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HYBRID CAPTURE-II AND LCR-E7 PCR ASSAYS FOR HPV TYPING IN CERVICAL CYTOLOGIC SAMPLES

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As part of an ongoing cohort study in the Hokuriku region of Japan, cervical cell samples from histologically confirmed normal ($n = 114$) or abnormal ($n = 286$) women were examined for the presence of HPV DNA using a second-generation hybrid capture assay (HCA-II) and LCR-E7 PCR. HCA-II detected low-risk (HPV-6, -11, -42, 43 and -44) and high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68) HPV types, while LCR-E7 PCR detected an additional 7 HPV types and some uncharacterized types. In screening of high-grade squamous intraepithelial lesions (HSILs) and invasive cervical cancer, the sensitivities of HCA-II and LCR-E7 PCR testing the high-risk HPV types were 83% and 81%, respectively, while the specificity of both assays was 93%. The sensitivity of LCR-E7 PCR increased to 87%, which was significantly higher than that in HCA-II, when testing both high-risk and other HPV types. Sixty-eight inconsistent results (17% of total tested) from HCA-II and LCR-E7 PCR were due to *(i)* low copy number of HPV genome (false-negative for HCA-II, 5.3% and for LCR-E7 PCR, 1.3%), *(ii)* infection with HPV types undetectable by HCA-II (4.8%), *(iii)* multiple HPV infections (5%) or *(iv)* unknown reasons (0.8%). LCR-E7 PCR revealed that infections with HPV-16, -18, -31, -33, -35, -51, -52, -56, -58 or -67 was a high risk for cancer since these types predominated in HSIL and invasive cervical cancer. Samples showing high relative light units (>20) with a high-risk probe in HCA-II also gave positive results in LCR-E7 PCR and were generally associated with abnormal cervical lesions. Thus, we propose that both HCA-II and LCR-E7 PCR are valuable screening tests for premalignant and malignant cervical lesions.

Key words: *hybrid capture assay II; LCR-E7 PCR; human papillomavirus; cervical cancer; high-grade squamous intraepithelial lesion*

Although deaths from cervical cancer in many developed countries have declined in recent decades, it remains the fifth most frequent cancer and the second most common cancer in women worldwide.[1] The adoption of routine cytologic testing for cervical cancer in many developed countries accounts, in large part, for the decrease in deaths from this disease. Problems remain with cytologic testing, however, particularly with the frequency of false-negatives, the high cost of repeat testing and diagnosis of equivocal cases using the Bethesda system.[2] Given these circumstances, cancer-screening programs using HPV tests are as effective at predicting disease as those using cytologic tests.[3] zur Hausen *et al.*[4] showed that infection with HPV was closely associated with cervical cancer development. In addition, several previous studies have shown that HPV-6 and -11 are associated with benign anogenital lesions, whereas HPV-16 and -18 are associated with cervical cancer.[5] Currently, more than 80 HPV types have been identified and of these, about 30 distinct HPV types are known to infect the genital tract,[6,7] at least 10 being associated with cancer.[8] Geographic differences in HPV types have been reported to exist between countries[8] and even within the United States.[9,10] The risk factors for cervical cancer and the prevalent HPV types in Japan[11,12] differ from those reported in Western countries.[8–10] HPV-51, -52 and -58 are more prevalent, whereas HPV-18 and -45 are less prevalent in Japan than in Western countries.[12] Therefore, the definition of high-risk HPV types in Japanese women is important with respect to both clinical management of HPV infection and unequivocal diagnosis of cervical pathology.

Highly sensitive HPV DNA tests have been developed as a supplements to cervical cancer screening and for follow-up in women with either low-grade cervical lesions or equivocal cytologic results, such as in cases of low-grade squamous intraepithelial lesions (LSILs)[13] or atypical squamous cells of undetermined significance (ASCUS).[14] Most interestingly, the introduction of a highly sensitive assay reveals HPV positivity in many women with normal cervical cytology, especially sexually active young women,[15,16] and some of these HPV-positive women are thought to develop cervical cancer.

To study the utility of HPV testing in a cervical cancer program in Japan, we tested randomly selected clinical samples for the presence of HPV DNA, using both the second-generation hybrid capture assay (HCA-II)[17,18] and a PCR-based assay (LCR-E7).[19] HCA-II is 1 of the most reliable assays commercially available and we have successfully used the LCR-E7 PCR assay to detect most mucosal HPV types. Comparing the results of these 2 assays, we detected significant numbers of HPV infections in cervical cytologic samples obtained for cancer screening. We discuss the applicability and potential limitations of both assays in cervical cancer-screening programs.

