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CONTRIBUTION IMMUNOCHEMICAL METHODS IN DIAGNOSIS AND PREVENTION OF CERVICAL DYSPLASTIC CHANGES

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Abstract

Cervical cancer is the world's fourth most frequent malignancy within the female population. Despite the established screening, incidences in the Czech Republic occur at a rate of about 20 cases per 100,000 women and with a mortality rate around 9 out of 100,000 women. The main factor of dysplasia and subsequent cervical cancer is the chronic infection with the human papillomavirus. This occurs when the oncogenic HPV types inactivate regulatory proteins of a cell and leads to uncontrolled cell proliferation. The aim of this study was to evaluate the benefits of immunochemical methods in the diagnostics of HPV infection in dispensarized patients with a finding of various grades of dysplasia. Immunocytochemical and immunohistochemical methods with an inhibitor of cyclin-dependent kinase $p16^{INK4a}$ and nuclear proliferation marker Ki-67 were used in a sample survey group of 47 women. The sample groups were also tested for the presence of the highly oncogenic HPV types by Hybrid Capture 2 method, but the relationship of the viral load and the grade of dysplasia was not proven. The control group consisted of five patients with normal findings, where the expected negativity of studied markers was confirmed. The results showed a correlation between the expression of the protein $p16^{INK4a}$ in cytological preparations with the morphological manifestations of the HPV infection in histological preparations, particularly with higher grades of dysplastic changes. This work confirmed that the detection of specific markers in the cytological and biopsy material contributes significantly to the specification of the degree of precancerous lesions on the cervix and, thus, their early detection.

Key words: *HPV; $p16^{INK4a}$; Ki-67; dysplasia; immunohistochemistry; immunocytochemistry; screening*

ORIGINAL ARTICLE

INTRODUCTION

Cervical cancer remains the world's second leading cause of death and the fourth most common malignancy among women (Wentzensen et al. 2012). The emergence of cervical cancer is preceded by a long-term stage of dysplastic changes, and, therefore, early diagnostics, treatment and check-ups are the only effective remedy against the process invasion. Dysplastic changes are most commonly diagnosed in young women between 25 and 35 years of age, and with the nationwide introduction of cervical cancer screening, the early onset of sexual activity and other general factors, including the age of women with a positive finding continues to decline (Globocan 2012). A crucial factor is the extensive time interval of the development

of dysplasia regarding invasive cancer. In the vast majority of cases, the dysplastic changes are asymptomatic and their presence can only be detected by histological or cytological examination. The standard cytological examination is relatively little sensitive and, therefore, more accurate methods of detection are still being investigated and developed. The work of both Prof. Wied and Prof. Schenck, as well as others, have shown that the morphological method – cytodiagnostics is a sensitive method that is highly dependent on the human factor, the technology of stem cells collection (in vaginal mirrors, adequate instruments for ektocervix and endocervix) and the transfer of the cells to glass.

The main etiological factor for the development of cervical cancer is a chronic infection involving the human papillomavirus. Thus far, 14 high risk viruses (HPV) involved in cervical intraepithelial neoplasia have been discovered. The most common include HPV 16 and 18, which substantially add to the formation of the cervical intraepithelial neoplasia (CIN 1–3) and cervical cancer (Ma et al. 2011). Upon contact with the endometrium with oncogenic papillomavirus, there occurs a loss of controlled balance between the tumor suppressor genes and proliferative markers on a molecular level. During the development of HPV infection, there is an interaction between the papillomavirus E6 and E7 oncogenes and regulatory tumor suppressor infected cell. The products of the major tumor suppressor genes are p53 and pRb, which under physiological conditions are involved in the inhibition, differentiation, and cell aging. Their inactivation or degradation lead to the DNA synthesis, uncontrolled cell proliferation and blocking apoptosis (van der Marel et al. 2014).

