p16^{INK4a} immunocytochemistry in liquid-based cervical cytology: Is it feasible for clinical use?

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Abstract. A consecutive series of 118 samples from patients referred to colposcopy assessment and follow-up with cytology and biopsies were analysed with immunocytochemical staining to determinate the expression of p16^{INK4a}. Accumulation of p16^{INK4a} antigen has been proposed as a biomarker helpful for the identification of dysplastic cervical cells. In our study all benign cases were negative for p16^{INK4a}, while more than half of the high grade lesions showed moderate or strong reactivity. There was a correlation between CIN grade and p16 ^{INK4a} expression levels with more advanced lesions showing stronger reactivity. The correlation between p16^{INK4a} immunoreactivity and the severity of cytological abnormality was stronger, when the diagnosis was based on simultaneous routine cytology ($p<0.001$, χ^2 exact test for trend). There was no or weak reactivity in benign cases, as well as almost all low-grade lesions, while two thirds of high-grade lesions showed moderate or strong staining for p16 ^{INK4a} antigen. Thus p16^{INK4a} expression analysis yielded information which is consistent with results from the histopathology and is a simple way of emphasizing the presence of premalignant cell reactive atypias. This staining can be applied to cytological samples, and might be a complement prognostic procedure in order to find women at risk for cervical cancer.

Introduction

Preinvasive lesions of the cervix are frequent in young women, with a peak in incidence between the age of 25 and 40 years (1). Cytopathological screening for precancerous cervical lesions has achieved a considerable reduction in cervical cancer incidence and mortality in industrialized nations (2). Nevertheless, it remains the principle female cancer in developing countries, mainly due to lack of screening (3).

Human papillomavirus (HPV) of high-risk types have been identified as the causative agent in cervical cancers (4). The presence of HPV has however a low predictive value since most infections are transient and only a small fraction of women positive for oncogenic types of HPV develop cervical cancer (5-7).

In Sweden, a combination of organized and opportunistic screening has reduced the incidence of squamous carcinoma substantially during the last decades (1,8). All abnormal samples are followed-up with colposcopy, biopsy and ultimately conization. Both cytological and histopathological assessment rely on defined morphological criteria, but are also associated with intra- and interobserver variation (9-11), and could therefore benefit from complementary, more objective procedures.

Various screening strategies have been proposed in which HPV testing is combined with the cytological examination to increase sensitivity. HPV testing has shown satisfying sensitivity, but it lacks specificity to be used as a primary screening tool (12,13). The ideal would be an objective test distinguishing women with non-progressive mild neoplasia from those with oncogenic transformation and at risk of developing invasive cancer, to facilitate decision on therapy and need of follow-up.

A strong nuclear accumulation of p16^{INK4a} has been observed in cervical dysplastic lesions (14). In normal cells, p16^{INK4a} acts as a negative regulator of cell proliferation through a negative feedback loop to down regulate cyclin-dependent kinase 4 and 6. This will lead to an accumulation of the inactive pRB-E2F complex, and less free amount of the transcription factor E2F.

High-risk HPV 16 oncoproteins E6 and E7 interact with two cellular proteins that play key roles in the cell cycle, pRb (retinoblastoma protein) and p53 (15,16). While E6 increases the degradation of p53 tumor suppressor protein, E7 interacts with pRb resulting in reduced binding of E2F (17). As a

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consequence, E2F is kept in active form and promotes the G1/S-phase progression of the cell cycle. Simultaneously the p16^{INK4a} expression increases in order to effectuate cell cycle control, but is hampered by the effect of E7 on pRb. The resulting nuclear accumulation of p16^{INK4a} allows immunocytochemical demonstration, and has therefore a potential to be used as an adjunct biomarker and a measure of HPV oncogene activity (17,18). Immunostaining of cytological samples for p16^{INK4a} may thus allow more precise identification of CIN-related lesions. Interpretation of staining patterns can be difficult since there is diffuse staining in metaplastic cells and inflammatory reactive cells (19). There has also been logistic difficulty when using samples of insufficient quantity or quality.

In the present study, we wanted to evaluate the use of p16^{INK4a} immunocytochemistry on liquid-based samples counterstained with Papanicolaou stain, and assess the relation to cytological and histological diagnosis.

