Original paper

Clinical efficacy of p16/Ki-67 dual-stained cervical cytology in secondary prevention of cervical cancer

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Cervical cancer is the third most common malignant neoplasm in women worldwide. HPV infection is the necessary factor for the cancer to develop. HPV DNA can be integrated into the genome of squamous epithelium and cause transcription of the viral oncoproteins and development of invasive cancer within 15-20 years. We assessed ICC co-expression of p16/Ki-67 proteins in smears collected from the uterine cervix and the association between p16/Ki-67 co-expression and cytologic and histologic results. Samples were collected from 93 women using liquid based cytology (LBC). Two microscopic slides were prepared: for Papanicolaou staining and ICC staining. Biopsy samples were collected from 43 women. Diagnosis of CIN 2+ was the endpoint of the study. p16/Ki-67 positive cells were found in women with: 1) a cytology result of ASC-US (3.59%), LSIL (2.22%), ASC-H (21.92%), HSIL (33.18%), SCC (72.22%) or NILM (3.44%); 2) a histopathologic result of CIN 1 (2.13%), CIN 2 (19.93%), CIN 3 (23.22%), SCC (69.72%) or normal histology (7.58%). p16/Ki-67 dual staining can increase the efficiency of screening methods and indicate women in whom further diagnostic procedures are required or those with extremely low risk of cancer. Sparing protocols will have a significant role in women of reproductive age.

Key words: cervical cancer, pap smear, cytology, p16/Ki-67, dual-stained cytology, HPV.

Introduction

Cervical cancer is the third most common malignant neoplasm in women worldwide [1]. A breakthrough in early diagnostics of cervical cancer was the discovery of human papilloma virus (HPV) [2]. Depending on the genotype of the virus we can distinguish low risk HPV types (LR HPV) and high risk HPV (HR HPV) types (16, 18, 31, 33, 41, 45, 51, 56). Most infections are acute and regress within 2 years [3]. In a persistent infection HR HPV DNA is integrated into the genome of the host squamous epithelial cells [4], which causes the transcription of the viral oncoproteins and development of intraepithelial neoplasia, and after 15-20 years invasive cancer. Persistent infections do not regress and the only treatment method is surgical removal of infected tissues. Since the 1980s in developed countries no decrease in mortality of cervical cancer has been observed, which proves that secondary prevention tools used now

(mainly the pap smear) are limited. There are trials in progress that aim to determine which alternative methods of cervical cancer screening are most useful, e.g. detection of HPV DNA as well as mRNA or dual-staining immunocytochemical (ICC) methods detecting p16 and Ki-67 protein co-expression [5].

As an effect of HPV integration into the host cell genome uncontrolled expression of E6 and E7 oncoproteins occurs (Fig. 1). E6 protein binds p53 protein and causes its ubiquitin-dependant proteolysis [6, 7, 8], which leads to lack of DNA repair and apoptosis and induces cell proliferation. E7 protein binds the unphosphorylated pRB, leading to its proteolysis, causing E2F dissociation and initiating DNA transcription and progression to S phase [7, 8, 9]. This also removes the suppressive effect on the p16INK4A gene promoter and p16 overexpression. Ki-67 is a marker of cell proliferation and is found in G1, S, G2 and M phases of the cell cycle; it is not found in G0 phase. This protein is responsible for ribosomal RNA transcription. p16/Ki-67 co-expression allows identification of cells in which the cell cycle has been disturbed as an effect of HR HPV infection.