MATERIAL AND METHODS

Study population

Ours was a case-control study nested in a larger screening cohort. More than 200,000 women were recruited to participate in a cervical cancer-screening program in the Hokuriku area of Japan (Fukui, Ishikawa and Toyama prefectures) from August 1995 to September 1999. Most women were asymptomatic and visited local private clinics, 4 big hospitals and a cancer-screening center for cancer screening. About 1,000 women were suspected of being abnormal in cytology and referred to outpatient clinics of the hospitals for further investigation. All cases showing equivocal cytologic findings were excluded in the abnormal group and most women participating in the cancer-screening program were older than 30 years. Of these abnormal women, 308 agreed to participate in this project; they were interviewed for past and current history and several demographic factors and underwent punch biopsy under the guidance of colposcopy by experienced gynecologists. Two hundred and eighty-six women were selected as eligible cases since they had histologically confirmed LSILs or high-grade squamous intraepithelial lesions (HSILs) and invasive cancer. Normal controls were defined as women who had no current evidence of cervical neoplastic lesions and sexually transmitted diseases and were randomly selected matched by age from the same population as cases were generated. All participants signed informed consent forms approved by Kanazawa University School of Medicine.

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Most cancer cases may have represented the parental cohort, whereas the control and SIL subjects may have been selected by residence or other factors since most preferred to be managed in local clinics.

Sample collection and cytologic and histologic evaluations

Cervical cells were obtained from all women. Cervical cell scrapings were collected with a cytobrush from the ectocervix and endocervix of the uterus. Samples were collected for a Pap test and 2 HPV tests at the same time. The latter samples were collected into a tube containing 1 ml of PBS and stored at –30°C until the HPV tests. For HPV tests, samples were divided into 2 and spun down at 500*g* for 1 min. A fraction of the cell pellet was resuspended in 1 ml of sample solution (Digene, Silver Springs, MD) for HCA-II and another fraction was subjected to DNA purification for PCR in our laboratory. Smears were screened by 1 cytotechnologist. The final clinical diagnosis of women with abnormal cytology was made by histologic evaluation of biopsy samples obtained at colposcopy. All possible abnormal smears and histologic slides were reviewed independently by 2 surgical pathologists. Final diagnoses were determined by agreement of both pathologists using the Bethesda system.[20] HPV detection and pathologic diagnosis were performed independently.

HPV detection and typing using HCA-II

HPV was detected at the Mitsubishi BCL Laboratory (Tokyo, Japan) according to the instruction manual provided by Digene. Each 1 pg/ml of HPV-11 and HPV-16 DNA was used as a positive control for both low-risk and high-risk probes, respectively. Low-risk probes included those for HPV-6, -11, -42, -43 and -44 and high-risk probes included those for HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68. Relative light units (RLUs) were calculated as follows: LU of sample/LU of positive control. The standard cut-off point (1 RLU) was considered positive for the presence of HPV DNA.

HPV detection and typing using PCR

Cervical cells were suspended in 50 mM Tris-HCl (pH 8.0) with 10 mM EDTA containing 200 μg/ml proteinase K and incubated for cell lysis overnight at 37°C or 1 hr at 55°C. DNA was extracted from this lysis solution by the phenol-chloroform-isoamylalcohol method. To avoid contamination, we used disposable utensils and discarded them immediately after a single use. A reaction mixture without template DNA was included in every set of PCR runs as a negative control.

Primers for a fragment of the *β-actin* gene served as an internal control to assess the quality and quantity of template DNA in each PCR specimen. The quality of DNA rendered 21 samples ineligible for study and these samples are not included in the numbers of case and control samples mentioned above. Four degenerate LCR forward primers (LCRF1, LCRF2, LCRF3 and LCRF4) and 4 E7 reverse primers (E7R1, E7R2, E7R3 and E7R4) were used to amplify E6 and E7 DNA of 36 mucosal HPV types, including HPV-6b, -11, -13, -16, -18, -26, -30, -31, -32, -33, -34, -35, -39, -40, -42, -43, -44, -45, -51, -52, -53, -54, -55, -56, -57, -58, -59, -61, -64, -66, -67, -68, -70, -71, -72 and -73. Sample DNA (100 ng) was added to a 50 μl PCR solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 μM of each dNTP, a mixture containing 0.2 μM of each primer and 0.25 units of KOD Dash DNA polymerase (Toyobo, Tokyo, Japan). PCR was then performed using an ASTEC (Fukuoka, Japan) PCR Thermal Cycler PC 707-02 with the following conditions. After a 1 min denaturing step at 95°C and cooling on ice, the next 30 to 35 cycles were at 95°C for 45 sec, 55°C for 20 sec and 74°C for 45 sec. There was a final step at 74°C for 5 min. Amplified DNA samples were run on 2% classic type ME agarose (Nakarai, Kyoto, Japan) in TBE buffer and transferred to nylon membranes (Hybond N^+; Amersham, Tokyo, Japan) using the alkaline-transfer method. The blotted membrane was hybridized with a mixture of 4 fluorescence-labeled, HPV-degenerated consensus oligoprobes. The se-

