High-risk HPV L1 capsid protein as a marker of cervical intraepithelial neoplasia in high-risk HPV-positive women with minor cytological abnormalities

INGRID NORMAN[1], ANDERS HJERPE[2] and SONIA ANDERSSON[3]

[1]Department of Clinical Science, Intervention and Technology, Division of Obstetrics and Gynecology, and [2]Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Karolinska University Hospital Huddinge, 141 86 Stockholm; [3]Department of Women's and Children's Health, Division of Obstetrics and Gynaecology, Karolinska Institutet, Elevhemmet H2:00, Karolinska University Hospital Solna, 171 76 Stockholm, Sweden

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Abstract. Human papillomavirus (HPV) L1 capsid protein is only produced during a productive HPV infection at the end of the natural viral life cycle and is a major target of the immune response in women with HPV-related squamous intraepithelial lesions. We evaluated the usefulness of L1 detection by immunocytochemistry in high-risk (HR) HPV-positive women with minor cytological abnormalities detected at organised population-based cervical cancer screening in Sweden, and assessed the relationship with histological diagnoses. Cytological slides were immunocytochemically stained using an HPV L1-specific monoclonal antibody for all known HPV types. HPV DNA analysis was performed using Linear Array test. Out of thirteen L1-positive women infected with HPV16, only two (15.0%) progressed to cervical intraepithelial neoplasia grade 2 or worse (CIN2$^+$); compared to four L1-positive women infected with other HR-HPV types. Among L1-positive women with CIN2$^+$, 35.7% harboured both HR and low-risk HPV types, 25.0% harboured HR-HPV types only and 13.3% were infected with HPV16. Loss of L1 expression could be a prognostic marker for the development of preinvasive cervical lesions. We show that different HPV types may initiate a parallel oncogenic process, but only loss of L1 expression predicts the development of CIN2$^+$, suggesting that HPV typing in combination with L1 detection could be used for more focused investigations of women with minor cytological abnormalities.

Correspondence to: Professor Sonia Andersson, Department of Women's and Children's Health, Division of Obstetrics and Gynaecology, Karolinska Institutet, Elevhemmet H2:00, Karolinska University Hospital Solna, 171 76 Stockholm, Sweden
E-mail: sonia.andersson@karolinska.se

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Introduction

Cervical cancer is the second most frequent neoplasm among women worldwide. However, in Europe, in the United States and in Japan there has been a marked decrease in cervical cancer incidence and mortality rates due to efficient mass screening programmes. Thus, since the introduction of population-based screening by Papanicolaou test, the incidence of and mortality from invasive cervical cancer has sharply declined in Sweden (1,2).

The presence of high-risk (HR) human papillomavirus (HPV) DNA identifies both women with neoplastic disease, and those who are at higher risk of developing disease (3). HPV16, 18, 45, 31 and 33 are the most frequently identified HR-HPV types in high-grade squamous intraepithelial lesions (HSILs) and squamous cell carcinomas (4), although HPV16 predominates (5).

HPV infection is a necessary, but not sufficient factor in the development of cervical neoplasia. Persistent infection with HR-HPV, especially HPV16, is regarded as a significant risk factor in the development of squamous cervical lesions and squamous cell carcinoma (6-9). However, most HPV infections clear spontaneously, and for those that do progress to cancer, a long period of latency is normally observed. Thus, HPV infections are prevalent, and often transient, among younger women, with a prevalence peak of 20-25% at 20-24 years of age. With increasing age, there is a decline in HPV prevalence to ~7% at 35 years of age (10). Failure of the host immune response to clear HPV infection is a prerequisite for persistence, thereby contributing to carcinogenesis, as suggested by the increased prevalence of HPV infection, cervical intraepithelial neoplasia (CIN) and cervical cancer in individuals with impaired cell-mediated immunity, such as HIV/AIDS patients, organ transplant recipients and those with iatrogenic immunosuppression. The increased incidence and progression of HPV infections in immunosuppressed patients emphasise the critical role of cell-mediated immune response in the clearance and control of HPV infections (11,12). The immunological barrier of the cervical mucosa is predominately regulated in the *lamina propria*, which contains plasma

cells, antigen-presenting cells (dendritic cells), natural killer cells and helper and cytotoxic T-lymphocytes. Successful clearance of HPV infection occurs in the presence of local pro-inflammatory (Th1) cytokine expression (13) and $CD4^+$ T-cells, as evidenced in immunological studies of HPV6- and 11-induced genital warts (14) and a systemic lymphoproliferative response to the HPV E7 capsid protein (15).

