

Human Papillomavirus L1 Capsid Protein and Human Papillomavirus Type 16 as Prognostic Markers in Cervical Intraepithelial Neoplasia 1

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Introduction: The aim of the study was to determine whether human papillomavirus (HPV) L1 capsid protein and the HPV genotype can predict the disease course as prognostic markers for cervical intraepithelial neoplasia 1 (CIN1).

Methods: Immunohistochemical staining was performed for HPV L1 capsid protein in 101 women who had been confirmed to have CIN1 by histologic examination and HPV high-risk infection by HPV genotyping. The disease course was analyzed by follow-up histologic examination according to the HPV L1 capsid protein and HPV genotype over a minimum of 12 months.

Results: The CIN1 regressed spontaneously in 60.4% of the women; most cases of regression occurred within 1 year (90.9% of regression cases). The HPV L1 capsid protein-positive patients had a spontaneous regression rate of 72.7% (48/66) and a rate of persistent disease or progression to higher grade disease of 27.3% (18/66). The HPV L1 capsid protein-negative women had a regression rate of 37.1% (13/35) and a rate of persistent disease or progression of 62.9% (22/35; $P < 0.001$). The HPV-16-infected patients had a regression rate of 38.6% (17/44) and a rate of persistent disease or progression of 61.4% (27/44), whereas the non-HPV-16-infected patients had a regression rate of 77.2% (44/57) and a rate of persistent disease or progression of 22.8% (13/57; $P < 0.001$).

Conclusions: The HPV L1 protein expression is closely related to spontaneous disease regression, but HPV-16 infection is related to persistent disease or progression to high-grade lesions in patients with CIN1.

Key Words: L1 capsid protein, HPV-16, CIN, Prognostic marker, Biomarker

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Cervical cancer of the uterus is the second most common cancer in women but is thought to be a preventable disease. Indeed, the incidence of cervical cancer has decreased

markedly in developed countries in the last 2 decades. The decreasing trend most certainly results from early diagnosis through effective screening programs and intervention of precursor lesions rather than a significant improvement in cervical cancer management.^{1–2}

Although most precursor lesions (cervical intraepithelial neoplasia [CIN]) are thought to regress spontaneously, there are potentially malignant lesions that may develop into cervical cancer over time; no reliable cytomorphologic criteria are available to accurately predict the fate of CIN.² Various possible prognostic markers of CIN have been suggested, including L1 capsid protein and human papillomavirus (HPV) genotype. The HPV L1 capsid protein is the major protein comprising the viral capsid and can assemble viruslike particles that are associated with immune responses, and some HPV genotypes are known to have more oncogenic properties than other HPV genotypes.^{3–11}

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In the present study, we have investigated the regression and progression rates of CIN1 based on the expression of HPV L1 capsid protein and the HPV genotype and ascertained whether the HPV L1 capsid protein and HPV genotype can serve as prognostic markers for CIN1.

MATERIALS AND METHODS

Subjects and Methods

The study population was selected from Korean women who had been referred to the Department of Obstetrics and Gynecology at Chonnam National University Hospital for further evaluation of abnormal cervical cytologic feature. Before colposcopic assessment, a cervical sample was obtained from all the patients for HPV DNA analysis and liquid-based cytologic analysis (ThinPrep; Cytec Corporation, Boxborough, Mass) by scraping across the entire transformation zone using a sterile cytobrush. After the cervical scraping was obtained, a colposcopic examination and cervical punch biopsy were performed to assess the grade of dysplasia.

The histologic diagnosis was divided into the following categories: normal (including cervicitis and squamous metaplasia), CIN1, CIN2/CIN3, squamous cell carcinoma, and adenocarcinoma. Infection with high-risk HPV types was determined using a commercially available HPV DNA chip test (MyHPV Chip; MyGene Co, Seoul, South Korea).

