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RNA (E6 and E7) Assays versus DNA (E6 and E7) Assays for Risk Evaluation for Women Infected with Human Papillomavirus[▽]

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In the majority of cases, high-risk human papillomavirus (HR HPV) infections regress spontaneously, with only a small percentage progressing to high-grade lesions. Current screening methods are based on DNA detection. An alternative would be to monitor expression of the E6 and E7 viral oncogenes continuously expressed by malignant phenotypes. In the work reported in this paper, we compared the two methods for a group of women with high-risk HPV infections. Cervical specimens from 400 women, previously found to be HPV DNA positive, were analyzed for HPV DNA by a liquid hybridization assay and typed by multiplex PCR (for types 16, 18, 31, and 33). Identification of HR HPV E6 and E7 RNA transcripts was performed using real-time reverse transcription-PCR and nucleic acid sequence-based amplification assays. Results were compared with concurrent cytological data. HR HPVs were found in 61.2% of patients. The most common genotype was HPV type 16 (HPV-16) (47.1%), followed by HPV-18, HPV-31, and HPV-33. Nine percent of cases involved other genotypes. Among 223 HPV DNA-positive samples, only 118 were positive in the RNA test. Among HPV DNA-positive patients with normal cytology, we detected E6 and E7 RNA transcripts in two cases (18.2%). The rate of detection increased gradually with the grade of the observed lesions, rising from 20% for patients with atypical squamous cells of undetermined significance to 48.1% for women with low-grade squamous intraepithelial lesions and 86.3% for those with high-grade squamous intraepithelial lesions. These results suggest that testing for HPV E6 and E7 transcripts could be a useful tool for screening and patient management, providing more accurate predictions of risk than those obtained by DNA testing.

Cancer of the cervix is the second most frequent gynecological malignancy in the world. It is well established that the main cause is infection with human papillomavirus (HPV) (30, 43, 46, 48). However, only certain specific types of virus lead to cancer. We can thus distinguish between high-risk and low-risk HPVs (HR HPV and LR HPV, respectively) (1, 6, 12, 31, 43). Epidemiological studies of HPV show that more than 70% of cervical cancers worldwide are caused by HPV type 16 (HPV-16) and HPV-18. The remaining cases of malignancy are associated with other types of HR HPV (mainly HPV-31, -33, and -45). Today, these viruses are among the most important known risk factors for human cancer (6, 20, 31, 38).

Most HR HPV infections regress spontaneously 6 to 12 months after their appearance, probably due to successful attack by the immune system (33). Only a small percentage of infections persist. Without surgical treatment, these infections can progress to high-grade lesions and to squamous cell carcinoma or adenocarcinoma of the cervix (37, 47, 48).

The introduction of the Pap smear test by Papanicolaou made it possible to identify precursor lesions and to significantly reduce mortality. However, the test cannot reliably predict whether a mild dysplasia will regress or progress (5, 36). Recently, it was suggested that cervical cancer screening could be improved by combining the cytological assay with testing for HPV DNA (2, 7, 13, 19, 21, 36). However, the high prevalence of papillomavirus infection means that such testing has a low positive predictive value (17, 25, 26).

It is known that the development of a malignant phenotype requires continuous expression of the E6 and E7 viral oncogenes. E6 and E7 viral oncoproteins bind and modulate cellular gene products (p53 and pRb) that play a key role in cell cycle control and DNA repair. The resulting genomic instability is a necessary condition for cell transformation and immortalization (29, 30, 46, 48). Increased expression of these transcripts has been described for both high-grade squamous intraepithelial lesions (HSIL) and clinical samples of cervical carcinoma (8, 20, 25, 30). It is logical, therefore, to hypothesize that E6 and E7 RNA transcripts could predict disease progression (4, 19, 26, 27). Although the value of RNA testing has yet to be assessed in large-scale clinical trials, recent studies (8, 9, 10, 22, 25, 44) are encouraging. Since DNA-based assays cannot distinguish between transient and potentially transforming infections, several studies (4, 11, 26, 40, 41) have suggested that testing for HPV E6 and E7 RNAs could be more specific. Taken together, these results suggest that RNA-based assays could have a higher prognostic value than DNA-based tests and that they could play an important role in future screening programs (2, 9).

