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Comparison of methods using paraffin-embedded tissues and exfoliated cervical cells to evaluate human papillomavirus genotype attribution

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Key words

Cervical intraepithelial neoplasia, exfoliated cervical cell, formalin-fixed paraffin-embedded tissue, HPV vaccine, human papillomavirus

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Monitoring the attribution of human papillomavirus (HPV) genotypes to cervical precancerous lesions is essential in assessing the efficacy of HPV vaccines. To resolve the lack of studies comparing the HPV genotyping procedures used to estimate HPV genotype attribution, we undertook a retrospective cross-sectional study to determine the appropriate genotyping procedures for evaluating the potential efficacy of HPV vaccines. Three procedures, including two different genotyping methods, Clinichip HPV test (C-Chip) and modified GP5+/6+ PCR coupled to fluorescent bead sorter detection (MGP), using exfoliated cervical cells (C-Chip and C-MGP, respectively) or formalin-fixed paraffin-embedded tissues (F-MGP), were compared. The overall agreement in detecting high-risk HPV was 88.5–92.1% among the three procedures, and genotype-specific agreement was 83.9–100% for all pairwise comparisons. In cervical intraepithelial neoplasia grade 2/3 specimens, HPV16/18 attribution estimated with the hierarchical attribution method was consistent among the procedures: 52.3% (45/86) for C-Chip, 54.7% (47/86) for C-MGP, and 52.3% (45/86) for F-MGP ($P = 0.81$). HPV16/18/31/33/45/52/58 hierarchical attribution was 88.4% (76/86) with C-Chip, 86.0% (74/86) with C-MGP, and 83.7% (72/86) with F-MGP ($P = 0.49$). In cervical intraepithelial neoplasia grade 3 specimens, the corresponding hierarchical attribution was 96.4% (53/55) with C-Chip, 89.1% (49/55) with C-MGP, and 94.5% (52/55) with F-MGP ($P = 0.27$). Although F-MGP is theoretically a reliable method for determining HPV genotype attribution, it is acceptable to use C-Chip or C-MGP, coupled to the hierarchical attribution formula to correct the bias of multiple infections. These approaches using exfoliated cervical cells are practical for monitoring the efficacy of HPV vaccines.

Persistent infection with oncogenic human papillomavirus (HPV) is the primary risk factor for the development of cervical cancer, and among at least 15 genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) recognized as high-risk HPV, HPV16/18 accounts for nearly 70% of cervical cancers and 50% of high-grade cervical lesions worldwide.[1] Accurate HPV genotyping is important for the clinical management of cervical cancer because the oncogenic potential of high-risk genotypes varies in the different processes of cervical carcinogenesis. In fact, the American Society for Colposcopy and Cervical Pathology released a clinical guideline for the management of individual cases of HPV16/18 infection or infection with other high-risk genotypes.[2] High-risk genotypes other than HPV16/18 should not be ignored in the prevention of cervical cancer, because the remaining 30% of cervical cancers are attributable to these non-HPV16/18 genotypes. Human papillomavirus genotyping is also crucial for epidemiological studies that evaluate HPV infection and monitor the efficacy of prophylactic HPV vaccines.[3] Two HPV vaccines, a quadrivalent vaccine targeting HPV6/11/16/18 and a bivalent vaccine targeting HPV16/18, are currently administered worldwide, and a nonavalent vaccine targeting five additional high-risk genotypes, HPV31, 33, 45, 52, and 58, together with HPV6/11/16/18, has been introduced in the USA, Canada, and Europe.[4,5] Because a higher prevalence of non-HPV16/18 genotypes is observed in precancerous cervical lesions and cervical cancers in East Asian countries, including Japan, the nonavalent HPV vaccine will be valuable if it is proven sufficiently effective.[6,7]

The most appropriate method for determining the causal attribution of individual HPV genotypes in cervical lesions when HPV prevalence is monitored in clinical and epidemiological settings is controversial. Some researchers use formalin-fixed paraffin-embedded (FFPE) tissues,[1,6,8] whereas others use exfoliated cervical cells.[9] The use of exfoliated cervical cells for HPV genotyping has been widely accepted for screening or vaccine clinical trials.[10,11] However, because a single cervical lesion generally arises from infection with

one genotype,[12] care should be taken regarding the co-detection of transient or non-causal infections that originate from the bulk population of exfoliated cervical cells. To overcome this limitation, an attribution formula for lesions infected with multiple HPV genotypes is incorporated, and subsequently used to estimate the HPV genotype attribution.[1,6,8,9,13] Theoretically, FFPE tissue specimens are a more reliable source of cancer cells for determining causative genotypes because these tissues contain small pathologically confirmed lesions. However, the recovery rate of DNA from FFPE tissue specimens is generally much lower than from exfoliated cervical cells,[13] and formalin fixation introduces DNA cross-linking and fragmentation. The extent of DNA damage might also be affected by the period of formalin fixation, a time interval between sampling to testing. Therefore, the preferred genotyping method is generally based on exfoliated cervical cells.