One hundred nine women who were confirmed to have CIN1 and were high-risk HPV positive were included in this study. After histologic confirmation of CIN1, the patients were closely followed with liquid-based cytologic analysis, HPV DNA chip testing, and colposcopic examination after 3 months and then biannually for at least 1 year. Disease progression was defined as a histologic worsening of the lesion within the observation period, that is, CIN1 to CIN2 or CIN3. Regression was defined as downgrading of either the histologic grade of dysplasia, that is, CIN1 to normal, or normalization of cytologic feature, for 2 or more follow-up examinations with confirmation by histologic examination through colposcopy-guided punch biopsy. Persistent disease was defined as the presence of a lesion for at least 12 months.

Assessment of Initial HPV Genotypes by the HPV DNA Chip Test

The HPV genotypes were assessed by the HPV DNA chip kit, a polymerase chain reaction (PCR)-based DNA microarray system (MyHPV Chip). The HPV DNA chip kit contains 24 type-specific probes: 15 probes are from high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68), and 9 probes are from low-risk HPV types (6, 11, 34, 40, 42, 43, 44, 54, and 70). Twenty-four type-specific 30-mer oligonucleotide probes containing an amine group at the 5' terminal are immobilized onto a chip slide glass. Each slide has 8 chambers, and each chamber is used for 1 test. Therefore, a slide simultaneously tests 8 samples. Briefly, DNA was isolated from swab samples using a DNA isolation kit (MyHPV Chip), and target L1 regions of HPV DNA were amplified and labeled by a single dye (indocarbocyanine-deoxyuridine-5'-triphosphate; MEN Life Science Products

Inc, Boston, Mass), using consensus GPd5+/GP6d+ primers. β -Globin was amplified using a PCR as an internal control. The PCR products of all the samples were detected by electrophoresis with a product size of 150 base pairs. Ten microliters of the HPV-amplified product was denatured for 5 minutes at 95°C. The samples were mixed with a hybridization solution and then applied onto the DNA chip. Hybridization was performed at 43°C for 90 minutes and followed by washing with 3× saline, sodium phosphate, EDTA buffer (SSPE; Bio Basic Inc, Markham, Ontario, Canada) for 5 minutes, and 1× SSPE for 5 minutes and then dried at room temperature. Hybridized HPV DNA was visualized using a DNA chip scanner (Scanarray Lite; GSI Lumonics, Ottawa, Ontario, Canada). The HPV amplicons can be hybridized with corresponding type-specific oligonucleotide probes and visualized on HPV DNA chip slides as double-positive spots when HPV DNA is present in the amplified PCR product. The samples that had a positive band of 150 base pairs on gel electrophoresis but negative on the HPV DNA chip slide were designated as "HPV others." None of the negative controls (without DNA) were HPV positive.

Immunohistochemical Stain for HPV L1 Protein

All of the biopsy specimens were immunohistochemically stained using a pan-reactive HPV L1 antibody (Cytoactiv; Cytoimmun Diagnostics Ltd, Pirmasens, Germany). Sections (4 μ m) were cut from a paraffin block. For immunohistochemical staining, the sections were deparaffinized and rehydrated. The slides were placed in 10-mmol/L citrate buffer (pH 6.0) and heated in a pressure cooker for 30 seconds at 125°C, then for 1 minute at 90°C. The antigen in the routinely stained specimens was revealed by boiling in citrate buffer without prior destaining. After immunostaining using a screening antibody directed against the HPV L1 capsid protein (Cytoactiv) and incubation using a biotin-linked anti-mouse antibody, the streptavidin-biotin complex enzymatic method was used for detection of HPV L1 capsid protein (SABC Detection System; Dako Ltd, Hamburg, Germany). Antigen visualization was achieved by applying 3-amino-9-ethyl carbazole chromogen (Dako Ltd, Glostrup, Denmark), followed by counterstaining with hematoxylin. Positive controls from the L1-positive specimens provided by the manufacturer of the HPV L1 capsid antibody were used for each staining series. The immunostained slides were studied using light microscopy and classed as positive when there was a clear nuclear staining. One positively stained cell was interpreted as positive.

