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Human Papillomavirus Genotyping and p16^{INK4a} Expression in Cervical Lesions: A Combined Test to Avoid Cervical Cancer Progression

SHORT COMMUNICATION

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Cervical cancer is a major public health problem in Morocco. The cervical cancer has a long precancerous period that provides an opportunity for the screening and treatment. Improving screening tests is a priority goal for the early diagnosis of cervical cancer. This study was conducted to evaluate the combination of p16^{INK4a} protein expression, human papillomavirus (HPV) typing, and histopathology for the identification of cervical lesions with high risk to progress to cervical cancer among Moroccan women. A total of 96 cervical biopsies were included in this study. Signal amplification in situ hybridization with biotinylated probes was used to detect HPV. Immunohistochemistry was used to evaluate the expression of p16^{INK4a} protein. HPV DNA was detected in 74.0% of the biopsies (71/96). Of the seventy-one positive HPV cases, we detected 67.6% (48/71) of high risk (HR)-HPV (HPV 16 and 18), 24% of low risk-HPV (HPV 6 and 11), 1.4% intermediate risk-HPV (HPV 31, 33, and 35), and 7% coinfections (HPV 6/11 and 16/18). Overexpression of p16^{INK4a} protein was observed in 72.9% (70/96) of the biopsies. In addition, p16^{INK4a} protein detection was closely correlated with recovery of HR HPV. Our result showed that p16^{INK4a} expression level is correlated with HR-HPV status.
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Key Words: Human papillomavirus, Biopsy, Cyclin-dependent kinase inhibitor p16, Immunohistochemistry, In situ hybridization

INTRODUCTION

Cervical cancer is the third most common cancer and the fourth cause of cancer related mortality among women worldwide.[1] In developing countries where access to screening is absent or limited, cervical cancer is the third cause of cancer mortality among women and is therefore much higher than in developed countries where this pathology is the seventh leading cause of cancer death.[1] In Morocco, cervical cancer is a major public health problem.[2,3] Fortunately, the cervical cancer has a long precancerous period that provides an opportunity for the screening and treatment before it progresses to cervical cancer. Therefore, improving screening tests is a priority goal for the early diagnosis of cervical cancer. p16^{INK4a} is a cyclin-dependent kinase inhibitor that regulates the activity of cyclin-dependent kinases 4 and 6. When the high risk (HR)-human papillomavirus (HPV) E7 oncoprotein inactivates retinoblastoma, p16^{INK4a} expression increases dramatically.[4-7] Moreover, overexpression of p16^{INK4a} protein in cervical cancer is used as a diagnostic tool and has been directly associated with infection by HR-HPV genotypes.[8,9] The aim of this study was to evaluate the combination of p16^{INK4a} protein expression, HPV typing, and histopathology for the early detection of cervical cancer among Moroccan women.

MATERIALS AND METHODS

1. Patients

A total of 96 cervical biopsies were collected. The study was conducted in the Laboratory of Histo-Cytopathology of Institut

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Pasteur du Maroc from January 2014 to October 2014 in Casablanca, Morocco under an approved human use protocol (Institutional Review Board, Ref: 013/14) to enroll and obtain tissue from the cervix of females to test for abnormal or precancerous conditions, or cervical cancer. The tissues were obtained by performing biopsies during routine physical examination to determine the health status of females ranging from 38 to 69 years of age. The biopsies were transferred to specimen collecting tubes containing 10% formalin and transported to the laboratory for processing and screening of precancerous or cancerous cells.

2. Histopathology and $p16^{INK4a}$ protein expression

The biopsy tissues were fixed in 10% formalin and embedded in paraffin. Tissue sections of 3 to 4 microns were prepared in paraffin and then placed on silanized slides. The pathology of the tissues was then examined using H&E conventional technique. $p16^{INK4a}$ protein expression was performed by immunohistochemistry using a CINtec Histology Kit (Biogen/Roche, Germany) according to the manufacturer's instructions.

