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A Cross-sectional Study of a Prototype Carcinogenic Human Papillomavirus E6/E7 Messenger RNA Assay for Detection of Cervical Precancer and Cancer

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Abstract

Purpose: To evaluate carcinogenic human papillomavirus (HPV) mRNA for E6 and E7 mRNA detection on clinical specimens to identify women with cervical precancer and cancer.

Experimental Design: We evaluated a prototype assay that collectively detects oncogenes E6/E7 mRNA for 14 carcinogenic HPV genotypes on a sample of liquid cytology specimens (n = 531), masked to clinical data and to the presence of HPV genotypes detected by PGMY09/11 L1 consensus primer PCR assay.

Results: We found an increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA with increasing severity of cytology ($P_{Trend} < 0.0001$) and histology ($P_{Trend} < 0.0001$), with 94% of cervical intraepithelial neoplasia grade 3 (CIN3) histology cases (46 of 49) and all five cancer cases testing positive for carcinogenic HPV E6/E7 mRNA. Overall, fewer specimens tested positive for carcinogenic HPV E6/E7 mRNA than for carcinogenic HPV DNA ($P < 0.0001$, McNemar's χ^2 test), especially in women with <CIN1 ($P < 0.0001$). We also found that using a higher positive cutpoint for detection of carcinogenic HPV E6/E7 mRNA improved the association of positive test results with cervical precancer and cancer by reducing the number of test positives in women without precancer without reducing clinical sensitivity for cervical precancer and cancer compared with detection of carcinogenic HPV E6/E7 mRNA using a lower positive cutpoint by the same assay and with detection of carcinogenic HPV DNA.

Conclusions: We found that carcinogenic HPV E6/E7 mRNA is a potentially useful biomarker for detection of cervical precancer and cancer and warrants further evaluation.

Cervical infections by ~15 carcinogenic HPV types cause virtually all cervical cancer and the immediate precursor (precancerous) lesion, cervical intraepithelial neoplasia grade 3 [CIN3; also known as carcinoma *in situ* (CIS); refs. 1–3]. Persistence of carcinogenic HPV is necessary for the development of cervical precancer and cancer (4); detection of carcinogenic HPV type-specific persistence strongly predicts the development of precancer and cancer (5, 6). Diagnoses of CIN3 or frank cancer require treatment; CIN2, a less certain diagnosis of cervical precancer (7), is also treated as a margin of safety.

Based on knowledge of the central role for persistent, carcinogenic HPV in cervical carcinogenesis, one pooled probe test for the carcinogenic types of HPV DNA has already been Food and Drug Administration (FDA) approved, and other tests will soon be widely available. HPV DNA negativity is associated with an extremely low risk of prevalent or incipient CIN3 or cancer (≥CIN3; refs. 8, 9). Carcinogenic HPV testing has now been approved in the United States as an adjunct to cytology for triage of equivocal cytology at all ages and for general screening in women ≥30 years old (10). There is now significant evidence that carcinogenic HPV DNA detection is significantly more sensitive but slightly less specific for detection of cervical precancer and cancer than cytology (11). Currently, there are no FDA-approved tests for detection of specific HPV genotypes, which could be used to measure persistent carcinogenic HPV infection, although repeatedly testing positive for carcinogenic HPV types in aggregate confers an elevated risk of precancer (12) presumably as surrogate for type-specific persistence.

During normal productive HPV infections, the expression of viral genes is tightly regulated and linked to the differentiation state of the epithelium. At the basal layer of the epithelium, where HPV infection is establish, the viral genome is maintained. Early proteins, such as oncoproteins E6 and E7, are expressed at low levels for genome maintenance and cell proliferation. As the basal epithelial cells differentiate, the viral life cycle goes through success stages of genome amplification, virus assembly, and virus release, with a concomitant shift in

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expression patterns from early genes to late genes, including L1 and L2 that assemble into viral capsid. However, if progression to a precancerous state occurs as the result of genetic and epigenetic changes, E6 and E7 expression is deregulated, leading to their overexpression throughout the full epithelial thickness of the precancerous (CIN3/CIS) lesion.

