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Research Article

Cancer Epidemiology, Biomarkers & Prevention

# Elevated Expression of Human Papillomavirus-16/18 E6 Oncoprotein Associates with Persistence of Viral Infection: A 3-Year Prospective Study in China

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## Abstract

**Background:** An association between high-risk human papillomavirus (HR-HPV) oncoprotein expression and viral persistence has been suggested by the outcome of etiology studies, but there are no epidemiologic studies evaluating that link.

**Methods:** We performed a 3-year prospective study in which 2,498 Chinese women ages 25 to 65 years were screened by six screening tests, including the Onco*E6* Cervical Test (Arbor Vita Corporation) in 2011 (baseline). Six-hundred and ninety women who were positive for any of the tests and a random sample of 164 women with all negative results received colposcopy, and cervical specimens for the cobas 4800 HPV test ("cobas," Roche Molecular Systems) were collected before colposcopy; of this group, 737 cervical specimens were collected to perform cobas and Onco*E6* Cervical Test in 2014 (follow-up). Twenty-four cases of HPV16/18 E6 positives and 204 selected controls at baseline, 13 cases of HPV16/18 E6 positive and another 204 selected controls at follow-up were analyzed separately using unconditional logistical regression models to estimate ORs and 95% confidence intervals (CI).

**Results:** Compared with women who were HPV16 E6 oncoprotein negative at baseline, women in the E6-positive group had a much higher risk of HPV persistence (adjusted OR, 54.64; 95% CI, 7.19–415.09) at 3-year follow-up; a statistically strong association was also found between HPV16/18 HPV persistence and E6 oncoprotein expression detected at follow-up (adjusted OR, 360.57; 95% CI, 28.30–4,593.55).

**Conclusions:** A single detection of HPV16/18 E6 oncoprotein expression was strongly associated with viral persistence.

**Impact:** HPV16/18 E6 oncoprotein constitutes a marker for risk of HPV persistence. *Cancer Epidemiol Biomarkers Prev; 25(7); 1167–74.* ©*2016 AACR.*

## Introduction

The implementation of organized screening programs has led to substantial reductions in cervical cancer incidence and mortality in developed countries. In China, however, cervical cancer screening is scarce, and therefore the burden of cervical cancer remains high, with an estimated 98,900 new cases and 30,500 deaths in 2015 (1). High-risk human papillomavirus (HR-HPV) infection is necessary for the development of invasive cervical cancer (ICC; ref. 2), especially HPV types 16 and 18 (3), which accounts for roughly 70% to 80% of all cervical cancer (4, 5). The establishment of an etiologic association between HPV and ICC drove the development of DNA-based tests for detection of HR-HPV; HPV testing is now being considered as a replacement for the Pap test in primary screening settings (6, 7). Although the prevalence of HR-HPV infections among women is very high in China [about 17% in rural China; ref. 8], most of these infections are transient and only a small proportion will progress and develop to ICC; persistent HR-HPV infection has been shown to constitute the single most important risk factor for development of cervical precancers—cervical intraepithelial neoplasia (CIN) grade 2 and 3 (9, 10). A single HPV test, however, cannot distinguish between transient and persistent infection, and it appears thus as an important clinical priority to identify clinical tests and markers that allow to better evaluate whether or not a given HPV infection detected at any time has a higher likelihood of being persistent. Several tests developed recently may aid risk stratification once high-risk HPV infection has been identified. Detection of viral oncoprotein mRNA precursors could describe enhanced risk for transforming infections, but outcome from several studies suggests that E6/E7 mRNA markers have characteristics similar to tests that detect presence of HPV by its DNA (11, 12); changed levels of cellular proteins implied in pathways affected by oncogenic transformation may be useful markers; examples are topoisomerase IIA (TOP2A) and minichromosome maintenance proteins (MCM) as well as p16INK4A kinase and Ki-67 nuclear antigen (13, 14). These markers may prove informative with regard to transforming HPV infections. These methods, however, either require biopsies (for histology) to be taken, which may result in physical and psychologic burden to the patient, or they associate with challenges characteristic for cytology, like subjectivity in interpretation and infrastructure requirements. Changes

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in epigenetic signatures (DNA methylation and histone acetylation) may be informative but tests are still in the development status. Noteworthy, none of the above risk stratification options directly detect changes in the levels of the E6 oncoprotein; E6 is an undisputed root cause for oncogenic transformation; this, and the fact that the E6-based test is commercially available prompted our choice to evaluate the association of E6 oncoprotein with HPV persistence.

