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Detection of Human Papillomavirus Infections at the Single-Cell Level

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Key Words
Human papillomavirus · Laser capture microdissection · Single cell

Abstract
Objective: To explore the possibility of single-cell analysis of human papillomavirus (HPV) infection. ***Methods:*** Two hundred and twenty cells were isolated by laser capture microdissection from formalin-fixed and paraffin-embedded cervical tissue blocks from 8 women who had HPV DNA detected in their cervical swab samples. The number of type-specific HPV copies in individual cells was measured by quantitative polymerase chain reaction with and without a prior reverse transcription. The cells were assayed and counted for more than once if the corresponding swab sample was positive for ≥2 HPV types. ***Results:*** Infection with HPV16, HPV39, HPV51, HPV52, HPV58, HPV59 and HPV73 was detected in 12 (5.5%) of 220, 3 (9.4%) of 32, 3 (5.8%) of 52, 11 (22.9%) of 48, 9 (18.8%) of 48, 3 (9.4%) of 32 and none of 20 cells, respectively. The numbers of HPV genome copies varied widely from cell to cell. The coexistence of multiple HPV types was detected in 6 (31.6%) of 19 positive cells from 1 of the 6 women who had 2 or 3 HPV types detected in their swab samples. ***Conclusion:*** Given the heterogeneity of HPV status in individual cells, further clarification of HPV infection at the single-cell level may refine our understanding of HPV-related carcinogenesis.

Introduction

Human papillomavirus (HPV) is a causative agent of cervical cancer and its precursor, high-grade cervical intraepithelial neoplasia (CIN) [1–6]. The virus belongs to the Papillomaviridae family, with more than 170 types having been fully characterized to date [7]. Genital infections with multiple HPV types are common, particularly among young women and women with cytological abnormalities [8–10]. It remains largely undetermined whether different HPV types can concurrently exist in the same cell in the natural history of the infection, although such a possibility has been suggested by a study showing the coexistence of two HPV types within the same primary keratinocyte in vitro [11] and a case report of double infection with HPV1 and HPV63 within the same nu-

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cleus [12]. Considering that the formation of neoplasia usually initiates from a single cell, mediated through clonal expansions [13–15], the clarification of coinfection with multiple HPV types at the single-cell level would refine our understanding of the intertype interaction and its association with carcinogenesis of the infection.

In the past 2 decades, considerable efforts have been made to investigate the clinical relevance of HPV DNA loads which were usually measured on cervical swab samples. The reported viral loads vary widely, ranging from a few to millions of copies per unit of cellular DNA [16–19]. These values reflect both the numbers of positive cells and copies of HPV genomes in individual cells, as swabbing collects a mixture of exfoliated cells. The consequences may not be the same for infections with a large number of positive cells but few copies of viral genomes in each compared to those with a small number of positive cells but many copies of viral genomes in each, although the overall viral loads could be similar. To better understand how the levels of HPV loads play a role in the development of cervical lesions, an approach for measuring viral loads at the single-cell level is desirable.

This study sought to explore the possibility of single-cell analysis of HPV infections using laser capture microdissection (LCM) followed by quantitative polymerase chain reaction (qPCR) with and without a prior reverse transcription (RT). The study protocol was approved by the Institutional Human Subjects Review Board of the University of Washington.

Materials and Methods

Specimens

Archived formalin-fixed and paraffin-embedded (FFPE) cervical tissue blocks from 8 women were retrieved from the University of Washington Biorepository. These women were participants of the Evaluation of Cervical Cancer Screening Methods (ECCSM), a study designed to evaluate screening strategies for identifying women with high-grade CIN. Women in the ECCSM underwent a routine pelvic examination and provided cervical samples for thin-layer Pap and HPV testing. Those with oncogenic HPV types detected in a cervical sample or a screening Pap indicating the presence of abnormal cytology were asked to return for colposcopy and biopsy. DNAs for HPV testing were isolated from cervical swab samples using the QIAamp DNA blood mini kit (Qiagen, Gaithersburg, Md., USA) and assayed by PCR-based reverse-line blot [20]. All 8 women had HPV16 DNA detected in their cervical swab samples; 6 of them were concurrently positive for other HPV types, including HPV39, HPV51, HPV52, HPV58, HPV59 and/or HPV73. A detailed description of the design and population of the ECCSM study has been presented elsewhere [21]. The use of specimens for the present study was approved by the Institutional Review Board of the University of Washington.

