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Short communication

Evaluation of p16^{INK4a} in Cervical Lesion of Premenopausal and Postmenopausal Women

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A B S T R A C T

Pap smears of postmenopausal women are often misdiagnosed because of the difficulty in distinguishing atrophic epithelial cells groups only by morphological criteria. In this study we investigated the diagnostic application of immunocytochemical staining of p16^{INK4a} on conventional Pap smear. A total of 137 cervical specimens were enrolled in this study, of which 77 and 60 cervical smears were taken from premenopausal and postmenopausal women, respectively. Two cervical smears were taken simultaneously in 68 women, one for conventional cytology and the other for immunostaining. Additional 69 cervical smears were taken from the archive, decolorized and then used for immunostaining. In premenopausal women 1 out of 14 (7.1%) with negative cytology, 7 out of 24 (29.2%) with low grade squamous intraepithelial lesion (LSIL), all 35 (100%) with high grade squamous intraepithelial lesion (HSIL) and all 4 (100%) with squamous cell carcinoma (confirmed by histopathology) had positive staining to p16^{INK4a}. In postmenopausal women p16^{INK4a} positivity was observed in 4 out of 7 (57.1%) cases of LSIL, 12 out of 14 (85.7%) cases of HSIL and all 4 out of 5 (80%) different cases of carcinoma (1 cervical adenosquamous carcinoma and 3 cervical squamous cell carcinoma in situ confirmed by histopathology), but none of 34 smears with normal cytology. Twenty smears with normal cytology chosen for the negative control in this study were from the group of postmenopausal women and were as expected negative for p16^{INK4a} immunostaining. In the group of postmenopausal women, 16 out of 60 (26.7%) cases the cytological diagnosis was established on the basis of p16^{INK4a} immunostaining as being HSIL. From our preliminary study on a limited number of samples, we can however conclude that p16^{INK4a} immunostaining is a very useful tool for cytological diagnosis enabling to distinguish HSIL from normal, reactive or inflammatory changes.

Key words: *postmenopausal, cervical, dysplasia, immunostaining, p16^{INK4a}*

Introduction

It is well established that persistant infection with oncogenic Human papillomavirus (HPV) causes preneoplastic and neoplastic changes of the cervical epithelium, leading towards carcinoma, especially in middle aged women[1]. In postmenopausal age, women have received little attention concerning this problem. Improvements in life standards and better health care have led to increasing population of elderly women who come to their regular gynaecological exam and their Papanicolaou (Pap) smears[2–3]. In spite of the fact that there is almost 20% of women infected with HPV[4], only a few of them develop preneoplastic lesion, and even fewer develop cancer. Smith and al.[5] found that 16% of persistent HPV infections exist in the elderly group of women, suggesting that one can predict development of a preneoplastic lesion that can progress toward cancer. However, problems in diagnosis such as aging-related changes, reparative changes and interpretation difficulties may lead towards an incorrect diagnosis. Pap smears of postmenopausal women are often misdiagnosed because of the difficulty in distinguishing atrophic epithelial cell groups only by the morphological criteria. Those criteria are more viable when premenopausal women are concerned, referred to better oestrogen effect, hence better maturation. In this study we investigated whether immunocytochemical staining of p16^{INK4a}, performed on conventional Pap smear

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could be of any diagnostic help in solving this dilemma. Thus, we performed p16^{INK4a} immunostaining on specimens taken from premenopausal and postmenopausal women in order to make an accurate diagnosis in those cases, which were impossible for us to distinguish on the basis of cytomorphological criteria, i.e. to distinguish a high grade cervical lesion from atrophy or reactive changes.

Examinees, Materials and the Methods

We analysed 137 cervical smears, which consisted in 69 archival Pap slides and 68 cervical specimens collected from women who attended our clinic in 2006 for their regular check-up. They were divided into two age groups. Seventy-seven premenopausal women were in group I (median age 34.5) and 60 postmenopausal women (median age 63) in group II, of which 20 women with normal cervical smears were used as a control group.