It had been suggested but not yet been demonstrated, that the HPV E6 and E7 oncoproteins could have a role in rendering a transitory HPV infection into a persistent one (15). HPV16/18 E6 and E7 are coordinately expressed (translated from a polycistronic pre-mRNA) oncoproteins, and expression of both E6 and E7 oncoprotein at elevated levels is required for initiation and maintenance of cervical epithelial cell transformation. Although expressed at minute levels in most HPV infections, certain molecular events (for example loss or mutation of the viral E2 gene product) can result in elevated expression of E6 and E7 oncoproteins. Both E6 and E7 are multifunctional oncoproteins that interfere with numerous cellular pathways and altogether drive cellular transformation (16). Chiefly, the E6 oncoprotein acts antiapoptotic, for example, by driving p53 tumor suppressor into the ubiquitin-dependent degradation pathway, whereas the E7 oncoprotein promotes proliferation, for example, by inactivating pRB (15, 17). Several known oncogenic activities of E6 and E7 oncoproteins could potentially also drive HPV persistence, but no study has directly demonstrated such an association. Here, we used the Onco*E6* Cervical test (Arbor Vita Corporation) in a clinical study cohort in China to demonstrate an association between presence of HPV16/18 E6 oncoprotein and HPV-persistent infection.

## Materials and Methods

### Study population

In 2011, 2,498 women ages 25 to 65 years living in Xinmi County were enrolled in a study named "Screening Technologies to Advance Rapid Testing for Cervical Cancer Prevention–Utility and Program Planning (START-UP)" Project. Women who were not pregnant, had a cervix, had not been previously diagnosed with cervical cancer, were physically able to undergo routine cervical cancer screening and were able to provide informed consent were eligible for this study. Details on participants' recruitment have been published elsewhere (18, 19). This study was registered with the U.S. National Institutes of Health and assigned the clinicaltrials.gov identifier: NCT01231945.The baseline study was approved by the institutional review boards (IRB) of the Cancer Institute/Hospital, Chinese Academy of Medical Sciences (CICAMS), of PATH (Program for Appropriate Technology in Health), and of the US National Cancer Institute; the follow-up study was approved by the IRB of CICAMS.

### Study description

Under START-UP, women were screened by 6 different screening tests in 2011: Onco*E6* Cervical Test on a clinician-collected (cc) specimen, HC2 (QIAGEN), and *care*HPV testing (QIAGEN), both on a second cc and self-collected (sc) specimens, and visual inspection with acetic acid (VIA). One month later, women who tested positive for any of the 6 screening tests and an approximately 9.8% of randomly selected women who tested negative for all 6 tests (screen-negative women) were referred to colposcopy using a biopsy protocol as previously described (20). Meanwhile, cervical specimens were recollected by physicians using a cervical brush and specimens were stored in the PreservCyt liquid cytology collection media (Cytyc Corporation); on these specimens, the cobas 4800 HPV test (Roche Molecular Systems Inc.) was performed. The referral population, except women who were histologically diagnosed as CIN2/3 and who received treatment at baseline as well as women who were out of contact, was followed and retested in 2014 by liquid-based cytology, cobas 4800 HPV test (to assess status of HPV infection) and by the Onco*E6* Cervical test (to assess status of HPV16/18 E6 expression). Women who tested positive for any of the three tests underwent colposcopic evaluation and biopsy. The association between HPV16/18 E6 oncoprotein expression and HPV persistence as detected by presence of HPV DNA was inspected from two angles: a prospective cohort design was used to (i) evaluate the risk of HPV 16/18–persistent infection in 3 years for women who were E6 oncoprotein positive at baseline; a retrospective cohort design was used to (ii) evaluate whether HPV16/18 E6 oncoprotein expression at follow-up was associated with existing persistent infection; cases and controls selection was described in details below.

### Analytic subject selection

HPV16/18 persistence was defined as HPV16/18 positive both in 2011 and in 2014. For the prospective cohort design ("analytic group 1"), women who were HPV16/18 E6 oncoprotein positive (24 subjects) at baseline were included as case group; the control group was selected according to the following criteria for comparability purposes: women who tested negative for all 6 screening tests (149 subjects) and 10% of women who tested negative for HPV E6 oncoprotein, but positive for any of the other 5 tests (55 subjects) at baseline; for the retrospective cohort design ("analytic group 2"), women who were HPV16/18 E6 oncoprotein positive (13 subjects) at follow-up were included as case group; women who tested negative for all 6 screening tests (149 subjects) at baseline and 10% of women who tested negative for HPV E6 at follow-up, but positive for any of the other 5 tests at baseline (55 subjects), were also included.

