RESEARCH COMMUNICATION

Combined p16INK4a and Human Papillomavirus Testing Improves the Prediction of Cervical Intraepithelial Neoplasia (CIN II-III) in Thai Patients with Low-Grade Cytological Abnormalities

Tipaya Ekalaksananan[1], Chamsai Pientong[1]*, Bunkerd Kongyingyoes[2], Arkom Chaiwongkot[1], Pissamai Yuenyao[3], Pilaiwan Kleebkaow[3], Onanong Kritpetcharat[4], Mark F Evans[5]

Abstract

Thailand is in the process of developing a national cervical screening program. This study examined p16INK4a staining and HPV prevalence in abnormal cervical samples with atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesion (LSIL), to evaluate the efficacy of combined HPV and p16INK4a detection to predict CIN II-III. Totals of 125 ASCUS and 87 LSIL cases were re-evaluated by Pap test and cervical cells of ASCUS and LSIL cases were prepared on slides for p16INK4a detection by immunocytochemistry. HPV genotyping of DNA extracts was performed by GP5+/6+ PCR and reverse line blot hybridization. Histopathologic tests were performed to identify cervical lesion. Total of 212 cases were diagnosed to normal (20), ASCUS (112), LSIL (78) and HSIL (2). HPV was detected in ASCUS (49/112, 43.8%), LSIL (60/78, 76.9%) and HSIL (2/2, 100%) cases. The majority of HPV positive samples typed for high-risk HPV. 55.7% (107/192) of abnormal cases (ASCUS, LSIL and HSIL) were positive p16INK4a. For the 111 HPV DNA positive cases, 34 of 49 (69.4%) ASCUS cases and 49 of 60 (81.7%) LSIL cases were p16INK4a positive. 140 biopsies were taken and histological classified: CIN negative (65 cases), CIN I (56 cases) and CIN II-III (19 cases). HPV DNA detection predicted CIN II-III with sensitivity and specificity of 84% and 49%, whereas p16INK4a staining showed higher sensitivity (89.5%) and specificity (56.2%). The prediction of CIN II-III was significantly better by combination of positive HPV DNA and p16INK4a with 93.8% sensitivity and 59.2% specificity. Detection of HPV DNA combined with p16INK4a in cervical cells can predict CIN II-III and may improve the screening diagnosis of Thai women at risk for CIN II-III or cancer.

Keywords: p16INK4a detection - HPVdetection - CIN - LSIL - ASCUS

Asian Pacific J Cancer Prev, **12**, 1777-1783

Introduction

Cervical cancer is the most common malignancy of women in the developing countries (Parkin, et al., 2005). It has a multi-step carcinogenetic progression from low to moderate and to high-grade epithelial lesion (Baseman and Koutsky, 2005).Cervical cancer prevention efforts worldwide have focused on screening sexually active women by conventional cytology, involving collection of cervical cell samples, followed by slide preparation, staining, reading, and reporting. The sensitivity of cytology in detecting cervical intraepithelial neoplasia (CIN) grades II and III is rather modest-around 60% and the specificity is about 75% (Fahey et al., 1995; Nanda et al., 2000). Borderline cytologic abnormalities, especially atypical squamous cells of undetermined significance (ASCUS) as well as low grade squamous intraepithelial lesion (LSIL), are the most common abnormalities observed in smears obtained for cervical carcinoma screening and are detected in 5-10% of women undergoing cervical cytologic screening (Kurman et al., 1994; Lytwyn, et al., 2000).

The practice guidelines from the American Society of Colposcopy and Cervical Pathology recommend three possible courses of action for women with ASCUS Papanicolaou (Pap) smear results: 1) recall the patient and repeat the Pap smear and only refer for colposcopy if the second result is not "normal," 2) refer the patient for immediate colposcopic evaluation, or 3) use an intermediate triage tool to detect the presence of "high-risk" human papillomavirus (HR-HPV) oncogenic deoxyribonucleic acid (DNA) subtype (s) from the