In this study, we evaluate this possibility, comparing DNA and RNA assays of cervical brush samples from women with a previous diagnosis of infection with HR HPV.

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MATERIALS AND METHODS

Patients and sampling. This study was conducted on cervical specimens from 400 Italian women (age range, 21 to 59 years) undergoing tests for HPV detection at the Outpatient Department of the Institute of Microbiology (Università

TABLE 1. Sequences of primers (F and R) and TaqMan probes (P) used for real-time PCR, target locations, and lengths of amplification products

Primer or probe	Sequence (5′ to 3′)	Location[a]	Product size (bp)
HPV 16 E6-F	GCACCAAAAGAGAACTGCAATGTT	85–108	152
HPV 16 E6-R	AGTCATATACCTCACGTCGCAGTA	197–236	
HPV 16 E6-P	GGACCCACAGGAGCGACCCAGAAAGTTA	112–139	
HPV 16 E7-F	CAAGTGTGACTCTACGCTTCGG	738–759	81
HPV 16 E7-R	GTGGCCCATTAACAGGTCTTCCAA	796–818	
HPV 16 E7-P	TGCGTACAAAGCACACACGTAGACATTCGT	763–792	
HPV 18 E6-F	CTATAGAGGCCAGTGCCATTCG	503–524	79
HPV 18 E6-R	TTATACTTGTGTTTCTCTGCGTCG	558–581	
HPV 18 E6-P	CAACCGAGCACGACAGGAACGACTCCA	530–556	
HPV 18 E7-F	TAATCATCAACATTTACCAGCCCG	721–744	113
HPV 18 E7-R	CGTCTGCTGAGCTTTCTACTACTA	810–833	
HPV 18 E7-P	CGAGCCGAACCACAACGTCACACAATGTT	745–774	
HPV 31, E6-F	AAGACCGTTGTGTCCAGAAG	428–447	106
HPV 31, E6-R	GTCTTCTCCAACATGCTATGC	511–534	
HPV 31, E6-P	CGTCCTGTCCACCTTCCTCCTATG	488–511	
HPV 31, E7-F	TGTGTTAGATTTGCAACCTGAG	592–613	78
HPV 31, E7-R	ACATCCTCCTCATCTGAGCT	669–649	
HPV 31, E7-P	CAACTGACCTCCACTGTTATGAGCAAT	615–641	
HPV 33, E6-F	TGCACGACTATGTTTCAAGAC	100–120	132
HPV 33, E6-R	CTCAGATCGTTGCAAAGGTTT	211–231	
HPV 33, E6-P	ATTCCACGCACTGTAGTTCAATGTTGT	179–205	
HPV 33, E7-F	TTGTAACCTGTTGTCACACTTG	733–754	88
HPV 33, E7-R	AGTAGTTGCTGTATGGTTCGTA	799–820	
HPV 33, E7-P	ACTTGCTGTACTGTTGACACATAAACGA	767–794	

[a] Nucleotide positions based on genome sequences for HPV-16, -18, -31, and -33 (GenBank accession numbers K02718, X05015, J04353, and M12732, respectively).

Cattolica del Sacro Cuore, Rome, Italy). Specimens were collected in the period from May 2006 to September 2007. All of the patients in the study had previously tested positive for HR HPV, in tests carried out at our own institution, 8 to 10 months before samples were taken. Patients who had tested positive only for LR HPV or who did not undergo concurrent cytological tests were not included in the study. All participants in the study gave their informed consent.

Cytological investigations were carried out at the Institute of Pathology (Università Cattolica del Sacro Cuore, Rome, Italy) in the same period that the DNA and RNA assays were performed. Cytology and RNA/DNA testing were conducted independently.