Therefore, cytokine-mediated immune responses may be a critical factor in HPV clearance. We focused on one of the major HPV-associated stimuli of the immune system, the L1 capsid protein. This major capsid protein is one of the eight known HPV-specific proteins (E1, E2, E4, E5, E6, E7, L1 and L2). L1 is only produced during a productive HPV infection at the end of the natural viral life cycle. Terminal differentiation of the epithelial host cell is required for this process and free viral particles are released from the L1-positive apical layers of keratinocytes to infect other cells and hosts (8). Earlier studies (16,17) have shown that the immunocytochemical evaluation of HPV L1 status is valuable for predicting the outcome of early dysplastic lesions, serving as a better risk factor than presence of HR-HPV DNA.

In the present study, our aim was to evaluate the usefulness of HPV L1 detection by immunocytochemistry in HR-HPV-positive women with minor cytological abnormalities (i.e., atypical squamous cells of undetermined significance, ASCUS and low-grade squamous intraepithelial lesions, LSIL) detected at organised population-based cervical cancer screening in Sweden and to assess the relationship with histological diagnoses.

Materials and methods

We consecutively enrolled 112 women with minor cytological abnormalities detected at population-based primary cervical cancer screening in Southern Stockholm, Sweden, between April 2007 and January 2009. The mean age of included women was 32 years (range, 23-57 years).

All cytological samples were collected by midwives who received training in liquid-based cytology (LBC) sampling, which was carried out using a plastic Ayre-like spatula and an endocervical brush. Cervical cells were suspended in PreservCyt media (ThinPrep®; Hologic, Boxborough, MA, USA).

LBC samples were prepared and evaluated at the Department of Clinical Pathology and Cytology, at Karolinska University Hospital, Sweden, using the ThinPrep® 2000 Processor (Hologic). The Bethesda classification (18) was modified according to the Swedish recommendations, which define samples with koilocytosis, but without cellular atypia, to be within normal limits (WNL). Therefore, LSIL includes only cases of mild dysplasia.

All women with signs of minor cytological abnormalities at screening underwent a follow-up pelvic examination and colposcopy by OMPI colposcope (Zeiss, Oberkochen, Germany) at the Department of Gynaecology, at Karolinska University Hospital, Stockholm, Sweden, 2-6 months after cytology screening results were recorded. At colposcopy, 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 was taken at the 12 o'clock position, close to the squamo-columnar junction.

Histological diagnoses were based on conisation specimens or colposcopically guided punch biopsies, which were preserved in formaldehyde and assessed by pathologists. Histological samples were evaluated and classified according to the CIN classification (19) and grouped into normal histology (i.e., WNL), CIN1 and CIN2 or worse ($CIN2^+$). The histological results were traced through the medical and laboratory records and through the Stockholm Oncology Center.

HPV DNA analysis. From each LBC sample, 2 ml of the remaining cell suspension was taken for HPV DNA analysis. DNA was extracted from the suspensions using the MagNA Pure LC Robot (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. HPV DNA detection and genotyping were carried out using the Linear Array (LA) HPV Genotyping test (Roche Diagnostics). Briefly, HPV DNA was amplified by PCR using a pool of biotin-labelled primers that hybridise in the L1 region (20). The 37 HPV types included in the LA test were divided into three categories: HR, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59/68/73 and 82; probable HR, HPV26, 53, 66; and low-risk or undetermined-risk (LR), HPV6, 11, 40, 42, 43,44, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108 (9).