TABLE I – PREVALENCE OF HPV INFECTION IN UTERINE CERVICES OF JAPANESE WOMEN

Diagnosis	Number of samples	HC-II			LCR-E7 PCR																						
		LR types[1]	HR types	Any types	Low-risk types				Other types							High-risk types											Any types
					6[2]	11	42	44	30	53	54	66	70	72	UC[3]	16	18	31	33	35	39	45	51	52	56	58	59	67	68	
NCX	114	2	9	9 (8%)	1[5]			1	1	4					3	4	1	1					1	2	1	1	1			13 (11%)
LSIL	108	14	67	72 (67%)	3	4	5	3		8	1	3	1[4]		12	12	4	9		2	3[4]		9	7	6	9	3	2	2[4]	84 (78%)
HSIL	110	4	94	94 (85%)		1[4]	1[4]	1[4]		2[4]		2[4]		2[4]	12	39	3	11	3	4		2[4]	7	11	6	16				103 (94%)
SCC	60	5	50	50 (83%)		3[4]	1[4]	1[4]		1[4]					4	29	6	4	1[4]	1[4]			2	8		2		1		56 (93%)
ADCA	8	0	6	6 (75%)		1[4]										1	6													7 (88%)

[1]Low-risk type in HCA-II test includes HPV6, -11, -42, -43 and -44; high-risk HPV types include HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68.–[2]HPV types.–[3]Uncharacterized types.–[4]All cases were detected as 1 infection of multiple HPV types.

TABLE II – SENSITIVITY AND SPECIFICITY OF HPV TYPING TEST FOR DIAGNOSIS OF CERVICAL ABNORMALITIES

Diagnosis	Total	HC-II									LCR-E7 PCR		
		Low-risk type[1]			High-risk type[2]			Either type			Low-risk		
		Positive	Sensitivity	Specificity	Positive	Sensitivity	Specificity	Positive	Sensitivity	Specificity	Positive	Sensitivity	Specificity
LSIL	108	14	13%	98%	65	**60%**	93%	70	**65%**	93%	12	11%	98%
HSIL	110	4	4%	98%	91	**83%**	93%	91	**83%**	93%	4	4%	98%
ICCA	68	5	7%	98%	56	**82%**	93%	56	**82%**	93%	6	9%	98%
HSIL/ICCA	178	9	5%	98%	147	**83%**	93%	147	**83%**	93%	10	6%	98%

LCR-E7 PCR												
Low-risk/other types[3]			High-risk type[4]			High-risk/other types			Any type[5]		
Positive	Sensitivity	Specificity	Positive	Sensitivity	Specificity	Positive	Sensitivity	Specificity	Positive	Sensitivity	Specificity
25	23%	95%	56	52%	93%	66	61%	89%	73	**68%**	89%
16	15%	95%	91	83%	93%	98	**89%**	89%	98	**89%**	89%
10	15%	95%	53	78%	93%	56	**82%**	89%	56	**82%**	89%
26	15%	95%	144	81%	93%	154	**87%**	89%	154	**87%**	89%

Cut off point of HCA-II was ×1 RLU of control HPV, as described in Material and Methods.–[1]Low-risk types in HCA-II test include HPV6, -11, -42, -43 and -44.–[2]High-risk HPV types in HCA-II include HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68.–[3]Other types include HPV30, -53, -54, -66 and unknown types.–[4]High-risk HPV types in LCR-E7 include HPV67 in addition to the high-risk types in HCA-II.–[5]Any HPV types detected with LCR-E7 PCR. Bold indicates highest sensitivity in detection of each disease.