*[1]Department of Microbiology, [2]Department of Pharmacology, [3]Department of Obstetrics and Gynecology, [4]Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, [5]Department of Pathology, University of Vermont, Burlington, USA *For correspondence: chapie@kku.ac.th*

cytologic sample, and refer only those who test positive for a colposcopic examination (Wright et al., 2002). Simply repeating the Pap smear obviates the expense and anxiety associated with providing colposcopy for all patients with minor Pap smear abnormalities (Kaufman, 2001). There is controversy regarding using the Pap smear in a diagnostic rather than a screening role (Lonky, 1999) and evidence from prior trials showed that triage based on repeat cytology alone may miss a significant number of cases of histologic low and high-grade premalignant disease (Slawson et al., 1994; Lytwyn et al., 2000; Morin et al., 2001). Colposcopy for ASCUS allows for lesion visualization, biopsy, clinical management, and possible treatment, and offers the highest practical probability of finding cervical cancer and its associated precursor lesions. Histological analysis of a biopsy sample, however, is more laborious for preparation and study as compared with that of a cytological smear, and is not absolutely efficient owing to a substantial rate of interobserver discrepancies among expert pathologists examining the same material (Klaes et al., 2002). The third option, obtaining a conventional or liquid-based Pap smear and testing with the commercially available Hybrid Capture II (HC-II) test for oncogenic HPV subtypes (includes HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) has been advocated (Manos et al., 1999; Solomon et al., 2001). Most high-grade CIN or cancer may be found if colposcopic evaluation is limited to women who test positive with the HC-II test.

Such management of ASCUS cases is not optimal. Although koilocytotic changes are accepted as HPV-induced abnormalities, their risk relative to LSIL, which are also called mild cervical intraepithelial neoplasia (CIN I) or early dysplasia. CIN I or for a subsequent diagnosis of cervical precancer still is debated (Casas-Cordero et al., 1981; Kuhler-Obbarius et al., 1994). Moreover, questions remain about the consistency of associations between cytologic findings and histologic diagnoses. The infection with HPV does not cause symptoms, but koilocytes can be induced by both low risk (LR) and HR-HPV and only HR-HPV is the main risk factor of cervical cancer (zur Hausen, 2002).

A variety of methods have been developed for the detection of HR-HPV. These include polymerase chain reaction (PCR) based assays and the HC-II test. However, HR-HPV detection has limitations as a marker of cervical carcinoma as many early stage lesion regress (zur Hausen, 2002). Additionally, the detection of HR-HPV does not distinguish between persistent and transient infections (Kjaer, et al., 2002) and the positive predictive value for future CIN III+ was highest among women with abnormal cytology and positive HPV test at baseline (Dillner et al., 2008). However, not all HPV infection will eventually progress to cancer, most of women can clear the viral infection. The women who cannot clear the viral infection and become persistent barriers constitutive the high risk group for progression to cervical cancer (Kjaer et al., 2002; Castle, 2005).

In recent years several research groups (Sano et al., 1998; Klaes et al., 2001; 2002; Volgareva et al., 2002) have proposed that the protein p16INK4a may be a possible supplementary marker of dysplastic and neoplastic cervical lesions. Although there is good evidence that p16INK4a immunostaining correlates with the severity of cytological/histological abnormalities, the reproducibility is limited due to insufficiently standardized interpretation of the immunostaining. Therefore, all stages of CINs analysed are heterogeneous and 20-25% of them were stained poorly or lacked any staining (Tsoumpou et al., 2009). Application of immunohisto-/cytochemical test for p16INK4a may be regarded as an additional (optional) test for early detection of precancerous lesions in cervical epithelium (Volgareva et al., 2004). However, HR-HPV or p16INK4a alone cannot be used as solitary markers for the assessment of LSIL (Tsoumpou, et al., 2009). The combination of p16INK4a and HPV DNA detection were in cervical lesion and demonstrated that the combination of HR-HPV and p16 (INK4a) has a higher diagnostic accuracy than HR-HPV or p16 (INK4a) alone in diagnosis of cervical lesions (Huang et al., 2010).

The objectives of the present study were to determine the infection of HPV regarding the role of LR- and HR-HPV and p16INK4a in ASCUS and LSIL samples from Thai women to evaluate the efficacy of combined HPV and p16INK4a cytology testing to predict CINII-III by comparing these data with the biopsy confirmed lesion diagnosis.

