ORIGINAL PAPER

Immunocytochemical expression of $p16^{INK4a}$ and HPV L1 capsid proteins as predictive markers of the cervical lesions progression risk

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Abstract

Genital HPV infections are extremely common but most of them are spontaneously cleared by the host immune response. The main problem is how to identify the HPV-HR positive patients who are at risk of progressive disease. *Aim*: The purpose of this study was to investigate the uterine cervix lesions concerning the HPV status appreciated through the immunocytochemical expression of the L1 HPV and $p16^{INK4a}$ proteins. *Material and Methods*: 76 women who tested positive for HPV were selected from a cohort of 374 patients. In this study were detected the immunochemical expression of HPV L1 capsid protein and $p16^{INK4a}$ in LBC samples. *Results*: The $p16^{INK4a}$ positive rate was expressed in 56.57% of all the cases. The percentage grew from 0% in NILM cases to 100% in SCCs cases (*p*-value <0.00001). The HPV L1 capsid protein positive was expressed in 12.50% of NILM cases, 33.33% of ASC-US, 50% of LSIL, 18.51% of HSILs cases, but 0% in the SCC group (*p*-value =0.01). The L1-/p16+ pattern was found in 21.87% of LSIL, 81.48% of HSIL, and 100% of SCC cases (*p*-value <0.00001). The association of these two markers (L1 and $p16^{INK4a}$) raises the accuracy of the diagnostic from 64% for HPV L1 capsid protein and respectively 87% for $p16^{INK4a}$ to 91% when they are associated. *Conclusions*: The combination of L1 capsid protein and p16 appears to be useful for an early diagnosis and may be able to identify the patients with risk of lesion progression.

Keywords: liquid-based cytology (LBC), human papillomavirus (HPV), L1 capsid protein, $p16^{INK4a}$, immunocytochemistry.

Introduction

The infection with human papillomavirus (HPV) is now recognized as a major cause of cervical cancer [1]. Although most women will have been some time infected with HPV, few of them will progress to invasive disease [2]. The main problem is how to identify, from the large number of HPV positive patients, the ones who are at risk of progressive disease. HPV genotyping can establish the presence of HPV-HR infection but cannot differentiate between latent, subclinical and clinical relevant infections. Neither cytological test nor HPV DNA typing could indicate whether there will be remission or progression to invasive disease [3, 4]. Therefore, it is necessary to identify some markers to offer information concerning the HPV infection status and the progression risk. The aim of our study is to detect the immunocytochemistry expression of $p16^{INK4a}$ and HPV L1 capsid protein and to investigate the combined expression of these markers in cervical lesions. HPV L1 capsid protein is expressed in the early, productive phase of cervical carcinogenesis and is progressively lost in the later phases, when p16 gets overexpressed [5, 6]. The combination of L1 capsid protein and p16 immunostaining in LBC appears to be useful for an early diagnosis of precancerous lesions, because the L1/p16 expression status may be able to identify individuals at risk of lesion progression and may also be helpful for subsequent follow-up of patients.

Material and Methods

From a cohort of 374 patients, we selected 76 women who are HPV positive, with normal cytological results (NILM) or an abnormal Pap test result (ASC-US, LSIL, HSIL and SCC). The Pap smears were immunocytochemical stained using two markers: $p16^{INK4a}$ and HPV L1 antibodies. All of the patients underwent colposcopy-guided biopsy to assess the grade of dysplasia.

Liquid-based cytology (LBC)

The samples were obtained from patients using a Wallach Papette that was immediately immersed into a vial with PapSpin Collection Fluid preservative. Liquid-based cytology (PapSpin®) was performed by using Cytospin 4 (Thermo Shandon), according to manufacturer's protocol. Three slides per case were prepared: one for Papanicolaou stain, respectively two slides for immunocytochemical staining. The cytological specimens were interpreted using the 2001 Bethesda reporting system [7].

HPV DNA testing

HPV genotyping was performed at the „Ştefan S. Nicolau" Institute of Virology, Bucharest. Biological samples were collected in Copan liquid medium. Two methods were used: Digene HC2 HPV DNA, a Test for differentiate between low and high-risk and LINEAR ARRAY HPV Genotyping, a Test (Roche Diagnostics) used for individual detection of 37 high-and low-risk human papillomavirus types, including: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108. HPV DNA Testing was performed according to manufacturer's protocol. Internal control and HPV negative and positive controls were included in each run.