Immunocytochemistry. Extra slides for each LBC sample were prepared for immunocytochemical staining and were stained using an HPV L1-specific monoclonal antibody (Cytoactive®; Cytoimmun, Pirmasens, Germany) according to the manufacturer's protocol. Briefly, slides were subjected to antigen unmasking by microwave treatment. Cytoactive screening antibody was applied to the slides, which were incubated for 30 min at room temperature, followed by incubation with the detection reagents for 10 min and AEC chromogen for 5 min. Slides were mounted with Aquatex (Merck, Darmstadt, Germany) and cover slipped. Stained slides were studied by light microscopy. Slides with at least 1 epithelial cell with distinctly positive nuclear staining were scored as positive (Fig. 1). This cut-off is recommended for the Cytoactive assay and has been used in all studies published so far (16). In each group analysis, a negative and a positive control were used.

All participants gave written informed consent and the study was approved by the Ethics Review Board at the Karolinska Institutet (no. 04-679/3). Participants diagnosed with CIN were treated in accordance with the Swedish national guidelines.

Statistical methods. Categorical data were summarised using frequency counts and percentages. The Chi-square test was used to analyse the association between L1 expression, different HR-HPV types and CIN grade. Both univariable and multivariable multinomial and binomial logistic regression were performed to study the association between predictors of CIN (both HR- and LR-HPV infection, HR-HPV infection only and HPV16 infection) and CIN grade, and these same predictors and L1 expression. Results are presented as odds ratios (OR) and 95% confidence intervals (CI). P-values <0.05 were considered statistically significant. SAS® System

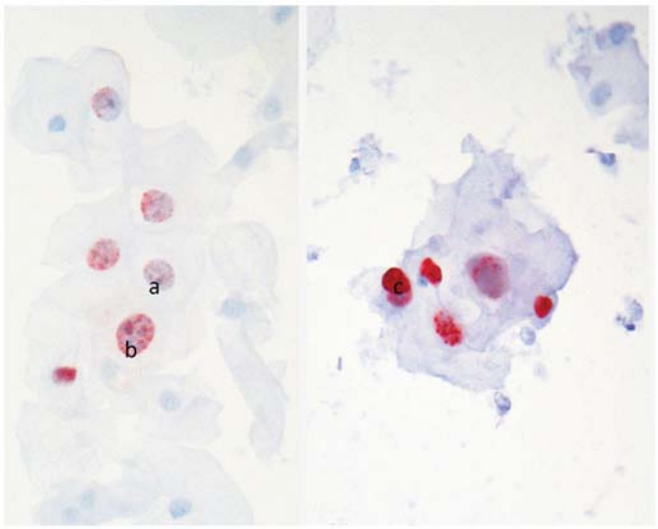

Figure 1. Immunocytochemical reactivity to HPV L1 capsid protein in squamous cells. Left frame show cells (a) with both week (+) and (b) moderate (++) reactivity while the reactivity in the right frame (c) is strong (+++).

9.1 (SAS Institute Inc., Cary, NC, USA) and Statistica 10.0 (StatSoft®, Inc., Tulsa, OK, USA) were used.

Results

Of the 112 women with minor cytological abnormalities enrolled, 108 (96.0%) had a cell suspension that was sufficient for immunocytochemistry. Four of these women were excluded due to insufficient tissue samples for histological examination, leaving a study population of 104 women in the final analyses.

Histological results. According to histology, 23 (22.1%) study women were scored as WNL, 43 (41.4%) had CIN1, 23 (22.1%) CIN2 and 15 (14.4%) CIN3+ (Table I).

Frequency of HR-HPV DNA detection. The frequency of HR-HPV detection by LA in the study population showed that HPV16 was the most common type (32.7%), followed by HPV51 (20.2%) and HPV31 (16.3%). HPV52 and 56 were equally frequent (11.5%). Multiple infections were found in 28 women.

L1 expression in HR-HPV infections. Forty-eight (46.0%) women were positive for L1 reactivity; the remaining 56 were L1-negative. However, the percentage of L1-positive HPV infections varied by HPV type. The HPV types that expressed L1 most frequently were HPV56 (80.0%), HPV45 (60.0%), HPV52 (58.3%) and HPV51 (52.4%). Only 13 of 34 HPV16 infections showed L1 expression (38.2%). On the other hand, the highest negative rate of L1 expression was found in HPV35 (80.0%), HPV16 (61.8%), HPV18 (60.0%) and HPV59 (60.0%) infections (Table II).