Patients were grouped based on cytological findings, as follows: 108 women showed normal cytology, in 84 we detected atypical squamous cells of undetermined significance (ASCUS), in 136 we found low-grade squamous intraepithelial lesions (LSIL), and in 72 we found HSIL. Cervical brush samples were collected using a cervical brush transport kit (Digene, Milan, Italy). Three identical aliquots were prepared, including one for DNA detection by the Hybrid Capture II HPV DNA test (hc2; Digene, Milan, Italy) and two for DNA and RNA extractions. Aliquots were stored appropriately for further processing.

HPV DNA detection by liquid hybridization assay. All samples were analyzed using the Hybrid Capture II HPV DNA test (hc2; Digene, Milan Italy) according to the manufacturer's directions. The hc2 assay, in routine use in our laboratory, effectively differentiates between LR HPV types (6, 11, 42, 43, 44) and HR HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, and 68) but does not allow precise identification of genotypes.

Nucleic acid isolation. Two identical aliquots were centrifuged, and the concentrated cell pellets were used for DNA and RNA extraction. Total RNA was extracted using a Qiagen Protect Mini kit (Qiagen SpA, Milan, Italy) and treated with DNase to eliminate DNA contamination according to the manufacturer's instructions. Extracted RNA was eluted with 60 μl of diethyl pyrocarbonate-treated water. DNA was purified using a spin column-based QIAamp DNA Mini kit (Qiagen SpA, Milan, Italy) and eluted in 60 μl of sterile water, following the manufacturer's directions.

HPV genotyping by multiplex PCR for HPV-16, -18, -31, and -33. Samples positive for HR HPV by the hc2 assay (Digene, Milan, Italy) were analyzed by a multiplex PCR assay using type-specific primer sequences as described by van den Brule et al. (24, 42), with some modifications. Each assay used 10 μl of DNA and 20 pmol of each of the four primer sets. The reaction mixture contained 25 μl of HotStar *Taq* master mix (Qiagen SpA, Milan, Italy) and 7 μl of RNase-free water in a final volume of 50 μl. The amplification profile consisted of 15 min at 95°C to activate the HotStar *Taq* DNA polymerase (Qiagen SpA, Milan, Italy), followed by 45 cycles of denaturation (94°C for 30 s), annealing (56°C for 40 s), and extension (72°C for 40 s). Assays were performed on an iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Amplicons were detected by electrophoresis of 20 μl of amplification products in a 2% agarose gel and visualized by ethidium bromide staining under a Fluor-S UV light transilluminator. Molecular sizes of amplicons (HPV-16, 152 bp; HPV-18, 216 bp; HPV-31, 513 bp; and HPV-33, 455 bp) were determined by matching the bands against commercial DNA molecular size markers (molecular weight markers V and VIII; Roche Diagnostics, GmbH, Mannheim, Germany).

To assess sensitivity, plasmids containing genomic DNAs of HPV-16 and -18, kindly provided by E. M. de Villiers (Deutsches Krebsforschungszentrum, Heidelberg, Germany), and specific PCR products of HPV-31 and -33 cloned into the pCR2.1 vector (Invitrogen, San Diego, CA) were quantified by optical density measurement. Constructs were serially diluted and subsequently amplified using type-specific primers. Multiplex PCR was shown to detect concentrations as low as five copies per sample.

