The role of the morphologic categorization of p16INK4a/Ki-67 dual stained cytology in detecting of high grade cervical intraepithelial neoplasia (CIN2+)

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Abstract

Background: The aim of the study is to analyze the morphological characteristics of p16INK4a/Ki-67 dual stained cells (DS) in cytology, to expand understanding of potential diagnostic value of integration of biomarkers in cytology and contribute to the existing knowledge on their utility in cervical cancer screening.

Materials and Methods: In our study included a total 162 woman, who had previous abnormal Pap screening results and were enrolled in an opportunistic screening program. For these participants 162 pap /p16INK4a/Ki-67 co- tests results and 29 histopathology results were available.

Results: In our study, sensitivity, specificity, PPV, NPV and accuracy of p16INK4a/Ki-67 DS of cervical smear, with abnormal morphology of stained cells to detect histologic high-grade cervical intraepithelial neoplasia (CIN2+) were 91%, 94%, 93%, 94% and 93%, respectively($p<0.01$). There occur 1 error.

Conclusions: Our study reveals that p16INK4a/Ki-67 DS cytology is superior in detecting CIN2+ to compare pap test, but morphologic categorization of p16INK4a/Ki-67 DS cytology is not superior over morphologic non-categorization of DS cytology in detecting high grade precancerous lesion during cervical cancer screening. **(TCM-GMJ December 2023; 8 (2):P25-P31)**

Keywords: p16INK4a/Ki-67 dual immunostaining (DS); CIN2+(CIN2 and CIN3) High-grade cervical Intraepithelial neoplasia; ASC-US; LSIL; HSIL; HR-HPV.

Introduction

Effective tool for timely detection of precancerous lesions may significantly reduce cancer mortality as well not necessary intervention with its adverse effect. Cervical cancer is fourth most common cancer among woman globally, with an estimated 604 000 new cases and 342 000 death in 2020, among them 90% of the new cases and death occurred in low- and middle-income countries.[1]

The existence of precursor lesions for invasive cervical cancer has been recognized for over a century.[2] Almost all carcinomas of the uterine cervix are derived from precancerous lesions or intraepithelial neoplasm (CIN),[3,4] but minority of woman with CIN develop cervical cancer.[5]

American Cancer Society (ACS) and European International Agency for Research on Cancer (IARC) approved three primary screening approaches for woman between 21-65 years old: pap test, HR-HPV DNA test and the co-testing (pap test plus HPV test).[6,7] Despite implementation of population-based pap test and HPV test in most developing countries, still cervical carcinoma is one of the common cancer of females throughout the world (WHO, 2019) and leading causes of death in many developing countries.[8]

For over 20 years, it has been evident that, high risk human papillomavirus (HR-HPV) cause almost all squamous cell carcinomas of the cervix as well as the vast majority of adenocarcinomas of the cervix.[2] It has been estimated, that at least half of all sexually active individuals will acquire HPV at some point in their lives, whereas at least 80% of women will acquire an HPV infection by age 50.9 Despite high prevalence, most HPV infections are transient10 and only small number of infected individuals develop disease in their life period.[11] Still much remains to be studied regarding the precise molecular pathways by which HPV produce tumors.

According to different studies pap test has low sensitivity. Not all persistent HR-HPV infections lead to cancer and regression of high grade cervical intraepithelial lesions also may develop.[12] According to many studies existent cytological and HPV screening recognizes mostly transient cervical lesions, investigation and treatment of which do not benefit the patient,[13] rather not necessary invasive diagnostics and excisional treatments may increase risk anxiety and stress on young woman, premature rapture of membranes and preterm delivery.[14-16]Furthermore, longevity of reproductive years and repeated recruitment of female with abnormal cytologic results, back into screening program, may affect logistics and financial resources especially in low income countries.

