Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2018, Vol. 46(3) 1033–1042

DOI: 10.1177/0300060517736913
journals.sagepub.com/home/imr
SAGE

Feasibility study of a human papillomavirus E6 and E7 oncoprotein test for the diagnosis of cervical precancer and cancer

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Abstract

Objective: To evaluate the clinical value of human papillomavirus (HPV) E6 and E7 oncoprotein (HPV E6/E7) detection in the early screening of cervical cancer.

Methods: This prospective study evaluated all patients with suspected cervical intraepithelial neoplasia (CIN) as identified by the presence of at least one positive indicator from a ThinPrep cytologic test (TCT) and/or a Hybrid Capture 2 (HC2) HPV DNA test. The levels of E6/E7 oncoproteins were determined using Western blot analysis. The diagnostic value of the HPV E6/E7 protein assay was compared with the clinical diagnosis from TCT, HC2 and the gold standard of cervical biopsy histology.

Results: A total of 450 patients were enrolled in the study and based on histological findings, 102 patients were diagnosed with CIN1 (22.7%), 241 with CIN2 (53.6%), 96 with CIN3 (21.3%) and 11 with squamous cell carcinoma (2.4%). For a diagnosis of CIN2+, although the sensitivity of the HPV E6/E7 assay was lower than HC2 (65.5% versus 96.6%, respectively), the specificity was higher (38.2% versus 5.9%, respectively). The sensitivity of the HPV E6/E7 assay was higher than TCT (65.5% versus 36.2%, respectively).

Conclusion: Measuring HPV E6/E7 oncoprotein levels is a potential new biomarker for HPV type 16.

Keywords

E6/E7 oncoproteins, HPV DNA, HPV mRNA, prognostic detection

Date received: 31 March 2017; accepted: 22 September 2017

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Introduction

Cervical cancer (CaCx) is closely associated with human papillomavirus (HPV) infection. More than half a million new cases of CaCx are diagnosed each year worldwide and the incidence in developing countries is much higher compared with developed countries.[1,2] More importantly, around half of all new cases are in China and India.[3] More than one-third of women of reproductive age in China have been infected with HPV, with a 5%–10% rate of continuous infection.[4] Among those with long-term HPV infections, approximately one-quarter will develop cervical intraepithelial neoplasia (CIN) and less than 1% will develop CaCx.[5] The technical developments in molecular biology and virology have enabled researchers to determine that integration of the viral HPV DNA into the host cell genome is the key step in the development of CaCx.[6] Two high-risk HPV E6/E7 oncoproteins, which are found in nearly all HPV-positive cases, are expressed from HPV DNA after it has inserted into the host cell genome.[7,8]

The current mainstream methods for screening for CaCx in clinical trials include the Papanicolaou test, ThinPrep cytological test (TCT), HPV DNA test (e.g. Hybrid Capture 2 [HC2]), HPV mRNA test (e.g. PreTect HPV-Proofer), colposcopy, and visual inspection with acetic acid. Women with abnormal results from cytological testing and/or HPV DNA detection will undergo a subsequent pathological biopsy to check for the existence and staging of CIN during long-term follow-up. Histology is recognized as the gold standard for diagnosing the pathological progress of CaCx development, while CIN2+ (CIN2, CIN3 or cancer) is the cut-off for intervention in clinical practice.[9] However, it is unknown which kind of lesions will finally develop into infiltrative cancers. In developed countries, primary screening based on cytological testing can prevent more than 80% of CaCx.[10] However, abnormal diseases are often missed or misdiagnosed due to the limitations of the testing sensitivity and sampling techniques.[11] Owing to the low sensitivity of the cytological test, females at a high risk are required to have regular retests to confirm the accuracy of the negative result.[9] Among those patients with a negative cytology result but a positive HPV DNA test, only a small proportion will eventually develop CaCx.[12] For the clinical management of these particular patients who are TCT–/HPV+, it would be useful to have new biomarkers to improve the prognostic abilities of the current screening methods.

The aim of this study was to investigate two new biomarkers, the E6/E7 oncoproteins, which are thought to be the main inducing factors for pre-CaCx lesions,[13] as prognostic indicators for CaCx development.

