

Human Papillomavirus (HPV) 16/18 E6 Oncoprotein Expression in Infections with Single and Multiple Genotypes



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Abstract

Factors that differentiate risk of cervical cancer associated with infection with single versus multiple HPV types are yet undefined. We hypothesize that E6 oncoprotein is one determining factor. This cross-sectional, multicenter study was performed between 2013 and 2017. A total of 1,781 women were recruited from six hospitals. Samples were tested for presence of 14 types of high-risk HPV DNA. HPV16/18-positive samples were also tested for HPV16/18-E6 oncoprotein. Of 1,781 subjects, 687 (38.6%) tested positive for HPV16/18. HPV16/18 single infections were associated with higher E6 positivity rates compared with multiple infections only for cancer cases (HPV16: 92.2% vs. 76.5%; HPV18: 93.9% vs. 62.1%) but not for normal histopathology or cervical intraepithelial neoplasia. In HPV16/18 coinfection subjects, the positivity rate was 42.9% for HPV16-E6 and 42.9% for HPV18-E6. The

combined positivity rate of either HPV16-E6 or HPV18-E6 among HPV16/18 coinfection subjects was 78.6%, similar with HPV16 (74.8%) and HPV18 (79.5%) single-infection subjects. The positivity rates of HPV16/18 E6 oncoprotein varied depending on the HPV-type composition in multiple infection ("clusters") including HPV types other than 16 and 18. Multiple infection clusters most likely to express HPV16-E6 and HPV18-E6 were HPV16/52 (61.5%) and HPV18/52 (66.7%), and the less were HPV16/45 (10.0%) and HPV18/51 (16.7%), respectively. Patterns of E6 oncoprotein expression varied depending on clustering types. However, expression was greatest in women with single HPV-type infections compared with those with multiple HPV types regardless of histopathology. Our findings provided new insight of natural history of cervical cancer.

Introduction

Cervical cancer is the fourth most common cancer in women worldwide, with an estimated 528,000 new cases causing 266,000 deaths each year (1). Infection with a HPV of the high-risk (hrHPV) group can result in HPV-driven cervical oncogenesis and constitutes thus the most important risk factor for cervical cancer, as almost all cervical

cancers result from hrHPV infection. HrHPV types 16 and HPV18 cause more than 70% to 80% of cervical cancer in most regions, while a total of 13 HPV types are considered to possess the capacity of oncogenic transformation, albeit most at lower rates than HPV 16 and 18 (2). Coinfection with multiple HPV types is common and can be observed in 20% to 50% of all HPV-positive women (3–6). Epidemiologic investigations have identified the risk factors of multiple infections (7–9), and longitudinal studies have shed light on the dynamics of viral acquisition and clearance (10–12). Other studies investigated the possible interactions of several HPV genotypes in multiple infections (6, 13–16). Virological, epidemiologic, and clinical significance of multiple HPV infection, however, is still the subject of debate. In particular, the biological significance of each individual infection in a multiple infection is difficult to establish. Some studies showed that every HPV type found in cervical intraepithelial neoplasia (CIN) is associated with a biologically separate independent CIN lesion (17–19), whereas others reported different HPV types can concurrently exist in one single cell (20). Almost all previous studies on multiple HPV infection were based on detection of HPV DNA. Because detection of multiple

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doi: 10.1158/1940-6207.CAPR-18-0343

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types of HPV DNA in cytology reflects a composite view of the infections present on the cervix, assessing the biological significance of every genotype in multiple infections is methodologically challenging. We assumed that clarification of HPV infection at cellular transformation level may refine our understanding of HPV-caused carcinogenesis.

The E6 oncoprotein of hrHPV is pivotal in initiation and maintenance of oncogenic transformation by HPV (21, 22). E6 is a multifunctional protein; most notably inhibiting apoptosis, for example, by shuffling the p53 tumor suppressor protein into the ubiquitin-dependent degradation pathway. E6 also interferes with certain cellular PDZ domain proteins (23, 24) that are involved in cell polarity, cell-cell contact, and other signaling pathways associated with oncogenic transformation. Elevated E6 protein expression correlates with the state of neoplastic transformation and with risk of progression to cervical cancer (25). Lacking appropriate methods to detect E6 oncoprotein, little epidemiology data on the E6 oncoprotein expression are available to date.