As Cytology based screening has weakness in terms

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of subjectivity and HPV-based testing identifies presence of infection and not presence of disease, introduction of integrated cytology marker concept may be beginning of improvement of cervical cancer screening strategy. Recently, studies on various biomarkers have confirmed their great importance in terms of diagnostics and personalized treatment. Their role is especially promising in the case of precancerous lesions and oncological diseases.

Several sufficient biomarkers have been proposed, among which p16INK4a and Ki-67 proteins were much extensively studied in different studies. Petry et al (2011) first proposed the concept of p16INK4a/Ki-67 dual staining cytology and its role in cervical cancer screening[17]. Since then, according to many published papers, based on cervical cancer screening data from different countries, there is significant improvement of sensitivity in to detect CIN2+ based on p16INK4a and Ki67 biomarker expression in cervical cytology.[18,19]

A number of studies have been conducted by different authors on the characteristics of p16INK4a/Ki-67 DS in cervical smear, the subject of our study was the correlation of double staining and morphology in detecting CIN 2+ in cervical cancer screening.

Pap test based Cervical cancer screening program implemented since 2008 with full coverage of country of Georgia since 2011. Since 2022 screening become population-based, and HPV test is added.20 HPV vaccination implemented since 2017. However, coverage with screening, moreover with vaccination is still lower. There are few peer reviewed scientific papers on cervical cancer screening, but recently have been published the first scientific paper on diagnostic performance on p16INK4a/Ki-67 dual immunostaining in Georgia.[21]

The aim of the study was to evaluate morphologic features of p16INK4a/Ki-67 dual stained cells of cervical smear in detecting high grade cervical intraepithelial neoplasia (CIN2+) and determine possibility of cytology and biomarker integration feature in cervical cancer screening.

Materials and methods

The study materials and staining methods are described in our previous article, were we analyzed diagnostic performance of p16INK4a/Ki-67 DS in detecting CIN2+, without considering morphology of stained cells.[21] In our current study, we specifically focus on evaluating the morphological features of p16INK4a/Ki-67 DS in cytology in the same study group. By examining the morphological characteristics of dual stained cells, we aim to expand our understanding potential diagnostic value of immunocytochemistry and contribute to the existing knowledge on their utility in cytologic screening.

Immunostaining Interpretations

All p16INK4a/Ki-67 DS cytology slides were stained and reviewed by trained cytotechnologist. Under light microscopic examination, presence of more than one cervical epithelial cells on the slide, irrespective to cell morphology, with a brown cytoplasmic and a red nuclear staining was categorized as a positive p16INK4a/Ki-67 DS (Fig. 1). Cases without double-immunostaining categorized as negative(Fig.3 and 4).

All stained slides after cytotechnologist review were referred to three independent pathologist. All immunocytostained cytology slides was assessed regarding morphology. According to the existing criteria, adopted for the morphological assessment,[2] cell with nuclear size ≤1/3 of the size of the whole cell was considered as a cell without atypia and cell with nuclear size >1/3 the size of the whole cell was considered as an atypical.

With considering together immunostaining results and morphology of epithelial cells, we made following categories of cases (Table N3; Figure 1-4)):

1) p16INK4a/Ki-67 dual staining ategory, stained cells with or without atypia
2) Only p16Inka staining category, stained cells with or without atypia
3) Only Ki-67 staining category, stained cells with or without atypia
4) p16INK4a stained cells with or without atypia in p16INK4a and Ki-67 staining category
5) Ki-67 stained cells with or without atypia in p16INK4a and Ki-67 staining category
6) No staining at all category, cells with or without atypia