Patients and methods

Patient population

This prospective study recruited patients with at least one positive indicator of cervical abnormalities either from a TCT or a HC2 HPV DNA test in the Department of Laboratory Medicine, Henan Province Chinese Medicine Hospital, Zhengzhou, Henan Province, China between October 2010 and September 2014. The patients had not undergone any treatment, hysterectomy, radiotherapy or chemotherapy. Each cervical cytology sample was analysed by TCT, HC2 HPV DNA test, Western blot analysis of HPV E6/E7 oncoproteins and a histological diagnosis to determine the stage of CIN as described below. The performance of the HPV E6/E7 assay was compared with the clinical diagnosis determined using the TCT and HC2 assays, as

well as the histological diagnosis to determine the stage of CIN. Women attending for routine gynaecological examinations who had a negative HPV DNA test were included as healthy control subjects.

This study was approved by the Ethics Committee of Henan Province Chinese Medicine Hospital, Zhengzhou, Henan Province, China (no. YC2010309316). Patients or their legal representative provided written informed consent prior to study enrolment.

Preparation and preservation of samples

Cervical cytology samples were prepared using PreservCyt® Solution (ThinPrep Pap Test; Hologic, Marlborough, MA, USA). Cervical cytology specimens (2 ml; approximately 75 µl of cell pellet per 1 ml of solution) were collected into vials containing 20 ml PreservCyt® Solution as specimens for TCT as described below. All samples were tested within 2 weeks after collection and stored at –80°C until tested.

Cervical cytology specimens with abnormal cytological findings (approximately 75 µl of cell pellet per 1 ml of solution) were rinsed in Specimen Transport Medium (Qiagen, Valencia, CA, USA) in preparation for the detection of HPV DNA by HC2 (Qiagen) according to manufacturer's instructions for PreservCyt® specimens as described below. Specimens were stored at 4°C and remained viable for analysis within 3 months of initial specimen collection. The threshold for positive HPV DNA detection was a relative light unit/cut-off ratio of $\geq$1.0. Any sample containing $<$ 5 µl of cell precipitate was discarded.

TCT testing

ThinPrep cytological testing was undertaken using a commercial kit according to the manufacturer's instructions (Beijing TCT Medical Technology, Beijing, China). Results of the cytological screening were classified into five grades according to The Bethesda System:[14] within normal limits (normal); atypical squamous cells with undetermined significance (ASC-US); low-grade squamous intraepithelial lesion (LSIL); high-grade squamous intraepithelial lesion (HSIL); atypical squamous cells–cannot exclude HSIL (ASC-H). TCT could also identify squamous cell carcinoma (SCC).

HC2 HPV DNA analysis

The *digene* HC2 HPV DNA Test (Qiagen) was used to quantitate the level of HPV DNA using a standardized test that has been approved by the US Food and Drug Administration.[15] Residual cervical cytological samples remaining after the TCT test were denatured to get single-stranded DNA, which was mixed and reacted with a cocktail of 13 full-length RNA probes designed to capture the following oncogenic HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. All samples were repeated in triplicate.

Western blot analysis of E6/E7 protein levels

Cervical cytology samples were collected and frozen for protein extraction, which was performed by adding RIPA rapid cell lysis buffer (Jierdun Biotechnology, Shanghai, China) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Rockford, IL, USA) as described previously.[16] The protein concentration of the cell lysate was determined using a BCA Protein Assay kit according to the manufacturer's instructions (Thermo Fisher Scientific). Total protein extracts (25 µg) were separated using 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) according to the molecular weight of the E6/E7

oncoproteins (17 kDa of HPV16 E6/E7) as described previously.[17] Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to confirm equal loading of cell lysates. After electrophoresis, the samples were transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific). The membranes were blocked with 5% skimmed milk powder in Tris-buffered saline Tween-20 buffer (TBST; pH 8.6; 20 mM Tris-HCl, 150 mM NaCl and 0.1 % Tween 20) for 1 h at room temperature. Mouse anti-human papillomavirus 16 (E7) monoclonal antibody (1:1500 dilution: Abcam, Cambridge, UK) and goat anti-human HPV16 (E6) polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with the membranes overnight at 4 °C. The primary and secondary antibodies against GAPDH were mixed with the antibody solutions for the target proteins and incubated under the same conditions (primary antibody: rabbit anti-human GAPDH polyclonal antibody, 1:5000 dilution, Abcam; secondary antibody: goat anti-rabbit IgG H&L horseradish peroxidase [HRP], 1:10 000 dilution, Abcam). After incubation with primary antibodies, the membranes were washed in TBST (pH 8.6) four times for 10 min each wash. The membranes were then incubated for 1 h at room temperature with the secondary antibodies, which were rabbit anti-mouse IgG H&L (HRP) (Abcam) for HPV16 E7 protein (1:10 000 dilution) and rabbit anti-goat IgG H&L (HRP) (Abcam) for HPV16 E6 protein (1:10 000 dilution). The membranes were washed in TBST (pH 8.6) four times for 10 min each wash. The protein bands on the membranes were detected using an Immun-Star™ HRP enhanced chemiluminescence kit (Bio-Rad) for 1 min according to the manufacturer's instructions and exposed to X-ray film (Beyotime Institute of Biotechnology, Shanghai, China). The extent of the grey value of the protein bands was determined using a Personal Densitometer SI (Amersham Pharmacia, Piscataway, NJ, USA) and densitometric analysis was taken using ImageJ Software.[18]

