High-Risk and Multiple Human Papillomavirus Infections Associated with Cervical Abnormalities in Japanese Women

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Abstract

To estimate the risk of human papillomavirus (HPV) infection for cervical malignancies, we conducted a case-control study in Japan. Abnormal cervical cell (366) and normal cell samples (1562) were tested for the presence of HPV DNA using a new PCR-based test (LCR-E7 PCR). When single HPV infections were considered, 26 different HPV types were identified in normal cervices and in low-grade squamous intraepithelial lesions (LSIL); whereas HPV-16, -18, -31, -33, -35, -45, -51, -52, -56, -58 and -67 were detected in high-grade squamous intraepithelial lesions (HSIL) and in squamous cell carcinoma (SCC) of the cervix, and HPV-16 and -18 were detected in cervical adenocarcinoma. HPV-6 and -11 were detected in condyloma acuminatum tissue. In HSIL and SCC, HPV-16 was the most prevalent type and HPV-51, -52, and -58 were the next most prevalent; whereas HPV-39, -59, and -68 were not detected. Analysis by odds ratio (OR) revealed that HPV-11, -39, -42, -44, -53, -59, -62, and -66 (HPV-66: OR,139; 95% confidence interval (CI) = 6.7–168) were associated with LSIL; HPV-16, -18, -31, -51, -52 and -58 (HPV-16: OR, 69; 95%CI = 36–131) were associated with SCC; and HPV-16 and -18 (OR, 94; 95%CI = 28–317) were associated with adenocarcinoma. Multiple HPV infection was associated with LSIL (OR, 24; 95%CI = 13–44), HSIL (OR, 16; 95%CI = 8.4–32), and SCC (OR, 8.3; 95%CI = 3.2–22), although the prevalence decreased with the grade of the lesions. All results suggest that HPV-6 and -11 are condyloma types, HPV-16, -18, -31, -51, -52, -58, and perhaps -33, -35, -45, -56, and -67, are the high-risk HPV types, and many other types are LSIL-associated types in Japan. HPV typing and detection of multiple HPV infections in clinical samples may be useful as surrogate markers for cervical cell abnormalities.

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Introduction

Cervical cancer is the fifth most frequently seen cancer and the second most common cancer in women worldwide (1). zur Hausen *et al.* (2) first showed that HPV[2] is closely associated with the development of cervical cancer. Many previous studies have shown that HPV-6 and -11 are associated with benign anogenital lesions, whereas HPV types 16 and 18 are associated with cervical cancer (3). Currently, more than 80 HPV types have been identified. More than 30 distinct HPV types are known to infect the genital tract, and at least 10 are associated with cancer (3). Therefore, the association between HPV infections and anogenital lesions is more complicated than expected. HPV types such as HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68 are thought to be high-risk types because these types are identified in HSIL (4, 5) or invasive cervical cancer (6), whereas HPV-6, -11, -40, -42, -43, -44, and -55 are considered low-risk types (3, 7). Geographical differences in HPV types have been reported to exist between countries (6) and even within the United States (7, 8). Therefore, the HPV types prevalent in Japan may differ from those in Western countries (5, 9).

Recent progress in molecular biology techniques prompted us to use a highly sensitive HPV DNA test as a supplementary test for cervical cancer screening, and to follow-up women with low-grade cervical lesions, such as LSIL (10), ASCUS, and AGUS (11–13). The clinicopathological grading of HPV types according to their ability to promote cancer is now the most important issue for many clinicians who use such HPV tests in cancer screening programs. However, the introduction of a highly sensitive assay revealed multiple HPV infections in women with abnormal cytology, and even in normal women (14, 15). Multiple HPV infections may cause confusion in determining which HPV types are responsible for the development of cervical lesions. We recently established a PCR-based system that is able to detect the E6-E7 DNA of more than 36 mucosal HPV types (16). Using this assay, we conducted a case-control study in Hokuriku, Japan, to elucidate the risk of individual HPV types for cervical cancer. In this study, we examined the prevalence of infection with a single HPV type or with multiple HPV types to clarify the association between HPV infection pattern and the stage of cervical lesions.

Materials and Methods

Study Population. Women were recruited to participate in a cervical cancer-screening program at four hospitals in the Hokuriku area of Japan (Fukui, Ishikawa, and Toyama prefec-

[2] The abbreviations used are: HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; UC, uncharacterized type; ADCA, adenocarcinoma; SCC, squamous cell carcinoma of cervix; LSIL, low-grade intraepithelial lesions; NCX, normal cervices; OR, odds ratio; CI, confidence interval; CIN, cervical intraepithelial neoplasia.

tures) from August 1995 to September 1999. The case group consisted of 366 cytologically abnormal women (aged 19 to 75), including 145 LSILs, 137 HSILs, 72 SCCs, 12 ADCAs of the cervix, and 16 condyloma acuminata. We randomly chose an eligible sample of 1,562 women (aged 16 to 72) from the same population (483,500) that generated the cases. The control subjects were defined as women who had no past or current evidence of cervical neoplastic lesions, and had no clinical symptoms of any sexually transmitted diseases. Women who agreed to participate then signed informed consent forms approved by Kanazawa University School of Medicine.