L1 expression by histological result. L1 expression was found in 13 out of 23 (56.5%) WNL cases and 26 of 43 (60.5%) CIN1 cases, but only 11 out of 38 (28.9%) women with CIN2+ (Table III). L1 expression was negatively correlated with CIN grade and the difference was statistically significant (P=0.012) by Pearsson's Chi-square test. In a univariate logistic regression model using CIN2+ as an outcome, absence of HR-HPV L1 expression at enrolment was a predictor, with an OR of 3.2 (95% CI, 1.081-9.417) for progression to CIN2+. When a stepwise logistic regression analysis was done, the OR for absence of L1 expression was 2.8 (95% CI, 0.92-8.679; P=0.07) when LR-HPV was also included in the model. Using lack of L1 expression as an outcome, only CIN grade was significant in a stepwise logistic regression analysis P=0.012 (data not shown).

Distribution of HR-HPV by histological diagnosis among L1-positive women. Distribution of HR-HPV infections among L1-positive women is shown in Table IV. Of 33 L1-positive women infected with both HR- and LR-HPV types, only 5 (15.2%) progressed to CIN2+.

Table I. Characteristics of the study population.

Characteristics	
Mean age (range), years	32 (23-57)
Histological results, n (%)	
CIN3+	15 (14.4)
CIN2	23 (22.1)
CIN1	43 (41.3)
WNL	23 (22.1)
Total	104

CIN3+, cervical intraepithelial neoplasia grade 3 or worse; CIN2, cervical intraepithelial neoplasia grade 2; CIN1, cervical intraepithelial neoplasia grade 1; WNL, within normal limits.

Table II. L1 expression in high-risk human papillomavirus (HPV) infections by HPV type.

	L1 expression			
HPV type	Positive N (%)	Negative N (%)	P-value	Total N
HPV16	13 (38.2)	21 (61.7)	0.16	34
HPV18	4 (40.0)	6 (60.0)	0.59	10
HPV31	8 (47.1)	9 (52.9)	0.92	17
HPV33	3 (50.0)	3 (50.0)	0.92	6
HPV35	1 (20.0)	4 (80.0)	0.19	5
HPV39	8 (47.1)	9 (52.9)	0.92	17
HPV45	6 (60.0)	4 (40.0)	0.43	10
HPV51	11 (52.4)	10 (47.6)	0.66	21
HPV52	7 (58.3)	5 (41.6)	0.45	12
HPV56	8 (80.0)	2 (20.0)	0.03	10
HPV58	4 (44.4)	5 (55.6)	0.81	9
HPV59	4 (40.0)	6 (60.0)	0.59	10
HPV53*	8 (61.5)	5 (38.5)	0.29	13

Table III. L1 expression by histological result.

	Histological results			
L1 expression	WNL N (%)	CIN1 N (%)	CIN2+ N (%)	Total N (%)
Negative	10 (43.5)	17 (39.5)	27 (71.1)	54 (51.9)
Positive	13 (56.5)	26 (60.5)	11 (28.9)[a]	50 (48.1)
Total	23	43	38	104

[a]L1 positivity correlated negatively with CIN grade and the difference was statistically significant (P=0.012) by using Pearson's Chi-square test. WNL, within normal limits; CIN1, cervical intraepithelial neoplasia grade 1; CIN2+, cervical intraepithelial neoplasia grade 2 or worse.

Table IV. Distribution of HR-HPV by histological diagnosis among L1-positive women.

	Histological diagnosis			
HPV type	WNL N (%)	CIN1 N (%)	CIN2+ N (%)	Total N (%)
Both HR and LR-HPV	10 (30.3)	18 (54.5)	5 (15.2)	33
HR-HPV only	3 (17.6)	8 (47.1)	6 (35.3)	17
HPV16	3 (23.1)	8 (61.5)	2 (15.4)[a]	13

[a]P=0.026 by Pearson's Chi-square test. HR-HPV, high-risk human papillomavirus; WNL, within normal limits; CIN1, cervical intraepithelial neoplasia grade 1; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; LR-HPV, low-risk and undetermined-risk HPV.

