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Immunocytochemical Expression of $p16^{INK4A}$ and Ki-67 in Cytologically Negative and Equivocal Pap Smears Positive for Oncogenic Human Papillomavirus

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Summary: This study was designed to analyze the cross-sectional comparison of the $p16^{INK4A}$ and Ki-67 immunocytochemical expression in negative and equivocal (atypical squamous cells of undetermined significance (ASC-US)) liquid-based cytology (LBC) samples testing positive for high-risk human papillomavirus (HPV) types with HC2 assay or polymerase-chain reaction (PCR). A series of 199 consecutive LBC specimens derived from the same number of women participating in the ongoing Latin American Screening Study at Leonor Mendes de Barros Hospital, São Paulo, were analyzed using immunocytochemistry for expression of $p16^{INK4A}$ and Ki-67 in negative and equivocal LBC samples testing positive for high-risk HPV types with hybrid capture II test (HC2) or PCR. All patients with at least one test positive (cytology, PCR, and/or HC2) were followed each 6 months for 3 years. The follow-up procedure consisted of visual examination, colposcopic inspection, cytology, and HC2 assay. Among the negative cytologic samples, 101 were HPV-positive and 55 HPV-negative. Of the HPV-positive group, 59 of 101 cases (58.4%) were positive for both p16 and Ki67 immunostaining, and 17 of 101 (16.8%) were negative for both. The proportion of Ki-67-positivity increased almost in parallel with the increasing grade of p16-positivity ($p = 0.0001$ for linear trend). In the HPV-negative group, both markers were negative in 41 of 55 cases (74.5%), and no statistical relationship was observed between the two markers (Pearson, $p = 0.595$). HPV-positive ASC-US samples demonstrated a simultaneous positive immunoreaction for p16 and Ki67 in 11 of 16 cases (68.7%), whereas 3 (18.7%) were concurrently negative. The relationship between the two markers was of borderline significance (Pearson, $p = 0.053$), but no linear relationship was found between the graded p16 and Ki-67 expression ($p = 0.065$ for linear trend). In the HPV-negative ASC-US group, there was no statistical association between the graded p16 and Ki-67 positivity (Pearson, $p = 0.281$). After 36 months of follow-up of the ASC-US patients, 6 women still displayed ASC-US smear, of which 4 of 6 were HPV-positive and expressed both p16 and Ki-67 markers. Two of 43 ASC-US smears had high-grade

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squamous intraepithelial lesions diagnosed (4.6%), and 1 had low-grade squamous intraepithelial lesion (2.3%). All of those were positive for HPV, p16 and Ki-67. Patients with ASC-US diagnosis and positive high-risk HPV status and positive for $p16^{INK4A}$ Ki67 should be carefully observed to exclude occurrence of a squamous intraepithelial lesion. The combination of these two markers can be a useful implement for management of women with equivocal cytology. **Key Words:** p16—Ki67—Cervical cancer—Human papillomavirus—Liquid-based cytology.

Persistent high-risk human papillomavirus (HPV) infection is probably the best predictor of increased risk for cervical cancer (1). The presence of HPV is an essential but not-sufficient cause of cervical cancer (2). Recent molecular biology data on the natural history of HPV infections and cervical cancer suggest that the viral infection critically interferes with the mechanisms of cellular growth, DNA repair, and immunologic responses (2). Part of these interferences is related to the action of oncoproteins encoded by E6 and E7 HPV genes that bind in the regulatory proteins of the cells, including those of p53 and RB-suppressor genes (3).

The protein p16 is a product of CDKN2A gene that suppresses the activity of CDK-4 and -6 cyclins, which regulates the G1 checkpoint (3). The p16 protein seems to interfere with the transforming activity of E6 but E7 interaction with RB can directly stimulates the cyclins inducing cell replication. The p16 is overexpressed, but its function is seriously affected as a consequence of the inactivation of suppressor proteins (4). For this reason, the overexpression of p16 protein is associated with the oncogenic potential of papillomaviruses in cervical and genital lesions (3,5). Moreover, p16 also is presumed to be a molecular biomarker for high-risk HVP in malignant and premalignant lesions of the vulva (6), squamous cell carcinoma of the anorectal region (7), penile carcinoma (8), and oral lesions (9). Additionally, p16 is overexpressed in cervical adenocarcinoma (both *in situ* and invasive) (10,11), and it is recognized as a strong predictor of poor prognosis in these lesions (12). Recently, immunohistochemical (IHC) expression of p16 has been used to discriminate cervical from endometrial adenocarcinomas if accompanied by high-risk HPV types (13).

