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Original Research

Human papillomavirus genotyping is a reliable prognostic marker of recurrence for high-grade cervical intraepithelial neoplasia (CIN2-3) with positive margins after loop electrosurgical excision procedure

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Summary

Objective: The study was performed to determine whether the human papillomavirus (HPV) genotype result from the HPV DNA chip test (HDC) was predictive of recurrent high-grade cervical intraepithelial neoplasia (CIN2-3) in patients with positive margins after a loop electrosurgical excision procedure (LEEP). *Methods*: A total of 184 patients with histologically confirmed CIN2-3 identified at the margin of a LEEP specimen were followed with HDC testing, hybrid capture II (HC2) analysis, and cytology examinations. Post-LEEP monitoring was conducted at 3, 6, 9, 12, 18, and 24 months during the first two years and annually thereafter. *Results*: Of the 184 patients, the HC2 test was positive in 179 patients (97.3%) and the HDC test was positive in 181 patients (97.6%) before the LEEP. The overall agreement between the HC2 and HDC tests was 98.9%. Forty-six (25.0%) patients developed a recurrence, and those who experienced a relapse tested positive for the same high-risk HPV genotype detected before the LEEP. Identifying the same high-risk HPV genotype by HDC testing during the follow-up period had a negative predictive value and a sensitivity of 100% in diagnosing recurrent lesions. HPV-18 was related to recurrent CIN2-3. A significant association between HPV-18 infection and recurrent CIN2-3 was found ($p < 0.05$). *Conclusions*: In patients with CIN2-3 identified at the margins of a LEEP specimen, the persistence of the same high-risk HPV infection, especially HPV-18, should be regarded as a risk factor for recurrent CIN2-3. After a LEEP, such patients require particular attention with short-term follow-up.

Key words: Loop electrosurgical excision procedure; High-grade cervical intraepithelial neoplasia; Cone margin; High-risk human papillomavirus testing.

Introduction

High-grade cervical intraepithelial neoplasia (CIN2-3) of the cervix is a premalignant lesion caused by high-risk human papillomavirus (HPV). The most common types of HPV are viral types 16 and 18 [1]. Persistent high-risk (HR) HPV infections are significantly related to the progression of CIN2-3 and are thought to progress to cervical cancer [2]. Conservative therapy with a loop electrosurgical excision procedure (LEEP) has been performed to diagnose or treat CIN2-3 [3]. However, the residual or recurrent disease rates after a LEEP range between 5-30%, receiving follow-up and retreatment once lesions have been identified [4]. After conization, close monitoring is necessary for the early detection of residual and recurrent lesions because recurrent CIN2-3 has the risk of developing into invasive carcinoma unless sufficient treatment is administered. New high-risk HPV diagnostic methods have been used to monitor for the persistence of HR HPV infections in patients treated for CIN2-3 at every visit as an important predictor of recurrent or residual CIN2-3 after a LEEP. Thus, there is mounting evidence that HR HPV and cervical cytology co-testing should be utilized for the follow-up of patients treated for CIN2-3 [5]. Thus, the purpose of this report was to determine if the HPV genotype results of the HPV DNA chip test (HDC) were clinically useful predictive markers of recurrent or residual lesions during the postoperative follow-up of CIN2-3 patients.

Materials and Methods

The medical records of all 206 patients with histologically confirmed CIN2-3 identified at the margin of a LEEP specimen in the Department of Obstetrics and Gynecology of Chonnam National University Hospital between January 2002 and February 2013 were reviewed retrospectively.

Altogether, 184 women were suitable for the study if they met the following inclusion criteria: (1) pathologically confirmed CIN2-3 by a LEEP; (2) patients with both pre- and post-LEEP HR HPV test results from the hybrid capture II assay (HC2) (Digene Co, Gaithersburg, MD) and the HDC test; and (3) women who received follow-up examinations for ≥ 5 years. We excluded 22 patients with a residual CIN2-3 lesion that was confirmed within three months

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following the LEEP. High-risk HPV test results from the HDC and HC2, histology data, and epidemiologic data were collected through the medical records. The LEEP was performed under local anesthesia utilizing wire loop electrodes with a diathermy apparatus set. An orienting mark was made at the 12 o'clock position of the LEEP specimen and the specimens for pathologic examination were fixed in 10% formalin. Endocervical cytology was performed on all patients immediately after the LEEP. The patients received postoperative follow-ups at 3, 6, 12, 18, and 24 months during the first two years, and annually thereafter. At all follow-up visits, all the patients underwent HPV DNA tests (HC2 and HDC), and cytology and colposcopy and endocervical sampling were performed if the HPV DNA test was positive or if repeat cytology revealed atypical squamous cells of undetermined significance or greater.

