five consecutive microscope fields (field diameter 490 μm, magnification ×40, corresponding to 1 mm^2 of neoplastic epithelium in the section), and the results were corrected to correspond the number of apoptotic cells per square millimeter of the neoplastic epithelium (volume-corrected AI). The criteria for identifying the apoptotic cells were those described previously (36): (*a*) cells showing marked condensation of the chromatin and cytoplasm, (*b*) cytoplasmic fragments containing condensed chromatin, and (*c*) intracellular and extracellular chromatin fragments down to diameter of ~2 μm (36). All counting was done by the same pathologist (R.S.) blinded by all the other data of the patients.

Statistical Analyses. Statistical analyses were done using the SPSS and Stata computer software packages (SPSS for Windows version 12.0.1 and STATA/SE version 9.1). Frequency tables were analyzed using the χ^2 test, with likelihood ratio being used to assess the significance of the correlation between the categorical variables. Differences in the means of continuous variables (i.e., percentages of the markers) between the strata were analyzed using nonparametric tests (Mann-Whitney or Kruskal-Wallis). Odds ratio (OR) and 95% CI were calculated using the exact method. Scale variables were correlated to each other using nonparametric (bivariate) correlation with Spearman ρ and its significance test. Interobserver agreement was measured using intraclass correlation coefficient, which is equivalent to weighted κ. Logistic regression models were used to analyze the power of different covariates as predictors of the outcome variables (high-grade CIN, virus clearance/persistence, integration) in both univariate (crude OR and 95% CI) and multivariate (adjusted OR) analysis using the stepwise backward approach and likelihood ratio statistic for removal testing ($P = 0.10$ for stepwise removal and $P = 0.05$ for stepwise entry). In all tests, Ps < 0.05 were regarded statistically significant.

Results

Table 2 summarizes the levels of the cell cycle markers in high-grade lesions (CIN3 and cancer) and low-grade lesions. MI and AI were the two factors with highest statistical difference between the two categories of lesions. In addition, Ki-67, p21, and cyclin A were up-regulated in high-grade lesions, whereas no statistically significant difference was found in the expression levels of E2F4, p105, p107, and p130. Interestingly, both E2F4 and p107 were expressed less in CIN3 and cervical cancer lesions than in low-grade lesions.

Logistic regression analysis (in univariate mode) was used to calculate the crude OR for the cell cycle markers, AI, MI, age, high-risk HPV, and viral load, as predictors of high-grade lesions (Table 3). AI and MI were significant predictors with the same power as age and high viral load ($P = 0.0001$), whereas Ki-67, high-risk HPV type, and cyclin A predicted high-grade lesions with lower statistical power.

All significant variables were entered in the multivariate logistic regression model to assess independent predictors of high-grade CIN. Only the MI remains an independent significant predictor ($P = 0.0001$) with OR (95% CI) of 1.77 (1.257-2.513) when adjusted for high-risk HPV type, viral load, AI, Ki-67, and cyclin A. The significance of MI was not confounded even by age, resulting in adjusted OR (95% CI) of 1.802 (1.198-2.712; $P = 0.005$).

Table 4 summarizes the levels of the cell cycle markers in high-risk HPV-positive and high-risk HPV-negative lesions. Of all markers analyzed, only cyclin A was proven to be significantly different in high-risk HPV-positive and high-risk HPV-negative lesions ($P = 0.002$). In regression analysis, cyclin A was a predictor of high-risk HPV with OR (95% CI) of 1.10 (1.02-1.20; $P = 0.011$). In multivariate analysis, cyclin A was not confounded by age or any of the other markers, and it remained the only significant independent predictor (OR, 1.09; 95% CI, 1.01-1.18; $P = 0.021$). This was true even when histology and Pap smear results were entered into the multivariate model, both being significant predictors of