In Japan, clinical HPV genotyping is part of the protocol for the management of patients with cervical intraepithelial neoplasia (CIN), and its cost is reimbursed by the National Health Insurance System for patients diagnosed with CIN1/2. The Clinichip HPV test (Sekisui Medical, Tokyo, Japan) is an HPV genotyping assay that has recently been validated[14–16] and is covered by the insurance system, but is only applicable to specimens of exfoliated cervical cells. Söderlund-Strand *et al.*[17] established a multiplex PCR method with modified GP5+/6+ primers to amplify high-risk HPV DNA, followed by its detection with a fluorescent bead sorter (hereafter referred to as MGP). The successful amplification of HPV DNA from FFPE tissue specimens was reported to correlate inversely with the amplicon length.[18,19] Because the amplicon size in MGP PCR is relatively small (140 bp), MGP appears to be suitable for genotyping both FFPE tissue specimens and exfoliated cervical cells.[20] However, no previous study has reported a comparison of the data obtained from FFPE tissue specimens and from exfoliated cervical cells from the same patients. Here, using an identical set of clinical specimens, we carried out a retrospective cross-sectional study to evaluate the HPV type attributions made with different genotyping procedures, involving combinations of Clinichip or MGP with exfoliated cervical cells or FFPE tissue specimens histologically diagnosed with CIN2/3 or CIN3, to assess the potential efficacy of HPV vaccines in Japan.

Materials and Methods

Human papillomavirus genotyping procedures. Two HPV genotyping methods, the Clinichip HPV test and PCR with modified GP5+/6+ primers followed by detection with a fluorescent bead sorter (MGP), were used to examine exfoliated cervical cells. The Clinichip HPV test amplifies HPV DNA of approximately 460 bp and distinguishes 13 high-risk genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) using loop-mediated isothermal amplification technology and an electric DNA-chip system. The MGP PCR amplifies HPV DNA of 140 bp and distinguishes 16 genotypes (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).[17] In addition to the 13 high-risk genotypes detected by the Clinichip HPV test, MGP detects possibly carcinogenic HPV66[21] and low-risk HPV6 and 11. Briefly, total genomic DNA was isolated from exfoliated cervical cells with proteinase K treatment, and extracted with phenol–chloroform. The extracted DNA (4 μg) was subjected to the Clinichip HPV test, and another 4 μg was subjected to MGP PCR, followed by detection with a Bio-Plex bead sorter (Bio-Rad Laboratories, Hercules, CA, USA). Human papillomavirus was genotyped in FFPE tissue specimens as follows. Total DNA was extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) from 30-μm-thick sections of FFPE tissues, in a final elution volume of 50 μL. An aliquot (5 μL) of the extracted DNA was analyzed with MGP. We designated the three genotyping procedures: (i) Clinichip with exfoliated cervical cells as C-Chip; (ii) MGP with exfoliated cervical cells as C-MGP; and (iii) MGP with FFPE tissues as F-MGP. High-risk HPVs were defined as HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 in this study, and when a patient was positive for both HPV6 and 16, their specimens were treated as a single high-risk HPV16 infection.

Study group. In total, 105 Japanese patients, 39 with CIN2 and 66 with CIN3 including adenocarcinoma *in situ* (AIS), treated at Keio University Hospital (Tokyo, Japan), between October 2008 and November 2011 were enrolled. Two cases of AIS were diagnosed as pure AIS and one was diagnosed as AIS coexisting with CIN3. The exclusion criteria included age <18 years, pregnancy, or previous treatment with chemotherapy or radiation for any cancer. The study was carried out with the approval of the institutional ethical committees. All patients were referred for follow-up with colposcopy for cytological abnormalities. The colposcopic examinations were undertaken by two gynecological oncologists (T.F. and T.I.) certified by the Japan Society of Gynecologic Oncology. The histological findings were confirmed with colposcopy-directed punch biopsies. Both exfoliated cervical cells and colposcopy-directed biopsy tissues were taken from all the patients for comparison (Fig. 1). Eighteen of the 105 patients with CIN2/3 were excluded from the statistical analysis because 13 patients had no data for C-MGP and five had insufficient quantities of DNA from the FFPE tissue specimens and therefore no data for F-MGP. The remaining 87 patients, who had results for all three procedures, were included in the analysis. The median patient age was 35 years (range, 21–63 years), with an age distribution of: 17 patients aged 21–29 years; 53 patients aged 30–39 years; 16 patients aged 40–50 years; and one patient aged 63 years.