The criteria for a recurrent or residual lesion was based upon positive histologic results in endocervical sampling or a colposcopy-directed biopsy. If CIN2-3 was confirmed within three months after a LEEP, it was regarded as a residual lesion. Patients with CIN2-3 detected on a colposcopic biopsy at a subsequent visit (from six months onward) were regarded as having recurrent lesions. For the statistical analysis, the cervical biopsy results during monitoring were categorized as positive for CIN2-3 findings, and negative for CIN1 findings, normal findings, or cervicitis. Positive histologic findings during monitoring were regarded as recurrent lesions. The study protocol was reviewed and approved by the Institutional Review Board at Chonnam National University Hwasun Hospital (CNUHH).

Hybrid capture II assay

All patients underwent HPV testing with the HC 2 system before the LEEP and at 3, 6, 9, 12, 18, and 24 months postoperatively. HPV DNA testing by the HC2 assay method was accomplished using an automated HC2 assay system in accordance with the manual of the manufacturer. Cervical samples for the HC 2 test were collected using a cytobrush (Digene Cervical Sampler; Digene Corporation, Gaithersburg, MD, USA) and placed in a vial containing Digene Specimen Transport Medium (STM). The samples were investigated only for the presence of HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. HPV type 16 (HPV-16) DNA (1 pg/mL) was utilized as a positive control (PC). The samples were identified as HR HPV DNA-positive if the relative light unit (RLU) reading from the luminometer was the same or greater than the mean value of the PC. The RLU/PC ratio was used as a semiquantitative measure of the amount of HR HPV DNA [6].

HPV DNA chip assay

HPV genotyping was performed using a DNA isolation kit (MyGene Co, Seoul, South Korea) and a commercially accessible HPV DNA chip PCR-based microarray system containing 24 type-specific probes (nine probes were low-risk types 6, 11, 34, 40, 42, 43, 44, 54, and 70 and 15 probes were HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) according to the manufacturers' instructions. Briefly, the amplified DNA was tagged with a single dye, indocardocyanine-dUTP (MEM Life Science Products, Inc., Boston, MA, USA). The PCR manufacture size of the HPV DNA was 150 base pairs (bp) on 2.5% agarose gel electrophoresis. The labeled PCR was hybridized onto the chip. Hybridization was conducted at 43 °C for 90 min and the product was rinsed with 3× saline-sodium phosphate-ethylenediamine tetraacetic acid (SSPE) for 5 min and with 1× SSPE for 5 min before dehydrating at room temperature. The hybridized signals were observed using a DNA chip scanner (Scanarray Lite; GSI Lumonics, Ottawa, Canada). The samples with a positive 150 bp band visualized on HPV PCR gel electrophoresis but a negative hybridized signal on the DNA chip scanner (15 HR HPV types were used in the HDC test) were designated HPV-other-type samples [7]. A lack of bands and samples that were negative for the 150 bp HPV-specific band on gel electrophoresis were considered HPV-negative.

Statistical analysis

The data are reported as the mean and standard deviation. p-values pf < 0.05 were regarded as statistically significant and all p-values were 2-sided. Agreement between the tests was determined by Cohen's kappa statistic and the p-value was assessed using McNemar's test, with values between 0.81-1.00 indicating near-perfect agreement, 0.61-0.80 substantial agreement, 0.41-0.60 moderate agreement, 0.21-0.40 fair agreement, and 0.00-0.20 indicating poor agreement. The correlation between other clinical factors and recurrence with post-LEEP HPV status was estimated by the Student's t-test or Fisher's exact test. The clinically most significant HR HPV cutoff value for viral load for predicting recurrent or residual lesions was determined by the receiver operating characteristic curve. The 95% confidence intervals (CIs) were estimated. The data were analyzed using SPSS for Windows, version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

The mean age of the patients was 42.8 years (range, 23-78 years). CIN2-3 was confirmed by histology. CIN2 was found in eight patients and CIN3 was found in 176 patients. Of the 184 patients, 46 (25%) progressed to recurrences during the hospital visit and histology disclosed 43 cases of CIN3 (93.5%) and three cases of CIN2 (6.5%). No invasive carcinoma was found. The mean lag time between the LEEP and the detection of a recurrent lesion was 23.5 months (range, 6-94 months). The HPV diagnosis rate by the HC2 assay was almost the same as that of the HDC test. Out of a total of 184 patients, 179 (97.3%) were positive on the HC2 test and 181 (97.6%) were positive on the HDC test. No significant differences in HR HPV status intraoperatively, former cytologic abnormalities, CIN grade, and cured patients with respect to age were found between the patients with recurrent lesions. Follow-up cytology, posi-

