

HPV-16 E2/E6 and POU5F1B as Biomarkers to Determine Cervical High-Grade Squamous Lesions and More

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Purpose: Human papillomavirus-16 (HPV-16) is the most carcinogenic HPV genotype. This study aimed to evaluate the clinical value of POU5F1B and HPV-16-E2/E6 by cervical cytology specimens to predict the cervical intraepithelial neoplasia two grade and more (CIN2+).

Methods: Finally, 248 patients with HPV-16 single infection were enrolled. Using cytology specimen by real-time quantitative PCR (qPCR), POU5F1B mRNA and HPV-16-E2/E6 were detected. The relationship of POU5F1B, HPV-16-E2/E6 and CIN2+ were analyzed, and the optimal cut-off values of POU5F1B and HPV-16-E2/E6 to predict CIN2+ were calculated.

Results: The mean HPV-16-E2/E6 decreased significantly with cervical lesions development, especially compared with CIN2+ ($p < 0.05$). And the POU5F1B demonstrated higher expression in CIN2+ than that of normal cervical tissue and CIN1 ($p < 0.05$). What is more, POU5F1B was negatively correlated with HPV-16-E2/E6. It demonstrated that the area under the receiver operating characteristic curve (AUC) for POU5F1B (0.9058) was higher than that for HPV-16-E2/E6 (0.8983), and the sensitivity and specificity of POU5F1B in the diagnosis of CIN2+ were higher than HPV-E2/E6. Furthermore, it demonstrated that the POU5F1B had the highest odds ratio (OR = 16.84; 95% CI (8.00–35.46)) for the detection of CIN 2+.

Conclusion: HPV-16-E2/E6 ≤ 0.6471 or POU5F1B ≥ 1.0310 in cervical exfoliated cells can be used as a reliable predictor of CIN2+. POU5F1B can be used as a new auxiliary biomarker to determine the HPV infection status and a reliable predictor of CIN2+. The expression of POU5F1B ≥ 1.0310 had the highest OR for the detection of CIN2+.

Keywords: cervical intraepithelial neoplasia, human papillomavirus type 16, POU5F1B, HPV integration, HPV-E2/E6

Introduction

Cervical cancer is the most common cancer of the lower female genital tract worldwide. Nearly 529,828 newly reported cases and 275,128 deaths were reported yearly.¹ China has 98,900 newly diagnosed patients and 30,500 deaths annually, with an increasing trend in China.² Therefore, identifying of new and reliable biomarkers involved in cervical intraepithelial neoplasia two grade and more (CIN2+) is urgent and highly demanded, predicting the pathogenesis and development of cervical lesions.

Previous reports showed that the occurrence of cervical cancer and cervical precancerous lesions is mainly caused by the persistent infection of one or

several high-risk human papillomaviruses (HR-HPV).^{3,4} And HPV-16 dominates 80–90% of all HPV-positive cases.⁵ However, Nearly 90% of women are likely to get HPV infections during their lifetime; only 10% will develop to persistent HPV infections, and only 1% will progress to cervical cancer.⁶ Therefore, the poor specificity of the HPV assay results in over-treatment and causes too anxious for patients. Fortunately, the increasing evidence has indicated that HPV integration into the host genome is considered a key step for the development of cervical intraepithelial neoplasia (CIN) and cervical cancer.⁷ So it is crucial to distinguish cervical lesions based on their progression ability if the biomarker is related to the HPV integration. However, the cause and consequences of HPV integration remain poorly understood.

It is reported that the hinge region of the HPV-E2 gene is unstable.⁸ It is the most common deletion or breakage site when the HPV DNA integrates into the host genome. And the deletion of the E2 gene makes the oncogenes E6, and E7 genes highly expressed.^{9–11} Therefore, the E2/E6 ratio has been utilized as surrogate markers to discriminate CIN2+.^{12–15} However, most published studies with HPV-E2/E6 are based on the cervical tissues by invasive biopsy procedures.