Ki-67 expression in early intraepithelial lesions is a strong predictor of progression (14). Expression of Ki-67 seems to increase progressively in parallel with the increasing aggressiveness of the lesions (15). However, Ki-67 is not an independent prognostic predictor (16).

Screening by cervical cytology suffers from inherent problems in sensitivity, and more recently, auxiliary techniques have been proposed to improve the performance of cervical cytology. Hybrid Capture II (HC2), used to detect high-risk HPV, seems to represent a remarkable improvement for cervical cancer screening, particularly in triaging equivocal cytological diagnoses (17). Because only persistent HPV infections can progress to carcinoma (1), different combinations of p16 overexpression, Ki-67 status, and HPV testing have been implicated as potential markers to predict the behavior of both glandular and squamous lesions of the cervix (5,18–20). Furthermore, the use of p16 and Ki-67 has been suggested as a useful tool in assessing the true nature of cytologic smears classified as atypical squamous cells of undetermined significance (ASC-US) (20,21).

This study was designed to analyze the cross-sectional comparison of the $p16^{INK4A}$ and Ki-67 immunocytochemical expression in negative and equivocal (ASC-US) liquid-based cytology (LBC) samples testing positive for high-risk HPV types with HC2 assay or polymerase-chain reaction (PCR).

MATERIALS AND METHODS

The present series comprised 199 (6.63%) from 3,000 consecutive women participating in the ongoing Latin American Screening (LAMS) Study, and attending Leonor Mendes de Barros Hospital, São Paulo, from 2002 to 2004. The women were eligible for the study if no previous history of HPV infection or any other cervical pathology was reported. The mean age of the women was 37 (range, 18–55) years. The patients had been sampled for LBC and subjected to HPV testing with HC2 assay (high-risk HPV types) at their first clinical visit. In addition, a multiplex PCR for HPV was performed in the residual LBC medium. The ethics committee of the hospital approved the project.

For this study we selected from the 3,000 patients, slides from 156 (5,2%) patients cytologically negative, and 43 (1.43%) classified as ASC-US in whom HPV-positive or -negative status were available by PCR and/or HC2 assay.

All patients with at least one test positive (cytology, PCR, and/orHC2) were followed every 6 months for

3 years. The follow-up procedure has consisted of visual examination, colposcopic inspection, cytology, and HC2 assay.

Cytologic Samples

Cervical samples were collected with a scored cervical brush included in the manual DNACitoliq® (Digene Brasil, São Paulo, Brazil) LBC system and stored in Universal Collection Medium. Details of the preparation of the slides were reported recently (22). The classification of cytology was performed according to the Bethesda 2001 system (23). The final cytological diagnoses were confirmed by consensus of two independent cytopathologists.

Immunocytochemistry for p16 and Ki-67

Immunocytochemical (ICC) analysis was performed directly on slides with LBC smears previously stained with Papanicolaou technique. Only the slides with a sufficient amount of cells were subjected to ICC analysis. The ICC procedures were performed after removing coverslips from the slides in xylene and rehydrating smears in decreasing concentrations of ethyl alcohol and in distilled water. Antigen retrieval was performed using a 10-mM concentration of citrate buffer (pH 6.0) in a pressure cooker for 4 minutes. The slides were allowed to cool until room temperature and were then subjected to immunostaining. The antibodies used in this study were $p16^{INK4A}$ (diluted 1:500), obtained from MTM Lab AG (Germany), and the Ki-67 antigen (diluted 1:100), supplied by Dako AS (Denmark), both amplified by labeled streptavidin-biotin-peroxidase system obtained from Dako Cytomation. The color of immunostaining was developed by chromogenic substrate diaminobenzidine (100 mg%) and hydrogen peroxide (0.1%). After light counterstaining in Harry's hematoxylin, the slides were mounted and analyzed using light microscopy.