### Laboratory tests

HC2 and *care*HPV were used to test for presence of HPV via detection of viral DNA at baseline; both methods apply signal amplification, thus requiring a defined cutoff point. The commercially used cutoff point of 1.0 RLU/CO (approximately equal to 1.0 pg of DNA/ml) was used as positive test outcome for both tests, and positives were referred to colposcopy.

The cobas 4800 HPV test is a fully automated PCR assay for the detection of viral DNA of 14 high-risk HPV types; this test simultaneously also differentiates HPV genotypes 16 and 18; the cobas 4800 HPV test was performed according to the recommendations of the manufacturer.

The Onco*E6* Cervical Test is an immunochromatographic test using a lateral flow format; HPV 16 and HPV 18 E6 oncoproteins are captured by monoclonal antibodies (mAb) immobilized on the porous membrane of the lateral flow strip, on distinct test lines for the respective HPV types. This test was designed for the detection of HPV16/18/45 E6 oncoprotein previously, and the baseline results of Onco*E6* Cervical Test consisted of these three HPV genotypes. In 2014, the manufacturer changed its protocol and removed HPV45 E6 oncoprotein detection from the test, so

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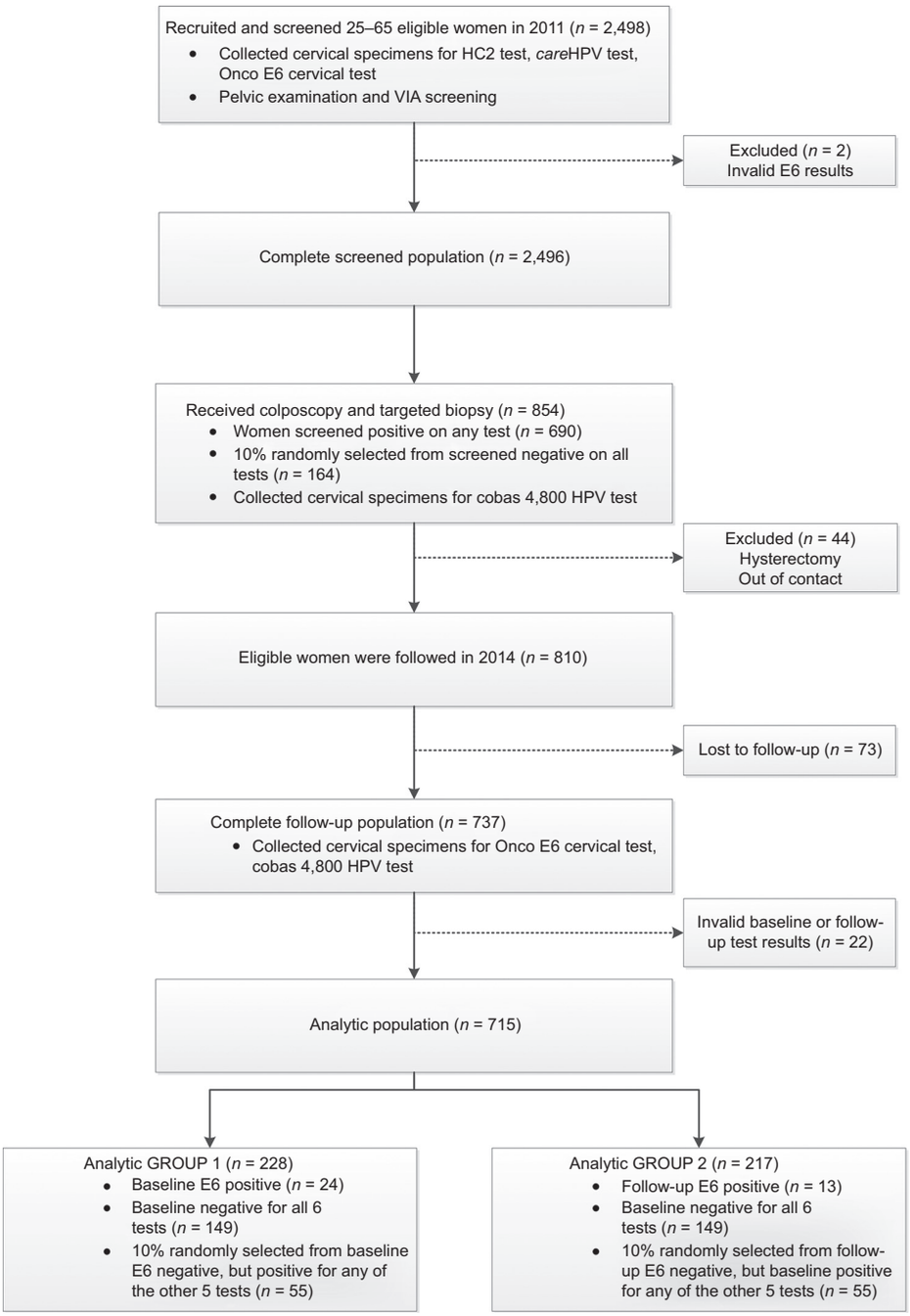