Materials and methods

We consecutively enrolled 118 women with any grade of cytological abnormality detected at population-based primary screening during 2004. The mean age of the women was 34.1 years (median 32 years, range 21-60 years). All women underwent a pelvic exam, cytological resampling, and colposcopy at the Gynaecological Department, Karolinska University Hospital Huddinge, Stockholm, 2-6 months after cytological screening diagnosis. From all women cells were obtained from the ecto- and endocervix with a spatula and a cervical brush and the cells were suspended in PreservCyt fixative (Cytyc® Corporation, Boxborough, MA, USA). The women underwent colposcopy and a Zeiss OMPI colposcope was used for magnification. The ectocervix and distal part of the endocervix were stained with 5% acetic acid. Punch biopsies were obtained from acetowhite areas. When no acetowhite area was observed, a biopsy at 12 o'clock was taken close to the squamo-columnar junction and fixed in buffered 4% formalin.

The cell suspension was subsequently prepared, using the ThinPrep® 2000 Processor (Cytyc Corporation). One slide was taken for routine Papanicolaou staining and cytological diagnosis, and another one for immunocytochemical demonstration of p16^{INK4a}. The biopsy material was embedded in paraffin, sectioned at 5 μm and stained with haematoxylin/eosin.

Slides taken for immunocytochemistry were post-fixed in acetone for 10 min at room temperature and air-dried for a minimum of 30 min. All slides were subject to 'Heat Induced Epitope Retrieval' (HIER), using the Epitope Retrieval Solution (Dako, Copenhagen, Denmark) at 95˚C-100˚C for 40 min. Staining for p16^{INK4a} was performed using the CINtec kit (Dako kit code K5339) in the DakoCytomation Autostainer in groups with five samples. In each staining group analysis one negative control on each sample and a positive control with strong reactivity were used.

Counterstaining after p16^{INK4a} staining was subsequently performed according to Papanicolaou in order to facilitate identification of transformed keratinocytes and to avoid counting false positive cells, i.e. metaplastic cells or inflammatory reactive cells. The cell material was considered sufficient if more than 3 reactive cells were present per slide. The p16^{INK4a} immunoreactivity was regarded as negative (-) if less than 3 reactive cells per slide were stained and categorized as increasingly positive (+, ++ and +++) depending on how intense the p16 reactivity was.

When the staining was completed, the manufacturer presented a supposedly more sensitive kit (Dako, cat. no. K5340). This kit was tested on 37 specimens with mild or moderate cellular lesions with previous weak or no immunoreactivity, and where the remaining cellular material sufficed for such a preparation.

The cytological and histological material was evaluated by different persons and independently of p16^{INK4a} analysis. Cytological diagnoses were defined according to the Bethesda 2001 system (20), and for statistical reasons grouped into WNL (within normal limits), ASCUS (atypical squamous cells - undetermined significance), low-grade lesions (LSIL) or high-grade lesions (HSIL) including ASC-H and cancer.

Histological samples were diagnosed according to WHO (ICD-10, Geneva 1990) and for statistical reasons grouped into benign (WNL), CIN I, and CIN II or worse. All patients gave their informed consent to participate and the study was approved by the Ethics Review Board at Karolinska Institute, Stockholm.

Table I. Intensity of p16^{INK4a} immunochemistry staining (in LBC) and histological diagnosis.

Intensity of p16^{INK4a} staining	Histological diagnosis			Total
	WNL	CIN I	CIN II+	
-	14 (74)	20 (77)	15 (25)	49
+	3 (16)	4 (15)	12 (20)	19
++	2 (11)	2 (7.7)	24[a] (39)	28
+++	0	0	10[b] (16)	10
Total	19	26	61	106

Data presented as number of cases (column percentage). [a]Two cases of adenocarcinoma *in situ*. [b]One case of squamous cell carcinoma. Spearman's rank correlation coefficient Rho 0.52, $p<0.001$. χ^2 exact test for trend, $p<0.001$.

Results

In 111 (94%) of the 118 cases the cell suspension sufficed for immunocytochemistry. Five of these cases were excluded because the tissue was insufficient for histological examination, leaving 106 cases with all three analyses performed.

Cytological findings. Thirteen (12%) of the 106 cases had benign findings in the cytological analysis, 40 (37%) cases had CIN I, 30 (28%) cases had CIN II, 14 cases CIN III and there were two cases of squamous cell carcinoma. Two cases were classified as ASCUS and three cases as ASC-H. Glandular atypia was rare with only one AGUS and one adenocarcinoma *in situ*.

