Co-expression of p16/ki-67 in Associated with Human Papilloma Virus Type 16

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The current trend in screening against cervical cancer is to improve the sensitivity of screening method and p16/Ki-67 dual-stained cytology, represents a promising approach. We performed a study to investigate the correlation between the p16/Ki-67 co-expression and HPV type on a group of patients with ASCUS and LSIL on Pap smear. On all patients, dual stain test and HPV genotyping were performed. In the group of patients with positive dual stain p16/ki-67 test, the number of patients with HPV type 16 was significantly higher than the number of patients with any other type of HPV. Also, all patients with CIN3 or above (cervical cancer) were positive for p16/Ki-67. Our study shows the potential use of those two tests to identify patients with high risk for severe dysplasia.

Keywords: HPV; immunocytochemistry; cervical cancer; p16

HPV is a DNA virus belonging to the family Papillomaviridae. There are over 150 genotypes of which 40 infect the squamous epithelium of the anogenital tract. Even though there is a variation between the most prevalent HPV types around the world, the most common types involved in cervical lesions are types 16 and 18, responsible for 60–80% of all cervical cancers.

The evolution of HPV-related disease is initiated with an acute infection followed by most frequently, a subclinical infection, or sometimes, a clinically evident infection. Subclinical infections usually resolve without symptoms with the virus being eradicated or suppressed to non-detectable levels. The development of clinically evident infection may produce either low-grade disease or the development of high-grade disease, a consequence of persistent HPV infection.

Recent data contradict what was originally believed that the HPV induced lesions have a natural progression from CIN I to CIN III or even cervical cancer and now it is a known fact that most of the low-grade lesions regress and most of the high-grade lesions progress. [1] Most untreated cases of LSIL regress within 2 years, as assessed by one or two normal Pap smears [2] whereas high-grade CIN has lower rates of spontaneous clearance (30–40%), and much higher rates of progression to cancer without treatment (> 12%) [3].

Different factors including ongoing HPV exposure, HPV genotype, the molecular variant of that genotype, the HPV viral load as well as the immune status of the host have been recognized as promotors of the infection progression. [4]

The carcinogenic activity of HPV is mediated through two viral oncoproteins, E6 and E7. The E6 and E7 proteins have the ability to bind host cell regulatory proteins causing anomalies in their function. These cellular dysregulations could be evidenced clinically by immunohistochemically study of some proteins, such as, p16 and Ki67 [5]. Detection of overexpression of these biomarkers has been shown to be an efficient tool in managing patients with atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL) cytology results, and for triaging HPV-positive women. [6]

Based on this background we aimed to investigate the correlation between the co-expression o immuno-cytochemistry of p16/ki-67 and the HPV type.

Experimental part

Materials and Methods

We performed a follow up study including all patients aged between 18 and 65 years with atypical squamous cells of undetermined significance(ASCUS) and low grade squamous intraepithelial lesions (LSIL) referred for colposcopy and biopsy to the department of gynecology of the county hospital Timisoara between January and December 2016. Inclusion criteria were ASCUS or LSIL on Pap smear, dual stain test P16/ki-67 positive and colposcopic findings that required biopsy or conization. Exclusion criteria: pregnancy, previous surgery of the uterine cervix, negative dual stain p16/ki-67 test, normal Pap smear. For each patient included in the study HPV genotyping was performed.

Conventional Papanicolau cytology was performed and evaluated according to the criteria of Bethesda 2001. All patients were evaluated by colposcopy and International Federation for Cervical Pathology and Colposcopy (IFCPC) criteria were used. All patients were evaluated by colposcopy and International Federation for Cervical Pathology and Colposcopy (IFCPC) criteria were used. All colposcopic examinations were performed by the same team, with expertise in colposcopy. Patients with colposcopic suspect lesions were referred for biopsy. The HPV genotyping and immunocytochemistry was performed on all patients with indication for biopsy before the procedure. All samples were examined using LINEAR ARRAY HPV Genotyping Test (CE-IVD), based on reverse hybridization of amplicons. The DNA of 37 HPV types (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, 81, 82, 83, 84, IS39 and CP6108) was detected in cervical samples by multiplex PCR targeted to the conserved L1 region of