quences of these probes were as follows: HPV-16R-AS, AATTGCTCATARCAGTAKAGRTCA; HPV-18R-AS, TCWYTAAAWGCAAATTCAWATACCTC; HPV-51/56-AS, AATTGYTCRTWGCATTGYAGGTCA; HPV-6b/11-AS, CAATGDAARCAGCGACCCTTCCA (R, A/G; K, G/T; W, A/T; Y, C/T; D, G/A/T). Hybridized HPV DNA was visualized using a CDP star detection module (Amersham). HPV typing was performed by an RFLP method using amplified DNA stained with ethidium bromide on agarose gel. Hybridization analysis was applied on some samples, which showed too faint signals, with a mixture of FITC-labeled E6 and E7 DNA probes of HPV-11, -16, -18, -31, -51, -52, -56, -58, -72 and -73. Most E6 and E7 DNA probes were PCR products from cloned wild-type HPV DNA and only E6 and E7 of HPV-51 was from a clinical sample. These E6 and E7 products were cloned into pGEM vector and confirmed using the autosequencer. Each E6 and E7 sequence was cut out and subjected to FITC labeling. Hybridization was performed under moderate-stringency conditions (Tm = –30°C). Labeling and detection of E6 and E7 DNA probes were performed using the ECL Random-Prime Labeling kit and the CDP star detection module (Amersham). Samples that could not be typed by this method were classified as uncharacterized types (UC).

Statistical analysis

The χ^2 test or Fisher's exact probability test was used to compare the prevalence of HPV infection. Quantitative HPV DNA levels (RLU levels) of each cervical lesion with HCA-II were compared using the Kruskal-Wallis or Mann-Whitney *U*-test. McNemar's χ^2 test was used to compare the sensitivity and specificity of HPV testing with those of cytology. The receiver operating characteristic (ROC) curve[21] was calculated to investigate the consequences of shifting cut-off values used to define HCA-II results as positive. The ROC curve weighed the estimated sensitivity of HCA-II at varying cut-off values to identify all severe diseases [HSIL or invasive cervical cancer (ICCA)] detected in the course of study against decreases in specificity.

RESULTS

Detection of various HPV types by LCR-E7 PCR

We examined the prevalence of HPV infection in cytologically abnormal cases and normal controls nested in an ongoing cohort study in the Hokuriku region of Japan. All HPV types identified as single infections or 1 of multiple infections are listed in Table I. Using HCA-II, prevalence rates of HPV infection were 8%, 67%, 85%, 83% and 75% in normal cervices (NCX), LSILs, HSILs, cervical squamous-cell carcinomas (SCCs) and cervical adenocarcinomas (ADCAs), respectively. In LCR-E7 PCR, HPV prevalence rates were 12%, 78%, 94%, 93% and 88% of NCX, LSILs, HSILs, SCCs and ADCAs, respectively. The prevalence of HPV for HSIL was higher in LCR-E7 PCR than in HCA-II ($p < 0.05$, χ^2 test) and that of LSIL and invasive cancer (SCC and ADCA) was marginally higher ($p < 0.1$), whereas no difference was observed in NCX.

Twenty-four distinct HPV types, including low-risk types (HPV-6, -11, -42, 43 and -44), other types (HPV-30, -53, -54, -66, -70 and -72), high-risk types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -67 and -68) and UC HPV types, were detected using LCR-E7 PCR (Table I). HPV-30, -53, -54, -66, -70 and -72 were not included as targets in HCA-II and were categorized as other HPV types.

In NCX and LSIL cases, 18 distinct types (HPV-6, -11, -42, -44, -30, -53, -54, -66, -16, -18, -31, -35, -51, -52, -56, -58, -59 and -67) were identified as single HPV infections. HPV-39, -68 and -70 were identified as 1 of multiple infections. In more severe diseases, 9 (HPV-16, -18, -31, -33, -35, -51, -52, -56 and -58) and 7 (HPV-16, -18, -31, -51, -52, -58 and -67) types were identified as single infections in HSIL and SCC of the cervix, respectively. Three cases infected with HPV-67, which is not included as a high-risk type in HCA-II, also were positive with the high-risk probe of HCA-II. Low-risk types (HPV-11, -42 and -44) and other types (HPV-53, -66 and -72) were coinfected with high-risk types in HSILs and cancer (SCC and ADCA). One of 2 HPV-45-positive HSIL cases was coinfected with another high-risk type and another was infected with low-risk type. HPV-33- and –35-positive cancer cases were coinfected with other low- or high-risk types, respectively.