Sample Collection and Cytological and Histological Evaluation. Cervical cells were obtained from all women of normal and abnormal cytology, and 9 of 16 condyloma patients. The cervical cell scrapings were collected with a cytobrush from the ecto- and endocervix of the uterus of each woman. After obtaining smeared cell slides for Pap test, the remaining cell samples on the cytobrush were suspended in PBS and stored at −70°C until DNA extraction. The final clinical diagnosis of the women with abnormal cytology was made by histological evaluation of biopsy samples obtained at colposcopy. The HPV detection and pathological diagnosis were performed independently. Smears were screened by two cytotechnologists. All possible abnormal smears and histological slides were reviewed independently by two surgical pathologists. The final diagnoses were determined by agreement of both pathologists using the Bethesda system (17).

HPV Detection and Typing Using the PCR. The cervical cells were suspended in 50 mM Tris-HCl (pH 8.0) with 10 mM EDTA containing 200 μg/ml proteinase K and incubated overnight at 37°C for cell lysis. 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 53 samples ineligible for the 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 four E7 reverse primers (E7R1, E7R2, E7R3, and E7R4) were used to amplify E6-E7 DNA of most mucosal HPV types. One hundred ng of sample DNA were added to a 50-μl PCR solution containing 20 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 7.5 mM DTT, 200 μM of each deoxynucleotide triphosphate, a mixture containing 20 pmoles each primer, and 0.25 units of KOD Dash DNA polymerase (Toyobo, Tokyo, Japan). For stable PCR amplification in clinical samples, we modified the procedure as follows: after denaturing the reaction solution containing no DNA polymerase for 2 min at 95°C, we cooled it on ice immediately, and then added the polymerase. PCR was then performed using an ASTEC PCR Thermal Cycler PC 707-02 (ASTEC, Fukuoka, Japan) with the following conditions. After a 1-min denaturing step at 95°C, the next 30–35 cycles were at 95°C for 45 s, 55°C for 20 s, and 74°C for 45 s. There was a final step at 74°C for 5 min. The amplified DNA samples were run on 2% classic type ME agarose (Nakarai, Japan) in Tris-borate EDTA buffer and transferred to nylon membranes (Hybond N+, Amersham, Japan) using the alkaline transfer method. The blotted membrane was hybridized with a mixture of four fluorescence-labeled, HPV-degenerated oligoprobes. The sequences of these probes were as follows: (*a*) HPV-16R-AS, AATTGCTCATARCAGTAKAGRTCA; (*b*) HPV-18R-AS, TCWYTAAAWGCAAATTCAWATACCTC; (*c*) HPV-51/56-AS, AATTGYTCRTWGCATTGYAGGTCA; and (*d*) 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 an enhanced chemiluminescence detection kit for fluorescence-labeled probes (Amersham, Japan). HPV typing was performed by an RFLP method using amplified DNA stained with ethidium bromide. HPV typing was performed by RFLP analysis on gels stained with ethidium bromide. The details of the RFLP typing method were as described previously (16). We performed RFLP analysis using hybridization on some samples, which had shown too-faint signals, with a mixture of E6-E7 DNA probes of HPV types 11, 16, 18, 31, 51, 52, 56, 58, 72, and 73. Most of the E6-E7 DNA probes were amplified with LCR-E7 PCR from cloned wild-type HPV DNA, and only E6-E7 of HPV51 from a clinical sample. An amplified DNA probe from each type was cloned into a pGEM vector, and the sequence was confirmed by autosequencer. Southern blot hybridization was performed under the moderate-stringency condition (Tm = −30°C). Labeling and detection of the E6-E7 DNA probe was performed using the ECF Random-Prime Labeling and Detection system (Amersham Pharmacia Biotech, Tokyo, Japan). Undetermined HPV types with RFLP analysis were cloned into a pGEM vector. HPV typing was performed on these clones by sequence analysis. The samples that could not be typed by RFLP and sequence analysis were classified as UCs. HPV 62 was determined with MY09/11-PCR and RFLP method established by Manos *et al.* (18) because we have no sequence data for E6-E7 genes of HPV62.

Statistical Analysis. The χ^2 test was used to compare the prevalence of HPV infection. We used the crude OR with the 95%CI to estimate the relative risk of each HPV type for cervical lesions. All cases and controls infected with single and multiple HPV infections were evaluated for this analysis.