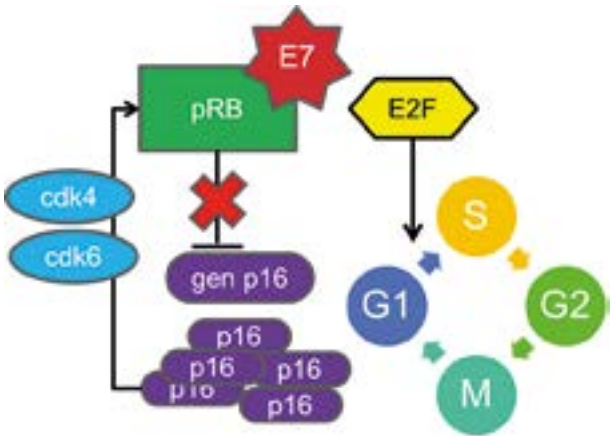

Fig. 1. Molecular basis of HPV infection.
HR HPV infection → HPV genome is built into host DNA → overexpression of E7 HPV and its binding to pRB → released E2F factor initiates S phase; no pRB suppressive effect on p16 gene promoter

Aim of the study

The aim of the study was to assess ICC co-expression of p16/Ki-67 proteins in smears collected from the uterine cervix and the association between co-expression of p16/Ki-67 and the cytological and histological results as well as efficacy of ICC methods for detecting precancerous lesions in the uterine cervix.

Material and methods

The samples were collected from 93 women aged 16-64 using the liquid based cytology (LBC) Thinprep Pap Test (Hologic Inc. Marlborough, USA) with the standard technique for collecting exfoliative cervical cytology. Patients attending the outpatient clinic of the Department of Gynecological Surgery and Gynecological Oncology of Adults and Adolescents PUM because of either a prior abnormal cytology result or without history of cervical dysplasia were enrolled. Pregnant patients and patients with diagnosed or with a history of cervical cancer were excluded from the study. Each patient gave informed consent to participate in the study. Microscopic slides were prepared using the Thinprep 2000 System (Hologic Inc. Marlborough, USA) according to the instructions of the manufacturer. The characteristics of the studied group are shown in Table I. From this material two microscopic slides were prepared using Thinprep 2000 Processor (Hologic Inc. Marlborough, USA). The first slide was stained by the Papanicolaou method for cytological examination according to the Bethesda system, and the second was stained by ICC for detection of co-expression of p16/Ki-67 proteins. FOR the ICC staining a Cintec plus kit (Roche Diagnostics, Basel, Switzerland) was used according to the instructions of the manufacturer. The kit consists of primary anti-$p16^{INK4A}$ mouse antibodies (clone E6H4), and monoclonal rabbit anti-Ki-67 antibodies (clone 274-11 AC3). DAB chromogen causes brown staining of the cytoplasm in presence of p16 overexpression, and

Table I. Clinical cytologic and histologic data in the studied group (n = 93)

Parameter		Number of women
Age (n = 93)	< 30 years	36
	30-64 years	57
	Minimal	16
	Maximal	64
	mean age	33,9
	Median age	31
Cytologic diagnosis (n = 93)	NILM*	34
	ASC-US	19
	LSIL	22
	ASC-H	7
	HSIL	9
	SCC	2
Histological diagnosis (n = 43)	Normal	17
	CIN 1	5
	CIN 2	10
	CIN 3	8
	SCC	3

**NILM – negative for intraepithelial lesions and malignancy*

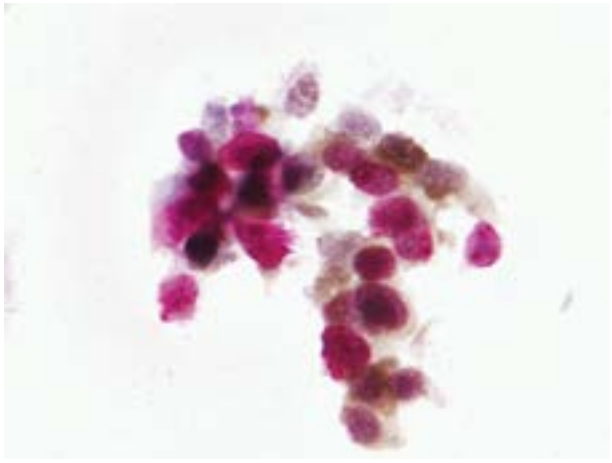

Fig. 2. p16/Ki-67 co-expression of in HSIL cells (p16/Ki-67 dual staining; magnification 400×). The presence of p16 is depicted by brown chromogen (DAB) in the cytoplasm and Ki-67 by red chromogen (Fast Red) in the cell nuclei