Recently, Becton Dickinson (BD Diagnostics) developed a new assay (BD Onclarity HPV assay) based on the HPV-type-specific detection of E6/E7 viral gene DNA of 14 hrHPV, providing information on six individual genotypes (16, 18, 31, 45, 51, and 52) and also on eight HPV genotypes in three distinct groups (33/58, 59/56/66, and 39/68/35; refs. 26–28). The type-specific PCR based on E6/E7 viral gene DNA seemed more suitable than the traditional broad-spectrum PCR that based on the L1 viral gene DNA for analysis of the significance of multiple infections (29, 30). Furthermore, another test that can detect HPV16 and 18 E6 proteins from the cervical specimen had also been developed (OncoE6 Cervical Test, Arbor Vita Corporation). The clinical performance of that test has been evaluated (31–33). Using these two tests, we aimed to characterize the likelihood of HPV genotypes 16 and 18 to express E6 protein in single and multiple infections, and we assessed the risk of these various patterns of multiple infections to cause CIN.

Materials and Methods

Study population

This cross-sectional, multicenter diagnostic study was performed between 2013 and 2017. Participants from six hospitals in China included outpatients referred for colposcopy and inpatients with CIN2 or worse (CIN2+) planned for treatment. Women who were not pregnant, had a whole cervix, had not been previously diagnosed with cervical cancer, and were able to provide informed consent were enrolled in this study. Excluded from this study were women who had hysterectomy or prior destructive therapy.

After informed consent form signed, the sociodemographics data were collected via a standardized inter-

view-based questionnaire. Two cervical exfoliated cells samples were collected before colposcopy or treatment: one was kept in PreservCyt Solution (Hologic, Inc.) for HPV E6/E7 DNA testing and for the liquid-based cytology assessment; the other sample was kept in a Dacron swab for HPV16/18 E6 protein detection. Both samples were sent to the central laboratory of Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China).

Institutional review board (IRB) approval was provided by Ethics Committee from Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China).

HPV DNA detection

The BD Onclarity HPV assay is a qualitative target-amplification test that utilizes RT-PCR and fluorescent probe technology. The primers for the 14 hrHPV genotypes are designed to target a region of 79 to 137 bases in the E6/E7 genome, whereas the internal control (IC) primers amplify a 75-base region in the human β -globin gene for detection of sample inadequacy or inhibition. The assay consists of three PCR assay wells and four optical channels for the detection of six individual HPV genotypes (16, 18, 31, 45, 51, and 52) and three groups of types (33/58, 59/56/66, and 39/68/35) and the IC in each well. All samples were tested on the BD Viper LT platform according to the manufacturer's instructions, which requires 500 μ L of sample input.

E6 protein detection

The OncoE6 Cervical Test was used for HPV16/18 E6 protein detection. Standard operation procedures were followed according to the specifications of the manufacturer. Briefly, swab samples were sequentially treated with 933 μ L of lysis solution (15 minutes), 87 μ L of condition solution (15 seconds), followed by clarification via centrifugation using a table-top microcentrifuge (10 minutes at >10,000 rpm). A 200- μ L aliquot of the specimen solution was then transferred into a vial with lyophilized detector mAb alkaline-phosphatase conjugate. The test strips with immobilized HPV16 and HPV18 capture mAbs were inserted into the specimen-conjugate mixture for 55 minutes. After a 12-minute wash step, the strips were immersed for 15 to 25 minutes (depending on the ambient temperature) into the developing solution containing the alkaline-phosphatase substrate and then placed on a reading guide for visual inspection of results. Appearance of test lines in the appropriate area indicated the corresponding HPV E6 protein to be presented in the sample. Disagreement between the two operators was resolved by a third operator. A control line was included on each strip, which allowed for verification of detector reagent activity and proper sample solution migration up the test strip.