Thus, overexpression of E6/E7 for carcinogenic HPV types may be a very specific marker of precancerous lesions that need clinical attention, perhaps more specific than HPV DNA that can be detected in both precancerous and milder lesions caused by HPV infection itself. Previous studies using one commercialized assay for the detection of E6/E7 mRNA have provided evidence of greater clinical specificity for cervical precancer and cancer detection (13–17). However, this assay (PreTect HPV-Proofer, Norchip AS) targets only the five riskiest carcinogenic HPV types, HPV16, HPV18, HPV31, HPV33, and HPV45, which may have contributed to greater specificity at a slight cost of sensitivity. Nevertheless, these studies suggest that detection of carcinogenic HPV E6/E7 mRNA may be a potentially useful molecular marker in cervical cancer screening. The purpose of exploring the role of pooled carcinogenic mRNA E6/E7 would be to exploit HPV biology, thus targeting the higher likelihood of identifying the lesions that need imminent treatment (≥CIN3).

Thus, we conducted a cross-sectional study to evaluate a prototype commercial assay [APTIMA Human Papillomavirus (HPV) Assay; Gen-Probe Incorporated] for detection of E6/E7 mRNA for 14 carcinogenic HPV types in aggregate. Using a convenience sample of cytologic specimens, we examined the correlation of carcinogenic HPV E6/E7 mRNA with cytologic and histologic end points.

Materials and Methods

Specimens and clinical data. We acquired anonymized residual PreservCyt specimens (N = 540, 536 with histologic results) after cytologic interpretation had been rendered for women participating in routine screening, from 125 women with normal cytology, 125 women with atypical squamous cells (ASC) cytology, 125 women with low-grade squamous intraepithelial lesions (LSIL) cytology, and 165 women with high-grade squamous intraepithelial lesions (HSIL) cytology or worse (≥HSIL), respectively, from the Medical University of South Carolina (Charleston, SC). The Institutional Review Board (IRB) of the Medical University of South Carolina approved the study, and the use of these specimens was deemed exempt from review by National Cancer Institute IRB. We subsequently excluded 12 specimens called ≥HSIL because of conditions unrelated to cervical abnormalities (e.g., endometrial carcinoma histopathology).

In addition to the original histologic diagnosis, based on the worst histology of biopsy or loop electrosurgical excision procedure/cone treatment, each case underwent a pathology review of all histology slides to ascertain histologic diagnosis. Six cases originally called CIN grades 2-3 and one case called CIN3 were reclassified as CIN2, and eight cases originally called CIN2-3 and one case called CIN2 were reclassified as CIN3. In addition, two cases called CIN2 and two cases called CIN1-2 were reclassified as CIN1.

To supplement the number of specimens from women severe disease, the University of Arizona (Tucson, AZ) supplied 12 anonymized specimens from women attending colposcopy (and, therefore, without cytologic results), 10 with CIN3/CIS and 2 with cancer. Specimens were collected under an IRB-approved protocol, and their use was deemed exempt from review by National Cancer Institute IRB. All specimens were processed by a central biorepository upon receipt in the same manner.

Thus, we had specimens from 540 women, 536 with histologic outcomes and 528 with cytologic interpretations. Specimens were stored at -20°C (median, 530 days; interquartile range, 482-553 days) before testing for carcinogenic HPV E6/E7 mRNA. We did not observe differences in testing performance with duration of storage (data not shown).

HPV testing. A set of 535 aliquots (531 with histologic results and 523 with cytologic results) were tested using the APTIMA HPV assay; 5 specimens (4 with <CIN1 histology or no biopsy and 1 with CIN2 histology) were missing results because no aliquots were available due to insufficient volumes. The cytologic and histologic results of tested specimens are summarized in Table 1.

