Title: The utility of p16 [sup]INK4a and Ki-67 to identify high-grade squamous intraepithelial lesion in adolescents and young women
Author(s): Diane Cavalcante , Paulo Giraldo , Iara Linhares and Margarida Pompeu
Source: ***Indian Journal of Pathology and Microbiology.*** 55.3 (July-September 2012): p339.
Document Type: Report

http://www.ijpmonline.org/
Full Text:

Byline: Diane. Cavalcante, Jose. Eleuterio, Paulo. Giraldo, Iara. Linhares, Margarida. Pompeu

Background: The repair of the immature squamous epithelium following HPV infection may mimic HSIL in adolescent women. Aim: to study the utility of p16 [sup]INK4a and Ki-67 in diagnosis of cervical squamous lesions in adolescents and young adults. Materials and Methods: In a cross-sectional study, the evaluation of p16 [sup]INK4a and Ki-67 immunohistochemistry was performed on 72 cervical biopsies of adolescents and young adults women diagnosed as negative for malignancy and intraepithelial lesion (NML) (n = 18) or positive for low grade (LSIL) (n = 31) and high grade (HSIL) (n = 23) squamous intraepithelial lesions in two references services in Fortaleza-Brazil. Data was evaluated using Fisher's test and Kappa index. Results: p16 [sup]INK4a was positive in 81% of HSIL, 19% of LSIL and in no NML (P < 0.0001). Ki-67 was positive in 74%, 32% and 5.5% of HSIL, LSIL and NML, respectively. p16 [sup]INK4a and Ki-67 in the diagnosis of HSIL showed high sensitivity and negative predictive value. Kappa index was very good for p16 [sup]INK4a (k = 0.72). Conclusions: In adolescents and young adults p16 [sup]INK4a alone or with Ki-67 represents important tool to reduce mistaken diagnosis of HSIL and to avoid overtreatment.

Introduction

According to recent studies almost 80% of women will be exposed to human papillomavirus (HPV) by the age of 50. [sup][1] It has been estimated that about 600 million women worldwide and approximately 20 million women in the United States are infected with HPV. Almost half of these populations are between 15 and 24 years old. [sup][2] Factors influencing HPV transmission are well known. Age, age at sexual debut, number of sexual partners, age of the first partner and smoking have important roles. [sup][3],[4]

Among adolescents, the HPV infection appears to be related to immunological and morphological aspects of genital tract immaturity. The finding of cervical ectopy, very prevalent in adolescence, is strongly associated with an elevated susceptibility to infection. [sup][5] On the other hand, repair of the immature squamous epithelium following exposure to a viral infection may result in morphological changes that mimic a cervical high-grade squamous intraepithelial lesion (HSIL). This can result in a false positive diagnosis. However, true HSIL also occurs in young women. [sup][6]

Although some studies have demonstrated an increased incidence of HSIL in adolescents and young women, the majority of these lesions regress spontaneously. [sup][6],[7] This raises the question: are all of these lesions actually high-grade lesions

or more severe morphological manifestations of immature squamous epithelium due to HPV infection? [sup][8]

According to Tsoumpou et al., [sup][9] even the histological assessment of cervical biopsies that is often considered as the "gold standard" can be significantly hampered by intra- and interobserver variability. This problem was confirmed by others. [sup][10],[11] Especially in adolescents and young women this is a concern. Overtreatment of a false positive lesion can affect future reproductive success and also have emotional consequences, in addition to its economic impact on public health policy. [sup][7]

Identifying true HSIL in adolescents is a challenge that may be helped by the use of biomarkers. A marker of proliferation (Ki-67) has been shown to be a sensitive and specific marker of HPV infection in mature squamous epithelia. [sup][12] p16 [sup]INK4a , a sensitive marker of cells with active expression of E7 oncoprotein, has also shown high sensitivity and specificity to HSIL in adults women. [sup][13]

Interaction of the high risk HPV E7 gene product with pRb, results in the liberation of E2F, inactivation of pRb and stimulation of the S-phase of the cell cycle. This is strongly associated with p16 [sup]INK4a expression. The p16 [sup]INK4a accumulates in the cell when the Rb gene is altered. Conversely, this protein is markedly reduced or missing from clinical specimens that contain the intact Rb gene. In other words, p16 tends not to be expressed in either normal proliferative epithelium cells or inflammatory lesions. [sup][13] Ki-67 is a proliferation-associated antigen, a nonhistone protein not specific for the cell cycle phase and usually expressed in the second or third parabasal layers and rarely in the basal layer of the cervical squamous epithelium. [sup][14]

The aim of this study was to identify the immunohistochemical profile of true high-grade lesions using p16 [sup]INK4a and Ki-67 in adolescent and young women and thus aid in prevention of false-positive diagnoses and misconduct.

