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Estimating Clinical Outcome of HPV Induced Cervical Lesions by Combination of Capsid Protein L1 and p16^{INK4a} Protein Detection

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ABSTRACT

The aim of this study was to investigate whether is possible to predict clinical outcome of cervical lesion by immunoassaying performed on cervical smears. During the two year study period the cervical smears of 81 patients were collected. All patients were tested for human papillomavirus (HPV) infections using Amplycor HPV test. Sixty-six of them were tested as positive for high risk types (hrHPV) and squamous intraepithelial lesion, and in those patients repeated cervical smears were taken every six months. The rest were hrHPV negative patients with normal smears which were used as a negative control in immunoassays with HPV L1 and p16^{INK4a} antibodies. The results of p16^{INK4a} staining in 66 hrHPV positive patients showed impairment of the cervical lesion in 22 (33.3%) and unchanged cytological finding in 21 (31.9%) p16^{INK4a} positive patients, respectively, while improving of cytological finding was seen only in three (4.5%) p16^{INK4a} positive patients. On the contrary, impairment of cytological finding was not seen in p16^{INK4a} negative patients, while in 17 out of 20 patients from that group improving or normalisation of cytological finding were detected ($p<0.01$). Correlation between L1/p16 pattern and cytological finding showed that only in L1–/p16+ cervical lesions was detected impairment of cytological finding during the study period. In L1+/p16+ group the cytological finding was the same during the follow up in all 11 patients, while in L1+/p16– group in most patients (9/11) downgrading or normalisation of Pap test were detected. The usage of p16 and HPV L1 markers can be useful in estimation of biologic potentiality and clinical outcome of cervical lesions.

Key words: *cervical dysplasia, clinical outcome, cytology, HPVL1 capsid protein, p16^{INK4a}*

Introduction

Cervical cancer is preventable by early detection of premalignant lesions. Over the years cervical cytology has become one of the most valuable methods in cancer prevention programs. Conventional Pap test is used not only to distinguish benign from malignant cases but also as a differential diagnostic method for predicting histological diagnosis and in that way it interferes with therapeutic procedures[1]. Cervical lesions are associated with persistent infection with oncogenic types of human papilloma viruses (HPV). HPV types 16 and 18 cause about 70% of cervical cancers and in more than 99% of cervical cancers one of 15 high risk HPV types (hrHPV) can be detected. Detection of hrHPV types is connected with the need for a larger number of HPV detection probes therefore larger expenses. Majority of low grade squamous intraepithelial lesion (LSIL) and HPV infections are prone to spontaneous regression especially in younger women[2], and repeated testing can produce unnecessary anxiety as well as expenses. On the other hand, HPV testing of the older women can replace frequent Pap

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smears, so postmenopausal women will have longer intervals between smears[3]. The epidemiological studies showed that minority of LSIL lesions progressed and that they were quite often over treated, incurring increased health costs because of the escalated number of check-ups as well as unnecessary treatments[4–5].

Because of subjectivity in Pap smear analyses, results can vary between investigators. Additional techniques are needed which are objective and interlaboratory reproducible in high rates.

Various biomarkers have been evaluated as specific markers for dysplastic cells on cytological as well as on histological specimens but with limited clinical use. Some of those markers in spite of rather high specificity have lower sensitivity, resulting in some cancer cells or their precursors being missed in analysis. The value of expression of p16^{INK4a} (p16) has already been demonstrated as a surrogate marker for the oncogenic activities of HPV in the cells of the cervical epithelium[3,6]. It is a regular cellular protein which gets overexpressed when specific viral oncogenes start to interfere with the host's cell-cycle regulation, although p16 expression is not absolute specific for hrHPV infection. Expression of p16 underlies a negative feedback control through pRB. Inhibited function of pRb should result in overexpression of p16 levels, making it a specific marker for cells with the expression of HPV oncogenes. HPV infection starts when a virus enters the epithelial stem cells usually at the squamo-columnar junction of the uterine cervix. During this early, so-called productive phase a high level of early (E2, E4) and late (L1, L2) viral genes occurs which results with virion particles release. Viral capsid protein L1 is expressed in that early phase and disappears in the later phases of carcinogenesis, suggesting its potential role in predicting the outcome of cervical intraepithelial lesion[4,7–8].