Materials and Methods

Patients and specimens

Patients recruited to participate in this study were women with ASCUS or LSIL cytology who were referred for colposcopy at Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand. Colposcopy was performed within 4-6 weeks from the date of referral. Written informed consent was obtained from the patients, and the study was approved by the ethics committee of Khon Kaen University (No. HE461115). Exclusion criteria of the study were known previous high grade cytological or histological lesions, previous treatments of the cervix, previously diagnosed cancers, and use of steroids or immunosuppressive agents. At the colposcopy visit, a conventional Pap test (CPT) slide and liquid based cervical cells were taken. In abnormal colposcopic finding cases, biopsy specimens were obtained from colposcopically suspect areas, fixed in 10% formalin and sent to pathology laboratory for tissue diagnosis.

Specimen preparation

The CPT was routinely performed to re-evaluate cervical cell diagnosis. The liquid based cervical cells were used to prepare a liquid-based Pap test (LBPT) slide as previously described (Pientong, et al., 2004). Briefly, cell suspension was centrifuged at 1,500 rpm, at 4ºC for 10 min. After washing twice with PBS, the cell pellet was re-suspended in PBS (50 μL) and aliquot of 10 μL cell suspension was dropped on the slide producing a 10 mm diameter. After being air-dried, LBPT was fixed in 0.1% formal saline (1,000 mL normal saline solution plus 2.5 mL of 40% formalin) for an hour, in 95% ethanol for 10 min, and then stored at 4ºC until used (or -20ºC for long

term storage). To the rest of the cell suspension, a cell lysis solution was added and the DNA was subsequently extracted using (and as per) Gentra DNA isolation kit (PUREGENE, Minneapolis, MN). The DNA was used for HPV DNA detection using primer and HPV genotyping using PCR and reverse line blot hybridization, respectively.

p16INK4a staining

The p16INK4a protein detection was performed using immunocytochemical techniques as previously described (Pientong, et al., 2004). The specificity of each staining run was confirmed using SiHa cells and human embryonic lung fibroblast (HEL) cells as the positive and negative controls, respectively. Positive cells with immuno-reactive staining displayed brown color in both the nucleus and cytoplasm. A slide sample (CPT or LBPT) was scored as positive when at least 1 to 2 immuno-reactive cells that showed cyto-morphology change were clearly detected under high power field.

HPV detection and genotyping

PCR was performed for HPV DNA detection using GP5+/6+ primers in the extracted DNA from cervical cell suspension. The quality of DNA preparation was checked by simultaneous amplification of human ß-globin gene (268 base-pairs) using GH20 (5'-GAA-GAG-CCA-AGG-ACA-GGT-AC-3') and PC04 (5'-CAA-CTT-CAT-CCA-CGT-TCA-CC-3') primers. Each PCR experiment was performed with positive (SiHa DNA) and negative (water) control. Amplification products (8 μL) were separated by electrophoresis using 1.5% agarose gel prepared with 0.5 times TBE buffer and visualized using UV-illumination after ethidium bromide staining. Biotin labeled PCR products of HPV positive samples were identified for 37 genotypes by Reverse Line Blot Hybridization (RLBH) as previously described (van den Brule, et al., 2002).

Statistic analysis

Statistical analysis was performed using SPSS software (SPSS for Windows, 12 Inc, Chicago, IL, USA). The correlation between HPV infection regarding LR and HR in ASCUS and LSIL was analyzed by chi-square test as appropriate. The sensitivity and specificity of HPV DNA detection and p16INK4a as well as combine HPV DNA with p16INK4a were calculated. P value of .05 or less was considered statistically significant.

Results

A total of 212 women who were identified as ASCUS (125 cases) and LSIL (87 cases) participated in this study. For re-evaluation by CPT, at time of recruitment, the cytology diagnosis consisted of normal cytology (20 cases), ASCUS (112 cases), LSIL (78 cases) and HSIL (2 cases) (Table 1). The patients with ASCUS and LSIL had mean (SD) age of 47.6 (8.3) years (range, 25-77 years) and 43.78 (8.1) years (range, 21-71 years), respectively. Seventy-three (65.2%) patients with ASCUS and 67 (85.9%) with LSIL showed abnormalities at colposcopy. The overall prevalence of HPV DNA detection in the cervical samples of these women was 53.8% (114/212). HPV DNA was detected in 43.8% and 76.9% of ASCUS and LSIL cases, respectively. LR- (HPV6, 11, 30, 42, 43 and 70) and HR- (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59 and 66) HPV genotypes were detected. In cases with a single infection, HPV16 was the most common followed by HPV11, 58, 18, 51 and 33. Two to four multiple infections were also detected in both groups. Table 1 shows how the frequency of HPV DNA detection increased significantly along with the grade of cellular abnormality (P = 0.000); HPV was significantly more common in LSIL than ASCUS samples. Table 1 also shows patterns of HPV infection. Among HPV positive samples, multiple infections were more common in ASCUS than in LSIL samples (P = 0.007).