The relationship between HPV-E2/E6 and cervical lesions is still controversial because the Cut-off is different from many studies. Therefore, it expedites to find a new and non-invasive method further to evaluate the relationship between HPV-E2/E6 and cervical lesions. It is reported that POU5F1B, the POU domain five transcription factored 1B gene (also known as OCT4-pg1, OTF3C, OTF3P1 and POU5F1P1), had the highest integration frequency when HPV integration happened to use the next-generation sequences (NGS).⁷ It is located on chromosome 8q24. Current research shows that POU5F1B is a tumor susceptibility gene and expressed highly in some cancer tissues, such as rectal cancer, chronic lymphocytic leukemia, bladder cancer, prostate cancer, and hepatocellular carcinoma.^{16–18} However, there are a few studies about its expression status and biological function in cervical cancer.

Here, we investigate whether the expressions of POU5F1B and measurement of HPV-E2/E6 are predictive of CIN2+ and if analyses of the cervical cytological specimens non-invasively collected yield sufficiently conclusive results.

Patients and Methods

Patients and Study Design

We analyzed 248 participants with cervical specimens tested positive for HPV-16 single infection from the Fujian Provincial Maternity and Children's Health Hospital, affiliated hospital of Fujian Medical University from December 2017 to September 2019. The population comes from one of the Fujian Cervical Lesion Screening Cohort (FCLSC), which has more than 120,000 cases involving the community population and hospital population. The populations must meet the following criteria: 1) HPV16 single infection; 2) age above 20 years; 3) sexually active; 4) no history of cervical cancer, CIN, or HIV infection; and 5) did not undergo a cervix surgery or hysterectomy. The Hospital Ethics Committee approved the study (FMCH2017J01232), and all individuals participating in this study provided written informed consent.

Specimen Collection and Management

Exfoliated cervical cells were collected from each participant using a cytobrush. The specimens were collected from the cervical canals. They were gathered in 2-mL vials containing preservation solutions for HPV DNA testing or in 20-mL bottles of ThinPrep® PreservCyt® solution (Hologic, Waltham, MA, USA) cytology examination. The samples for HPV testing need to be stored at –20°C before DNA extraction, and the examples for cytology needs to be stored at 4°C.

Liquid-Based Cytology, HPV Genotyping Test

Cytological specimens were blindly reviewed, independent of the other assays' results, by two experienced cytopathologists. The results were reported following the Bethesda 2001 system.¹⁹ If the diagnosis was different, the cervical samples were evaluated again, and a consensus diagnosis was obtained. The PCR-RDB HPV genotyping kit (Yaneng Limited Corporation, Shenzhen, China) can detect 18 HR-HPV types and 5 LR-HPV types. All examination procedures were performed according to the manufacturer's instructions provided by the kit.²⁰

Histology

Women who were HPV-16 positive with or without abnormal cytological results (with a grade higher than atypical squamous cells of undetermined significance (ASC-US))

need to be referred for colposcopy and punch biopsy. Women with a punch biopsy diagnosis greater than high-grade squamous intraepithelial lesions and more (HSIL) received loop electrosurgical excision procedure cone biopsy (LEEP) or conization by the cold knife. Specimens were fixed in 10% formalin and were routinely processed for paraffin embedding. Then, 4 μ m thick histological sections were cut and stained with hematoxylin and eosin using standard methods. According to the 2014 World Health Organization (WHO) Classification of Tumors of the Female Genital Tract, the pathological diagnostic standard is defined.²¹ An independent expert reviewed all samples with a primary histology result of CIN2+. If the review reading is inconsistent, conduct a second histological review. If two-thirds of the diagnoses are the same, the result is considered the final result.

