

Evaluation of the HPV L1 Capsid Protein in Prognosis of Mild and Moderate Dysplasia of the Cervix Uteri

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ABSTRACT

Cervical intraepithelial neoplasia (CIN) can be detected in the cytologic smears years before invasive squamous cancer arises, but no reproducible morphologic criteria exist to predict behavior of cervical lesions. The possibility of predicting the clinical course of cervical lesions could be of high value in clinical practice and some women will spare of unnecessary treatment. HPV L1 capsid protein represents about 90% of the total protein on the surface of the virus and can be detected in mild to moderate dysplasia and rarely in severe dysplasia. The purpose of the study was to evaluate the use of immunodetection of HPV L1 protein on archival Pap smears with findings of mild and moderate dysplasia in predicting its clinical course. Immunochemical analyses with L1 antibody revealed positively stained nuclei of squamous epithelial cells in 56 of 114 smears (49.1%). The staining results were correlated with follow-up smears or with histologic verification. Regression (negativisation of the Pap smear for 24 months or longer) was noticed in 31 of 56 (55.4%) L1-positive cases and in 20 of 58 (34.5%) L1-negative cases. Persistent disease occurred in 13 (23.2%) L1-positive cases and in 14 (24.1%) L1-negative cases. Progressive disease occurred in 12 (21.4%) L1-positive cases and in 24 (41.4%) L1-negative cases. The difference in the clinical course between the L1-positive and L1-negative patients was statistically significant ($p=0.025$). Also, the difference in the clinical course of the L1-negative staining in the under-30 and over-30 years age group was statistically significant ($p=0.04$). For conclusion, our data confirm that immunostaining for HPV L1 capsid protein could offer prognostic information about mild and moderate intraepithelial cervical squamous lesions.

Key words: cervical dysplasia, Pap smear, human papillomavirus, L1 capsid protein, immunostaining

Introduction

Epidemiological studies have pointed out that human papillomaviruses (HPV) are the main aetiological factor for cervical cancer¹. In the past 30 years, DNA of specific HPV types has been found in almost all cervical cancer biopsies². Over 100 HPV types have been characterized molecularly and 40 types are able to infect the genital tract³. HPV can be classified by risk of causing cervical cancer into low-risk (lr) and high-risk (hr) types^{4,5}. HPV 16 is the most frequent and HPV 18 the second most frequent hr HPV types contributing to 50–55%, and 15–20% of invasive cervical cancer cases, respectively⁶. Women

with positive hr HPV DNA have an increased risk of cervical cancer. Up to 59% of low grade squamous intraepithelial lesions (LSIL) are HPV DNA positive, but most of this lesions regress spontaneously, due to natural history of HPV infection and host immunity, with clearance of the viral genome within 1–2 years^{1,4,7}.

Persistency of the hr HPV DNA infection, viral integration and morphologic progression of squamous intraepithelial lesions are markers of neoplastic progression and are used for determining the patients requiring a treatment^{1,8}.

Cervical intraepithelial neoplasia (CIN) can be detected in the cytologic smears years before invasive squamous cancer arises. Appearance of koilocytes in cervical smears indicates the presence of the papillomavirus infection⁹, but no reproducible morphologic criteria exist to predict behavior of cervical lesions. Routine molecular genetic methods also can not be helpful, but recently developed biomarkers may be useful in the evaluation of the biologic potential of these lesions^{10–12}.

Because of the high regression rates of low grade dysplastic lesions, the possibility of predicting the behavior of cervical lesions could be of high value in clinical practice¹⁰. In Germany¹², and also in Croatia¹³, the accepted guidelines for management of cytology diagnoses of CIN 1 and CIN 2 lesions is expectant for a certain time of follow up with repeat smears and knowing the progression risk some women would be spared of unnecessary treatment.

HPV L1 capsid protein represents about 90% of the total protein on the surface of the virus. Together with the minor capsid L2 protein, encapsidates the viral DNA to built new infectious viral particles which are released in the upper epithelial layer². HPV L1 capsid protein is a nuclear protein that is rapidly transported into the nucleus of the cell after it is synthesised in the cytoplasm. In CIN 1 and CIN 2 lesions virus replication is evident in the upper squamous epithelial layer⁵ and allows immunochemical detection of infected cell nuclei¹⁴. HPV L1 capsid protein can be detected in mild to moderate dysplasia and rarely in severe dysplasia⁷. The immunostaining for L1 capsid protein can be performed on native smears, Papanicolaou stained smears, monolayer slides and histological sections.