Statistical Analysis

The data were analyzed using the Statistical Package Service Solution software (SPSS for Windows, standard version 12.0; SPSS, Inc, Chicago, Ill). The data were dichotomized for statistical purposes for patients with regression versus patients with nonregression, including persistent or progressive disease. The Pearson χ^2 test (2-sided) was used to identify significant differences between the HPV L1-positive and HPV L1-negative cases. A $P < 0.05$ was considered significant. The odds ratio estimation was calculated for

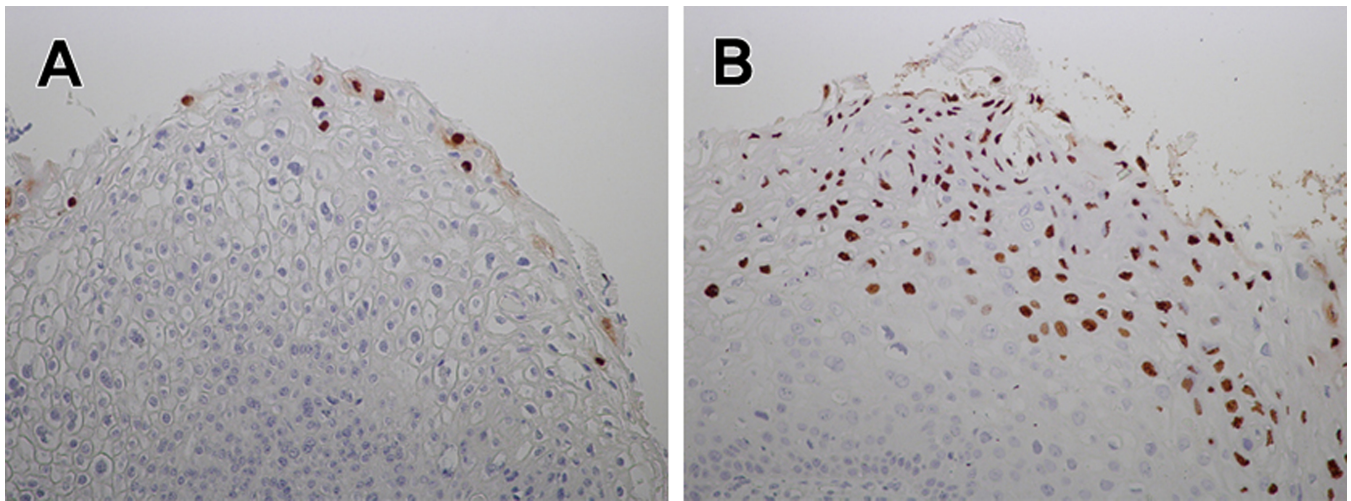


FIGURE 1. Immunohistochemical stain for HPV L1 capsid protein. A, Focal positive nuclear staining with HPV L1 antibody (uterine cervix; original magnification: A, $\times 200$; immunohistochemical stain with L1 capsid antibody). B, Diffuse positive nuclear staining with HPV L1 antibody (uterine cervix; B, $\times 200$; immunohistochemical stain with L1 capsid antibody). The immunohistochemical stain was interpreted as positive when there was a clear nuclear staining. One positively stained cell was interpreted as positive.

the probability of progression within the follow-up period. Ninety-five percent confidence intervals were calculated.

RESULTS

Characteristics of the Subjects

One hundred nine women who had been confirmed to have CIN1 by histologic examination and high-risk HPV infection by HPV DNA chip test were selected. Eight women were lost to follow-up and thus were excluded from the analysis. The final study population comprised 101 eligible women who met the above-mentioned conditions. The mean age of the subjects was 42.7 years (range, 26–77 years). The mean follow-up period was 21.3 months (range, 12.0–48.1 months) in the entire study population; the mean follow-up period was 20.7 months in the HPV L1 capsid protein–positive women and 21.7 months in the HPV L1 capsid–negative women. The mean age of the regression and progression groups was 41.6 years (range, 26–65 years) and 44.1 years (range, 28–77 years), respectively. The mean age of the regression and progression groups was 40.9 years (range, 26–65 years) and 44.2 years (range, 31–77) in the HPV L1 capsid protein–positive women, whereas 43.7 years (range, 26–62 years) and 44.9 years (range, 28–67 years) in the HPV L1 capsid protein–negative women, respectively.

