Up-Regulation of 14-3-3σ (Stratifin) Is Associated With High-Grade CIN and High-Risk Human Papillomavirus (HPV) at Baseline but Does Not Predict Outcomes of HR-HPV Infections or Incident CIN in the LAMS* Study

Stina Syrjänen, DDS, PhD,[1] Paulo Naud, MD, PhD,[2] Luis Sarian, MD, PhD,[3] Sophie Derchain, MD, PhD,[3] Cecilia Roteli-Martins, MD, PhD,[4] Adhernar Longatto-Filho, PhD, MIAC,[5] Silvio Tatti, MD, PhD,[6] Margerita Branca, MD, PhD, MIAC,[7] Mojca Eržen, MD, PhD, MIAC,[8] Luciano S. Hammes, MD, PhD,[2] Silvano Costa, MD, PhD,[9] and Kari Syrjänen, MD, PhD, FIAC[10]

Key Words: High-risk human papillomavirus; Stratifin; 14-3-3σ; Cell cycle control; Inactivation; Overexpression; Epigenetic; Cervical intraepithelial neoplasia; Viral outcomes; Disease progression; Longitudinal predictive values; Surrogate end points

DOI: 10.1309/AJCP49DOITYDCTQJ

Abstract

To assess whether the potentially high-risk (HR) human papillomavirus (HPV)-related up-regulation of 14-3-3σ (stratifin) has implications in the outcome of HPV infections or cervical intraepithelial neoplasia (CIN) lesions, cervical biopsy specimens from 225 women in the Latin American Screening Study were analyzed for 14-3-3σ expression using immunohistochemical analysis. We assessed its associations with CIN grade and HR HPV at baseline and value in predicting outcomes of HR-HPV infections and the development of incident CIN 1+ and CIN 2+.

*Expression of 14-3-3σ increased in parallel with the lesion grade. Up-regulation was also significantly related to HR-HPV detection (*P = .004*; odds ratio, 2.71; 95% confidence interval, 1.37-5.35) and showed a linear relationship to HR-HPV loads (*P = .003*). 14-3-3σ expression was of no value in predicting the outcomes (incident, persistent, clearance) of HR-HPV infections or incident CIN 1+ and CIN 2+.*

14-3-3σ is not inactivated in cervical carcinoma and CIN but is up-regulated on transition from CIN 2 to CIN 3. Its normal functions in controlling G_1/S and G_2/M checkpoints are being bypassed by HR HPV.

Practically all cervical carcinomas (CCs) are caused by high-risk human papillomavirus (HR-HPV) infections, whereas the low-risk (LR) HPV types are rarely found in CC or its precursor (cervical intraepithelial neoplasia [CIN]) lesions.[1-6] This divergent oncogenic potential of LR HPV 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.[1,4,7-11] In addition to this G_1/S checkpoint, E6 and E7 (together with several cellular proteins) also control the G_2/M checkpoint.[7-9,11] One of the cellular G_2/M checkpoint–controlling proteins is 14-3-3σ, also known as stratifin.[12]

The 14-3-3σ gene was originally characterized from normal mammary epithelium as a human mammary epithelial marker (HME1), and its expression was markedly reduced in breast cancer cells.[13] 14-3-3 proteins represent a family of highly homologous proteins and have been described in all eukaryotic organisms.[12] The mammalian 14-3-3 isoforms β, γ, ε, η, σ, τ, and ξ are encoded by 7 individual genes.[12,14] 14-3-3σ expression is mediated by p53 and p21, following various types of DNA damage, and 14-3-3σ has an important role in preventing mitotic catastrophe after DNA damage, sequestering the cyclin-B1 and cyclin-dependent kinase 1 (CDK1) complexes from the nucleus to the cytoplasm, resulting in G_2/M cell cycle arrest.[15,16] Furthermore, 14-3-3σ binds to CDK1, CDK2, CDK4, and their related complexes and inhibits the cells from entering the cell cycle.[17,18]

In addition to this G_2/M checkpoint control,[12-18] 14-3-3 proteins are important regulators of many cellular processes,[19-21]

including signal transduction, apoptosis, transcriptional regulation, and coordination of cell adhesion and motility.[19-21] Thus, 14-3-3 proteins in human carcinogenesis have attracted considerable interest.[14,22] Despite the alleged tumor suppressor activity of 14-3-3σ, this gene has been shown to be inactivated in most human malignancies.[14,22-24] It is interesting that this inactivation is not the result of mutation or gene deletion but is due to epigenetic inactivation by promoter methylation.[14,22-24]

In contrast with most other malignant tumors,[14,22-24] CC and CIN lesions seem to continue normal expression of 14-3-3σ, which shows even up-regulation as compared with the normal epithelium.[25] The reasons for this paradoxical behavior of 14-3-3σ in CC and CIN are not understood, but interactions with HR HPV are implicated. Until now, however, expression of 14-3-3σ in CC and CIN lesions has been analyzed in 1 study only.[25] While strong and diffuse immunoreactivity for 14-3-3σ was uniformly observed in all CIN and squamous cell carcinoma lesions, some (HPV–) adenosquamous carcinomas and adenocarcinomas did not express 14-3-3σ. Concomitant inactivation of 14-3-3σ and p16 was never observed, suggesting that inactivation of 14-3-3σ or p16 might have an effect equivalent to the expression of HR HPV E6 and E7 oncoproteins.