In this study the expression of L1 and p16 in abnormal cervical smears with LSIL (CIN 1) and HSIL (CIN 2) diagnosis were investigated and compared the results with the cytological findings of these lesions during the two years of follow-up. The prognostic relevance in predicting the clinical outcome of lesions in comparison with L1/p16 pattern on the basis of repeated cytological smears is estimated.

Examinees, Materials and Methods

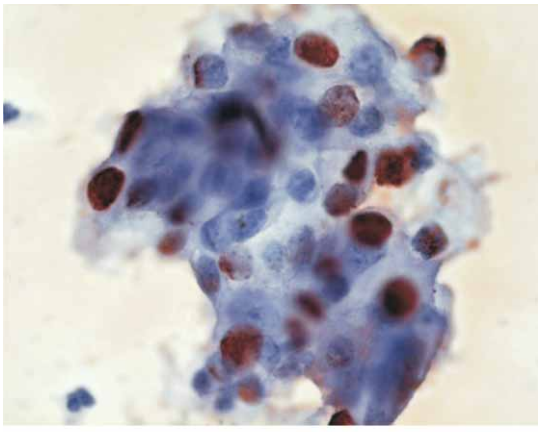
In two year study period the cervical smears from 81 patients were collected in Department of Gynaecology and Obstetrics at the University Hospital »Merkur«. Patients were chosen by random selection according to age, parity and clinical findings. According to cytological diagnosis we chose patients with normal Pap smear and those patients with CIN I (LSIL) or CIN II (HSIL) lesions. Patients with CIN III lesions as initial diagnosis were excluded.

Cervical swabs were taken by a wooden spatula and cytobrush. After making a smear for cytological examination which was immediately fixed in 95% ethanol, two additional smears were made for immunochemical staining from each patient. Simultaneously, extra smear was taken for HPV testing.

All patients were tested for hrHPV infection at the Dr. Andrija Stampar Institute for Public Health by the polymerase chain reaction based AMPLICOR HPV test (Roche Molecular System, Roche). Sixty-six patients tested hrHPV positive, while in 15 patients hrHPV infection was not confirmed.

Cytology diagnosis was made according to the Bethesda classification. In 66 hrHPV positive patients, the initial cytology result revealed LSIL (CIN 1) in 52, and high grade lesions (only CIN II) in 14 patients. Patients without HPV infection, 15 of them had a normal cervical smear throughout two years, and they represented the control group in immunostaining procedure.

For immunostaining p16 monoclonal antibody (clone E6H4, CINTec p16^{INK4a} cytology kit, MTM-laboratories, Heidelberg, Germany) and a monoclonal antibody L1 (HPV L1, Cytoactive Diagnostics GmBH, Pirmasens, Germany) were used, and slides were stained according to the manufacturer's protocol. Stained smears were studied under the light microscope and classified as positive when there was a clear red nuclear staining in just one cell for HPV L1 assaying (Figures 1, 2), and at least



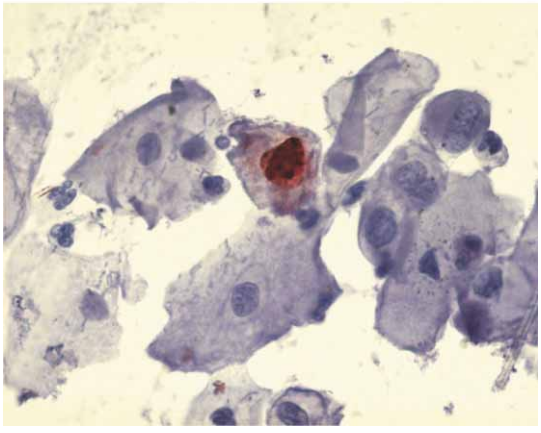


Fig. 1 and 2. HPVL1 in LSIL (x1000).