Results

Detection and Typing of HPV in Clinical Samples Using LCR-E7 PCR. To determine the prevalence of HPV infection in different cervical lesions in Japanese women, we examined the presence of HPV DNA in cervical cytology samples using the LCR-E7 PCR method, which we recently developed. This new method is able to amplify the E6-E7 DNA of more than 36 mucosal HPV types (16). We tested 1562 normal controls and 366 cases with abnormal cervical cells. In the study, 9.7% (151 of 1562), 77% (111 of 145), 91% (125 of 137), 93% (67 of 72), and 67% (8 of 12) were positive for any HPV type in NCX, LSILs, HSILs, SCCs, and ADCAs, respectively (Table 1). When we tested condyloma tissues, 100% (16 of 16) of condyloma samples were positive for HPV-6 or -11 DNA. HPV typing using RFLP and sequence analysis revealed two patterns of infection: single HPV infection and multiple HPV infection. Multiple HPV infection was suspected in some samples that showed an overlay of type-specific bands in the RFLP analysis. Correct typing with RFLP was sometimes very difficult in the samples with multiple infection if the band signals were weak or if the samples contained more than three distinct HPV types. HPV types of such cases were determined by sequence analysis on cloned HPV DNA from the PCR products.

In condyloma tissue, HPV-6 was the predominant type (88%, 14 of 16), and only two cases were HPV-11. Nine of 10 (90%) condyloma patients also showed positive for HPV DNA in their cervices too. Surprisingly, the HPV types identified in

Table 1 The prevalent HPV types in the genital areas of Japanese women

Diagnosis[a]	The prevalence of HPV infection				A10	A10	A1	A10	A6	A7	A6	AX	A10	A7	A3	A3	A6	A7	A7	A3	A11	A9	A7	A9	A9	A9	A7	A5	A9	A6	A9	A9[a]	
	Any types		Multiple infection		Condyloma, LSIL-associated types, and other types																	High-risk types											
	No.	%	No.	%	6	11	42	44	30	39	53	54	55	59	61	62	66	68	70	72	73	16	18	31	33	35	45	51	52	56	58	67	UC[b]
Vulva and vagina																																	
Con. T (n = 16)[c]	16	100%	0	0%	14[d]	2																											
Cervix																																	
Con. CX (n = 10)	9	90%	4	44%													1						1					1			2		
					(1)[e]		(1)	(1)																(1)					(1)	(1)			(1)
NCX (n = 1562)	151	9.7%	17	1.1%		2	7	1	2	1	3	1	1	1	1	4	1	1		1	1	19	13	8	7	3	1	6	16	4	7		22
					(2)	(3)	(1)	(1)			(2)		(1)	(1)				(1)		(5)		(1)	(9)		(1)		(2)	(3)	(2)	(1)	(2)		(1)
LSIL (n = 145)	111	77%	30	21%		1	1	1		1	4	1		2	1	4	2				1	11	5	8	2	1		7	10	3	5	2	8
					(2)	(3)	(5)	(3)		(4)	(11)			(1)		(1)	(4)	(2)	(1)	(2)		(2)	(2)	(4)		(1)	(1)	(3)	(2)	(3)	(5)		(8)
HSIL (n = 137)	125	91%	21	15%																		40	2	9	1	2		9	13	2	16	10	
					(1)	(1)	(1)	(1)			(2)						(1)			(1)		(9)	(1)	(4)	(2)	(2)	(3)	(1)	(2)	(6)	(2)		(5)
SCC (n = 72)	67	93%	9	13%																		32	3	3			1	2	7		3	1	6
					(1)	(2)	(1)	(1)			(2)											(2)	(2)	(2)	(1)	(1)		(2)	(2)				(2)
ADCA (n = 12)	8	67%	1	8%																		1	6										
						(1)																(1)	(1)										

[a] Phylogenetic classification of HPV types by Bernard *et al.* (19).
[b] HPV type was not characterized.
[c] Con. T, condyloma tissue; Con. CX, cervical cells from condyloma patients.
[d] Number of the women with single HPV infection.
[e] Number of women with multiple HPV infections.