Statistical analysis. The causal attributions of HPV genotypes were analyzed in patients with CIN2/3 and CIN3 including

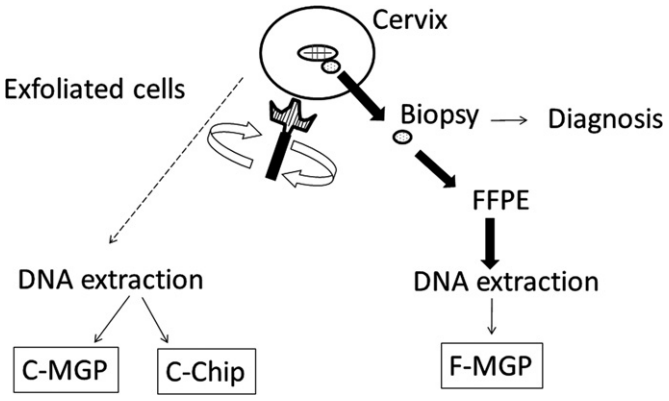

Fig. 1. Preparation of clinical specimens and human papillomavirus (HPV) genotyping. Dotted and bold arrows indicate the flow of material preparation from exfoliated cervical cells and formalin-fixed paraffin-embedded (FFPE) tissue specimens, respectively. HPV genotyping was carried out using the Clinichip HPV test using exfoliated cervical cells (C-Chip), a modified general-primer PCR system using exfoliated cervical cells (C-MGP), and a modified general-primer PCR system using FFPE tissues from biopsied specimens (F-MGP).

AIS. Patients with results for all three procedures were eligible for further statistical analysis. To determine the attribution of the HPV genotypes, the distributions of single and multiple infections were analyzed with the three procedures. Based on a previous report,[13] the categories of agreement among the three procedures were classified as: (i) identical, when exactly the same genotypes or none were identified with both methods; (ii) compatible, when at least one HPV genotype was found in common; and (iii) discrepant, when no HPV genotype was identified in common or HPV was negative with either method. The agreement in HPV genotyping between paired methods was also evaluated with the κ-value and uneven distributions of HPV genotyping were evaluated with the McNemar test.[22]

The crude coverage of the bivalent and quadrivalent vaccine types was calculated based on the detection rate of either HPV16 or 18 without other high-risk genotypes. For example, when a multiple infection with HPV16 and 31 was detected in a specimen, this case was not considered to be covered by the bivalent or quadrivalent vaccine. The crude coverage of the nonavalent vaccine (HPV16/18/31/33/45/52/58) was calculated in a similar manner. To more logically analyze the HPV genotypes attributed to the CIN2/3 or CIN3 lesions, we used proportional and hierarchical attribution methods.[8,9] Briefly, in the proportional method, in a specimen containing HPV16, 31, and 52, the genotype attribution was split between the three types in proportion to the overall frequencies of the respective genotypes detected in a specific disease category (e.g., if the prevalence of HPV16, 31, and 52 were 50%, 11%, and 15%, respectively, in the CIN category, then we allocated 50/76 to HPV16, 11/76 to HPV31, and 15/76 to HPV52 in this case). In the hierarchical method, the abovementioned case was exclusively attributed to HPV16, which was the most prevalent ("hierarchical") genotype detected in the CIN2/3 samples (n = 87) (the hierarchical order was HPV16, 52, 31, 51, 58, 18, and 33 with F-MGP) (Fig. 2, left panel).[9] The type distributions used to calculate the proportional or hierarchical method are described in Table S1. The HPV genotype attribution was calculated and classified into two categories: (i) HPV16/18 for the bivalent or quadrivalent vaccine; and (ii) HPV16/18/31/33/45/52/58 for the nonavalent vaccine. The Cochran Q test was used to test the differences among the three procedures. The level of significance was set at $P < 0.05$. To apply the McNemar test with Bonferroni's correction, data were paired in combinations of C-Chip, C-MGP, and F-MGP for each patient. A two-sided value of $P < 0.05$ was set as the level of significance. All statistical analyses were undertaken with SPSS version 21 (SPSS, Chicago, IL, USA).