To further delineate the role of 14-3-3σ in HR HPV–associated cervical carcinogenesis, we analyzed a series of cervical biopsy specimens derived from 225 women included in the Latin American Screening (LAMS) Study cohort (n = 12,114) in Brazil and Argentina.[26-29] The study aimed to assess whether the expression of 14-3-3σ is associated with the grade of CIN and HR HPV type at baseline and predict the outcome of these HR-HPV infections or development of incident CIN 1+ and CIN 2+ during prospective follow-up of the women.[26,27]

Materials and Methods

General Study Design

The ongoing LAMS Study is a multicenter screening trial targeting female populations at different risk for CC in 2 Latin American countries, Brazil and Argentina.[26] At their baseline visit, a total of 12,114 consecutive women attending the 4 partner clinics, Campinas, Brazil; Sao Paulo, Brazil; Porto Alegre, Brazil; and Buenos Aires, Argentina, were screened for HPV and CIN using 8 different diagnostic tools, as detailed before.[26-29] 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 (Pap) smear–negative women were recalled for a new Pap test at 12 months, as were 20% of women testing negative with the Hybrid Capture II (HC2; Digene, Gaithersburg, MD) assay, to assess the rates of incident Pap smear abnormalities and HPV infections, respectively.[26,27] The women with biopsy-confirmed low-grade CIN comprise the prospective cohort (n = 1,011) and were 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.[26-29] For the present analysis of stratifin, baseline biopsy samples taken from 225 of these women were available.

Prospective Follow-up

According to 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.[27-29] The mean follow-up time as of 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

In the present study, biopsies from 225 women included in this subcohort of 1,011 women were analyzed for the surrogate end points of progressive disease: progression to CIN 1+ and progression to CIN 2+, and for outcomes of their HR-HPV infections, including incident infections, virus persistence, and HPV clearance. Progression to CIN 1+ was based on detection, in baseline biopsy-negative women, of 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 a baseline negative, flat HPV lesion with no CIN, or CIN 1 biopsy was established in the subsequent follow-up-visits, as recently detailed.[30] 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 involved the appearance of a positive HC2 test (at 1pg/mL relative light units/cutoff ratio) among baseline HR HPV–negative 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. Persistent HR-HPV infections were infections in which 2 or more subsequent HC2 assays

DOI: 10.1309/AJCP49DOITYDCTQJ

were HR HPV–positive and the infection was not cleared at the last follow-up visit. Times to the 3 outcomes were also calculated and expressed as cases per 1,000 WMR.

LAMS Study Methods

Because they are detailed in a series of recent reports,[26-30] the methods used in the LAMS Study are described here only 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 CC. This structured questionnaire contained questions exploring reproductive history, sexual history, current sexual practices, sexual hygiene, medical history, smoking habits, and contraception.[26,27]

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, Sao Paulo; and SurePath, TriPath, Durham, NC).[26,27] In the present analysis, only the results of the conventional Pap test were used.

Directed Punch Biopsy

Directed punch biopsy 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 the routine procedures. All biopsy specimens were examined within the daily routine in the pathology departments of the partner clinics in both studies and diagnosed using the commonly agreed-on CIN nomenclature. The pathologists were also asked to report the HPV-suggestive morphologic changes in flat lesions with no CIN (ie, HPV NCIN [flat condyloma]).[26-29] The slides from two of the centers (Campinas and Sao Paulo) were subjected to reexamination by a panel of pathologists from the European Community partners (M.E. and K.S.). The consensus diagnosis of the panel was considered the final diagnosis.

Detection of HPV DNA by the HC2 Assay

Primary HPV testing was done by using the HC2 assay, using cervical swabs (collected by a physician) and self-sampling devices (tampons), as described previously.[26,29] The HC2 assay (n = 4,694 tests) was performed 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 (cutoff), ie, samples were classified as HR HPV–positive with a relative light units/cutoff ratio of 1.0 pg/mL or more.

Immunohistochemical Detection of Stratifin (14-3-3σ)

A total of 225 slides from the same number of women were available for immunohistochemical analysis of stratifin. In brief, 4-µm-thick sections were cut on ChemMate Capillary Gap Microscope slides (DAKO, Glostrup, Denmark), kept overnight at 55°C, deparaffinized in xylene, and rehydrated in graded alcohol. Before immunohistochemical analysis, stratifin antigen retrieval was done by heating the tissue sections in a buffer of 10 mmol/L tris(hydroxymethyl)aminomethane (Tris) and 1 mmol/L EDTA (pH 9.0) with a microwave oven (600 W) for 10 minutes. Immunohistochemical staining for stratifin was performed with the DAKO TechMate 500 Plus Autostainer using monoclonal 14-3-3σ antibody (ab14123; Abcam, Cambridge, MA) diluted 1:75 and the reagents from the DAKO REAL-kit (DAKO). The sections were washed with distilled water and Tris-buffered saline. Then, the sections were stained with the primary antibody and the secondary biotinylated antibody (antimouse IgG) for 30 minutes. Endogenous peroxidase activity was blocked by using 5% hydrogen peroxide 3 times for 2 minutes, 30 seconds each. This was followed by incubation with streptavidin peroxidase for 30 minutes. The counterstaining was performed with hematoxylin for 1 minute, and the immunoperoxidase reaction was developed using 3,3'-diaminobenzidine 3 times for 5 minutes each. Finally, the sections were washed with distilled water and mounted with Aquamount (BDH Laboratory Supplies, Poole, England). 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 Immunohistochemical Staining for Stratifin (14-3-3σ)