Sensitivity and specificity of LCR-E7 PCR and HCA-II

To compare the sensitivity and specificity of LCR-E7 PCR and HCA-II in the screening of HSILs or cancer, we first assigned a cut-off point for HCA-II. We could not estimate true sensitivity and specificity since a few women with disease may have been missed by cytologic screening. Therefore, we defined estimated sensitivity and specificity: estimated sensitivity was 84.3% and specificity was 92.5% at the cut-off point of 0.84 RLU, whereas sensitivity was 68% and specificity was 95% at the cut off point of 1.5 RLU. When we set the standard cut-off point of 1.0 RLU, HCA-II reached an estimated sensitivity plateau around 83% detection of HSILs or cancer, at which point estimated specificity was 93% in ROC analysis. Thus, we adopted this standard cut-off point in screening of severe cervical lesions.

TABLE III – HPV TYPES DETECTED BY HCA-II AND LCR-E7 PCR

HCA-II	Number of cases	LCR-E7 PCR				
		LR types[1]	HR types[2]	LR + HR types[3]	Other types[4]	Negative
Low-risk types[1]	6	**2 (33%)**[5]	2 (33%)	0 (0%)	2 (33%)	0 (0%)
High-risk types[2]	205	1 (0.5%)	**170 (83%)**	9 (4%)	20 (10%)	5 (2%)
LR + HR types[3]	19	3 (16%)	8 (42%)	**7 (37%)**	1 (5%)	0 (0%)
Negative	170	0 (0%)	19 (11%)	2 (1%)	19 (11%)	**130 (76%)**
Number of positive cases	400	6 (2%)	199 (50%)	18 (5%)	42 (11%)	135 (34%)

[1]Low-risk (LR) types (HPV6, -11, -42, 43, -44) or coinfection of LR types and other types.–[2]High-risk (HR) types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -67, 68) or coinfection of HR types and other types.–[3]Coinfection of LR and HR types.–[4]Single and multiple infection of other types (HPV types 30, 53, 54, 66, 70 and 72 and uncharacterized types).–[5]Number positive in LCR-E7 PCR/number positive in HCA-II. Bold indicates concordant results between both assays.

We compared the sensitivity and specificity of both HCA-II and LCR-E7 PCR for detecting different stages of cervical lesions (Table II). We calculated both sensitivity and specificity by counting cytologically normal women as disease-free controls. In low-risk HPV types, both assays showed very low sensitivity for detecting any cervical lesions (<13%) compared to cytologically normal types. However, detection of either low- or high-risk HPV in HCA-II and of any HPV types in LCR-E7 PCR showed the highest sensitivities (65% in HCA-II and 68% in LCR-E7 PCR) for LSILs. When targeting high-risk HPV infection, both HCA-II and LCR-E7 PCR showed equivalently high sensitivity (83% in HCA-II and 81% in LCR-E7 PCR) and specificity (93%) in screening of HSILs or ICCA. However, the sensitivity of LCR-E7 PCR for detecting HSILs and ICCA increased to 87% when both high-risk and other types were counted as targets and this sensitivity was significantly higher than that of HCA-II ($p < 0.02$, McNemar's test).

Practically, many clinicians wish to screen HSIL and cervical cancer cases from others using simple cancer screening. When we counted both cytologically normal and LSIL women as disease-free controls, the sensitivities of detecting high-risk types in the screening for HSIL and SCC were 83% and 81% and the specificities were 67% and 71% in HCA-II and LCR-E7, respectively. When detecting high-risk and other types with LCR-E7 PCR, the sensitivity increased to be 87%, while the specificity was 64%.

Comparisons of HPV typing results using HCA-II and LCR-E7 PCR assays

We compared HPV-typing results from HCA-II and LCR-E7 PCR (Table III) and found that samples that scored positive for hybridization with high-risk probes in HCA-II were also positive for the high-risk types (83%), the low- and high-risk types (4%) and the other types (10%) in LCR-E7 PCR. These samples were rarely positive (0.5%) for the low-risk HPV types defined by LCR-E7 PCR. In contrast, one-third of cases positive with the low-risk probe in HCA-II were either low-risk, high-risk or other types in LCR-E7 PCR. In samples positive for low- and high-risk types in HCA-II, 37% had low- and high-risk types, 16% had low-risk types, 4% had high-risk types and 5% had other types in LCR-E7 PCR. Agreement of the results of HCA-II and LCR-E7 PCR was therefore observed in 86% (309/358) of all cases, if the other type of infection was excluded.