Pathology

Cervical biopsies were used for histopathologic diagnosis. The primary histopathologic diagnosis was provided by

Table 1. Sociodemographics and risk factors for study population (n, %)

| | Total (n = 1,781) | HPV Positive (n = 1,069) | HPV16 (n = 621) | | P | HPV18 (n = 94) | | P |
|----------------------|----------------------|-----------------------------|----------------------------------|------------------------------------|--------------|---------------------------------|-----------------------------------|--------------|
| | | | Single infection (n = 424) | Multiple infection (n = 197) | | Single infection (n = 44) | Multiple infection (n = 50) | |
| Age, mean (range) | 48.2 (20–75) | 48.1 (20–75) | 47.1 (23–69) | 48.5 (27–75) | | 49.5 (31–68) | 48.4 (25–67) | |
| <30 | 56 (3.1) | 41 (3.8) | 17 (4.0) | 3 (1.5) | 0.100 | 0 (0.0) | 2 (4.0) | 0.598 |
| 30~39 | 249 (14.0) | 166 (15.5) | 71 (16.7) | 35 (17.8) | | 5 (11.4) | 5 (10.0) | |
| 40~49 | 674 (37.8) | 371 (34.7) | 159 (37.5) | 71 (36.0) | | 15 (34.1) | 21 (42.0) | |
| 50~59 | 569 (31.9) | 341 (31.9) | 135 (31.8) | 56 (28.4) | | 19 (43.2) | 18 (36.0) | |
| ≥60 | 233 (13.1) | 150 (14.0) | 42 (9.9) | 32 (16.2) | | 5 (11.4) | 4 (8.0) | |
| Marital status | | | | | | | | |
| Married/cohabitation | 1,764 (99.0) | 1,055 (98.7) | 416 (98.1) | 193 (98.0) | 0.904 | 44 (100.0) | 50 (100.0) | -- |
| Divorced/widowed | 17 (1.0) | 14 (1.3) | 8 (1.9) | 4 (2.0) | | 0 (0.0) | 0 (0.0) | |
| Smoking | | | | | | | | |
| No | 1,737 (97.5) | 1,034 (96.7) | 402 (94.8) | 191 (97.0) | 0.203 | 41 (93.2) | 50 (100.0) | 0.061 |
| Yes | 44 (2.5) | 35 (3.3) | 20 (4.7) | 4 (2.0) | | 3 (6.8) | 0 (0.0) | |
| Drinking | | | | | | | | |
| No | 1,430 (80.3) | 797 (74.6) | 330 (77.8) | 144 (71.6) | 0.236 | 36 (81.8) | 40 (80.0) | 0.823 |
| Yes | 351 (19.7) | 272 (25.4) | 91 (21.5) | 54 (27.4) | | 8 (18.2) | 10 (20.0) | |
| Gravidity | | | | | | | | |
| ≤2 | 627 (35.2) | 360 (33.7) | 134 (31.6) | 67 (34.0) | 0.551 | 16 (36.4) | 14 (28.0) | 0.385 |
| ≥3 | 1,154 (64.8) | 709 (66.3) | 290 (68.4) | 130 (66.0) | | 28 (63.6) | 36 (72.0) | |
| Parity | | | | | | | | |
| ≤2 | 1,257 (70.6) | 725 (67.8) | 289 (68.2) | 136 (69.0) | 0.827 | 31 (70.5) | 38 (76.0) | 0.544 |
| ≥3 | 524 (29.4) | 344 (32.2) | 135 (31.8) | 61 (31.0) | | 13 (29.5) | 12 (24.0) | |
| Oral contraceptive | | | | | | | | |
| No | 1,773 (99.6) | 1,062 (99.5) | 422 (99.5) | 196 (99.5) | 0.952 | 43 (97.7) | 50 (100.0) | 0.284 |
| Yes | 8 (0.4) | 5 (0.5) | 2 (0.5) | 1 (0.5) | | 1 (2.3) | 0 (0.0) | |
| Menopause | | | | | | | | |
| No | 1,131 (63.5) | 655 (61.3) | 273 (64.4) | 123 (62.4) | 0.638 | 25 (56.8) | 31 (62.0) | 0.609 |
| Yes | 650 (36.5) | 414 (38.7) | 151 (35.6) | 74 (37.6) | | 19 (43.2) | 19 (38.0) | |