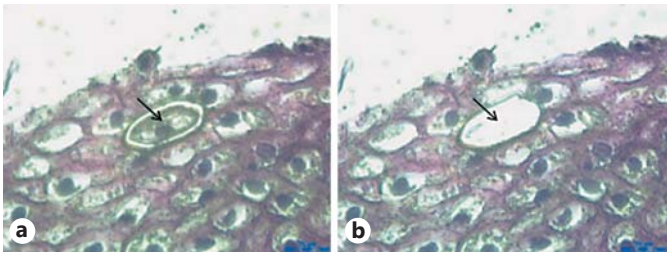

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Fig. 1. LCM of cells from a cervical tissue section. Arrows indicate the position of a single cell before (**a**) and after (**b**) its removal. Magnification ×100.

The CaSki cell line was used to assess degrees of DNA recovery by single-cell analysis. As reported previously by others [22, 23], there are about 500–600 integrated HPV16 genome copies per cell, estimated by Southern blot analysis of CaSki cellular DNA with a HPV16 probe. This cell line, initially obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA), has been routinely maintained in our laboratory. The cells were cultured and harvested at ~85% confluence, fixed with formaldehyde, pelleted and paraffin-embedded for sectioning as other FFPE samples.

Isolation of Single Cells by LCM

Serial 5-μm sections were cut from FFPE cervical tissue blocks and mounted on polyethylene naphthalate membrane-coated slides (Leica Biosystems, Buffalo Grove, Ill., USA). Hematoxylin and eosin staining was used to guide microdissection. The slides were incubated at 60°C for 30 min in a drying oven. Tissue sections were deparaffinized in 2 changes of xylene for 30 s each, followed by rehydration in a series of graded ethanol to deionized water and then counterstained using HistoGene staining solution (Arcturus, Mountain View, Calif., USA). Finally, the tissues were dehydrated in a series of graded ethanol, cleared in xylene, and dried at room temperature for 10 min.

LCM was performed on a Leica LMD6000 platform, a noncontact system using a 355-nm UV laser beam. The cells were identified morphologically; margins of the cells were microscopically circumscribed with a digital pen. The selected cells were dissected with the laser beam. Figure 1 exemplifies photomicrographs (100×) of a section before and after the removal of a single cell. The microdissected cell was transferred into a 0.2-ml PCR tube containing 20 μl of proteinase K buffer (1 mM EDTA, 20 mM Tris, 0.5% Tween-20, 0.2 mg/ml proteinase K and 10 ng/ml carrier RNA). Cells in individual tubes were incubated in a thermocycler at 55°C for 8 h for digestion and then at 95°C for 10 min for inactivation of proteinase K. Following that, the samples were transferred into 1.5-ml microcentrifuge tubes, dried with a Vacufuge™ (Eppendorf, Hauppauge, N.Y., USA), resuspended in 5 μl of distilled water, and stored at –20°C until further assay.

To minimize a potential of sample-to-sample carryover, all instruments for tissue sectioning (such as microtome, tweezer and slide warmer) were thoroughly cleaned with isopropyl alcohol pads and DNA Away (Molecular Bio Products, San Diego, Calif., USA) after the completion of each block. In addition, we kept a space of around 30–50 μm between the selected cells to minimize a potential of cell-to-cell carryover.

