Association of Elevated E6 Oncoprotein With Grade of Cervical Neoplasia Using PDZ Interaction–Mediated Precipitation of E6

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■ **Abstract**

Objective. To determine the expression of human papillomavirus (HPV) type 16 E6 oncoprotein in cervical specimens of women with and without cervical intraepithelial neoplasia (CIN).

Materials and Methods. Cervical specimens from 2,530 unscreened women aged 30 to 54 years from Shanxi, China, were obtained. All women were assessed by liquid-based cytology, high-risk HPV DNA tests, and colposcopy with directed biopsy and endocervical curettage as necessary. Women with abnormal cytologic results or positive HPV DNA results were recalled for colposcopy, 4-quadrant cervical biopsies, and endocervical curettage. Women with biopsy-proven CIN and cancer and a convenience sample of HC2-positive, disease-negative women were tested for the presence of HPV-16 infection via HPV-16 E6 DNA-specific polymerase chain reaction. A PDZ interaction–mediated E6 oncoprotein precipitation method followed by E6-specific Western blot was performed on specimens from women with HPV-16 infections. Associations between elevated expression of E6 oncoprotein and CIN 2 and 3 were determined using logistic regression and a reference category of CIN 1 and disease-negative.

Results. A significant trend for the detection of HPV-16 E6 oncoprotein in specimen of women with proven HPV-16 infection was determined: 0% (0/12), 12.5% (1/8), 36.4% (4/11), and 42.9% (3/7) of those with negative findings, CIN 1, 2, and 3, respectively ($p = .01$). Compared with the category combining negative findings and CIN 1, detection of E6 oncoprotein was associated with CIN 2 (odds ratio = 10.9, $p = .05$) and CIN 3 (odds ratio = 14.3, $p = .04$).

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Conclusions. There is a significant association between elevated expression of E6 oncoprotein and grade of CIN. This finding seems consistent with the role played by E6 oncoprotein in carcinogenesis. ■

Key Words: biomarker, cervical intraepithelial neoplasia, E6 oncoprotein, natural history, screening

Although the role played by human papillomavirus (HPV) in the pathogenesis of cervical cancer has not yet been fully elucidated, it has been shown that both high-risk HPV E6 and E7 oncoproteins are necessary and sufficient for initiation and maintenance of cervical epithelial cell transformation [1–4]. Both E6 and E7 oncoproteins are expressed by cervical epithelial cells infected by carcinogenic types of HPV, and each oncoprotein plays a multifunctional and synergistic role in cervical carcinogenesis. Most notably, E6 has been shown to result in degradation of the tumor suppressor p53 and certain PDZ domain proteins, whereas E7 leads to inactivation of the tumor-suppressor protein Rb [5, 6]. Elevated E6 oncoprotein expression in HPV-infected cervical cells was hypothesized to correlate with the state of neoplastic transformation and thus the risk of progression to cervical cancer [6].

Infections by carcinogenic types of HPV tend to be relatively common for a lifetime, but most often, they are resolved by the body's immune system without pathological consequences. Several lines of evidence suggest that elevated quantities of virally encoded oncoproteins E6 and E7, rather than the simple presence of the viral DNA, would indicate more specifically that a woman is at an increased risk of cervical cancer [7, 8]. Data are lacking regarding the molecular epidemiology of E6 oncoprotein expression or the potential clinical utility of an E6 oncoprotein-based cervical cancer diagnostic assay, mostly because of the technical difficulties in detecting E6 oncoprotein. On the basis of the potential as a screening tool for the detection of cervical neoplasia and thus cervical cancer prevention, PATH collaborated with Arbor Vita Corp (Sunnyvale, CA) to investigate the prevalence of E6 oncoprotein overexpression among women being screened for cervical cancer and to further develop an E6 oncoprotein–based diagnostic assay for cervical cancer that is suitable for use in low-resource settings [9–11]. We report a prospective study using the previously developed E6 pull-down assay to detect E6 oncoprotein among women with and without cervical neoplasia in collaboration with the Cancer Institute, Chinese Academy of Medical Sciences (CICAMS).