**Figure 1.**
Flow diagram showing procedures of the study.

the follow up results of this test only consisted of detection of E6 oncoproteins of HPV genotypes 16 and18; the procedures are the same for both versions of the test (E6 of HPV16/18/45 vs. 16/18) and were described previously (21).

Briefly, a cervical specimen collected using a polyester swab was stored in a tube without buffer until tested. The swab specimen was treated in a two-step process, first with 933 μL of lysis solution and next with 87 μL of conditioning solution, both with 15-minute incubation under gentle agitation. Next, the specimen solution was clarified from insoluble components by centrifugation in a table-top microcentrifuge for 10 minutes at >10,000 rpm. A 200 μL aliquot of the sample solution was then transferred into a vial with lyophilized detector mAb; the test unit was next inserted into the detector mAb vials, and the specimen solutions ran up the test strips by capillary action. After 55 minutes, the test unit was transferred into vials with wash solution, and after a 12-minute wash the test unit was immersed into another set of vials containing developing solution. After 15 to 25 minutes (depending on the ambient temperature), the test unit was removed from the developing solution vials and placed onto a reading guide, allowing for visual inspection. Appearance of one or more test lines indicated elevated E6 oncoprotein of the corresponding HPV

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type present in the initial cervical swab specimen. The time from sample collection to test results is typically approximately 2.5 hours.

### Pathology

The primary histopathologic diagnosis was provided by two CICAMS pathologists after reaching agreement and the worst of the biopsies or, if available, histology from the surgical specimen was used for the final diagnosis. Additional sections of all initial biopsy diagnoses with $CIN2^+$ were cut and tested for $p16^{INK4a}$ by immunohistochemistry as previously described (22).

### Statistical analysis

We compared the means of continuous variables between cases and controls using *t* tests. Categorical variables were compared using $\chi^2$ tests. Associations between HPV E6 expression and HPV persistence were examined using unconditional logistic regression models. Variables (age at baseline, education level, parities, and menopause), associated HPV DNA persistence, and HPV E6 positivity were included as adjustment variables in unconditional logistic regression models. Statistical significance was assessed by two-tailed tests at a level of 0.05. SPSS 17.0 (SPSS, Inc.) was used to analyze the data.

## Results

The study design is shown in Fig. 1. Of note, 2,498 women were recruited in the study in 2011, of which 2,496 (99.9%) were eligible and had valid test results. Of note, 725 (29.0%) women tested positive by at least one of the screening tests and were referred to colposcopy; 1,771 (71.0%) women tested negative for all the tests, and 174 (9.8%) of those were also referred to colposcopy. Of note, 690 (95.2%) of 725 and164 (94.3%) of 174 referral women completed the colposcopy and biopsy, and a total of 854 specimens were submitted to the cobas 4800 HPV test. In 2014, 810 of 854 referral women were to be followed, 23 (2.7%) women of which 17 tested positive for HPV E6 oncoprotein were excluded due to hysterectomy, and 21 women were excluded because they were out of contact. Of note, 737 (91.0%) women completed the follow-up and 715 (97.0%) with valid baseline and follow-up test results were included for analysis.

Table 1 shows the positivity rates of HPV infection and of women with elevated E6 oncoprotein expression at baseline and at follow-up. In analytic group 1, 19 (8.3%) and 7 (3.1%) women were positive for HPV16 and HPV18 E6 at baseline, respectively. The positive rates were 10.5% and 4.4% for HPV16 DNA at baseline and follow-up, and 10 (4.4%) women had HPV16 DNA–persistent infection, 3.1% and 0.4% women tested positive for HPV18 DNA at baseline and follow-up, respectively, but no persistent infection for HPV18 DNA was found. In analytic group 2, 13 (6.0%) women were positive for HPV 16/18 E6 at follow-up. 20 (9.2%) women tested positive for HPV16 DNA, and 13 (6.0%) had persistent infections, 3 (1.4%) women tested positive for HPV18 DNA and 2 (0.9%) had persistent infection. In total, 15 (6.9%) women had HPV16/18–persistent infections.