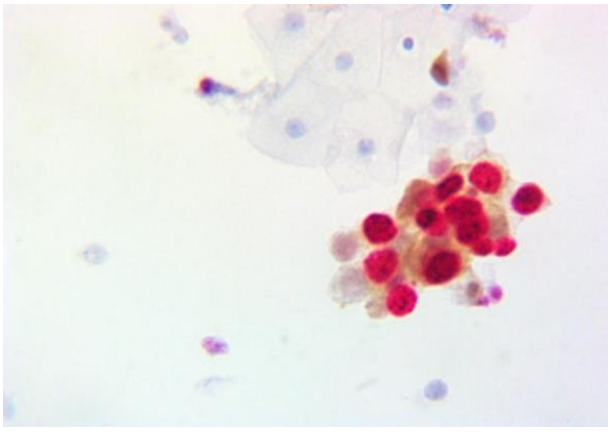

Figure 1: Dual p16INK4a/Ki-67 immunostaining positive cells with atypia

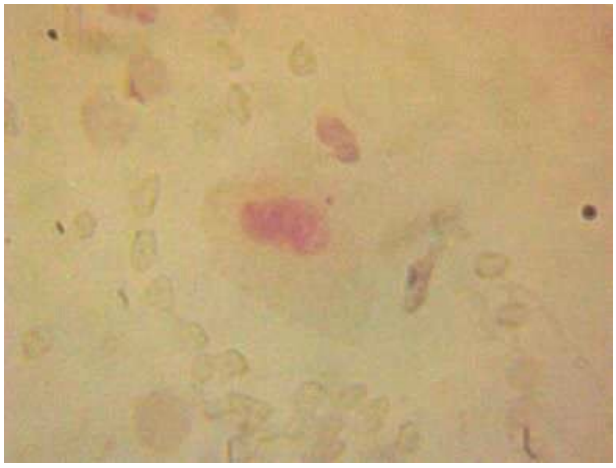

Figure 2: Dual p16INK4a/Ki-67 immunostaining positive cells without atypia

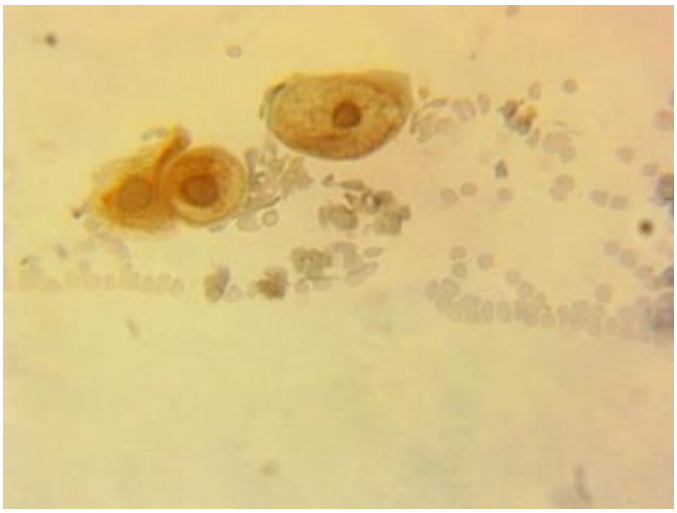

Figure 3: p16INK4a positive cells with/ without atypia during dual p16INK4a/Ki-67 immunostaining