MATERIALS AND METHODS

Patients and Procedures

Previously unscreened 30- to 54-year-old nonpregnant women with no history of cervical neoplasia and living in rural villages in Shanxi Province, China, were enrolled and screened for cervical cancer from May 10 to June 15, 2007 (Figure 1), as described previously [12]. This study was approved by the institutional review board of the CICAMS and by the Human Subjects Protection committee of PATH. After informed written consent was obtained, each woman was asked for sociodemographic, behavioral, and reproductive data by a health worker. A vaginal brush specimen (Cervical Sampler; Qiagen, Gaithersburg, MD) and 2 nylon-swab specimens for storage were collected by each woman. A nurse-midwife inserted a vaginal speculum with water as lubricant and collected 2 flocked nylon swabs (Seacliff Packaging, Inc, Newport Beach, CA) from the ectocervix, a cervical brush (Cervical Sampler) specimen for the *care*HPV Test (Qiagen), and a cervical specimen for storage in medium for liquid-based cytology (LBC, SurePath; Becton Dickinson, Franklin Lakes, NJ) and HC2 testing (Qiagen). Each woman was assessed by colposcopy with directed biopsy and endocervical curettage, as indicated. The flocked nylon swabs from the ectocervix were preserved dry at −80°C and one was used in the E6 analysis.

Women who were negative on colposcopy but had abnormal LBC results (atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion, low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, or cancer), or unsatisfactory cytology according to the Bethesda System, positive HC2, or positive *care*HPV testing were recalled for repeat colposcopy and 4-quadrant cervical biopsies at the squamocolumnar junction and endocervical [12].

Digene HC2 HPV DNA and *care*HPV Tests

The laboratory personnel performing these tests were blinded to all other results. As previously described, the *care*HPV Test is based on the *Digene* HC2 HPV DNA Test but only takes 2.5 hours to perform rather than 6 hours for the *Digene* HC2 HPV DNA Test [12]. Both tests are signal amplification assays, and the *care*HPV Test detects target HPV DNA from HPV type 66 in addition to the 13 carcinogenic types detected by the *Digene* HC2 HPV DNA Test (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The procedures for both tests have been described [12]. The *Digene*