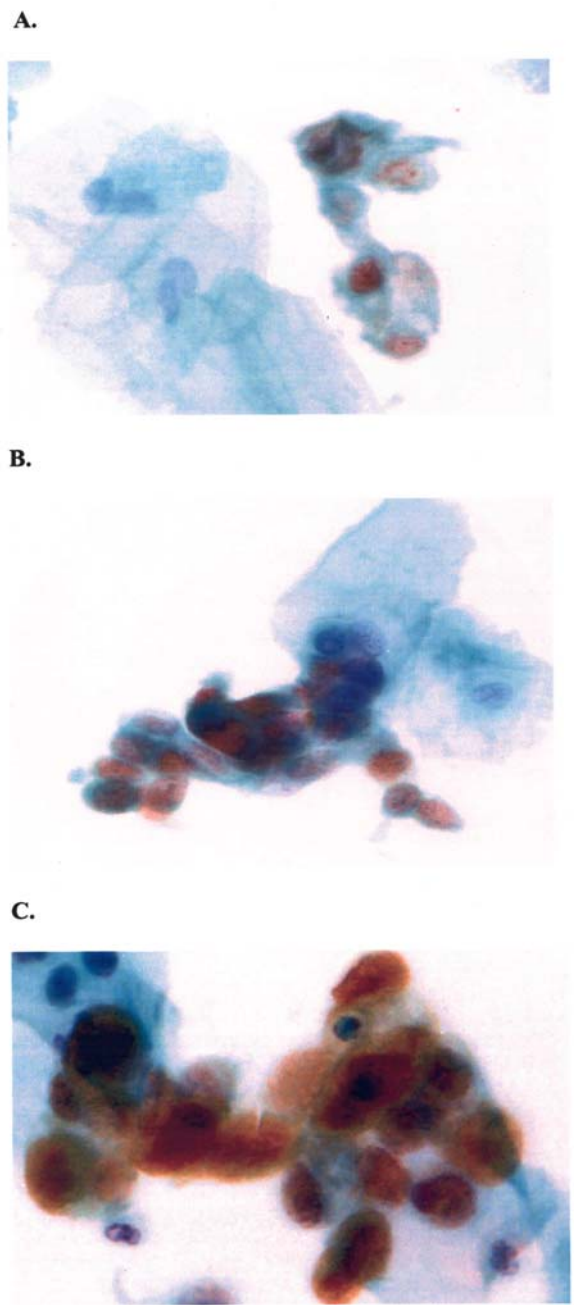

Figure 1. Immunocytochemical reactivity to $p16^{INK4a}$ in squamous cells with (A) CIN I, (B) CIN II, and (C) CIN III. The intensity of nuclear staining strongly correlates to the severity of the lesion.

Histological findings. In the histological assessment, one of the suspected squamous cell carcinomas was considered CIN II, and one ASC-H was considered as an adenocarcinoma *in situ*. In total 19 (18%) of the 106 cases were benign, 26 (25%) women had CIN I, 28 (26%) CIN II, 30 (28%) CIN III, one squamous cell carcinoma, and two were found to have adenocarcinoma *in situ*. These carcinomas are included in the group of high-grade lesions, CIN2+ (Table I).

$p16^{INK4a}$ expression in relation to CIN grade in histology. The immunocytochemical staining for $p16^{INK4a}$ was readily performed together with the conventional Papanicolaou as counterstain (Fig. 1). Out of 106 patients, 10 (9%) showed strong $p16^{INK4a}$ reactivity, 28 (26%) moderate, and 19 (18%) weak reactivity. In 49 samples reactivity was absent. Some of the benign cases showed weak reactivity. Of the CIN2+ 75% was identified by $p16^{INK4a}$ staining and more than half showed moderate or strong reactivity (Table I). The degree of reactivity to the $p16^{INK4a}$ antigen correlated to the severity of cervical intraepithelial neoplasia as determined histologically (Table I). The sensitivity of $p16^{INK4a}$ immunostaining to detect a histological diagnosis of CIN II or worse was 0.59 (95% CI. 0.49-0.69), the specificity 0.94 (95% CI 0.68-1.0), and positive predictive value 0.98 (95% CI 0.89-1.0).

Table II. Intensity of $p16^{INK4a}$ immunostaining in LBC (Dako kit code 5339) correlated to cytological diagnosis.