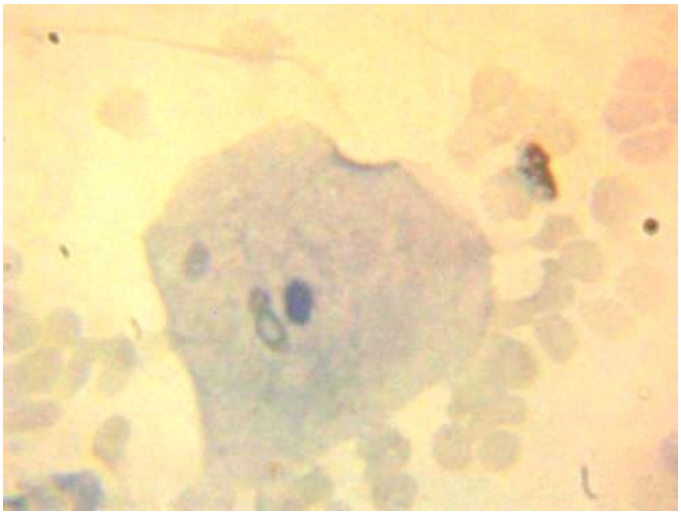

Figure 4: Cell without staining during dual p16INK4a/Ki-67 immunostaining

Statistical analysis

All collected data were entered into the database and underwent statistical analysis. The data were analyzed with the program SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). $\chi 2$ test or Fisher exact test was used when it was appropriate for comparisons between categorical variables. The accuracy of clinical performance of p16INK4a/Ki-67 DS results with considering morphologic features of stained cells for the diagnosis of CIN2+ was evaluated as sensitivity, specificity, positive predictive value, negative predictive value (NPV) and accuracy, considering histomorphology as a gold standard.

Results

Out of 162 immunocytochemistry stained cases 9,8% (16) was p16INK4a/Ki-67 DS positive (Table N 1.), out of which cellular atypia of stained cells were 87,5% (14) (Table N3). Out of all 162 pap test, 80,9% (131) cases were with different epithelial cell abnormality, according The Bethesda System: ASC-US was 27(20,6%); ASC-USH 5(3,8%); LSIL 93 (70,9 %); HSIL 6 (4,6 %) (Table N1);

Out of 29 histopathology results 11 were CIN2+(two equivocal CIN2/CIN3); 5 case were CIN1; one case was equivocal for CIN1/metaplasia; 5 case was Chronic lymphocytic Cervicitis; We did not have histological results for all cytological smears. Out of 16 with positive double p16INK4a/Ki-67 staining cytology, 11 women had histologic CIN2+, and one woman had histologic CIN1; All Histologic CIN2+cases had p16/Ki67 DS positive cytology results. No other histologic results were detected with p16INK4a/Ki-67 DS positive cytology results. In all positive cases with dual p16INK4a/Ki-67 immunocytostaining, with histologic result CIN2+, stained cells showed cellular atypia, except for one CIN2 case, where p16INK4a/Ki-67 DS was positive without atypia. One dual p16INK4a/Ki-67 positive result without atypia was identified in one woman with a histologic CIN1 result (Table N2).

CIN2+ was not detected in any of the immunocytochemical cytological categories, except for the DS categories, of which 87% were atypical cells. Staining categories and morphological features of DS cytology are given in the Table N3.

In our study, out of all women sensitivity, specificity, PPV, NPV and accuracy of dual p16INK4a/Ki-67 positive immunocytostaining with atypia of stained cells, to detect histologic CIN2 + lesion, considering histology as the gold standard were 91%, 94%, 91% and 93% ($p=5,75\times10- 6<<0.01$); Results of statistical analysis are given in the Table N4. and Table N5. Morphologic finding in positively stained cells revealed improved specificity, but same accuracy and decreased sensitivity to compare with DS stained cell irrespective to their morphology; There occur Type 1 error. Statistical results of p16INK4a/Ki-67 DS without considering morphology in detecting CIN2+ was considered in our previous study (Table N6).[21]

Interpretative variability was not observed between cytotechnologists and pathologists in the assessment of p16INK4a/Ki-67 biomarker expression, although there was no consensus between cytotechnologists and pathologists in the assessment of morphological features of the staining cells.

Discussion

Our study includes analysis of p16INK4a/Ki-67 DS in detecting of CIN2+, with considering morphologic features of stained cells in cytology. Analysis of p16INK4a/Ki-67 DS in detecting of CIN2+, irrespective to morphology of stained cells in the same study group was recently published in our previous study.[21]