Results

Agreement between the three HPV genotyping procedures. In all CIN2/3 cases (n = 87) analyzed with the three genotyping procedures, the percentage of identical results ranged from 52.9% for C-MGP *versus* F-MGP to 58.6% for C-Chip *versus* C-MGP, and the percentage compatible results ranged from 25.3% for C-MGP *versus* F-MGP to 32.2% for C-Chip *versus* F-MGP (Table 1). Discrepant results were usually associated with negative test results for one procedure (Table S2a), with the highest discrepancy observed for C-MGP *versus* F-MGP (21.8%) and the lowest for C-Chip *versus* C-MGP (11.5%). The genotyping results of four HPV-positive discrepant cases between C-MGP and F-MGP are shown in Table S2b. Among

Table 1. Human papillomavirus (HPV) genotyping agreement among three procedures

CIN2/3 (n = 87)	C-Chip/C-MGP		C-Chip/F-MGP		C-MGP/F-MGP	
	n	%	n	%	n	%
Identical	51	58.6	48	55.2	46	52.9
Compatible	26	29.9	28	32.2	22	25.3
Discrepant	10	11.5	11	12.6	19	21.8

C-Chip, cervical exfoliated cells evaluated by Clinichip HPV test; CIN, cervical intraepithelial neoplasia; C-MGP, cervical exfoliated cells evaluated by PCR with modified general primers; F-MGP, formalin-fixed paraffin-embedded tissues evaluated by PCR with modified general primers.

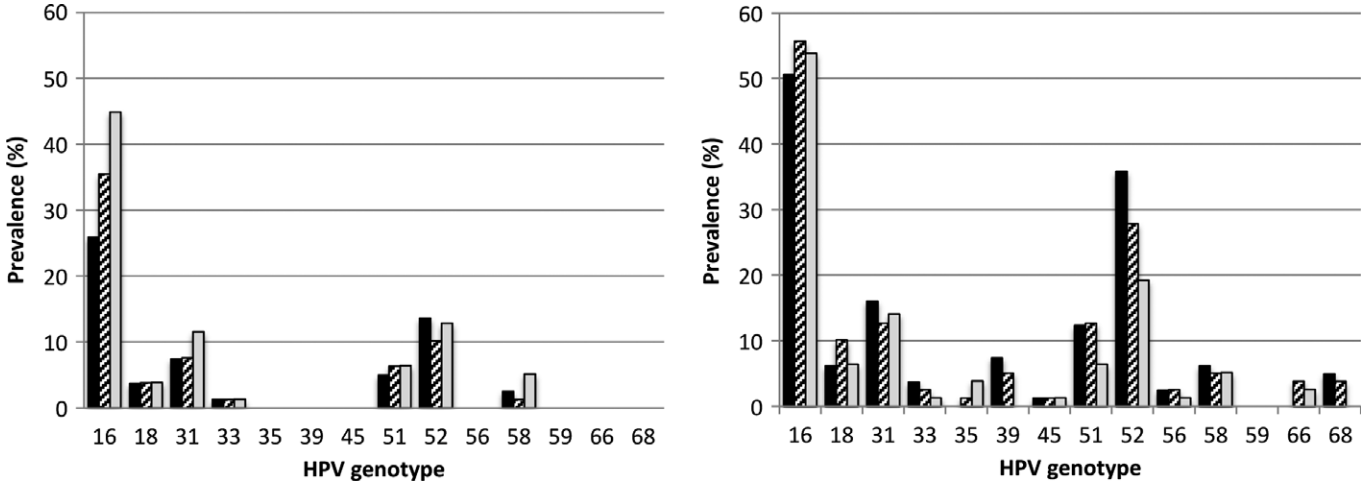

Fig. 2. Distribution of human papillomavirus (HPV) genotypes detected with three procedures in cases of cervical intraepithelial neoplasia grade 2/3 (n = 87). Left panel, prevalence (%) of single infections; right panel, prevalence of both single and multiple infections. Black column, HPV genotyping with the Clinichip HPV test using exfoliated cervical cells; hatched column, HPV genotyping with a modified general-primer PCR system using exfoliated cervical cells; gray column, HPV genotyping with a modified general-primer PCR system using formalin-fixed paraffin-embedded tissues from biopsied specimens.

the four cases, two cases showed multiple infections in C-Chip results between the types detected with C-MGP or F-MGP, which may suggest biased detection of one genotype with either procedure.