Table 1. — *Patient characteristics.*

	No recurrence N = 138	Recurrence N = 46	*p*
Age (years)			0.3
Mean + SD	38.5 + 8.4	47.1 + 12.2	
Range	23-70	26-78	
Menopause			< 0.01
No	122	28	
Yes	16	18	
Initial cytology			0.31
ASCUS	16	2	
LSIL	5	1	
HSIL	117	43	
CIN at LEEP			0.42
CIN2	5	3	
CIN3	133	43	
Pre-LEEP HC2			> 0.99
Negative	4	1*	
Positive	134	45	
Pre-LEEP HDC			0.57
Negative	3	0	
Positive	135	46	
Endocervical cytology at LEEP			< 0.01
Negative	114	21	
Positive	24	25	
Follow-up cytology			< 0.01
Negative	118	7	
≥ ASC	20	39	
Post-LEEP HC2			< 0.01
Negative	125	1**	
Positive	13	45	
Post-LEEP HDC			< 0.01
Negative	124	0	
Positive	14	46	

*SD, standard deviation; ASCUS, atypical squamous cells of undetermined significance; LSIL, low squamous intraepithelial lesion; HSIL, high squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia; LEEP, loop electrosurgical excision procedure; HC2, Hybrid Capture II test; HDC, HPV DNA chip test. *HR-HPV genotype by the HPV DNA chip test is HPV-53. ** HR-HPV genotype by the HPV DNA chip test is HPV-53.*

tive endocervical cytology, and HR HPV diagnosis by the HDC and HC2 tests postoperatively were correlated with a higher risk of recurrent or residual lesions. The genotyping of one HDC-positive/HC2-negative patient with a recurrent lesion revealed HPV-53 (Table 1). The CIN grade at the point of the conization and former cytologic abnormalities were not related to postoperative HR HPV genotypes ($p = 0.31$ and 0.42, respectively). Positive intraoperative endocervical cytology was statistically significant ($p < 0.01$). The discordant results and concordance between both HPV tests are summarized in Table 2. The overall agreement between the two tests was 98.9%, with a kappa value of 0.745 ($p < 0.001$). Of the 179 HR HPV-positive specimens by the HDC test, the HC2 assay was positive in 179 patients (100%). Among the five specimens that were negative by the HC2 test, the HDC test was negative in three specimens (60%). In a comparison between the HDC and the HC2 test results, discordant results were seen in two (1.1%) specimens, two HC2-negative/HDC-positive samples and no HC2-positive/HDC-negative results. All 46 patients who experienced recurrences were positive for the same HR HPV genotype by the HDC test. In contrast, none of the seven patients with dissimilar HR HPV genotypes experienced a recurrence (Table 3). The area under the receiver operating characteristic curve for the HR HPV viral load predicting recurrent or residual lesions was 0.547 ($p = 0.371$). Table 4 shows the positive predictive value, negative predictive value, sensitivity, and specificity for postoperative recurrent or residual lesions with the same HR HPV genotype by the HDC test, HR HPV detection by both the HC2 and HDC tests after a LEEP, endocervical cytology, and follow-up cytology. Finding the same high-risk HPV genoype by HDC testing during monitoring as was identified before the LEEP had a negative predictive value and sensitivity of 100% for diagnosing recurrent or residual lesions. The distribution of prevalent HR HPV genotypes by the HDC test in recurrent lesions is summarized in Table 5. Among the 46 patients who had recurrent lesions, 43 patients (93%) tested positive for infection with a single HR HPV infection. HPV-16 (53.5%, 23/43) was the most common single HR HPV infection type. The next most prevalent types were HPV-18 (18.6%, 8/43) and HPV-58 (11.6%, 5/43). HPV-18 correlated more with the recurrence of CIN2-3 ($p < 0.05$).

Discussion

All patients who undergo a LEEP are at increased risk for invasive carcinoma of the cervix for a minimum of 10 years postoperatively compared to the general public [8]. The identification of a predictor that can identify the patients at increased risk for recurrent CIN or cervical cancer after a LEEP could enable the delivery of proper therapy depending upon the patient‘s individual risk, thereby reducing patient anxiety and avoiding overtreatment. The size of the cervical excision is also known to correlate with the risk of preterm birth, so balancing the risk of accurate therapy with iatrogenic complications is critical [9].