3. Human papillomavirus detection

Signal amplification in situ hybridization (CSA-ISH, Genpoint, DAKO, Glostrup, Denmark) with biotinylated probes for HPV-6/11, 16/18, and 31/33/51 (ENZO, New York, NY, USA) was used according to manufacturer protocols. After dewaxing and hydration, the 96 tissue sections were treated with proteinase K solution for 5 minutes at room temperature. The denaturation of target DNA was carried out on a hotplate at 95°C and transferred to 37°C for hybridization with probes. The revelation of reaction product was performed using the system 'GenPoint DAKO', which created an additional level of the signal amplification. Revelation of the hybridization signal was produced by a chromogenic indicator, diaminobenzidine. The slides were counterstained with Mayer's hematoxylin, rinsed in ethanol and xylene, mounted by the Permanent Mounting Medium (DAKO), and then examined by light microscope.

4. Statistical analysis

Data were entered into a database using IBM SPSS ver. 20.0 for windows (IBM Co., Armonk, NY, USA). Associations between HPV status, histopathological diagnosis, and $p16^{INK4a}$ expression were analyzed using χ^2 test or Fisher's exact test. Spearman's rank correlation coefficients were analyzed to investigate the possible correlations between HPV status, histopathological diagnosis, and $p16^{INK4a}$ expression. All statistical tests were two-sided and results with values of $P < 0.05$ were considered statistically significant.

RESULTS

The histopathological diagnosis of the 96 studied biopsies revealed 14 cervicitis, 22 low-grade squamous intraepithelial lesions (LSIL), 36 high-grade squamous intraepithelial lesions (HSIL), and 24 invasive cancers. Elevated expression of $p16^{INK4a}$ protein was observed in 72.9% (70/96) of the biopsies. The $p16^{INK4a}$ positivity was more observed in HSIL (100%) as compared to LSIL (50%). In addition, the $p16^{INK4a}$ positivity rate (96%) was very high in invasive cancers as compared to LSIL (50%). Immunohistochemical analyses revealed that $p16^{INK4a}$ protein expression was diffuse in 18%, 64%, and 87% of LSIL, HSIL, and invasive cancer, respectively. Taken together, these data indicate that $p16^{INK4a}$ protein is more frequently expressed in invasive cancers than in LSIL and HSIL tissues (Fig. 1A).

HPV DNA was detected in 74.0% (71/96) of the biopsies. The positivity of the reaction resulted in a purple blue perinuclear precipitate (Fig. 1B and 1C).

Our result showed significant difference between HPV detection rate for cervicitis and LSIL ($P < 0.001$). However, no significant differences between HSIL and LSIL ($P > 0.05$) nor between HSIL and invasive cancer ($P > 0.05$) were observed. Of the seventy-one HPV positive cases, we detected 67.6% (48/71) of HR-HPV (HPV 16 and 18), 24% of low risk-HPV (HPV 6 and 11), 1.4% intermediate risk-HPV (HPV 31, 33, and 35), and 7% coinfections (HPV 6/11 and 16/18). Moreover, correlation between $p16^{INK4A}$ expression and histopathological grade showed a positive correlation (correlation coefficient = 0.470, $P < 0.05$). Furthermore, the HR-HPV status was significantly correlated with histopathological grade (correlation coefficient = 0.364, $P < 0.05$) (Fig. 2).