Intensity of $p16^{INK4a}$ staining	Cytological diagnosis				
	WNL	ASCUS	LSIL	HSIL	Total
-	12 (92)	2 (67)	30 (73)	5 (10)	49
+	1 (7.7)	1 (33)	7 (17)	10[a] (20)	19
++	0	0	4 (9.8)	24[b] (49)	28
+++	0	0	0	10[c] (20)	10
Total	13	3	41	49	106

Data are presented as number of cases (column percentage). [a]One case of suspected squamous cell carcinoma not confirmed in histology. [b]One case of adenocarcinoma *in situ*. [c]One case of squamous cell carcinoma. Spearman's rank correlation coefficient 0.70, $p<0.001$. χ^2 exact test for trend, $p<0.001$.

$p16^{INK4a}$ expression in relation to cytological abnormality. The correlation between $p16^{INK4a}$ immunoreactivity and the severity of cytological abnormality was stronger, when the diagnosis was based on simultaneous routine cytology (Table II). There was only one benign case with weak reactivity (7.7%). Some reactivity, usually weak, was seen in the low-grade lesions, while two thirds of high-grade lesions were moderately or strongly stained (Table II). The sensitivity of $p16^{INK4a}$ immunostaining to detect a high grade lesion on cytology was 0.60 (0.50-0.70), the specificity 1.0 (0.77-1.0) and positive predictive value 1.0 (0.92-1.0) using Dako test kit K5339.

Sensitivity of different test kits. When a supposedly more sensitive Dako test (kit K5340) was applied to a subset of 39 cytologically abnormal non-reactive cases, there was strong staining in 4 cases (Table III). Among women with high-grade lesions in histological biopsies, but normal cytology, 6 had sufficient material for $p16^{INK4a}$ analysis also with this latter kit. All of these samples were, however, negative also with this reagent, thus the improved reagents did not identify women with false negative reports on cytology. The sensitivity for Dako kit K5340 was 0.59 (0.42-0.75). The second generation Dako kit code K5340 showed no increased sensitivity compared to K5339 (Table IV, $p=0.116$, χ^2 exact test for trend).

Table III. Intensity of p16INK4a immunochemistry with improved Dako kit (K5340) in 39 cases of cytological abnormality, but weak or no staining with ordinary kit (K5339).

Intensity of p16INK4a staining	Cytological diagnosis			
	ASCUS	L-SIL	H-SIL	Total
-	3 (100)	10 (37)	1 (11)	14
+	0	12 (44)	3 (33)	15
++	0	2 (7.4)	4 (44)	6
+++	0	3 (11)	1 (11)	4
Total	3	27	9	39

Data presented as number of cases (column percentage). Spearman's rank correlation coefficient Rho 0.44, p=0.005.

Table IV. Correlation of immunocytochemical reactivity to p16INK4a in high grade lesions and other (Dako K5340).

Intensity of p16INK4a staining	Cytological diagnosis		
	High grade lesion	Other	Total
-	1	13	14
+	3	12	15
++	4	2	6
+++	1	3	4
Total	9	30	39

Data presented as number of cases. Spearman's rank correlation coefficient Rho 0.35, p=0.03. χ^2 exact test for trend, p=0.116.

Discussion

The implementation of the Papanicolaou test in the 1960s, and its use as a tool in primary health screening of cervical cancer, brought a revolution to the clinical management of this disease. Since then, the quality and reliability of the test has been gradually improved. Still the conventional screening procedure has a limited sensitivity, which necessitates narrower screening intervals for preventing the development of an invasive lesion, and the cytological smear still lacks objective measures, resulting in an inter-observer variability that may cause uncertainty for the clinical handling of these women. This is a particular problem at the borders between normal and abnormal, where the numerous reports of ASCUS or CIN I call for resource-demanding follow-up. New techniques in the form of liquid-based cytology and HPV testing are now awaiting its implementation in the established screening process, and their possible role in an organized cancer-preventing program is of substantial interest.