Table 1. Primers and probes for qPCR

HPV types	Forward primer (5′–3′)	Probe (5′–3′)	Reverse primer (5′–3′)
16	TTCGGTTGTGCGTACAAAGC	VIC-CACACGTAGACATTCGT-MGB	GCCCATTAACAGGTCTTCCAAA
39	TGACCTTGTATGTCACGAGCAAT	VIC-AGGAGAGTCAGAGGATG-MGB	TGCATGGTCGGGTTCATCTA
51	GCTCCGTGTTGCAGGTGTT	VIC-AAGTGTAGTACAACTGGC-MGB	GGGTGTCTCCACTGCTTTCC
52	GACAGCTCAGATGAGGAGGATACA	VIC-ATGGTGTGGACCGGC-MGB	TGGCTTGTTCTGCTTGTCCAT
58	GACAGCTCAGACGAGGATGAAA	VIC-AGGCTTGGACGGGC-MGB	TGGCCGGTTGTGCTTGT
59	TGACTCCGACTCCGAGAATGA	VIC-AAAGATGAACCAGATGGAGT-MGB	TCGTCTAGCTAGTAGCAAAGGATGAT
73	ACCAACAACCGAAATTGACCTT	VIC-CATGTTACGAGTCATTGGA-MGB	CTGTTTCATCCTCATCCTCTGAGTT

Detection of the HPV Genomes by qPCR

Levels of type-specific HPV DNA/RNA and DNA alone in individual cells were measured by qPCR with and without a prior RT, respectively. Types chosen for testing were based on what was detected in the corresponding cervical swab sample. The cells were assayed and counted for more than once if the corresponding swab sample was positive for 2 or more HPV types. For example, if a woman had HPV16 and HPV31 detected in her cervical swab sample, the cells dissected from this woman's tissue block would be tested for both types; if her swab sample was positive for HPV16 alone, the cells from this woman would be tested only for HPV16.

An aliquot of a 2-μl sample (equivalent to ~2/5 of a cell) was subjected to RT in a reaction volume of 3 μl using a SuperScript® VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, Calif., USA). The mixture was incubated at 25°C for 10 min and 42°C for 60 min followed by 85°C for 5 min for inactivation of the reverse transcriptase. DNAs were not removed from RT samples because this procedure was used to maximize the sensitivity of the detection rather than quantify the amount of HPV transcripts. SiHa RNA (9 ng/μl) and sterile water were included in each run of the RT as a positive and negative control, respectively. The success of the RT was indicated by the positive control which tested positive for HPV16 by qPCR with RT, with a mean (SD) of 255,181 copies (±13,922), but negative by qPCR without RT.

The qPCR assay was set up in a reaction volume of 10 μl with TaqMan Universal PCR Master Kit (Applied Biosystems, Foster City, Calif., USA), 0.09 μM of primers, 0.06 μM of probe and 1 μl of sample (equivalent to 1/5 of a cell for a sample without a prior RT or 1/7.5 of a cell (1/3 × 2/5) for a sample with a prior RT). The sequences of the primers and probes for 7 HPV types (HPV types 16, 39, 51, 52, 58, 59 and 73) are listed in table 1. The specificity of each set of primers and probes has previously been verified by testing with plasmids containing DNAs of other HPV types at a concentration of 10^5 copies/μl [19]. PCR amplification was carried out on an Applied Biosystems 7900 HT Sequence Detection System with a cycling program of holding at 50°C for 2 min and then at 95°C for 10 min followed by a two-step cycle of 10 s at 95°C and 1 min at 60°C for 40 cycles. A log-phase 6-point standard curve (ranging from 10^6 to 10^1 copies) for each type of HPV was implemented in each set of the assay. We manually checked and confirmed the amplification curves for all positive tests. The numbers of HPV copies were determined by linear extrapolation of the cycle threshold values using the equation derived from the standard curve. The observed values were converted to copy number per cell by multiplying by 7.5 and 5 for those obtained by RT-qPCR and qPCR without RT, respectively.

Verification of Multiple HPV Types in Single Cells by DNA Sequencing

Cells positive for more than 1 type of HPV by qPCR were further assayed by PCR-based DNA sequencing to verify the presence of multiple types. Briefly, a regular PCR in a reaction volume of 25 μl was performed with 1 μl of qPCR products as sample input. The PCR products were resolved by electrophoresis and visualized on an ethidium bromide-stained 1.5% agarose gel. The PCR-generated DNA fragments were cloned into pSC-B plasmid using a StrataClone Ultra Blunt PCR cloning kit according to the protocol recommended by the manufacturer (Stratagene, La Jolla, Calif., USA). The accuracy of the target inserts was confirmed by DNA sequencing from both directions with a pair of M13 primers (forward: 5′-GTAAAACGACGGCCAGT; reverse: 5′-AACAGCTATGACCATG).