Table 2. Cell cycle markers in high-grade and low-grade cervical lesions

Marker	CIN3 or cancer, OR (95% CI)	Low-grade lesions, OR (95% CI)	Significance*
MI†	9.04 (5.55-12.53)	1.91 (1.41-2.40)	0.0001
AI‡	4.14 (2.44-5.84)	1.44 (1.12-1.76)	0.0001
Ki-67§	26.70 (8.43-44.96)	13.14 (10.64-15.64)	0.044
p21§	18.12 (8.25-27.99)	11.52 (8.24-14.80)	0.033
Cyclin A§	30.75 (16.32-45.18)	15.75 (13.79-20.39)	0.021
E2F4§	0.33 (0.21-0.87)	0.66 (0.31-1.00)	0.704
p105§	62.22 (36.50-87.94)	40.84 (33.84-47.84)	0.061
p107§	8.12 (1.85-18.10)	13.65 (9.98-17.31)	0.200
p130§	42.50 (4.38-80.61)	36.70 (28.17-45.34)	0.757

*Mann-Whitney *U* test.
†No. mitotic figures/mm^2.
‡No. apoptotic cells/mm^2.
§Percentages of stained cells in 10 randomly selected fields (×40).

Table 3. Cell cycle markers and other predictors of CIN3 and invasive cancer in univariate regression analysis

Marker	Crude OR (95% CI)	Significance*
MI[†]	1.342 (1.172-1.535)	0.0001
AI[‡]	1.431 (1.191-1.728)	0.0001
Ki-67[§]	1.056 (1.011-1.102)	0.013
p21[§]	1.023 (0.984-1.063)	0.247
Cyclin A[§]	1.042 (1.005-1.080)	0.025
E2F4[§]	0.788 (0.369-1.682)	0.538
p105[§]	1.023 (0.999-1.049)	0.066
p107[§]	0.967 (0.903-1.036)	0.343
p130[§]	1.004 (0.986-1.022)	0.693
Age	1.090 (1.065-1.116)	0.0001
High-risk HPV	2.887 (1.122-7.427)	0.028
High-risk HPV viral load[‖]	1.227 (1.116-1.349)	0.0001

*Mann-Whitney *U* test.
†No. mitotic figures/mm^2.
‡No. apoptotic cells/mm^2.
§Percentages of stained cells in 10 randomly selected fields (×40).
‖Measured as HCII RLU/CO index.

high-risk HPV in univariate analysis [OR (95% CI), 3.19 (1.83-5.54) and 19.81 (7.84-50.02), respectively].

Similar analysis was made to assess the expression levels of the cell cycle markers and AI and MI in lesions with integrated or episomal HPV16. None of these nine markers was significantly related to HPV16 integration status (data not shown).

Table 5 gives the values of the markers in relation to clinical and virological outcomes during the follow-up. The levels of AI, MI, and all markers were similar in lesions where HPV persisted and in those undergoing HPV clearance as well as in cases with persistent versus cleared Pap smear abnormality, implicating that none of these markers is a reliable predictor of clinical and virological outcome in this series. In logistic regression, p105 expression predicted HPV clearance with borderline significance (P = 0.042; OR, 0.984; 95% CI, 0.969-0.999), whereas none of the other markers predicted either clinical or viral outcomes.

The correlation matrix was calculated for the cell cycle markers analyzed. The significant bivariate correlations (Spearman ρ) are shown in Table 6. AI and MI were significantly related to each other (P = 0.0001). MI and cyclin A were correlated to each other at P = 0.0001 level as were Ki-67 to p21, Ki-67 to cyclin A, and p21 to cyclin A. Of the other correlations, the most significant one was that between p105 and cyclin A. All other correlations not shown in Table 6 did not reach statistical significance.

Discussion

Papillomaviruses replicate and complete their life cycle in terminally differentiating cells. Acting in concert with other early proteins (E5 and E6; refs. 1-3, 9, 10), the role of E7 is to stimulate their target cells (programmed to differentiation) to reenter the cell cycle and progress through S phase to create an environment for efficient DNA replication (37). Biological functions of E7 are important for this transition and the protein acts on pathways that include (*a*) those regulated by the Rb family, which have effects on cell arrest, differentiation, and transcription of proteins regulating the G_1-S-phase transition, (*b*) the pathways that control the chromatin structure, which in turn will determine the transcriptional pattern in the cells, and (*c*) pathways stimulated by the G_1 cyclin kinases (CDK). All these pathways interact at different stages, so the E7 oncoprotein has multiple input signals to bypass the arrest and stimulate the G_1-S-phase progression (37), allowing also the replication of viral DNA. Interestingly, not only E7 but also E6 induced cellular proliferation, pRb phosphorylation, and accumulation of products of genes that are negatively regulated by pRb, such as p16, E2F1, and cyclin A. All these events seemed to be independent of p53 inactivation (38).