Inconsistent results of HCA-II and LCR-E7 PCR

To clarify potential factors producing discrepancies between HCA-II and LCR-E7 PCR, we classified the above cases by other means. False-negatives and differences in HPV-typing results led to discrepancies between HCA-II and LCR-E7 PCR. Cases that were negative in HCA-II but positive in LCR-E7 were considered false-negatives for HCA-II. Similarly, cases that were negative in LCR-E7 PCR but positive in HCA-II were designated false-negatives for LCR-E7 PCR. Of 68 inconsistent cases, 40 (59% in inconsistent results, 10% in totals) were HCA-II false-negatives, whereas there were 5 (7.4%, 1.3% in totals) false-negatives in LCR-E7 PCR. Nineteen (28%, 4.8% in total) false-negative cases in HCA-II were due to undetectable HPV types (other HPV types) in HCA-II. HPV-30, -53 and -66 as well as some UC HPV types detected by LCR-E7 PCR were included in this group. Of all women infected with other HPV types, 45% (19/42) were undetectable with any probes of HCA-II, 48% (20/42) were positive with the high-risk probe and 7% (3/42) were positive with the low-risk or the low-/high-risk ones. Four such cases, infected with HPV-53, -54, -66 and –72, were positive with the high-risk probe and 1 case with HPV-53 was positive with the low-risk probe in HCA-II; other positive samples with the low- or high-risk probe were UC HPV infection.

Discordance in HPV-typing results was classified into 3 categories, using factors such as discordance by multiple infection and discordance for unknown reasons. Discordance by multiple infection (29%, 20/68) included 2 different categories: samples ($n = 11$) positive for either low- or high-risk types in HCA-II and for both risk types in LCR-E7 PCR and samples ($n = 9$) positive for both risk types in HCA-II but only for the high-risk types in LCR-E7 PCR. Discordant results from unknown factors were observed in 3 LSIL cases (4.4%, 3/68). Two of the 3 were positive for low-risk HPV in HCA-II and for high-risk HPV types (HPV-18, -58 and -51) in LCR-E7 PCR; the other was positive for high-risk HPV in HCA-II and for a low-risk HPV type (HPV-6) in LCR-E7 PCR.

One of the advantages of HCA-II is the ability to estimate viral genome copy number from the magnitude of the RLU obtained. The magnitude of all missed types with LCR-E7 PCR in false-negative or multiple-infection cases was from 1.0 to 6.0 RLU with either low-risk or high-risk probes in HCA-II. From these findings, low copy number of the HPV genome in a sample may be a major cause of many discrepant results.

RLU in HCA-II and stage of cervical lesions

By comparing RLU values, we estimated HPV genome copy numbers in various pathologic lesions. The RLU values obtained with HCA-II *vs.* HPV-typing results with LCR-E7 PCR are shown in Figure 1. All HPV-positive samples in LCR-E7 PCR were plotted. The highest RLU of high-risk HPV infection cross-reacted to the low-risk probe was 19.3 RLU, whereas the highest RLU of low-risk HPV infection cross-reacted to the high-risk probe in HCA-II was 4.16. Therefore, samples showing more than 20 RLU in HCA-II appear to be concordant with low-risk or high-risk HPV type determined with LCR-E7 PCR. Such high RLU values (>20 RLU) of high-risk probes were observed in no cases of low-risk infection and in 78 of 199 (39%) cases of high-risk HPV infection, in 7 of 18 (39%) low- and high-risk HPV infections and in 8 of 42 (19%) infections involving other HPV types. Six of 8 (75%) samples in the other HPV type group were infected with UC HPV types and the remaining 2 were HPV-53 and -66. Testing with the low-risk HPV probe in HCA-II, high RLU (>20) values were observed in 1 HPV-42- and 1 HPV-44-positive LSIL case, 1 HPV-44-positive normal woman and 3 HPV-6- or –42-positive LSIL cases coinfected with high-risk types. In contrast, no high RLU values with the low-risk HPV probe were observed in the