Table V. The distribution of L1 expression in women with CIN2+ by HR-HPV group.

	L1 expression		
HPV type	Negative N (%)	Positive N (%)	Total N (%)
Both HR and LR-HPV	9 (64.3)	5 (35.7)	14
HR-HPV only	18 (75.0)	6 (25.0)	24
HPV16	13 (86.7)	2 (13.3)	15

CIN2+, cervical intraepithelial neoplasia grade 2 or worse; HR-HPV, high-risk human papillomavirus; LR-HPV, low-risk and undetermined-risk HPV.

Table VI. L1-positive and L1-negative HPV types among four CIN2+ cases.

Case no.	L1-positive types	L1-negative types
1	HPV16, HPV33, HPV39	HPV31
2	HPV31	HPV18 and 58
3	HPV16	HPV52
4	HPV51	HPV16

CIN2+, cervical intraepithelial neoplasia grade 2 or worse; HPV, human papillomavirus.

L1-positive women infected with HR-HPV types included 13 infected with HPV16, and another 4 infected with other HR-HPV types; all 4 of the latter progressed to CIN2+, compared to only 2 of the former. Progression to CIN2+ among L1-positive women infected with HR-HPV, including HPV16, was found to be 35.3%, compared to only 15.4% in the group infected with both HR- and LR-HPV types (Table IV). This was confirmed in a stepwise logistic regression analysis (OR 4.46, CI, 1.4-14.212; P=0.0115).

Since HPV16 was the most predominant type detected in this study population, L1 expression was examined separately for HPV16 in correlation to CIN grade. Among 13 HPV16-positive CIN1 cases, L1 expression was found in 8 (61.5%), whereas L1 expression was only in found in only 2 of 15 (13%) HPV16-positive CIN2+ cases (P=0.026).

L1 expression in women with CIN2+ by HR-HPV. We found that among women with CIN2+, HPV16 L1 expression was rare (13.3%). Interestingly, 25.0% of women infected with HR-HPV only progressed to CIN2+. In the group of CIN2+

cases infected with both HR- and LR-HPV types, we found a much higher proportion of women that progressed to CIN2+ (35.7%) (Table V).

We analysed four CIN2+ cases with multiple HPV infections after staining with type-specific antibodies for different HPV types (Table VI). We demonstrated in this limited material that lack of L1 expression may initiate an oncogenic process that evolves over time. Most likely different HR-HPV types in combination with loss of L1 expression have different oncogenic potential for the development of CIN2+.

Discussion

HPV L1 is a major target of the immune response in HPV-infected squamous intraepithelial lesions and is only produced during a productive HPV infection at the end of the natural viral life cycle (12,21).

Different authors (21) have reported that the majority of HR-HPV-related LSIL expresses HPV L1, whereas HR-HPV-related HSIL fails to synthesise L1. Therefore, loss of L1 expression could be used as a prognostic marker for the development of preinvasive lesions. Since reports on L1 expression in women with minor cytological abnormalities are lacking, we focused on the expression of L1 in HR-HPV-positive women with ASCUS and LSIL.

In the present study L1 expression was detected in 48 (46.2%) women, whereas in 56 this expression was absent. This is in accordance with a previous report (22) where L1 expression was detected in 44% of HR-HPV-positive samples with mild or moderate dysplasia. L1 expression was associated with smears showing normal cytology (i.e., WNL) or LSIL, but was mostly absent in cases where a high-grade lesion was histologically confirmed. In the present study, we were able to confirm for the first time that previous findings from a group of HR-HPV-positive women with mild and moderate dysplasia are also valid for women with minor cytological abnormalities. Indeed, it seems that loss of L1 expression is a very early event in HPV-related carcinogenesis that can be found in samples from women with these minor abnormalities. Disturbed viral cellular interactions on transcriptional, translational and/or genomic levels interfere with squamous epithelial cell differentiation, and are responsible for the lost capability to produce L1.