Statistical Analyses

A McNemar test with continuity correction was used to compare frequencies of positive detections by qPCR with versus without a prior RT assay. The difference in $\log_{10}$-transformed viral copy numbers measured by qPCR with, compared to without, a prior RT was assessed by Student's t test. Fisher's exact test was used to examine the frequencies of coexistent HPV types in single cells. Statistical tests were at the 5% two-sided significance level.

Results

Recovery of the HPV16 Genomes from Microdissected CaSki Cells

We pooled 20 dissected CaSki cells together to get an expected total of ~10,000 HPV16 genome copies. The pooled sample was diluted to a series of 3 concentrations (500, 100 and 10 HPV16 genome copies/μl) and assayed by qPCR without RT. The number of HPV16 copies detected was 66.4 for a sample input of 500 copies and 21.5 for a sample input of 100 copies, accounting for 13.3 and 21.5% of the expected number, respectively. The HPV16 genome for a sample input of 10 copies was undetectable.

Table 2. Type-specific HPV infections detected in individual cells by qPCR with and without a prior RT

Case No.	Histological diagnosis	Types detected in swab sample	Cells, n	Tests, n	Type-specific positive cells by qPCR, n (%)		
					without RT	with RT	either
42	CIN2	HPV16	16	16	0	0	0
147	CIN2	HPV16	32[a]	32	1 (3.1)	2 (6.3)	2 (6.3)
		HPV39		32	0	3 (9.4)	3 (9.4)
249	CIN1	HPV16	48[b]	48	3 (6.3)	5 (10.4)	6 (12.5)
		HPV52		48	9 (18.8)	8 (16.7)	11 (22.9)
		HPV58		48	5 (10.4)	4 (8.3)	9 (18.8)
427	CIN1	HPV16	32[a]	32	1 (3.1)	1 (3.1)	1 (3.1)
		HPV59		32	1 (3.1)	3 (9.4)	3 (9.4)
735	CIN1	HPV16	20	20	0	1 (5.0)	1 (5.0)
		HPV51		20	2 (10.0)	1 (5.0)	2 (10.0)
842	CIN2	HPV16	20	20	0	1 (5.0)	1 (5.0)
		HPV73		20	0	0	0
891	CIN2	HPV16	20	20	0	0	0
1,055	CIN1	HPV16	32	32	0	1 (3.1)	1 (3.1)
		HPV51		32	1 (3.1)	1 (3.1)	1 (3.1)
Total			220	452	23 (5.1)	31 (6.9)	41 (9.1)

Either: positive by qPCR with and/or without RT; if positive by both, only one was counted.
[a] Including 12 cells initially collected and 20 recollected.
[b] Including 12 cells initially collected, 8 by the 1st repeat and 28 by the 2nd repeat.

Type-Specific HPV Infections Detected at the Single-Cell Level

The number of cells dissected from FFPE cervical tissue blocks ranged from 16 to 48 per woman, with a total of 220 obtained from 8 women (4 with a histological diagnosis of CIN2 and 4 with CIN1). The LCM was repeated on different tissue blocks for 3 women to increase the number of cells (cases No. 147 and 427, with an initial collection of 12 cells and a recollection of 20 for each) or to confirm the presence of multiple HPV types in single cells (case No. 249, with an initial collection of 12 cells and a recollection of 8 and then 28).

Overall, infection with HPV types 16, 39, 51, 52, 58, 59 and 73 was detected in 12 (5.5%) of 220, 3 (9.4%) of 32, 3 (5.8%) of 52, 11 (22.9%) of 48, 9 (18.8%) of 48, 3 (9.4%) of 32 and none of 20 cells, respectively (18 positive by RT-qPCR alone, 10 by qPCR without RT alone and 13 by both). The proportions of positive detections were comparable between the procedures with and without RT (table 2; 6.9 vs. 5.1%, p = 0.19).