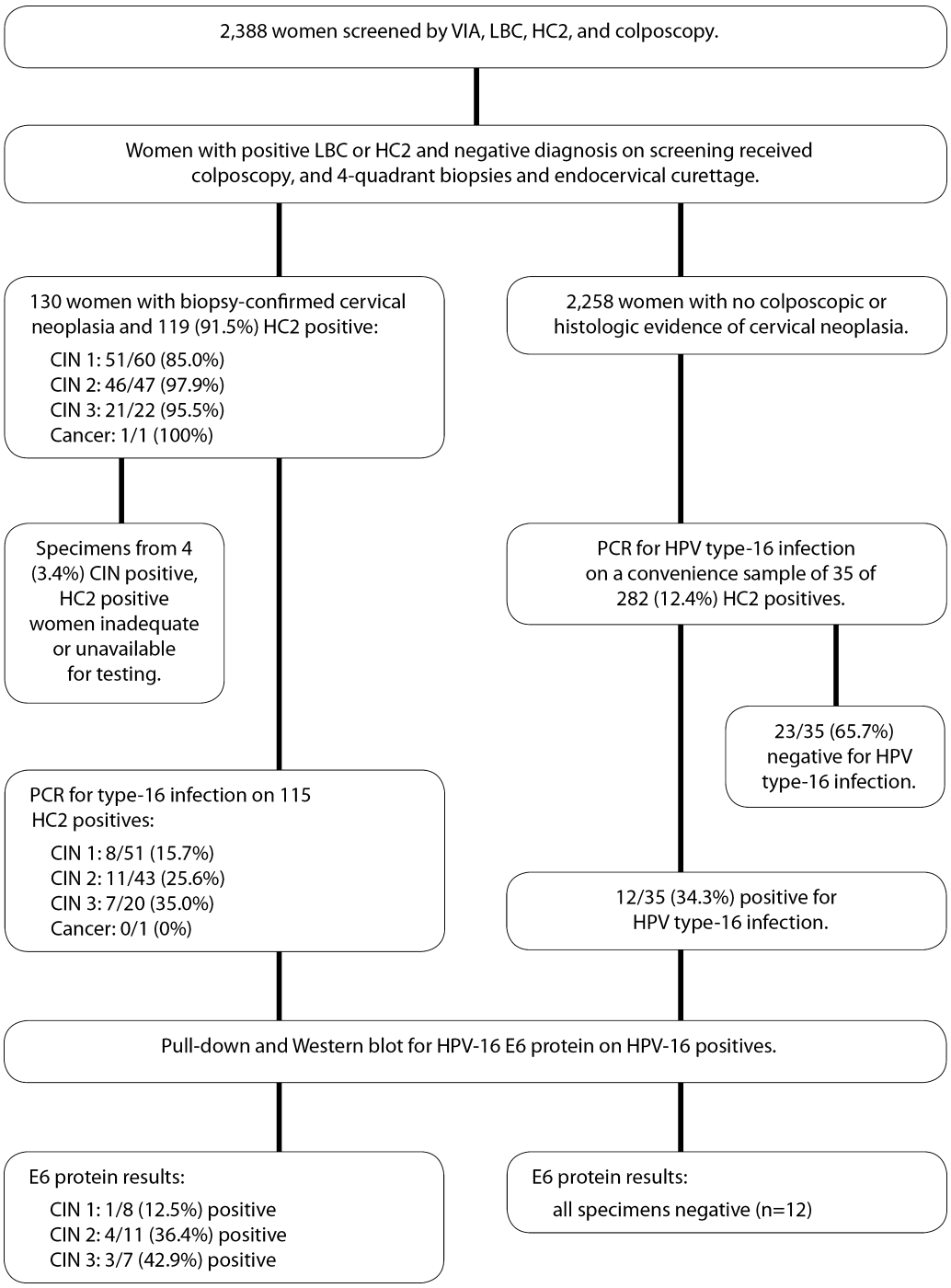

Figure 1. Flow diagram showing the 2,388 women, for whom all data were available, from screening, diagnosis, and subsequently laboratory testing for HPV-16 infection and the overexpression of HPV-16 E6 oncoprotein. Results are shown in Table 1.

HC2 HPV DNA Test was performed within 2 weeks at CICAMS central laboratory in Beijing on the residual storage medium after LBC had been performed, and the *care*HPV Test was performed within 3 hours at the screening site laboratory while the women waited. Test results are expressed in relative light units (RLUs) and compared with the mean RLU from a positive control of 1 pg/mL of HPV-16 DNA (cutoff) resulting in the RLU/cutoff ratio. An RLU/cutoff ratio of 1.0 or higher indicated a positive result on the *Digene* HC2 HPV DNA Test and an RLU/cutoff ratio of 0.5 or higher indicated a positive result on the *care*HPV Test.

Pathology

Histological and cytological diagnoses were based on consensus assessments by a panel of Chinese and Canadian pathologists blinded to all other results, using the cervical intraepithelial neoplasia (CIN) and the Bethesda classification systems, as previously described [12]. The final diagnosis for each woman was based on the highest reading across all histology results including directed and 4-quadrant biopsies and endocervical curettage. If a biopsy had not been indicated or the histologic finding result was negative for a woman, she was assessed as negative for cervical neoplasia.

Specimen Preparation

Cervical swab samples were thawed for 10 minutes at room temperature then lysed (50 mM Tris pH 8.0, 2% Triton X-100, 150 mM NaCl, 50 mM EDTA, 0.1% sarcosine, 0.02% sodium azide, 2% bovine serum albumin, 5% rat serum) for 1 hour at room temperature. Lysates were transferred into strainer spin tubes (Costar Spin-X Centrifuge Tube with insert, catalog nos. 3213 and 9301, respectively) and cleared via centrifugation for 10 minutes at 13,000 rpm in an Eppendorf 5415C tabletop centrifuge. Sample lysis was done in the CICAMS laboratory. Samples were frozen after lysis and shipped to the United States and stored at −20°C or colder until used. Human papillomavirus typing of lysates and E6 pull-down and detection were done at Arbor Vita Corp.

Specimens from all women with histologically proven CIN or cervical cancer and a convenience sample of women with normal cervices who were positive on the *Digene* HC2 HPV DNA Test were chosen by personnel at PATH. These specimens were assessed for the presence of HPV-16 infection using HPV-16 E6 DNA-specific polymerase chain reaction (PCR), and positives were tested for E6 oncoprotein. Laboratory personnel at Arbor Vita Corp performing the HPV typing and the E6 pull-down were blinded to all other clinical and laboratory data including the grade of CIN but were aware of which specimens were from women without cervical neoplasia.