In normal and metaplastic squamous epithelium, expression of stratifin was invariably present, being predominantly cytoplasmic and confined to the cells in the lowermost layers of the epithelium **Image 1**. In CIN lesions and CC, cytoplasmic and nuclear stratifin expression was markedly increased, almost in parallel with the increasing grade of the lesion **Image 2**, **Image 3**, and **Image 4**. In original grading of the stratifin staining, a semiquantitative scoring of 4 categories was used: 0, no expression; 1, weak staining (equivalent to normal squamous epithelium); 2, moderately increased staining (intermediate cells stained); and 3, strongly increased staining (all layers diffusely positive for intense stratifin staining). In statistical analysis, the staining results were also treated as dichotomous variables (negative-weak vs moderate-strong), or 3-tier grading was used: negative-weak, moderate, and strong.

DOI: 10.1309/AJCP49DOITYDCTQJ

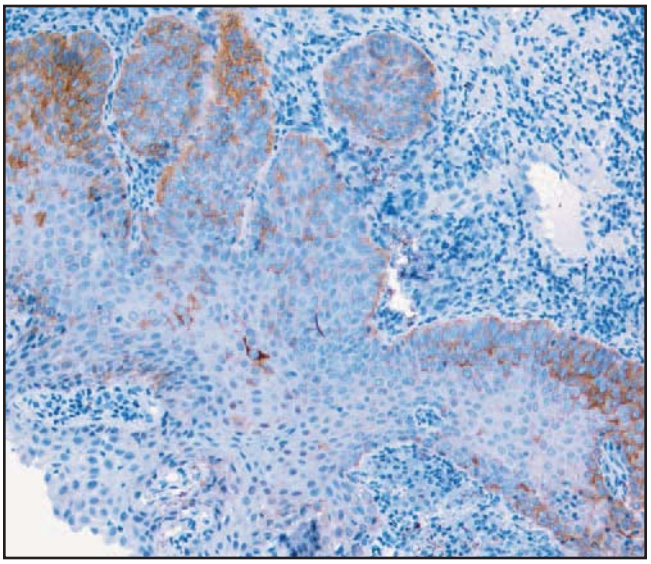

Image 1 Normal cervical epithelium undergoing physiologic squamous cell metaplasia. Stratifin expression is equivalent to that in the normal squamous epithelium. Stratifin expression is focal, predominantly cytoplasmic, and confined to the cells in the lower (and scattered in the intermediate) layers of the metaplastic epithelium (immunohistochemical analysis for stratifin, ×50).

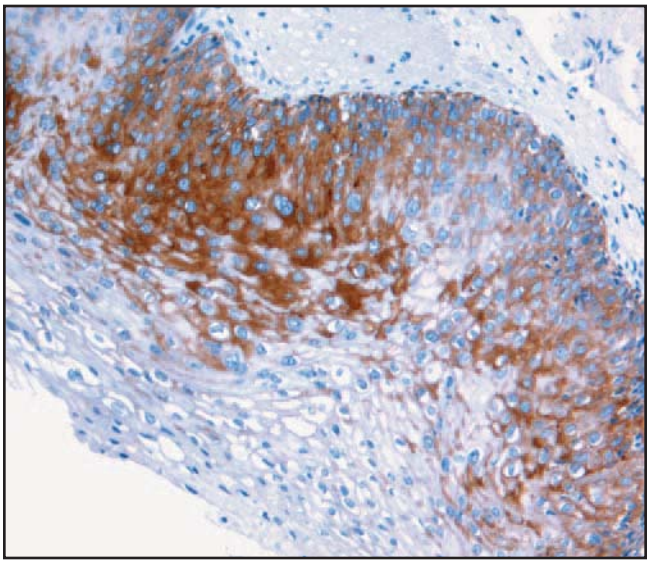

Image 2 A low-grade cervical intraepithelial neoplasia-1 lesion with characteristic features of human papillomavirus infection (koilocytes), from intermediate layers upwards. As compared with the metaplastic epithelium, cytoplasmic and nuclear expression of stratifin is increased in intensity, and positive staining is also present at higher levels of the epithelium. Yet, lacking the expression in the uppermost layers makes a major difference to high-grade lesions (immunohistochemical analysis for stratifin, ×100).

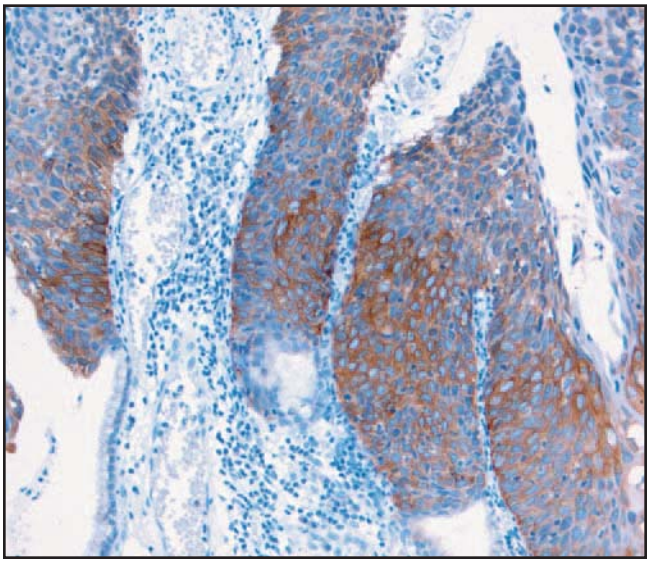

Image 3 A high-grade cervical intraepithelial neoplasia-3 lesion penetrating into the underlying glandular openings. Stratifin-positive cells are found throughout the full thickness of the epithelium, indicating marked up-regulation. The staining intensity shows some variability, with some cells showing intense cytoplasmic and/or nuclear expression, while in the rest of the cells, stratifin expression is less intense (immunohistochemical analysis for stratifin, ×100).