Evaluation of the ICC Staining

Evaluation of the ICC staining was performed counting the positive reactions in 1,000 cells at magnification of $\times 400$. All positive cells were counted in whole slide (DNA-Citoliq® System slide, with 2.5 cm of diameter of cell area). The p16 reaction was evaluated as positive if nuclear or cytoplasmic immunostaining was clearly demonstrated. For Ki-67, only nuclear reaction was considered positive. Positive immunostaining (per 1,000 cells) was graded as follows: negative (0); faintly positive, if between ≥ 1 and ≤ 10 cells stained; sporadic if >10 and ≤ 50; intermediate >50 and ≤ 100; and diffuse if >100 cells were positive. In statistical calculations, positive reactions of both markers also were considered as positive or negative.

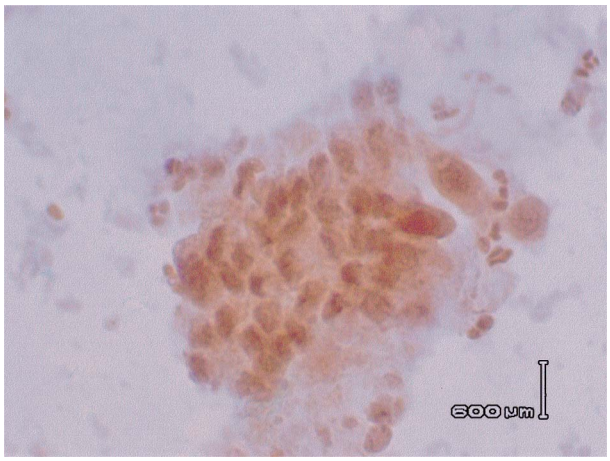

FIG. 1. Positive nuclear and cytoplasmic immunoreaction for $p16^{INK4A}$ is observed in metaplastic and glandular cells ($\times 400$).

Hybrid Capture II and PCR in LBC Medium

The Hybrid Capture II (HC2) assay was performed according to the instructions of the manufacturer (Digene Co., Gaithersburg, MD). In estimation of positive reactions, samples were considered positive if the relative light units cutoff (RLU/CO) were >1 (24). Only high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) were tested. The method of PCR in LBC medium (Universal Collection Medium, Digene Co.) has been described previously (22). In brief, DNA purification was performed with GFX® (Genomic Blood DNA Purification Kit, Amersham cat #27-9603-01, Piscataway, NJ) and PCR amplification was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PGMY09/11 L1 consensus primers to detect HPV and GH20/PCO4 primers for human β-globin were co-amplified.

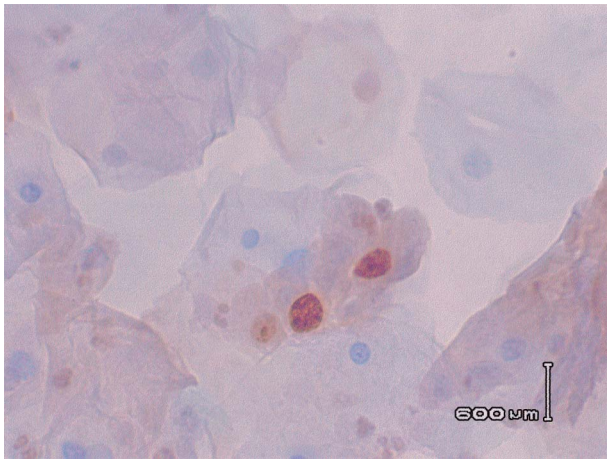

FIG. 2. Positive nuclear immunoreaction for Ki67 is observed in reactive squamous cells ($\times 400$).