The present study attempted to dissect some of these mechanisms by targeted analysis of the core proteins acting in the pathways regulating the G_1-S transition: the three pocket proteins of the Rb family (p105, p107, and p130), transcription factor E2F4 (capable of binding to all these pocket proteins), cyclin A (forming complexes with CDK2 during the S phase), and a potent CDK inhibitor, $p21^{CIP1/WAF1/SDI1}$, the transcriptional target of p53 and a critical determinant of the G_1 arrest on DNA damage (6, 11, 12, 21, 37). In addition, Ki-67 expressed in all phases of the cell cycle (except G_0) was analyzed to monitor cell proliferation (32). The rationale for selecting these particular proteins is that E7 of the oncogenic HPV types is capable of interacting with all of them (37). Thus, E7 binds to the pocket proteins (p105, p107, and Rb2/p130), leading to abrogation of their growth-suppressive properties (4, 7, 11-13, 16). Similarly, E7 increases free E2F by disruption of E2F-pocket protein-complexes and phosphorylation and degradation of Rb, leading to anchorage-independent cell proliferation (21, 23). The effects of E7 on cyclin A-CDK2 are similar as for cyclin E-CDK2 (i.e., E7 binds to cyclin A-CDK2 in a complex involving E2F and p107; ref. 39), but the details of the E7 activities on this complex are not fully elucidated as yet (23, 37). Furthermore, high-risk HPV E7 is capable of blocking the ability of $p21^{CIP1/WAF1/SDI1}$ to inhibit CDK activity and proliferating cell nuclear antigen–dependent DNA replication through direct binding to the COOH terminus of $p21^{CIP1/WAF1/SDI1}$, a function necessary for HPV DNA replication and G_1-S transition (6, 25).[12]

Until now, incomplete data are available on the interactions of E7 with most of these proteins at tissue level. Earlier studies on cell cycle proteins and their associations with HPV infections in cervical lesions were focused on interactions between E7 and p105 (7, 37, 40-42) followed by the recent interest in p107 and p130 (11-16, 43). The HPV interactions with $p21^{CIP1/WAF1/SDI1}$ have received some attention only recently (5, 6, 44-48), and even less reports are available on cyclin A expression and its eventual prognostic value in cervical cancer (8, 49-51). Thus, it was of interest to elucidate how the differential expression of these cell cycle regulators relates to the different intermediate end point markers in cervical carcinogenesis based on immunohistochemical staining of the baseline biopsies from Pap smear–positive and/or HCII-positive women followed-up as part of the original New Independent States Cohort study (26-31).

For routine use, one of the most important aspect is which cell cycle proteins could be used to predict the progression of CIN. Here, we compared their expression in low-grade and high-grade lesions, and the two most powerful discriminators

Table 4. Cell cycle markers in high-risk HPV-positive and high-risk HPV-negative lesions

Marker	HCII positive, OR (95% CI)	HCII negative, OR (95% CI)	Significance*
MI[†]	2.87 (2.10-3.64)	2.81 (0.05-5.68)	0.959
AI[‡]	1.82 (1.43-2.22)	1.62 (0.13-3.11)	0.743
Ki-67[§]	14.04 (11.27-16.80)	10.40 (3.80-16.99)	0.303
p21[§]	12.40 (8.88-15.91)	9.80 (4.40-15.19)	0.525
Cyclin A[§]	19.88 (16.18-23.59)	6.78 (3.80-9.77)	0.002
E2F4[§]	0.49 (0.26-0.72)	0.68 (0.40-1.76)	0.609
p105[§]	42.98 (35.50-50.46)	35.00 (21.51-48.48)	0.351
p107[§]	12.72 (9.40-16.05)	11.31 (6.34-16.28)	0.705
p130[§]	40.12 (31.08-49.17)	22.35 (3.50-41.20)	0.085

*Mann-Whitney *U* test.
†No. mitotic figures/mm^2.
‡No. apoptotic cells/mm^2.
§Percentages of stained cells in 10 randomly selected fields (×40).