The genotype-specific agreement ranged from 83.9% to 100% for all pairwise comparisons (Table 2), yielding κ-values between 0.477 and 1.000, indicating moderate or perfect correlations among the three procedures, except for HPV66. The detection rate of high-risk HPV ranged from 89.7% for F-MGP to 93.1% for C-Chip (Table 3), although the three procedures did not differ significantly ($P = 0.662$).

The type-specific prevalence of high-risk HPV in the CIN2/3 cases were similar among the three procedures, although there were some differences (Fig. 2). Overall, HPV16 was most frequently detected by all three procedures, followed by HPV52, 31, 51, and 18, consistent with a previous report in Japan.[7] Looking at the distribution patterns in more detail, single HPV16 infections were more often detected with F-MGP (44.9%) than with C-Chip (25.9%) or C-MGP (35.4%). In contrast, when both single and multiple infections are considered, similar levels of HPV16 infection were observed with the three procedures, indicating that more HPV16 was detected as multiple infection with C-Chip than with C-MGP or F-MGP. Multiple infections of HPV52 were also more frequently detected with C-Chip and C-MGP than with F-MGP, accounting for the higher proportions of HPV52 in the total genotyping results obtained with C-Chip and C-MGP.

The percentage agreement for HPV16 detection ranged from 83.9% for C-Chip *versus* C-MGP to 91.7% for C-Chip *versus* F-MGP (Table 2). The discrepant results are shown in Table S3. Among the 13 cases that were inconsistent between C-MGP and F-MGP (green in Table S3), nine cases showed multiple infections with both procedures (yellow in Table S3), suggesting that competitive PCR amplification might have occurred between high- and low-load HPV DNAs coexisting in some samples. In contrast, the 15 discrepant results between C-Chip and C-MGP showed no apparent trend in inconsistency (orange in Table S3), although HPV16 was more frequently detected with C-MGP (10 with C-MGP *vs*. six with C-Chip), which may suggest a slightly higher sensitivity of C-MGP for the detection of HPV16 in cases of multiple infections (Fig. 2, right panel).

Overall, the detection rates for multiple infections were 12.6% for F-MGP, 31.0% for C-MGP, and 36.8% for C-Chip (Table 3). Although the difference between C-Chip and C-MGP was not significant ($P = 0.96155$), the detection rate for multiple infections with F-MGP was significantly lower than those with the other two procedures ($P = 0.00021$ for C-Chip *vs*. F-MGP; $P = 0.00864$ for C-MGP *vs*. F-MGP). The use of exfoliated cervical cells as a specimen source resulted in a higher detection rate of multiple infections, suggesting that transient infections or infections unrelated to the causality of the CIN2/3 lesion might have been detected.

Estimation of HPV genotype attribution with different calculation methods. Although the crude estimation of HPV16/18 attribution to CIN2/3 lesions using either exfoliated cervical cells or FFPE tissue specimens yielded significantly different values among the three procedures ($P = 0.00125$), the HPV16/18 attribution calculated with the hierarchical attribution method was consistent, ranging from 52.3% for C-Chip to 54.7% for C-MGP (Table 3). When restricted to the CIN3 specimens ($n = 55$), the HPV16/18 attribution estimated with the hierarchical method was also similar among the three procedures, ranging from 63.6% for F-MGP to 65.5% for C-Chip and C-MGP ($P = 0.931$). This means that there were no significant

Table 2. Human papillomavirus (HPV) genotype-specific agreement among three procedures for cases with cervical intraepithelial neoplasia grade 2/3 ($n = 87$)