local pathologists. If the primary diagnosis result was (i) CIN, (ii) adenocarcinoma (ADC) or adenosquamous carcinoma, (iii) atypical glandular cells or adenocarcinoma, or (iv) undetermined diagnosis, then the associated hematoxylin & eosin staining and p16^{INK4a} IHC-staining slides were submitted to a panel of five pathologists, and underwent a diagnostic blind review for consensus.

Statistical analyses

Because OncoE6 Cervical Test was targeting HPV16/18 E6 protein, only HPV16/18 DNA-positive women were included into final analysis. Potential confounders included age (<30, 30–39, 40–49, 50–59 and ≥60), marital status (married/cohabitation or divorced/widowed), smoking (yes or no), drinking (yes or no), gravidity (≤2 or ≥3), parity (≤2 or ≥3), oral contraceptive (yes or no) and menopause (yes or no). In the analysis of multiple infections, to study all of the clustering patterns is impossible; we just focused on the hierarchical pair cluster regardless of a third genotype. χ^2 tests were used to compare categorical variables between single and multiple infections groups. Statistical significance was assessed by two-tailed tests with α level of 0.05. The SPSS 17.0 (SPSS Inc.) was used for statistical analyses.

Results

In total, 1,005 women with normal histopathology/CIN1, 284 with CIN2/3, and 492 women with squamous

cell carcinoma (SCC)/ADC were eligible and had valid test results. Of these 1,781 subjects, 687 (38.6%) tested positive for HPV16 and/or 18 DNA. The sociodemographics and risk factors for study population are shown in Table 1. Age, marital status, smoking, drinking, gravidity, parity, oral contraceptive use, and menopause were not statistically different between the single and multiple infections groups in HPV16- or HPV18-positive women.

Table 2 shows the distribution of histopathology and presence of E6 oncoprotein in HPV16 and/or HPV18 DNA-positive women. Overall, the positivity rate was 69.4% for HPV16-E6 and 64.9% for HPV18-E6 oncoproteins. The frequency of E6 oncoprotein expression was significantly higher in single infections than in multiple infections for both HPV16-E6 and HPV18-E6 (HPV16: 74.8% vs. 57.9%, $\chi^2 = 18.083$, $P < 0.001$; HPV18: 79.5% vs. 52.0%, $\chi^2 = 7.795$, $P = 0.005$). In HPV16/18 coinfection, the positivity rate was 42.9% for HPV16-E6 oncoprotein and 42.9% for HPV18-E6. There were only 2 cases showing expression of HPV16-E6 and of HPV18-E6 at the same time, and the overall positivity rate of either HPV16 or HPV18 oncoprotein expression in HPV16/18 coinfection subjects was 78.6%, almost the same as in the corresponding single infections (HPV16: 74.8%; HPV18: 79.5%).

In coinfection of HPV16 with any of the other 12 hrHPV types assessed, HPV16-E6 expression (regardless

Table 2. Distribution of histology and presence of E6 oncoprotein in HPV16/18 DNA-positive women (E6 protein/DNA, %)