The aim of this study was to determine whether HPV L1 immunoreactivity could help us to discriminate between regressing and persistent/progressing mild and moderate cervical dysplasia in cytological specimens. We immunostained archival Pap smears from hr HPV DNA positive squamous lesions with mild or moderate dysplasia. The staining results were correlated with follow-up smears for lesions which are going to remission or for persistent lesions with mild dysplasia. Progressive or persistent lesions with moderate dysplasia were correlated with histologic verification on Large Loop Excision of the Transformation Zone (LLETZ) or conization.

Materials and Methods

This retrospective study has been made on archival material and data of the Department of Gynecological Cytology, University Department of Gynecology and Obstetrics, Rijeka University Hospital Center, Croatia. From January 1, 2005 till January 1, 2006 routinely stained archival Pap smear slides from 114 lesions with mild (85 smears) and moderate (29 smears) dysplasia were included. Pap smears were classified according to »The Classification of cytologic findings of cervix uteri »Zagreb 2002« (The Modification of the »Zagreb 1990« and »NCI Bethesda System 2001« Classifications)¹⁵. The

HPV status was assessed using the digene Hybrid Capture 2 test (hc2 High-Risk HPV DNA Test, Qiagen Gaithersburg, USA) and all samples were positive for hr HPV DNA.

The Pap smears were immunochemically stained using a panreactive HPV L1 antibody. L1 capsid protein is synthesized in differentiated squamous epithelia of the superficial layer that are easy to obtain during the smear test. From women with remission (continued absence of dysplastic or malignant epithelial cell changes), follow-up smears were available. Most of the cases of persistent mild dysplasia were correlated with follow-up smears, too. Histologic results of LLETZ or conization from patients with progression or persistent lesions with moderate dysplasia are known. The clinical course were compared for two defined groups: under-30 and over-30 years of age.

Immunocytochemistry

From Papanicolaou stained slides the coverslip was removed (slides were remained in xylene for few days). After rehydration, for antigen demasking, the slides were transfer in the water bath, cover with citric buffer and boiled for 20 minutes. Then the slides were incubated with the anti-HPV L1 capsid protein screening antibody (Cytoactiv screening antibody, Cytoimmun diagnostic GmbH, Pirmasens, Germany) for 30 minutes, the detection reagent for 10 minutes and the chromogen for 5 minutes. After each step the slides were washed for 1 minute in washing buffer. Non-diluted haematoxylin was used for counterstaining. The slides were covered with aqueous mounting medium (Faramount Aqueous Mounting Medium, DakoCytomation) and coverslipped. Positive controls from L1 positive smears provided by the manufacturer of the L1 capsid antibody were used for each staining series. Stained slides were studied by light microscopy independently by two cytologist. Epithelial cells with a clear nuclear staining were scored as positive. Staining within the cytoplasm was neglected. The follow-up data for a dysplastic lesions were not known at the time of slide evaluation.

Progression was defined as a cytological or histological verified upgrading of the lesion (CIN1 to HSIL, or CIN2 to CIN3). Persistent disease was defined as persistence of the histological grade of CIN or persistence of the abnormal Pap smear for 18 months or longer. Regression was defined as negativisation of the Pap smear for 24 months or longer and in some cases also with negative control Hybrid Capture II test.

Statistical analysis was performed using computer software package »Statistica 7.1«. Data have been dichotomized for statistical purposes for patients with regression versus patients with persistent disease and progression. The differences within the groups were tested using χ^2 -test. Significance was assumed at a p value less than 0.05.

Results

Immunocytochemical analyses with L1 antibody revealed positively stained nuclei of squamous epithelial cells in 56 of 114 smears (Table 1). We notice a well-preserved cytologic features after the immunocytochemical staining procedure. The L1 capsid protein is a nuclear protein and L1 positive smears revealed strong, red to brown nuclear staining. It was observed in one, few or sometimes many nuclei of dysplastic squamous epithelial cells (Figure 1 and 2). Median age of women was 33 (range 20–70 years) and median observation period was 26.8 months (range 4–60). Regression was noticed in 31 of 56 (55.4%) of L1-positive cases and in 20 of 58 (34.5%) of L1-negative cases. Persistent disease occurred in 13 (23.2%) and progressive disease in 12 (21.4%) of L1 positive cases (7 CIN2, 5 CIN3). Of the 58 L1-negative cases, 14 (24.1%) persisted and 24 (41.4%) went into progression (9 CIN2, 14 CIN3, 1CIM). The difference in the clinical course between the L1-positive and L1-negative patients was statistically significant ($p=0.025$) (Table 2).

TABLE 1

TOTAL NUMBER OF L1 POSITIVE AND L1 NEGATIVE SMEARS

Total number of smears	L1 positive	L1 negative
114	56 (49.1%)	58 (50.9%)

Women under the age of 30 had L1-positive staining in 29 of 59 cases (49.2%). Regression was noticed in 19 cases, persistent disease in 4 cases and progression in 6 cases. In the over-30 age group, L1 positive staining were seen in 27 of 55 women (49.1%). The regression was ob-



Fig. 1. Positive staining in a koilocytic cell, L1 capsid immunostain, x400.