The sociodemographics and risk factors for two analytic groups are shown in Table 2. Marital status, second-hand smoking, drinking, number of family member, lifetime sexual partners, age at first intercourse, contraception method, history of trichomonas infection/vaginomycosis/cervicitis, and family history of cervical cancer did not associate with differences between cases and controls in the two groups. HPV16-persistent infection, however, and positivity of HPV E6 oncoprotein expression varied significantly by age at baseline, educational level, parities, and menopause.

**Table 1.** Number and proportion of women who were HPV DNA/E6 positive at baseline and follow-up

| | Group 1 (*N* = 228) | | Group 2 (*N* = 217) | |
|---|---|---|---|---|
| | **Positive** | **%** | **Positive** | **%** |
| Baseline | | | | |
| HPV16 DNA | 24 | 10.5 | 20 | 9.2 |
| HPV18 DNA | 7 | 3.1 | 3 | 1.4 |
| HPV16/18 DNA | 28 | 12.3 | 22 | 10.1 |
| HPV16 E6 | 19 | 8.3 | 10 | 4.6 |
| HPV18 E6 | 7 | 3.1 | 2 | 0.9 |
| HPV16/18 E6 | 24 | 10.5 | 11 | 5.1 |
| Follow-up | | | | |
| HPV16 DNA | 10 | 4.4 | 14 | 6.5 |
| HPV18 DNA | 1 | 0.4 | 4 | 1.8 |
| HPV16/18 DNA | 11 | 4.8 | 17 | 7.8 |
| HPV16 E6 | 7 | 3.1 | 11 | 5.1 |
| HPV18 E6 | 0 | 0.0 | 4 | 1.8 |
| HPV16/18 E6 | 7 | 3.1 | 13 | 6.0 |
| HPV16 persistence | 10 | 4.4 | 13 | 6.0 |
| HPV18 persistence | 0 | 0.0 | 2 | 0.9 |
| HPV16/18 persistence | 10 | 4.4 | 15 | 6.9 |

Table 3 shows the associations between HPV16 E6 positivity at baseline and risk of HPV16 persistence in 3 years. Compared with HPV16 E6 negatives at baseline, women who were E6 positive had a much higher risk of HPV16 persistence (OR, 54.64; 95% CI, 7.19–415.09) after adjustments for age at baseline, education level, parities, menopause. As shown in Table 4, a strong association (OR, 360.57; 95% CI, 28.30–4593.55) was found between HPV16/18 persistence and E6 expression at follow-up after adjustment for age at baseline, education level, parities, and menopause.

## Discussion

The definite recognition of HPV as the etiologic agent of cervical cancer has greatly focused the interest on the development and implementation of prophylactic HPV vaccine and on HPV testing as important tools toward prevention of cervical cancer. Although HPV prophylactic vaccines are recommended by the WHO and have been approved in >140 countries, they are not yet available in China (23), underlining the importance of cervical cancer screening. The introduction of HPV DNA tests as primary screening modality is of great importance to China because cytology-based screening has been proven difficult to implement in rural areas due to its requirement for high-quality laboratory and cytotechnologist support, coupled with the need for multiple visits. 80% to 90% of women with a positive HPV DNA test, however, have transient infections and will not have concurrent disease. The challenge of using HPV testing for primary screening is thus to find complementary clinical tests and markers that can discriminate clinically relevant infection; this is of special importance when limited resources need to be prioritized for women at highest risk for harboring cancer. Previous proposals for the triage of HPV-positive women include Pap cytology, genotyping for HPV16 and HPV18, immunostaining for p16, with or without ki-67, and molecular tests assessing viral or host DNA methylation (24–26). Each method has specific limitations, including relatively low sensitivity, low PPV, complex procedure, or subjectivity