Two simultaneous cervical smears were taken by a cytobrush and a wooden spatula, one for conventional cytology and the other for immunostaining. Archival Pap slides were decolorized in 0.5% HCl, rinsed with distilled water and then immunocytochemically stained with CINtec™ p16^{INK4a} Kit (DAKOCytomation, Denmark) according to the manufacturer instructions. There were no differences in staining reaction or intensity of reaction between decolorized and freshly taken smears. According to DAKO instructions at least 1% of cells should be stained for the reaction to be considered positive. Cells are regarded as stained if brownish-like granules were found in nuclei and/or in a cytoplasm.

Pap smears were classified by the »Bethesda system«[6] into a normal epithelium, low (LSIL) and high grade intraepithelial cervical lesion (HSIL) and squamous cell carcinoma (SCC). Among postmenopausal women, there was one case of recurrent adenosquamous carcinoma and metastatic transitional cell carcinoma. In 16 postmenopausal women we could not diagnose based on the morphological criteria; in those women diagnosis was established after the p16^{INK4a} immunostaining.

In total, fifty-five women underwent colposcopy and biopsy, and a consequent cold knife conisation or hysterectomy was performed; in each case histological verification was made. Unfortunately, the patient with metastatic transitional cell carcinoma died.

The distribution of positivity rate versus diagnosis was analyzed with the standard Chi–square (χ^2) test. The statistical significant differences was set at $p<0.025$.

Results

The distribution of cytological diagnoses and p16^{INK4a} immunostaining in premenopausal women is as follows: 18.2% (14/77), 31.2% (24/77), 45.5% (35/77) and 5.2% (4/77) women had a negative cytology, LSIL, HSIL, SCC, respectively (Table 1). P16^{INK4a} immunostaining was observed in 7.1% (1/14), 29.2% (7/24) and 100% (35/35 and 4/4) of premenopausal women with negative cytology, LSIL, HSIL and SCC, respectively. The observed frequencies were significantly different ($\chi^2=52.24$, $p<0.0001$). The positive p16^{INK4a} immunostaining of one of the 4 SCC later histologically confirmed, and of normal metaplastic cells is shown in Figure 1 and 2. In this age group we succeed to diagnose all smears according to viable morphological signs and well established criteria for every grade of intraepithelial lesion.

TABLE 1
P16^{INK4a} FINDINGS ACCORDING TO DIAGNOSIS OF PREMENOPAUSAL WOMEN

p16^{INK4a}	N (%) of cytological diagnosis				
	Normal	LSIL	HSIL	Carci-noma	Total
Negative	13 (92.9)	17 (70.8)	0	0	30 (39)
Positive	1 (7.1)	7 (29.2)	35 (100)	4 (100)	44 (61)
Total	14 (18.2)	24 (31.2)	35 (45.5)	4 (5.2)	77 (100)

LSIL – low grade squamous intraepithelial lesion, HSIL – high grade squamous intraepithelial lesion, *cervical squamous cell carcinoma *in situ* confirmed by histopathology, $\chi^2 = 52.24$, $p < 0.0001$

The distribution of cytological diagnoses and p16^{INK4a} immunostaining in postmenopausal women is as follows: 52.3% (34/60), 10.8% (7/60), 21.5% (14/60) and 7.7% (5/60) women had a negative cytology, LSIL, HSIL, SCC, respectively (Table 2). P16^{INK4a} immunostaining was observed in 57.1% (4/7), 85.7% (12/14) and 80% (4/5) of postmenopausal women with LSIL, HSIL and SCC, respectively, while none with those with normal cytology. The observed frequencies were significantlly different ($\chi^2=40.97$, $p<0.0001$).

In 16 postmenopausal women we had difficulties for classifying a certain cell groups as neoplastic because of their resemblance to mucosal atrophy or because of the presence of strong degenerative, inflammatory or reactive changes. In 8 women, after p16^{INK4a} immunostaining we were able to distinguish HSIL from reactive changes (Figure 3). Among these women one case of previously diagnosed adenosquamous carcinoma, was p16^{INK4a} positive, indicating the possibility of recurrent carcinoma.