| | Normal/CIN1 | CIN2/3 | SCC/ADC | Total |
|---------------------------|--------------|---------------|----------------|----------------|
| HPV16 (E6 protein/DNA, %) | 11/75 (14.7) | 85/166 (51.2) | 335/380 (88.2) | 431/621 (69.4) |
| Single HPV16 infections | 6/42 (14.3) | 51/100 (51.0) | 260/282 (92.2) | 317/424 (74.8) |
| Multiple HPV16 infections | 5/33 (15.2) | 34/66 (51.5) | 75/98 (76.5) | 114/197 (57.9) |
| HPV16 & 18 | 0/5 (0.0) | 0/3 (0.0) | 12/20 (60.0) | 12/28 (42.9) |
| HPV16 & 52 | 2/9 (22.2) | 7/14 (50.0) | 15/16 (93.8) | 24/39 (61.5) |
| HPV16 & 59/56/66 | 0/10 (0.0) | 14/21 (66.7) | 17/23 (73.9) | 31/54 (57.4) |
| HPV16 & 51 | 1/8 (12.5) | 4/5 (80.0) | 7/8 (87.5) | 12/21 (57.1) |
| HPV16 & 33/58 | 2/11 (18.2) | 8/21 (38.1) | 22/28 (78.6) | 32/60 (53.3) |
| HPV16 & 39/68/35 | 1/13 (7.7) | 9/15 (60.0) | 11/14 (78.6) | 21/42 (50.0) |
| HPV16 & 31 | 0/6 (0.0) | 4/7 (57.1) | 9/13 (69.2) | 13/26 (50.0) |
| HPV16 & 45 | 0/4 (0.0) | 0/2 (0.0) | 1/4 (25.0) | 1/10 (10.0) |
| HPV18 (E6 protein/DNA, %) | 9/26 (34.6) | 3/6 (50.0) | 49/62 (79.0) | 61/94 (64.9) |
| Single HPV18 infections | 3/9 (33.3) | 1/2 (50.0) | 31/33 (93.9) | 35/44 (79.5) |
| Multiple HPV18 infections | 6/17 (35.3) | 2/4 (50.0) | 18/29 (62.1) | 26/50 (52.0) |
| HPV16 & 18 | 1/5 (20.0) | 1/3 (50.0) | 10/20 (50.0) | 12/28 (42.9) |
| HPV18 & 52 | 2/2 (100.0) | 1/1 (100.0) | 1/3 (33.3) | 4/6 (66.7) |
| HPV18 & 59/56/66 | 3/7 (42.9) | 0/0 (0.0) | 3/5 (60.0) | 6/12 (60.0) |
| HPV18 & 31 | 2/3 (66.7) | 0/0 (0.0) | 1/2 (50.0) | 3/5 (60.0) |
| HPV18 & 39/68/35 | 1/6 (16.7) | 0/0 (0.0) | 4/4 (100.0) | 5/10 (50.0) |
| HPV18 & 45 | 0/0 (0.0) | 0/0 (0.0) | 1/2 (50.0) | 1/2 (50.0) |
| HPV18 & 33/58 | 3/6 (50.0) | 0/0 (0.0) | 1/3 (33.3) | 4/9 (44.4) |
| HPV18 & 51 | 0/4 (0.0) | 0/0 (0.0) | 1/2 (50.0) | 1/6 (16.7) |

of histology) was detected in 61.5% of HPV16/52 coinfection, 57.4% for HPV16/59/56/66, 57.1% for HPV16/51, 53.3% for HPV16/33/58, 50.0% for HPV16/39/68/35 and for HPV16/31, and 10.0% for HPV16/45. For HPV18-E6 expression when detected in coinfection, prevalence was 66.7% for HPV18/52, 60.0% for HPV18/31 and for HPV18/59/56/66, 50.0% for HPV18/39/68/35, 50% for HPV18/45, 44.4% for HPV18/33/58, and 16.7% HPV18/51; note that the sample size of HPV18-E6 expressors, however, was limited, thus potentially reducing significance of the above listed measured rates of HPV18-E6 expression in the context of multiple infections.