RT-PCR and Analysis of HPV16-E2/E6

For the HPV DNA extraction, we centrifuge the remaining cervical cells and resuspend them in a digestion solution (10 mmol/L Tris, one mmol/L ethylenediaminetetraacetic acid, 200 μ g/mL proteinase K) for 3 hours at 56°C. It was incubated at 95°C for ten minutes to inactivate proteinase K. Real-time quantitative PCR (qPCR) was carried out with modifications in the protocol. E2 PCR was utilized with primer pairs 5'-AGT AAC TGT GGT AGA GGG TCA AG-3' and 5'-TCA GGA GAG GAT ACT TCG TTG TC-3', while E6 PCR was performed with primer pairs 5'-CGT GAG GTA TAT GAC TTT GCT TTT C-3' and 5'-TGT ATT GCT GTT CTA ATG TTG TTC C-3'. And the POU5F1B PCR was reproduced with primer pairs 5'-TGG CAT TCT TAT CCA CAA AGT GA-3' and 5'-CTG TGA CCG TAT GGC TGT GT-3'. β -globin was applied as an experimental control for sample quality and adequacy during the PCR process. β -globin PCR was reproduced with primer pairs 5'-GCA CCG TCA AGG CTG AGA AC-3' and 5'-TGG TGA AGA CGC CAG TGG A-3'. Relative levels of ER α and ERR α mRNA were quantified by qPCR and calculated by the $2^{-\Delta\Delta CT}$ method. According to the PCR results, E2/E6 of cervical cells were calculated to assess HPV integration status. E2/E6 of 1 was regarded as "episome state," and that ranging from >0 to <1 shown "mixed state"; a ratio of 0 was considered as a "fully integrated state." The "episomal state" means that HPV is not integrated into the host genome. Conversely, the "fully integrated state" will result in the loss of E2.²²

Statistical Analysis

The counting data were analyzed with t-tests, and the measurement data were Chi-square tests. The data were calculated using the IBM SPSS statistical package version 22.0 (IBM Corporation, Armonk, NY, USA). To evaluate the association between the severity of cervical pathology and physical status of HPV integration, linear by linear analysis was performed. Diagnostic accuracy of POU5F1B and E2/E6 ratio for CIN2+ was expressed as the area under the receiver operating characteristic (ROC) curve (AUC). The odd's ratios (ORs) with 95% confidence intervals (CIs) were used to estimated different risks. In all statistical tests, the differences were considered statistically significant at P-values<0.05

Result

Clinical Presentation and Associations Between Cervical Lesions and Clinicopathological Factors

Analysis of the 248 cervical tissue samples with HPV16 single infection indicated that 53 (21.37%) were normal, 54 (21.77%) were classified as CIN1, 87 (35.08%) were categorized as CIN2/3, and 54 (21.77%) were diagnosed as CC. The clinicopathologic features of the study population are presented in Table 1. The age, cytology, E2/E6 and the POU5F1B were significantly related to cervical lesions. It revealed that older age and abnormal cytology promoted the development of CIN2+. What is more, With the development of cervical lesions, HPV-E2/E6 decreased, but POU5F1B expression increased significantly, especially compared with CIN2+. It suggested that HPV-E2/E6 and POU5F1B could predict the development of CIN2+ lesions (p<0.01).

We found a significant difference in means of HPV-E2/E6 in four groups (p<0.001, ANOVA test) (Figure 1A and B). No significant difference was found between normal pathology and CIN1 in terms of means of HPV-E2/E6 (p=0.16). In particular, HPV-E2/E6 of CIN2/3 (mean, 0.52) and CC (mean, 0.38) specimens were significantly lower than normal (mean, 1.14) or graded as CIN1 (mean, 1.01) (p<0.05). Furthermore, HPV-E2/E6 of CIN2/3 and CC specimens were significantly different (p<0.05). The relation between HPV-E2/E6 and POU5F1B with cytology was summarized in Figure 1C and D. It revealed that HPV-E2/E6 decreased significantly according to the development of the cytology with a higher ASCUS group, and POU5F1B expression increased significantly in the ASCUS group and higher ASCUS group.