TABLE 1. *Quantified p16 expression related to Ki-67 expression and HPV status in cytologically normal LBC smears*

	HPV positive			HPV negative		
p16 immunostaining	Ki-67 negative	Ki-67 positive	Total	Ki-67 negative	Ki-67 positive	Total
0	17 (16.8)	15 (14.8)	32 (31.7)	41 (74.5)	8 (14.5)	49 (89.1)
≥1 and ≤10	3 (2.9)	7 (6.9)	10 (9.9)	0 (0)	0 (0)	0
>10 and ≤50	4 (3.9)	31 (30.7)	35 (34.6)	2 (3.6)	1 (1.8)	3 (5.4)
>50 and ≤100	2 (1.9)	9 (8.9)	11 (10.9)	2 (3.6)	1 (1.8)	3 (5.4)
>100	1 (0.9)	12 (92.3)	13 (12.8)	0 (0)	0 (0)	0
Total	27 (26.7)	74 (73.3)	101 (100)	45 (81.7)	10 (18.2)	55 (100)

HPV, human papillomavirus; LBC, liquid-based cytology.
Data are numbers with percentages in parentheses.

Statistical Analysis

Statistical analyses were performed using the SPSS (for Windows, version 11.5) software package (SPSS, Chicago, IL), and the tables of frequencies were analyzed with the chi-square test, with a likelihood ratio used to assess the significance between the categorical variables. HPV status was considered positive if HC2 and/or PCR were positive.

RESULTS

The LBC slides from 156 patients were classified cytologically negative and 43 as ASC-US. Only one case was insufficient for technical problems. The number of cells was sufficient (at least 5,000 cells as recommended by TBS 2001) and well preserved (25). p16 immunostaining was localized both in the nucleus and cytoplasm (Fig. 1). According to recent reports, both nuclear and cytoplasmic staining was considered positive (26). Only nuclear reaction for Ki-67 was observed (Fig. 2).

Table 1 shows the correlation between p16 and Ki67 staining in both HPV-negative and HPV-positive, cytologically negative smears. Among these negative cytologic samples, 101 were HPV-positive and 55 HPV-negative. Of the HPV-positive group, 59 of 101 cases (58.4%) were positive for both p16 and Ki67 immunostaining, and 17 of 101 (16.8%) were negative for both. The proportion of Ki-67 positivity increased almost in parallel with the increasing grade of p16 positivity ($p = 0.0001$ for linear trend). In the HPV-negative group, both markers were negative in 41 of 55 cases (74.5%). Not unexpectedly, only 3 of 55 (5.4%) cytologically negative smears were simultaneously positive for both markers. No statistical relationship was observed between the grade of p16 positivity and Ki-67 expression in this HPV-negative group (chi-square, Pearson, $p = 0.595$).

The same correlation of marker expression in ASC-US (equivocal) smears is depicted in Table 2. HPV-positive ASC-US samples demonstrated a simultaneous positive reaction for p16 and Ki67 in 11 of 16 cases (68.7%), whereas 3 (18.7%) were concurrently negative. The relationship between the two markers was of borderline significance (chi-square, Pearson, $p=0.053$), but no linear relationship was found between graded p16 and Ki-67 expression ($p = 0.065$ for linear trend). In the HPV-negative ASC-US group, 7 of 27 cases (25.9%) cases were completely negative, and 9 (33.3%) were positive for both markers. In this group, there was no statistical association between the graded p16 and Ki-67 positivity (chi-square, Pearson, $p = 0.281$).

In Table 3, a quantified Ki-67 expression is related to p16 expression and HPV status in cytologically normal smears. In HPV-positive group, the proportion of p16 positivity increased in parallel with the increasing grade

TABLE 2. *Quantified p16 expression related to Ki-67 expression and HPV status in cytologically equivocal (ASC-US) LBC smears*