APTIMA HPV assay. All specimens were tested in a standardized manner as described and masked to cytology and histology results. Specimens were diluted 1:2.9 in specimen transport media (STM; a phosphate-buffer solution containing detergent and a chelating agent) before being run in the assay. After dilution in STM, specimens were tested within 24 h on the fully automated TIGRIS DTS System (Gen-Probe Incorporated; ref. 18) for detection of carcinogenic HPV E6/E7 for 14 carcinogenic HPV types collectively. The assay was designed to detect E6/E7 mRNA from the 14 high-risk types based on the known sequences of these high-risk types and the mutations in those sequences. Specifically, capture probes, primers, and detection probes were designed to not be negatively impacted by known mutations.

Each diluted specimen (0.4 mL) was transferred to a reaction tube, to which 0.1 mL of Target Capture Reagent (TCR) was added, and the mixture was incubated 30 min at 62°C. TCR contains magnetic

Table 1. A summary of cytologic and histologic results of tested specimens

	<CIN1/no biopsy	CIN1	CIN2	CIN3	Cancer	Missing	Total
Negative	125	0	0	0	0	0	125
	100%	0%	0%	0%	0%	0%	
ASC	102	17	3	0	0	0	122
	84%	14%	2%	0%	0%	0%	
LSIL	64	52	5	3	0	1	125
	51%	42%	4%	2%	0%	1%	
HSIL	48	18	43	36	3	3	151
	32%	12%	28%	24%	2%	2%	
Missing	0	0	0	10	2	0	12
	0%	0%	0%	83%	17%	0%	
Total	339	87	51	49	5	4	535
	63%	16%	10%	9%	1%	1%	

NOTE: Row percentages are present below the number in each cell.

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microparticles, capture oligomers, internal control (IC), buffer, salts and detergent that lyse all cells in the specimen and allow for release and stabilization of HPV mRNA, which then hybridizes to chimeric capture oligomers. The 5′ ends of the capture oligomers contain sequences complementary to conserved regions of carcinogenic HPV E6/E7 mRNA and the IC. The capture oligonucleotides also have 3′ dA sequences that are complementary to dT sequences attached to the magnetic microparticles. The reactions were moved to a 40°C incubator and allowed to equilibrate for ~20 min. The microparticles with the captured E6/E7 mRNA (when present) and IC molecules bound to them were isolated from the solution by pulling them to the side of the reaction tube by magnets, and the residual fluid was aspirated by the instrument. One milliliter of wash buffer (a buffered detergent solution) was added to the tube, and the solution was mixed by vortexing to wash the magnetic particles. The particles were pulled to the side of the tube by magnets, the residual fluid was aspirated, and the wash step was repeated.

The analyte and IC were amplified by transcription-mediated amplification (TMA), which relies on two enzymes, Moloney murine leukemia virus reverse transcriptase and T7 RNA polymerase (19). The reverse transcriptase generates a DNA copy of the target sequence that contains a promoter sequence for the T7 RNA polymerase. T7 RNA polymerase produces multiple copies of the RNA amplicon from the DNA copy template, which are then cycled through the same process to achieve exponential amplification of the target. Targeted amplification is achieved using primers that hybridize to conserved regions of high-risk E6/E7 mRNA. To each reaction tube, 75 μL of amplification reagent (a buffered solution containing deoxynucleotide triphosphates, nucleotide triphosphates, metals, salts, and oligonucleotide primers) and 200 μL of silicone oil were added, and the mixture was heated to 62°C for ~10 min. The reaction tubes were subsequently transferred to a 40°C incubator for ~6 min to allow annealing of the specific primers to the target and IC. Finally, 25 μL of enzyme reagent (a buffered solution containing T7 RNA polymerase, reverse transcriptase, detergent, and glycerol) was then added, and the amplification reaction was allowed to proceed at 42°C for 1 h.