The likelihood of HPV16 and 18 to express E6 oncoprotein showed increasing trends in both single and multiple infections as histopathology status changed from normal/CIN1 to SCC/ADC. For HPV16, the E6 positivity rate of single and multiple infections were not significantly different in both normal/CIN1 (single: 14.3%; multiple: 15.2%, $\chi^2 = 0.011$, $P = 0.916$) and CIN2/3 (single: 51.0%; multiple: 51.5%, $\chi^2 = 0.004$, $P = 0.948$), while in SCC/ADC, the positivity rate of single infections was significantly higher than multiple infections (single: 92.2%; multiple: 76.5%, $\chi^2 = 17.101$, $P < 0.001$). The positivity rate of HPV18 was similar to HPV16 in each histopathology status except for normal/CIN1, which was slightly but not significantly higher than HPV16 in both HPV18 single and multiple infections. Also, the HPV18-E6-positivity rate in single infections was significantly higher than multiple infections (single: 93.9%; multiple: 62.1%, $\chi^2 = 9.461$, $P = 0.002$) in SCC/ADC but not in normal histopathology (single: 33.3%; multiple: 35.3%, $\chi^2 = 0.010$, $P = 0.920$) and precancer lesion (single: 50.0%; multiple: 50.0%, $P = 1.000$).

Discussion

To our knowledge, this study was the first to detect type-specific HPV E6 oncoprotein instead of HPV DNA or mRNA in the study of single and multiple HPV infection. Chiefly, this study provided three distinctive outcomes: first, the positivity rates of E6 oncoprotein of HPV 16 and 18 varied depending on the HPV-type composition in multiple HPV infection ("clusters") including HPV types other than 16 and 18. For example, the HPV16/52 cluster was most likely to express HPV16-E6, whereas the HPV16/45 cluster was least likely to do so. Second, we found that single infections showed higher HPV16-E6 and HPV18-E6-positivity rates than multiple infections in SCC/ADC but not for normal histopathology and precancer lesion. Third, we found that the positivity rate of HPV16-E6 and HPV18-E6 was similar in HPV16/18 coinfection, and the combined positivity rate of either HPV16 or HPV18 oncoprotein expression in HPV16/18 coinfection subjects was almost the same as the rate of expression seen in the corresponding single infections.

The fact that the positivity rate of HPV16-E6 and HPV18-E6 was similar in HPV16/18 coinfection was unexpected, because HPV16 and HPV18 show significantly different biological behavior in their correlation to progression to cancer. First, integration of HPV16 and HPV18 into the cellular genome may show differences (34). Second, differences are also found in the trends seen for presumptive viral loads from a normal cervix to SCC (35, 36). Although evidence above has confirmed that HPV16 acts in malignant transformation differently from HPV18, our data show that the combined positivity rates of HPV16-E6 and HPV18-E6 in HPV16/18 coinfection were similar to their single infections counterparts, suggesting HPV16 and HPV18 might cause cervical lesion independently.

Several studies had also concluded that cervical disease was caused by single-HPV genotype, from tissue-based genotyping and from modeling aspects. Study addressed by Quint and colleagues (17) using laser capture micro-dissection with HPV PCR genotyping technology in cervical lesions have demonstrated that most lesions contained only one genotype of HPV, suggesting one virus to one lesion. Chaturvedi and colleagues (4) calculated ORs pooled and compared with pair-specific ORs to identify genotype combinations that deviated from the pooled OR, found that coinfecting HPV genotypes occur at random and lead to cervical disease independently.

HPV45 is the third to fifth most common HPV genotype present in cervical cancer (37–39), and it is genetically most closely related to HPV18. In one study, HPV45 was shown to be most prone to express oncogenic mRNA (E6/E7), both in high-grade and in low-grade lesions, even more commonly than HPV16 (40). Other than the association between HPV16 and HPV18, HPV18 and HPV45 are genetically so close that all factors required for E6 expression are the same for both types. The drawback of this explanation is that we do not know whether HPV45 was expressed as well in the HPV16/45 and HPV18/45 cases.

HPV51 and HPV52 are also commonly found in cervical cancer (37–39). Studies of HPV-type clustering observed that HPV16/52 was one of the most frequent genotype combinations in multiple infections (41). Clustering of certain HPV genotypes, however, does not necessarily mean a direct biological interaction. Our findings on the activity of HPV16-E6 and HPV18-E6 oncoprotein expression in multiple infections are interesting in this context. HPV16/52, HPV18/52, and HPV16/51 were most likely to express HPV16-E6 and HPV18-E6, whereas HPV18/51 was least likely to express HPV18-E6. The biological behavior of E6 oncoprotein suggested that in addition to genetically relationship, other biological mechanism may also be important, such as the influence of viral DNA integration or the host's immunity (42).