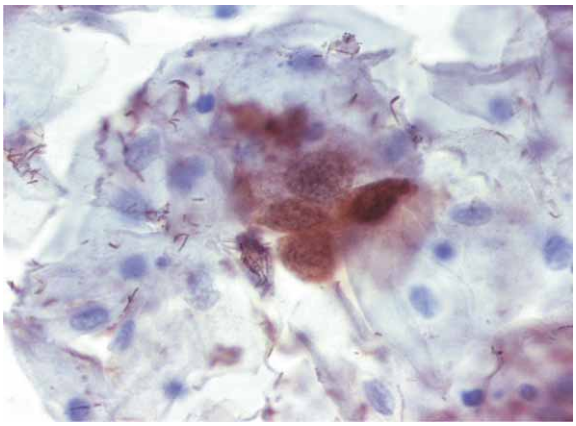


Fig. 3. p16INK4a in LSIL (x1000).

1% of stained cells for p16 assaying according to manufacturers. Cells treated with the p16 antibody were considered stained if brownish granules were found in the nuclei and in the cytoplasms of the cells (Figure 3).

Control cervical smears were taken every six months. For the study purpose impairment of lesion was defined as an elevated Pap test resulted in the higher group i.e., LSIL to HSIL (CIN 1 to CIN 2 or from CIN 2 to CIN 3) group, and the stable disease was defined as unchanged lesion as it was at the initial smear. Improving was defined as normalisation of the Pap test for at least two consecutive cervical smears within one year period or down-grading of dysplasia.

In eight patients with an elevated Pap result histological confirmation of cytological diagnoses was made as well as treatment according to the guidelines, accepted by the Croatian Society for Colposcopy and Cervical Pathology[9].

For statistic analysis χ^2-test was used and p values <0.01 were defined as significant.

Results

During two years cervical smears of 66 hrHPV positive and 15 HPV negative patients were analysed. Simultaneously with the initial cervical smear, additional smears were taken and stained with p16 and L1 monoclonal antibodies.

As was expected, the p16^{INK4a} expression was negative in the 15 hrHPV negative patients, and smears collected from those patients served as negative control for p16^{INK4a} immunostaining procedure. The results of p16^{INK4a} staining in 66 hrHPV positive patients (Table 1) showed impairment of the cervical lesion in 22 (33.3%) and unchanged cytological finding in 21 (31.9%) p16^{INK4a} positive patients, respectively, while improving of cytological

TABLE 1
COMPARISON OF P16^{INK4A} POSITIVITY AND CYTOLOGICAL FINDING (N=66)

P16^{INK4a}	Impairment	Improving	Unchanged	TOTAL
Positive	22 (33.3%)	3 (4.5%)	21 (31.9%)	46 (69.7%)
Negative	– (0.0%)	17 (25.8%)	3 (4.5%)	20 (30.3%)
TOTAL	22 (33.3%)	20 (30.3%)	24 (36.4%)	66 (100.0%)

$\chi^2=41.49$, $p<0.01$

TABLE 2
COMPARISON OF L1 POSITIVITY AND CYTOLOGICAL FINDING (N=66)

L1	Impairment	Improving	Unchanged	TOTAL
Positive	– (0.0%)	9 (13.6%)	13 (19.7%)	22 (33.3%)
Negative	22 (33.3%)	11 (16.7%)	11 (16.7%)	44 (66.7%)
TOTAL	22 (33.3%)	20 (30.3%)	24 (36.4%)	66 (100.0%)

$\chi^2=16.91$, $p<0.01$

TABLE 3
CORRELATION BETWEEN L1/ p16 PATTERN AND CYTOLOGICAL FINDING (N=66)

L1/p16	Impairment	Improving	Unchanged	TOTAL
L1+/p16+	– (0.0%)	– (0.0%)	11 (16.7%)	11 (16.7%)
L1+/p16–	– (0.0%)	9 (13.7%)	2 (3.0%)	11 (16.7%)
L1–/p16–	– (0.0%)	8 (12.1%)	1 (1.5%)	9 (13.6%)
L1–/p16+	22 (33.3%)	3 (4.6%)	10 (15.1%)	35 (53.0%)
TOTAL	22 (33.3%)	20 (30.4%)	24 (36.3%)	66 (100.0%)

finding was seen only in three (4.5%) p16^{INK4a} positive patients. On the contrary, impairment of cytological finding was not seen in p16^{INK4a} negative patients, while in 17 out of 20 patients from that group improving or normalisation of cytological finding were detected (χ^2= 41.49, p<0.01).