The cyclin-dependent kinase inhibitor $p16^{INK4a}$ has the key role in controlling the cell cycle, which, in immunochemical examination, can be used as an indicator of an active HPV infection in cytological and histological preparations. The antibody Ki-67, which is expressed during active phases of the cell cycle, serves to determine cell proliferation activity. Based on these findings, the aim of our study was to evaluate the benefits of setting $p16^{INK4a}$ and the nuclear proliferation antigen Ki-67 in the diagnostics of HPV infection in cytological and histological preparations in patients with CIN 1 to 3.

MATERIAL AND METHODS

The study included 58 patients at the Department of Gynecologic Oncology Centre at the University Hospital in Ostrava.

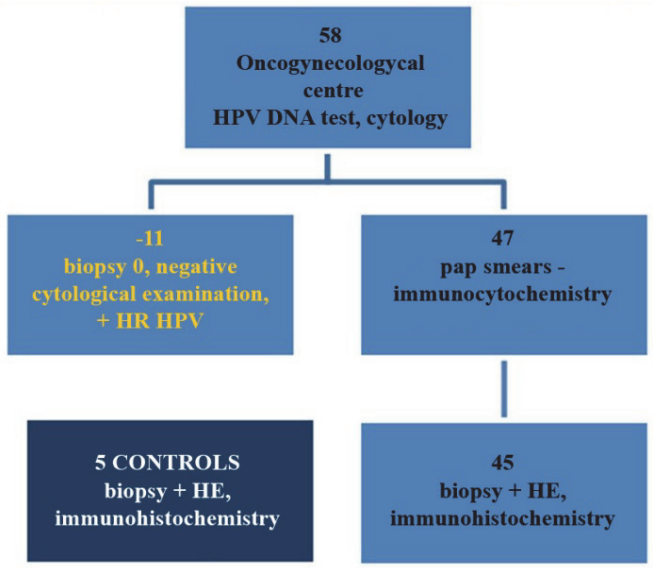

Chart 1. Group of patients

They were patients with previous positive cytological findings and positive HPV findings. Eleven patients were excluded during monitoring because of the negativity of cytological findings and the absence of biopsy samples. The sample group, thus, consisted of 47 women who were examined cytologically, where biopsy was performed and who were tested for the presence of HPV. The control group consisted of women with negative cytological and biopsy finding (Chart 1).

Specimen collection

In the first phase, the women took the Pap test for cytological samples using immunocytochemical methodology and detection of HPV DNA using the Hybrid Capture II methodology. To obtain specimen for biopsy, punch biopsy or cervical conization were used.

Basic cytological and histological examination of intraepithelial lesions

Cytological preparations were stained using the Pap methodology and evaluated according to the standard cytological criteria. The following conclusions were determined: positive findings, negative cytological findings or atypical glandular changes.

Histological material was processed by standard histological techniques, that involves embedding in paraffin and preparing sections with the thickness of about 3 μm. The basic staining with hematoxylin-eosin (HE) was used in all preparations. The microscopic evaluation of the degree of dysplastic changes of preparations stained by HE was built on the changes present in the basal third of the epithelium in CIN 1, in the basal and middle layer of the epithelium in CIN 2 and in all layers of the epithelium in CIN 3.

Immunocytochemistry and immunohistochemistry

To detect markers $p16^{INK4a}$ and Ki-67 in the cytological preparation, the dual immunocytochemical method (CINtec Plus kit, fa Roche-mtm Laboratories), which was carried out manually according to the instructions included in the kit, was used. In the completed preparations, the positivity of both markers visualized by different chromogens was monitored with an optical microscope. The expression of $p16^{INK4a}$ was shown as a brown precipitation in the cytoplasma and the nuclear positivity of Ki-67 was labelled red (Figs 1–3).