Table 1 Clinical Characteristics of the Participants of the Study

Item	Pathology				X ²	P
	Normal (n=53)	CIN I (n=54)	CIN 2-3 (n=87)	CC (n=54)		
Age					47.258	<0.001
<30 (N=31)	14 (26.42%)	18 (33.33%)	10 (11.49%)	1 (1.85%)		
30-50 (N=130)	28 (52.83%)	33 (61.11%)	58 (66.67%)	25 (46.30%)		
≥50(N=61)	11 (20.76%)	3 (5.56%)	19 (21.84%)	28 (51.85%)		
Cervical cytology					117.005	<0.001
NILM (N=75)	41 (77.34%)	18 (33.33%)	11 (12.64%)	5 (9.26%)		
ASCUS (N=33)	5 (9.43%)	13 (24.07%)	12 (13.79%)	3 (5.56%)		
>ASCUS (N=107)	3 (7.734%)	20 (37.04%)	57 (65.52%)	27 (50.00%)		
E2/E6	1.53+0.92	1.01+0.89	0.52+0.49	0.38+0.34	54.263	<0.001
POU5F1B	0.36+0.32	0.71+0.69	2.19+1.69	2.87+1.89	37.239	<0.001

Note: P<0.05.

Abbreviations: CIN, cervical intraepithelial neoplasia; NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cells of undetermined significance; LEEP, loop electrosurgical excision; CC, cervical cancer.

The Physical Status of HPV Integration in Cytological Samples

The results of the physical status of HPV integration in 248 samples revealed that the proportion of episome, mixed, and fully integrated state was 22.58%, 75.40%, and 2.02%, respectively (Figure 2A). The episomal status of HPV was found in 62.3% (33 of 53) of normal pathology but 1.9% (1 of 54) in SCC, whereas the fully integrated form of HPV was noted in 7.48% (4 of 54) of SCC but not found in normal pathology and CIN1.

Prediction of HPV-E2/E6 and POU5F1B for ≥CIN2

To predict CIN2+ lesions, ROC analyses of HPV-E2/E6 and POU5F1B mRNA were performed. HPV-E2/E6 and POU5F1B mRNA were evaluated individually (Figure 2A). We observed that AUC for POU5F1B mRNA (0.9058) was higher than that for HPV-E2/E6 (0.8983) (Figure 2B). Also, an optimal cut-off of POU5F1B mRNA was 1.0310, and an optimal cut-off of HPV-E2/E6 was 0.6471. The sensitivity of HPV-E2/E6 for women with CIN2+ was 80.35%, and specificity was 88.79%. The sensitivity and specificity of the combination of POU5F1B mRNA in the differential diagnosis of CIN2+ was 82.98% and 90.65%, respectively (Table 2). The PPV of the POU5F1B mRNA was significantly higher than HPV-E2/E6 (92.13% vs 77.87%). There are similar results in the CIN3+.

HPV-E2/E6 and POU5F1B is a Predictive Factor for the Detection of CIN 2+

As shown in Table 3, the independent factors associated with the diagnosis of high-grade CIN were identified in the multiple logistic regressions. It is demonstrated that the positivity for POU5F1B had the highest OR (OR= 16.84; 95% CI (8.00–35.46)), as well as the following: HPV-E2/E6 (OR = 3.43; 95% CI (1.07–9.53)) and TCT (OR = 1.72; 95% (1.46–2.04)).

Discussion

This study indicated that cervical cytology samples with over-expression of POU5F1B and a decreased HPV-E2/E6 showed significant association with CIN2+. Hence, our study may provide information with a new and possible biomarker to predict the pathogenesis and development of CIN2+ with fewer invasive methods.

Co-test screening with cytology and HPV test has been routinely used for cervical screening. However, the subjective interpretation of cytology is associated with a high degree of variability,²³ which depends on the quality of the laboratory, experience of the cytologist, adequacy of the sample, and technique of fixation.²⁴ It is reported that the sensitivity of HR-HPV testing is higher, and the negative predictive value (NPV) is better.²⁵ HPV detection needs to balance clinical sensitivity and specificity and maximizes the protection of low-risk populations and discrimination of high-risk groups but avoids over-treatment and causing too much anxiety. This study aims to

