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

Lu-Lu Yu¹, Le-Ni Kang², Fang-Hui Zhao¹, Xiao-Qin Lei¹, Yu Qin¹, Ze-Ni Wu¹, Hong Wang³, Wen Chen¹, and You-Lin Qiao¹

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 OncoE6 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 OncoE6 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.


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 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 II A (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 acetyla-
tion) 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

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 molec-
ular events (for example loss or mutation of the viral E2 gene
product) can result in elevated expression of E6 and E7 onco-
proteins. 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 OncoE6 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-persis-
tent 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
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 base-
line 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 Technol-
ogy 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 screen-
ing tests in 2011: OncoE6 Cervical Test on a clinician-collected
(cc) specimen, HC2 (Qiagen), and careHPV 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 approxi-
ately 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 (Cytec Corporation); on these specimens, the
cobas 4800 HPV test (Roche Molecular Systems Inc.) was
performed. The referral population, except women who were
historically 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
OncoE6 Cervical test (to assess status of HPV16/18 E6 expres-
sion). 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 persist-
ence as detected by presence of HPV DNA was inspected from two
angles: a prospective cohort design was used to (i) evaluate the
risk of HPV16/18 persistence 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 com-
parability purposes: women who tested negative for all 6 screen-
ing 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 oncopro-
tein 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 careHPV 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 com-
mercially 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 recommen-
dations of the manufacturer.

The OncoE6 Cervical Test is an immunochromatographic test
using a lateral flow format; HPV 16 and HPV 18 E6 oncoproteins
are captured by monoclonal antibodies (mAbs) 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 OncoE6 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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the follow up results of this test only consisted of detection of E6 oncoproteins of HPV genotypes 16 and 18; 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 strain.

Figure 1.
Flow diagram showing procedures of the study.
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 p16INK4a 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 (7.8%) of those were also referred to colposcopy. Of note, 690 (95.2%) of 725 and 164 (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 test results. Of note, 725 (91.0%) of 725 and 164 (94.3%) of 174 referral women 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 HPV E6 expression. The positive rates were 10.5% and 4.4% for HPV16 DNA at baseline and follow-up, respectively, but no persistent infection for HPV18 DNA was found. In analytic group 1, 19 (8.3%) and 7 (3.1%) women were positive for HPV16/18 DNA at baseline and follow-up, respectively, but no persistent infection was found. In analytic group 2, 13 (6.0%) women were positive for HPV16/18 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 trichomoniasis infection/vaginamysis/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 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 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...
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 E7 may 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. To increase 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.

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group 1 (N = 228)</th>
<th>Group 2 (N = 217)</th>
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<td>HPV16 persistent</td>
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<td><strong>(n = 218)</strong></td>
<td><strong>(n = 10)</strong></td>
<td><strong>(n = 204)</strong></td>
<td><strong>(n = 13)</strong></td>
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<td>Divorced/widowed (%)</td>
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<tr>
<td>Education level</td>
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<td>≥23 (%)</td>
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<td>No (%)</td>
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<td>—</td>
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<tr>
<td>Yes (%)</td>
<td>—</td>
<td>200 (98.0%)</td>
<td>2 (15.4%) &lt;0.001</td>
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Table 2. Sociodemographics and risk factors for women included in this study.
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 OncoE6 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 OncoE6 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 OncoE6 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 (“cut-off”); however, we deem that the outcome of this study has clinical relevance for field use of the OncoE6 Cervical Test, because a single positive test result at 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 OncoE6 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 (“cut-off”); however, we deem that the outcome of this study has clinical relevance for field use of the OncoE6 Cervical Test, because a single positive test result at 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.

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 OncoE6 Cervical Test as a tool for stratification of risk of cervical malignancies are warranted.

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

<table>
<thead>
<tr>
<th>HPV16 E6</th>
<th>Subjects in analysis</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted a OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>209</td>
<td>− (−)</td>
<td>− (−)</td>
</tr>
<tr>
<td>Positive</td>
<td>19</td>
<td>75.27 (14.26–297.45)</td>
<td>54.64 (7.19–415.09)</td>
</tr>
</tbody>
</table>

aAdjusted for age at baseline, education level, parities, and menopause.

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

<table>
<thead>
<tr>
<th>HPV16/18</th>
<th>Subjects in analysis</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted a OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-persistent infection</td>
<td>202</td>
<td>− (−)</td>
<td>− (−)</td>
</tr>
<tr>
<td>Persistent infection</td>
<td>15</td>
<td>275 (45.34–1668.06)</td>
<td>360.57 (28.30–4,593.55)</td>
</tr>
</tbody>
</table>

aAdjusted for age at baseline, education level, parities, and menopause.

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**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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