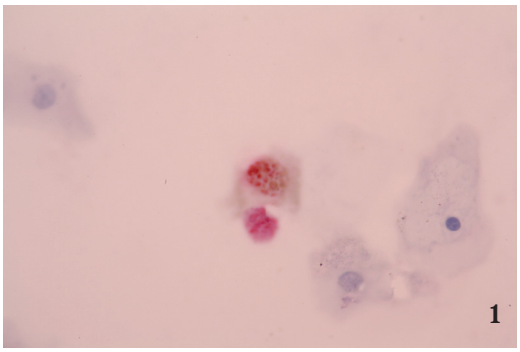

Fig. 1. The nuclear positivity in the cytological preparation with the used Ki-67 antibody with red coloration, 1000×

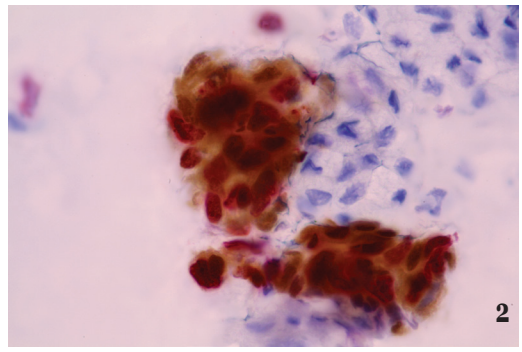

Fig. 2. The positivity of the $p16^{INK4a}$ protein (brown precipitation in the plasma) and Ki-67 (red nuclear positivity), 1000×

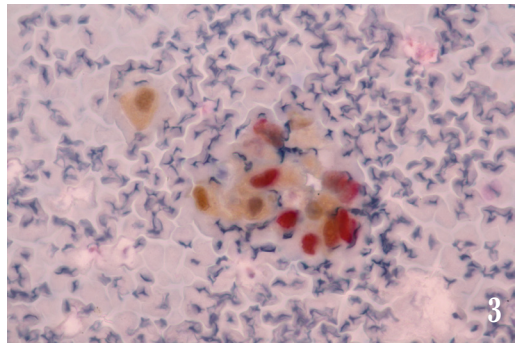

Fig. 3. The positivity of the $p16^{INK4a}$ protein (plasmatic positivity) and Ki-67 (nuclear positivity), 1000×

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Regarding the needs of the immunohistochemical examination, paraffin sections were prepared from tissue blocks with a thickness of 3 μm. The method and application of the antibodies $p16^{INK4a}$ (CINTec p16 Histology, Roche, incubation 30 minutes) and Ki-67 (M7240, dilution 1:50, Dako, incubation 30 minutes) were carried out in the machine Ventana Benchmark XT after the previous adjustments of CC1 buffer. To determine the degree of dysplasia in the optical microscope, the cytoplasmic and nuclear expression of $p16^{INK4a}$ and nuclear expression of Ki-67 in individual layers of the epithelium were evaluated, in both cases resulting in brown color (Figs 4–6).

Detection and genotyping of HPV DNA

The basis of the Hybrid Capture 2 technology is the direct hybridization of HPV DNA with the amplification of the signal. This method was performed in microplates. For a qualitative detection of HPV types, the principle of chemiluminescence was used in the methodology, and the values are expressed in relative light units (RLU). The test distinguished the presence of LR HPV and HR HPV types, including the semi-quantitative determination of the amount of the viral DNA in the sample.

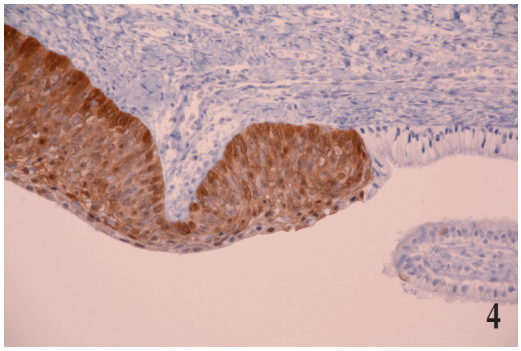

Fig. 4. The cytoplasmatic expression of the $p16^{INK4a}$ protein in the transitional zone of the cervix, 400×