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**Table 2.** Sociodemographics and risk factors for women included in this study

| | Group 1 ($N = 228$) | | | Group 2 ($N = 217$) | | |
|---|---|---|---|---|---|---|
| | **HPV16 non-persistent ($n = 218$)** | **HPV16 persistent ($n = 10$)** | ***P*** | **HPV16/18 E6$^-$ ($n = 204$)** | **HPV16/18 E6$^+$ ($n = 13$)** | ***P*** |
| Characteristics | | | | | | |
| Age at baseline (y) | | | | | | |
| Continuous | 46.4 ± 7.5 | 55.2 ± 5.9 | ≤0.001 | 46.1 ± 7.5 | 52.2 ± 8.1 | 0.005 |
| ≤50 (%) | 154 (70.6%) | 1 (10.6%) | ≤0.001 | 151 (74.0%) | 4 (31.8%) | 0.002 |
| ≥51 (%) | 64 (29.4%) | 9 (90.0%) | | 53 (26.0%) | 9 (69.2%) | |
| Marital status | | | | | | |
| Married/cohabitation (%) | 207 (95.0%) | 10 (100.0%) | 1.000 | 197 (96.6%) | 13 (100.0%) | 1.000 |
| Divorced/widowed (%) | 11 (5.0%) | 0 (0.0%) | | 7 (3.4%) | 0 (0.0%) | |
| Education level | | | | | | |
| Primary school or below (%) | 99 (45.4%) | 8 (80.0%) | 0.049 | 88 (43.1%) | 11 (84.6%) | 0.004 |
| Middle school or above (%) | 119 (54.6%) | 2 (20.0%) | | 116 (56.9%) | 2 (15.4%) | |
| Second-hand smoking | | | | | | |
| No (%) | 79 (36.2%) | 5 (50.0%) | | 74 (36.3%) | 4 (30.8%) | 0.774 |
| Yes (%) | 139 (63.8%) | 5 (50.0%) | 0.504 | 130 (63.7%) | 9 (69.2%) | |
| Drinking | | | | | | |
| No (%) | 204 (93.6%) | 10 (100.0%) | 1.000 | 7 (3.4%) | 13 (100.0%) | 1.000 |
| Yes (%) | 14 (6.4%) | 0 (0%) | | 197 (96.6%) | 0 (0%) | |
| No. of family member | | | | | | |
| ≤4 (%) | 117 (53.7%) | 4 (40.0%) | 0.522 | 113 (55.4%) | 6 (46.2%) | 0.516 |
| ≥5 (%) | 101 (46.3%) | 6 (60.0%) | | 91 (44.6%) | 7 (53.8%) | |
| Lifetime sexual partners | | | | | | |
| 1 (%) | 204 (93.6%) | 10 (100.0%) | 1.000 | 187 (91.7%) | 12 (92.3%) | 1.000 |
| ≥2 (%) | 14 (6.4%) | 0 (0%) | | 17 (8.3%) | 1 (7.7%) | |
| Age at first intercourse (y) | | | | | | |
| ≤22 (%) | 139 (63.8%) | 7 (70.0%) | 1.000 | 129 (63.2%) | 10 (76.9%) | 0.386 |
| ≥23 (%) | 79 (36.2%) | 3 (30.0%) | | 75 (36.8%) | 3 (23.1%) | |
| Parities | | | | | | |
| ≤2 (%) | 133 (61.0%) | 2 (20.0%) | 0.017 | 121 (59.3%) | 4 (30.8%) | 0.043 |
| ≥3 (%) | 85 (39.0%) | 8 (80.0%) | | 83 (40.7%) | 9 (69.2%) | |
| Menopause | | | | | | |
| No (%) | 145 (66.5%) | 1 (10.0%) | 0.001 | 142 (69.6%) | 3 (23.1%) | 0.001 |
| Yes (%) | 73 (33.5%) | 9 (90.0%) | | 62 (30.4%) | 10 (76.9%) | |
| Contraception method | | | | | | |
| No (%) | 8(3.7%) | 0(0%) | 1.000 | 4 (2.0%) | 0 (0%) | 1.000 |
| Yes (%) | 210 (96.3%) | 10 (100.0%) | | 200 (98.0%) | 13 (100.0%) | |
| History of trichomonas infection | | | | | | |
| No (%) | 183 (85.5%) | 9 (90.0%) | 1.000 | 172 (85.6%) | 11 (84.6%) | 1.000 |
| Yes (%) | 31 (14.5%) | 1 (10.0%) | | 29 (14.4%) | 2 (15.4%) | |
| History of vaginomycosis | | | | | | |
| No (%) | 182 (85.0%) | 9 (90.0%) | 1.000 | 171 (85.5%) | 12 (92.3%) | 0.699 |
| Yes (%) | 32 (15.0%) | 1 (10.0%) | | 29 (14.5%) | 1 (7.7%) | |
| History of cervicitis | | | | | | |
| No (%) | 145 (67.4%) | 9 (90.0%) | 0.177 | 141 (69.8%) | 10 (83.3%) | 0.516 |
| Yes (%) | 70 (32.6%) | 1 (1.0%) | | 61 (30.2%) | 2 (16.7%) | |
| Family history of cervical cancer | | | | | | |
| No (%) | 214 (98.2%) | 9 (90.0%) | 0.203 | 195 (95.6%) | 13 (100.0%) | 1.000 |
| Yes (%) | 4 (1.8%) | 1 (10.0%) | | 9 (4.4%) | 0 (0.0%) | |
| Baseline HPV 16 E6 | | | | | | |
| Negative | 207 (95.0%) | 2 (20.0%) | ≤0.001 | — | — | — |
| Positive | 11 (5.0%) | 8 (80.0%) | | — | — | |
| HPV 16/18 persistent infection | | | | | | |
| No (%) | — | — | — | 200 (98.0%) | 2 (15.4%) | ≤0.001 |
| Yes (%) | — | — | | 4 (2.0%) | 11 (84.6%) | |