DISCUSSION

The current screening tests for cervical cancer (histology and cytology) are unable to distinguish between the lesions which progress to cancer and those that do not.[10] To overcome these limitations, an additional test is required to determine if oncogenic HPV virus has already increased genetic instability and made the infected cells sensitive to processing, leading eventually to the development of cancer. This test relies on the HR-HPV detection that exerts its oncogenic potential in infected woman.[10] In this study, the HPV DNA was detected in 74% of the biopsies. This prevalence was very high compared to that previ-

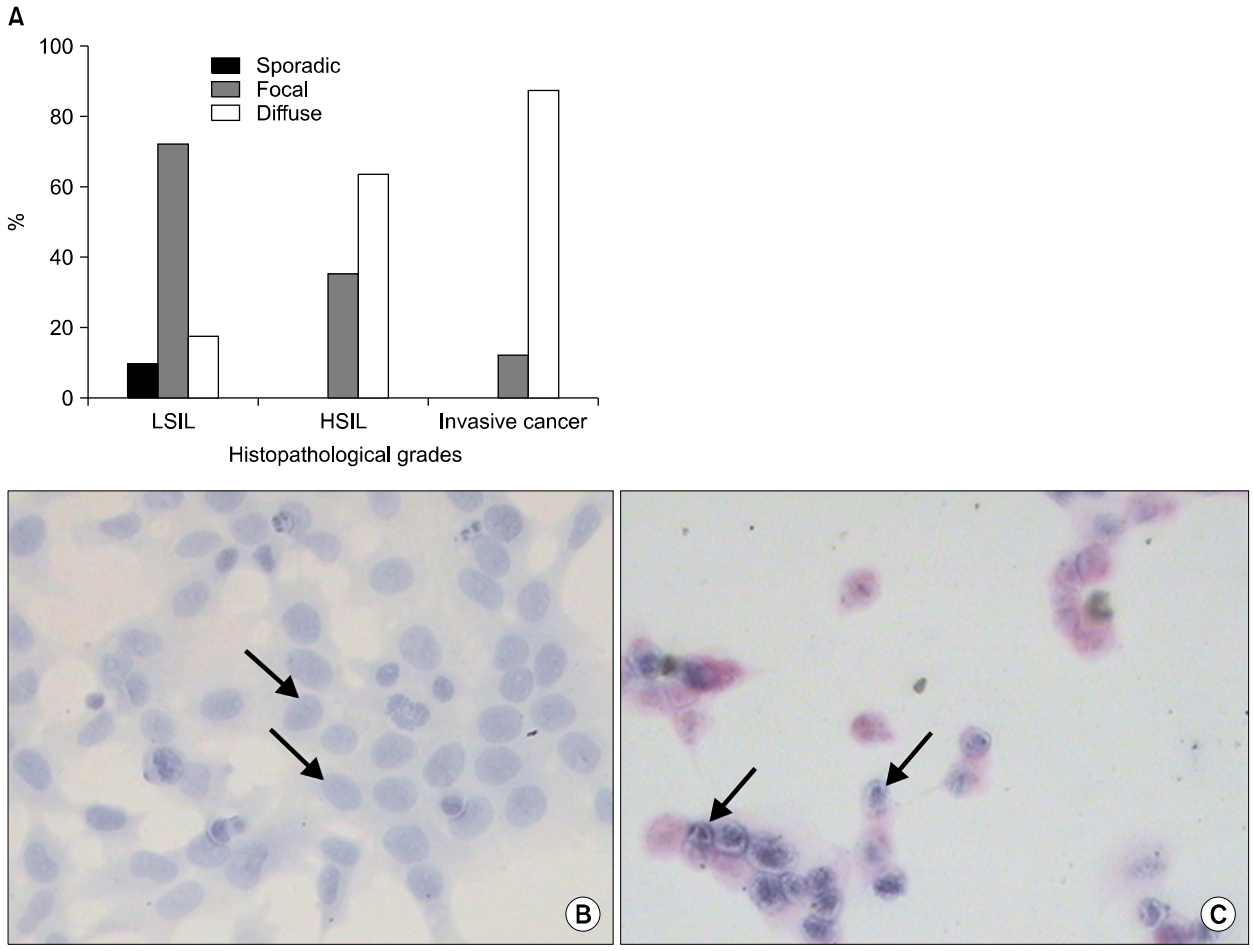

Figure 1. p16^{INK4a} protein expression in human papillomavirus (HPV). (A) p16^{INK4a} expression according to HPV types and histopathological grades. (B, C) Detection of high risk-HPV and low risk-HPV in HPV positive cases by in situ hybridation with catalyzed signal amplification (× 100). (B) HPV 6/11: diffuse signal (arrows). (C) HPV 16/18: punctuated signal (arrows). LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.