Cervical biopsy specimens

Colposcopically guided punch biopsies were fixed in neutral buffered formalin, embedded in paraffin, sectioned and then stained with Hematoxylin–Eosin. Histopathological specimens were interpreted using Richart terminology (CIN) [8].

Immunostaining for $p16^{INK4a}$ and L1 capsid protein

For the immunochemical analysis, we used a p16 monoclonal antibody (CINtec® Cytology Kit clone E6H4TM MTM) and a L1 monoclonal antibody (Cytoactiv® Screening Set Cytoimmun Diagnostics GbmH). The testing was performed according to the manufacturer's protocol. Positive and negative controls were included in each run.

The evaluation of $p16^{INK4a}$ and L1 capsid protein immunostaining

The CINtec® Cytology Kit uses the cyclin-dependent kinase inhibitor $p16^{INK4a}$ as a biomarker; the specific staining is nuclear and/or cytoplasmatic. Immunostaining results were evaluated as positive when the smear included atypical cells that showed specific immunoreactivity for p16.

The cytoactiv HPV L1 Screening antibody is directed against epitopes of the L1 capsid protein. The specific staining is nuclear, but sometimes a vesicular staining can be observed in cytoplasm.

When the expression of L1 capsid protein was analyzed in relation with p16 expression status, the staining pattern was divided in four groups:

- L1 negative/p16 negative (L1-/p16-);
- L1 positive/p16 negative (L1+/p16-);
- L1 positive/p16 positive (L1+/p16+);
- L1 negative/p16 positive (L1-/p16+).

Statistical analysis of immunocytochemistry results

To analyze the diagnostic efficiency of the L1, $p16^{INK4a}$ and of their combination, sensitivity (Sn), specificity (Sp), positive predictive values (PPV), negative predictive values (NPV) and accuracy were calculated, using the histopathological "gold standard" and CIN II + were considered positive results. Statistical analysis was evaluated using the diagnostic test and the Fisher exact test.

Results

Cytological results

The cytological diagnosis of the 76 patients included in this study were: eight cases (10.52%) negative for intraepithelial lesions or malignancy (NILM), six cases (7.89%) with atypical squamous cells of undetermined significance (ASC-US), 32 (42.10%) with low-grade squamous intraepithelial lesions (LSIL), 27 (35.52%) presented high-grade squamous intraepithelial lesions (HSIL) and in three cases (3.94%) squamocellular carcinoma (SCC).

Histopathological results

The histopathological results showed: 12 cases (15.78%) were benign, 31 (40.78%) – CIN I, 17 (22.36%) – CIN II, 13 (17.10%) – CIN III, and three cases (3.94%) – SCC. The distribution of cytological diagnosis, according to the histological diagnosis, can be seen in Figure 1.

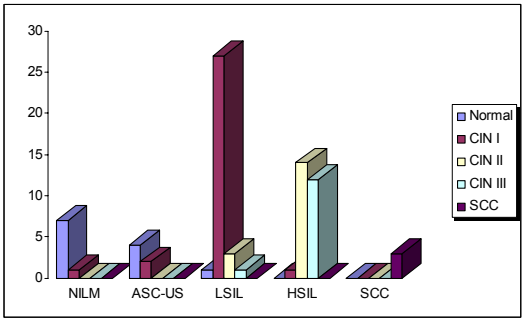

Figure 1 – ***Distribution of cytological results according to the histological diagnosis.***

HPV genotyping results

We found single HPV infections in 50 cases (65.78%) and multiple HPV types (HPV-multiple) in other 26 cases (34.21%). In the group with single HPV infection, 17 cases presented HPV-LR and 33 cases HPV-HR. As all cases with multiple type HPV infections included at least one HR type, all of them were included for evaluation in HPV-HR group. The HPV genotyping results were grouped according to the HPV risk group and to the diagnostic category (Figure 2). HR-HPV infection was found in 25% of NILM cases (2/8), 66.66% of ASC-US cases (4/6) and 78.12% of LSIL cases (25/32). All HSIL and SCC cases displayed HR-HPV genotypes.