C-Chip/C-MGP	% Agreement	McNemar	κ	SD	C-Chip/F-MGP	% Agreement	McNemar	κ	SD	C-MGP/F-MGP	% Agreement	McNemar	κ	SD
16	83.9	0.424	0.679	0.078	16	91.7	0.453	0.835	0.060	16	84.7	0.581	0.694	0.078
18	95.4	0.625	0.690	0.146	18	100.0	1.000	1.000	0.000	18	95.4	0.625	0.690	0.146
31	96.6	0.250	0.850	0.084	31	96.5	0.250	0.850	0.084	31	97.7	1.000	0.887	0.079
33	97.7	0.500	0.491	0.306	33	97.7	0.500	0.491	0.306	33	100.0	1.000	1.000	0.000
35	98.9	NA	NA	NA	35	100.0	NA	NA	NA	35	98.9	NA	NA	NA
39	97.7	0.500	0.821	0.123	39	95.4	0.125	0.580	0.186	39	95.4	0.625	0.477	0.222
45	100.0	1.000	1.000	0.000	45	98.9	NA	NA	NA	45	98.9	NA	NA	NA
51	97.7	1.000	0.887	0.079	51	93.1	0.219	0.590	0.150	51	93.1	0.219	0.590	0.150
52	88.5	0.021	0.722	0.080	52	83.9	0.000	0.588	0.092	52	86.2	0.146	0.583	0.106
56	100.0	1.000	1.000	0.000	56	98.8	1.000	0.661	0.317	56	98.8	1.000	0.661	0.317
58	95.4	0.625	0.577	0.190	58	95.4	0.625	0.577	0.190	58	97.7	1.000	0.738	0.178
59	100.0	NA	NA	NA	59	100.0	NA	NA	NA	59	100.0	NA	NA	NA
66	96.6	NA	NA	NA	66	98.9	NA	NA	NA	66	95.4	0.625	−0.018	0.013
68	96.6	1.000	0.554	0.230	68	95.4	NA	NA	NA	68	96.6	NA	NA	NA
High risk	88.5	0.754	0.225	0.166	High risk	89.7	0.508	0.346	0.168	High risk	92.1	1.000	0.022	0.210

Cervical exfoliated cells evaluated by PCR with modified general primers (C-MGP) and formalin-fixed paraffin-embedded tissues evaluated by PCR with modified general primers (F-MGP): 14 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) were analyzed. Cervical exfoliated cells evaluated by the Clinichip HPV test (C-Chip): 13 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) were analyzed. NA, not applicable.

Table 3. High-risk human papillomavirus (HPV) detection rates and expected coverages for prophylactic vaccines

Method	CIN2/3 (n = 87)			Cochran Q	C-Chip/ C-MGP	C-Chip/ F-MGP	F-MGP/ C-MGP	CIN3 (n = 55)			Cochran Q	C-Chip/ C-MGP	C-Chip/ F-MGP	F-MGP/ C-MGP
	C-Chip†	C-MGP†	F-MGP‡					C-Chip	C-MGP	F-MGP				
High-risk HPV-positive rate (%)	81/87 (93.1)	79/87 (90.8)	78/87 (89.7)	0.662	NA	NA	NA	53/55 (96.4)	50/55 (90.9)	52/55 (94.5)	0.459	NA	NA	NA
HPV-negative	6	8	9					2	5	3				
Single infection	49	52	67					27	32	47				
Multiple infection	32	27	11					26	18	5				
Multiple infection rate (%)	32/87 (36.8)	27/87 (31.0)	11/87 (12.6)	0.00019*	0.96155	0.00021*	0.00864*	26/55 (47.3)	18/55 (32.7)	5/55 (9.1)	0.00001*	0.19223	0.00000*	0.00787*
Crude coverage for vaccine for HPV16/18	26/87 (29.9)	33/87 (37.9)	40/87 (46.0)	0.00125*	0.20300	0.00770*	0.20300	18/55 (32.7)	25/55 (45.5)	32/55 (58.2)	0.00044*	0.14800	0.00025*	0.14800
Adjusted coverage for vaccine for HPV16/18 by hierarchical attribution	45/86 (52.3)	47/86 (54.7)	45/86 (52.3)	0.81000	NA	NA	NA	36/55 (65.5)	36/55 (65.5)	35/55 (63.6)	0.93100	NA	NA	NA
Adjusted coverage for vaccine for HPV16/18 by proportional attribution	40.8/86 (47.4)	45.0/86 (52.3)	44.6/86 (51.8)	NA	NA	NA	NA	32.6/55 (59.3)	34.8/55 (63.2)	34.6/55 (62.9)	NA	NA	NA	NA
Crude coverage for vaccine for HPV16, 18, 31, 33, 45, 52, and 58	60/87 (69.0)	58/87 (66.7)	68/87 (78.2)	0.04500*	1.00000	0.17800	0.05500	40/55 (72.7)	37/55 (67.3)	49/55 (89.1)	0.00381*	1.00000	0.04847*	0.00402*
Adjusted coverage vaccine for HPV16, 18, 31, 33, 45, 52, and 58 by hierarchical attribution	76/86 (88.4)	74/86 (86.0)	72/86 (83.7)	0.49400	NA	NA	NA	53/55 (96.4)	49/55 (89.1)	52/55 (94.5)	0.27300	NA	NA	NA
Adjusted coverage vaccine for HPV16, 18, 31, 33, 45, 52, and 58 by proportional attribution	73.7/86 (85.7)	71.5/86 (83.1)	71.7/86 (83.4)	NA	NA	NA	NA	53/55 (96.4)	49/55 (89.1)	50.9/55 (92.5)	NA	NA	NA	NA