Our study revealed that, the sensitivity, specificity, PPV, NPV and accuracy of p16INK4a/Ki-67 DS with

morphologic atypia of stained cells to detect CIN2 + lesion, considering histology as the gold standard, were 91%, 94%, 91% 94% and 93%, respectively (p=5.75×10-6<<0.0001). The sensitivity, specificity, PPV, NPV and accuracy of p16INK4a/Ki-67 DS regardless of morphologc categorization of stained cells were 100%, 89%, 85%,100% and 93% respectively (p=2,5×10-6<<0.01) in our previous study, within the same study group.21 Our previous study also revealed sensitivity, specificity and accuracy of conventional pap test to detect CIN2+ lesions was 9%, 100%,85% and 64% respectively(p=0,6)[21]. Schmidt et al reported in their study that p16INK4a/Ki- 67 immunostaining has a high sensitivity for detecting CIN2+ irrespective to morphology of the stained cells.[22] According Allia et al. and Prevodnik et al. double p16INK4a/Ki-67 immunostaining cells with morphologic atypia have a higher accuracy in detecting high-grade dysplasia than staining irrespective to morphology.[23,24]Nkwabong et al reported in their study, that sensitivity, specificity, positive PPV and NPV values of Pap test were 55.5%, 75%, 88.2% and 33.3%, respectively.[25]

Our study revealed improved specificity of morphologic categorization of p16INK4a/Ki-67 DS cytology in detecting of CIN2+, but lowered sensitivity and the same accuracy, to compare morphologic non-categorization of p16INK4a/Ki-67 DS cytology. This suggests that, the inclusion of morphological evaluation in addition to p16INK4a/Ki-67 DS can help improve identifying true positive cases. However, sensitivity of p16INK4a/Ki-67 DS, when considering morphology of stained cells was lower and accuracy was the same compared to p16INK4a/Ki-67 DS without considering morphology. There has occurred Type 1 error. While high specificity can help minimize false-positive results, it is crucial to maintain an acceptable level of sensitivity to avoid missing true positive cases. Therefore, based on our results, dual p16INK4a/Ki-67 immunostaining is superior in detecting CIN2+ among LSIL/ASCU-US category of pap test result, irrespective of morphology of stained cells. It may be related to the cellular biochemical changes that precede to the morphological alterations, that can be detected in exfoliated cells in the smear of the cervix. Based on our study, prevalnece of H&E confirmed histopathologic SIL was 55%, out of which CIN2+ was 37.9%; CIN1 was 17.2%; From remaining category CIN1/metaplasia equivocal 3.4%; Prevalence chronic lymphocytic cervicitis 17.2% and normal histology was 24.1%; Pap test result distribution based on our previous study was following: NILM 31(19.1%); ASC-US 27 (16.7%); ASC-USH 5 (3.1%); LSIL 93 (57.4%); HSIL 6 (3.7%).[21] Pap test results vary among different studies, For ASC-US it varies between 4,3%-40%, for ASC-USH 2-20.9%; for LSIL 2%-22%; for HSIL 0,5%-15,6%.[26–28] In our study LSIL result was most prevalent result. Machalek DA, Poynten IM, Jin F, et al in their study revealed that, the prevalence of cytologically predicted high- grade SIL (HSIL, 17.9%) was lower than histologically diagnosed HSIL (31.7%, P < 0.001)[29].

In or study 9.8% was double p16INK4a/Ki-67 positive staining results, however Pap test based cytologic abnormality (SIL, ASC) rate was 80.9%; In our study, 12% of all women with abnormal pap test showed positive results with double p16INK4a/Ki-67 immunocytostaining; This results is low compared to the study, in which 67% of all women with epithelial abnormality had positive dual staining.30 The weakness related to the sample size and possible selection bias, as well as the strengths of our study is detailed in the previous our article.21 TBS Categories of pap test results such as ASC-US and LSIL are challenging for women and for doctors as well. In our study performance of p16INK4a/Ki-67 DS of cervical smear shows, that it can improve triage of woman within LSIL and ASC category, that can improve detection of underling cervical high-grade dysplasia. New findings in molecular biology may have positive impact regarding decreasing unnecessary colposcopy referral or "find and treat" action for females involving in cervical cancer screening. Further, the results from large sized, randomized sample are interesting, as well the long-term prognosis of dual p16INK4a/Ki-67 positive and negative cytology results and relationship between the expression of p16INK4a/Ki-67 biomarkers and the age of a woman. Morphology based cytology results have played a important role in cancer screening, although once sufficient results will accumulated, in the knowledge of molecular biology and integration of biomarkers in cytology, it may result to improve cancer screening outcomes.