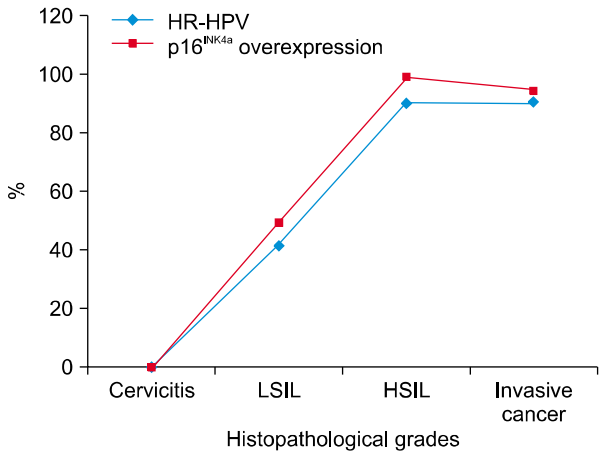

Figure 2. p16^{INK4a} expression and high risk (HR)-human papillomavirus (HPV) rates according to histopathological grades. LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.

ously reported among Moroccan women with normal and abnormal cytology (15.7%).[3] However, another study conducted by Khair et al.[11] on cancer biopsies of the cervix found a higher incidence rate 92% close to our study. Worldwide, HPV prevalence was very variable: in Africa 22.1%, Central America and Mexico 20.4%, Northern America 11.3%, Europe 8.1%, and Asia 8.0%.[12]

In our study, we found some convergence of HR-HPV in HSIL and invasive cancer. This result is consistent with those reported in Nigeria that showed the presence of 100% of HR-HPV in all samples with abnormal cytology.[13] In addition, HR-HPV detection was related to HSIL and invasive cancer among Chinese and Hungarian populations.[14,15]

In this report, HPV 16 and 18 were the most detected. This finding seems to be in line with previous data and highlighted that HPV16 and HPV18 are the most common types associated with cervical cancer worldwide.[16-22]

The $p16^{INK4a}$ protein overexpression was detected in 72.9%. In previous Moroccan study, the overexpression of $p16^{INK4a}$ was detected in 92.45% of cervical cancer specimens.[23] Our results were similar to those reported in many studies, where nearly 100% of high grade lesions and invasive cancers showed a strong overexpression of $p16^{INK4a}$ protein, while non-dysplastic lesions remained negative.[24,25] Interestingly, our finding highlighted a trend positive correlation between cervical lesions severity, $p16^{INK4a}$ expression, and HR-HPV prevalence. So these two markers are likely indicators of the severity of cervical lesions, and may be used in case of diagnostic's doubt particularly between LSIL and HSIL. In fact, many reports revealed $p16^{INK4a}$ overexpression by immunohistochemistry in a very high proportion of neoplasia intraepithelial high grade, 80% to 100% of cervical intraepithelial neoplasia (CIN)2 and almost all CIN3, and in nearly all invasive cervical cancers, with diffuse expression throughout the height of squamous epithelium.[26-29] Thus, these data indicate that $p16^{INK4a}$ protein expression can be used to improve the histopathological diagnosis of precancerous cervical lesions.[30]

Our findings highlighted a strong link between HR-HPV and HSIL and/or invasive cancers. Moreover, we found that $p16^{INK4a}$ protein is a promising marker for the early diagnosis of precancerous and cancerous lesions of the cervix. The results of this study provide an improved screening approach for the diagnosis of the risk of lesions progressing to cervical cancer.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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