Materials and Methods

The surgical pathology records in the Department of Pathology, Federal University of Ceara-Brazil, were searched from 2005 to 2009 to identify cervical biopsies in women ages 13-24 years, with either a negative diagnosis for malignancy and intraepithelial lesions (NML), low grade squamous intraepithelial lesions (LSIL) and HSIL. To be included in the study there had to be sufficient material to prepare slides for analysis and age between 13 and 18 (adolescents) and 19 and 24 (young adults), as defined by the Medical Subject Headings-MeSH/NIH.

Paraffin blocks of tissue were processed for 4-[micro]m sections and stained with hematoxylin and eosin for morphological diagnosis after concordance of a double-blind evaluation by two independent pathologists. The cases were classified as NML, LSIL encompassing cervical intraepithelial neoplasia grade 1 (CIN 1), and HSIL, encompassing cervical intraepithelial neoplasia grade 2 and 3 (CIN 2 and 3), as described by Kurman [sup][14] . Cases with either dissimilar diagnoses or with unsatisfactory material for evaluation were excluded from the study.

Immunohistochemical staining for p16 [sup]INK4a was performed using the CINtec[sup][R] histology kit (MTM Laboratories, Heidelberg, Germany) on 4-[micro]m sections of formalin-fixed, paraffin-embedded specimens on slides with 10% poly-L-lysine (Sigma, USA). As a final step, the slides were stained with a light hematoxylin counterstain. Squamous cell carcinoma cells were used as a positive control for p16 [sup]INK4a .

Cervical biopsies were scored for p16 immunostaining according to criteria described by Eleuterio et al. [sup][13] are:

I- negative: <1% of cytoplasmic and nuclear positivity.

II- sporadic: 1-10% of cytoplasmic and nuclear positivity.

III- moderate: 10-30% of intense cytoplasmic and nuclear positivity, in areas of epithelium.

IV- diffuse: >30% of intense cytoplasmic and nuclear positivity, in areas of epithelium.

To achieve a more uniform interpretation of p16 [sup]INK4a immunostaining, slides were classified into only two categories (p16 positive vs. p16 negative), considering positive only those samples with moderate and diffuse staining.

Ki-67 antigen was identified using the mouse monoclonal antibody MIB1 (Dakocytomation, CA, USA) at a dilution of 1:200, after heat-induced antigen retrieval. Strong nuclear staining for Ki-67 was scored as positive when it extended beyond the lower one-third of the epithelium. [sup][15],[16]

The performance of the immunohistochemical tests for p16 [sup]INK4a and Ki-67, in the detection of squamous intraepithelial lesions was evaluated by means of conventional contingency tables to calculate sensitivity, specificity, positive and negative predictive values. Kappa (k) index was used to determine the agreement of the markers with histological diagnosis.

This research project was approved by the Ethics and Research Committee at the Faculty of Medicine of Federal University of Ceara (UFC)-Brazil (protocol 201/10).

Results

The expression of p16 [sup]INK4a was studied in 72 cases (NML = 18; LSIL = 23; HSIL = 31). According to previously defined criteria (13) 80.9% (20/23) of women with HSIL and 19.4% (6/31) of women with LSIL were positive. All (18/18) NML samples were negative for p16 [sup]INK4a [Table 1] [Figure 1]. Considering only HSIL, the expression of p16 [sup]INK4a demonstrated a sensitivity of 86.9%, a specificity of 87.7% and positive and negative predictive values of 76.6% and 93.4%, respectively. The Kappa index was 0.72 [Table 2].{Figure 1}{Table 1}{Table 2}

Ki-67 was positive in 74% (17/23) of women with HSIL, 32% with LSIL (10/31) and 5.5% (1/18) of the NML cases [Table 1] [Figure 1]. For HSIL this marker had a

sensitivity of 74%, specificity of 77.7%, positive and negative predictive values of 60.7% and 86.3%, respectively. The Kappa index was 0.49 [Table 2].

Evaluating the expression of the two markers simultaneously, in HSIL the sensitivity was 86.9%, the specificity was 77.5% and positive and negative predictive values were 64.5% and 92.7% respectively. The Kappa was 0.59 [Table 2].