Immunocytochemistry results

Positive result for $p16^{INK4a}$ was found in 56.57% (43/76) from all the cases, namely: 16.66% from ASC-US cases (1/6), 40.62% from LSIL cases (13/32), 96.29% from HSIL cases (26/27), and 100% from SCC cases. In NILM cases, no positive result was detected for $p16^{INK4a}$. The correlation between cytological diagnosis, $p16^{INK4a}$ staining and histological results are shown in Figure 3.

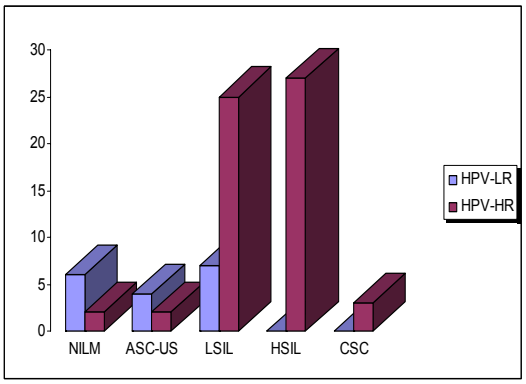

Figure 2 – ***Distribution of HPV risk group according to cytodiagnostic category.***

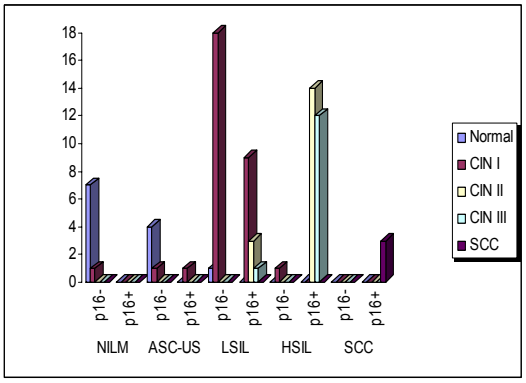

Figure 3 – ***The histological follow-up results of the liquid-based cytology samples in correlation with p16INK4a protein staining results.***

The positive rate of HPV L1 capsid protein was identified in 30.26% (23/76) of cases. L1 capsid protein was expressed in one of the eight NILM cases (12.5%), two of the six ASC-US cases (33.33%), 16 of the 32 LSIL cases (50%), five of the 27 HSIL cases (18.51%), and no positive cases in the group of SCC. The link between the cytological diagnosis, the histological results and the HPV L1 capsid protein staining is presented in Figure 4.

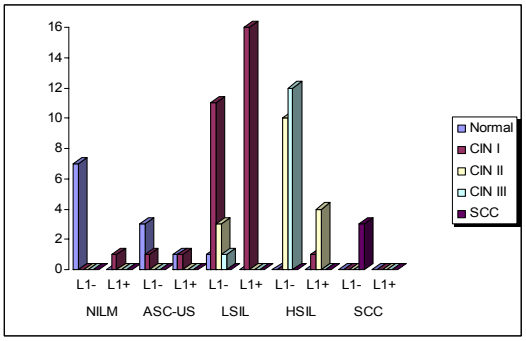

Figure 4 – ***The histological follow-up results of the liquid-based cytology samples in correlation with HPV L1 capsid protein staining results.***

Relation between the L1 capsid protein and p16^{INK4a}

L1-/p16- represented 26.31% (20/76) of all cases. This pattern was found in 87.5% of NILM cases, in 66.66% of ASC-USs, in 28.12% of LSILs and 0% in the HSIL and SCC cases.

L1+/p16- represented 18.42% (14/76) of all the cases. This pattern was found in 12.5% in NILM cases, 33.33% of ASC-USs ones, 31.25% in LSILs, one case (3.70%) in HSIL, and 0% in SCC cases.

L1+/p16+ represented 13.15% (10/76) of all the cases. This pattern was found in 18.75% in LSIL cases (Figure 5, A–D), 14.81% in HSILs and 0% in SCC cases.