Discussion

In spite of good screening methods based on Pap smear and well organized screening programmes in many countries, women all around the world continue to develop cervical disease. The incidence rate of cervical cancer in developing countries is increasing due to the aging population, while in Western Europe first peak is between 30 and 40 years of age and the second peak occurs around 60 years of age[7]. Genital infection with oncogenic or high-risk (hr) HPV is established as one of the main events in pathogenesis of cervical and other genital tumours[1]. After the first contact with the virus,

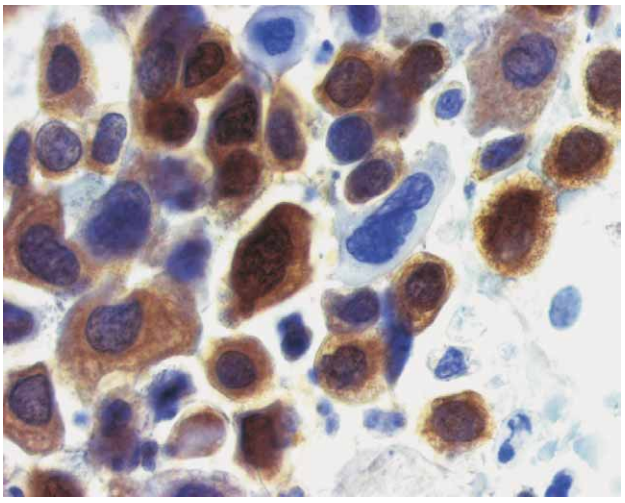

Fig 1. p16^{INK4a} staining of malignant squamous cells found in a smear of a premenopausal woman

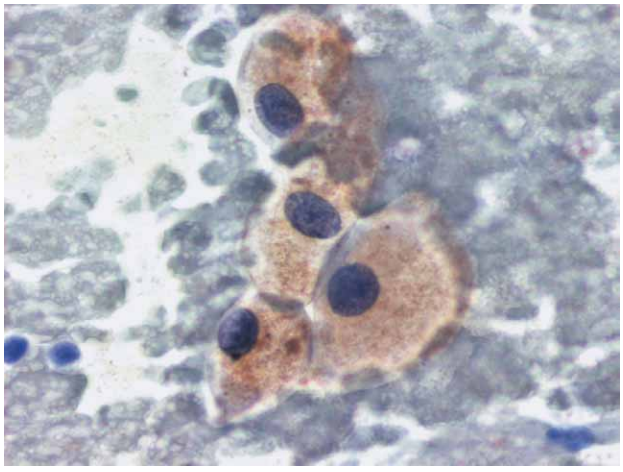

Fig 2. p16^{INK4a} staining of normal metaplastic cells found in a smear of a premenopausal woman

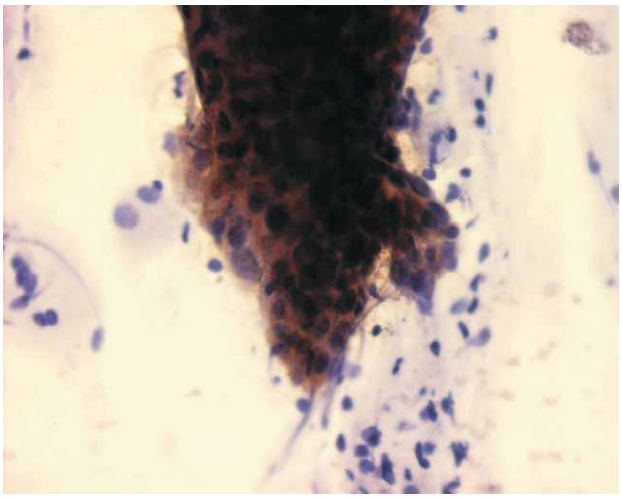

Fig 3. p16^{INK4a} staining of high grade squamous intraepithelial lesion (HSIL) found in a smear of a postmenopausal woman

TABLE 2
P16^{INK4a} FINDINGS ACCORDING TO DIAGNOSIS OF POSTMENOPAUSAL WOMEN

p16^{INK4a}	N (%) of cytological diagnosis				
	Normal	LSIL	HSIL	Carcinoma	Total
Negative	34 (100)*	3 (42.9)	2 (14.3)	1** (20.0)	40 (66.7)
Positive	0	4 (57.1)	12 (85.7)	4*** (80.0)	20 (33.3)
Total	34 (52.3)	7 (10.8)	14 (21.5)	5 (7.7)	60 (100)