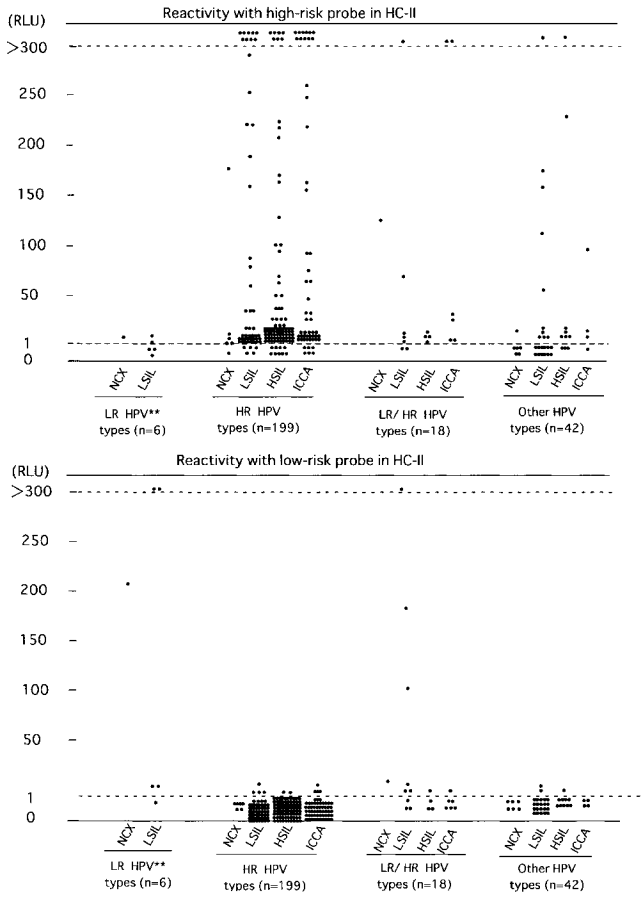

FIGURE 1 – Difference in RLU values in HCA-II of clinical samples. RLU values for samples were plotted. Low-risk HPV, single infection and multiple infections of low-risk HPV types or single infection and multiple infection of high-risk HPV types. Low-risk/high-risk HPV, infection with multiple types of both low- and high-risk HPV. Other HPV, single infection and multiple infections of HPV types other than low-risk or high-risk. A cut-off point (×1 RLU of control) and >300 RLU are shown as dotted lines.

high-risk and other HPV type infections. From these findings, HPV types showing high RLU values (>20) in HCA-II appear to be concordant with HPV types determined using LCR-E7 PCR.

It is generally accepted that the HPV genome replicates in differentiated squamous epithelium, suggesting that benign cervical lesions (LSILs), which are more differentiated than malignant cervical lesions (HSILs and ICCAs), are more likely to support HPV replication. Moreover, when viral genes are integrated into the host genome, *e.g.*, in carcinoma cells, replication of HPV DNA may not occur. In all cases infected with high-risk types (high-risk types and low-risk/high-risk types in Fig. 1), a high RLU (>20) was observed in 29% (2/7) of NCX, 45% (26/58) of LSILs, 29% (27/93) of HSILs and 51% (30/59) of ICCAs. RLU values differed among NCX, LSILs, HSILs and ICCAs ($p = 0.015$, Kruskal-Wallis test). Values of the HSIL group were lower than those of the LSIL group ($p = 0.015$, Mann-Whitney *U*-test), whereas no such difference was observed between NCX and LSILs or between HSILs and ICCAs.

DISCUSSION

Our aim was to demonstrate the significance of HPV typing in cervical cell samples using a commercially available HCA and LCR-E7 PCR. HCA has been used in many clinical and epidemiologic studies in the United States and its reliability has been reported.[16,17] In screening of HSILs and ICCA, the estimated sensitivities by detection of high-risk HPV types were 81% and 83% in LCR-E7 PCR and HCA-II, respectively, while the specificity was 93% in both assays. These sensitivities were equivalent to the level reported in a previous study,[22] whereas our specificity was higher. The higher specificity in our study may be due to selection of our control samples from which we had excluded any women with current evidence of sexually transmitted diseases. When we counted both cytologically normal and LSIL women as a disease-free group, the sensitivities of high-risk HPV screening of HSILs and SCCs were 83% and 81% and the specificities decreased to 67% and 71% in HCA-II and LCR-E7, respectively. The sensitivity of LCR-E7 PCR for detecting HSILs or ICCA was higher than that of HCA-II ($p < 0.02$, McNemar's test) when both high-risk and other HPV types were tested.

In our study, 68 inconsistent results between assays were observed, which might be due to various factors. Differences in the sensitivity of the 2 assays may be due to different spectra of detectable HPV types. This type of difference was observed in 28% (19/68) of samples infected with the other HPV types, including HPV-30, -53 and -66. However, the failure to detect HPV-30, -53 and -66, which are not included as targets in HCA-II, is not critical for cancer screening since they do not represent high-risk types in our study and others.[6–8,12] Many UC HPV infections may be responsible for this result. In fact, 6 UC HPV infections showed high RLU (Fig. 1).