HPV Typing

Lysed cervical swab samples were typed for presence of HPV-16 E6 DNA via real-time PCR. A 40-μL fraction of cervical sample lysate was boiled at 95°C for 10 minutes and adjusted to 22.5 mM $MgCl_2$. Three serial dilutions (1:10) of template were subjected to real-time PCR. Human papillomavirus type 16 E6-specific primers were designed using Primer Express (version 2.0; Applied Biosystems, Foster City, CA) software and obtained from IDT DNA Corp (Coralville, IA). Real-time PCR was performed using the ABI PRISM 7000 system (Applied Biosystems, Foster City, CA) in combination with SYBR green technology according to the manufacturer's instructions.

Controls

Human papillomavirus type 16 E6-expressing CaSki cervical cancer cells were titrated and used as positive controls. The level of E6 expression was previously determined to be 1 ng/1 × 10^6 cells by growing the cells to approximately 70% confluence and establishing a correlation between cell numbers and total lysate protein quantities. Next, a titration of lysate was performed on Western blot. Using a Fuji LAS3000 camera (Fujifilm Luminescent Image Analyzer, Fujifilm Corp, Tokyo, Japan), band intensities for E6 were quantified. Recombinant HPV-16–(MBP)-E6 protein preparations of known quantities served as standards. E6 band intensities showed a linear relation to quantities of cell lysate per quantity of recombinant E6 protein. Infrequently, E6 expression diminished on overgrowing the cells or keeping the cells in culture for extended periods (>8 wk). Therefore, the cells were controlled for confluence and time in culture.

PDZ Interaction–Mediated E6 Precipitation (E6 Pull-Down) From Cervical Swab Samples

High-risk HPV E6 proteins specifically contain a PDZ domain-binding motif at the C-terminal to which the PDZ domain 1 of the cellular protein MAGI-1 binds [3, 13–15]. This interaction is the basis of an E6 pull-down approach, by which high-risk E6 proteins can be precipitated from lysed cervical swab samples using bead-bound MAGI-1 PDZ domain 1. For the E6 pull-down, recombinant MAGI-1 PDZ was coupled to Glutathione Sepharose GS4 beads (GE Healthcare, Waukesha, WI). PDZ-coupled beads were washed in 1x phosphate-buffered saline before use in pull-down. To pull down E6 protein from cervical swab sample lysates, 20 μL of PDZ-coupled GS4 beads was mixed with 300 μL of precleared lysate. The final reaction volume was brought up to 1 mL with lysis buffer. Binding occurred overnight at 4°C. Beads were washed in phosphate-buffered saline before Western blot analysis.

Western Blot

Beads recovered from the E6 pull-down were boiled for 10 minutes in reducing sample buffer (50 mM Tris

pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.145% bromphenol blue, 100 mM dithiothreitol, and 250 mM β-mercaptoethanol) before separation on a 10%/20% gradient criterion Tris-HCl polyacrylamide gel. Gel blotting onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA; pore size, 0.45 um) occurred at a 15-V constant voltage for 40 minutes under semidry conditions followed by appropriate blocking of the membrane (1× Tris-buffered saline [TBS]–0.05% Tween-20 with 1% casein and 2% albumin, overnight at 4°C).

Blocked membrane was then probed with primary antibodies against HPV-16 E6 for 2 hours at room temperature with rocking (monoclonal antibody [mAb] 4C6; licensed from University Louis Pasteur, Strasbourg, France) in 1.5 μg/mL in 1× TBS-T with 1% bovine serum albumin, followed by 4 washes before incubating with the secondary antimouse immunoglobulin G mAb (goat antimouse immunoglobulin G/horseradish peroxidase, 115-035-062; Jackson Immunoresearch, West Grove, PA) at 1:12,500 dilution in 1× TBS-T and 3% blocking solution. Incubation with secondary mAb occurred for 1 hour at room temperature followed by 6 washes. Western blot development occurred with chemiluminescent substrate (ECL Advance; GE Healthcare) according to the manufacturer's protocol. Visualization and quantification occurred via Fujifilm Luminescent Image Analyzer LAS-3000 (Fujifilm Corp, Tokyo, Japan).

Statistical Analyses

The association between the presence of E6 and cervical histology (negative, CIN 1, CIN 2, and CIN 3) was examined by calculating the odds ratio (OR) using multinomial logistic regression with a 3-category outcome of negative/CIN 1, CIN 2, and CIN 3. Potential confounders of age, years since sexual initiation, and menopausal status were examined in single-variable analysis using the Fisher exact test. None of these variables was statistically significantly associated with a category of cervical histology; therefore, the analysis of E6 and cervical histology was not adjusted. The criterion of statistical significance (α level) was set at $p < .05$ (2-sided). Analyses were conducted using Intercooled STATA (version 8.0; STATA Corp LP, College Station, TX).