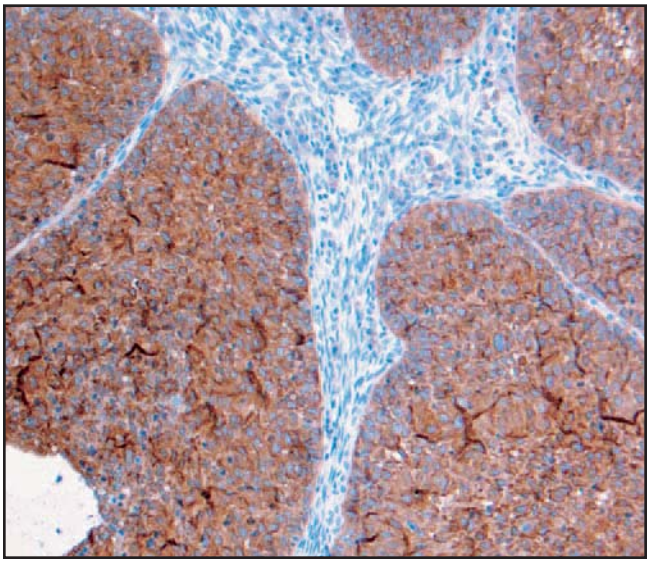

Image 4 An invasive squamous cell carcinoma with intense expression of stratifin. Positive immunostaining is detected in practically all cancer cells, being an indicator of a marked overexpression of stratifin, even more diffuse than in the cervical intraepithelial neoplasia-3 lesion shown in Image 3 (immunohistochemical analysis for stratifin, ×100).

DOI: 10.1309/AJCP49DOITYDCTQJ

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. Differences in the means of continuous variables were analyzed using nonparametric tests (Mann-Whitney) or analysis of variance. Performance indicators (sensitivity, specificity, positive predictive value, negative predictive value, and their 95% confidence intervals [CIs]) for 14-3-3σ as a predictor of baseline high-grade CIN or HR HPV and the longitudinal predictive values for the 3 viral outcomes and incident CIN 1+ and CIN 2+ were calculated by using Stata/SE software and the algorithm introduced by Seed and Tobias,[31] which also calculates the area under the receiver operating characteristic curve (AUC). In all tests, values of *P* of less than .05 were considered statistically significant.

Results

Expression of 14-3-3σ (stratifin) in cervical biopsy specimens as related to lesion grade is summarized in **Table 1**. There was a significant linear trend of increasing up-regulation of 14-3-3σ ($P = .0001$) in parallel with increasing grade of CIN. When dichotomized (negative-weak vs moderate-strong), up-regulated 14-3-3σ expression predicted CIN 3+ with an odds ratio (OR) of 3.39 (95% CI, 1.25-9.14; $P = .007$) and CIN 2+ with an OR of 2.70 (95% CI, 1.27-5.76; $P = .006$).

Table 2 depicts the association of 14-3-3σ expression with HR-HPV detection and semiquantitative viral load detected with the HC2 assay. Stratifin was clearly up-regulated more often in HR-HPV+ lesions than in those remaining HC2– ($P = .008$). Dichotomized expression was associated with HR-HPV detection with an OR of 2.71 (95% CI, 1.37-5.35; $P = .004$). The log-transformed semiquantitative HR-HPV viral loads were also directly related to up-regulation of 14-3-3σ ($P = .003$). When the performance indicators were calculated, 14-3-3σ was not a particularly good predictor of HR HPV, with an AUC of 0.608 (95% CI, 0.533-0.683).

Expression of 14-3-3σ in the baseline biopsy specimens was related to outcome of the HPV infection **Table 3**. Up-regulation of 14-3-3σ was a 100% sensitive marker in predicting incident HR HPV (AUC, 0.718) but was slightly less sensitive in predicting persistent HR-HPV infection or virus clearance using the longitudinal performance indicators. As to the time to clearance and time to incident HR HPV

Table 1
Expression of Stratifin (14-3-3σ) as Related to Lesion Grade*

	Stratifin Expression		
Lesion Grade	**Negative-Normal Expression**	**Moderate Up-Regulation**	**Strong Up-Regulation**
Normal or HPV-NCIN (n = 94)	34 (36)	29 (31)	31 (33)
CIN 1 (n = 64)	23 (36)	23 (36)	18 (28)
CIN 2 (n = 21)	5 (24)	6 (29)	10 (48)
CIN 3 (n = 36)	5 (14)	5 (14)	26 (72)
SCC (n = 1)	0 (0)	0 (0)	1 (100)

CIN, cervical intraepithelial neoplasia; HPV-NCIN, flat HPV lesion with no CIN; SCC, squamous cell carcinoma.
* Data are given as number (percentage). $P = .002$ (Fisher exact test); $P = .0001$ for linear trend.