The results of HPVL1 staining are summarised in Table 2. Overall L1 was expressed in 33.3% cases (22/66). While not one case showed impairment of cytological finding when lesion was L1 positive, in half of L1 negative patients (22/44) impairment of cytological finding was detected (χ^2=16.91, p<0.01).

In Table 3 correlation between L1/p16 pattern and cytological finding showed that only in L1 negative/p16 positive cervical lesions was detected impairment of cytological finding during the study period. Statistically significant difference was observed when L1–/p16+ and L1–/p16– groups were compared (χ^2=8.9, p<0.01), with predominant improvement of cytological finding in L1–/p16– group. In L1+/p16+ group the cytological finding was the same during the follow up in all 11 patients, while in L1+/p16– group in most patients (9/11) downgrading or normalisation of Pap test were detected.

Discussion

Although HPV infection is the most common sexual transmitted infection, not many women develop cervical cancer. Cervical cancer can be curable if treated in an early stage. Early detection of a precancerous lesion is of importance in reducing mortality. Implementation of the Pap test as a method of triaging patients in screening programs all over the world has lead to the reduction of morbidity and mortality from cervical cancer, especially in countries with organized screening, and less in countries with opportunistic screening such as Croatia. Despite all advantages, Pap test has some limitations due to subjectivity of the method, inadequate sample collection or interpretation errors[10–13]. All of these contribute to a false negative result of Pap test up to 50%[12]. To overcome these problems new methods and technologies are introduced: like LBC (liquid based cytology) or computerised image analysis system, but because of higher costs their implementation into public health screening programs is limited only to developed countries. Alternative screening techniques suitable for developing countries are being investigated in various parts of the world but still not recommended outside research programs[1,9].

It was established that cervical premalignant lesions and cancer are HPV-induced diseases, and hrHPV nucleic-acid can be detected in almost all HSIL and cervical cancers. On the other hand, HPV infections exist among women with no cervical pathology which means that the positive predictive value of detecting HPV is rather low[3].

Until nowadays no worldwide consensus exists with respect to the choice of the primary screening tests. HPV tests are more reproducible and sensitive than cervical cytology but they are also less specific. If HPV test is negative, a longer screening interval should be considered. In Europe the standard screening method is still cytology-based. However this policy was reviewed and combination of cytology and HPV testing was approved for primary screening in women over 30 years of age and for the triage of women with abnormal cytology [15]. As von Doeberlitz suggested[3], the optimal screening test would be the test which can identify true dysplasia in which regression of the lesion is not possible because of the genomic instability and cellular dysregulation that takes place after incorporation of HPV oncogenes into the host genome.

In present study overall L1 positivity was low, only 33.3% among which in 13.6% and 19.7% lesions improvement of cytological finding or unchanged finding were detected, but in none of L1 positive lesion impairment of cytological finding was recorded. Relatively low proportion in L1 positive lesion was found in other studies as well. Griesser et al. reported 34.5% of L1 positive lesions, similar to Hilfrich and Negri et al., but Rauber et co-workers reported 61.3% positive nuclear staining[4,7,8,16]. It has been found that L1 protein begins to disappear as the disease progresses. Explanation probably lies in the maturation process of epithelial cells and it's linkage to the synthesis of HPV L1 capsid protein in HPV infected cells. L1 is strongly expressed in the superficial level of epithelium[12,17], and since maturation process in dysplasia is disturbed, L1 expression tends to decline probably, as Hilfirch pointed,[7] because of the transcriptional and genetic alterations within the epithelium. Morphologically changed cells can run away from the immune system since the L1 is one of the main targets for cell mediated immune defence and proceed to malignant transformation. In present study statistically significant association between negative L1 and cytological impairment of lesion during the time was demonstrated.

The result of p16 staining seems to be even more important for estimating progression of cervical lesions. In cervical precancerous and cancerous lesion high level of overexpressed p16 was found, suggesting it as a specific biomarker for cells with expression of HPV oncogenes, as many studies confirmed[18,19]. During the last decade, the expression pattern of p16 in dysplastic squamous cells has been extensively investigated. In general the proportion of p16 positive cases is closely associated with the severity of cytologic diagnoses. Relatively low proportion of positivity (18% to 27%) was found in normal cervical smears[20,21]. Positive staining can be found in metaplastic cells because of their unfinished maturation, endosalpingiosis, or fallopian tube epithelium. A wide range of studies indicate that p16 is highly expressed in HSIL as well in squamous cell carcinomas and not only that but its positivity may achieve an interobserver reproducibility in cytological as well as in a histological specimens[20,22,23].