Another problem may arise in the high rates of true HCA-II false-negatives (21/68, 31% of discordant results) observed in HCA-II, not the least because more than half of these cases (16/21) involved HSILs and cancer. This may suggest that HCA-II is less sensitive than LCR-E7 PCR at detecting high-risk HPV infection under certain conditions. For false-negative cases in LCR-E7 PCR, a possible reason may be low copy number of viral genome since RLU values of these cases were low (1–6 RLU). Low RLU values in HCA-II may be caused by 2 factors: low level of HPV gene replication in lesions and small size of lesions. We did not perform repeated PCRs on any samples that were completely negative in the first screening step. We sometimes experience discordant results in the same samples under the same conditions in the PCR-based assay. Therefore, we may have missed some positive samples in the first screening, if they had a low copy number of HPV genome. This is also likely in the false-negative samples in HCA-II. To reduce false-negative rates, repeated analyses may be necessary to reconcile false-negative results in both HCA-II and PCR-based methods.

In our study, 20 discordant results (30%) were due to multiple HPV infections. We have previously shown that multiple infection is 1 of the major factors accounting for discordant results in different PCR-based assays.[19] Discordant results arising for unknown reasons were observed in 2 cases of LSIL and 1 normal case, which appeared to be high-risk HPV infection in 1 assay but the opposite in another assay. It is likely that in samples containing low-copy mixtures of low- and high-risk HPV types, either the low- or high-risk probe hybridizes with 1 HPV type alone in HCA-II or 1 HPV type alone is amplified in LCR-E7 PCR. This hypothesis may be supported by the evidence that positive results with high RLU values detected by HCA-II were concordant with those of LCR-E7 PCR (Fig. 1). High RLU values in HCA-II are therefore important predictors of the predominant HPV type in cases of infection with multiple HPV types.

In theory, viral replication occurs in differentiated cervical epithelium, such as LSILs, but not in undifferentiated cervical lesions, such as HSILs and ICCA. It has also been postulated that viral gene replication does not occur in HSILs and ICCA, where the viral genome is often integrated into the host genome. In the present study, RLU values in HSIL samples were significantly lower than in LSIL samples ($p = 0.015$, Mann-Whitney test).

However, RLU values in cancer samples were not lower than those in LSIL samples. This result may partially support the above theory. Viral replication may occur more frequently in LSILs than in HSILs. However, not only replication status but also the population of HPV-positive cells included in clinical samples may influence RLU values. For the latter reason, it is likely that not all RLU values in cancer samples were lower than those in LSIL samples. Further investigations using *in situ* hybridization or histochemic analysis of tissues containing various stages of cervical lesions are necessary to resolve this issue. In conclusion, our results corroborate the findings of other groups[22,23] in showing that RLU values have no diagnostic value for predicting the grade of cervical lesions.

HPV typing has gained acceptability not only as a supplement to cytologic tests but also as the first choice for testing self-collected vaginal cytologic samples[3] in areas where cytologic tests are not readily available[22] or for elderly women who experience difficulty in undergoing routine gynecologic examinations with vaginal forceps. Cancer-screening programs using self-collected vaginal cytologic samples require highly sensitive and specific methods to detect high-risk HPV infection.[3] The present results also show the usefulness of HCA-II and LCR-E7 PCR in screening of HSILs and ICCA because of the higher sensitivity (82% for HSILs and 83% for cancer in HCA-II, 82% for HSILs and 87% for cancer in LCR-E7 PCR) at the specificity levels of 93% in HCA-II and 89% in LCR-E7 PCR.

The present results suggest that LCR-E7 PCR, which we have recently established, may also be useful for identifying infection with multiple HPV types. However, HPV typing by RFLP in this assay is a little complicated and repeated tests are necessary to decrease false-negative results. In the future, LCR-E7 PCR may be adapted, in combination with reverse hybridization[24] or DNA enzyme-linked immunosorbent assay[25] techniques, to the simultaneous detection of multiple HPV types immobilized on membranes or plates. Our PCR method also has the problem that some HPV types identified in the present study were UC types since the E6 and E7 regions of many HPV types have not been sequenced. Accumulated data for many HPV sequences may resolve this problem in the future.

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