Chemiluminescent detection of analyte and IC amplification products was achieved using the hybridization protection assay technique and dual kinetic assay (20–22). Analyte amplicons were detected using 2-methyl acridinium ester–labeled probes that hybridized to conserved regions of carcinogenic HPV genotypes. An *o*-fluoroacridinium ester–labeled probe was used to detect the IC amplicon. For detection, 0.1 mL of hybridization reagent (a buffered solution containing detergent, salts, and acridinium ester–labeled oligonucleotide probes) was added to the amplicons, mixed, and incubated for 20 min at 62°C. Then, 0.25 mL of selection reagent (a borate buffered solution containing detergent) was added, mixed, and incubated for 10 min at 62°C. The reactions were moved to a room temperature incubator and allowed to equilibrate for at least 10 min. The chemiluminescent signal was then measured in a luminometer for 2 s and was reported in relative light units (RLU). Deconvolution of analyte chemiluminescent signal from IC signal was done using a proprietary algorithm.

Three negative calibrators (0.4 mL of STM) and three positive calibrators (400 copies of *in vitro* transcribed HPV 16 E6/E7 RNA in 0.4 mL STM) were placed at the beginning of each run. Calibrators establish run validity and the IC cutoff value. The IC cutoff for an invalid specimen reaction was an IC signal less than half the mean IC signal in the three negative calibrators. Calibrators will also establish the analyte cutoff value, once the analyte cutoff calculations are defined and confirmed by receiver operating characteristic analysis. A negative TIGRIS control (0.4 mL of STM) and a positive TIGRIS control (400 copies of *in vitro* transcribed HPV 16 E6/E7 RNA in 0.4 mL STM) were placed at the end of each run, as required by the TIGRIS software.

HPV genotyping by linear array. Aliquots of these specimens were previously tested using Linear Array HPV Genotyping Test (LA; Roche Molecular Systems) as previously described (23). LA uses the PGMY09/11 L1 consensus primer system and includes coamplification of a human cellular target, β-globin (24), as an IC. Detection and HPV genotyping are achieved using a reverse line-blot method (25, 26), and this test includes probes to genotype for 37 anogenital HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51-56, 58, 59, 61, 62, 64, 66-73, 81, 82 subtype [IS39], 82 subtype [W13b], 83, 84, and 89). HPV testing results were categorized as negative or positive for carcinogenic HPV DNA if positive for 1 of the 14 carcinogenic HPV types and hierarchically according to cancer risk (HPV risk group; HPV16 > HPV18 > carcinogenic HPV types except HPV16 and HPV18 > noncarcinogenic HPV types > PCR negative). A total of 527 women had both carcinogenic HPV E6/E7 mRNA and HPV genotyping results.

Hybrid capture 2. Some women (n = 86, 85 with APTIMA results) had been previously tested by Hybrid Capture 2 (HC2; Digene Corporation), a signal amplification DNA test that targets 13 carcinogenic HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). HC2 is well known to cross-react with HPV66 (27).

Statistics. We tested the association of carcinogenic HPV E6/E7 mRNA by the APTIMA HPV assay as detected with the severity of the cytologic interpretation (negative < ASC < LSIL < HSIL; n = 523), and the histologic diagnosis (less than CIN1 [which included women with no biopsy taken based on colposcopic impression of normality] < CIN1 < CIN2 < CIN3 < cancer; n = 531), and with the HPV risk group (n = 531) using a Pearson χ^2 test and Cochran-Armitage trend test. We compared the percentage of test positives by LA and HC2 for carcinogenic HPV versus testing positive for carcinogenic HPV E6/E7 mRNA using an exact McNemar's χ^2 test.

In a *post hoc* analysis, we compared the testing results of APTIMA using a higher positive cutpoint, 750,000 RLU, versus 125,000 RLU. Overall and within each cytology and histology category, by carcinogenic HPV DNA status by LA, within HPV risk group, and by HC2 test result, we compared the percentage of test positives at the two different thresholds using an exact McNemar's χ^2 test.