	HPV positive			HPV negative		
p16 immunostaining	Ki 67 negative	Ki 67 positive	Total	Ki 67 negative	Ki67 positive	Total
0	3 (18.7)	1 (6.2)	4 (25)	7 (25.9)	7 (25.9)	14 (52)
≥1 and ≤10	0 (0.0)	1 (6.2)	1 (6.2)	0 (0)	0 (0)	0 (0)
>10 and ≤50	0 (0.0)	7 (43.7)	7 (43.7)	5 (18.5)	4 (14.8)	9 (33.3)
>50 and ≤100	1 (6.2)	1 (6.2)	2 (12.5)	0 (0.0)	2 (7.4)	2 (7.4)
>100	0 (0.0)	2 (12.5)	2 (12.5)	0 (0.0)	2 (7.4)	2 (7.4)
Total	4 (25)	12 (75)	16 (100)	12 (44.4)	15 (55.5)	27 (100)

HPV, human papillomavirus; LBC, liquid-based cytology; ASC-US, atypical squamous cells of undetermined significance.
Data are numbers with percentage in parentheses.

TABLE 3. *Quantified Ki-67 expression related to p16 expression and HPV status in cytologically normal LBC smears*

	HPV positive			HPV negative		
Ki67 immunostaining	p16 negative	p16 positive	Total	p16 negative	p16 positive	Total
0	17 (16.8)	10 (9.9)	27 (26.7)	41 (74.5)	3 (5.4)	44 (80)
≥1 and ≤10	12 (11.9)	40 (39.6)	52 (51.4)	7 (12.7)	1 (1.8)	8 (14.5)
>10 and ≤50	2 (1.9)	18 (17.8)	20 (19.8)	2 (3.6)	1 (1.8)	3 (5.4)
>50 and ≤100	0 (0)	1 (0.9)	1 (0.9)	0 (0)	0 (0)	0
>100	0 (0)	1 (0.9)	1 (0.9)	0 (0)	0 (0)	0
Total	31 (30.7)	70 (69.3)	101 (100)	49 (89.1)	6 (10.9)	55 (100)

HPV, human papillomavirus; LBC, liquid-based cytology.
Data are numbers with percentages in parentheses.

of Ki-67-positivity (chi-square, Pearson, p = 0.001, and p = 0.0001 for linear trend). Among HPV-negative samples, no statistical association could be established between graded Ki-67 expression and p16 positivity (Pearson, p = 0.284).

Table 4 gives the same data for ASC-US smears. In HPV-positive group, the proportion of p16 positivity increased in parallel with the increasing grade of Ki-67 positivity, albeit the two highest categories are missing (chi-square, Pearson, p = 0.026, and p = 0.018 for linear trend). In the HPV-negative group, there was no statistical relationship between the grade of Ki-67 expression and p16 positivity (Pearson, p = 0.636).

After 12 months of follow-up of the ASC-US patients, 6 women still displayed ASC-US smear, of which 4 of 6 were HPV-positive and expressed both p16 and Ki-67 markers. Two of 43 (4.6%) ASC-US smears had high-grade squamous intraepithelial lesions (HSIL) diagnosed, and 1 (2.3%) had a low-grade squamous intraepithelial lesion (LSIL). All of those were positive for HPV, p16, and Ki-67.

DISCUSSION

The HPV infection by the high-risk types might identify women who are at increased risk for development of a significant cervical lesion and, conversely, a negative HPV test has a high negative predictive value. This potential risk of developing a cervical lesion does not necessarily mean that HPV infection would progress (2). The use of p16 marker has been suggested to compensate this lack of specificity of an HPV test, and advocated as an adjunct tool in routine use, because it is overexpressed in cases in which E7 oncogenes block the activity of pRB that regulates p16 (5). For this reason, the pivotal work of Klaes and colleagues (5) was crucial to determinate the importance of p16 overexpression as a specific markers for malignant and premalignant lesions of the cervix in both histologic and cytologic samples. These authors observed that $p16^{INK4A}$ is expressed in dysplastic but not in normal cells. Moreover, low-grade abnormalities testing positive for low-risk HPV did not express detectable overexpression of $p16^{INK4A}$.