in interpretation. It has been shown that persistence of HR-HPV infection is a major risk factor for development of transforming activities; thus, assessment of the risk of a HR-HPV infection to be a transforming one consist in the determination of HPV persistence. It has been suggested, that expression of viral oncoproteins E6 and E7may also play a role for HPV persistence, but no studies have been presented to date to demonstrate this.

Here, we present the first prospective study testing for an association between elevated HPV16/18 E6 oncoprotein expression and HPV persistence. Under the START-UP clinical study, we followed women over a 3-year time period for HPV 16/18 status and expression of HPV 16/18 E6 oncoprotein expression. For increased validity, we analyzed data in two distinct approaches: applying a prospective approach, we determined the predictive value of HPV16/18 E6 oncoprotein expression at baseline for HPV persistence; in a retrospective approach, we investigated the association between HPV16/18 HPV persistence and E6 oncoprotein positivity at 3 year follow-up. Furthermore, we selected controls based on equal principles in both approaches to improve study efficiency.

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**Table 3.** Crude and adjusted OR and 95% CI for the associations between HPV16 E6 positive at baseline and DNA persistence in 3 years

| HPV16 E6 | Subjects in analysis | Crude OR (95% CI) | Adjusted[a] OR (95% CI) |
|---|---|---|---|
| Negative | 209 | — (—) | — (—) |
| Positive | 19 | 75.27 (14.26–297.45) | 54.64 (7.19–415.09) |

[a]Adjusted for age at baseline, education level, parities, and menopause.

We observed that HPV16 E6 oncoprotein expression was strongly associated with a high risk of DNA persistence; compared with the HPV16 E6 oncoprotein negative group; subjects in the positive group had a much higher risk of HPV16 HPV persistence (OR, 54.64; 95% CI, 7.19–415.09). We also found that HPV16/18 DNA persistence was strongly associated with E6 oncoprotein expression at follow-up, with an OR of 360.57 (95% CI, 28.30–4,593.55).

The multifunctional activities of HR-HPV E6 and E7 oncoproteins have been subject to extensive research throughout the past decades, and it has been suggested that some of these activities may act in favor of HPV persistence; examples of E6/E7 oncoprotein functions that may promote HPV persistence are: modulation of cell-cycle regulators to maintain long-term replication (27–29), repression of the function of immune system (15, 30, 31), degradation of p53 tumor-suppressor protein (16, 32), telomerase activation (33) and enhanced genomic instability, which could result in stabilization of the viral genome by genomic integration. Although it remains subject to further elucidation if and how these activities contribute to HPV persistence, it is of clinical relevance to establish the association between HPV persistence and presence of elevated expression of the E6 oncoprotein of HPV types 16 and 18. Considering that HPV infection is a prerequisite for expression of HPV E6 oncoprotein, the outcome of this study suggests that detection of elevated E6 oncoprotein at a single time point is an indicator for a high risk of persistent HPV infection in the past and in the future; consequently, detection of HPV E6 oncoprotein will allow to identify those women who have no visible disease (< CIN2), but who may be at elevated risk for future disease.