We also calculated percent agreement and κ values, and tested for differences in testing positive by McNemar's χ^2 for carcinogenic HPV DNA as detected by LA versus carcinogenic HPV E6/E7 mRNA for each cutpoint, overall and for each histologic diagnosis.

Results

Carcinogenic HPV E6/E7 mRNA versus cytology. We found a strong trend of an increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA at a positive cutpoint of 125,000 RLU with an increasing severity of cytologic interpretation (P_{Trend} < 0.0001; Table 2). The percentages of test positives were 8% for cytologic negative, 43% for ASC, 71% for LSIL, and 87% for HSIL.

The strong trend of increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA with the severity of cytology was also observed using a higher positive cutpoint of 750,000 RLU (P_{Trend} < 0.0001). The percentages of test positives were 6% for cytologic negative, 34% for ASC, 62% for LSIL, and 84% for HSIL. The decrease in test positives at the higher positive cutpoint versus the lower positive cutpoint was significant for ASC (43% versus 34%; P = 0.0005) and LSIL (71% versus 62%; P = 0.0005) cytology.

Carcinogenic HPV E6/E7 mRNA versus histology. We found a strong trend of an increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA at a positive cutpoint of 125,000 RLU with an increasing severity of histologic diagnosis with (P_{Trend} < 0.0005; Table 3). The percentages of test positives were 40% for histology of <CIN1 or no biopsy, 70% for CIN1, 90% for CIN2, 94% for CIN3, and 100% for cancer [note that there was no statistical difference in carcinogenic HPV E6/E7

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Table 2. Testing positive for carcinogenic HPV E6/E7 mRNA at two positive cutpoints compared with the severity of the cytologic interpretation

	CO = 125,000		CO = 750,000		Total	Δ	*P*
	Negative	Positive	Negative	Positive			
Cytology negative	115	10	118	7	125	3	0.3
	92%	8%	94%	6%		2%	
ASC	69	53	81	41	122	12	0.0005
	57%	43%	66%	34%		10%	
LSIL	36	89	48	77	125	12	0.0005
	29%	71%	38%	62%		10%	
HSIL	20	131	24	127	151	4	0.1
	13%	87%	16%	84%		3%	
Total	240	283	271	252	523	31	<0.0001
P_{Trend}	<0.0001		<0.0001				

NOTE: Row percentages are shown beneath the raw numbers. *P* values for trend (P_{Trend}) of testing positive at two positive cutpoints with the severity of cytologic interpretation are shown in the last row. *P* values for an exact McNemar's χ^2 test for differences in percentage of test positives at two positive cutpoints within a cytologic category are presented in the rightmost column. Δ, difference in the number and % of test positives.

mRNA signal strength (P = 0.4, Kruskal-Wallis test) among women with ≥CIN3 between the two clinical sites].

The strong trend of increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA with the severity of histology was also observed using a higher positive cutpoint of 750,000 RLU (P_{Trend} < 0.0005). The percentages of test positives were 34% for histology of <CIN1 or no biopsy, 60% for CIN1, 86% for CIN2, 94% for CIN3, and 100% for cancer. The decrease in test positives at the higher positive cutpoint versus the lower positive cutpoint was significant for <CIN1/no biopsy (40% versus 34%; P < 0.0001) and CIN1 (70% versus 60%; P = 0.004) histology. There were no differences in the number of test positives using either cutpoint for CIN3 or cancer histology.

Carcinogenic HPV E6/E7 mRNA versus HPV DNA. Fewer specimens were called positive carcinogenic HPV E6/E7 mRNA at a positive cutpoint of 125,000 RLU than were called positive for carcinogenic HPV DNA by LA (P < 0.0001). We also found a strong trend of an increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA at a positive cutpoint of 125,000 RLU with riskier HPV risk group (P_{Trend} < 0.0001; Table 4), due to the increasing strength of association of HPV risk groups with cervical precancer and cancer. The percentages of test positives were 3% for PCR negative, 17% for DNA positive for noncarcinogenic HPV types, 84% for DNA positive for carcinogenic HPV types excluding HPV16 and HPV18, 86% for HPV18 DNA positive, and 91% for HPV16 DNA positive.