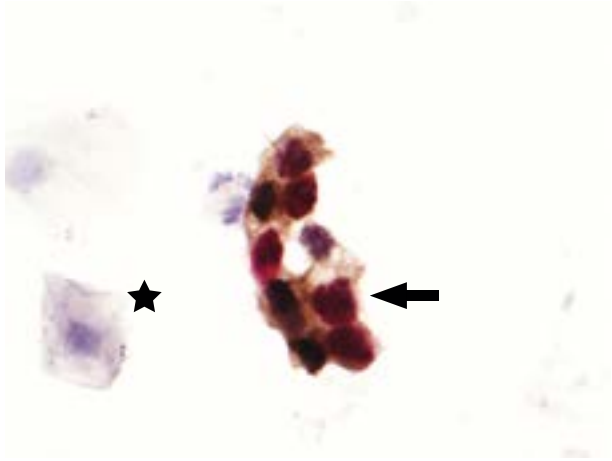

Fig. 3. p16/Ki-67 co-expression (→) in SCC cells. In a normal squamous cell, the p16/Ki-67 dual-staining is negative (*); (p16/Ki-67 dual staining; magnification 400×)

FastRed chromogen causes red staining of the cell nuclei in case of Ki-67 overexpression. The cytology result was considered positive if changes of ASC-US or higher were diagnosed. ICC staining was considered positive if at least 1 cell on the whole slide displayed a red nucleus (Ki-67) and brown cytoplasm (p16). Photographs of p16/Ki-67 dual-stained positive cells are shown in Figs. 2 and 3. Polish Society of Gynecologists and Obstetricians recommendations for assessing abnormal cytology results were used in deciding whether the patient required further diagnostic procedures, e.g. colposcopy, colposcopy guided biopsy or repeated cervical cytology. The ICC result was not taken into account in deciding what further diagnostic procedures would be performed. From 93 enrolled patients 55 underwent colposcopy and 43 had colposcopy-guided biopsy samples collected. Histological diagnosis of CIN 2, CIN 3 or SCC in biopsy samples was the clinical endpoint in this study. All cytological and histological analyses were performed in the Department of Pathology of Pomeranian Medical University. Cytological analysis was performed by a cytotechnician and approved by a pathomorphologist. Another pathomorphologist, blinded to the cytology result, assessed the p16/Ki-67 dual-stained cytology.

Statistical analysis was performed using Statistica 12 (StatSoft Inc.) (license no. JPZP-602C295824AR-V). Sensitivity, specificity, PPV and NPV for the whole studied group, patients aged < 30 years (36) and those aged 30-64 years (57) were calculated for cytology results, p16/Ki-67 dual-staining method, and combined cytology results +p16/Ki-67 dual-staining method approach. Statistical analyses were performed using a nonparametric Mann-Whitney U test. P values were determined, and $p < 0.05$ was considered to be statistically significant.

The Pomeranian Medical University bioethics committee agreed to this study being performed (KB-0012/66/12 decision date 28.05.2012).

Results

Cells with co-expression of p16/Ki-67 were found in women with:

- cytologic result of ASC-US (3.59%), LSIL (2.22%), ASC-H (21.92%), HSIL (33.18%), SCC (72.22%) as well as in those with NILM (3.44%) (Table II),
- histologic diagnosis of CIN 1 (2.13%), CIN 2 (19.93%), CIN 3 (23.22%), SCC (69.72%) as well as in those with normal histology (7.58%) (Table III).