Human Papillomavirus Genotype Analysis

The initial prevalence of the HPV genotype detected by the HPV DNA chip test was as follows: HPV-16 (43.6% [44/101]) was the most prevalent genotype in all the specimens, followed in order of prevalence by HPV-58 (18.8% [19/101]), HPV-18 (16.8% [17/101]), HPV-53 (13.9% [14/101]), HPV-33 (11.9% [12/101]), HPV-66 (8.9% [9/101]), HPV-31 (6.9% [7/101]), HPV-52 (6.9% [7/101]), HPV-56

(6.9% [7/101]), HPV-39 (4.9% [5/101]), HPV-35 (2.9% [3/101]), HPV-45 (1.9% [2/101]), HPV-51 (1.0% [1/101]), HPV-68 (1.0% [1/101]), and all other types (6.9% [7/101]). A single infection type was identified in 48.5% (49/101) of the women, and infection by 2, 3, 4, and 5 different genotypes occurred in 33.7% (34), 7.9% (8/101), 1.9% (2/101), and 1% (1/101) of the subjects, respectively. The prevalence of HPV genotypes was determined by calculating the percentage of each HPV genotype within the study population.

Immunohistochemical Stain for HPV L1 Capsid Protein

Positive nuclear staining was observed in 65.3% (66/101) of those women with HPV L1 capsid antibody, and no immunohistochemical staining was noted in 34.7% (35/101) of the women (Fig. 1). The HPV L1 capsid protein was expressed in 63.6% (28/44) of the women positive for HPV-16, 57.9% (11/19) of the women positive for HPV-58, 76.5% (13/17) of the women positive for HPV-18, 85.7% (12/14) of the women positive for HPV-53, 75.0% (9/12) of the women positive for HPV-33, and 57.1% (5/7) of the women positive for HPV-31.

TABLE 1. Overall disease course based on L1 capsid protein

L1 Capsid Protein (n)	Regression		Persistent Disease or Progression		P
	Case	Rate, %	Case	Rate, %	
Positive (66)	48	72.7	18	27.3	<0.001
Negative (35)	13	37.1	22	62.9	
Overall	61	60.4	40	39.6	

TABLE 2. Disease course based on a combination of HPV genotype and L1 capsid protein

HPV Genotype (n)	n	L1 Protein Positive/L1 Protein Negative				P
		Regression		Persistent Disease or Progression		
		Case, n	Rate, %	Case, n	Rate, %	
HPV-16 (44)	28/16	14/3	50.0/18.8	14/13	50.0/81.2	0.041
HPV-58 (19)	11/8	8/4	72.7/50.0	3/4	27.3/50.0	0.377
HPV-18 (17)	13/4	10/1	76.9/25.0	3/3	23.1/75.0	0.099
HPV-53 (14)	12/2	10/0	83.3/0	2/2	16.7/100	0.066
HPV-33 (12)	9/3	7/1	77.8/33.3	2/2	22.2/66.7	0.236
HPV-31 (7)	4/3	4/2	100/66.7	0/1	0/33.3	0.429

Analysis of Disease Course

As shown in Table 1, the overall regression rate was 60.4% (61/101), and the rate of persistent disease or progression was 39.6% (40/101). Most cases in this study with regression (90.2% of regression cases [55/61]) occurred within 12 months. Seven of the patients with progression had CIN3 or carcinoma in situ; there were no cases of invasive cancer. The HPV L1 capsid protein–positive patients revealed spontaneous regression in 72.7% of the cases (48/66) and persistent disease or progression to high-grade lesions in 27.3% of the cases (18/66); the HPV L1 capsid protein–negative patients had regression in 37.1% of the cases (13/35) and persistent disease or progression in 62.9% of the cases (22/35). The difference in disease course between HPV L1–positive and HPV L1–negative patients was statistically significant (sensitivity, 78.7%; specificity, 55.0%; positive predictive value, 72.7%; negative predictive value, 62.9%; odds ratio, 4.51; 95% confidence interval [CI], 1.88–10.8; *P* < 0.001).