Similarly, fewer specimens were called positive carcinogenic HPV E6/E7 mRNA at a positive cutpoint of 750,000 RLU than were called positive for carcinogenic HPV DNA by LA (P < 0.0001). A similar trend with HPV risk groups and testing positive for carcinogenic HPV E6/E7 mRNA was observed using the higher positive cutpoint. The higher cutpoint significantly

Table 3. Testing positive for carcinogenic HPV E6/E7 mRNA at two positive cutpoints compared with the severity of the histologic diagnosis

	CO = 125,000		CO = 750,000		Total	Δ	*P*
	Negative	Positive	Negative	Positive			
Histology <CIN1 or no biopsy	204	135	224	115	339	20	<0.0001
	60%	40%	66%	34%		6%	
CIN1	26	61	35	52	87	9	0.004
	30%	70%	40%	60%		10%	
CIN2	5	46	7	44	51	2	0.5
	10%	90%	14%	86%		4%	
CIN3	3	46	3	46	49	0	1.0
	6%	94%	6%	94%		0%	
Cancer	0	5	0	5	5	0	n/a
	0%	100%	0%	100%		0%	
Total	238	293	269	262	531	31	<0.0001
P_{Trend}	<0.0001		<0.0001				

NOTE: Row percentages are shown beneath the raw numbers. *P* values for trend (P_{Trend}) of testing positive at two positive cutpoints with the severity of histologic diagnosis are shown in the last row. *P* values for an exact McNemar's χ^2 test for differences in percentage of test positives at two positive cutpoints within a histologic category are presented in the rightmost column. Δ, difference in the number and % of test positives. Abbreviation: n/a, not applicable.

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Table 4. Testing positive for carcinogenic HPV E6/E7 mRNA at two positive cutpoints compared with detection of carcinogenic HPV DNA and HPV risk group (HPV16 > HPV18 > carcinogenic HPV except HPV16 and HPV18 [Carcinogenic (example HPV16 and HPV18)] > noncarcinogenic > PCR negative) by the LA HPV genotyping assay

LA results	CO = 125,000		CO = 750,000		Total	Δ	*P*
	Negative	Positive	Negative	Positive			
Carcinogenic HPV DNA							
Negative	196	14	204	6	210	8	0.008
	93%	7%	97%	3%		4%	
Positive	42	279	65	256	319	23	<0.0001
	13%	87%	20%	80%		7%	
Total	238	293	269	262	531	31	<0.0001
P	<0.0001		<0.0001				
HPV risk group (DNA)							
PCR Negative	153	5	154	4	158	1	1.0
	97%	3%	97%	3%		1%	
Noncarcinogenic	43	9	50	2	52	7	0.02
	83%	17%	96%	4%		13%	
Carcinogenic (example HPV16 and HPV18)	29	153	45	137	182	16	<0.0001
	16%	84%	25%	75%		9%	
HPV18	3	19	4	18	22	1	1
	14%	86%	18%	82%		5%	
HPV16	10	107	16	101	117	6	0.03
	9%	91%	14%	86%		5%	
Total	238	293	269	262	531	31	<0.0001
P_{Trend}	<0.0001		<0.0001				

NOTE: Row percentages are shown beneath the raw numbers. *P* values for the McNemar's χ^2 test for differences in percentage of test positives at two positive cutpoints versus carcinogenic HPV DNA detected by LA are shown in the row below the carcinogenic HPV DNA section. *P* values for trend (P_{Trend}) of testing positive at two positive cutpoints with the HPV risk group are shown in the last row. *P* values for an exact McNemar's χ^2 test for differences in percentage of test positives at two positive cutpoints by carcinogenic HPV DNA status or within a HPV risk group are presented in the rightmost column. Δ, difference in the number and % of test positives.

reduced the number of test positives for women with noncarcinogenic HPV (17% to 4%, $P = 0.02$), carcinogenic HPV (84% to 75%, $P < 0.0001$), and HPV16 (91% to 86%, $P = 0.01$). The trend of increasing likelihood of testing positive for carcinogenic HPV E6/E7 mRNA at either positive cutpoint with the riskier HPV risk group remained strong even among women who had less than CIN2 histology (data not shown).