Table 5. Cell cycle markers related to different viral and clinical outcomes

Marker	Viral outcome		Significance*
	Persistent HPV, OR (95% CI)	HPV cleared, OR (95% CI)	
MI[†]	1.92 (1.19-2.65)	1.86 (1.13-2.60)	0.909
AI[‡]	1.56 (1.15-1.97)	1.43 (0.96-1.90)	0.670
Ki-67[§]	13.55 (8.48-18.62)	14.51 (10.80-18.21)	0.756
p21[§]	12.29 (7.18-17.39)	11.38 (6.59-16.18)	0.793
Cyclin A[§]	19.48 (13.14-25.81)	19.50 (13.81-25.18)	0.996
E2F4[§]	1.77 (0.15-3.70)	0.48 (0.10-0.85)	0.173
p105[§]	35.71(22.01-49.41)	49.02 (38.04-60.01)	0.131
p107[§]	11.55 (6.92-16.18)	13.23 (7.48-18.98)	0.661
p130[§]	47.66 (32.39-62.94)	33.88 (21.04-46.73)	0.161
	Clinical outcome		
	Persistent abnormal Pap smear, OR (95% CI)	Abnormal Pap smear cleared, OR (95% CI)	
MI[†]	2.50 (1.63-3.36)	2.00 (1.20-2.79)	0.392
AI[‡]	1.44 (0.92-1.95)	1.60 (1.10-2.11)	0.648
Ki-67[§]	14.68 (10.18-19.18)	16.53 (11.62-21.44)	0.573
p21[§]	12.85 (7.55-18.15)	10.88 (6.03-15.74)	0.583
Cyclin A[§]	17.29 (11.01-23.57)	20.20 (13.83-26.56)	0.509
E2F4[§]	2.01 (0.67-4.70)	0.67 (0.05-1.41)	0.266
p105[§]	40.86 (28.85-52.88)	40.00 (25.05-54.94)	0.926
p107[§]	9.92 (5.84-14.00)	9.85 (5.25-14.46)	0.981
p130[§]	27.85 (11.83-43.87)	42.00 (28.25-55.74)	0.173

*Mann-Whitney *U* test.
†No. mitotic figures/mm^2.
‡No. apoptotic cells/mm^2.
§Percentages of stained cells in 10 randomly selected fields (×40).

between the two were AI and MI, both being significantly elevated in high-grade lesions. The three other markers significantly elevated in high-grade lesions were Ki-67, p21$^{CIP1/WAF1/SDI1}$, and cyclin A, although with less statistical significance. Of the Rb family members, only p105 reaches a borderline statistical significance ($P = 0.061$) although distinctly up-regulated in high-grade lesions. In a previous study, p105 was invariably expressed in cervical cancer (41) but had no prognostic value (40, 41). In another report, p107 was shown to increase and p130 was shown to decrease with increasing lesion grade (43), but we could not confirm such a trend in our study. E2F4 was less stained in CIN3 and cervical cancer lesions than in low-grade lesions, but the difference was statistically not significant. It is known that the majority of the cellular E2F activity is due to E2F4 and that E2F4 is relatively constantly expressed throughout the cell cycle. E2F4 is expressed only in undifferentiated cells (52).

Up-regulation of Ki-67 in high-grade lesions is not unexpected, because this protein is one of the most useful cell proliferation markers in CIN and cancer (32, 47, 48, 53-55). The close correlation among Ki-67, p21$^{CIP1/WAF1/SDI1}$, and cyclin A lets us anticipate that also the latter two are related to lesion grade, which was the case. Our data that overexpression of p21$^{CIP1/WAF1/SDI1}$ is closely correlated with CIN grade are consistent with the observations reported in most of the previous studies (44, 46-48), except one (45). Previous data on the expression of cyclin A in CIN are scanty but more uniform in confirming that cyclin A expression increases with increasing grade of CIN and further on progression to invasive disease (8, 50, 51). We confirm these results showing that the proportion of p21$^{CIP1/WAF1/SDI1}$ and cyclin A–expressing cells was usually 2-fold higher in CIN3 than in low-grade lesions. In logistic regression analysis, in addition to age, high-risk HPV type, and high-risk HPV viral load, the most powerful predictors of CIN3 were AI and MI followed by Ki-67 and cyclin A, whereas the predictive power of p21$^{CIP1/WAF1/SDI1}$ does not reach statistical significance. In a multivariate model, only MI remains a significant independent predictor of CIN3 with OR (95% CI) of 1.777 (1.257-2.513; $P = 0.0001$).