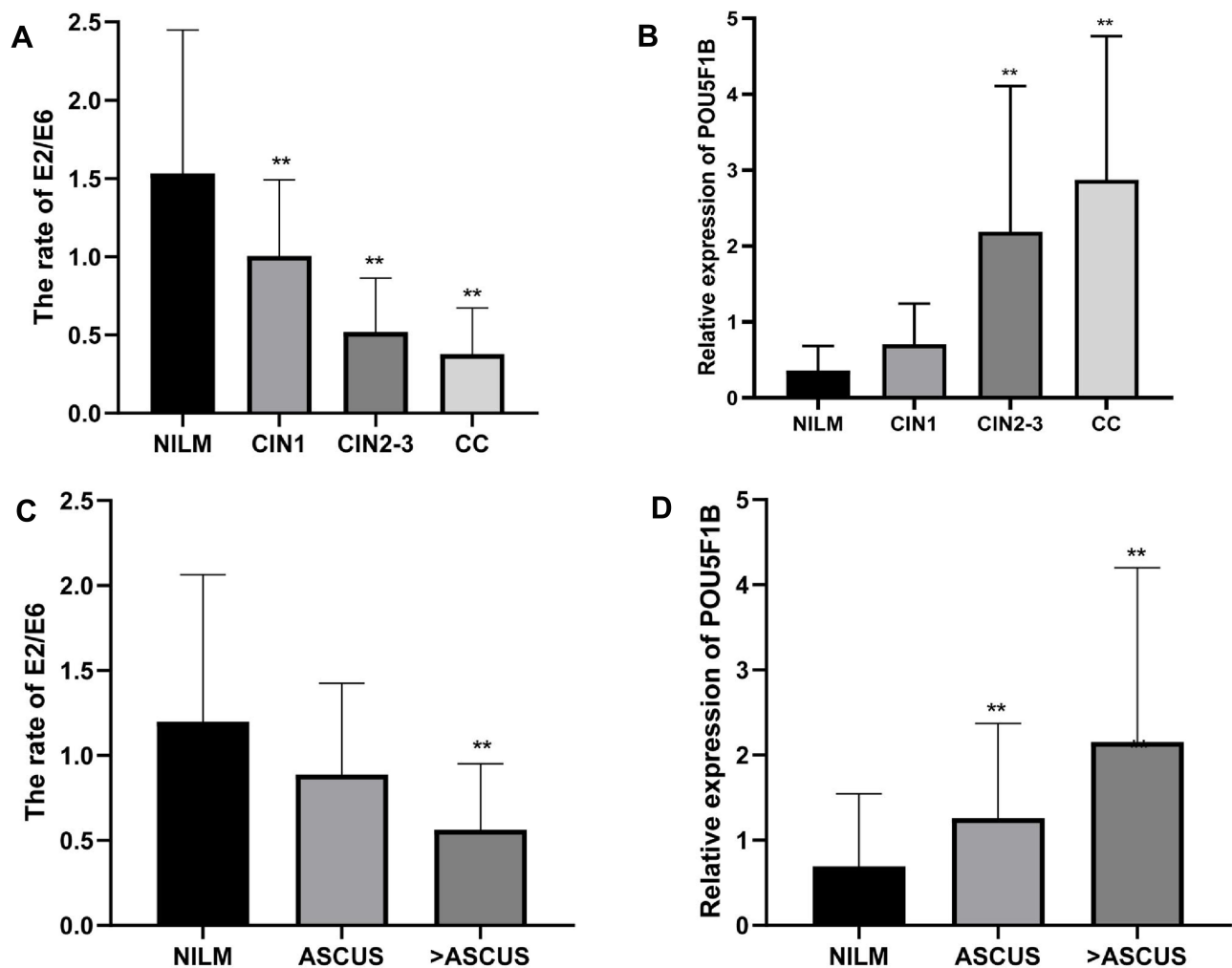


Figure 1 HPV-E2/E6 and POU5F1B in HPV16-positive cervical cytology. (A) HPV-E2/E6 by the cervical lesion. (B) POU5F1B by the cervical lesion. (C) HPV-E2/E6 by cervical cytology. (D) POU5F1B by cervical cytology.

Notes: **Asterisks indicate cervical lesions and cytology results with significantly different HPV-E2/E6 and POU5F1B.