The $\log_{10}$-transformed HPV copy number in individual cells is plotted in figure 2. The majority of detectable infections were at a level of <2 logs. The mean (SD) of the $\log_{10}$-transformed HPV copy number was 1.22

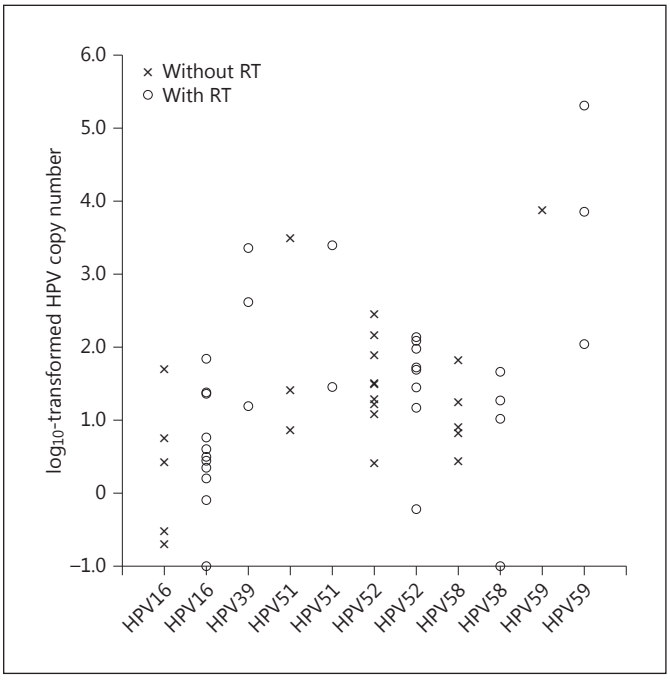

Fig. 2. $\log_{10}$-transformed type-specific HPV copy number in individual positive cells. The mean (SD) of the $\log_{10}$-transformed copy number detected by qPCR with and without a prior RT was 1.44 (±1.35) and 1.35 (±1.05), respectively (p = 0.79).

(±1.27) for cells positive by RT-qPCR alone and 0.91 (±0.73) for those positive by qPCR without RT alone. For cells positive by both, the mean (SD) was 1.73 (±1.45) and 1.68 (±1.16) detected by qPCR with and without a prior RT, respectively. There were no appreciable differences in type-specific copy numbers by qPCR with versus without RT for all types (p = 0.79) except for HPV59 (case No. 427). Of 32 cells tested for HPV59, 2 were negative by qPCR without RT but positive by RT-qPCR at a level of 2.05 and 3.86 logs, respectively; 1 was positive by both but with a substantially greater number of copies detected by qPCR with, compared to without, RT (5.31 vs. 3.87 logs).

Coexistence of Multiple HPV Types in Single Cells

Six women had 2 or 3 HPV types detected in their cervical swab samples. By analysis of the corresponding types of HPV in individual cells, the presence of more than 1 type of HPV in single cells was found in 1 woman (table 3; case No. 249). In the initial analysis of 12 cells, 3 were positive for HPV16 and/or HPV52 with the coexistence of both types detected in 2. We repeated LCM on different FFPE blocks from this woman twice with another 36 cells collected. Infection with more than 1 type of HPV was detected in 3 (1 with HPV16 and HPV52, 1 with HPV52 and HPV58, and another with all 3 types) in the first repeat of 8 cells and the coexistence of HPV52 and HPV58 in 1 in the second repeat of 28 cells.

Figure 3 shows an example of reamplification of qPCR products of 3 positive cells from the initial test of case No. 249, including 2 each positive for HPV16 and HPV52 (cells No. 2 and 12) and 1 positive for HPV52 (cell No. 1). The PCR products were cloned into pSC-B vector; sequences of the target region (66 bp for HPV16 and 64 bp for HPV52) were confirmed by sequencing with the M13 primer (data not shown). The confirmatory analysis (reamplification of qPCR products) was performed on all cells from the repeated test showing multiple HPV types in each and a set of cells tested negative by qPCR. All of the positive tests were confirmed; none of the negative cells displayed any visible bands (data not shown).