RESULTS

Results are based on 2,388 (94.4%) of 2,530 women for whom all data were available (Figure 1). One hundred thirty women (5.4%) had histologic evidence of cervical neoplasia: CIN 1 in 60 (2.5%), CIN 2 in 47 (1.9%), CIN 3 in 22 (0.9%), and squamous carcinoma (cancer) in 1 (0.04%). The prevalence of carcinogenic types of HPV by HC2 testing was 16.8% (401/2,388) overall, 12.5% (282/2,258) in women with no evidence of cervical neoplasia, 85.0% (51/60) in CIN 1, 97.9% (46/47) in CIN 2, 95.5% (21/22) in CIN 3, and 100.0% (1/1) in cancer. All of the women were currently or had been married, the mean (SD) age was 43.4 (6.2) years, the mean (SD) age of first sexual intercourse was 20.5 (2.4) years, 98.7% (2,356/2,388) had never smoked, 1.1% had ever used hormonal contraceptives, 16.9% were postmenopausal (403/2,388), and the mean (SD) number of live births was 2.7 (1.1).

Human papillomavirus type 16 PCR analysis was conducted on cervical specimens from 150 women: 115 with cervical neoplasia and a convenience sample of 35 HC2-positive women from the 282 women without cervical neoplasia (Figure 1). Of these, 38 (25.3%) were positive for HPV-16 infection and had valid E6 oncoprotein results (Figure 1). Of the 115 women with CIN, 22.6% (26/115) were positive and 77.4% (89/115) were negative for HPV-16 infection. Among 35 CIN-negative women tested for HPV-16, 65.7% (23/35) were negative and 34.3% (12/35) were positive for HPV-16 infection. None of the 12 HPV-16 positive, disease-negative women was positive for E6 oncoprotein.

Table 1 shows the association of E6 oncoprotein, age, years since sexual initiation, and menopausal status among women with and without cervical neoplasia. In contrast to detectable E6 oncoprotein, menopausal status, age, and years since sexual initiation were not

Table 1. E6 Western Blot Results and Potential Confounders Compared With Clinical Diagnosis for the 38 Women With HPV-16 Infection

	Disease-negative or CIN 1, % (*n*)	CIN 2, % (*n*)	CIN 3, % (*n*)	*p* value
E6 oncoprotein				
Not detected	95.0 (19)	63.6 (7)	57.1 (4)	
Detected	5.0 (1)	36.4 (4)	42.9 (3)	.02
Age, y				
<44	60.0 (12)	63.6 (7)	28.6 (2)	
≥44	40.0 (8)	36.4 (4)	71.4 (5)	.32
Years since sexual initiation				
<20	45.0 (9)	18.2 (2)	14.3 (1)	
≥20	55.0 (11)	81.8 (9)	85.7 (6)	.23
Menopausal status				
Premenopausal	85.0 (17)	90.9 (10)	85.7 (6)	
Postmenopausal	15.0 (3)	9.1 (1)	14.3 (1)	1.00

All CIN lesions were confirmed by histology of colposcopically directed biopsies and women with negative findings were confirmed by colposcopy with biopsy, as required. Women with disease-negative status and those with CIN 1 are combined into 1 category for analysis.