Potential risk factors for HPV persistence that were determined previously in various studies are age, age at first intercourse, number of sex partners (lifetime and recent), smoking, contraception methods, chlamydia and herpes simplex virus infection, chronic inflammation, immunosuppressive conditions, and menopause (34–43). Results from these studies, however, were inconsistent partly because different populations had been studied. In our study, age at baseline, educational level, parities, and menopause varied significantly in both analytic groups; however, these factors were adjusted in regression models.

Although this is the first study demonstrating a strong association between HPV-persistent infection and E6 oncoprotein elevated expression, potential limitations of this study should also be considered. First, the Onco*E6* Cervical Test only targets HPV16 and 18 infections, which restricts the study of correlation to these two types. Second, HPV-persistent and E6-positive cases were limited in numbers. There was no HPV18-persistent subjects in analytic group 1, this can be accounted to the low positivity rate of E6 oncoprotein in the screening population (18); Next, women who screened positive for any of the 6 screening tests and showed high-grade disease based on histology outcome received treatment at baseline and were not followed further. Moreover, a limited number of $CIN2^+$ cases presented at follow-up (only 1 and 4 cases of $CIN2^+$ in analytical groups 1 and 2, respectively), and we thus abstained from evaluating the clinical performance of the Onco*E6* Cervical Test for detection of high-grade lesions in this study population. As presented above, however, a correlation between elevated E6 oncoprotein expression and HPV persistence was detected even in the absence of cervical high-grade disease. We consequently conclude that elevated E6 oncoprotein describes a "true" risk factor for HPV persistence and therefore a risk for future high-grade disease, rather than a manifestation of existing high-grade disease, which could present a bias for HPV persistence (we assumed with confidence that there is virtually no cervical cancer without expression of the viral oncoproteins). In the future, a larger study using disease ($CIN2^+$) endpoints may be warranted. Another potential limitation consists in the definition of HPV persistence used in our study; there is variation in definition of HPV persistence in the literature (9, 44–46), but repeat HR-HPV testing at 12 month is recommended in a meta-analysis (47), whereas the time interval between the two HPV tests in our study was 3 years; consequently, we cannot determine whether all cases of infection detected at follow-up were persistent infections or reinfection, and the conclusions may therefore have some bias. Finally, the Onco*E6* Cervical Test is qualitative, not quantitative. Consequently, we could not elucidate how E6 protein expression at levels below the test's limit of detection (LoD) relates to HPV persistence; there is no other E6 oncoprotein test that can surpass the currently achieved LoD. We recognize that the here described correlation between HPV persistence and E6 oncoprotein may thus depend in part on the current test's LoD ("cutoff"); however, we deem that the outcome of this study has clinical relevance for field use of the Onco*E6* Cervical Test, because a single positive test results describes a risk of persistence, and thus may facilitate clinical management.

**Table 4.** Crude and adjusted OR and 95% CI for the associations between HPV16/18 DNA persistence and E6 expression in 3 years

| HPV16/18 | Subjects in analysis | Crude OR (95% CI) | Adjusted[a] OR (95% CI) |
|---|---|---|---|
| Non-persistent infection | 202 | — (—) | — (—) |
| Persistent infection | 15 | 275 (45.34–1,668.06) | 360.57 (28.30–4,593.55) |

[a]Adjusted for age at baseline, education level, parities, and menopause.

In conclusion, our study provides the first prospective evaluation of an association between elevated HPV E6 oncoprotein expression and HPV persistence; we observed that women who were HPV E6 oncoprotein positive had a much higher risk of viral persistence in 3 years, and there was a strong association between HPV16/18 persistence and E6 expression. This association suggests that women who test HPV16/18 E6 oncoprotein positive in the absence of visible disease are at a higher risk for developing high grade disease in the future, and a single detection HPV16/18 E6 oncoprotein expression may predict persistence of HPV16/18 infection. The outcome of this study suggests that more extensive studies to evaluate the Onco*E6* Cervical Test as a tool for stratification of risk of cervical malignancies are warranted.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** W. Chen, Y.-L. Qiao
**Development of methodology:** W. Chen
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L.-L. Yu, L.-N. Kang, F.-H. Zhao, X.-Q. Lei, Y. Qin, Z.-N Wu, H. Wang
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L.-L. Yu, L.-N. Kang
**Writing, review, and/or revision of the manuscript:** L.-L. Yu, L.-N. Kang, W. Chen
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** L.-L. Yu, X.-Q. Lei, Y. Qin, Z.-N Wu, H. Wang
**Study supervision:** L.-N. Kang, W. Chen, Y.-L. Qiao

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