Discussion

Adolescents and young women have high rates of HPV infection. Its persistence can lead to development of premalignant lesions and cervical cancer. Fortunately, the diagnosis of HSILs is uncommon (0.12-3%) in this age group. [sup][6]

Discordance on histological diagnosis of cervical cancer precursor lesions have been documented in several studies, suggesting a need to identify biological markers that could help the pathologist make a correct diagnosis in equivocal lesions. [sup][10],[17],[18] In adolescents and young adult women atypical immature metaplasia of the cervix, an immature metaplastic epithelium with mild cytological atypia but with strong reaction phenomenon, sometimes, mimics the morphology of a high-grade lesion. [sup][12],[18] Therefore, an "HSIL"-diagnosed lesion in this age group could in reality be a false-positive. Testing for p16 [sup]INK4a expression appears to be a good addition to more accurately diagnose HSIL. [sup][19]

In the present study, p16 [sup]INK4a was positive in 81% of HSIL lesions and in 19% of LSIL lesions in adolescents and young adult women. All NML cases were negative for p16 [sup]INK4a . This is in accordance with the findings of other investigations in adult and adolescent women. [sup][13],[20],[21] Eleuterio et al. , [sup][13] evaluating p16 [sup]INK4a biopsies from Brazilian adult women, observed positive staining in 15% of LSIL cases, 93% of HSIL cases and in none of the negative cases. Similar findings were observed in Russian women by Volgareva et al. [sup][22] Hu et al. [sup][20] evaluating an adolescent group observed basal and diffuse expression of p16 [sup]INK4a in the epithelium in 74% of women with HSIL, 9% with LSIL and 0% in women with cervicitis/normal cervix.

Ki-67 is a cell proliferation marker demonstrated in some studies to aid in the diagnosis of HSIL. [sup][18],[21],[23] However, reactive and reparative epithelial phenomena are also associated with positivity for this marker. In the present study Ki-67 had significant greater expression in HSIL (74%) than LSIL (32%) and NML (5.5%) ($P < 0.0001$).

Keating et al. [sup][23] observed that despite the expression of Ki-67 in the upper third of the epithelium being a strong indicator of HSIL, it was less reliable for LSIL, especially in immature condylomas. In other situations, such as immature metaplasia and an inflammatory/reactive process, positive nuclei can be seen in the upper layers of the epithelium. Although Ki-67 may be useful in the diagnosis of squamous intraepithelial lesions by histology, its use as a potential marker is problematic because it is expressed in all proliferating cells and is, therefore, not a specific marker for cervical neoplasia. [sup][18]

The performance of both markers, independently and together, to diagnosis of HSIL showed a high sensitivity (p16 [sup]INK4a = 87%, Ki-67 = 74% e p16 [sup]INK4a /Ki-

67 = 87%), high specificity (p16 [sup]INK4a = 88%, Ki-67 = 78% e p16 [sup]INK4a /Ki-67 = 77%) and high negative predictive value (p16 [sup]INK4a = 93%, Ki-67 = 86% e p16 [sup]INK4a /Ki-67 = 93%). The agreement index (Kappa) was good for Ki-67 and for both markers simultaneously, but best for p16 [sup]INK4a alone.Evaluating p16 [sup]INK4a as biomarkers in adult women Eleuterio et al. [sup][13] observed similar high values of sensitivity, specificity and predictive values, besides an excellent agreement index. Srivastava [sup][24] had similar findings in adult women showed that p16 and MIB-1 markers in tissue sections can be used as an adjunct to definitively diagnose preneoplastic and neoplastic lesions in the cervix. Others authors evaluated the performance of p16 [sup]INK4a without stratification of squamous intraepithelial lesions (SIL) and observed high sensitivity and specificity, but very low negative predictive value. [sup][25],[26] This reinforces the importance of utilizing p16 [sup]INK4a to identify HSIL, but not LSIL.

The association between the expression of Ki-67 with HSIL but with a lower performance than p16 [sup]INK4a was also observed by other authors. [sup][19] The use of both markers did not increase the association with a diagnosis of HSIL, when compared to p16 [sup]INK4a alone in the present study.

Much remains to be studied on the usefulness of diagnostic markers, particularly p16 [sup]INK4a and Ki-67, especially in adolescent and young adult women. Nevertheless, our results suggest that given the strong association of the two markers, especially p16 [sup]INK4a , with high-grade lesions, its use represents a potentially important tool for the pathologist, reducing equivocal diagnosis of suspect lesions, especially among adolescents and young adult women and avoiding the overtreatment.

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