†In the CIN2+ samples in the cervical exfoliated cells evaluated by Clinichip HPV test (C-Chip) and cervical exfoliated cells evaluated by PCR with modified general primers (C-MGP) groups, one case of HPV39+56 was observed. This resulted in a denominator change from 87 to 86 by the attribution formula. ‡In the CIN2+ samples in the formalin-fixed paraffin-embedded tissues evaluated by PCR with modified general primers (F-MGP) group, one case of HPV39+56+66 was observed. This resulted in a denominator change from 87 to 86 by the attribution formula. The Clinichip HPV test detected 13 high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). PCR with MGP primers detected 14 high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). The statistical analyses were carried out using the Cochran Q and McNemar tests. *Two-sided values of $P < 0.05$ were considered significant. In the cervical intraepithelial neoplasia grade 2+ (CIN2+) samples, high-risk HPV(−) included one case of HPV11. NA, not applicable.

differences in HPV16/18 attribution among the procedures when estimated with the hierarchical attribution method.

As shown in Table 3, HPV16/18/31/33/45/52/58 attribution to the CIN2/3 lesions estimated with the hierarchical attribution method ranged from 83.7% for F-MGP to 88.4% for C-Chip ($P = 0.494$), whereas the corresponding attribution to CIN3 lesions ranged from 89.1% for C-MGP to 96.4% for C-Chip ($P = 0.273$). Again, no significant differences among the procedures were observed in the values estimated with hierarchical attribution.

Finally, calculation with the proportional attribution method generated similar attribution values for HPV16/18 and HPV16/18/31/33/45/52/58 among the three procedures (Table 3), and no apparent differences in the extent of coverage of the vaccine types were observed between the hierarchical and proportional attribution methods in any categories, although statistical analyses were not applicable to these values.

Discussion

Human papillomavirus genotyping is the basis for monitoring the efficacy of currently licensed prophylactic HPV vaccines, and a practical and reliable genotyping procedure is critical for establishing effective vaccine programs. The expected genotype coverages of HPV vaccines have been reported in Europe and North America.[6,23,24] A variety of factors influence the estimation of vaccine type coverage, including the cervical disease category, clinical specimen source, genotyping method, HPV positivity, ethnicity, and correction formulae. Care should be taken when using DNA specimens extracted from exfoliated cervical cells to estimate HPV genotype attributions because multiple infections may be present. Although FFPE tissue specimens are thought to be a more reliable source of DNA for monitoring HPV type attribution to CIN2/3 lesions,[8] the sensitivity of HPV detection is low, and the work is time-consuming. In the present study, we showed that genotyping procedures using exfoliated cervical cells can generate similar attribution values to those obtained with FFPE tissue specimens when attribution formulae are used to exclude the influence of multiple infections.

We first showed that the genotyping agreement, including identical and compatible categories, ranged from 78.2% to 88.5% (Table 1). The paired analysis of HPV genotype specificity showed strong correlations, indicating that the three procedures reliably detected HPV genotypes, although with some variation. To compensate for this variation, we investigated two methods of assessing the contribution of individual HPV infections to CIN because multiple infections were detected, to greater or lesser extents, with all three procedures. Hierarchical attribution tended to overestimate the contribution of HPV16, as the most dominant genotype. In contrast, proportional attribution, which estimates the attribution of individual genotypes by applying weights that are proportional to the observed frequencies of the genotypes, requires large numbers of samples for accuracy, and the calculation is complex. There were no obvious differences in the calculated values between the hierarchical and proportional attribution methods (Table 3), suggesting that hierarchical attribution is a promising and convenient method.