Of the remaining 5 women with 2 HPV types detected in their cervical swab samples, 4 (cases No. 147, 427, 735 and 1,055) had both types detected by qPCR, but in different cells (table 2). None of 14 positive cells from these 4 women, in contrast to 6 (31.6%) of 19 positive cells from case No. 249, displayed multiple types of HPV within the single cell (p = 0.03).

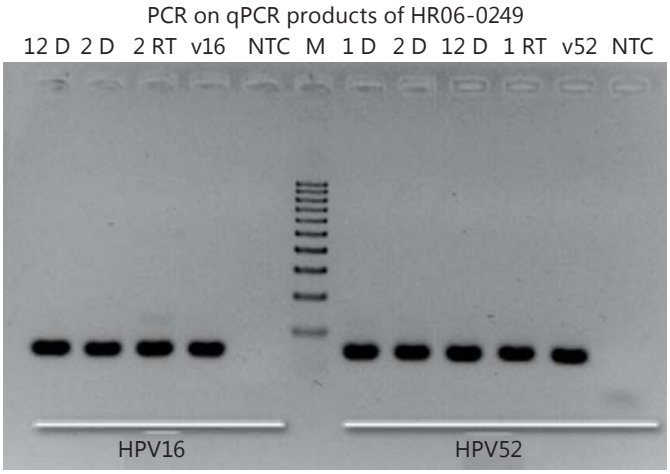

Fig. 3. Reamplification of HPV16 and HPV52 with an aliquot of qPCR products as sample input, visualized on a 1.5% agarose gel. RT = Products from qPCR with RT; D = products from qPCR without RT; v16 = HPV16-positive control; v52 = HPV52-positive control; NTC = negative control; M = marker (100-bp DNA ladder). Three positive cells were identified by the initial qPCR analysis of 12 cells from case No. 249, including 1 positive for HPV52 (cell No. 1) and 2 positive for both HPV16 and HPV52 (cells No. 2 and 12).

Table 3. Multiple HPV types detected in single cells from a woman (case No. 249) who had HPV16, HPV52 and HPV58 detected in her cervical swab sample

Test	Cells, n	HPV-positive cells, n	Identification No. for cells positive for multiple types	Viral copies in single cells, n		
				HPV16	HPV52	HPV58
Initial test	12	3	2	0.3	16.5	
			12	0.2	26.2	
1st repeat	8	5	5		45.7	34.2
			8	4.0	14.8	
			3	5.8	94.5	6.6
2nd repeat	28	11	24		31.8	8.1

Discussion

In this proof-of-principle study, we demonstrated the feasibility of single-cell analysis of HPV infection by LCM followed by qPCR. Although the use of LCM for analyses of HPV status was reported before [24–26], previous studies focused on infections in individual lesions or individual components of the lesions. Data from this study revealed the heterogeneity of HPV status in individual cells, showing that most cells dissected from tissue blocks

from women with HPV DNA detected in their cervical swab samples did not have a detectable HPV infection; in a small set of HPV-positive cells, the numbers of viral copies varied substantially from cell to cell.

Considering the depth of tissue sections and the potential loss in sample extraction, we expected that DNAs retrieved from single cells might represent only a fraction of the tissue's entire content. Thus, we used CaSki cells to assess the degrees of recovery. The lower than expected number of HPV16 copies observed from CaSki cells suggests that the actual number of viral copies in cells from clinical samples might have been larger than what we observed. This may in part explain why the type-specific infection was detected only in a small fraction of cells. We chose not to adjust for the number of viral copies according to percentages of the recovery. This is because the assay-related DNA loss may vary from cell to cell; ratios of the observed to expected copies may not necessarily be linear. Nevertheless, data from the present study clearly indicated that the number of viral copies in individual positive cells was not a unique value but a distribution of values. One interpretation for the broad range of viral copies pertains to cellular differentiation and proliferation as HPV utilizes the host cell DNA replication machinery for its own replication. Alternatively (or in addition), cells may differ in numbers of initially acquired HPV copies and/or physical statuses of the viral genomes, thereby resulting in differing accumulation of viral copies.