As summarized in Table 2, HPV type-specific behaviors were analyzed according to a combination of HPV L1 protein and HPV genotype, but the analysis did not demonstrate a correlation between type-specific behavior of disease based on the combination of HPV L1 protein and HPV genotype because the data did not have statistical signifi-

cance, except for cases that were HPV-16 positive and HPV L1 protein positive.

As summarized in Table 3, the disease course was analyzed according to each HPV genotype, with the exclusion of HPV L1 capsid protein in 3 major genotypes in Korean women with CIN1, through classification into small groups as follows: HPV-16 versus non–HPV-16, HPV-58 versus non–HPV-58, and HPV-18 versus non–HPV-18. In the HPV-16–positive group, the rate of disease regression was 38.6% (17/44) and persistent disease or progression occurred in 61.4% of the cases (27/44). On the other hand, in the non–HPV-16 group, the rate of disease regression was 77.2% (44/57) and persistent disease or progression was present in 22.8% (13/57) of the cases. The HPV-16 genotype infection was associated with a tendency of more frequent persistent lesions and progression to high-grade lesions (ie, poor prognosis) than other genotypes (sensitivity, 65.9%; specificity, 72.1%; positive predictive value, 61.4%; negative predictive value, 77.2%; odds ratio, 5.38; 95% CI, 2.26–12.79; *P* < 0.001). However, the other genotypes were not statistically significant.

DISCUSSION

It is well known from previous studies that the spontaneous regression rate of biopsy-confirmed CIN1 is

TABLE 3. Analysis of the disease course based on HPV genotypes in the following 3 major types: HPV-16/non–HPV-16, HPV-58/non–HPV-58, and HPV-18/non–HPV-18

Genotype	Patients	Major Types (n)	Regression		Persistent Disease or Progression		OR	95% CI	P
			Case	Rate, %	Case	Rate, %			
HPV-16	Case	HPV-16 (44)	17	38.6	27	61.4	5.38	2.26–12.79	<0.001
	Control	Non–HPV-16 (57)	44	77.2	13	22.8			
HPV-58	Case	HPV-58 (19)	12	63.2	7	36.8	0.86	0.31–2.43	0.785
	Control	Non–HPV-58 (82)	49	59.8	33	40.2			
HPV-18	Case	HPV-18 (17)	11	64.7	6	35.3	0.80	0.27–2.38	0.691
	Control	Non–HPV-18 (84)	50	59.5	34	40.5			

OR, odds ratio; CI, confidence interval.

60% to 85%.^{2,11,12} Most cases of regression occur within 2 years.^{2,11,12} In the present study, 60.4% of CIN1 cases regressed spontaneously without any treatment, and most regressions occurred within 1 year (90.9% of regression cases). In previous reports, the rate of disease regression was 49.1% to 90.6% in HPV L1 protein-positive patients.³⁻⁷ These rates compared with the regression rate of 72.7% in the present study. The differences in regression rates can likely be attributed to differences in the study populations. In the present study, the study population was restricted to patients who had biopsy-confirmed CIN1 and were high-risk HPV positive, whereas in several previous reports, CIN1, CIN2, and CIN3 were included in the study population and persistent disease were classified as stable disease, that is, the stable/regression group.³⁻⁷ However, persistent disease should not be classified as regression or stable disease because persistent high-risk HPV-positive women have a higher risk for developing CIN or invasive cervical cancer in comparison with HPV-negative patients and persistent CIN has malignant potential.^{2,13-15} In addition, the present study differed from the above-mentioned studies in that this study was not based on cytologic diagnosis but rather on histologic diagnosis. Indeed, colposcopy-guided histologic diagnosis is more reliable than cytologic diagnosis in women with CIN.³⁻⁷ The present study cope with the debate concerning CIN 1 diagnosis. It is a dilemma that CIN1 lack interobserver and intraobserver reproducibility and the most lack of reproducibility occur between koilocytosis and CIN1. Therefore, misinterpretation is within the bounds of possibility in the interpretation of disease behavior. We should have overcome the dilemma, but topic of the present study has not been focused on CIN1 triage but on the role of L1 and HPV genotype as prognostic markers in CIN1, and we have tried to verify the errors. Colposcopic cervical biopsy was undertaken by an experienced gynecologic oncologist, and cervical specimen was interpreted by multiple experienced histopathologists blind to L1 protein expression data and reviewed retrospectively.