Histological diagnosis

Histological examination of cervical biopsy samples is deemed the gold standard for the diagnosis of CaCx and it is used to clarify the staging of CIN. The main steps have been reported previously.[19] Cervical biopsies were only feasible to those patients with abnormal colposcopy. A cervical biopsy would usually be undertaken when collecting the cervical cytology specimen as part of standard care. Under certain conditions, biopsies were performed during the subsequent follow-up; and in this situation, histological diagnosis of biopsies taken within 1 year were included. The samples were randomly and blindly evaluated by at least two pathologists (X.Y.Z. and H. Y.W.) and were recognized as the end-point of this study.

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Statistical significance was evaluated by paired Student's *t*-test or one-way analysis of variance followed by a Student–Newman–Keuls *post-hoc* test when a statistically significant improvement of outcome was observed. A P-value < 0.05 was considered statistically significant.

Results

A total of 450 patients had at least one positive indicator of cervical abnormalities and were recruited to the study. All patients were Asian women from China aged 20–45 years. According to the histological findings, 102 patients were diagnosed with

Table 1. Characteristics of female patients ($n = 450$) with cervical intraepithelial neoplasia (CIN) stratified according to the histological diagnosis.

	CIN2+ group $n = 348$	CIN2– group $n = 102$	Statistical significance[a]
Age, years	39.7 ± 8.9	42.2 ± 9.7	NS
20 to <30	207 (59.5%)	14 (13.7%)	$P < 0.001$
30 to <40	84 (24.1%)	22 (21.6%)	NS
≥40	57 (16.4%)	66 (64.7%)	$P < 0.001$
HPV E6/E7 oncoproteins			
Positive	228 (65.5%)	63 (61.8%)	NS
Negative	120 (34.5%)	39 (38.2%)	
High-risk HC2			
Positive	336 (96.6%)	96 (94.1%)	NS
Negative	12 (3.4%)	6 (5.9%)	
TCT			
HSIL+	126 (36.2%)	12 (11.8%)	$P < 0.001$
LSIL	108 (31.0%)	39 (38.2%)	
ASC-H	48 (13.8%)	6 (5.9%)	
ASC-US	45 (12.9%)	21 (20.6%)	
Normal	21 (6.0%)	24 (23.5%)	

Data presented as mean ± SD or *n* of patients (%).

[a]Statistical significance was evaluated by paired Student's *t*-test or one-way analysis of variance followed by a Student–Newman–Keuls *post-hoc* test.

HPV, human papillomavirus; HC2, Hybrid Capture 2; TCT, ThinPrep cytological test; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells–cannot exclude HSIL; ASC-US, atypical squamous cells with undetermined significance; NS, no significant between-group difference ($P \geq 0.05$).

CIN1 (22.7%), 241 with CIN2 (53.6%), 96 with CIN3 (21.3%) and 11 with SCC (2.4%). As shown in Table 1, there was no significant difference between the CIN2+ and CIN2– groups in terms of the proportion of E6/E7 oncoprotein-positive patients. Similar findings were observed in the high-risk HC2-positive patients, although the sensitivity of HC2 was higher than that of the E6/E7 oncoprotein test, with a value of 96.6% in the CIN2+ group. When the patients were stratified by age, CIN2+ cases were mainly younger patients aged 20 to <30 years. In contrast, the majority of CIN2– cases were patients aged ≥40 years.