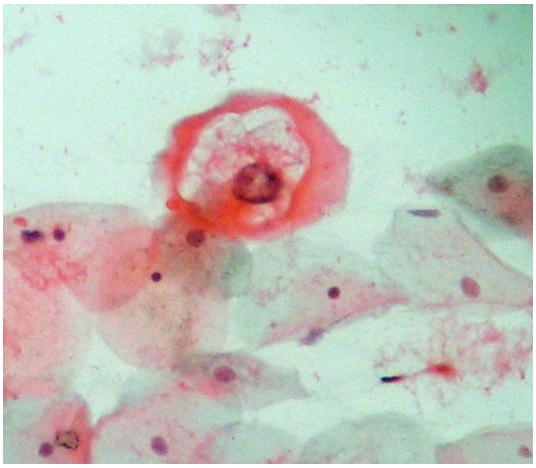

Figure 5 – ***(A) p16+/L+ LSIL case: typical koilocyte–eosinophylic cytoplasm, perinuclear halo surrounded by cytoplasmic thickening, moderate nuclear enlargement. PapSpin, 400×.***

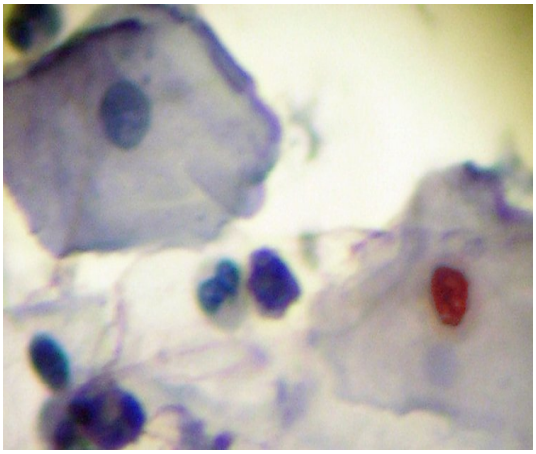

Figure 5 – ***(B) p16+/L+ LSIL case: positive staining for p16^{INK4a} in the nucleus and cytoplasm of a dysplastic cell. PapSpin, 400×.***

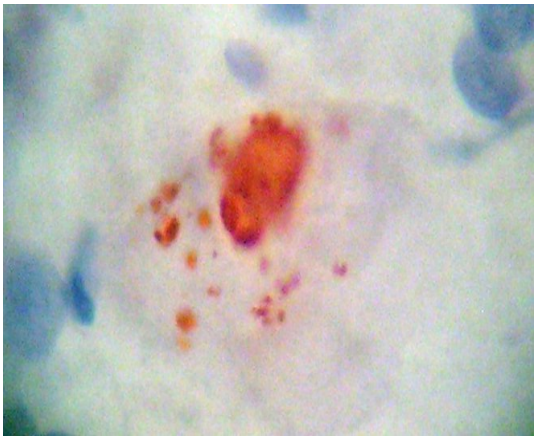

Figure 5 – ***(C) p16+/L+ LSIL case: positive staining for HPV L1 capsid protein in the nucleus of a dysplastic cell. PapSpin, 400×.***

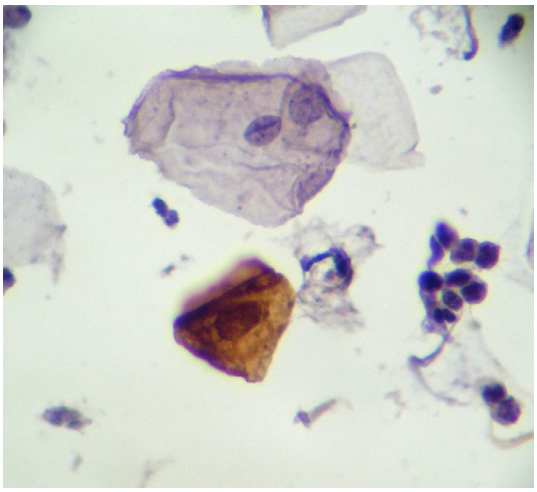

Figure 5 – ***(D) p16+/L+ LSIL case: positive staining for HPV L1 capsid protein in the nucleus and a vesicular staining in cytoplasm. PapSpin, 100×.***

L1-/p16+ represented 42.10% (32/76) of all the cases including seven cases (21.87%) LSIL, 22 (81.48%) HSIL (Figure 6, A–C), and 100% in SCC cases. The histological follow-up results of the liquid-based cytology samples in correlation with combination of $p16I^{NK4a}$ and L1 capsid protein immunostaining results are shown in Figure 7.