Table 2 The estimated risk of each HPV type for cervical lesions

HPV types	LSIL		HPV types	HSIL		HPV types	SCC		HPV types	ADCA	
	OR[a]	95% CI		OR	95% CI		OR	95% CI		OR	95% CI
HPV30	NE[c]	NE	HPV30	NE	NE	HPV30	NE	NE	HPV6	NE	NE
HPV55	NE	NE	HPV39	NE	NE	HPV39	NE	NE	HPV30	NE	NE
HPV67	NE	NE	HPV54	NE	NE	HPV54	NE	NE	HPV31	NE	NE
HPV18	2	0.9-6.4	HPV55	NE	NE	HPV55	NE	NE	HPV33	NE	NE
HPV33	2.7	0.57-13	HPV59	NE	NE	HPV56	NE	NE	HPV35	NE	NE
HPV45	3.6	0.37-35	HPV61	NE	NE	HPV59	NE	NE	HPV39	NE	NE
HPV72	3.6	0.72-18	HPV62	NE	NE	HPV61	NE	NE	HPV42	NE	NE
HPV61	11.0	0.67-18	HPV67	NE	NE	HPV62	NE	NE	HPV44	NE	NE
HPV54	11.0	0.67-18	HPV68	NE	NE	HPV67	NE	NE	HPV45	NE	NE
HPV73	11.0	0.67-18	HPV73	NE	NE	HPV68	NE	NE	HPV51	NE	NE
HPV42	7.0	2.3-22	HPV42	1.4	0.2-12	HPV72	NE	NE	HPV52	NE	NE
HPV35	7.3	1.2-44	HPV18	1.5	0.44-5.1	HPV73	NE	NE	HPV53	NE	NE
HPV11	7.4	2.1-26	HPV72	1.8	0.2-15	HPV66	NE	NE	HPV54	NE	NE
HPV16	7.6	3.7-16	HPV11	1.9	0.2-16	HPV33	2.7	0.33-22	HPV55	NE	NE
HPV52	7.7	3.6-16	HPV6	5.7	0.52-64	HPV42	2.7	0.33-22	HPV56	NE	NE
HPV58	10	4.4-25	HPV66	5.7	0.52-64	HPV35	7.3	0.75-71	HPV58	NE	NE
HPV6*	10.9	1.5-78	HPV44	5.7	0.52-64	HPV45	7.3	0.75-71	HPV59	NE	NE
HPV68*	11	1.5-78	HPV53	4.6	0.9-24	HPV6	11	0.98-123	HPV61	NE	NE
HPV56	13	4.1-45	HPV33	4.3	1.1-17	HPV44	11	0.98-123	HPV62	NE	NE
HPV51	13	5.1-32	HPV52	11	5.2-21	HPV18	5.0	1.84-14	HPV66	NE	NE
HPV62	14	3.7-52	HPV45*	12	2.3-58	HPV11*	7.4	1.5-37	HPV67	NE	NE
HPV44	16	2.7-99	HPV35	16	3.5-71	HPV51	7.4	1.5-37	HPV68	NE	NE
HPV59	16	2.7-99	HPV51	14	5.4-34	HPV53*	8.9	1.7-47	HPV72	NE	NE
HPV31	17	6.9-43	HPV56	19	6.2-60	HPV58	9.7	2.4-38	HPV73	NE	NE
HPV53	36	13-100	HPV31	19	7.5-46	HPV52	10	4.1-26	HPV11*	24	2.6-212
HPV39	56	6.5-481	HPV58	21	9.8-46	HPV31	14	4.6-46	HPV16	15	3.2-75
HPV66	139	6.7-168	HPV16	43	24-75	HPV16	69	36-131	HPV18	94	28-317
Multiple	24	13-44	Multiple	16	8.4-32	Multiple	13	5.6-30	Multiple	18	3.7-89

[a] ORs were calculated by comparison with control group (OR, 1).
[b] NE, Not evaluated; shaded area, HPV types showing significantly high odds ratio for each lesion; *, types not identified as single infection in each lesion.

their cervices differed from those detected in the condyloma tissue in most of these cases (Table 1). HPV-18, -51, -58, and -66 were detected as a single HPV types, and HPV-6, -31, -42, -44, -52, and -56 were detected as multiple HPV infections in the cervices (Table 1).

In the cervical samples that were positive for HPV infection, single infection was found in 8.6% (134 of 1562) of normal, 56% (81 of 145) of LSILs, 76% (104 of 137) of HSILs, and 81% (58 of 72) of SCCs (Table 1). HPV-11, -16, -18, -30, -31, -33, -35, -39, -42, -44, -45, -51, -52, -53, -54, -55, -56, -58, -59, -61, -62, -66, -67, -68, -72, and -73 were identified as single HPV types in NCX and in LSILs, whereas HPV-16, -18, -31, -33, -35, -45, -51, -52, -56, -58, and -67 were identified in HSILs and SCCs, and HPV-16 and -18 were identified in ADCA of the cervix. Of the HPV types thought to be cancer-associated types in a previous study (6, 8), HPV-39, -59, and -68 were not identified in HSILs and SCCs, and HPV 56 were not detected in SCCs.