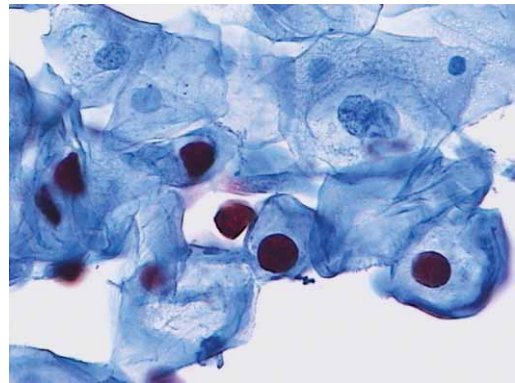


Fig. 2. Positive staining in groups of dysplastic cells (LSIL), L1 capsid immunostain, x400.

served in 12 cases, persistent disease in 9, and progression in 6 cases. The difference was not statistically significant ($p=0.11$). However, in the group of L1-negative staining we noticed a statistically significant difference in the clinical course between woman under-30 age group and over-30 age group ($p=0.04$). In the younger group we noticed 14 cases of regression, 5 cases of persistent disease and 11 cases of progression. In the over-30 age group the regression was observed in 6 cases, persistent disease in 9 and progression in 13 cases (Table 3).

The significantly different clinical course of the L1 negative cases is present in two different age groups. We noticed 14 cases of regression in the under-30 age group, and only 6 in the over-30 age group. Progression was observed in 16 cases in the under-30 age group and in 22 cases in the over-30 group (Figure 3).

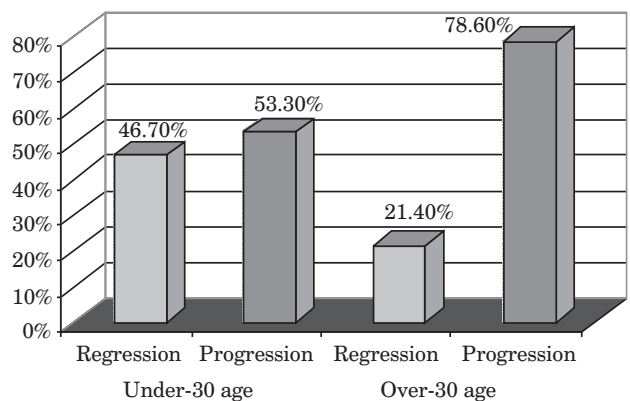


Fig. 3. The difference in the clinical course of the L1 negative staining in the under-30 and over-30 age group.

TABLE 2
CLINICAL COURSE OF DISEASE FOR L1 POSITIVE AND HPV L1 NEGATIVE CASES

Clinical course	L1 positive staining	L1 negative staining	Total
Regression	31 (55.4%)	20 (34.5%)	51
Persistent disease/ Progression	25 (44.6%)	38 (65.5%)	63

$p=0.025$

TABLE 3
CLINICAL COURSE OF DISEASE FOR HPV L1 POSITIVE AND HPV L1 NEGATIVE CASES FOR UNDER-30 AND OVER-30 AGE GROUPS

Clinical course		L1 positive	L1 negative	Total
Under-30 age group	Regression	19 (65.5%)	14 (46.7%)	33
	Persistent disease/ Progression	10 (34.5%)	16 (53.3%)	26
Over-30 age group	Regression	12 (44.4%)	6 (21.4%)	18
	Persistent disease/ Progression	15 (55.6%)	22 (78.6%)	37

p = 0.11

p = 0.04

Discussion

The main purposes of Pap test are early diagnosis of cervical precancerous lesions in cervical cancer screening programmes¹⁶, predicting grade of the lesions, as well as follow-up after conservative surgical treatment^{17,18}. One major issue in the management of cervical precancerous lesions is the evaluation of the progression risk of dysplastic lesions¹⁰. L1 capsid protein is synthesized in the nucleus during the productive phase of the viral life cycle in upper epithelial layers. Within this phase, morphological epithelial changes occur presenting as mild or moderate dysplasia in cytological smears¹⁹.