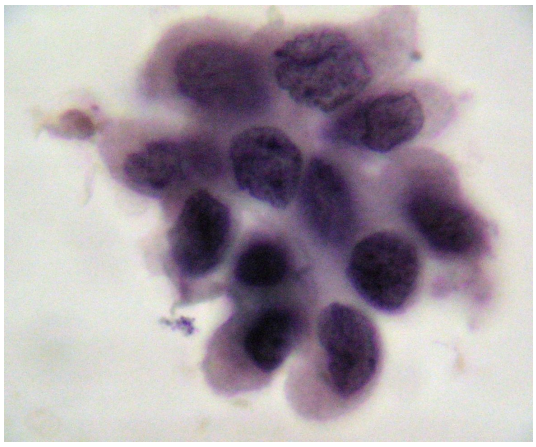

Figure 6 – ***(A) p16+/L1- HSIL case: small group of basal cells with an enlarged, hyperchromatic nuclei, irregular nuclear outlines and an irregular chromatin. PapSpin, 400×.***

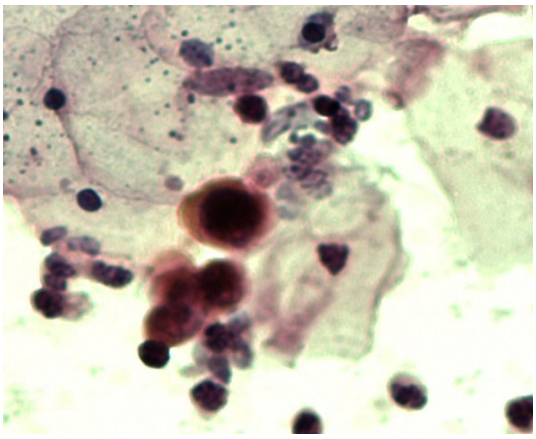

Figure 6 – ***(B) p16+/L1- HSIL case: positive staining for $p16^{INK4a}$ in the nucleus and cytoplasm of a small group of dysplastic cell. PapSpin, 200×.***

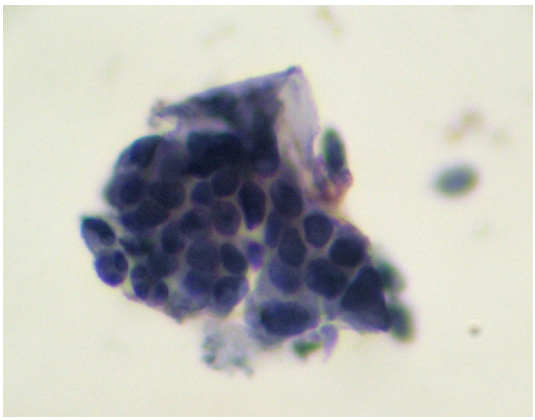

Figure 6 – ***(C) p16+/L1- HSIL case: negative staining for HPV L1 capsid protein. PapSpin, 200×.***

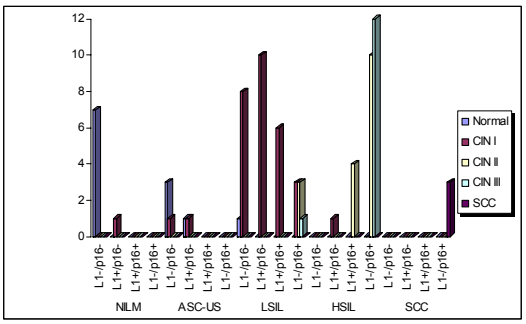

Figure 7 – ***The histological follow-up results of the liquid-based cytology samples in correlation with combination of p16INK4a and L1 capsid protein immunostaining results.***

Statistical results

For the L1 capsid protein, as a positive result was considered the absence of the immunostaining. The diagnostic test results were: Sn=87.88%, Sp=46.51%, PPV=55.8%, and PPN=83.3%. For this situation, the accuracy of the diagnosis was of 64%. For this antibody, the results was statistically significant (p=0.014318).

For the $p16^{INK4a}$, as a positive result was considered the presence of the immunostaining. The diagnostic test results were: Sn=100%, Sp=76.74%, PPV=76.7%, PPN=100%, the accuracy was 87%. For this antibody, the log-rank test was statistically significant (p<0.00001).