Table 1. HPV DNA Detection with Revised Cytological Grades from ASCUS (125) and LSIL (87) Cases

HPV DNA	Cytological grades				P value
	Normal N = 20	ASCUS N = 112	LSIL N = 78	HSIL N = 2	
HPV-ve	17 (85%)	63 (56.3%)	18 (23.1%)	0	
HPV+ve	3 (15%)	49 (43.8%)	60 (76.9%)	2	0.000
Single infection					
LR type	3 (100%)	3 (12%)	15 (36.6%)	0	
HR type	0	22 (88%)	26 (63.4%)	2	
Multiple infections					
	0	21 (42.9%)	11 (18.3%)	0	0.007
Unknown	0	3 (6.1%)	8 (13.3%)	0	

Table 2. HPV DNA and p16INK4a Detection in ASCUS, LSIL and HSIL Cases

Detection	ASCUS N = 112	LSIL N = 78	HSIL N = 2	Total
HPV+ve	49	60	2	111
p16INK4a+ve	34 (69.4%)	49 (81.7%)	2	85
p16INK4a-ve	15 (30.6%)	11 (18.3%)	0	26
HPV-ve	63	18	0	81
p16INK4a+ve	15 (23.8%)	7 (38.9%)	0	22
p16INK4a-ve	48 (76.2%)	11 (61.1%)	0	59

For the detection of p16INK4a, both methods of slide preparation (LBPT and CPT slides) interpreted by both slide-readers showed a high concordance in assessing p16INK4a stained cells. Total of 192 abnormal cervical cases was performed. One hundred-seven of 192 cases (55.7%) were p16INK4a positive as shown in Table 2. For the 111 HPV DNA positive cases, 34 of 49 (69.4%) ASCUS cases and 49 of 60 (81.7%) LSIL cases were p16INK4a positive. However, 15 ASCUS cases (30.6%) and 11 LSIL cases (18.3%), which were negative for p16INK4a immunostaining, were positive for HPV DNA. P16INK4a was also expressed in 23.8% and 38.9% of HPV DNA negative ASCUS and LSIL cases, respectively (Table 2).

The value of HPV DNA or p16INK4a staining for predicting CINII-III grade was also assessed. Seventy-three of 112 ASCUS patients with colposcopic abnormalities were biopsied and graded histologically. The results were 44 cases (60.3 %) of no squamous intraepithelial lesion (no CIN), 20 cases (27.4%) of CIN I and 9 cases of CIN II-III (12.3%). Of the LSIL cases, 67 of 78 cases with colposcopic abnormalities were also

Table 3. Histology Diagnosis of Cases with Combined p16INK4a and HPV Test

Detection	Histology		P value	Sens	Spec
	CIN II-III (19 cases)	No CIN/CIN I (121 cases)			
HPV DNA					
positive	16 (84.2%)	62 (51.2%)	0.007	84%	49%
negative	3 (15%)	59 (48.8%)			
P16INK4a					
positive	17(89.5%)	53(43.8%)	0.000	89.5%	56.2%
negative	2(10.5%)	68(56.2%)			
HPV + p16 INK4a					
+ve/+ve	15 (78.9%)	31 (25.6%)	0.000	93.8%	59.2%
-ve/-ve	1 (5.3%)	45 (37.2%)			
+ve/-ve	1 (5.3%)	31 (25.6%)			
-ve/+ve	2(10.5%)	14 (11.6%)			