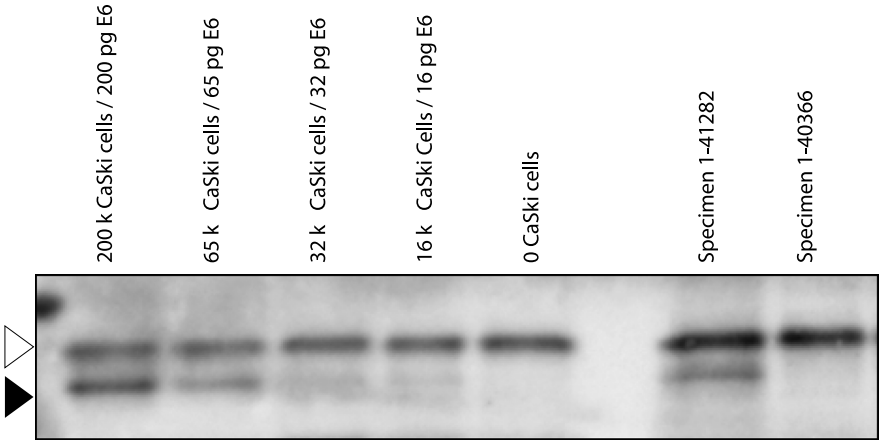

Figure 2. Detection of HPV-16 E6 oncoprotein from clinical cervical swab specimen via Western blot. Human papillomavirus type 16 E6 protein of HPV-16 E6 DNA–positive clinical cervical swab specimen and CaSki HPV-16 cervical cancer cell lines was ''pulled down'' via bead-bound GST-MAGI-1 PDZ domain 1 and detected via an anti–HPV-16 E6 mAb and Western blot (filled triangle); the band above the E6-specific band (open triangle) represents irrelevant cross-reactivity. Titration of CaSki cells allows quantification of E6 present in clinical samples, as the E6 content of CaSki cells was previously determined at 1 ng E6/1 $\times$ 10^6 cells. Half of the total clinical cervical swab samples were analyzed in this experiment. Specimen 1-41282 was diagnosed CIN 3, specimen 1-40366 was diagnosed CIN 2. See text for details.

statistically significantly associated with CIN 2 or 3. In multinomial logistic regression analysis, using women with negative findings and those with CIN 1 as the reference category, E6 oncoprotein has a borderline association with CIN 2 (OR = 10.9, 95% confidence interval = 1.03–114.58, p = .05) and a significant association with CIN 3 (OR = 14.3, 95% confidence interval = 1.16–174.80, p = .04). A nonparametric test for trend is significant (z = 2.58, p = .01) when the 0% (0/12), 12.5% (1/8), 36.4% (4/11), and 42.9% (3/7) prevalences of E6 oncoprotein are compared among women with negative findings, CIN 1, 2, and 3, respectively.

Figure 2 illustrates the detection of E6 oncoprotein from samples via E6 pull-down followed by Western blot assay. Comparison of E6 oncoprotein-specific signal intensity with positive control signals demonstrated a limit of detection of approximately 30 pg of E6 using this experimental system; if HPV-16 E6 oncoprotein was detected in clinical specimens using the E6 pull-down method, HPV-16 E6 oncoprotein levels were at approximately 50 pg/specimen or higher, as determined via signal intensity comparison from clinical specimens (e.g., see specimen no. 1-41282 in Figure 2) with positive control titration of HPV-16 E6 oncoprotein from CaSki cells.

DISCUSSION

Our approach to detect HPV-E6 oncoprotein takes advantage of the specific molecular interaction between oncogenic E6 and cellular PDZ domains, in combination with the availability of novel, high-affinity anti–HPV-E6 mAbs. This is the first detection of HPV-16 E6 oncoprotein from clinical samples. Results from this study demonstrate that the expression of E6 oncoprotein is associated with the degree of neoplastic change seen in the cervical histology of women being screened for cervical cancer for the first time. Among women with HPV-16 infection, HPV-16 E6 protein was not detectable in those without cervical neoplasia, with a significant trend toward increasing prevalence of 12.5%, 36.4%, and 42.9% among those with CIN 1, 2, and 3 lesions, respectively. To our knowledge, this is the first prospective study of the molecular epidemiology of E6 protein, and the results are consistent with what others have suggested about the role of E6 in the initiation and maintenance of epithelial cell transformation in vivo [6, 16–18]. Using immunohistochemical staining methods for E6 protein in a case-control study, Pillai et al. [19] showed that among HPV-16–infected women, histologically normal cervical tissues were negative for E6, but E6 was found in 6 (37.5%) of 16 low-grade lesions. Among high-grade cervical lesions that were positive for HPV-16 or HPV-18, 52 (76.4%) of 68 specimens were positive for E6 and 164 (87.2%) of 188 cervical cancer specimens were positive. The 1 woman in this study with cervical cancer was negative for HPV-16 infection and was not tested for E6 protein. Other studies have used a commercially available assay for E6/E7 mRNA detection [7, 8, 20]. Castle et al. found that E6/E7 mRNA was detected in 34% of women with