The levels of E6/E7 oncoproteins in all cytological samples were measured using Western blot analysis (Figure 1). According to the densitometric analysis, patients were considered to be positive for the HPV oncoproteins if the ratio of E6 or E7 to GAPDH was greater than the positive threshold value of 20%. Cytological samples from healthy volunteers were used as negative controls. Representative photomicrographs of the typical appearance of four subtypes of cervical epithelial lesions as they appear during the TCT are shown in Figure 2. The prevalence of abnormal cytological findings ≥ ASC-US in CIN2+ patients was 94.0% (327/348) compared with 76.5% (78/102) in CIN2– patients. In total, 45 HC2+ patients tested as normal in the TCT.

For the diagnosis of CIN2+, although the sensitivity of the E6/E7 oncoprotein test was lower than HC2, the specificity

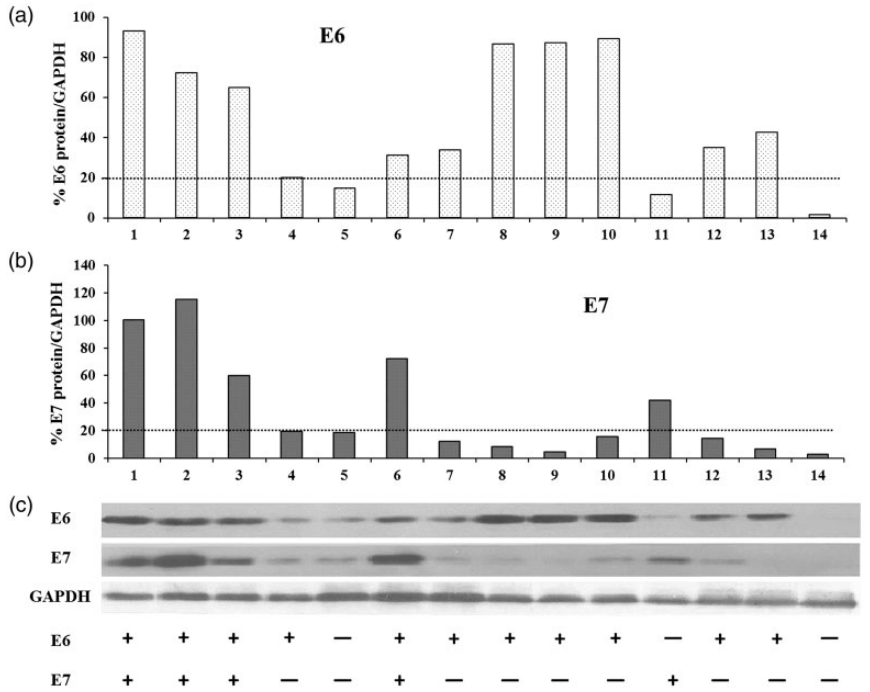

Figure 1. Densitometric analysis of human papillomavirus (HPV) E6/E7 oncoproteins was performed on Western blots to quantify the E6/E7 oncoprotein-positivity in cervical cytological specimens using a positive threshold value of 20% (ratio of E6 or E7 to glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). (A) Percentage value of E6/GAPDH; (B) percentage value of E7/GAPDH; (C) Western blot analysis of E6/E7 oncoproteins in representative samples with definition of protein expression (+ or –). 1–13: samples of HPV-infected patients, 14: negative control sample from a healthy volunteer.

was greater (Table 2). Compared with TCT, the sensitivity of the E6/E7 oncoprotein test was much higher, but had lower specificity.

Table 3 presents the sensitivity and specificity data for the E6/E7 oncoprotein test and the HC2 HPV DNA test for different cervical lesions based on TCT. The specificity of the E6/E7 oncoprotein test was higher than the HC2 test for patients with ASC-US, LSIL or HSIL+.