To assess the association of these two markers, positive cases were considered those with L1-/p16+ immunostaining. The diagnostic test results were the following: Sn=87.88%, Sp=93.02%, PPV=90.6%, PPN=90%, accuracy 91%. Also, in this case the results was statistically significant (p<0.000001) (Table 1).

Table 1 – ***Diagnostic efficiency of combining L1 with $p16^{INK4a}$ vs. L1 or $p16^{INK4a}$ alone***

	Sn [%]	Sp [%]	PPV [%]	NPV [%]	Acc [%]	*p*-value
L1	87.88	46.51	55.8	83.3	64	0.01
p16	100	76.74	76.7	100	87	<0.000001
L1/p16	87.88	93.02	90.6	90.9	91	<0.000001

Discussion

Organized cervical cancer screening based on the Papanicolaou smear has been proven to prevent 80% of

cervical cancer deaths [1, 2]. However, several studies have shown that cytology has a limited sensitivity for detecting cervical intraepithelial neoplasia [9, 10].

More recently, testing for human papillomavirus (HPV) has been adopted for the management of unclear and mildly abnormal Pap Tests. The sensitivity for disease of HPV testing is superior to that of a Pap Test [11, 12]. The majority of acute productive infections spontaneously resolve within several months [13, 14]. That is why use of HPV DNA tests in primary cervical cancer screening has a lower specificity which results in more women needing to go through unnecessary follow-up procedures, including repeat testing, colposcopy [15].

A more efficient approach to cervical cancer early detection and diagnosis is to detect specific biomarkers that indicate the presence or absence of cervical cancer or its precursors.

The life cycle of HPV is related to the biology of the host cells. It is known that the human papillomavirus contributes to neoplastic progression predominantly through the action of two viral oncoproteins, E6 and E7, which interact with various host regulatory proteins to influence the function or expression levels of host gene products, eventually leading to the disruption of the cell cycle. p16^{INK4a} is a cyclin-dependent kinase inhibitor that negatively regulates cell proliferation by inhibiting hyperphosphorylation of pRb via the cdk4/6 complex [16]. It has been proposed that, rather than viral presence only, p16^{INK4a} is a useful biomarker for the identification of cervical intraepithelial lesions because it is a measure of active HPV gene expression. Overexpression of p16^{INK4a} has been directly correlated to the oncogenic activity of high-risk HPV types [17]. p16^{INK4a} staining marks those persistently HR-HPV-infected cells that display deregulated expression of the viral oncogenes.

Numerous prospective studies using p16^{INK4a} immunostaining in liquid-based cytology specimens have observed a good agreement between p16^{INK4a} positive staining and the grade of intraepithelial lesion [18–22].

A meta-analysis makes by Tsoumpou I *et al.* [22] showed that the proportion of cervical smears overexpressing p16^{INK4a} increases with the severity of cytological abnormality. Among normal smears, only 12% were positive for this biomarker compared to 45% of LSIL and 89% of HSIL smears. Similarly, in histology only 2% of normal biopsies and 38% of CIN I compared to 68% of CIN II and 82% of CIN III.

In the present study p16^{INK4a} positivity was assessed within each cytological category and was shown to increase from 16.66% in ASC-US samples to 40.62% in LSILs, 96.29% in HSILs respectively to 100% in SCCs. Our results showed that p16^{INK4} positivity has 100% negative predictive value for presence of CIN II+ lesions (p<0.000001).

Recently, some reports showed that L1 capsid protein can be a new powerful and useful marker for revealing the status of productive and/or active HPV infections [23–25]. The positive staining for L1 capsid protein was detected only in the productive phase. Loss of L1 capsid protein expression may show two statuses of viral DNA: one is the integration of viral DNA into the host genome and the other is latent infection with low or no synthesis of HPV oncoproteins and no HPV production.

In 2003, Melsheimer P *et al.* [23] have shown that most of the HPV high risk associated LSIL are expressing HPV L1 capsid protein, but in most of the HPV high risk associated HSIL the HPV L1 capsid protein is missing. They suggested that this disturbed viral cellular interaction in HPV infected HSIL with loss of viral L1 capsid protein could function as prognostic marker to predict the prognosis of CIN lesions.