normal cervices and 60%, 86%, 94%, and 100% of those with histologic evidence of CIN 1, 2, 3, and cancer, respectively. Association of E6/E7 mRNA as determined in the previously mentioned studies and of HPV-16 E6 oncoprotein with cervical cancer and precancer, as reported in this study, are congruent; however, even in CIN 3, the HPV-16 E6 oncoprotein was found in less than half of the specimens, using the E6 pull-down method with a level of detection threshold of approximately 30 pg of HPV-16 E6 oncoprotein. It is possible that more than the detected 43% of the CIN 3 specimens had elevated HPV-16 E6 oncoprotein expression, but that levels were underneath the approximately 30-pg level of detection. Under this scenario, a more sensitive E6 oncoprotein assay might have likely resulted in a rate higher than 43 % for HPV-16 E6 oncoprotein in CIN 3. It is plausible that E6/E7 mRNA expression is less discriminative than E6 oncoprotein levels for disease state and risk of progression than E6 oncoprotein, depending on the chosen signal cutoff value for the E6/E7 mRNA test. Presumably, progression of neoplastic lesions to more severe states requires certain quantities of E6 and E7 oncoproteins, which are associated with certain levels of corresponding E6/E7 mRNA levels. No elevated HPV-16 E6 oncoprotein levels were detected in healthy women in this study, whereas 34% of healthy women were reported to express E6/E7 mRNA [7]. This discrepancy may be due to the detection of E6/E7 basic mRNA expression levels. E6/E7 basic mRNA expression may not be sufficient to result in the elevated E6 oncoprotein levels required for disease progression. Future studies will be necessary to address the question of whether detection of elevated E6 oncoprotein levels is a better predictor of risk of progression than E6/E7 mRNA measurements.

Given the absence of HPV-16 E6 protein detection in normal cervices infected with HPV-16 and the increasing proportions of HPV-16 E6 protein–positive women in each of the 3 HPV-16–infected groups with CIN 1, 2, and 3, the presence of E6 protein may reflect a progression of CIN, similar to that reported in natural history studies [21–23]. On the other hand, one must be cautious in drawing parallels between our prevalences of the E6 biomarker and the risk of progression derived from natural history studies because the current study is not longitudinal. In addition, women with several types of HPV, in addition to HPV-16, are included in natural history studies, and consequently, such studies would tend to underestimate the risk for women with HPV-16 infection [24].

Our study design and conduct were methodologically rigorous and, to the extent possible, adhered to current recommendations on how to evaluate a new screening test [25]. To increase the external validity of the findings, recruitment was in an unscreened population and included women who were typical of those on whom a suitable E6 test eventually would be used. Expectation bias could have been introduced because the personnel conducting the HPV-16 typing and Western blots were aware of whether specimens were from women in a group with or without cervical neoplasia but otherwise were blinded to the clinical data. To reduce verification bias, each participant had a colposcopic examination with directed biopsy and endocervical curettage as required. Those who had been negative on colposcopy but positive on HPV testing, LBC, or the *Digene* HC2 HPV DNA Test were recalled for 4-quadrant cervical biopsies and endocervical curettage to maximize ascertainment of disease. Pathology was assessed by independent observers to reach a consensus on categorizing abnormal specimens and to minimize misclassification bias. In view of small numbers, it seemed reasonable to combine the results of women without cervical neoplasia and those with CIN 1 into 1 category because their natural history is similar [26].

These findings support the idea that E6 oncoprotein could be a useful biomarker for a cervical cancer and precancer diagnostic test. A diagnostic test that detects E6 has potential utility for triaging women with positive HPV results. Specifically, women who are positive for HPV with normal histological finding or CIN 1 who would not normally be treated under current treatment algorithms would be tested for the presence of E6 protein. The women who are positive for E6 protein can receive treatment or can be monitored more frequently for disease progression. Additional studies are necessary to determine the analytical sensitivity required for such a test to be suitable for primary screening.

REFERENCES

1. Klaes R, Woerner SM, Ridder R, Wentzensen N, Durst M, Schneider A, et al. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res* 1999;59:6132–6.

2. Münger K. The role of human papillomaviruses in human cancers. *Front Biosci* 2002;7:641–9.

3. Nguyen ML, Nguyen MM, Lee D, Griep AE, Lambert PF. The PDZ ligand domain of the human papillomavirus type 16

E6 protein is required for E6's induction of epithelial hyperplasia in vivo. *J Virol* 2003;77:6957–64.