Data Availability

The authors confirm that all data underling the results are available as part of the article and no additional source of data are required.

Ethical approval

Ethical approval of the study was obtained on March 2011 from David Tvildiani Medical University (DTMU) Committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

	Dual p16INK4a/Ki-67 positive with atypia	Dual p16INK4a/Ki-67 positive without atypia
NILM 31		
ASC-US 27	1	
ASC-USH 5	2	
LSIL 93	9	2
HSIL 6	2	
Total 162	Total 14	Total 2

Table 1. Distribution of the Pap test and p16INKA4/Ki-67 DS results:

	Dual p16INK4a/Ki-67 immunocyto-chemistry positive with atypia	Dual p16INK4a/Ki-67 immuno-cytochemistry positive without atypia
N		
CIN1; 6 case		1
CIN1/Metaplasia; 1 case		0
CIN2; 2 case	1	1
CIN3; 8 case	8	0
CIN2/3; 2 case	2	0
Chronic lymphocytic Cervici-tis; 5 case	0	0

Table N2. Distribution of H&E histopathology and p16INKA4/Ki-67 DS results:

	With atypia	Without atypia	Categories re-garding stain-ing	Total
p16INK4a/Ki-67dual staining	14(88%)	2	16 (6,8%)	16(9,8%)
p16INK4a stained cells in p16INK4a and Ki67 staining category (without dual staining)	11 (18%)	50	61(26,18%)	
Only p16INK4a staining	4 (26,7%)	11	15 (6.43%)	
Ki-67 stained cells in p16INK4a and Ki-67 staining cate-gory (without dual staining)	10 (17,2%)	48	58 (24,9%)	
Only Ki67 staining	3 (23,7%)	10	13 (5.6%)	76(46,9%)
No staining at all	4 (5.7%)	46	70 (30.4%)	70(43,2%)
Total	46	167		162

Table N3. Categories of cases based on immunostaining results and morphology of epithelial cells:

		Positive p16INKA4/Ki-67 DS		Total
		Without atypia	With atypia	
CIN2+	No	17	1	18
	Yes	1	10	11
	Total	18	11	29

Table N5. Results of statistical analysis of p16INK4a/Ki-67 DS and CIN2+, considering morphology of DS A. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.17. B. Computed only for a 2x2 table

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi- Square	21.127[a]	1	4,29794267072079E-06		
Continui ty Correctio n[b]					
Likelihoo d Ratio	17,657	1	0,000026449651474 1014		
Fisher's Exact Test					
Linear- by- Linear Associati on	24,070	1	9,28985169077428E-07		
N of Valid Cases				0,00000575189559 6447	0,0000057 5189559 6447
	20,399	1	6,28746989236552E-06		
	29				

Table N6. Results of statistical analysis of P16INKA4/Ki-67 DS irrespective of morphology of stained cells and CIN2+[21] A. 1 cell (25.0%) have expected count less than 5. The minimum expected count is 4.93. B. Computed only for a 2x2 table

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1- sided)
Pearson Chi- Square	21.812a	1	3,00718842851831 E-06		
Continuity Correctionb	18,366	1	0,00001822820059 69676		
Likelihood Ratio	27,334	1	1,71203259956547 E-07		
Fisher's Exact Test				2,2545118418234 5E-06	2,2545118418 2345E-06
Linear-by- Linear Association	21,060	1	4,45163412822054 E-06		
N of Valid Cases	29				

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