Our data demonstrate that L1 positive patients with mild or moderate dysplastic cervical lesions have reduced risk of disease progression, compared to L1 negative patients, because in 31 (55.4%) L1 positive patients we noticed regression of the lesions. Griesser et al.¹⁴ demonstrate regression in 69%, Rauber et al.¹² in 38.7% and Lee et al.²⁰ in 46.1%. In L1 negative patients we noticed progression in 38 (65.5%) cases, which is similar to results of Griesser et al.¹⁴ (76.3%), but Rauber et al.¹² notice progression only in 25.9% cases. This different data may be due to different grouping of the cases, because patients with persistent disease some authors added to regression and others to progression cases. Krivak Bolanča et al.²¹ notice progression in 33.3% and persistent disease in 16.7% of cases. The exact cause for the significantly reduced or lacking L1 capsid protein synthesis in progressive intraepithelial lesions, is not known. Melsheimer et al.⁷ demonstrates that the expression of L1 capsid proteins are significantly reduced in HPV 16 DNA positive HSIL and HPV hr DNA positive HSIL. They thought that explanation may be found in a disturbed viral cellular interaction and loss of L1 expression due to integration. Integration of the virus DNA into the host cell genome is detectable in some HSIL lesions, but development of viral capsid antigen L1 also depends upon transcriptional factors. This factors are result of abnormal E6/E7 gene activation and can be expressed during the

maturation process from basal to superficial epithelial cell⁵. In HSIL the maturation is disturbed and predominant cell type are dysplastic basal epithelial cells with reduced L1 capsid protein expression. Also, control mechanisms exist at the translational level (mRNA), or molecular changes in the episomal L1 gene^{14,19}. However, despite a detectable antigenic L1 capsid synthesis of the HPV, the immune system is not capable of eliminating infected host cells in rare cases¹⁹. Our data confirm that 12 cases (21.4%) of L1 positive patients went into progression, and other authors obtained similar results^{12,14}.

HPV L1 capsid proteins are considered to be a major target of cellular immune response in CIN. A reduction or loss of capsid antigen production therefore might result in a reduction of cellular immune response^{7,20} and promote further transformation of immature epithelial cells⁵. Lack of T-cell response also leads to reduced antibody synthesis for protecting against endogenous and exogenous reinfection¹⁴. Griesser et al. observed that different clinical course of L1 antigen-positive and L1-antigen negative groups became increasingly apparent with rising age and in the age group >45, most lesions regressed¹⁴. Lee and al. noticed that L1 antigen-negative rate was higher than the positive rate in the over-40 age group and conclude that increased L1 capsid negative rate might reflect the increasing prevalence of cervical cancer in the old age group²⁰. Our data demonstrate that progression is significantly more often observed in over-30 age group comparing to under-30 age group in the L1 antigen-negative cases.

We can conclude, that under-30 years age patients with mild and moderate dysplastic lesions and L1 antigen-positive smears have low risk for disease progression and watch-and-wait management with regular control smears is optimal. If over-30 age patients with persistent mild or moderate dysplasia have L1 antigen-negative smears and are hr HPV positive, short-terms controls and histological verification is justified. Large prospective follow-up studies is needed to confirm the prognostic value of L1 protein.

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VRIJEDNOST HPV L1 PROTEINA U PROGNOZI BLAGE I UMJERENE DISPLAZIJE VRATA MATERNICICE

SAŽETAK

Cervikalna intraepitelna neoplazija (CIN) može se dijagnosticirati u citološkim razmazima godinama prije nastanka invazivnog karcinoma, ali ne postoje morfološki kriteriji koji bi ukazivali na prognozu takve promjene. Mogućnost predviđanja kliničkog tijeka bolesti važna je u kliničkoj praksi i neke bi žene poštedjela nepotrebnih kirurških zahvata. Ekspresija HPV L1 proteina nađena je kod blage i umjerene displazije, a rijetko kod teške displazije. Cilj rada je procjena vrijednosti imunocitokemijskog bojenja HPV L1 proteina u predviđanju kliničkog tijeka bolesti. Korišteni su arhivski Papa razmazi s nalazom blage i umjerene displazije. Imunocitokemijska analiza pokazala je pozitivnu reakciju kod 56 od 114 uzoraka (49,1%). Usporedbom rezultata bojenja s kontrolnim citološkim razmazima ili patohistološkom potvrdom, regresija (normalizacija Papa razmaza tijekom 24 mjeseca ili dulje) je nađena kod 31 od 56 (55,4%) L1-pozitivnih razmaza, te kod 20 od 58 (34,5%) L1-negativnih razmaza. Stagnacija bolesti uočena je kod 13 (23,2%) L1-pozitivnih razmaza i kod 14 (24,1%) L1-negativnih razmaza. Progresija bolesti nađena je kod 12 (21,4%) L1-pozitivnih razmaza i 24 (41,4%) L1-negativnih razmaza. Razlika u kliničkom tijeku bolesti između L1-pozitivnih i L1-negativnih pacijentica je statistički značajna ($p=0,025$). Također, razlika u kliničkom tijeku bolesti L1-negativnih pacijentica mlađih od 30 i starijih od 30 godina je statistički značajna ($p=0,04$). Naši rezultati ukazuju da imunocitokemijsko bojenje HPV L1 proteina može ukazati na prognozu blage i umjerene displazije vrata maternice.