HPV DNA detection: PPV=20%, NPV=96%; p16 INK4a detection: PPV=24.3%, NPV=96%; HPV+ p16 INK4a detection: PPV=32%, NPV=97.1%

biopsied for histological diagnosis; CIN I (40/67 [59.7%]) was the most common finding; no CIN and CIN II-III were found in 17 (25.4%) and 10 (14.9%) cases, respectively. The 140 biopsied cases (73 ASCUS and 67 LSIL) with histological diagnosis demonstrated HPV DNA in 44.3% (27/61) of no CIN cases, 58.3% (35/60) of CIN I cases and 84.2% (16/19) of CIN II-III cases. The sensitivity and specificity of HPV detection for prediction the 'CIN II-III' group were significantly different (P = 0.007) from the 'no CIN + CIN I' group with 84% and 49%, respectively (Table 3). The pattern of HPV infection (single vs. multiple infections) did not correlate with severity of cervical lesions (CIN I and CIN II-III) (data not shown). P16INK4a was detected in 43.8% (53/121) of 'no CIN + CIN I' group, and 89.5% (17/19) of 'CIN II-III' group (Table 3). The sensitivity and specificity of P16INK4a detection for prediction the 'CIN II-III' group were significantly different (P = 0.000) with 89.5% and 56.2%, respectively (Table 3). This result showed slightly higher sensitivity (89.5%) and specificity (56.2%) of p16INK4a than HPV DNA detection for the prediction of CIN II-III group. Also indicate whether HR or LR-HPV was associated with CIN I and with CIN II-III lesions and the sensitivity/specificity of HR-HPV for CIN II-III. Multiple infections including a HR type could be classed as HR for this purpose.

The results of p16INK4a and HPV DNA detection were combined and evaluated for the prediction of CIN II-III histopathology (Table 3). The sensitivity of CIN II-III prediction significantly increased to 93.8%, as well as the specificity increased to 59.2%. There was a significant association between CIN II-III and p16INK4a combined with HPV DNA positivity (P = 0.000).

Discussion

The prevalence of HPV in women with ASCUS in the present study (43.8%) is comparable to other studies (39.5%, 31.0–59.7%, and 51.1%) (Manos et al., 1999; Solomon et al., 2001; Pientong et al., 2004). In LSIL samples, the prevalence of HPV was 76.9%, which was higher than our previous study of Thai samples (57.1%) (Pientong et al., 2004) but it is lower than the ASCUS-LSIL Triage Study (ALTS) (~89%) that screened of HR-HPV using the HC-II test, HPV testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: baseline data from a randomized trial (The Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group," 2000). A number of factors may account for these, many of our patients are from rural Thailand rather than a high-risk inner-city population, and there also was a higher prevalence of HPV positivity in women <30 years of age (80.0%) compared with women >30 years of age (52.3%)29; the mean age of our patient population was 47.6. Additionally, the different methods used in different studies may have different sensitivities for HPV (Chaiwongkot et al., 2007). There may also be differences in cytopathological diagnoses of ASCUS and LSIL made across different institutes. In agreement with other studies, HPV16 was the most common type in both of ASCUS and LSIL consistent with its oncogenic potential.

Among ASCUS patients undergoing a cervical biopsy, CIN I and CIN II-III were diagnosed in 27.4% (20/73) and 12.3% (9/73) of the patients, respectively, whereas they were diagnosed in 59.7% (40/67) and 14.9% (10/67) of biopsied LSIL patients, respectively. These findings demonstrate the risk of women with low-grade cytological abnormalities to have a high-grade histologically defined disease. Gonzalez-Bosquet et al. (2010) found that HPV infection and duration of follow-up are predictive factors for the detection of CIN II-III in follow-up case for women with CIN I. Correspondingly, our study demonstrated that HPV DNA detection could significantly predict cervical lesion as CIN II-III (P = .007) (Table 3), specificity, however, was quite low (49%). Further, Zuna et al. (2006) found that the absolute risks of CIN I and HPV cellular changes for cumulatively detected CIN III ranged from 12% to 16% for CIN I and from 6% to 9% for HPV-associated cell changes; koilocytotic atypia, and Castle et al. (2005) reported that women with ASCUS or LSILs who were HPV16-positive had the highest 2-year risk for CIN > III compared with women who were HPV-negative (OR, 38; 95% CI, 22-68 [P < .001]), five-fold greater than the increased risk in women who were positive for other oncogenic HPV types (OR, 7.2, 95% CI, 4.2-13 [P <.001]).