In the next step, the cell cycle protein expression was related to the high-risk HPV status of the lesions tested with HCII assay (26, 34). We found that only cyclin A was significantly differently expressed in high-risk HPV-positive than high-risk HPV-negative lesions. In the previous studies, either no correlation was established between HPV status and p21$^{CIP1/WAF1/SDI1}$ expression (47, 48, 56) or it was shown to be lower in HPV-negative lesions (45, 46). Our data did not disclose any difference in p21$^{CIP1/WAF1/SDI1}$ expression between high-risk HPV-positive and high-risk HPV-negative lesions. The same was true with Ki-67, confirming some previous data (47, 48). Concerning the expression of the Rb family pocket proteins, our data implicate that one cannot determine whether the lesion harbors high-risk HPV by using immunohistochemical staining for p105, p107, or p130 (Table 5). No previous data are available on this subject.

This is the first report to confirm a strong association between high-risk HPV and cyclin A expression in CIN lesions. This issue was not addressed in two of the previous reports on cyclin A and CIN (49, 50), and in another study, cyclin A was equally expressed in high-risk HPV-positive and low-risk HPV-positive lesions (8). In multivariate analysis, cyclin A remained as the only significant independent predictor of high-risk HPV (OR, 1.09; 95% CI, 1.01-1.18; $P = 0.021$) even when lesion histology and Pap smear results were entered into the model. This cyclin A activity was unrelated to HPV integration status, however. This close association between oncogenic HPV and cyclin A could be explained by its complex up-regulation by both E7 (during the G_1-S transition) and E6 (in G_2-M transition) oncoproteins, making cyclin A active in both S phase and late G_2 phase of the cell cycle (6, 11, 12, 36). Recently, it was shown that cyclin A and Ki-67 were elevated in E6-expressing cells and that E6 activates the cyclin A promoter in serum-deprived NIH3T3 cells (38, 57). E2F4-p130 complexes have been shown to contribute to repression of cyclin A transcription. Thus, we might speculate that high-risk HPV can interfere with this repression. This

Table 6. Bivariate correlations between the different cell cycle markers

	AI	MI	MIB-1	p21	Cyclin A	E2F4	p105	p107	p130
AI	—	0.491 (0.0001)		0.248 (0.05)	0.230 (0.044)				
MI	0.491 (0.0001)	—	0.356 (0.002)	0.291 (0.013)	0.447 (0.0001)				
Ki-67		0.356 (0.002)	—	0.381 (0.0001)	0.418 (0.0001)				
p21	0.248 (0.05)	0.291 (0.013)	0.381 (0.0001)	—	0.384 (0.0001)		0.236 (0.034)		
Cyclin A	0.230 (0.044)	0.447 (0.0001)	0.418 (0.0001)	0.384 (0.0001)	—		0.338 (0.001)		
E2F4						—			
p105				0.236 (0.034)	0.338 (0.001)		—		0.326 (0.002)
p107								—	
p130							0.326 (0.002)		—

NOTE: Only statistically significant correlations are shown [r (P); r = Spearman ρ].

hypothesis is supported by our results that of all markers analyzed, only cyclin A was proven to be significantly different in high-risk HPV-positive and high-risk HPV-negative lesions ($P = 0.002$). In regression analysis, cyclin A was a predictor of high-risk HPV with OR (95% CI) of 1.10 (1.02-1.20; $P = 0.011$).