Primers and probes. Type-specific reverse transcription-PCR (RT-PCR) and real-time PCR primers and probes for the E6 and E7 regions of the four HPV types were designed using DNASIS, version 2.1, for Windows (Hitachi Software Genetic System, San Francisco, CA) and synthesized by Applied Biosystems (Monza, Milan, Italy). Fluorescent probes were labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine. The

TABLE 2. HPV DNA test and cytological findings for cervical brush specimens from 400 women who tested positive for HPV DNA 8 to 10 months previously

Cytology result	Age (yr) (range [mean])	Total no. of patients	No. (%) of patients with hc2 HR HPV test result	
			Positive	Negative
Normal	23–49 (33.7)	108	18 (16.7)	90 (83.3)
ASCUS	21–59 (34.4)	84	46 (54.7)	38 (45.3)
LSIL	21–57 (35.2)	136	113 (83.1)	23 (16.9)
HSIL	24–56 (36.3)	72	68 (94.5)	4 (5.5)
Total		400	245 (61.25)	155 (38.75)

TABLE 3. Cytology and HR HPV genotyping by multiplex PCR for cervical brush samples from 245 HPV DNA-positive patients

Cytology result	No. of HR HPV-positive samples by hc2 test	No. (%) of positive samples by multiplex PCR (for HPV-16, -18, -31, and -33)
Normal	18	11 (61.1)
ASCUS	46	40 (86.9)
LSIL	113	106 (93.8)
HSIL	68	66 (97.1)
Total	245	223 (91.0)

sequences of primers and TaqMan probes, GenBank accession numbers, and the lengths of the amplification products are listed in Table 1.

Real-time PCR and real-time RT-PCR assays. Positive samples were analyzed to determine DNA and RNA copy numbers for HPV genotypes 16, 18, 31, and 33. Real-time PCR and real-time RT-PCR were performed using the iCycler iQ detection system (Bio-Rad Laboratories Inc., Hercules, CA). Quantitative PCR amplification for viral DNA detection was performed in a reaction volume of 50 μl containing 10 μl of DNA, 5 μl of primers (5 μM), 5 μl of probe (1.5 μM), and 25 μl of Platinum Quantitative PCR Super-Mix-UDG buffer (Invitrogen, San Diego, CA). Thermal cycling conditions were 2 min at 50°C and 15 min at 95°C followed by 50 cycles of 15 s at 95°C and 30 s at 57°C for DNA detection by real-time PCR. Real-time RT-PCR for E6 and E7 mRNA detection used the same reaction conditions and thermal profile except for an initial 30 min at 50°C for RT (RT-PCR Super-Mix-UDG; Invitrogen, San Diego, CA).

Specific PCR products for each target sequence were cloned into the pDRive cloning vector (Invitrogen, San Diego, CA), quantitated in triplicate by measurement of UV absorption, mixed with HPV-negative human genomic DNA, and serially diluted to generate a standard curve ranging from 10 to 10×10^7 copies. After confirmation of the accuracy of the standard dilution series, standards were stored at −20°C in small aliquots. Amplification was measured as an increase in reporter fluorescence. Data were collected in real time and analyzed by sequence detection system software (Bio-Rad Laboratories Inc., Hercules, CA). A standard curve was constructed by plotting the log of the starting plasmid concentration against the cycle threshold, that is, the first cycle number at which reporter fluorescence was significantly higher than the mean background fluorescence. This curve was subsequently used to determine DNA and RNA concentrations in specimens. Preliminary studies showed that both real-time PCR assays were able to detect 10 to 10^7 plasmid copies per reaction. For each sample, we computed the ratio between the numbers of mRNA and DNA copies (RNA/DNA copy number ratio). This method allowed us to correct for the highly variable numbers of cells in cervical samples.

HPV RNA detection by real-time multiplex nucleic acid sequence-based amplification. DNA-positive samples were retested for the presence of E6 and E7 transcripts of HR HPV types 16, 18, 31, 33, and 45 by use of the NucliSENS EasyQ HPV test (bioMerieux, Rome, Italy) according to the manufacturer's directions.

Controls. Specimen quality was assessed with a primer set that amplifies a 268-bp fragment coding for human beta-globin (35) and with a glyceraldehyde-3-phosphate dehydrogenase primer set that amplifies a 240-bp product from human cDNA (39).

The specificity of HPV primer pairs was evaluated by testing them against specimens negative for the targeted type but positive for other types. No PCR product was generated from these DNA and RNA amplifications.