In the present study, the prevalence of p16INK4a staining in ASCUS and LSIL cases were 43.8% (49/112 cases) and 71.8% (56/78 cases), which is corresponded to previous studies where staining ranges from ~50-75% (Bibbo et al., 2002; Saqi et al., 2002). Nasioutziki et al. (2011) studied in women with the mean age was 29 years and reported that expression of p16INK4a was detected in the cytological samples of 44% of ASCUS and 46% of LSIL.

The variation may be because of an incorrect morphological interpretation of the routine conventional Pap smear which has the relatively high positive rate in the range of 5–70% (Sherman et al., 1994). In most laboratory experience in the Netherlands and Germany, false positive cytology is confined to the diagnosis of borderline smears known as ASCUS and inflammation.

The reason for this is the lack of morphological criteria for distinguishing between repair or inflammation-induced reactive atypia and LSIL.

Among HPV positive cases, 69.4% of ASCUS and 81.7% of LSIL cases were p16INK4a positive, whereas 30.6% HPV positive ASCUS cases and 18.3% HPV positive LSIL cases were negative for p16INK4a (Table 2). This result demonstrates the high sensitivity but low specificity of HPV testing for use in cervical cell screening. A previous study reported that, in ASCUS categorized smears, 60% (18/30) of the smears that were negative for p16INK4a staining were positive for HR-HPV and that all p16INK4a-expressing smears demonstrated the presence of SIL in the follow-up biopsies of those classified as ASCUS or ASC-H. However, p16INK4a negative, SIL positive samples were also noted (Sung et al., 2009). This study showed that about one half of the ASCUS classified smears were p16INK4a-negative but HR-HPV positive, possibly indicative of the sensitivity or specificity of methods. Nasioutziki et al. (2011) reported that the concordance between the 2 tests in cervical sample, HPV DNA and p16, was 59% regarding infection-positive cases (Nasioutziki, et al., 2011). In addition, p16INK4a regulation may rely on more than HPV infection. Other reports demonstrated an alternative pathway, where the hypermethylation of the p16INK4a promoter lead to a loss or decreased p16INK4a expression irrespective of HPV/RB status (Kang, et al., 2006; Virmani, et al., 2001).

Patients with ASCUS or LSIL Pap smears exhibit a wide spectrum of histological findings ranging from no pathologic abnormality to high grade CIN. In the present study, 73 of 112 ASCUS cases underwent colposcopy, 20 (27.4%) had CIN I and 9 (12.3%) had CIN II-III. Another study reported that many of the ASCUS cases harbored initially unrecognized CIN I (33%) and CIN II-III (12.6%), which was present in the specimen at the time of rendering the ASCUS diagnosis (Tarkkanen et al., 2007). A lower prevalence was reported in a study from Tehran University, in the years 1998-2001. Of the 266 patients who underwent colposcopy, 28 (11%) had CIN I, 16 (6.3%) had CIN II-III, 2 (0.8%) had squamous cell carcinoma (SCC), and 48 (18.8%) had flat condyloma (Yarandi et al., 2004). From the histologic finding in LSIL cases in our study, 25.4% had CIN I and 14.9% had CIN II-III. In Thai women from a region with a high incidence of cervical cancer, the pathological results of initial colposcopic evaluations were: 63 (30.3%) with CIN II-III; 62 (29.8%) with CIN I; 4 (1.9%) with cervical cancer; and 79 (38.0%) with no epithelial lesion (Kiatiyosnusorn et al., 2010). These results suggested false positive cytology diagnoses and discrepancy of cytology and histology diagnosis. Nyirjesy, et al. (1998) reported that cervical cytology was falsely negative in 8% of patients with CIN II–III and in 14% of those with CIN I and also suggested that virological data did not increase test sensitivity for CIN II and CIN III among women with LSIL and HSIL cytology.

For histologically confirmed cases, the present study showed that 17 of 19 CIN II-III (89.5%) were positive by p16INK4a immunocytochemical staining, whereas 2 cases were negative, suggesting that p16INK4a immunohistochemistry alone may not be a good biomarker for high-grade CIN. Previous studies have reported p16INK4a positive staining in 75.8% (50/66) and 87.5% (21/24) cytological specimens with CIN II found following a biopsy (Holladay et al., 2006; Juric et al., 2010).