Although the positivity rates of E6 varied among multiple HPV infection clusters, they were lower than in single infections with HPV16 or HPV18 in SCC/ADC but not in normal histopathology and precancer lesion. In previous HPV DNA-test-based studies, it was not demonstrated that multiple HPV infection harboring HPV16/18 and other HPV types are associated with higher risk of carcinogenesis than single HPV16/18 infection. Some studies reported that simultaneous presence of multiple HPV genotypes was associated with an increased risk of high-grade lesions or cytologic abnormalities (43, 44). In other studies, the difference between the single and multiple HPV infection in terms of risk for neoplastic transformation was not significant (4, 45), or reduced high-grade lesions rates were observed in vari-

ous patterns of multiple infections compared with single infections (5, 13). We thought that the arguments resulted from inconsistencies in study design and disease assessment. If studies did not evaluate risk of the precancer and SCC/ADC separately, it might misestimate the role of single and multiple infections in carcinogenesis. In addition, the similar sensitivity for detecting the E6-protein expression in single infection and coinfection in normal histopathology and precancer lesion eliminated the doubt whether E6-protein detection was interfered when multiple genotypes were present. If being interfered, the E6-protein positivity rates in multiple infections would be lower than single infection.

Several potential limitations of this study should be considered. First, although the BD Onclarity assay was performed in an automated, clinically validated platform and target the E6/E7 oncogene, it was limited in the ability to inform on the characteristic of every genotype of hrHPV due to the 3-group report of eight of them. The knowledge that different hrHPV genotypes confer different risks is in the process of being scientifically established, and to study their impact on E6 oncoprotein expression may be helpful in the future. One benefit of the combination, however, was that it can provide a larger sample size of the less common genotypes for analysis. As shown in our data, some pooled positivity rates of the combinatorial genotypes were still lower than HPV16/52 and HPV16/51. Another potential limitation of the study consists in the identification of the actual number of genotypes coinfecting in HPV pair analysis. Although the definition of multiple HPV infection is simple, the clustering patterns vary among genotypes. Most clusters comprise more than two genotypes. To study all of the clustering patterns is impossible, not only because a large sample size is required to identify all of them, especially for some rare genotypes, but also is complex in the mathematical calculation. Therefore, we just focused on the hierarchical pair cluster regardless of a third genotype, and the pooled effects of the underlying genotypes may have some bias. Furthermore, the cross-sectional design of our study is unable to clarify the dynamics of the E6 oncoprotein expression. Elevated expression of E6 protein was reported associated with persistence of viral infection (25, 46), but its impact on multiple HPV infection was not clear yet. Finally, the OncoE6 Cervical Test only targets HPV16-E6 and HPV18-E6, which restricts the study of correlation to these two types. To better elucidate how E6 oncoproteins express in different clustering patterns, the next generation of E6 test targeting more genotypes is needed.

In conclusion, detection of E6 oncoprotein in single and multiple genotypes pattern can give new insights into the field of natural history of cervical cancer, which also suggests the variable oncogenic risk of single HPV type and different pattern of multiple infections.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Wu, L. Yu, W. Chen, Y. Qiao

Development of methodology: L. Yu, W. Chen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Wu, T.-Y. Li, M. Jiang, L. Yu, H. Wang, X. Zhang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Wu, W. Chen

Writing, review, and/or revision of the manuscript: Z. Wu, M. Jiang, H. Wang, W. Chen, Y. Qiao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Jiang, J. Zhao, Y. Qiao
Study supervision: W. Chen

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Acknowledgments

We thank all the staff of Cancer Hospital, Chinese Academy of Medical Sciences, pathology review group members, the local doctors from Beijing, Shanxi, Tianjin, Sichuan and Henan Province, as well as other colleagues who assisted in conducting this work. This work was supported by National Natural Science Foundation of China (grant no. 81272337).

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Received September 3, 2018; revised December 1, 2018; accepted December 31, 2018; published first January 3, 2019.

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