As positive controls for HPV-18 and HPV-16, we used HeLa cells (American Type Culture Collection) and CaSki cells (Interlab Cell Line Collection, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy); for HPV types 31 and 33, we used cloned DNAs from previously typed cervical biopsies. In each amplification run, we used distilled water as a negative control. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at all stages.

RESULTS

Initial screening with the hc2 DNA assay detected HR HPV infection in 245 of 400 women (61.25%). In 67, we detected concurrent infection with LR types. The remaining 155 women tested negative (Table 2). HR HPV DNA was found in 16.7% of patients with normal cytology (18/108 patients), 54.7% of women with ASCUS (46/84 patients), 83.1% of those with LSIL (113/136 patients), and 94.5% of those with HSIL (68/72 patients) (Table 2).

All specimens were positive for beta-globin DNA, confirming the quality of the DNA preparations.

The 245 samples positive for HR HPV by hc2 assay were typed by multiplex PCR. HR genotypes (types 16, 18, 31, and 33) were detected in 223 cases (91.0%) (Table 3). The commonest genotype was HPV-16 (105 cases [47.1%]), followed by HPV-18 (30 cases [13.4%]), HPV-31 (18 cases [8.1%]), and HPV-33 (15 cases [6.7%]). In 55 cases (24.6%), we detected infection with multiple types. Twenty-two of these involved mixed infections with HPV-16 and HPV-18. In 22 (9%) cases, we detected no HPV of types 16, 18, 31, and 33 (Table 4).

The lowest rate of prevalence for HR genotypes was for patients with normal cytology or ASCUS. A total of 93.8% of patients with LSIL and 97.1% of patients with HSIL displayed simple or mixed infections with HPV-16, -18, -31, and -33 (Table 3).

Viral DNA loads, estimated by "in-house" RT-PCR, ranged from 10^2 to 10^6 copies per specimen. These data correlated well with relative light unit values from the hc2 HPV DNA test (data not shown); minor differences were probably due to differences in the methods used (amplification versus hybridization). Quantities of HPV DNA varied widely within and between patient groups. There was no significant correlation between copy number and grade of lesion.

In our RNA assay, RT-PCR (for HPV-16, -18, -31, and -33) detected E6 and E7 oncogene transcripts in 118 patients (52.9%) who had tested positive for specific HR genotypes (Table 5). Amplification with a glyceraldehyde-3-phosphate dehydrogenase cDNA primer set confirmed the adequacy of RNA-negative samples. These results were replicated using the NucliSENS EasyQ HPV test (bioMerieux, Rome, Italy). The results matched for all except two samples.

For patients with normal cytology, E6 and E7 transcripts were detected in 18.2% of cases (Table 5). The proportion of

TABLE 4. Cytology and HR HPV genotypes for 223 HPV-positive patients

Cytology result	No. (%) of patients testing positive for HPV type(s) by multiplex PCR										Total
	16	18	31	33	16 and 18	16 and 31	16 and 33	18 and 31	18 and 33	31 and 33	
Normal	3	2	1	2		1	1			1	11
ASCUS	24	3	3	1	2	2	2	1		2	40
LSIL	43	14	9	8	14	5	5	2	3	3	106
HSIL	35	11	5	4	6	2		1	1	1	66
Total	105 (47.1)	30 (13.4)	18 (8.1)	15 (6.7)	22 (9.9)	10 (4.5)	8 (3.6)	4 (1.8)	4 (1.8)	7 (3.1)	223 (100)

TABLE 5. Cytology, HR genotypes, and presence of E6 and E7 transcripts for patients previously classified as HPV DNA positive

Cytology result	No. of patients positive for HPV-16, -18, -31, and -33	No. (%) of patients positive for E6 and E7 transcripts by real-time RT-PCR and EasyQ HPV test
Normal	11	2 (18.2)
ASCUS	40	8 (20.0)
LSIL	106	51 (48.1)
HSIL	66	57 (86.3)
Total	223	118 (52.9)