The combined result of HPV and p16INK4a detection enabled the prediction of CIN II-III with sensitivity of 93.8%, corresponding with 88.9% (8/9) of ASCUS cases, 90% (9/10) in LSIL cases and 100% (2/2) in HSIL cases. In ASCUS cases, there were three cases of positive HPV DNA that was negative for p16INK4a. Sung et al. (2009) studied ASCUS categorized smears and found that 60% (18/30) of the ASCUS smears that were negative for p16INK4a staining were positive for HR-HPV and reported that the expression of p16INK4a did not correlate with the presence of SIL in ASCUS smears, although p16INK4a expression did correlate with the presence of SIL in ASC-H samples (Sung et al., 2009). Denton et al. (2010) analyzed the performance of p16(INK4a) immunocytochemistry on a series of 810 retrospectively collected ASC-US and LSIL cases with available biopsy follow-up data, HPV testing was also performed from the same residual liquid-based cytologic specimen, and results for both tests were correlated with histologic follow-up data. Sensitivity values for high-grade CIN (HGCIN) confirmed on biopsy within 6 months were 92.6% (ASC-US) and 92.2% (LSIL) for cytotechnologists' reviews of p16 cytology and 90.1% (ASC-US) and 95.7% (LSIL) for HPV testing.

However, it is important to note that women who have negative p16INK4a expression still may progress to CIN III. More importantly, negative p16INK4a immunostaining does not exclude the possibility that CIN I is associated with HR-HPV infection.

The present study has demonstrated that cervical cell screening with positive p16INK4a immunocytochemical staining combined with a positive HPV DNA test detects high grade lesions (CIN II-III) with sensitivity of 93.8%, therefore simultaneous p16INK4a staining and HPV detection in cervical cells may be useful in cervical cancer screening approach for the prediction of precancerous lesions in Thailand. However, of 15.8% (3/19) CIN II-III patients with controversy result is a matter for concern and further studies are required to examine whether these patients are HPV positive with an alternative HPV assay or indeed represent true HPV negatives, especially in p16INK4a positive cases. We also hypothesize that CIN I cases positive both by p16INK4a and by HPV DNA are at an increased risk for cervical cancer progression.

In conclusion, our data suggested that combining the detection of HPV and p16INK4a could help to predict high-grade CIN and provide the basis for early treatment and prevention of cervical cancer in a substantial proportion of Thai women.

Acknowledgements

Funding for this study was obtained from a Khon Kaen University project grant (47017). The authors declare that there is no conflict of interest with this work.

References

Baseman JG, Koutsky LA (2005). The epidemiology of human papillomavirus infections. *J Clin Virol*, **32** Suppl 1, S16-24.

Bibbo M, Klump WJ, DeCecco J, et al(2002). Procedure for immunocytochemical detection of P16INK4A antigen in thin- layer, liquid-based specimens. *Acta Cytol*, **46**, 25-9.

Casas-Cordero M, Morin C, Roy M, et al(1981). Origin of the koilocyte in condylomata of the human cervix: ultrastructural study. *Acta Cytol*, **25**, 383-92.

Castle PE, Solomon D, Schiffman M, et al(2005). Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities. *J Natl Cancer Inst*, **97**, 1066-71.

Chaiwongkot A, Pientong C, Ekalaksananan T, et al (2007). Evaluation of Primers and PCR Performance on HPV DNA Screening in Normal and Low Grade Abnormal Cervical Cells. *Asian Pac J Cancer Prev*, **8**, 279-82.

Dillner J, Rebolj M, Birembaut P, et al (2008). Long term predictive values of cytology and human papillomavirus testing in cervical cancer screening: joint European cohort study. *British Medical Journal*, **377**, a1754.

Denton KJ, Bergeron C, Klement P, et al (2010). The sensitivity and specificity of p16(INK4a) cytology vs HPV testing for detecting high-grade cervical disease in the t riage of ASC-US and LSIL pap cytology results. *Am J Clin Pathol*, **134**, 12-21.

Fahey MT, Irwig L, Macaskill P (1995). Meta-analysis of Pap test accuracy. *Am J Epidemiol*, **141**, 680-9.