In this study, viral loads were measured by qPCR with and without a prior RT. It should be pointed out that the copy number detected by RT-qPCR represented both HPV DNA and RNA. We expected that qPCR with, compared to without, a prior RT would identify more infected cells if the virus was transcriptionally active; the observed difference, however, was not substantial. We noted that type-specific infection was not detected in 10 cells by RT-qPCR but was detected by qPCR without RT. This discrepancy could be in part explained by a lack of HPV RNA due to either inactive transcription or RNA degradation and/or a loss of few DNA copies in the RT procedure as the number of HPV copies detected by the latter was quite small. We did not see appreciable differences in HPV copy numbers detected by RT-qPCR versus qPCR without RT for all types except for HPV59. The number of HPV59 copies detected by RT-qPCR was substantially greater than that by qPCR without RT, suggesting an active transcriptional status of the virus. The identification of viral transcription in individual cells is important as the detection of HPV RNA compared to DNA in cervical swab samples has been shown to be more specific for predicting high-grade CIN [27–29].

In agreement with a previous case report of double infection with HPV1 and HPV63 within the same nucleus identified by fluorescence in situ hybridization [12], we repeatedly detected the coexistence of two or three HPV types in single cells dissected from different tissue blocks from 1 woman. Our results cannot be explained by assay-related cross-reaction as the specificity of primers and probes was well verified previously [19]. Also, all sequences of multiple types detected in single cells were confirmed by direct sequencing of PCR products. One concern, however, is whether types detected were from different cells rather than the same cell. Although the cells selected displayed clean margins and there was space (~30–50 μm) between the selected cells, a possibility of cell-cell overlap could not be excluded. If 1 type of HPV is in an upper cell and another type in a cell beneath, our results could be misinterpreted. Arguing against this is the fact that coexistence was repeatedly detected in 1 woman. Had the overlap been a reason for the detection of multiple types, it would have been expected to occur in the other 4 women who had both types detected by qPCR. However, none of 14 positive cells from these 4 women, compared to 6 of 19 positive cells from case No. 249, was positive for multiple types. Furthermore, had a detection of multiple HPV types in single cells resulted from cell-to-cell carryover, it would have been more likely to have been detected in women with a larger number of viral copies in single cells, such as case No. 427. In fact, our finding of the coexistence of multiple HPV types in single cells is in part supported by a study in vitro showing that a subset of high-risk HPV types can be stably maintained in the same cell, and interactions between types do occur [11].

Genital infection with multiple HPV types is common, detected in 15–40% of women with CIN of all grades [9, 24]. An increased risk of cervical lesions among women infected with multiple types versus only 1 type of HPV has been observed in some studies [30–34] but not in others [9, 35–37]. Previous studies of HPV DNA in microdissected cervical lesions have demonstrated that most lesions contained only 1 type of HPV; the lesions with 2 types appeared to morphologically represent the collision of two lesions each with a single type of HPV, suggesting one virus to one lesion [24, 26]. On the other hand, the presence of multiple types of HPV in microdissected cervical lesions has also been reported from a vaccine trial [38]. The finding of coexistent HPV types in single cells is intriguing, as it may bring about a new area in HPV research. It would be interesting to know the frequency, du-

ration and clinical relevance of multiple HPV types in single cells, whether different types enter into cells concurrently or sequentially, and how they interact with each other. Clearly, additional studies are warranted to examine HPVs in individual cells over the course of the infection, as well as studies to identify mechanisms of type-type interaction and host-virus interaction.

The major limitation of the present study was the limited sample size with only 8 women included. Thus, our results should be interpreted with caution. In addition, the image of individual cells prior to microdissection was not recorded. Consequently, the relationship between HPV status and morphological features of the cell remained undetermined. Finally, in analyses of HPV16 DNA from dissected CaSki cells, the viral genome was undetectable for a sample with an expected number of 10 copies, suggesting that we might miss infections with few HPV genome copies. It is likely that the actual number of positive cells might be larger than that observed in the present study.

In conclusion, the present study demonstrated the feasibility of single-cell analysis of HPV infections. Given the heterogeneity of HPV status in individual cells (in terms of copy number, viral transcription and coexistence of multiple types), further studies of HPV status at the single-cell level may refine our understanding of HPV-related carcinogenesis.

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Disclosure Statement

All authors have no commercial or other associations that might pose a conflict of interest.

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