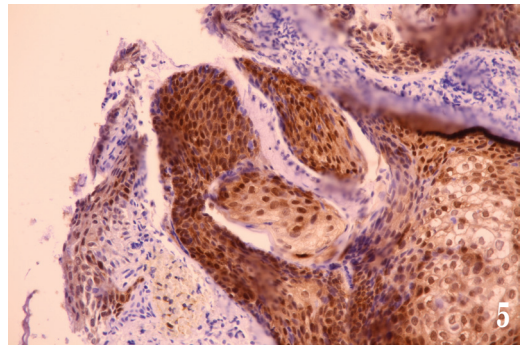

Fig. 5. The cytoplasmatic expression of the $p16^{INK4a}$ protein in the field of koilocytotic light dysplasia, 400×

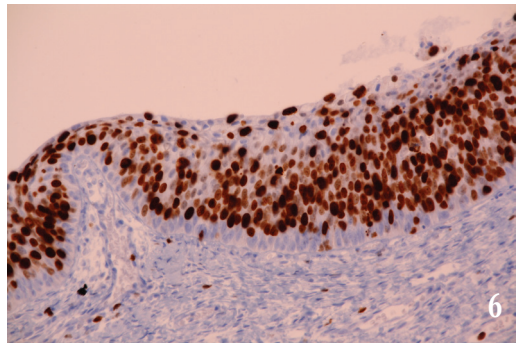

Fig. 6. The nuclear expression of Ki-67 with the expression of proliferative activity, 400×

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RESULTS

The cytological preparations evaluated LSIL in 18/47, HSIL in 23/47, AGC-NOS in 3/47 and the cytological examination was assessed as negative 3×. Histopathologically, CIN 1 was set in 14/47, CIN 2 in 12/47 and CIN 3 in 10/47 cases. In 11 biopsy specimen, there were other lesions diagnosed (metaplasia, inflammation, polyp). The comparison of the results of the basic cytological and histological evaluations in the examined group of 47 women is illustrated in Table 1.

Table 1. Comparison of cytological and histopathological findings

Histological diagnosis	Cytological diagnosis			
	AGC-NOS	***LSIL***	***HSIL***	***negative***
CIN 1	1/14	10/14	2/14	1/14
CIN 2	0/12	1/12	11/12	0/12
CIN 3	0/10	2/10	8/10	0/10
Other findings (metaplasia, polyp, inflammation)	2/11	5/11	2/11	2/11
	3	18	23	3

In cytological and histological preparations, an increasing positivity of both markers was monitored in correlation with the degree of the lesion. In cytology and histology, the positivity of $p16^{INK4a}$ was significant, particularly in the higher grades of dysplasia (Table 2).

Table 2. Comparison of the results of immunocytochemistry, immunohistochemistry and HR HPV positivity

			Dual immunocytochemistry		***Immunohistochemistry***	
Histological diagnosis	**Age**	**HR HPV DNA +**	**$p16^{INK4a}$ +**	**Ki-67 +**	**$p16^{INK4a}$ +**	**Ki-67 +**
CIN 1 14×	≥ 30 yrs	6	2	1	3	6
	≤ 30 yrs	1	1	2	1	6
	total	**7/14**	**3**	**3**	**4**	**12**
CIN 2 12×	≥ 30 yrs	5	2*	2*	5	5
	≤ 30 yrs	7	7	4	7	7
	total	**12/12**	**9**	**6**	**12**	**12**
CIN 3 10×	≥ 30 yrs	3	3	3	3	3
	≤ 30 yrs	6	5*	5*	6*	6*
	total	**8/10**	**8**	**8**	**9**	**9**
Other findings (metaplasia, inflammation, polyp) 11×	≥ 30 yrs	4	0	0	0	4
	≤ 30 yrs	1	0	0	1	3
	total	**5/11**	**0**	**0**	**1***	**7***

* The number of non-diagnostic cytology preparations: two preparations in patients with histological diagnosis of CIN 2, two preparations in patients with histological diagnosis of CIN 3. The number of non-diagnostic histological preparations: 1 case in a patient with CIN 3 exscinded, 1 case in a patient with chronic inflammation is invalid because of the absence of squamous epithelium.