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the viral genome. The Gene Amp PCR System 9700 was used for genotyping test according to the manufacturer's instructions. Automated hybridization and detection of HPV DNA was done on the ProfiBlot 48 (Tecan Trading AG, Zurich, Switzerland). Immunocytochemistry analysis was performed using the CINtec PLUS Cytology kit (Roche MTM Laboratories, Heidelberg, Germany) according to the manufacturer's instructions. The kit contains a ready-to-use primary antibody combination: a mouse mono-clonal antibody (clone E6H4) directed to p16 protein and a rabbit monoclonal antibody (clone 274-11 AC3) directed against Ki-67 protein. One section for each selected case was stained with p16/Ki-67 dual test. Ki-67 expression within the nucleus was marked with red chromogen and p16 cytoplasmic expression was marked with brown chromogen. Each sample was considered positive when both markers were observed within the same cells (Figure 1a). Cases without any double-immunoreactive cell were considered negative. The specimens for HPV genotyping and dual stain test were obtained using the cervical brush and deposited in Sure Path collection vials. Informed consent was obtained from every patient prior to their inclusion in the study. All procedures have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and were approved by the Institutional Review Board and Ethical Committee of Victor Babes University of Medicine and Pharmacy Timisoara.

For the statistical significance of our study we computed a chi square test.

Results and discussions

A total of 116 with ASCUS and LSIL on Pap smear were referred for colposcopy to our service. 54 patients presented colposcopic lesions with indication for biopsy and were included in our study. The dual stain test and HPV genotyping were performed on all 54 patient's prior biopsy. 42 (77.8%) patients had positive dual stain test. 53 (98.1%) presented HPV infection. HPV type 16 was found in 23 patients (54.76%), 8 patients had HPV 18 (19.04%), 3 patients had HPV 52 (7.14%), 2 patients had HPV 33 (4.76%), 2 patients had HPV 16 combined with HPV 33 and HPV 56 (4.76%), 2 patients had HPV 18 combined with HPV 52 (4.76%), 1 patient had HPV 18 combined with HPV 33 and HPV 52 (2.38%) and 1 patient had HPV 33 combined with HPV 35 (2.38%) (table 1).

The pathological exam found in our group 35 mild dysplasia (CIN1), 15 cases moderate dysplasia (CIN2) 3 cases of severe dysplasia (CIN 3) and one case of in situ carcinoma. The patients with CIN3 and carcinoma in situ were all found positive using the dual stain test, and all 4 cases presented infection with HPV type 16. The number of patients with HPV type 16 was significantly higher than the number of patients with any other type of HPV, in the group of patients with positive dual stain p16/ki-67 test ($p<0.001$) (table 2).

Development of cervical intraepithelial neoplasia (CIN) and cervical cancer is almost always preceded by a persistent oncogenic HPV infection [7]. The risk of developing CIN 3 is 14 times higher for women who have had at least three positive tests for high risk HPV compared with women who have had negative tests [8].

In the last years different factors have been identified in association with an increased risk of HPV infection persistence. Infections with high-risk HPV types are more persistent than those with low-risk types, and among those, HPV 16, is more likely to persist than any other HPV type [9]. Besides being the most frequent in most of the studies, at rates ranging from 8 to 63%, Campion et al. found this type to be present in 85% of the lesions that progressed. [10] Also, Wheeler et al. noted that among women with LSIL infected with genotype 16, the cumulative risk for HSIL was 50% over a two-year period. [11] Same results were observed after LEEP excision [12].

Persistence was also associated with multiple coinfections. According to Rousseau et al. the presence of more than one HPV type, either low or high risk, at the same time or at different times (sequential infections) may increase the HPV persistence rate, by increasing the oncogenic viral potential. [13] Infections with multiple HPV types may occur at rates ranging from 2.2 to 63% [14].

One of the most studied is the age of the women and is a subject of controversy. Some studies found a higher proportion of persistent infection at older ages (> 35 years) [15-17], results also observed in several studies regarding persistence of infection after excisional treatment [18, 19]. On the other hand, Grainge et al. and Rijkaart et al. found no correlation [20, 21].

In the literature, several other factors influencing persistence have been cited including: use of oral contraceptives, glucocorticoids, both by suppressing the immune response to infection and hormonal therapy, which has the opposite effect, lowering the risk [22].