TABLE 7. Cytology and mean RNA/DNA copy number ratios

Cytology result	Mean RNA/DNA copy no. ratio for positive samples				
	HPV types				All types[a]
	16	18	31	33	
Normal	2.6	2.3	0.8		1.92
ASCUS	2.6	3.5		0.5	2.21
LSIL	12.9	5.8	12.0	7.5	9.56
HSIL	22.2	11.6	7.2	10.2	12.8

[a] The mean for all types for samples with normal cytology and ASCUS was 2.27 (SD, 1.44), and that for samples with HSIL and LSIL cytology was 14.43 (SD, 12.63). The difference between these means was statistically significant (Student's $t = 12.57$; $P < 0.001$).

patients with detectable HPV transcripts increased progressively with the grade of observed lesions, rising from 20% for patients with ASCUS (8/40 patients) to 48.1% for those with low-grade lesions (51/106 patients) and 86.3% for those with high-grade lesions (57/66 patients) (Table 5).

The results for the prevalence of specific genotypes were similar to those from the earlier DNA assay. In 56.8% of cases, we detected HPV-16. The second commonest genotype was HPV-18 (17%), followed by HPV-31 (10.2%) and HPV-33 (5.9%) (Table 6). The assay detected six mixed infections with HPV-16 and -18, three with HPV-16 and -31, two with HPV-18 and -31, and one with HPV-16 and -33. In two cases (one mixed infection of HPV-16 and HPV-18 and one of HPV-16 and HPV-33), the NucliSENS EasyQ HPV test (bioMerieux, Rome, Italy) failed to detect the genotypes detected by RT-PCR. For two other patients, the test detected the presence of HPV-45 in association with HPV-18. Our RT-PCR assay did not include primers for HPV-45 and could not detect this virus.

E6 and E7 RNA transcripts ranged from 10^2 to 10^7 copies per specimen. The RNA/DNA copy number ratio was higher for patients with LSIL and HSIL (mean = 14.43; standard deviation [SD] = 12.63) than for patients with normal cytology or ASCUS (mean = 2.27; SD = 1.44). This difference was statistically significant (Student's $t = 12.57$; $P < 0.001$) and was observed for all genotypes (Table 7).

Among the 55 samples with multiple HPV infections, 12 (21.8%) were positive for E6 and E7 transcripts. The level of expression was always significantly higher for one of the two genotypes. This finding contrasts with the DNA findings, which showed no significant differences between genotypes.

DISCUSSION

Given the importance of HPV in the etiology of cervical cancer (38, 43, 45, 46, 48), many groups have developed methods for detecting the virus (34). These tests have been shown to have a higher sensitivity for high-grade cervical lesions than that of cytology (5, 7, 21, 36). It has therefore been suggested that DNA-based HPV detection should play a routine role in screening (3, 10, 11, 14, 37).

Ninety percent of HPV infections clear spontaneously over a period of 6 to 24 months (33). Generally speaking, only persistent infections lead to cancer (6, 18, 28). This implies that although DNA testing has a high negative predictive value, its positive predictive value is relatively low (26, 27, 33), and tests need to be repeated periodically, with a negative impact on health service costs and patient comfort.

Research in recent years has found that histologically proven cases of cervical intraepithelial neoplasia 3 (CIN3) and invasive cervical carcinoma are associated with the presence of transcripts from the E6 and E7 oncogenes (8, 19, 20, 30, 32). It has been suggested that tests for these transcripts or for other biomarkers, such as p16 (16), could be more effective than DNA detection in predicting disease progression (25–27, 31, 44).

In this study, therefore, we compared the two approaches (DNA and RNA testing) with clinical specimens from patients previously diagnosed with HR HPV.