No link with respect to the degree of dysplasia has been proven and, therefore, the quantification of the viral load of HPV cannot be considered valid (Chart 2), which is confirmed in the work of Iaconis et al. (2007). Another conclusion follows: therefore, it is not possible to establish a blanket screening based on the detection of HPV. HR HPV effect on the tissue is a long struggle with the immune system of the carrier. The actual detection of HR HPV DNA does not have to mean the necessary presence of precancerous changes of the cervix, but it does present a greater risk of developing.

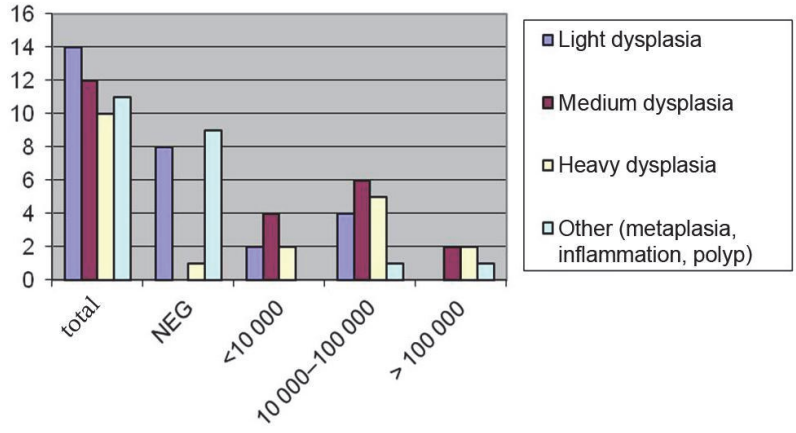

Chart 2. Positive viral load of HPV and its relationship to histology

DISCUSSION

Cytological and histological criteria degrees of dysplastic changes are well known. By default, the evaluation of cytological preparations is performed by Pap staining and the evaluation is carried out according to set cytological criteria. The grade of dysplasia in histological preparations stained by HE is determined on the basis of morphological changes: nucleoplasmatic mismatch, the number of mitoses, disorders of stratification of the epithelium and also the presence of koilocytes. It is generally known that especially low-grade lesions and small specimens are difficult to diagnose (Pinto et al. 2012). Histopathological interpretation of CIN is struggling with high variability and significant numbers of falsely negative and falsely positive results. IHC interpretation of the results requires certain experience of the pathologist and there are studies suggesting the variability of the evaluation, inaccuracies and difficulties in setting an accurate diagnosis without the use of other methods (Bergeron et al. 2010, Gupta et al. 2010, Pinto et al. 2012). The standard is formed by the biopsy findings within the cytological biopsy correlations (Odile et al. 2009, Gustinucci et al. 2012).

The grade differentiation of CIN has a major impact on the fate of the patient. CIN 2 diagnosis is the borderline when surgical removal of the lesion is indicated. If it is not possible to clearly determine the grade of CIN from HE staining, $p16^{INK4a}$ examination shows a diffuse, highly positive and highly sensitive finding of CIN 2 and CIN 3 (Dušková 2012). Our results confirm that the immunochemical examination allows a more accurate interpretation of cytological and histological findings, and that the degree of dysplasia correlates with the expression of $p16^{INK4a}$ (Agoff et al. 2003, Redman et al. 2008, del Pino et al. 2009, Gertych et al. 2012, Pinto et al. 2012). The grading of cervical biopsies using $p16^{INK4a}$ is much easier, especially for small biopsies. Additionally, the determination of $p16^{INK4a}$/Ki-67 in cytological examinations increases the sensitivity of determination of CIN 2 when compared with Pap test (Iaconis et al. 2007).