The Relative Risk of Each HPV Type for Cervical Lesions. Table 2 shows the crude OR for the prevalence of each HPV type associated with various cervical lesions. The OR was calculated for all cases of single and multiple HPV infection. For LSIL, the OR was significantly high for HPV types 6, 11, 16, 31, 35, 39, 42, 44, 51, 52, 53, 56, 58, 59, 62, 66, and 68, with magnitudes between OR, 7.0 (95% CI = 2.3–22) for HPV-42 and OR, 139 (95% CI = 6.7–168) for HPV-66. For HSIL, statistically significant associations with risk were detected for HPV types 16, 31, 33, 35, 45, 51, 52, 56, and 58, with magnitudes between OR, 4.3 (95% CI = 1.1–17) for HPV-33 and OR, 43 (95% CI = 24–75) for HPV-16. For SCC, statistically significant risk estimates were observed for HPV types 11, 16, 18, 31, 51, 52, 53, and 58, with magnitudes between OR, 5.0 (95% CI = 1.84–14) for HPV-18 and OR, 69 (95% CI = 36–131) for HPV-16. For ADCA, statistically significant risk estimates were observed for HPV types 11, 16, and 18, with magnitudes between OR, 24 (95% CI = 2.6–212) for HPV-11 and OR, 94 (95% CI =, 28–317) for HPV-18. Because HPV-6 and -68 in LSIL, HPV-45 in HSIL, HPV-11 and -53 in SCC, and HPV-11 in ADCA were not identified as single HPV types, these types are not considered to be associated with these lesions. From these findings, we conclude that HPV-11, -39, -42, -44, -53,

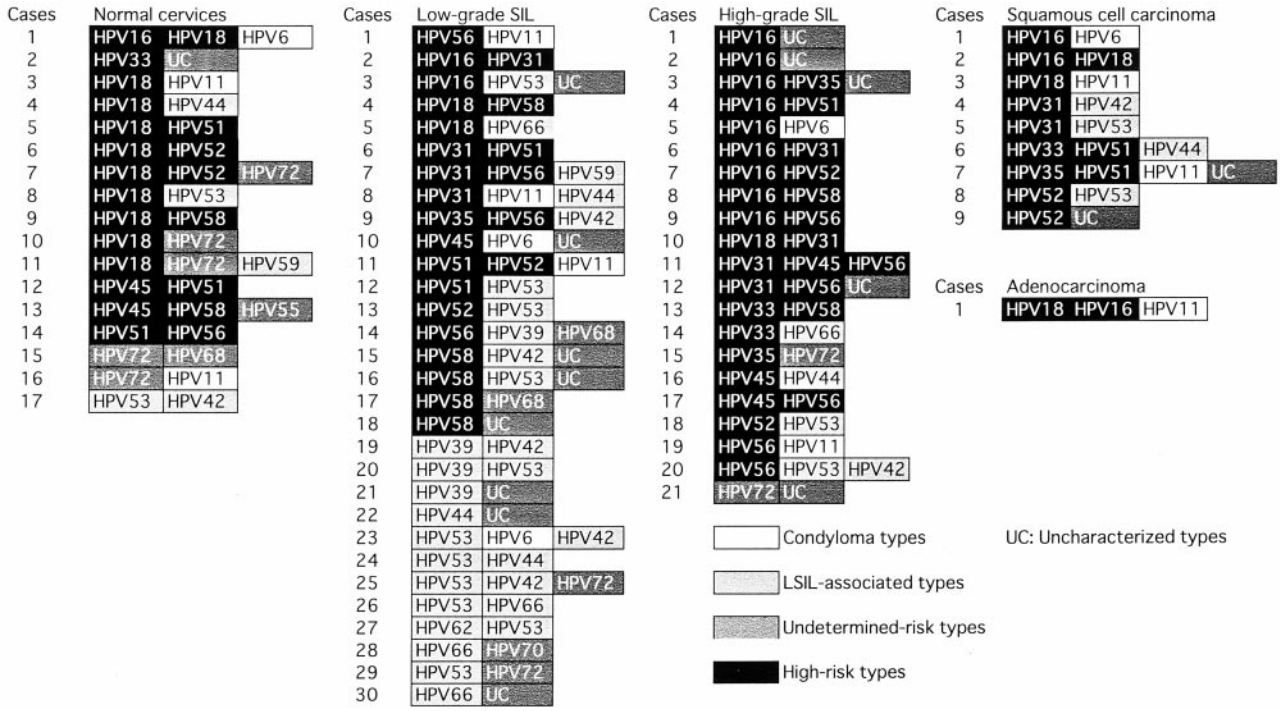

Fig. 1. HPV types identified as multiple HPV infection in cervical cell samples form different cervical lesions. Condyloma types are HPV-6 and -11 because they were identified in condyloma tissues. LSIL-associated types are HPV-39, -42, -44, -53, -54, -59, -61, -62, -66, and -73, identified as single type in LSIL. High-risk types are HPV-16, -18, -31, -33, -35, -45, -51, -52, -56, and -58, identified as single type in HSIL or invasive cervical cancer. *UC*, undetermined HPV types by our assay.