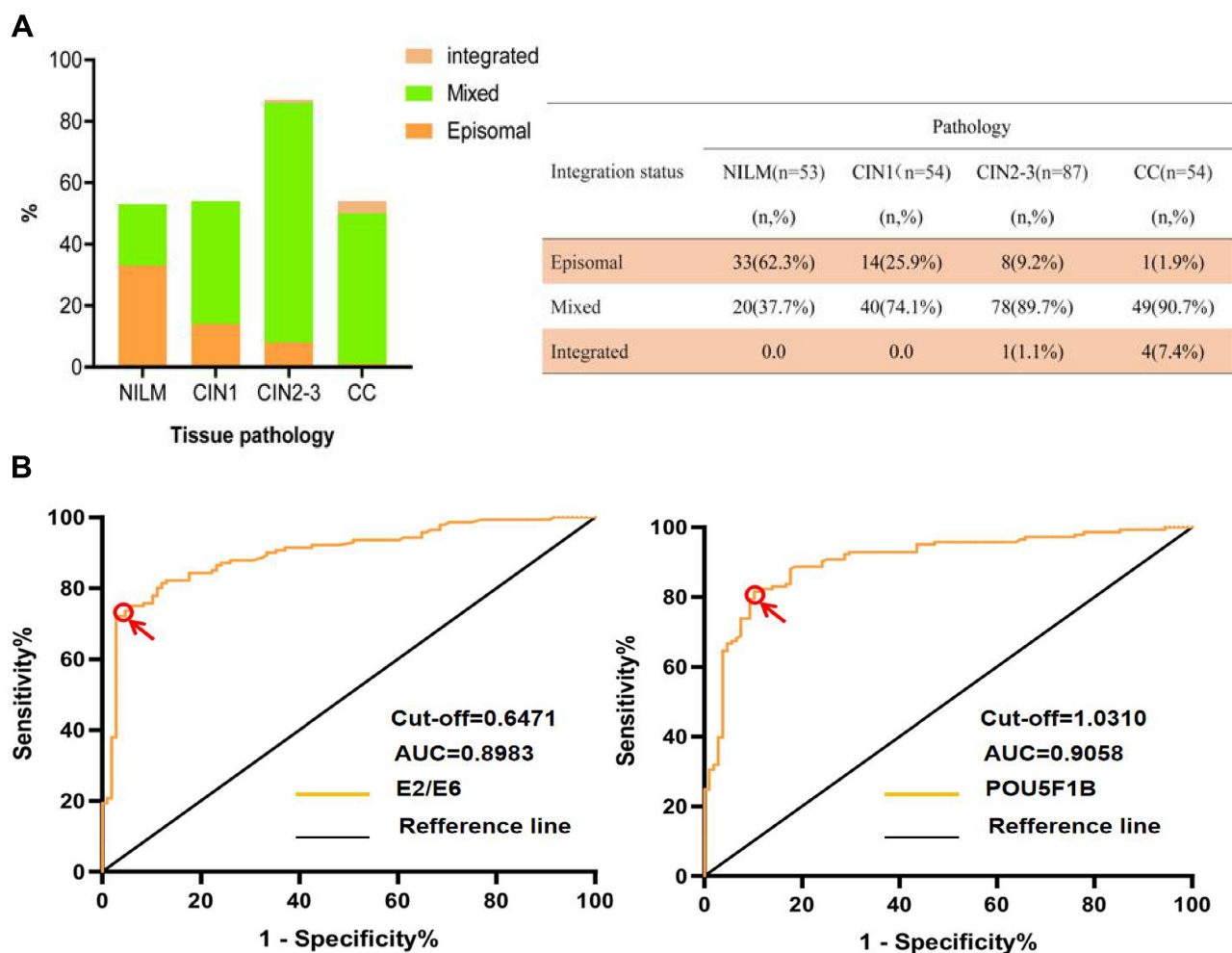
Abbreviations: CIN, cervical intraepithelial neoplasia; NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cells of undetermined significance; CC, cervical cancer.

evaluate the biomarker concerning the HPV integration to predict the CIN2+.