Table 2
Expression of Stratifin as Related to Detection of HR-HPV With the Hybrid Capture II Assay and Its Viral Load*

	Stratifin Expression		
	Negative-Normal Expression	**Moderate Up-Regulation**	**Strong Up-Regulation**
High-risk HPV result†			
HPV+ (n = 111)	25 (22.5)	34 (30.6)	52 (46.8)
HPV– (n = 59)	26 (44)	17 (29)	16 (27)
Viral load‡	1.30 (0.38-2.22) (n = 51)	2.85 (1.89-3.81) (n = 51)	3.32 (2.58-4.07) (n = 68)

CI, confidence interval; HPV, human papillomavirus.
* Performance indicators of dichotomous (moderate to strong vs negative to weak [normal]) stratifin are as follows: sensitivity, 77.5% (95% CI, 68.6-84.9); specificity, 44.1% (95% CI, 31.2-57.6); positive predictive value, 72.3% (95% CI, 63.3-80.1); negative predictive value, 51.0% (95% CI, 36.6-65.2); area under the receiver operating characteristic curve, 0.608 (95% CI, 0.533-0.683).
† Data are given as number (percentage). $P = .008$ (χ^2, log rank); $P = .002$ for linear trend.
‡ Data are given as mean (± 95% confidence interval). Semiquantitative viral load determined by the relative light units/cutoff ratio in the Hybrid Capture II assay, log-transformed. $P = .003$ (analysis of variance); $P = .004$ (Kruskal-Wallis, Monte-Carlo simulation with the 10,000 sample and 99% confidence interval).

DOI: 10.1309/AJCP49DOITYDCTQJ

(not calculable), there were no differences related to 14-3-3σ expression. All these calculations are hampered by the small number of events.

Table 4 gives the data on 14-3-3σ as a predictor of the 2 surrogate end points of progressive disease (incident CIN 1+ and CIN 2+). The 3-tier grading of 14-3-3σ expression was practically identical in the baseline biopsy specimens that subsequently progressed to incident CIN1+, with no significant difference. Longitudinal performance indicators did not provide any useful values, with an AUC of 0.503. The same was true with 14-3-3σ as a predictor of incident CIN 2+ (AUC, 0.472). However, the negative predictive value approached 90%, implying that negative-weak 14-3-3σ expression precludes progression to CIN 2+ with high accuracy (95% CI, 75.8%-97.1%). Times to progression to CIN 1+ or CIN 2+ were identical in different categories of 14-3-3σ expression, with no significant differences (data not shown).

Discussion

Recent studies implied that the role of 14-3-3σ in cell cycle control is more complex than previously anticipated. In addition to controlling the G_2/M checkpoint, 14-3-3σ also seems to control the G_1/S checkpoint.[12,14,22] In both cases, the effects are mediated by complex binding of 14-3-3σ with a multitude of cellular proteins.[12] In controlling the G_2/M checkpoint, the association with 14-3-3 proteins requires a specific phosphorylation of the protein ligand, and 14-3-3 binding may lead to cytoplasmic sequestration of the protein

Table 3
Expression of Stratifin as Predictor of Different Outcomes of HR-HPV Infection*

	Stratifin Expression		
End Point	**Negative-Normal Expression**	**Moderate Up-Regulation**	**Strong Up-Regulation**
Incident HR HPV†			
Yes (n = 1)	0 (0)	0 (0)	1 (100)
No (n = 55)	24 (44)	19 (35)	12 (22)
HR HPV cleared‡			
Yes (n = 14)	5 (36)	6 (43)	3 (21)
No (n = 42)	19 (45)	13 (31)	10 (24)
HR HPV persistence§			
Yes (n = 11)	2 (18)	5 (45)	4 (36)
No (n = 45)	22 (49)	14 (31)	9 (20)

AUC, area under the receiver operating characteristic curve; CI, confidence interval; HPV, human papillomavirus; HR, high risk; M-S/N-W, moderate to strong vs negative to weak (normal); NPV, negative predictive value; PPV, positive predictive value.
* Data are given as number (percentage).
† $P = .235$ (Fisher exact test); $P = .235$ for linear trend. Longitudinal performance indicators of dichotomous (M-S/N-W) stratifin were as follows (95% CI in parentheses): sensitivity, 100.0% (2.5-100); specificity, 43.6% (65.0-88.2); PPV, 3.1% (0.1-16.2); NPV, 100.0% (85.8-100); AUC, 0.718 (0.000-1.000).
‡ $P = .726$ (Fisher exact test); $P = .846$ for linear trend. Longitudinal performance indicators of dichotomous (M-S/N-W) stratifin were as follows (95% CI in parentheses): sensitivity, 64.3% (35.1-87.2); specificity, 45.2% (29.8-61.3); PPV, 28.1% (13.7-46.7); NPV, 79.2% (57.8-92.9); AUC, 0.548 (0.397-0.698).
§ $P = .175$ (Fisher exact test); $P = .095$ for linear trend. Longitudinal performance indicators of dichotomous (M-S/N-W) stratifin were as follows (95% CI in parentheses): sensitivity, 81.8% (48.2-97.7); specificity, 48.9% (33.7-64.2); PPV, 28.1% (13.7-46.7); NPV, 91.7% (73.0-99.0); AUC, 0.654 (0.513-0.794).