The presence or absence of high-risk HPV in smears with mild lesions may provide a basis for the decision if the observed lesion has a pre-neoplastic potential or not, i.e., if the woman should be offered gynecological follow-up, or if she should be retained in the ordinary screening program. With liquid-based cytology this ancillary testing can be performed without calling the patient for additional sampling (reflex screening). During such reflex screening one may directly demonstrate presence of the viral genome, show expression of the viral oncogenic E6 or E7 RNA, or trace proteins like p16INK4a, which are synthesized as a result of viral effects on host cell p53 or pRB. In a previous study (21), we demonstrated that viral expression of E7 RNA was not a sensitive marker for presence of high-risk HPV in patients with high-grade lesions. The accumulation of p16INK4a in the squamous cell nuclei is supposed to indicate a disturbed pRB function in these cells in association with the expression of the E7 gene from an oncogenic HPV. Information on such accumulation of p16 INK4a might then replace HPV analysis as a less resource-demanding marker for oncogenic potential. We noted that a quarter of the histological high-grade lesions completely lacked p16INK4a reactive cells. These may represent cases with false negative cytology, i.e., samples devoid of diagnostic cells, in which reflex testing of any sort would be useless.

With immunocytochemistry we can visualize the accumulation of p16INK4a in cells from the same suspension that has shown cellular abnormalities on routinely stained material. This means that we can avoid much of the sampling errors associated with repeated sampling, and the ancillary analysis becomes a possible tool to sharpen the primary cytological evaluation. We can also evaluate the nature of immunoreactive cells by counterstaining with Papanicolaou. This is supported by another study (22), confirming our notion that transformed keratinocytes stain in the nucleus, as opposed to cytoplasmatic staining seen in metaplastic and glandular cells in inflammatory conditions, unrelated to a precancerous process. Wentzensen *et al* have approached the difficulty of staining interpretation by proposing a nuclear scoring system to identify transformed cells (19,23). Papanicolaou counterstain could be less time consuming as it integrates cytological evaluation and immunocytochemistry.

The p16INK4a immunoreactivity correlated with the presence of dysplastic lesion. Expression of p16INK4a was mostly absent in normal cells, with strong reactivity confined to the highly neoplastic cells alone, in concurrence with previous studies (24,25). There was a statistically significant correlation between immunocytochemistry and the severity of the lesion, the intensity of p16 INK4a reactivity increasing with CIN grade on histology and cytology. Where p16INK4a immunostaining was negative in high-grade lesions, we assume that diagnostically important cells did not end up on the slides, perhaps as a result of a low proportion of abnormal cells in the cell suspension.

The vast majority of low-grade lesions showed no or only faint p16INK4a reactivity, which could mean that these cells were not expressing the protein at the time of sampling, due to regression of the lesion or due to being non-transformed. There is theoretical support that the level of p16INK4a immunoreactivity correlates with integration of virus DNA (26). Still, this does not exclude that the lesion contains high-risk HPV. In a previous study, by our group (21), only a third of high-risk HPV-containing samples showed distinct p16INK4a immunoreactivity. Ideally, presence of p16INK4a accumulation

demonstrates oncogenic transformation by HR-HPV, although absence of such immunoreactivity will not exclude an infection with long-term oncogenic potential.

One possible use for $p16^{INK4a}$ immunostaining in the context of reflex screening of minder cytological abnormalities could be as a first step detecting some potentially precancerous lesions, but leaving the negatives to be analyzed by other means such as the analysis of HPV DNA. Another desirable use for $p16^{INK4a}$ immunostaining would be to decrease the false negative rate, i.e., to detect precancerous cells that otherwise would have been left undetected with conventional staining. This is unfortunately not supported by the present study, where the 6 examined cases with high-grade lesions but simultaneous normal smear, all remained negative on $p16^{INK4a}$ immunocytochemistry. Yet, it is known that a majority of histological high-grade lesions will spontaneously resolve (27-29) and it is plausible that $p16^{INK4a}$-expression could actually be more specific of lesions with invasive potential.

The demonstration of $p16^{INK4a}$ accumulation in the cell nucleus is a simple way of emphasizing the presence of dysplastic cells. This staining may have a role as ancillary screening test, but the sensitivity is insufficient to replace molecular biology techniques demonstrating HPV DNA. These tests are preferably used in reflex screening, which requires liquid-based sampling.

In conclusion, the demonstration of $p16^{INK4a}$ accumulation in the cell nucleus is an efficient way of emphasizing the presence of dysplastic cells, and this staining can be applied to cytological samples, particularly when used on liquid-based samples. It is possible that such staining will not only improve our chances of detecting premalignant cells, but also aid in the distinction between premalignant and reactive atypias. Although vaccination might prevent up to 70% of cervical cancers worldwide, the need for screening is likely to remain.

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