There was a statistically significant association between the mean percentage of p16/Ki-67 (+) cells and the:

- cytologic results (ASC-US vs. ASC-H, ASC-US vs. HSIL, LSIL vs. ASC-H, LSIL vs. HSIL) (Fig. 4),
- histologic diagnosis (normal vs. CIN 2, normal vs. CIN 3, normal vs. SCC, CIN 1 vs. CIN 2, CIN 1 vs. CIN 3, CIN 1 vs. SCC) (Fig. 5).

In the whole studied group sensitivity (90%) was the same for ICC and cytologic examination in detection of CIN 2+ changes but the specificity was higher for ICC (63%) than for cytologic examination (44%). When the ICC was analyzed in the case of an abnormal cytology result the sensitivity was lower (86%) but with higher specificity (74%). In women aged < 30 years the sensitivity of such a protocol reached 80% with specificity of 70%. Efficacy for different methods is shown in Table IV.

Discussion

In this study, we found similar efficacy in detecting CIN 2+ changes for cytology samples collected

Table II. Correlation between different cytological results and CIN 2 histological diagnosis based on p16/Ki67 dual stain positive and negative cases (n = 93)

Cytologic result	Number of p16/Ki-67 (+) cases	CIN 2+ histopathological diagnosis	Number of p16/Ki-67 (–) cases	CIN 2+ histopathological diagnosis
NILM	9/34 (26.5%)	1	25/34 (73.5%)	1
ASC-US	10/19 (53%)	2	9/19 (47%)	0
LSIL	11/22 (50%)	2	11/22 (50%)	1
ASC-H	5/7 (71%)	4	2/7 2 (29%)	0
HSIL	9/9 (100%) 0	8	0/9 (0%)	0
SCC	2/2 (100%)	2	0/2 (0%)	0

Table III. Mean percentage of p16/Ki-67 (+) cells in relation to histologic diagnosis (n = 43)

Histologic diagnosis	p16/Ki-67 (+)	Mean percentage of p16/Ki67 (+) cells (%)
normal	9/17 (53%)	7,58
CIN 1	2/5 (40%)	2,13
CIN 2	9/10 (90%)	19,93
CIN 3	7/8 (87,5%)	23,22
SCC	3/3 (100%)	69,72

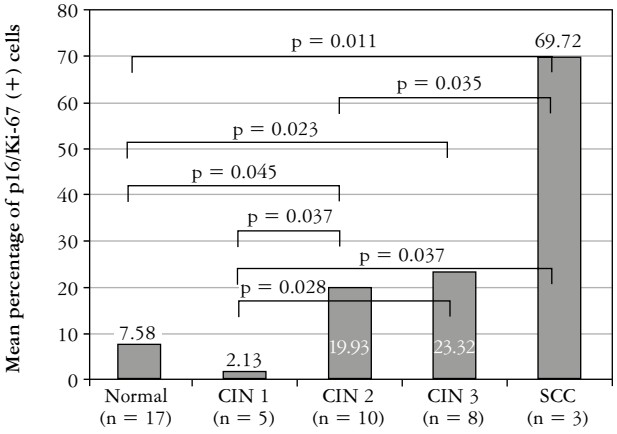

Fig. 5. Percentage of p16/Ki-67 (+) cells in relation to histologic result from the cervical biopsy (n = 43)

on a liquid medium and the p16/Ki-67 dual-staining method. Sensitivity reached 90% and specificity 44% for LBC and 63% for ICC methods. We have not found any data in international articles concerning combination of these two methods, as the sample collected from one patient can be used to create additional cytologic slides for ICC assessment. We have also calculated the efficacy for a joined protocol, where ICC result was taken into account only if there was an abnormal cytology result. As shown above, it achieves the best efficacy for women aged 30-64. In this group, the sensitivity reached 91% and specificity 76%. In the PALMS study [10], conducted on