In this study, 46 of 184 patients (25%) had recurrent lesions, consistent with the reports of previous studies [10-12]. According to the recent American Society for Colposcopy and Cervical Pathology Consensus guidelines [5, 13], when margins are positive for CIN 2-3 at the time of the excisional procedure in patients 25 years or older who are not concerned about the potential effect of treatment on future pregnancy outcomes, repeat excision or observation is acceptable. For observation, HPV-based testing in six months is preferred and it is also acceptable to perform a colposcopy and endocervical sampling at six months. For

Table 2. — *The level of concordance between HR-HPV tests.*

	No. of specimens (%) with HDC*		Total no. of specimens (%)
	Negative	Positive	
HC2*			
Negative	3	2	5 (2.7)
Positive	0	179	179 (97.3)
Total	3 (1.6)	181 (97.6)	

HR, high risk; HPV, human papillomavirus; HC2, Hybrid Capture II test; HDC, HPV DNA chip test.
** Absolute agreement = 98.9%, kappa = 0.745 ($p < 0.001$). Agreement between tests was assessed by Cohen's kappa statistic. p value was calculated using McNemar's test.*

Table 3. — *Comparison of the two groups divided according to HR-HPV genotype infection after a LEEP.*

	Persistent HR-HPV infection		
	Different subtype (%) N = 7	Same subtype (%) N = 53	*p*
Menopause			0.02
No	1 (14.3)	34 (64.2)	
Yes	6 (85.7)	19 (35.8)	
Initial cytology			0.1
ASCUS	1 (14.3)	2 (3.8)	
LSIL	1 (14.3)	1 (1.9)	
HSIL	5 (71.4)	50 (94.3)	
CIN at LEEP			> 0.99
CIN2	0 (0)	3 (5.7)	
CIN3	7 (100.0)	50 (94.3)	
Positive endocervical cytology at LEEP	0 (0)	25 (47.2)	0.01
Recurrence	0 (0)	46 (76.5)	< 0.01

HR, high risk; HPV, human papillomavirus; LEEP, loop electrosurgical excision procedure; ASCUS, atypical squamous cells of undetermined significance; LSIL, low squamous intraepithelial lesion; HSIL, high squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia.

Table 4. — *Sensitivity, specificity, and predictive values of the different tests in predicting recurrent CIN2-3.*

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Menopause	39.1 (25.4-54.6)	88.4 (81.6-93.0)	52.9 (35.4-69.8)	81.3 (73.9-87.0)
Endocervical cytology	54.3 (39.2-68.8)	82.6 (74.0-88.3)	51.0 (36.5-65.4)	84.4 (77.0-89.9)
Follow-up cytology	84.8 (70.5-93.2)	85.5 (78.3-90.7)	66.1 (52.5-77.6)	94.4 (88.4-97.5)
Post-LEEP HC2	93.8 (67.7-99.7)	90.6 (84.1-94.7)	53.6 (34.2-72.0)	99.2 (95.0-99.9)
Post-LEEP HDC	100.0 (90.4-100.0)	89.9 (83.3-94.1)	76.7 (63.7-86.2)	100.0 (96.2-100.0)
Same HR-HPV genotype by HDC	100.0 (90.4-100.0)	94.9 (89.4-97.8)	86.8 (74.0-94.1)	100.0 (96.4-100.0)

HR, high risk; HPV, human papillomavirus; LEEP, loop electrosurgical excision procedure; HC2, Hybrid Capture II test; HDC, HPV DNA chip test; CI, confidence interval.

patients younger than 25 years or those concerned with the potential effect of treatment on future pregnancy outcomes, observation is recommended. If recurrent CIN2-3 develops after excisional treatment and repeat excision is not feasible or not desired, hysterectomy is recommended. The results of a meta-analysis found that the HPV test diagnosed residual lesions sooner with a higher sensitivity [14]. In this report, patients with persistent HR HPV infections detected by the HDC test postoperatively had a 76.7% (46/60) risk of recurrence, whereas HPV-negative women had no risk of recurrence. Fifty-three (28.8%) of the 184 of patients showed a persistent infection with the same HR HPV type and it was the most important risk factor for recurrence (46/53; 86.8%). The same HR HPV genotype identified by the HDC test during postoperative monitoring showed a negative predictive value and a sensitivity of 100% for di-

Table 5. — *The correlation between pre-LEEP HR-HPV genotypes by HDC and recurrent disease.*

	No recurrence N = 138	Recurrence N = 46	Total N = 184
None (N = 3)	3	0	3
Single infection (N = 164)			
16	57	23	80
18*	8	8	16
58	19	5	24
31	7	1	8
33	7	1	8
45	2	1	3
51	1	1	2
52	5	1	6
53	1	1	2
56	4	1	5
Other types†	10	0	10
Multiple infection (N = 17)			
16 + 18	5	1	6
16 + 58	4	1	5
18 + 58	1	1	2
Other mixed types	4	0	4

LEEP, loop electrosurgical excision procedure; HR, high risk; HPV, human papillomavirus; HDC, HPV DNA chip test.