Table 4
Expression of Stratifin as a Predictor of the Two Surrogate End Points of Disease Progression*

	Stratifin Expression		
End Point	**Negative-Normal Expression**	**Moderate Up-Regulation**	**Strong Up-Regulation**
Incident CIN 1+†			
Yes (n = 18)	7 (39)	4 (22)	7 (39)
No (n = 81)	32 (40)	26 (32)	23 (28)
Incident CIN 2+‡			
Yes (n = 9)	4 (44)	1 (11)	4 (44)
No (n = 90)	35 (39)	29 (32)	26 (29)

AUC, area under the receiver operating characteristic curve; CI, confidence interval; CIN, cervical intraepithelial neoplasia; M-S/N-W, moderate to strong vs negative to weak (normal); NPV, negative predictive value; PPV, positive predictive value.
* Data are given as number (percentage).
† $P = .643$ (Fisher exact test); $P = .641$ for linear trend. Longitudinal performance indicators of dichotomous (M-S/N-W) stratifin were as follows (95% CI in parentheses): sensitivity, 61.1% (35.7-82.7); specificity, 39.5% (28.8-51.0); PPV, 18.3% (9.5-30.4); NPV, 82.1% (66.5-92.5); AUC, 0.503 (0.375-0.631).
‡ $P = .376$ (Fisher exact test); $P = .838$ for linear trend. Longitudinal performance indicators of dichotomous (M-S/N-W) stratifin were as follows (95% CI in parentheses): sensitivity, 55.6% (21.2-86.3); specificity, 38.9% (28.8-49.7); PPV, 8.3% (2.8-18.4); NPV, 89.7% (75.8-97.1); AUC, 0.472 (0.293-0.652).

DOI: 10.1309/AJCP49DOITYDCTQJ

ligand. Indeed, the 14-3-3σ gene is induced by p53, and its product inhibits G_2/M progression by cytoplasmic sequestration of the CDC2-cyclin B complexes.[12]

To increase the complexity, 14-3-3 proteins are also involved in the regulation of G_1/S-phase transition by several mechanisms.[12] They bind to and negatively regulate CDC25 phosphatases, which are involved in regulating the CDK complexes critical for G_1/S-transition. Furthermore, a direct association of 14-3-3σ with the G_1-specific kinases CDK2 and CDK4 is most likely mediated by a cyclin-CDK2 binding motif, which 14-3-3σ seems to share with several other cell cycle regulators, including p107, p130, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$.[12] It is important to note that 14-3-3 proteins may also directly interact with the CDK inhibitor $p27^{Kip1}$, which mediates G_1 arrest by inhibiting cyclin E–CDK2 complexes.[12] In addition, 14-3-3 proteins have been implicated in the transcriptional regulation of CDK inhibitors as they modulate the transcription factors p53, FOXO, and MIZ1.[12]

Considering the known complexity of the mechanisms whereby HR-HPV types regulate the cell cycle and other key cellular functions,[1-3,7-9,11] there is little doubt that multiple potential sites of interaction between 14-3-3 proteins and HR-HPV oncoproteins E6 and E7 could exist. Until now, only 3 studies have analyzed expression of 14-3-3σ and/or genetic and epigenetic changes in the gene in HPV-associated cancers (oral, vulvar, cervical).[23-25] In oral and vulvar cancer (and their precursors) but not in CC and CIN, coincident loss of expression of 14-3-3σ and $p16^{INK4a}$ was commonly detected, particularly in HPV– lesions.[23-25] In contrast, CC and CIN ubiquitously expressed 14-3-3σ, and concomitant inactivation of 14-3-3σ and p16 was never detected.[25] This leaves room for speculations that oral, vulvar, and cervical cancer might differ in details of how HR HPV[1-3,7-9,11] and 14-3-3σ[12,14,15,19-22] interfere with the normal cell functions and, particularly, how these two might interact.

In fact, several mechanisms could explain this detected up-regulation of 14-3-3σ in CC and CIN.[25] Expression of 14-3-3σ is up-regulated directly by interaction with HR HPV, or the presence of HR HPV is just incompatible with the epigenetic silencing of 14-3-3σ. Is this up-regulated 14-3-3σ impaired (inhibited) in its function (by HR HPV) to enable normal control of the cell cycle checkpoints? If still functional, there must be a mechanism whereby HR HPV can bypass these two 14-3-3σ–controlled cell cycle checkpoints. Under any of these circumstances, one could expect to see some differences in the 14-3-3σ expression as related to the following: (1) the grade of CIN, (2) HR-HPV detection, (3) the outcome of these HR-HPV infections, and (4) development of incident CIN 1+ and CIN 2+, all examined in the present study.

Using the normal squamous epithelium as a reference, the expression of 14-3-3σ seems to increase in parallel with the increasing grade of CIN. This is fully consistent with the data of Sano et al,[25] describing ubiquitous expression of 14-3-3σ in CIN and CC lesions, with increasing intensity among high-grade CIN and CC. In our series, some 14% of the CIN3 and 24% of CIN2 lesions still retained only weak expression, equivalent to normal epithelium. This obvious major up-regulation (from 47.6% to 72.2%) on transition from CIN 2 to CIN 3 is most feasibly explained by the fact that practically all CIN 3 lesions contain HR HPV DNA, implying an intimate association between 14-3-3σ and HR HPV. This is in alignment with the recent observations on oral and vulvar cancer (and precursors) in which 14-3-3σ was silenced typically in HPV– lesions only, not in HPV+ lesions.[23,24] However, in CC and CIN, this association was not perfect, but 14-3-3σ was inactivated by promoter methylation also among a few HR-HPV+ lesions, implying that these 2 events are not mutually exclusive.[25]

The present data (Table 2) indicate that up-regulation of 14-3-3σ was closely associated with HR-HPV detection (P = .008; P = .002 for linear trend). It is interesting that expression of 14-3-3σ was also linearly related to the semiquantitative (HC2) viral load of HR HPV (P = .003), which increased in parallel with the increasing intensity of 14-3-3σ expression, being almost 3 times higher among the lesions with intense overexpression of 14-3-3σ as compared with the reference category (Table 2). However, unlike some other markers recently analyzed ($p16^{INK4a}$, survivin, and hTERT),[32] 14-3-3σ was not a useful marker of HR HPV (AUC, 0.608).