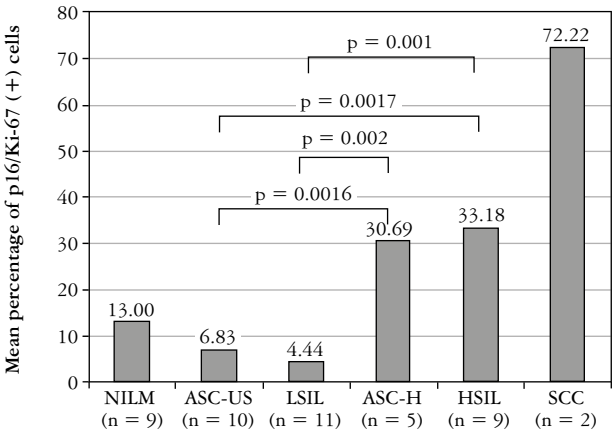

Fig. 4. Mean percentage of p16/Ki-67 (+) cells in relation to cytologic result in women with positive p16/Ki-67 dual staining (n = 46)

a group of 27 349 women, the p16/Ki-67 dual-staining method was compared with conventional cytology and LBC. Sensitivity was 63.5% and specificity 97.5% for conventional cytology. Higher sensitivity was observed for LBC and ICC methods (84.7% and 91.4% respectively), with similar specificity (95.0% and 94.8% respectively). The PALMS study divided women into age groups <30 years and 30+ years. In women < 30 years old HPV DNA presence was tested, but was not an indication for further diagnostics unless an abnormal cytology result was obtained. This is important, as HPV infections which can result in remission are very common at this age [11, 12]. In the PALMS study 20.2% of women aged < 30 years had a positive HPV DNA result, which means that dual-staining methods can be an alternative for diagnosing transforming HPV infection in this age group.

Discrepancies between results obtained in our study and other authors may originate from the fact that most authors include women who prior to dual-staining sampling have had an abnormal cytology result.

Fuji *et al*. [13] conducted their study on women with a prior ASC-US or LSIL result, who had cervi-

Table IV. Sensitivity, specificity, PPV, NPV of detecting CIN 2+ in different age groups

Parameter	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Women aged 16-64 years (n = 93)				
Cytology	90	44	32	94
ICC p16/Ki-67	90	63	41	96
Cytology + p16/Ki-67	86	74	49	95
Women aged < 30 years (n = 36)				
Cytology	78	48	33	87
ICC p16/Ki-67	89	52	38	93
Cytology + p16/Ki-67	80	70	50	91
Women aged 30-64 years (n = 57)				
Cytology	100	41	29	100
ICC p16/Ki-67	91	67	40	97
Cytology + p16/Ki-67	91	76	48	97

cal biopsy performed, which raised the specificity of their study. They reported 87.3% sensitivity of dual-staining methods, 76.4% specificity, PPV 45.7% and NPV 96.4%. Wentzensen *et al*. [14] included in their study women with ASC-US and LSIL cytology results, and used the dual-staining method later confirmed by histopathological result. He discovered presence of p16/Ki-67(+) cells in 26.8% of women with a normal histological result, 46.5% with CIN 1, 82.8% with CIN 2, 92.8% with CIN 3 and 100% with squamous cancer. Sensitivity for dual-staining methods was 85.8%, specificity 59.4%, PPV 48.4% and sensitivity 90.2%. Schmidt *et al*. [15] compared p16/Ki-67 dual staining with only p16 staining of cytology slides. With p16 staining sensitivity in diagnosing CIN 2+ changes rose from 77.9% (ASC-US) and 79.6% (LSIL) to 92.4% and 94.2% when p16/Ki-67 was applied. Specificity was 63.4% for ASC-US and 37.1% for LSIL when only p16 was assessed. It reached 80.6% and 68% with p16/Ki-67 dual staining. Interestingly, when only p16 was applied, cytotechnicians' assessment had higher sensitivity than that of pathologists, with a lower specificity. In another study Wentzensen *et al.* [16] examined repetitiveness of p16/Ki-67 assessment in cytology. 480 HPV DNA positive women were enrolled in this study and microscopic analysis was performed by cytotechnicians, senior cytotechnicians and pathologists. Each slide was assessed multiple times, to allow for easier statistical analysis. Personnel who performed ICC assessment had no prior experience with this method and underwent standard orientation training in this field. The authors confirmed the repetitiveness and high agreement ratio between personnel and referral diagnosis as well. The disease threshold was established at CIN 2 and sensitivity reached 82% with 63.9% specificity, with similar values for the newly trained staff and referral personnel. What can be questioned is the presence of p16/Ki-67(+) cells in women with an NILM result in cytology (13%) or histology (7.98%). Similar findings were reported by other authors. Negri *et al*. [17] studied the usefulness of p16 immunohistochemical (IHC) staining as a predictor of CIN 1 progression, and found that 71.4% of p16(–) and 37.8% of p16(+) CIN 1 cases regressed spontaneously. In 28.6% of p16(–) and 62.2% of p16(+) cases progression to CIN 3 was observed within 4 years.