**Significantly higher than the results for other HR-HPV genotype infection (chi-square test; $p < 0.05$).*

†High risk human papillomavirus types 33, 35, 39, 45, 51, 59, 66, and 68.

agnosing recurrent or residual lesions. Among 46 patients with recurrent CIN2-3, 43 patients were infected with a single HR HPV genotype, but only three patients (HPV-18/58, HPV-16/18, and HPV-16/58) with infections with multiple HR HPV genotypes had recurrences. Moreover, HPV-18 is primarily associated with the recurrence of CIN2-3 ($p < 0.05$). Persistence of the same HR HPV infection by the HDC test detects recurrent lesion faster. Patients with persistent postoperative infections with the same HR HPV must be followed carefully to monitor for a recurrence. Positive margin status is the conventional predictive factor for treatment failure [15]. However, a negative margin does not always indicate complete lesion excision owing to the possibilities of multifocal CIN or insufficient removal of the lesion due to ablative conization [16]. Up to 40% of all patients treated by loop excision have histologic margin involvement [17]. In a recent study, among 184 women with intraoperative positive section margins, 46 (25%) patients developed a recurrence, the intraoperative endocervical cytology was positive in 49 (26.6%), and 25 (51.0%) patients developed a recurrence. The newly introduced HPV diagnosis technique using cervical swabbing, the HPV DNA chip test, has the merit of being able to type different HPV infections at once [18]. The accuracy of HR HPV testing did not demonstrate the difference in the accuracy by the kind of test assay, when limited to HDC for the diagnosis and typing of HPV in cervical lesion (91.1%) [9]. In this report, the degree of concordance between the HC2 and the HDC tests was 98.9% (Cohen's kappa was 0.745, indicating substantial agreement), and the HPV detection rate by the HC2 test was similar to that of the HDC test [19]. Former studies suggested a relationship between a high HC2 viral load with the risk of recurrent CIN2-3. However, this study found that HR HPV viral loads detected by the HC2 test were not correlated with recurrent CIN2-3 [16]. In this study, the area under the receiver operating characteristic curve for the high-risk viral load estimated by the HC2 test for predicting recurrent or residual CIN2-3 was 0.547 ($p = 0.371$). The HR HPV load using different cutoff values (1, 10, 100, and 1000 RLU) before conization did not predict recurrent or residual CIN2-3. At least two hypotheses may account for the discordant results. One main bias of the HC2 viral load testing is that this diagnostic method cannot estimate cell numbers, which can differ considerably from one sample to another. Second, the total high viral load detected by the HC2 test is quite uninformative since it might demonstrate multiple or single HPV types among the 13 HR types detected by the kit [20].

The major strength of our present study was the long follow-up of women with CIN2-3 with positive margins using an HPV genotyping test. The detailed stratification by histological diagnosis and HPV genotype is also an innovative contribution to the knowledge of the natural history of HPV infections. The limitations of our study include the retrospective nature of the design and the data collected by classifying the endocervical and endocervical margins.

However, it was difficult to classify both because some data were not identified as having endocervical or exocervical origins. In addition, the use of the HDC test for verifying HR HPV genotypes has not been approved by the Food and Drug Administration (FDA).

The most remarkable finding of this report was that the persistence of the same high-risk HPV infection, especially HPV-18, significantly predicted a recurrence, in comparison with persistent high-risk HPV infection associated to recurrences in former reports. In conclusion, our study performed HR HPV genotyping with a systematic pathologic control and showed that the clearance or persistence of an HR HPV genotype was a trustworthy prognostic marker for cure or recurrence after a LEEP for CIN2-3 with a positive margin. Although standardized PCR techniques for HPV diagnosis deserve further investigation, in patients with CIN2-3 identified at the margins of the LEEP specimen, persistent postoperative infections with the same HR HPV genotype, especially HPV-18, should be regarded as a strong risk factor for developing recurrent or residual CIN2-3, and such patients should be assessed with short-term follow-ups.

Ethics approval and consent to participate

The protocol was approved by the Institutional Review Board of CNUHH (approval number: CNUHH-2020-188).

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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