Paired test results for carcinogenic HPV DNA and E6/E7 mRNA by histologic diagnosis and overall are shown in Table 5. There was 89% agreement and a κ of 0.78 for a carcinogenic HPV E6/E7 mRNA positive cutpoint of 125,000 RLU, and 87% agreement and a κ of 0.72 for a carcinogenic HPV E6/E7 mRNA positive cutpoint of 750,000 RLU. Percent agreement (98%) and κ values (0.79) were greatest for

Table 5. A comparison of paired carcinogenic HPV DNA detection by LA and carcinogenic HPV E6/E7 mRNA detection at a cutoff of 125,000 RLU (A) and 750,000 RLU (B) for each histologic diagnosis

	DNA-/ mRNA-, *n* (%)	DNA+/ mRNA-, *n* (%)	DNA-/ mRNA+, *n* (%)	DNA+/ mRNA+, *n* (%)	DNA+, *n* (%)	mRNA+, *n* (%)	Total	Δ, *n* (%)	%Agree	κ	*P*
(A) Cutoff: 125,000 RLU											
<CIN1 or no biopsy	171 (51)	31 (9)	6 (2)	129 (38)	160 (47)	135 (40)	337	25 (7)	89	0.78	<0.0001
CIN1	17 (20)	9 (10)	6 (7)	55 (63)	64 (74)	61 (70)	87	3 (3)	83	0.57	0.6
CIN2	4 (8)	1 (2)	2 (4)	43 (86)	44 (88)	45 (90)	50	-1 (-2)	94	0.69	1.0
≥CIN3	2 (4)	1 (2)	0 (0)	50 (94)	51 (96)	50 (94)	53	1 (2)	98	0.79	1.0
Total	194 (37)	42 (8)	14 (3)	277 (53)	319 (61)	291 (55)	527	28 (5)	89	0.78	0.0002
(B) Cutoff: 750,000 RLU											
<CIN1 or no biopsy	176 (52)	46 (14)	1 (0)	114 (34)	160 (47)	115 (34)	337	45 (13)	86	0.72	<0.0001
CIN1	19 (22)	16 (18)	4 (5)	48 (55)	64 (74)	52 (60)	87	12 (14)	77	0.49	0.01
CIN2	5 (10)	2 (4)	1 (2)	42 (84)	44 (88)	43 (86)	50	1 (2)	94	0.74	1.0
≥CIN3	2 (4)	1 (2)	0 (0)	50 (94)	51 (96)	50 (94)	53	1 (2)	98	0.79	1.0
Total	202 (38)	65 (12)	6 (1)	254 (48)	319 (61)	260 (49)	527	59 (11)	87	0.72	<0.0001

NOTE: An exact McNemar's χ^2 test was used to test for differences in testing positive. Δ, difference in the number and % of test positives; %Agree = % total agreement.

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histologic diagnoses of CIN3 or cancer (≥CIN3), and the difference in number of test positives was negligible when a higher carcinogenic HPV E6/E7 mRNA positive cutpoint (P = 1.0). Fewer women tested positive for carcinogenic HPV E6/E7 mRNA at the 125,000 RLU (40%; $P < 0.0001$) and at 750,000 RLU (34%; $P < 0.0001$) cutpoints than for carcinogenic HPV DNA (47%) among women with <CIN1 histology or no biopsy taken. Fewer women tested positive for carcinogenic HPV E6/E7 mRNA at the 750,000 RLU cutpoint (60%) than for carcinogenic HPV DNA (74%) among women with CIN1 histology (P = 0.01).