The intention of our work was to observe whether the p16 overexpression can occur in cases that are cytologically negative or equivocal (ASC-US), but test positive for high-risk HPV. We wanted to test our hypothesis that $p16^{INK4A}$ overexpression can be present in cytologic samples before any morphologic abnormalities are detectable. Conversely to some previous reports (5,26), we could detect $p16^{INK4A}$ immunostaining in normal-looking cervical cells. There are several recent reports on similar findings (27–29). Interestingly, Sahebali and colleagues (29) have found a close correlation between the numbers

TABLE 4. *Quantified Ki-67 expression related to p16 expression and HPV status in cytologically equivocal (ASC-US) LBC smears*

	HPV positive			HPV negative		
Ki67 immunostaining	p16 negative	p16 positive	Total	p16 negative	p16 positive	Total
0	3 (18.7)	1 (6.2)	4 (25)	7 (25.9)	4 (14.8)	11 (40.7)
≥1 and ≤10	1 (6.2)	7 (43.7)	8 (50)	6 (46.2)	7 (25.9)	13 (48.1)
>10 and ≤50	0 (0.0)	4 (25)	4 (25)	2 (7.4)	1 (3.7)	3 (11.1)
>50 and ≤100	0 (0.0)	0 (0.0)	0	0 (0.0)	0 (0.0)	0 (0.0)
>100	0 (0.0)	0 (0.0)	0	0 (0.0)	0 (0.0)	0 (0.0)
Total	4 (25)	12 (75)	16 (100)	15 (55.5)	12 (44.4)	27 (100)

HPV, human papillomavirus; LBC, liquid-based cytology; ASC-US, atypical squamous cells of undetermined significance.
Data are number with percentages in parentheses.

of p16-stained cells and the HPV type, being most intense in HPV16 type and lowest in low-risk HPV lesions. They also reported that this correlation is also a significant diagnostic sign, with higher mean count of p16-positive reactions detectable in HSIL than in other diagnostic categories.

As we principally studied cytologically negative cases, we used Ki67 proliferation marker to disclose an eventual correlation between the insensitivity of p16 immunostaining and the expression of this cell cycle marker. We observed that the majority of the cases concurrently positive for both p16 and Ki67 markers occurred in the group of patients testing positive for high-risk HPV. The mean count of p16 immunoreactive cells falls between 10 and 50 of 1,000 cells, whereas that of Ki67-positive cells were predominantly between 1 and 10 of 1,000 cells. The value of Ki67 as a predictor in cervical lesions seems to depend on the number of positive cells counted (19). As an independent variable, Ki67 has a strong predictive value for progression for cervical intraepithelial neoplasia (CIN) 1 and CIN 2 lesions (30). Similarly, there seems to be a significant correlation between p16 and Ki67 immunoreactions among significant lesions (20). This is consonant with the present observations, in which the proportion of Ki-67 positivity increased almost in parallel with the increasing grade of p16 positivity ($p = 0.0001$ for linear trend). Furthermore, in our HPV-positive group, 59 of 101 cases (58.4%) were positive for both p16 and Ki67 immunostaining, whereas only 17 of 101 (16.8%) were negative for both.

Whether the combination of p16 and Ki67 would be useful as an adjunct tool to predict the development of CIN lesions in women with normal or equivocal cytology remains to be seen by the follow-up of our patients. They all are under strict surveillance and attend the control visits regularly. Among the women with ASC-US cytology at the onset, we have already detected three cases with significant lesions: two HSIL, and one case of LSIL. Similarly, among the cases cytologically classified as negative, one case (positive for HPV by biomolecular assay) revealed at cytologic examination slight atypias classified as ASC-US after 36 months of follow-up.

As stated earlier, the rationale for the use of the p16 in cervical pathology examination is based on the increasing expression of viral oncogenes in dysplastic cells reflected by the increased expression of $p16^{INK4A}$. The potential use of $p16^{INK4A}$ in routine cytology certainly depends on additional long-term studies, including molecular testing for high-risk HPV. The main unanswered questions in our study are 1) whether all high-risk HPV-positive, cytologically negative or equivocal, patients who express $p16^{INK4A}$ and Ki67 will eventually develop cervical lesion, and 2) whether such women with ASC-US cytology will progress for a squamous intraepithelial lesion. The three detected cases of squamous intraepithelial lesions among these women suggest that this might be the case, and using these potential predictors might be seriously considered in the management of patients with equivocal cytology.

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