If the crude coverage of vaccine types is considered, the combination of the specimen source with the HPV genotyping method is a critical factor. For instance, there was a difference between the values for F-MGP (46%, 40/87) and C-Chip (29.9%, 26/87; $P = 0.0077$) for HPV16/18 attribution in CIN2/3 (Table 3), and similar results were obtained in CIN3 (Table 3). To monitor vaccine efficacy, F-MGP is theoretically more reliable because possible disease-unrelated multiple infections are more likely to be excluded. However, there was no apparent difference between the values obtained for F-MGP (52.3%, 45/86) and C-Chip (52.3%, 45/86) when HPV16/18 attribution was calculated with hierarchical attribution. The hierarchical attribution seems to be beneficial in excluding non-causal HPV infections.

Although the detection rate of high-risk HPVs in cervical cancer is reported to be 99.7%,[25] small amounts of recovered DNA or degenerated genomic DNA can produce false-negative results. Therefore, the quality of specimens potentially affects the outcomes of HPV detection. In fact, there were inconsistent findings for the presence of HPV DNA among the three procedures. To monitor HPV vaccine efficacy, Hariri *et al.*[1] additionally used the INNO-LiPA HPV Genotyping Extra Assay (Innogenetics, Ghent, Belgium) when clinical samples were HPV-negative according to the Linear Array HPV Genotyping Assay (Roche Molecular Systems, Inc., Branchburg, NJ), and omitted the specimens found to be HPV-negative in both assays from the analyses. However, HPV-negative cases could be attributed to untested genotypes.[26] In the present study, HPV-negative cases were included in the analysis, although their inclusion would result in an underestimation of vaccine efficacy. Adjustment for the presence of multiple HPV infections also influences the estimated attribution of HPV genotypes to CIN, especially for genotypes other than HPV16/18.[8] [Corrections added on 17 October 2016, after first online publication: 'the (Innogenetics, Ghent, Belgium)' and 'the (Roche Molecular Systems, Inc., Branchburg, NJ)' have been corrected to 'the INNO-LiPA HPV Genotyping Extra Assay (Innogenetics, Ghent, Belgium)' and 'the Linear Array HPV Genotyping Assay (Roche Molecular Systems, Inc., Branchburg, NJ)' respectively in the above statement.]

The present study had some limitations. The formula for the contribution of HPV infections to CIN was established by considering multiple infections, and consequently, is a theoretical method for identifying the genotypes that are responsible for CIN. Because the number of samples was limited, it was difficult to estimate the attribution for less frequently determined genotypes, such as HPV59, 66, and 68. Rare genotypes, such as HPV66, 70, and 82, which have been detected in a percentage of high-grade squamous intraepithelial lesions in Japan,[27] were not analyzed.

Our ultimate goal was to establish an appropriate method for monitoring HPV infections and estimating the efficacy of HPV vaccines in a practical manner. Because the diagnosis of CIN3 is more reproducible and reliable than that of CIN2,[28] CIN3 is the best surrogate marker for invasive cancer, and overall protection against CIN3 is an important consideration when assessing vaccine efficacies.[29] Of particular note, the large-scale monitoring of HPV infections in young Japanese women is now ongoing, with the collection of exfoliated cervical cells.[30] The present study provides information necessary for such efforts to monitor vaccine efficacy in the future.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Calculation formula of the attribution of the modified general-primer PCR system using formalin-fixed paraffin-embedded tissues from biopsied specimens (F-MGP) for human papillomavirus genotyping in cases with cervical intraepithelial neoplasia grade 2+ (n = 87).

Table S2. (a) Discrepant results of human papillomavirus genotyping in cases with cervical intraepithelial neoplasia grade 2/3 (n = 87) using the Clinichip HPV test using exfoliated cervical cells (C-Chip), a modified general-primer PCR system using exfoliated cervical cells (C-MGP), and a modified general-primer PCR system using FFPE tissues from biopsied specimens (F-MGP).

Table S2. (b) Four discrepant cases of human papillomavirus genotyping identified by two modified general-primer PCR systems using either exfoliated cervical cells (C-MGP) or formalin-fixed paraffin-embedded tissues from biopsied specimens (F-MGP)

Table S3. Discrepant results for human papillomavirus 16 genotyping among three procedures using the Clinichip HPV test using exfoliated cervical cells (C-Chip), a modified general-primer PCR system using exfoliated cervical cells (C-MGP), and a modified general-primer PCR system using FFPE tissues from biopsied specimens (F-MGP).