4. Shai A, Brake T, Somoza C, Lambert PF. The human papillomavirus E6 oncogene dysregulates the cell cycle and contributes to cervical carcinogenesis through two independent activities. *Cancer Res* 2007;67:1626–35.

5. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes degradation of p53. *Cell* 1990;63:1129–36.

6. Münger K, Baldwin A, Edwards K, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus–induced oncogenesis. *J Virol* 2004;78:11451–60.

7. Castle PE, Dockter J, Giachetti C, Garcia FAR, McCormick MK, Mitchell AL, et al. A cross-sectional study of a prototype carcinogenic human papillomavirus E6/E7 messenger RNA assay for detection of cervical precancer and cancer. *Clin Cancer Res* 2007;13:2599–605.

8. Molden T, Nygård JF, Kraus I, Karlsen F, Nygård M, Skare GB, et al. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV proofer and consensus PCR: a 2-year follow-up on women with ASCUS or LSIL Pap smear. *Int J Cancer* 2005;114:973–6.

9. Peck RB, Weigl BH, Jeronimo JA, Sellors JW. Human papillomavirus testing today and tomorrow. New assay platforms promise detection leading to treatment for women in underserviced regions. *IVD Technology* 2008;14:47.

10. Sellors JW. Towards an affordable HPV test. *HPV Today. Newsletter on Human Papillomavirus* 2006;8:4–5.

11. Gravitt PE, Coutlée F, Iftner T, Sellors JW, Quint WGV, Wheeler CM. New technologies in cervical cancer screening. *Vaccine* 2008;26(suppl 10):K42–52.

12. Qiao YL, Sellors JW, Eder PS, Bao YP, Lim JM, Zhao FH, et al. A new HPV-DNA test for cervical cancer screening in developing regions: a cross-sectional study of clinical accuracy in rural China. *Lancet Oncol* 2008;9:929–36.

13. Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T, Ishibashi M. Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the *Drosophila* discs large tumor suppressor protein. *Proc Natl Acad Sci U S A* 1997;94:11612–6.

14. Thomas M, Glaunsinger B, Pim D, Javier R, Banks L. HPV E6 and MAGUK protein interactions: determination of the molecular basis for specific protein recognition and degradation. *Oncogene* 2001;20:5431–9.

15. Schweizer J, Somoza C, Belmares M, Chen C, Carrick D, Khanna R, et al. Development of a diagnostic assay based on binding of high risk HPV-E6 oncoproteins to PDZ. Presented at: 22nd International Papillomavirus Conference 2005, Vancouver, Canada, April 30 to May 6, 2005. Abstract (poster no. 482).

16. Duensing S, Münger K. Mechanisms of genomic instability in human cancer: insights from studies with human papillomavirus oncoproteins. *Int J Cancer* 2004;109:157–62.

17. Lee C, Laimins LA. Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. *J Virol* 2004;78:12366–77.

18. Yim EK, Park JS. Biomarkers in cervical cancer. *Biomark Insights* 2007;1:215–25.

19. Pillai MR, Lakshmi S, Sreekala S, Devi TG, Jayaprakash PG, Rajalakshmi TN, et al. High-risk human papillomavirus infection and E6 protein expression in lesions of the uterine cervix. *Pathobiology* 1998;66:240–6.

20. Kraus I, Molden T, Ernø LE, Skomedal H, Karlsen F, Hagmar B. Human papillomavirus oncogenic expression in the dysplastic portio; an investigation of biopsies from 190 cervical cones. *Br J Cancer* 2004;90:1407–13.

21. Ostor AG. Natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol* 1993;12:186–92.

22. Melnikow J, Nuovo J, Willan AR, Chan BKS, Howell LP. Natural history of cervical squamous intraepithelial lesions: a meta-analysis. *Obstet Gynecol* 1998;92:727–35.

23. Holowaty P, Miller AB, Rohan T, To T. Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999; 91:252–8.

24. Bosch FX, Burchell AN, Schiffman M, Giuliano AR, Sanjose S, Bruni L, et al. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. *Vaccine* 2008;26(suppl 10):K1–16.

25. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al. Towards complete and accurate reporting of studies of diagnostic accuracy: STARD initiative. *Br Med J* 2003;326:41–4.

26. Moscicki AB, Schiffman M, Kjaer S, Villa LL. Updating the natural history of HPV and anogenital cancer. *Vaccine* 2006;24(suppl 3):S3/42–5.