-59, -62, and -66 are LSIL-associated types, HPV-16, -18, -31, -51, -52, and -58 were cervical cancer (SCC) -associated types, and HPV-33, -35, and -56 were HSIL-associated types. HPV types 16, 18, 31, 51, 52, 58, and perhaps 33, 35, and 56 are considered high-risk types. The magnitude of OR for HPV-16 dramatically increased with malignancies of cervical lesions, whereas ORs for other HPV types did not, suggesting that HPV-16 is the most malignant type. The risk of other HPV types, such as HPV-6, -30, -54, -55, -61, -67, -68, -70, -72, and -73, were not determined in the present study. However, HPV-6 was identified in condyloma tissue, HPV-54, -61, and -73 were detected as single-infection in LSIL, HPV-45 in SCC and HPV-67 was in LSIL and SCC, suggesting that HPV-6 is condyloma type, HPV-54, -59, -61, and -73 may be LSIL-associated types, and HPV-45 and -67 may be high-risk types. HPV-11 is likely to be associated with both condyloma and LSIL of the cervix. On the other hand, HPV-30, -55, -68, -70, and -72 were undetermined for the risk of any cervical lesions.

High-Risk and Multiple HPV Infections in Cervical Lesions. Multiple HPV infection in LSIL (27%, 30 of 111) was a little more frequent than in HSIL (17%, 21 of 125; $P = 0.056$) and significantly more frequent than in SCC (13%, 9 of 67; $P = 0.033$; Table 1). The OR for multiple HPV infection was significantly higher for LSIL (OR, 24; 95%CI = 13–44), HSIL (OR, 16; 95%CI = 8.4–32), and SCC (OR, 8.3; 95%CI = 3.2–22), suggesting that multiple infection is associated with all stages of cervical lesions (Table 2).

Next, we tried to identify all of the HPV types involved in multiple infection, although we failed to characterize HPV types in 16 cases (1 normal, 8 LSILs, 5 HSILs, and 2 SCCs; Fig. 1). HPV-6 and -70 were not identified as single types, but as one of multiple types in cervical cell samples. In multiple infection, the high-risk HPV types were identified as one of multiple types in 82% (14 of 17) of NCX, 60% (18 of 30) of LSILs, 95% (20 of 21) of HSILs, all SCCs (9 of 9), and all ADCAs (1 of 1). In contrast, condyloma types (HPV-6 and -11), LSIL-associated types (HPV-39, -42, -44, -53, -54, -59, -61, -62, -66, and -73), and the undetermined risk types (HPV-30, -55, -68, -70, -72) were identified in 65% (11 of 17) of NCX, 90% (27of 30) of LSILs, 57% (12 of 21) of HSILs, 89% (8 of 9) of SCCs, and all (1 of 1) ADCAs. This analysis also revealed that the high-risk types included in multiple infection cases increased in percentage according to the grade of cervical lesions.

The proportion of HPV infection patterns in each lesion is demonstrated according to the classification of HPV types with the risk for cancer in this study (Fig. 2). The proportion of single high-risk HPV infections increased with the malignant grade of the lesion and was 56, 49, 75, and 78% for NCX, LSIL, HSIL, and SCC, respectively. In contrast, condyloma, LSIL-associated, and undetermined-risk types were positive in 19% (28 of 151) and 17% (19 of 111) of NCX and LSILs, respectively, although none of these types was found as single infections in HSILs and SCCs. Combining single and multiple infections, high-risk types were identified in 91% (114 of 125) of HSILs, 91% (61 of 67) of SCCs, and 100% (8 of 8) of ADCAs, whereas they were only detected in 65% (72 of 111) of LSILs, and 65% (98 of 151) of NCX. In either analysis of single infection or the combining one, high-risk HPV types were significantly prevalent in HSIL and SCC than in NCX and LSIL ($P < 0.03$).