It is tempting to speculate that the presence of HR HPV is the reason why 14-3-3σ is not inactivated in CIN and CC lesions, in contrast with practically all non-HPV–related carcinomas studied so far.[14,22] This view is also consistent with the data on oral and vulvar cancers,[23,24] in which 14-3-3σ inactivation was a frequent phenomenon. In contrast with CIN and CC, only a minority of these lesions are associated with HR HPV, which would feasibly explain this difference. Noteworthy in all these studies,[23-25] however, was the fact that 14-3-3σ inactivation by promoter methylation also occurred in a small proportion of HR-HPV+ lesions, indicating that these are not mutually exclusive. The observed weak expression in 22.5% of the HR-HPV+ lesions in our series could be consistent with this.

Considering the well-established tumor suppressive function of 14-3-3σ,[12,14,15,19-22] one would expect that its abundant expression should have some favorable impact on the outcome of HR-HPV infections or the progression to CIN. In the present study, we were unable to provide any confirmatory data to support either of these concepts. Thus, incident HR-HPV infections, virus clearance, or HR-HPV persistence did not show any direct association with 14-3-3σ expression (Table 3), and longitudinal predictive indicators were not useful in predicting the 3 viral outcomes. Similarly, there was practically no difference in 14-3-3σ expression patterns

DOI: 10.1309/AJCP49DOITYDCTQJ

among progressive and nonprogressive lesions (Table 4), and longitudinal predictive indicators were of no value in discriminating the incident CIN 1+ and CIN 2+ cases from cases that did not progress.

The observations in our study are based on use of immunohistochemical analysis only, and we did not look at the epigenetic or genetic changes in the 14-3-3σ gene, as recently done in 2 other HPV-related cancers.[23,24] This notwithstanding, however, the data from the present study are in full agreement with the observations of these previous studies,[23-25] all implying that most likely it is the presence of HR HPV that explains the paradoxical observations of 14-3-3σ expression in CC (up-regulated) and all other carcinomas (inactivated by epigenetic mechanisms) studied so far.[14,22] The molecular mechanisms explaining these paradoxical observations in cervical lesions have been discussed to some extend by Sano et al.[25] These authors linked their observations to the status of p53 and $p16^{INK4a}$, concluding that inactivation of 14-3-3σ or $p16^{INK4a}$ has an effect equivalent to the expression of HR HPV E6 and E7 proteins because these proteins cooperate to immortalize primary keratinocytes.[25]

As to the role of p53, Sano et al[25] speculated that abrogation of p53-dependent induction is, at least in part, the mechanistic basis for silencing of 14-3-3σ, predominantly seen in HPV– CCs. In the present study, we did not look at p53 or $p16^{INK4a}$, but Branca et al[33] showed an intimate association between HR HPV and $p16^{INK4a}$. It is most likely that also in CC, more than one mechanism is available to interfere with the $p16^{INK4a}$/cyclin-D/Rb pathway. Apart from HR HPV E6, one seems to be the silencing of the $p16^{INK4a}$ gene by promoter methylation, characteristic of HPV– lesions,[25,33,34] as recently shown in oral and vulvar cancers.[23,24]

As to the established role of 14-3-3σ as one of the key control mechanisms of the G_1/S and G_2/M checkpoints, it seems obvious that HR HPV must be capable of bypassing both to resume the cell cycle.[7-9] The participation of E6 and E7 in the abrogation of normal cell cycle control at these 2 checkpoints is a highly complex process, involving several cellular control proteins.[11] Of interest in this respect are the recent observations that a direct association of 14-3-3σ with the G_1-specific kinases CDK2 and CDK4 is mediated by a cyclin-CDK2 binding motif, which 14-3-3σ seems to share with several other cell cycle regulators, including p107, p130, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$.[12] In addition to the interesting links to HR HPV of p107 and p130,[11] we should give attention to the $p21^{Cip1}$ role[11] in HR HPV–driven cell proliferation.[35] It is well known that $p21^{Cip1}$ simultaneously controls CDK activity and proliferating cell nuclear antigen (PCNA)-dependent DNA replication, and it is important to note that HR HPV E7 is capable of overriding this $p21^{Cip1}$ control, which leads to loss of normal cell cycle control.[35] Like 14-3-3σ in the present study, PCNA also was shown to be intimately linked with CIN 2/3 and HR-HPV infection, feasibly explained by the actions of E7 oncoprotein reverting the $p21^{Cip1}$-mediated control of PCNA.[35] It is tempting to speculate that something analogous might take place that explains this similar association of PCNA[35] and 14-3-3σ overexpression with high-grade CIN and HR-HPV detection.