LSIL – low grade squamous intraepithelial lesion, HSIL – high grade squamous intraepithelial lesion, *include 20 women as control group, **transitional cell carcinoma confirmed by histopathology, ***1 case of cervical adenosquamous carcinoma and 3 cases of cervical squamous cell carcinoma *in situ* confirmed by histopathology, χ^2=40.97, p<0.0001

viral replication is limited to superficial layers of the cervical epithelium that represents the acute, transitory phase. If viral E6 and E7 oncogene products are over-expressed in a basal or parabasal layers, they will interfere with the regulation of the host cell cycle, inducing genetic instability. The probability of progression of cervical precancerogenic lesions is considered greater with the longer persistence of papillomavirus infection. Almost 100% of all cervical cancers can be attributed to certain hrHPV types[8]. The highest proportion of cervical squamous cancers is associated with HPV type 16, followed by type 18 which is more common in adenocarcinomas, than HPV types 31, 33, 45, 52, 58 and others[1,7].

There are lots of regulating proteins involved in the cell cycle control. One of them, p16^{INK4a}, has been shown to be over-expressed after the cell has been infected by hrHPV types. Expression of the viral E7 oncogene leads to functional inactivation of tumour suppressor retinoblastoma protein (pRB), which in turn, results in strong over-expression of the cyclin-dependent kinase inhibitor p16^{INK4a}. This indicates that an active expression of the viral E7 oncogene is present in dysplastic cells[4].

There are a lot of data about HPV prevalence in younger women, but not much about elderly population. Smith et al.[5] showed that, opposite to a common belief, the prevalence rate of HPV in postmenopausal women was almost as high as in younger women and the association between hrHPV and consecutive abnormal Pap smear was 36%. Bosch and Harper[7] on the other hand summarised the results from cohort studies and found that hrHPV infection persisted in 50% cases. Bruner and Davey[9] had similar results in their group of women aged 60 years and over in contrast with 20% of hrHPV positive infection in younger groups (40 and 50 year old women). That leads towards conclusion of Massad et al.[10] that older women more frequently have a higher abnormality grade, while younger women had more lesions connected with transient infection.

In our study, following »Bethesda recommendations« we classified lesions as normal including inflammatory and reactive changes, as LSIL presented with or without koilocytotic changes, as HSIL and as carcinoma. Among premenopausal women with normal cervical smears p16--positive metaplastic cells were noted in one patient. It is known that some metaplastic and endocervical normal cells could be positively stained due to unfinished differentiation of the cells. Staining intensities of the normal, metaplastic cell are weaker than in dysplastic[10,12], and staining was observed only in the cytoplasm, while the nucleus showed no staining reaction. After p16^{INK4a} immunostaining, most (70.8%) of dysplastic cells in LSIL were stained negative in a group of premenopausal women. There were only 7 (29.2%) LSIL smears with a stained reaction in the nucleus and cytoplasm of the cells (Figure 1). All of these premenopausal women with HSIL were hrHPV positive (data not shown). Our results showed p16^{INK4a} overall positivity of 61% that is similar to the results of Bose et al.[13], although higher percentage up to 78% were reported previously[14,15]. We agree with the statement of Bose that p16^{INK4a} does not have a role in the diagnosis of LSIL but it might be useful for confirming the diagnosis of HSIL since our study showed positive staining in all HSIL. The role of p16^{INK4a} as a predictive and prognostic factor is to be confirmed by additional large scale studies.

Correct cytological diagnosis of cervical abnormalities in older women is a much greater problem because of the morphological similarities between atrophic epithelial cells and cells from high-grade squamous lesion. Therefore one should keep in mind several cytomorphologic findings such as: clean smear background, maturation index, nuclear/cytoplasmatic ratio, presence of hyperchromasia and then incorporate that in physiologic and pathologic changes of women's genital tract. Saad et al.[16] divided impact factor analysis to individual (degree of cell maturation, patient age, and smear background) and the other ones that dramatically influenced accurate diagnosis. The following factors were suggested: nuclear membrane features, hyperchromasia with nuclear/cytoplasmatic ratio favouring nucleus and abnormal single cell arrangement. These cytomorphologic findings lead us to an »uncertain« cytological diagnosis in postmenopausal smears with profound inflammatory or degenerative changes. By performing p16^{INK4a} immunostaining, in case that it is positive in the nucleus and cytoplasm, we will be able to classify some of these »uncertain« lesions as likely to be HSIL, which could be further confirmed by histology. In cytologically negative smears of postmenopausal women, p16^{INK4a} staining was negative as well.