Discussion

Human papillomavirus E6/E7 oncoproteins are overexpressed after HPV invasion into the host cervical cells in the form of episomal HPV DNA or viral integration into the host's genome, and are closely and directly related to the development of cancers.[16] There are numerous studies that have diagnosed HPV and the occurrence of CaCx via E6/E7 mRNA detection,[20] but only a few studies have determined E6, but not E6/E7, expression at the protein level.[21,22] After cervical cells have been infected with HPV, the presence of HPV DNA or mRNA is only a relatively low risk factor for CaCx progression because the major cause of pre-CaCx lesions is the functional expression of high-risk E6/E7 proteins.[23] So E6/E7 protein expression might be more directly associated with cervical cancer risk. A pilot clinical study that used the OncoE6 assay demonstrated that it had

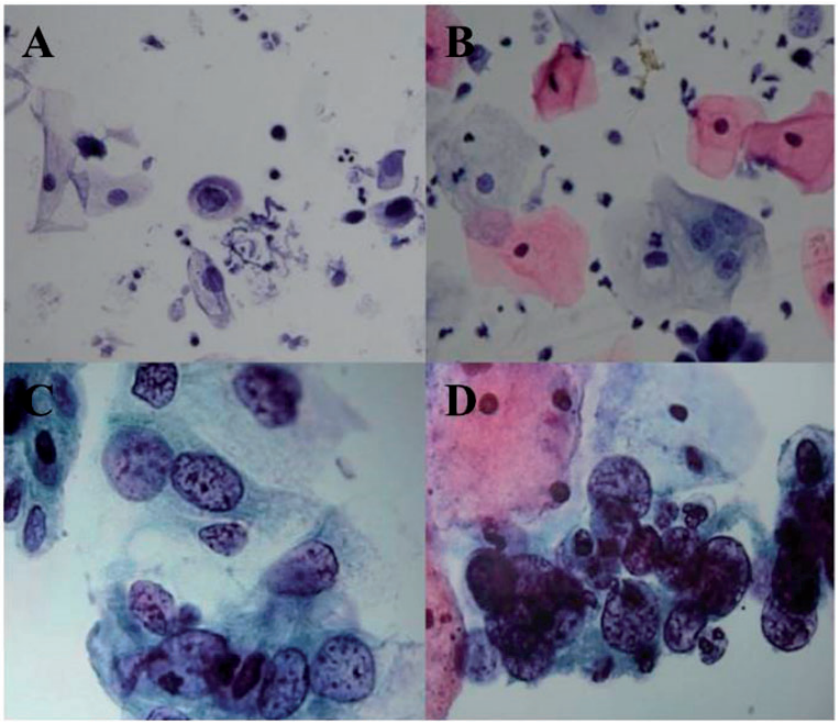

Figure 2. Representative photomicrographs of the typical appearance of four subtypes of cervical epithelial lesions as they appear during the ThinPrep cytological test: (A) atypical squamous cells with undetermined significance; (B) low-grade squamous intraepithelial lesion; (C) high-grade squamous intraepithelial lesion; (D) squamous cell carcinoma. The colour version of this figure is available at: http://imr.sagepub.com. Scale bar 10 μm.

Table 2. Diagnostic value of the human papillomavirus (HPV) E6/E7 oncoprotein test, Hybrid Capture 2 (HC2) HPV DNA test, and ThinPrep cytological test (TCT) for high-grade cervical lesions (CIN2+).

Diagnostic tests	Sensitivity	Specificity	PPV	NPV
HPV E6/E7	65.5 (60.5, 70.5)	38.2 (28.6, 47.8)	78.4 (73.6, 83.1)	24.5 (17.8, 31.3)
HC2	96.6 (94.6, 98.5)	5.9 (1.2, 10.5)	77.8 (73.8, 81.7)	33.3 (9.2, 57.5)
TCT	36.2 (31.1, 41.3)	88.2 (81.2, 94.6)	91.3 (86.5, 96.1)	28.8 (23.8, 33.9)

Data presented as percentage (95% confidence interval).
PPV, positive predictive value; NPV, negative predictive value.
Sensitivity = true positive/(true positive + false negative); specificity = true negative/(true negative + false positive); PPV = true positive/(true positive + false positive); NPV = true negative/(true negative + false negative).

higher specificity than the HPV DNA test for CIN3+ detection.[21] In a previous study, the sensitivity of single E6 protein detection (OncoE6 testing) was only 42.4%,[24] which was lower than the 65.5% that was observed for the HPV E6/E7 oncoprotein test used in this present study. This new technology for cervical cancer screening was evaluated in another clinical trial and it demonstrated higher specificity

Table 3. Diagnostic value of the human papillomavirus (HPV) E6/E7 oncoprotein test and Hybrid Capture 2 (HC2) HPV DNA test for different cervical lesions.