Finally, we compared the results of the assay for carcinogenic HPV E6/E7 mRNA to HC2, a FDA-approved DNA test for carcinogenic HPV in a subset of 85 women who had both testing results. HC2 was more likely to test positive (67%) than the APTIMA HPV assay at a cutpoint of 125,000 RLU (58%)(P = 0.08) and at a cutpoint of 750,000 RLU (49%) (P = 0.0007). Among the 57 women who were HC2 positive, fewer women tested positive for carcinogenic HPV E6/E7 mRNA at the higher threshold compared with the lower threshold (53% versus 47%, respectively; P = 0.06).

Discussion

Here, we showed that detection of E6/E7 mRNA for 14 carcinogenic HPV types collectively is strongly correlated with the severity of cytologic interpretation and, more importantly, with histologic diagnosis. More than 90% of CIN3 cases and all five cancers included in this study were positive for carcinogenic HPV E6/E7 mRNA. Using the preliminary, manufacturer-recommended positive cutpoint, mRNA detection performed very similarly to previous results for carcinogenic HPV DNA detection using a HPV genotyping assay on this same specimen set (23). In a *post hoc* analysis using a higher positive cutpoint, the carcinogenic HPV E6/E7 mRNA assay was less likely to test positive among women with mild abnormalities caused by HPV infection, without significantly reducing test positives in true cervical precancer, CIN3, and cancer. The reduced number of test positives was the result of increased analytic specificity, as fewer specimens with noncarcinogenic HPV types and fewer positive specimens with carcinogenic HPV types but without concurrent high-grade cervical neoplasia were called positive.

We suggest, based on our data, that detection of carcinogenic HPV E6/E7 mRNA may achieve similar clinical sensitivity and negative predictive value as, while possibly achieving better clinical specificity and positive predictive value than, the detection of carcinogenic HPV DNA. One of the main criticisms of carcinogenic HPV DNA testing has been its lower specificity compared with cytology screening. It remains to be determined whether detection of carcinogenic HPV E6/E7 mRNA is sufficiently specific for precancer and cancer that it can be used as a primary, stand-alone screening test or will be included with cytologic screening.

At the higher threshold, only two CIN2 lesions were classified negative using a higher threshold and were called positive at the lower threshold, one of which was negative for carcinogenic HPV DNA. Although CIN2 represents the clinical threshold for treatment by ablation or excision, it is an equivocal and heterogeneous precancer category composed of cases of HPV infection and true preneoplastic disease. Moreover, CIN2, often missed by cervical cytology (11) in routine screening, can also be caused by noncarcinogenic HPV genotypes (28), with virtually no potential for progression to cancer.

Importantly, HPV mRNA was sufficiently well preserved in liquid cytology medium for retrospective testing using this assay. That is, although the specimens were eventually stored at -20°C, no special precautions were taken at the clinical center, and the specimens were stored at ambient temperature for several weeks until clinical decisions were rendered. Thus, it would seem that stability of mRNA in these specimens may be sufficient for detection of carcinogenic HPV E6/E7 mRNA by the APTIMA HPV assay in a routine clinical laboratory setting. Formal specimen stability studies are needed to support this observation.

Based on these and other promising results (13–17), additional studies are needed and warranted to evaluate/validate this assay and, in general, the detection of carcinogenic HPV E6/E7 mRNA for cervical cancer screening. Specifically, for the APTIMA HPV assay, studies composed of large population-based samples of women and with rigorous disease ascertainment, including longitudinal follow-up, are needed to establish the optimal positive cutpoint based on receiver-operator characteristics. Studies are also needed to fully characterize the assay performance including demonstration of its intra-laboratory and interlaboratory reproducibility. Together, these studies will establish the clinical utility of this assay for cervical cancer screening (29).

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