Conclusion

From our preliminary study on a limited number of samples, it appears that although p16^{INK4a} is not helpful in the diagnosis of LSIL in younger women, in postmenopausal women it may be useful for confirming cervical lesion even if cytological diagnosis was mild dysplasia. To avoid »uncertain« diagnosis when the rate of possible false-negative diagnoses as well as false-positive cytology is high, we think that the use of p16^{INK4a} staining would be helpful for identification of adequate diagnosis.

For elderly women with squamous atypia an oestrogen treatment is often suggested, maturation effect can make an abnormal cell more viable and abnormal reactive changes disappear. That involves repeated patient's discomfort of physical exam as well as a certain dose of stress. To minimize that we suggest taking two simultaneous smears during Pap sampling; it will allow to perform normal Pap and p16^{INK4a} immunostaining. In this way, we will be able to distinguish more accurately normal, non-specific reactive or inflammatory changes from neoplastic changes in postmenopausal women Furthermore, p16^{INK4a} immunostaining would be useful in a routine laboratory practice for analysis and follow-up of abnormal cervical smears in general. Further population-based studies will be necessary to analyse predictive value of p16^{INK4a} protein in cervical smears in both groups of women.

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PROCJENA P16^{INK4a} U LEZIJAMA VRATA MATERNICE KOD ŽENA U GENERATIVNOJ DOBI I POSTMENOPAUZI

S A Ž E T A K

Česte su pogrešne citološke dijagnoze cervikalnih razmaza žena u postmenopauzi zbog sličnosti u morfološkom izgledu stanica dubljih slojeva epitela i neoplastično promijenjenih stanica. U našoj studiji istražili smo primjenu imunocitokemijskog bojanja sa p16^{INK4a} na cervikalnim razmazima u svrhu rješavanja tog problema. Imunocitokemijski smo obojali uzorke 77 žena u generativnoj dobi i 60 uzoraka žena u postmenopauzi. Kontrolnu skupinu su sačinjavali 20 urednih citoloških razmaza bez izraženih upalnih ili degenerativnih promjena iz skupine žena u postmenopauzi. Kod 68 pacijentica prilikom uzimanja uzoraka učinili smo 2 razmaza: jedan za citološku analizu a drugi za imunocitokemijsko bojanje. Šezdesetdevet predhodno uzetih uzoraka smo odbojali i pripremili za imunoreakciju. Kod žena generativne dobi 14 pacijentica je imalo uredan Papa-test i opažen je p16^{INK4a} pozitivitet kod jednog uzorka (7,1%); 24 uzoraka je ocijenjeno kao SIL (skvamozna intraepitelna lezija) niskog stupnja i među njima je bilo 7 (29,2%) p16^{INK4a} pozitivno obojenih uzoraka. Svih 39 uzoraka SIL visokog stupnja, od kojih 4 karcinoma pločastih stanica *in situ* naknadno histološki utvrđenih, pozitivno se obojilo. Pozitivitet p16^{INK4a} kod razmaza starijih žena je rastao u slijedećem nizu: 0%, 57,1%, 85,7% i 80% kod 34 urednih razmaza, 4 razmaza niskog i 12 visokog SIL te 4 karcinoma naknadno histološki utvrđenih (1 adenokarcinom, 3 karcinoma pločastih stanica *in situ*). U skupini od 16 pacijentica, točna citološka dijagnoza SIL-a visokog stupnja postavljena je tek nakon imunocitokemijske reakcije. Na temelju našeg preliminarnog istraživanja na malom broju uzoraka, možemo zaključiti da uključivanje p16^{INK4a} imunocitokemijskog bojanja u redovitu laboratorijsku praksu može pomoći u razlikovanju reaktivnih ili degenerativnih promjena od neoplastičnih lezija cervikalnog epitela.