In the present study, HPV-16 infection was related to persistent disease or progression to high-grade lesions. This is in agreement with the epidemiologic evidence and experimental studies that have shown that the HPV-16 genotype has more oncogenic properties than other genotypes.^{2,13,15,16} However, the disease behavior of other HPV genotypes did not have statistical significance. Etherington et al¹⁷ reported that HPV-16 intratypic variants are detected significantly more often in high-grade CIN, and the variants suggest the lack of an antibody response to HPV-16 virus-like particles and an altered base sequence of E6 gene. Thus, the natural variants of HPV-16 may have differences in pathogenicity, and HPV has numerous mechanisms by which to escape immune responses.^{17,18} Some of the HPV L1 protein-positive patients progressed to high-grade CIN, some of the HPV L1-negative patients regressed spontaneously, and some of the HPV-16-positive patients regressed. It has been suggested that complex immune responses play a role in determining disease course, but it is not possible to dissect the precise relevance of innate or adaptive responses, or humoral or cell-mediated immunity in women with HPV infections.^{2,19,20}

In the 2006 American Society for Colposcopy and Cervical Pathology consensus guidelines for the management of women with CIN or adenocarcinoma in situ, a watch-and-wait approach was recommended; that is, cytologic follow-up and optional HPV DNA testing was recommended for women with biopsy-confirmed CIN1 who have low-grade referral cervical cytology, regardless of whether the colposcopic examination is satisfactory. If CIN1 persists for at least 2 years, either continued follow-up or treatment is acceptable.²¹ As shown in the present study, HPV L1 capsid protein and HPV-16 may offer prognostic information regarding regression or disease progression. Thus, the management modality to either continue follow-up or treatment may be determined beforehand by the results of cytologic analysis and clinical judgment, including the results of HPV L1 capsid protein and HPV typing. The above-mentioned facts are essential to support the basis for the clinical strategy that overtreatment and see-and-treat should be avoided in women with CIN1, particularly in women who are HPV L1 protein-positive, but close observation is required in HPV-16-infected women in clinical settings.^{2,3-7,21} To clarify the clinical role of HPV L1 protein and HPV genotype, technical improvements are essential with sensitive, reliable, and simple techniques for detecting HPV L1 protein and HPV genotype. Recently, simple commercial detection kits became available, such as Cytoactiv for HPV L1 capsid protein and MyHPV Chip for HPV typing. The HPV L1 capsid protein and HPV are detected within the superficial layer of the cervical epithelium, and the cells are easily collected by a routine cervical smear.^{4,22,23} However, HPV detection at a single point in time does not provide reliable prognostic information because high-risk HPV is frequently present in normal cervical smears and HPV infections may be transient. Serial monitoring of HPV infection every 6 to 12 months may help to identify CIN lesions at high risk for progression.^{2,13,21,24}

The initial aim of this study had the following 2 goals: (1) determine the correlation between HPV L1 capsid protein and disease course and (2) determine the difference between genotype-specific disease behavior in accordance with HPV L1 capsid protein. The presumption was based on the greater oncogenic properties of HPV-16 and HPV-18 compared with other genotypes, and there may have been differences in type-specific behavior with HPV L1 capsid protein. Unfortunately, the latter aim was not achieved because there was no statistical significance, except for the HPV-16 and HPV L1 protein-positive combination, which may have resulted from the small sample size in the non-HPV-16 genotypes. Meaningful outcomes may be derived through a large-scale study, such as a meta-analysis.

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