As reported by Singh et al. (2014), when $p16^{INK4a}$ is used, it exceptionally leads to an overevaluation of CIN 2. When combined with the method of Ki-67, however, a high degree of specificity is ensured. In some cases of CIN 1, there may be a positive expression of $p16^{INK4a}$, but the negativity of Ki-67 in the lower third of the epithelium in this case, is essential for expressing the diagnosis of CIN 1 (Pinto et al. 2012). However, some works have shown that the method of determining Ki-67 in cytological preparations is sensitive, but for CIN 2 and CIN 3, much less specific than $p16^{INK4a}$ (Cavalcante et al. 2012, Dušková 2012). Although in higher grades of CIN there is a significantly higher expression of Ki-67 than in the earlier ones, there is positivity in other lesions such as atypical inflammatory,

reactive and reparative changes (Dušková 2012). Ki-67 marker does not seem to be a significant marker for determining the grade of CIN in cytological preparations. Some studies have also shown that the diffusive expression of $p16^{INK4a}$ in CIN 1 have a greater risk of progress of high-grade CIN lesions than a negative CIN 1 (Ordi et al. 2014, Singh et al. 2014).

Although the HPV is a major risk factor for cervical cancer, morphological changes or HPV genotyping are not 100% capable of anticipating the clinical remission or progression of the disease (Mikyšková et al. 2003). In addition to other works, our study was unable to prove any connection between a viral load and dysplasia grades (Ikenberg et al. 2013). Another study showed that in women younger than 30 years of age, the method of Hybrid Capture 2 is more sensitive than immunocytochemistry ($p16^{INK4a}$/Ki-67), but less specific, and the number of falsely positive screening cytological examination were halved through immunocytochemistry (Iaconis et al. 2007). For the determination of viral etiology of dysplastic lesions, therefore, the combination of both methods, Hybrid Capture 2 and immunochemistry, seems preferable (Redman et al. 2008).

Just like some authors, we have also met a weak positivity of $p16^{INK4a}$ in several cases of CIN 1 in cytological (Horáček and Kobilková 2014) and histological preparations (Galgano et al. 2010). In cytological preparations, there was faint reddish coloration present, which was classified as an artefact. This conclusion was formulated on the basis of the additional method Ki-67, which, in these cases, was the crucial factor. Generally, in the examined group, it was observed that in the upper epithelial rails, Ki-67 primarily correlated with the positivity of cytological preparations. If the positivity in the histological preparation of Ki-67 was present only in the basal layer, in cytological preparations, Ki-67 was rarely present (3 of 14 cases of CIN 1).

The cause of the mismatch between the immunochemical examination and the HPV DNA test can be a reparative and regenerative processes, a laboratory error or the previously mentioned incorrect specimen collection by a clinician. The issue of the accuracy of the evaluation of immunochemical examinations also involves setting boundaries for positivity and requires the use of HPF for detailed scans of cytological and histological preparations.

CONCLUSION

The implementation of cervical cancer screening using the available investigative methods, including biopsy, is an important tool in early detection of dysplastic changes in the cervix and, thus, leads to the reduction of the incidence of cervical cancer.

In clear findings and in cases of large specimens where there is plenty of biopsy material, HE staining base is sufficient for an experienced pathologist. In smaller specimens, in the evaluation of resection margins of biopsy specimens and atypical findings, in order to determine the grade of dysplasia, it is necessary to apply the mentioned immunohistochemical examination. Evaluating $p16^{INK4a}$ is beneficial for reducing the variability in the evaluation of unclear or atypical cytological and biopsy specimens from the cervix. Although the determination of the protein $p16^{INK4a}$ gives precision to the diagnosis of CIN, it serves as an additional marker and it cannot be considered as a substitute for conventional cytological or histological examination. Our results demonstrate that immunohistochemical detection of $p16^{INK4a}$ is proving to be more specific and useable in diagnosing HPV infections than genic typing of papillomavirus using PCR methods.

Our results showed a correlation between the expression of $p16^{INK4a}$ protein in cytological preparations with morphological manifestations of HPV infection. The immunocytochemical identity card of $p16^{INK4a}$/Ki-67 could be beneficial in the screening programme of cervical cancer and of HPV infection ID. However, it is necessary to consider the issue of costs and high-quality requirements pertaining to the cytological smear.

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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