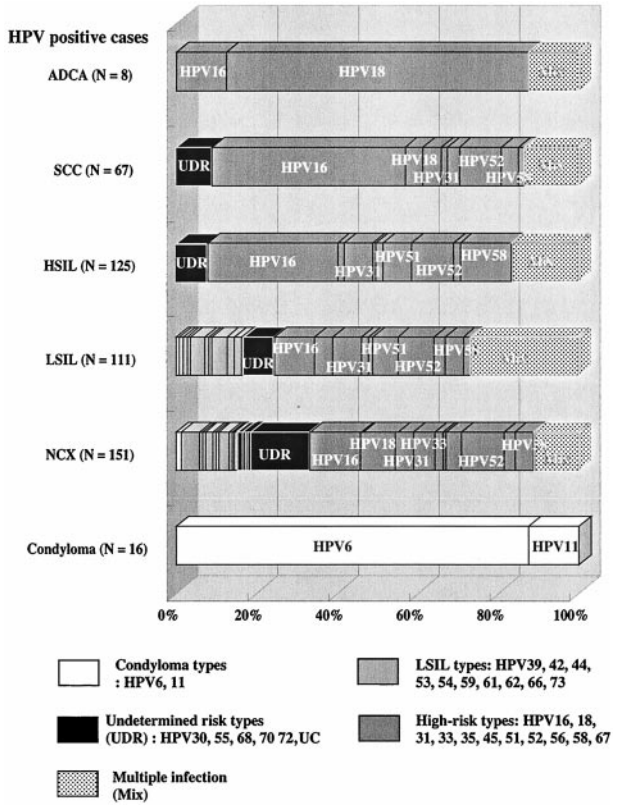

Fig. 2. Proportion of HPV types associated with different genital lesions. HPV types shown here were detected as single infections. Multiple infection (*Mix*) and undetermined risk types (*UDR*) include HPV-30, -55, -68, -70, and -72 and uncharacterized types (*UC*).

Discussion

Our new PCR test (16) identifies most mucosal HPV types; both the spectrum of detectable HPV types and the sensitivity are equivalent to the MY09/11-PCR method (15). Using this test, we conducted a case-control study in the Hokuriku area of Japan. We found that 9.7% (151 of 1562), 77% (111 of 145), 91% (125 of 137), 93% (67 of 72), and 67% (8 of 12) of NCX, LSILs, HSILs, SCCs, and ADCAs were positive for HPV, respectively (Table 1). The prevalence of HPV in various cervical lesions in this study was quite similar to the results of a recent population-based study conducted in Costa Rica (15).

In our study, only HPV-6 (88%) and -11 (12%) were detected in condyloma tissues, suggesting that both types are condyloma-specific. Women with condyloma acuminata in their vulva or vagina were concomitantly infected with other HPV types in their cervices. This suggests that the preferred infection sites of HPV-6 and -11 might be the vagina and vulva, whereas the other mucosal types prefer the cervix. HPV-6 is more likely to infect the vagina or the vulva than HPV-11 because HPV-6 was not identified as a single type in the cervix in this study. Liaw *et al.* have first reported that HPV-52 and -58 are the most prevalent types in HSILs and SCCs in China (20). In our study, HPV-16 was the most prevalent type in HSILs and in SCCs. HPV-52 was the next most common in SCCs (12%, 7 of 58), and HPV-58 was the next most common in HSILs (15%, 16 of 104). Therefore, HPV-52 and -58 appear to be the most prevalent HPV types in East Asia. When the prevalence of each HPV type in cervical cancer was compared with the results in a worldwide study conducted by Bosch *et al.* (6), HPV-51 and -52 were more prevalent in Japan, whereas HPV-18 and -45 were less prevalent in Japan than in other areas (Table 3). However, we have to be careful in comparison of the prevalence of HPV infection between both studies, because we tested cytological samples from cervical cancer, and the former study used cancer tissue.

The relative risk of each HPV type for cervical lesions was estimated using the OR. This analysis showed that HPV types 16, 18, 31, 51, 52, and 58 were associated with SCC, and HPV types 16, 31, 33, 35, 45, 51, 52, 56, and 58 were associated with HSIL. Therefore, HPV-16, -18, -31, -51, -52, and -58, and perhaps HPV-45 and -67, are considered as the high-risk types in Japanese women, inasmuch as these types were identified as single-type in invasive cervical cancer. In contrast, HPV-33 and -56 were not detected as single-type in invasive cancer, although they were associated with HSIL in the present analysis. Our results showed that HPV types 11, 39, 42, 44, 53, 54, 59, 61,62, 66, and 73 were associated with LSIL, whereas they were not associated with HSIL and invasive cancer. Therefore, these types are LSIL-associated types and may be low-risk for cancer. HPV-6, -11, -42, and -44 are thought to be the low-risk types from many previous studies. Most of our results concur with the results of many previous studies. However, HPV-39, -59, and -68 were not identified in HSIL and SCC, although these types are thought to be associated with cancer (6). The risk of HPV-30, -55, -68, -70, and -72 for cancer were not determined in the present study. Additional study is needed to clarify whether these uncommon types are actually high-risk types or others.

There were many multiple HPV infections in our clinical samples. Multiple infection appears to be a characteristic of women with cervical abnormalities, inasmuch as it showed statistically significant associations with the risk for LSIL, HSIL, and SCC in this study. Multiple infection was more frequent in LSILs than in malignant lesions (HSILs and SCCs; $P < 0.05$). This may represent a segregation of certain HPV types in cancer cells.