These findings are consistent with our present data that show expression of L1 capsid protein in 33.33% of ASC-US cases, 50% of LSILs, 18.51% of HSILs. No positive cases were found in group of SCC, thus indicating that L1 capsid protein expression tends to decline with increasing severity of the lesions. The presence of HPV L1 presumably represents the episomal stage of a transient HPV infection and correlates well with the regression of the abnormal cytology. Loss of HPV L1 capsid protein in HPV-HR positive women is associated with a higher risk of progression to CIN 2+ lesions. The lack of HPV L1 capsid protein in higher-grade lesions was likely to indicate host cell integration of HPV DNA.

As expressed in different phases of the cervical carcinogenesis, HPV L1 capsid protein and p16^{INK4a} are potentially promising markers of progression risk of cervical lesions. HPV L1 capsid protein is expressed in the early, productive phase of cervical carcinogenesis and is progressively lost in the later phases, when p16 become overexpressed [5, 6]. The combination of L1 capsid protein and p16 immunostaining in LBC appears to be useful for an early diagnosis of precancerous lesions because the L1/p16 expression status may be able to identify individuals at risk of lesion progression and may also be helpful for subsequent follow-up of the patients [26, 27].

Yoshida T *et al.* [26] have demonstrated that the combination of p16^{INK4a} and L1 capsid protein immunostaining is a very useful and powerful test as a prognostic marker. The L1(-)/p16(+) results of their study represented 74.6% of all lesions, including 45%, 88%, and 100% of LSILs, HSILs, and SCCs, respectively. In our study, the L1(-)/p16(-) pattern was found in 20 of all cases, including seven NILM, four ASC-US, nine LSIL and none of the HSIL, respectively SCC. The histopathological diagnostics in this cases were normal or CIN I. This pattern means that viral DNA is present without either viral replication or alteration of the cell cycle, thus indicating that the lesion is in a latent state without risk of progression in near future.

The L1(+)/p16(-) pattern was found in 14 cases, including: one NILM, two ASC-US, 10 LSIL, respectively 1 HSIL. The histopathological diagnoses, including HSIL, have been CIN I. This pattern showed that viral DNA is present as a productive state without alteration of the cell cycle, which indicates a low risk of developing CIN II+ lesion. In 10 cases, the pattern was L1(+)/p16(+) with the following distribution: six LSIL and CIN I at biopsy and four HSIL and CIN II. All this cases presented HPV-HR infection. This pattern showed a productive status of HPV infection and is associated

with an alteration of the cell cycle. It indicates that the lesion is in a virus-producing state with immediately risk of progression.

L1(-)/p16(+) pattern was identified in seven (21.87%) cases with LSIL (three CIN I, three CIN II and one with CIN III), 22 (81.48%) HSIL cases (10 CIN II, 12 CIN III) respectively three (100%) SCC cases that have been histological confirmed. The L1(-)/p16(+) pattern showed the integration of HPV DNA into the host genome with alteration of the cell cycle. This pattern might be defined as "high-risk" pattern, which is typically found also in high-grade lesions of the cervix.

The minor cytological lesions (ASC-US and LSIL) are most often related to transient HPV infections that regress spontaneously and do not require treatment. In these cases, the L1/p16 expression status may be able to identify individuals at risk of lesion progression. Cases with L1(-)/p16(-) pattern can be followed with a longer time interval, because this pattern means that the lesion is in a latent state. L1(+)/p16(-) cases need follow-up within a short time interval, because this pattern means a productive status of HPV infection that may produce a high-grade lesion in the future. Cases with L1(+)/p16(+) and L1(-)/ p16(+) patterns need strict follow-up with colposcopy and biopsy, because p16 positivity is a indicator of dysplastic lesions.

Conclusions

This study has investigated the uterine cervix lesions concerning the HPV status appreciated through the immunocytochemical expression of the L1 and $p16^{INK4a}$ proteins. As expressed in different phases of cervical carcinogenesis, p16 and L1 are potentially promising markers of progression risk of LSIL. The combination of p16 and L1 capsid protein immunostaining in LBC appears to be useful for an early diagnosis of precancerous lesions and for an appropriate clinical attitude.

Acknowledgements

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