In this study, 78.50% >ASC-US showed CIN2+, which was higher than that reported in our previous study.²⁶ Higher progression for CIN2+ among >ASC-US may be explained by the effect of specific HPV genotypes such as HPV16 during infection. Moreover, of all HR-HPV genotypes, HPV-16 showed the strongest oncogenic capacity for cervical cancer development. Similarly, with our results, many studies reported that HPV-16 was significantly associated with a higher incidence of CIN2/3 in LSIL.²⁷

HPV-16 persistent infection has been attributed partially to its oncogenic potency and is proposed to be regarded as a reliable marker for cervical precancerous lesion progression.²⁸ Although the mechanism that causes HPV-

16, persistent infection is not clear, it has been associated with HPV integration into the human genome.²⁹ HR-HPV E2, E6 and E7 proteins play an important role in the suppressing and activating of HPV promoters.^{30,31} The expression levels of these proteins can affect HPV integration.³² The E2 protein plays various roles, such as promoting apoptosis,³³ causing DNA breaks and chromosomal instability during mitosis⁹ and inhibiting the expression of E6 and E7 proteins.^{34,35} HPV E6 and E7 genes are transcriptions start sites for most HPV types, and they play a key role in the viral transformation and cell carcinogenesis. A previous study has pointed out that HPV integration results in deleting of the open reading frame (ORF) of the E2 gene and releases its inhibitory on E6 and E7 gene.³⁶ The elimination of E2 expression leads to augmented transcription of E6 and E7 oncogenes



and is thought to confer a selective growth advantage to the infected cell, which presented the full integrated state. In a word, the function of E2 seems to depend on their relative ratio to E6. Thus, determining of the relative quantities of E2 genes (or PCR products) and their respective rates to E6 could estimate the HPV integration degree. Therefore, the information about the level of integration of HPV DNA with a host genome could be important and probably useful in the prognosis of cervical cancer's progression and outcome.

This study demonstrated that HPV-E2/E6 evaluated by qPCR with the cervical cytological specimen is predictive of CIN2+ and allows discrimination of cancer from cervical precursors (Figure 1). We also found that HPV-E2/E6 had an optimal cut-off of 0.6471 to predict the CIN2+. Thus, the measurement of HPV-E2/E6, which represents the integration status of HPV16 in cervical cancer, may be

useful for normal and low-grade lesions (CIN1) from high-grade lesions (CIN2+). It showed that HPV16-E2/E6 was significantly distinct according to the severity of cervical lesions and was regarded as a surrogate marker for HPV integration in cervical tissues. In accordance with our results, Cricca M and Lorenzi A also revealed similar conclusions.^{12,15} As we had known, it takes a long process from the HPV infection to the occurrence of cervical lesions, so the HPV integration status from the episome to the fully integrated is a dynamic process. However, currently, there is no consensus to distinguishing the status of the episome, mixed and fully integrated. Yoshinouchi M defined HPV-E2/E6 of 1 as "episome state," whereas that is ranging from >0 to <1 as "mixed state"; HPV-E2/E6 of 0 as "fully integrated state".¹⁴ Based on the Manawapat A's result, HPV-E2/E6 of 1 is regarded as "episome state,"

Table 2 Diagnostic Accuracy of the Rate of E2/E6 and POU5F1B for the Detection of CIN 2+ and CIN 3+

	E2/E6	POU5F1B
CIN2+		
Sensitivity	80.35 (74.36–87.35)	82.98 (76.76–89.18)
Specificity	88.79 (82.81–92.76)	90.65 (85.14–96.17)
PPV	77.87 (70.50–85.24)	92.13 (87.44–96.81)
NPV	90.48 (85.35–95.60)	80.17 (73.06–87.27)
Misdiagnosis	19.15 (12.65–25.64)	17.02 (10.82–22.22)
CIN3+		
Sensitivity	80.37 (72.85–87.90)	86.92 (80.53–93.31)
Specificity	71.63 (64.19–79.07)	75.89 (68.83–82.95)
PPV	68.25 (60.13–76.38)	73.23 (65.53–80.93)
NPV	82.79 (76.09–89.49)	88.43 (82.73–94.13)
Misdiagnosis	19.63 (12.10–27.15)	13.08 (6.70–19.47)