When we examined the HPV types involved in multiple infection, high-risk types were identified as one of the types in most HSILs and in all cervical cancers, whereas 60% of LSILs had high-risk types. For single-HPV infection and combining single and multiple infections, high-risk types were more prevalent in HSILs and in SCCs than in NCX and in LSILs ($P < 0.03$). All of our findings support the idea that there are two different groups of cervical lesions: (*a*) low-risk HPV-associated lesions; and (*b*) high-risk HPV-associated lesions. A previous study showed that the pathological grade shift from CIN I to III in the same cases was associated with infection by different HPV types (21). The higher-grade lesions (CIN III) were infected with HPV types 16 or 18, whereas the lower grade lesions (CIN I) were infected with HPV 11 in the report. Thus, lower-grade cervical lesions can be induced by low-risk or high-risk HPV types or both, whereas high-risk HPV infection alone promotes cancer. Two recent studies also showed that high-risk HPV infection persists longer than low-risk HPV types (14, 22). The cervix may deal with low-risk HPV-associated lesions easily, whereas high-risk HPV-associated lesions may resist host immunity and persist longer. In the present study, high-risk types alone appear to be segregated in HSIL and in SCC, suggesting that high-risk HPV-associated lesions are able to persist and progress to HSIL and SCC. This may fit the model of multistep carcinogenesis of the uterine cervix supported by many pathologists. In contrast, the low-risk HPV-associated lesions are merely HPV-induced transient lesions.

Table 3 Comparison of the prevalent HPV types in cervical cancer between Japan and the world[a]

Author	Bosh *et al.* (6)												Sasagawa *et al.*		
Areas	Africa		Central South Africa		South East Asia		Europe		North America		All the world		Japan		
No. positive	186 *n*	%	505 *n*	%	98 *n*	%	86 *n*	%	57 *n*	%	932 *n*	%	84 *n*	%	
HPV types															
6/11	0	0.0%	2	0.4%	0	0.0%	2	2.3%	0	0.0%	2	0.2%	4	6%	
16	79	42.5%	255	50.5%	42	42.9%	56	65.1%	33	58%	465	49.9%	36	50%	
18	33	17.7%	48	9.5%	31	31.6% [c]	7	8.1%	9	16%	128	13.7%	12	14%	a
26	0	0.0%	4	0.8%	0	0.0%	0	0.0%	0	0%	4	0.4%	0	0%	
31	5	2.7%	35	6.9%	1	1.0%	5	5.8%	3	5%	49	5.3%	5	7%	
33	5	2.7%	18	3.6%	2	2.0%	1	1.2%	0	0.0%	26	2.8%	1	1%	
35	4	2.2%	10	2.0%	1	1.0%	1	1.2%	0	0.0%	16	1.7%	1	1%	
39	0	0.0%	13	2.6%	1	1.0%	0	0.0%	0	0.0%	14	1.5%	0	0%	
45	23	12.4%	37	7.3%	8	8.2%	2	2.3%	8	14%	78	8.4%	1	1%	b
51	2	1.1%	5	1.0%	0	0.0%	0	0.0%	0	0.0%	7	0.8%	4	6%	
52	4	2.2%	16	3.2%	2	2.0%	3	3.5%			25	2.7%	9	13%	
55	0	0.0%	2	0.4%	0	0.0%	0	0.0%	0	0.0%	2	0.2%	0	0%	
56	6	3.2%	3	0.6%	3	3.1%	2	2.3%	2	4%	16	1.7%	0	0%	
58	5	2.7%	11	2.2%	2	2.0%	1	1.2%	0	0.0%	19	2.0%	3	4%	
59	0	0.0%	14	2.8%	1	1.0%	0	0.0%	0	0.0%	15	1.6%	0	0%	
68	4	2.2%	2	0.4%	1	1.0%	3	3.5%	1	2%	11	1.2%	0	0%	

[a] HPV type which was more prevalent in Japan than in the other countries shown with dark gray.
[b] HPV type which was less prevalent in Japan than in the other countries shown with light gray. Difference in the prevalence between different countries was evaluated with χ^2 test.
[c] The reference data showing significant difference with Japanese data are shown with open box.

The reasons why a high-risk HPV infection is able to persist longer than a low-risk HPV infection remain to be investigated in the future.

A recent study found that multiple HPV infection is a factor in persistent HPV infection in normal young women (14). Some studies also indicate that persistent HPV infection is important for the development of cervical dysplasia (14, 23). Combining these results, multiple HPV infection is likely to be associated with the development of cervical neoplasia. Multiple HPV infection may produce conditions that confer immunological protection for persistent infection of HPV, or women with multiple HPV infections may be susceptible to HPV infection, although additional study is needed to elucidate these hypotheses.

Our findings suggest that it is important to identify HPV types and HPV infection patterns to predict the outcome in women with HPV DNA in their cervices. Accumulated data for the high-risk HPV types from different countries help determine the clinicopathological grading of HPV types. Under a consensus of clinicopathological grading of HPV types, HPV tests could be clinically useful for laboratory quality control and adjudication of ASCUS.

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