Ideally, absence of L1 expression would demonstrate the oncogenic transformation initiated by HR-HPV and help identify HPV infections with long-term oncogenic potential, whereas presence of L1 expression would be indicative of a transient infection in the majority of cases, although it would not be able to exclude the existence of an HR-HPV infection.

Most probably the correlation between L1 expression and reduced risk of high-grade CIN can be explained in two different ways. First, L1 expression indicates a productive infection, i.e., the cells harbour the virus episomally, and are not integrated in a way that makes them able to release complete variants and will therefore not progress to a higher-grade lesion. However, it may also be that L1 evokes an immune response that results in clearance of the infection. In this case, L1 expression would also have prognostic value, pinpointing a chance for spontaneous clearance. The latter possibility is supported by the findings of a recent study, which showed an antagonistic interaction between HPV6/11 antibodies and decreased the risk of squamous cell carcinoma from simultaneous infection with HPV16 (23), whereas in the present study we showed that the risk of developing of CIN2+ was the same for L1-positive women infected with both HR and LR-HPV types and those infected with HPV16 (15%). The low proportion of L1-positive women infected with HPV16 that progressed to CIN2+ in our study (15%) is really surprising, as it is known that HPV16 has the highest risk of progression to CIN2+ compared to all other HPV types. Therefore, it could be speculated that as long as L1 is produced, HPV16 infection can be cleared as effectively as infection with other HR-HPV types, but that HPV16 may be able to turn off L1 expression more rapidly to escape immune activation, and clearance of the infection. Among L1-positive CIN2+ cases, we found an increasing proportion infected with both HR and LR-HPV types (35.7%).

In addition to this, we showed for the first time that L1-positive minor cytological abnormalities were associated with multiple HPV infections. In addition, women with minor cytological abnormalities who were later diagnosed with CIN2+ in our study showed different L1 expression profiles for different HR-HPV types. Using type-specific L1 antibodies for different HPV types, it became obvious that progression to CIN2+ was associated with a mixture of L1-positive and L1-negative HR-HPV infections. Most probably the different L1-negative HPV types were responsible for the development of CIN2+ in those women. Interestingly, we found that not all high-grade lesions were associated with loss of HPV16 or HPV18 L1 expression. One may speculate that these types were present, but not overrepresented in CIN2+ cases with multiple HR-HPV infections and lack of L1 expression. It is likely that the different HPV types initiated a parallel oncogenic process that evolved during the course of follow-up, but that only the L1-negative type prompted the development of CIN2+.

Our data suggest that L1 detection in combination with HPV typing could be used for a more focused investigation of women with minor cytological abnormalities. Using our data, the clinical management of these women could be improved, and treatment recommended according to the individual risk profile of the woman rather than the present practice of basing treatment on the generalised risk of groups with similar histological changes.

In the present study, we found that minor cytological abnormalities (ASCUS and LSIL) were mixtures of distinct biological stages that resulted in either clearance or progression of HPV infection. Taking these data into account it seems reasonable to consider L1-positive ASCUS and LSIL as lesions with low malignant potential, calling for a 'wait and see' strategy with follow-up. However, in women infected with HR-HPV types, L1-negative ASCUS/LSIL has a precancerous character and special attention should be paid to these women.

The implications of this would be a new way of thinking about the clinical management of women with minor cytological abnormalities. Various screening strategies have been proposed in which HPV testing is combined with cytological examination to increase sensitivity. HPV testing has shown satisfactory sensitivity, but it lacks specificity to be used as a primary screening tool especially in younger women (24). The ideal would be an objective test that distinguishes between

women with non-progressive mild neoplasia and those at risk of oncogenic transformation that could lead to invasive cancer, to facilitate therapy decisions and determine the need for follow-up. In this scope HPV L1 detection can probably help clinicians in the future. Although in the future, HPV vaccination might prevent up to 80% of cervical cancers worldwide, the need for a marker is likely to remain.

The present study shows that HPV L1 capsid protein detection is able to predict the clinical outcome of early dysplastic lesions, allowing for discrimination between transient HPV infections and risk of progression to cancer, and could be used as an objective standard to optimise the clinical management of squamous intraepithelial lesions.

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