Abbreviations: CIN, cervical intraepithelial neoplasia; PPV, positive predictive value; NPV, negative predictive value.

whereas that is ranging from >0 to <1 is considered as a “mixed state”; HPV-E2/E6 of 0 indicated “fully integrated state”.³⁷

Recently, Hu et al.,⁷ using sequencing, reported frequent HPV integration sites in genes such as POU5F1B. So we hypothesized that POU5F1B can be used as an auxiliary indicator with HPV-E2/E6 to predict the CIN2+ lesions. POU5F1B is involved in many important biological processes. Multiple studies have shown that POU5F1B plays a role as a tumor oncogene in the progression of various cancers. Bellanger S et al.³⁸ revealed that POU5F1B was amplified in gastric cancer and promoted the invasion phenotype of gastric cancer cells. Steger G et al.³⁹ found that the expression of POU5F1B was significantly up-regulated in human hepatocellular carcinoma tissues and promoted the proliferation of hepatocellular carcinoma by activating the AKT gene. The mechanism of POU5F1B is not clear. Senapati R et al.⁴⁰ hypothesized that POU5F1B might be used as a microRNA sponge to competitively regulate the microRNA of OCT4A by regulating the

Table 3 Independent Predictors for Detection of CIN2+ Lesions

Variables	Category	N	OR	95% CI
TCT	≤ASCUS	108	1.0	Reference
	>ASCUS	107	1.72	1.46–2.04
E2/E6	Low	126	1.0	Reference
	High (<0.7119)	122	3.43	1.07–9.53
POU5F1B	Low	121	1.0	Reference
	High (≥ 1.0067)	127	16.84	8.00–35.46

Note: The cutoff of PMR values were defined by ROC curve analysis.

Abbreviations: TCT, Thinprep cytologic test; ASC-US, atypical squamous cells of undetermined significance; OR, odds ratio; 95% CI, 95% confidence interval.

expression of OCT4 protein. In our study, we demonstrated that POU5F1B mRNA was significantly increased with the development of the cervical lesions, especially in the CIN2+. And it is an optimal cut-off of 1.0310 to predict the CIN2+. What is more, the sensitivity, specificity and PPV are superior to HPV E2/E6 to predict the CIN2+/CIN3+ lesions. Furthermore, the POU5F1B has the highest OR (16.84) (95% CI 8.00–35.46) using multiple logistic regressions to detect CIN 2+.

As a result of the continuous efforts to find cervical markers, p16 immunostaining positivity and an increase in Ki-67 expression may predict a higher risk progression in low-grade lesions.^{41,42} However, the indirect correlations of the combined markers have limitations to explain the progression or regression of cervical lesions. To solve this limitation, we utilized the expression of POU5F1B, which is the most frequent HPV integration sites in POU5F1B genes when HPV integration into the host genome.

As we knew, these are a few studies to test and verify the utility of the viral marker of HPV integration. We utilized POU5F1B mRNA and integration methods such as HPV-E2/E6 for the diagnosis of CIN2+ lesions. The accuracy of the identification of CIN2+-was increased in the POU5F1B mRNA versus measurement of HPV-E2/E6 (AUC=0.9058 vs AUC=0.8983). There are some limitations to our research. First, the size of the specimens was not big enough. For a more accurate evaluation, increasing the sample size may be necessary for future studies. Second, in our study, we did not further study the mechanism of the POU5F1B of promoting the development of cervical lesions, which is worth further studying.

In conclusion, our results demonstrate that POU5F1B expression increased, and HPV-E2/E6 decreased in cervical cytology are strongly related to a higher grade of cervical lesions. We advocate cervical cytology from non-invasive procedures and combined measurement of POU5F1B and HPV-E2/E6 as a suitable method to predict CIN2+. Our results may offer a sound basis for the accurate cytological diagnosis of cervical pre-malignancy and cancer in future transitionally research.

Ethics Approval and Consent to Participate

The study was approved by the Hospital Ethics Committee (FMCH2017J01232) and complied with the Declaration of Helsinki. All individuals participating in this study provided written informed consent.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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