We also analyzed whether any of these cell cycle proteins could be of value as predictors of clinical and virological outcomes during the follow-up. It is generally agreed that disclosing reliable markers to predict the women likely to develop a persistent high-risk HPV infections would have important clinical implications (1, 2, 31). In this same cohort, only the smoking status was proven to be such a predictor of the 70 epidemiologic variables analyzed (31). Unfortunately, the present results were proven to be almost equally disappointing in this respect, marker expression being similar in persisting lesions and in those regressed. Somewhat unexpectedly, p105 was disclosed as a significant predictor of HPV clearance in regression analysis, with OR (95% CI) of 1.016 (1.001-1.032; $P = 0.042$). This suggests that higher expression of p105 is linked with high-risk HPV clearance. The mechanism explaining this remains obscure, and we did not yet analyze whether this marginal predictive power of p105 can resist the multivariate analysis, where the previously tested clinical and epidemiologic variables are entered (31).

We also found several significant correlations between the individual variables analyzed. AI and MI are significantly interrelated as reported before in other carcinomas (35) as well as in cervical cancer (53). MI (but not AI) was also closely correlated with other markers of cell proliferation, Ki-67 and cyclin A, the latter being not induced until S phase. In line with previous studies, we could confirm an intimate relationship between Ki-67 and $p21^{CIP1/WAF1/SDI1}$ expression ($P = 0.0001$; refs. 5, 6, 25, 35, 44-46).[12] On the other hand, AI and MI do not seem to be as sensitive markers of $p21^{CIP1/WAF1/SDI1}$ up-regulation, albeit related to it with a lower statistical power ($P = 0.05$ and 0.013, respectively). Not unexpectedly, $p21^{CIP1/WAF1/SDI1}$ and cyclin A also are closely interrelated, implicating that both are useful markers of cell proliferation (6, 11, 12, 21, 22, 24),[12] most notably orchestrated by the capability of E7 to abrogate the inhibitory activity of $p21^{CIP1/WAF1/SDI1}$ on CDK and proliferating cell nuclear antigen–dependent DNA replication necessary for the G_1-S transition (37). E6 protein is shown to induce strong down-regulation of the $p21^{CIP1/WAF1/SDI1}$ gene. It is also known that cyclin A of $p21^{CIP1/WAF1/SDI1}$ complexes may be able to directly affect E2F transcription activity (52).

Of no less interest are the interactions between the three pocket proteins (p105, p107, and p130) and their relationships to the other cell cycle proteins (40, 41, 43). Interestingly, only p105 (but not the others) showed a weak but significant association to $p21^{CIP1/WAF1/SDI1}$ and more strong to cyclin A. The former weak association ($P = 0.034$) might be purely incidental, reflecting the G_1-S transition in general, because these two proteins affect the pathways seemingly unrelated to each other (37). However, the more intimate ($P = 0.001$) association between p105 and cyclin A might be of more direct nature and related to the close interaction of p105 with cyclin A in S phase. Additionally, HPV oncoproteins could stabilize CDK2-E2F-p105 complex (37, 38). It seems that E7 is unable to disrupt the cyclin A-CDK2-E2F-p107 complex (37), which could explain why p107 levels are not directly proportional to cyclin A. In our analysis, p105 was not shown to be related to MI (40) or Ki-67 (39), and no association between p107 and p130 was found (43). E2F4 can induce both p130 and p107, but this association was not found in the protein levels in the present study. Clearly, the current data implicate that the three pocket proteins are active in their complexes at different times of the cell cycle (12) and not necessarily activated simultaneously in a sample taken at a single time point. In addition, the stability and half-time of these proteins are of crucial importance when quantification is made by immunohistochemistry.

In conclusion, the expression of the ''proliferation markers'' tested here were mostly up-regulated in high-grade lesions compared with low-grade disease, but none of them could compete with MI as an independent predictor of CIN3. Cyclin A is a powerful independent predictor of high-risk HPV in multivariate analysis. All markers, however, are disappointing while not providing any useful information about the clinical or virological outcomes during the follow-up. From the practical point of view, these results offer new insights in the highly complex mechanisms of cell cycle regulation and helps understanding the interference of those by oncogenic HPV types at multiple points. As always, transfer of these research data into clinical practice is not straightforward. Valuable as they might be as research tools, the role of these markers (e.g., as potential screening tools) remains to be established by another type of study design devoid of any verification bias (i.e., all women are tested, which is only realistic in samples taken for liquid-based cytology but not by using biopsies).

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