Unlike in most other human cancers, 14-3-3σ (stratifin) in CC and CIN is rarely inactivated, but, in contrast, seems to be overexpressed in high-grade lesions associated with HR-HPV infection. Thus, rather than being inactivated (by epigenetic promoter methylation), 14-3-3σ is expressed in CIN and CC, but, evidently, its normal control functions on the G_1/S and G_2/M checkpoints are being bypassed by HR HPV using some as yet unknown molecular mechanisms.

From the [1]Department of Oral Pathology, Institute of Dentistry, and [10]Department of Oncology and Radiotherapy, Turku University Hospital, Turku, Finland; [2]Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil; [3]Universidade Estadual de Campinas, Campinas, Brazil; [4]Hospital Leonor M de Barros, Sao Paulo, Brazil; [5]Instituto Adolfo Lutz, Sao Paulo, Brazil, and University of Minho, Braga, Portugal; [6]First Chair Gynecology Hospital de Clinicas, Buenos Aires, Argentina; [7]Unit of Cytopathology, National Centre of Epidemiology, Surveillance and Promotion of Health, National Institute of Health (ISS), Rome, Italy; [8]SIZE Diagnostic Center, Ljubljana, Slovenia; and [9]Department of Obstetrics and Gynecology, S. Orsola-Malpighi Hospital, Bologna, Italy.

[*] *LAMS, Latin American Screening Study, funded by European Commission, INCO-DEV contract ICA4-CT-2001-10013.*

Address reprint requests to Dr Kari Syrjänen: Dept of Oncology & Radiotherapy, Turku University Hospital, Savitehtaankatu 1, FIN-20521 Turku, Finland.

Acknowledgment: The generous contribution of former DIGENE (Gaithersburg, MD) by donating the HC2 tests that were at our disposal is gratefully acknowledged.

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.

DOI: 10.1309/AJCP49DOITYDCTQJ

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. Buitrago-Pérez A, Garaulet G, Vázquez-Carballo A, et al. Molecular signature of HPV-induced carcinogenesis: pRb, p53 and gene expression profiling. *Curr Genomics*. 2009;10:26-34.
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 Prevent*. 2006;15:1250-1256.
12. Hermeking H, Benzinger A. 14-3-3 proteins in cell cycle regulation. *Semin Cancer Biol*. 2006;16:183-192.
13. Prasad GL, Valverius EM, McDuffie E, et al. Complementary DNA cloning of a novel epithelial cell marker protein, HME1, that may be down-regulated in neoplastic mammary cells. *Cell Growth Differ*. 1992;3:507-513.
14. Lodygin D, Hermeking H. Epigenetic silencing of 14-3-3sigma in cancer. *Semin Cancer Biol*. 2006;16:214-224.
15. Hermeking H, Lengauer C, Polyak K, et al. 14-3-3 sigma is a p53-regulated inhibition of G_2/M progression. *Mol Cell*. 1997;1:3-11.
16. Chan TA, Hermeking H, Lengauer C, et al. 14-3-3sigma is required to prevent mitotic catastrophe after DNA damage. *Nature*. 1999;401:616-620.
17. Conklin DS, Galaktionov K, Beach D. 14-3-3 proteins associate with cdc25 phosphatases. *Proc Natl Acad Sci U S A*. 1995;92:7892-7896.
18. Laronga C, Yang HY, Neal C, et al. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol Chem*. 2000;275:23106-23112.
19. Hermeking H. The 14-3-3 cancer connection. *Nat Rev Cancer*. 2003;3:931-943.
20. Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol*. 2000;40:617-647.
21. van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays*. 2001;23:936-946.
22. Lodygin D, Hermeking H. The role of epigenetic inactivation of 14-3-3sigma in human cancer. *Cell Res*. 2005;15:237-246.
23. Gasco M, Sullivan A, Repellin C, et al. Coincident inactivation of 14-3-3sigma and $p16^{INK4a}$ is an early event in vulval squamous neoplasia. *Oncogene*. 2002;21:1876-1881.
24. Gasco M, Bell AK, Heath V, et al. Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. *Cancer Res*. 2002;62:2072-2076.
25. Sano T, Shimooka H, Weixa P, et al. Immunohistochemical expression of 14-3-3 sigma protein in various histological subtypes of uterine cervical cancers. *Pathol Int*. 2004;54:743-750.
26. 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.
27. 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.
28. 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.
29. 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.
30. Syrjänen K, Shabalova I, Naud P, et al. Persistent high-risk human papillomavirus (HPV) infections and other endpoint markers of progressive cervical disease among women prospectively followed-up in the NIS and LAMS cohorts. *Int J Gynecol Cancer*. 2009;19:934-942.
31. Seed PT, Tobias A. Summary statistics for diagnostic tests. *Stata Technical Bull*. 2001;59:9-12.
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. 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.
34. Ferreux E, Lont AP, Horenblas S, et al. Evidence for at least three alternative mechanisms targeting the p16INK4A/cyclin D/Rb pathway in penile carcinoma, one of which is mediated by high-risk human papillomavirus. *J Pathol*. 2003;201:109-118.
35. Branca M, Giorgi C, Ciotti M, et al. Up-regulation of proliferating cell nuclear antigen (PCNA) is closely associated with high-risk human papillomavirus (HPV) and progression of cervical intraepithelial neoplasia (CIN), but does not predict disease outcome in cervical cancer. *Eur J Obstet Gynecol Reprod Biol*. 2007;130:223-231.

DOI: 10.1309/AJCP49DOITYDCTQJ