Multiplex PCR detected DNA from HR HPV in 91% of the patients who had tested positive by the hc2 assay. The most common genotype was HPV-16, followed by HPV-18, HPV-33, and HPV-31. The majority of the patients who tested positive showed lesions classified as LSIL or HSIL (93.8% and 97.1%, respectively). These findings match prevalence data in the literature and confirm the importance of HPV-16 and -18 as highly specific markers for risk (6). The value of genotyping for other HR types (6, 7, 14, 18) is limited by their low frequencies.

A total of 38.8% of the 400 women tested negative for HPV DNA after 8 to 10 months. This result matches usual rates of

TABLE 6. Cytology and prevalence of E6 and E7 transcripts from HPV types 16, 18, 31, and 33 in cervical brush samples

Cytology result	No. (%) of samples positive for HPV type(s) by RNA genotyping by real-time RT-PCR								Total no. of positive samples
	16	18	31	33	16 and 18	16 and 31	16 and 33	18 and 33	
Normal		1				1			2
ASCUS	6	1					1		8
LSIL	26	9	6	6	2			2	51
HSIL	35	9	6	1	4	2			57
Total	67 (56.8)	20 (17.0)	12 (10.2)	7 (5.9)	6 (5.1)	3 (2.5)	1 (0.8)	2 (1.7)	118 (100)

clearance for HPV infections, as reported in previous studies (33).

Quantitation showed large variations in viral load among patients with lesions of the same grade and a certain degree of overlap between women with and without high-grade lesions. The variability in the data is possibly due to the highly variable nature of cervical specimens (2). Although the methods used provide no data on the number of infected cells or the number of HPV genomes per cell, these findings support suggestions from other authors that viral load may be a poor predictor of acquisition or progression of disease (2, 37, 45), with limited applicability in the clinic (10, 11, 15).

Compared to the DNA test, the RNA assay produced fewer positive results. E6 and E7 transcripts were detected in just 52.9% of patients who tested positive for HR HPV. The lack of RNA transcripts possibly reflects an episomal state of the virus in which regulation of the transcription process is still effective, creating a higher probability that the infection will clear spontaneously. Negative test results were common for women with normal cytology, ASCUS, and LSIL. In contrast, 86.3% of patients with HSIL tested positive. The high frequency of E6 and E7 transcripts in high-grade dysplasia confirms that the integration of HR HPV into the host genome, loss of control over oncogene transcription, and consequent expression of E6 and E7 gene products are necessary conditions for the development and maintenance of malignant phenotypes.

The remaining 13.7% of HPV DNA-positive HSIL cases were E6 and E7 mRNA negative. It is possible that these negative results may have been due to a very low level or a lack of transcriptional viral activity. Alternatively, the viruses detected by the DNA assays may have belonged to types we did not consider in our study. However, the cytological diagnosis of HSIL includes lesions, such as CIN2, that sometimes regress (9, 20, 22). For reasons of safety, patients with these lesions undergo surgical resection. Other studies have suggested, however, that some of these women may be overtreated. The results of our own studies support this hypothesis (23).

In our study, we detected E6 and E7 transcripts not only in specimens showing high-grade lesions but also in cytologically normal or borderline samples. This indicates that HR HPV may be oncogenically active even before it produces detectable changes in cells. Given the significant association between RNA/DNA copy number ratio and the severity of lesions, E6 and E7 transcripts could provide a sensitive, early predictor of persistent infection and subsequent severe dysplasia.

In sum, the findings from our study support the hypothesis that, especially for patients with normal cytology or low-grade lesions, E6 and E7 mRNA detection may be a more specific diagnostic tool and a better predictor of disease progression than DNA-based assays. We are aware that these results on their own are not enough to demonstrate the usefulness of mRNA testing in the clinic. This would require a comparative analysis of the sensitivities and specificities of DNA- and RNA-based methods for HPV-positive and -negative patients followed up by colposcopy, cytology, and histological examination. To meet this need, we recently began a longitudinal study in which we will assess the value of the RNA assay as a predictor of clinical outcomes.

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