P16 expression using IHC methods can be focal or diffused. Focal p16 expression is not considered as a positive result [11]; only diffused p16 staining is considered abnormal. Ki-67 is a protein that can also be found in benign cells, not only in precancerous and cancerous cells. When a pap smear is taken from the uterine cervix we can find cells from any layer of the epithelium, and the layer from which the cells originate is not taken into account when assessing p16/Ki-67 co-expression. In our study 26.5% of cases diagnosed as NILM revealed p16/Ki-67(+) cells.

In another study [16] the authors found p16/Ki-67(+) cells in 23.4% of women with no indications for cervical biopsy, 25.64% with a normal cytology result and 25.71% with CIN 1. This percentage rose to 80% in CIN 2 and 66.7% in CIN 3.

The main limitation of the study was the small number of enrolled patients. Another limitation was the lack of HPV DNA tests in the studied group. Tests for presence of HPV are not a routine screening tool used in Poland. We did not collect data on whether HPV DNA was positive in the enrolled group. We did not use p16 immunohistochemical (IHC) staining for biopsy samples according to ASCCP/CAP LAST. The Polish Society of Gynecologists and Obstetricians recommendations regarding

p16 IHC staining in cervical biopsy samples were released in December 2016.

The material collected during routine cervical cytology sampling can be used to acquire a LBC slide and HPV DNA test or a p16/Ki-67 dual-stained cytology slide. What is worth noting is the improved efficacy of p16/Ki-67 dual-stained cytology over HPV DNA tests observed in the PALMS study [10], as it helped reduce the number of false-positive screening test results by almost 50%, maintaining similar costs for both methods.

Conclusion

Immunocytochemistry methods detecting co-expression of p16/Ki-67 proteins can help improve cervical cancer screening by offering additional tools to stratify women with abnormal cytological results (ASC-US, LSIL). As most HPV infections causing low grade lesions in cytology results are transient, patients with negative p16/Ki-67 dual-stained cytology may be observed rather than qualified for colposcopy targeted cervical biopsy. These patients will not require repeated cytology and in consequence it can help reduce the number of false-positive cases thanks to increased specificity of p16/Ki-67 dual-stained cytology in comparison to LBC only. Women demonstrating co-expression of p16/Ki-67 and ASC-US or LSIL cytology results may be admitted for colposcopy without waiting for further diagnostics (e.g. repeated cytology after 6-12 months), thus reducing the time for diagnosis of precancerous lesions. Sparing protocols instead of invasive diagnostic procedures in women without co-expression of p16/Ki-67 in cytology results will have a significant role in women of reproductive age. Assessment of p16/Ki-67 cells can lead to increased efficiency of cervical cancer screening through higher sensitivity and specificity in detection of precancerous lesions at an early stage, also because the cost/ benefit ratio may be higher in comparison to HPV DNA or mRNA detection.

The authors declare no conflict of interest.

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