Table 1
THE COMPUTED RISK ANALYSIS FOR DETERMINING IF HPV 16 CAN BE CONSIDERED A RISK FACTOR FOR TESTING POSITIVE AT P16/KI67

Exposure / Test	p16/ki67 present	p16/ki67 absent	Total	Results
HPV 16 present	25	4	29	p>0.001 RR+1.92; RR ∈ (1.32; 2.82) OR=7.72; OR ∈ (2.24; 26.52)
HPV 16 absent	17	21	38	
Total	42	25	67	

HPV types	Number of patients	Percentages
HPV 16	23 patients	54.76%
HPV 18	8 patients	19.04%
HPV 52	3 patients	7.14%
HPV 33	2 patients	4.76%
HPV 16 + HPV 33 + HPV 56	2 patients	4.76%
HPV 18 + HPV 52	2 patients	4.76%
HPV 18 + HPV 33 + HPV 52	1 patients	2.38%
HPV 33 + HPV 35	1 patients	2.38%

Table 2
THE DISTRIBUTION OF HPV TYPES

Given the evidence available, knowledge about HPV persistence or HPV infections that are likely to resolve spontaneously, is essential for a rational HPV testing into cervical cancer screening programs.

Regular cervical screening has had a significant impact on the incidence and mortality associated with cervical cancer. Approximately 92% of women diagnosed with, and treated for, early-stage invasive cervical cancer survive 5 years. [23] Although cervical screening has significantly decreased the mortality associated with HPV infection, the sensitivity of the Pap smear is generally less than many believe it to be. The current trend in screening against cervical cancer is to improve the sensitivity of screening with new methods and to propose new algorithms for diagnostic and therapeutic decisions.

In recent decades, great advances have been made in understanding the molecular biology of HPV, and the importance of HPV genotyping. In 2012, American Cancer Society guidelines for the early detection of cervical cancer began including HPV DNA testing as a method to be used in conjunction with cytology [24]. This recommended use of HPV DNA testing has been incorporated into current clinical practice. Cotesting with the Pap and HPV tests is a first-line cervical cancer screening method, and it is recommended that women aged 30 to 65 years have these tests performed every 5 years [25]. The importance of HPV type 16 in the development of cervical cancer has been underlined by many studies. Type 16 has the lowest clearance rate after cone excision and is associated with disease recurrence [13, 18].

Immunohistochemistry is used for accurate assessment of gynecological malignancies [26-28] Recently, p16/Ki-67 dual-stained cytology has emerged as an interesting candidate of a biomarker for cell-cycle deregulation. The simultaneous detection of the overexpression of the p16-protein, which under normal physiological conditions induces cell cycle arrest in the course of cellular differentiation, and the expression of the proliferation marker Ki-67 within the same cervical epithelial cell points to HPV-induced deregulation of the cell cycle. [29] The high sensitivity and high specificity of dual-stained cytology testing in women with ASC-US and LSIL were observed in multiple studies and confirmed in a large, prospective, multicenter cervical cancer screening trial conducted in 5 countries (PALMS) [30] Regarding the detection of high-grade CIN The p16/Ki-67 dual-stained cytology positivity rates were comparable with the prevalence of abnormal Pap cytology results and less than 50% of the positivity rates observed for HPV testing. In women of all ages, dual-stained cytology was more sensitive than Pap cytology (86.7% vs 68.5%; $P < .001$) for detecting CIN2+, with comparable specificity (95.2% vs 95.4%; $P = .15$). The relative performance of the tests was similar in both groups of women: younger than age 30 and 30 years or older. HPV testing in women 30 years or older was more sensitive than dual stained cytology (93.3% vs 84.7%; $P = .03$) but less specific (93.0% vs 96.2%; $P < .001$). [6]

The importance of our study is that it shows a correlation between the infection with HPV type 16 and the co-expression of p16/ki-67. The fact that all patients with severe dysplasia were picked up by HPV genotyping and dual stain test opens a window of opportunity for the use of those two tests in order to identify the patients with risk for severe dysplasia before referral to colposcopy. Further studies including larger numbers of patients are needed to validate our conclusion.

Conclusions

In our study the presence of infection with HPV type 16 is correlated with a positive dual stain p16/ki-67 test. Since all cases with severe dysplasia were positive at the dual stain test and presented infection with HPV type 16, patients positive at the dual stain test and presenting infection with HPV type 16 should be included in a high risk group.

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