	Sensitivity	Specificity	PPV	NPV
ASC-US, $n = 66$				
HPV E6/E7	48.4 (38.0, 58.7)	55.6 (35.5, 75.6)	78.9 (68.0, 89.9)	23.8 (13.0, 34.6)
HC2	93.5 (88.5, 98.6)	11.1 (–1.6, 23.8)	78.4 (70.6, 86.2)	33.3 (–5.1, 71.8)
LSIL, $n = 147$				
HPV E6/E7	77.8 (69.8, 85.8)	15.4 (3.5, 27.2)	71.2 (63.5, 80.1)	20 (4.8, 35.2)
HC2	97.2 (94.1, 100)	7.7 (–1.1, 16.4)	74.5 (67.2, 81.8)	7.7 (–1.1, 16.4)
HSIL+, $n = 138$				
HPV E6/E7	88.1 (83.6, 91.9)	66.7 (48.9, 86.2)	83.3 (74.8, 92.9)	27.3 (18.4, 36.7)
HC2	98.4 (96.9, 100)	6.3 (–1.0, 14.2)	72.7 (65.6, 80.1)	6.9 (–1.4, 13.8)

Data presented as percentage (95% confidence interval).
PPV, positive predictive value; NPV, negative predictive value; ASC-US, atypical squamous cells with undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.
Sensitivity = true positive/(true positive + false negative); specificity = true negative/(true negative + false positive); PPV = true positive/(true positive + false positive); NPV = true negative/(true negative + false negative).

compared with HC2 (98.9% [95% confidence interval (CI) 98.6%, 99.2%] versus 86.8% [95% CI 85.9%, 87.7%], respectively), but lower sensitivity (67.3% [95% CI 52.5%, 80.1%] versus 98.0% [95% CI 89.1%, 99.9%], respectively) for CIN3+ detection only.[22]

This current study provided further evidence to show that the E6/E7 oncoprotein test is a potential new biomarker that has a satisfactory diagnostic value for CIN2+ CaCx screening. It demonstrated a better sensitivity than TCT and a better specificity than HC2 HPV DNA testing. For diagnosing ASC-US or LSIL, the sensitivity and specificity of the E6/E7 oncoprotein test were similar to E6/E7 mRNA for the same HPV types, but better with a higher PPV.[25–27] These novel indicators (E6/E7 oncoproteins) could be quantified using standard detection methods established in the clinical screening of cervical cancer and this would reduce the limitations of other recent technologies used in this current study.

This current study had several strengths and limitations. The strengths included the prospective design and contemporaneous data collection with a large sample size. One limitation of this study was that it did not use all of the E6/E7 monoclonal antibodies with high specificity that are currently commercially available.[28] In addition, the study only included Asian women from mainland China, so the usefulness of this screening test for pre-cancerous cervical changes in different human populations remains to be evaluated. Further multicentre clinical studies with larger samples sizes are needed to evaluate the effectiveness of E6/E7 oncoprotein detection as a new biomarker for the screening and diagnosis of cervical pre-cancer and cancer.

Regarding HPV-related cancers, the overexpression of E6/E7 oncoproteins is closely associated with the malignant transformation of tumours.[29] E6/E7 oncoproteins can inactivate two tumour suppressor proteins p53 and pRB and thereby promote atypical cell growth.[30] The expression level of E6/E7 oncoproteins might differentiate the types of HPV infection; with HPV types with low E6/E7 expression being transient with low-risk of cancer induction.[16] In contrast, HPV types with a high level or long-standing expression of E6/E7 oncoproteins are probably

associated with a higher risk of inducing cancer.[31] Based on this hypothesis, the screening threshold of E6/E7 oncoproteins should be quantified and used to test abnormal females in the future.[32]

In conclusion, this current study provided further evidence that measuring the levels of E6/E7 oncoproteins might be a potential new biomarker with satisfactory diagnostic values for HPV type 16. The relative diagnostic value might be further improved if the number of HPV oncogenic types was increased.

Author contributions

The authors were involved as follows: J.J.Z., H. Y.W., Y.W.L. – conception and design of the study; J.J.Z., H.Y.W., X.C.C, X.Y.Z., Y.W.L. – performance; H.Y.W., X.C.C – analysis and interpretation; J.J.Z., Y.W.L.– input into drafting the manuscript; J.J.Z., H.Y.W., X.C.C, X.Y. Z., Y.W.L. – responsible for revision of the manuscript.

Acknowledgements

We would like to thank all of the people involved in this study including the patients, healthy control subjects, clinicians and healthcare professionals.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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