In present study overall positivity for p16 was 69.7%, showing mainly unchanged or impairment of the cytological finding during the time, and regression in only three (4.5%) cases. The expression pattern results may be summarised in four categories as was shown in Table 3. In the groups with double positive (p16 +/L1+) result and

positive p16 and negative L1 (p16+/L1–) result, impairment of cytological finding was detected, so these pattern may be defined as »high-risk«, especially p16+/L1– pattern, and should be followed by cytology or by colposcopy and accordingly biopsy. Groups with p16 negative and L1 positive (p16–/L1+), or double negative (p16–/L1–) result may be defined as the »low-risk« pattern. In the double negative pattern was found that cytological finding improved in eight out of nine such lesions. Repeated analysis showed that probably because of the inflammatory or degenerative changes, smears were mistakenly evaluated as dysplastic. The p16–/L1+ pattern means that virus is present in the epithelial cells in productive phase, producing new virions and all p16–/L1+ findings were unchanged or improved during the time of follow up, and no impairment of cytological finding was detected, which indicate that only regular cytology control is needed.

In conclusion, the combination of these two biomarkers along with the cytology could be useful for identification of the patients at risk of lesion progression and can contribute to better understanding of biological potentiality of cervical lesions. Present two year prospective study indicated association between p16+/L1– immunocytochemistry and impairment of cytological finding. Further, larger studies are needed to confirm the need of detecting HPV L1 and p16 as well as other biomarkers in laboratory and clinical routine for better planning of the clinical management of patients with cervical abnormalities.

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PROCJENA KLINIČKOG ISHODA HPV-OM INDUCIRANIH LEZIJA VRATA MATERNICE DETEKCIJOM KAPSIDNOG PROTEINA L1 I STANIČNOG PROTEINA p16^{INK4a}

SAŽETAK

Svrha istraživanja bila je istražiti mogućnost predviđanja kliničkog ishoda lezije vrata maternice uz pomoć imunoloških testova provedenim na obriscima vrata maternice. Tijekom dvogodišnjeg razdoblja istraživanja prikupljani su obrisci u 81 pacijentica. Sve su testirane na humane papiloma viruse pomoću Amplycor HPV testa.U 66 pacijentica je detektiran HPV visokog rizika (hrHPV) i intraepitelna lezija, pa su obrisci vrata maternice u tih pacijentica ponavljani svakih šest mjeseci. Ostale su bile hrHPV negativne pacijentice s normalnim obriscima koji su upotrijebljeni kao negativna kontrola u imunološkim testovima s HPV L1 i p16^{INK4a} protutijelima. Rezultati p16^{INK4a} bojanja u 66 hrHPV pozitivnih pacijentica pokazali su pogoršanje cervikalne lezije u 22 (33,3%) a nepromijenjen citološki nalaz u 21 (31,9%)

pacijentice, dok je poboljšan citološki nalaz opažen u samo tri (4,5%) p16^{INK4a} pozitivne pacijentice. Naprotiv, pogoršanje citološkog nalaza nije viđeno u p16^{INK4a} negativnih pacijentica, dok je u 17 od 20 pacijentica iz te grupe nalaz bio poboljšan ili se normalizirao ($p<0,01$). Korelacija između L1/p16 uzorka i citološkog nalaza pokazala je da je u samo L1–/p16+ cervikalnih lezija detektirano pogoršanje citološkog nalaza u razdoblju istraživanja. U L1+/p16+ skupini citološki nalaz nije se mijenjao za vrijeme praćenja u svih 11 pacijentica, dok je u većine pacijentica (9/11) iz L1+/p16– grupe došlo do snižavanja stupnja lezije ili normalizacije Papa testa. Upotreba p16^{INK4a} i HPV L1 markera može biti korisna u procjeni biološkog potencijala i kliničkog ishoda cervikalnih lezija.