Gonzalez-Bosquet E, Selva L, Sabria J, et al (2010). Predictive factors for the detection of CIN II-III in the follow-up of women with CIN I. *Eur J Gynaecol Oncol*, **31**, 369-71.

Huang MZ, Huang S, Li DQ, et al (2010). The study of the combination detection of HPV-DNA and p16(INK4a) in cervical lesions. *Med Oncol*. Nov 4 [Epub ahead of print].

Holladay EB, Logan S, Arnold J, et al(2006). A comparison of the clinical utility of p16(INK4a) immunolocalization with the presence of human papillomavirus by hybrid capture 2 for the detection of cervical dysplasia/neoplasia. *Cancer*, **108**, 451-61.

The Atypical Squamous Cells of Undetermined Significance/ Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group (2000). Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: baseline data from a randomized trial. *J Natl Cancer Inst*, **92**, 397-402.

Juric D, Mahovlic V, Rajhvajn S, et al (2010). Liquid-based cytology--new possibilities in the diagnosis of cervical lesions. *Coll Antropol*, **34**, 19-24.

Kang S, Kim J, Kim H B, et al (2006). Methylation of p16INK4a is a non-rare event in cervical intraepithelial neoplasia. *Diagn Mol Pathol*, **15**, 74-82.

Kaufman RH (2001). Is there a role for human papillomavirus testing in clinical practice? *Obstet Gynecol*, **98**, 724-25.

Kiatiyosnusorn R, Suprasert P, Srisomboon J, et al (2010). High-grade histologic lesions in women with low-grade squamous intraepithelial lesion cytology from a region of Thailand with a high incidence of cervical cancer. *Int J Gynaecol Obstet*, **110**, 133-6.

Kjaer S K, van den Brule A J, Paull G, et al (2002). Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. *Bmj*, **325**, 572.

Klaes R, Benner A, Friedrich T, et al (2002). p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol*, **26**, 1389-99.

Klaes R, Friedrich T, Spitkovsky D, et al (2001). Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer*, **92**, 276-84.

Kuhler-Obbarius C, Milde-Langosch K, Loning T, et al(1994). Polymerase chain reaction-assisted evaluation of low and high grade squamous intraepithelial lesion cytology and reappraisal of the Bethesda System. *Acta Cytol*, **38**, 681-6.

Kurman RJ, Henson DE, Herbst AL, et al (1994). Interim guidelines for management of abnormal cervical cytology. The 1992 National Cancer Institute Workshop. *Jama*, **271**, 1866-9.

Lonky N M (1999). Perspectives in cervical cancer prevention and health services management. *J Lower Genital Tract Dis*, **3**, 123–30.

Lytwyn A, Sellors JW, Mahony JB, et al (2000). Comparison of human papillomavirus DNA testing and repeat Papanicolaou test in women with low-grade cervical cytologic abnormalities: a randomized trial. HPV Effectiveness in Lowgrade Paps (HELP) Study No. 1 Group. *Cmaj*, **163**, 701-7.

Manos MM, Kinney WK, Hurley LB, et al (1999). Identifying women with cervical neoplasia: using human papillomavirus DNA testing for equivocal Papanicolaou results. *Jama*, **281**, 1605-10.

Morin C, Bairati I, Bouchard C, et al (2001). Managing atypical squamous cells of undetermined significance in Papanicolaou smears. *J Reprod Med*, **46**, 799-805.

Nanda K, McCrory DC, Myers ER, et al (2000). Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: a systematic review. *Ann Intern Med*, **132**, 810-9.

Nasioutziki M, Daniilidis A, Dinas K, et al (2011). The evaluation of p16INK4a immunoexpression/immunostaining and human papillomavirus DNA test in cervical liquid-based cytological samples. *Int J Gynecol Cancer*, **21**, 79-85.

Nyirjesy I, Billingsley FS, Forman MR (1998). Evaluation of atypical and low-grade cervical cytology in private practice. *Obstet Gynecol*, **92**, 601-7.

Parkin DM, Bray F, Ferlay J, et al(2005). Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.

Pientong C, Ekalaksananan T, Kongyingyoes, B, et al (2004). Immunocytochemical staining of p16INK4a protein from conventional Pap test and its association with human papillomavirus infection. *Diagn Cytopathol*, **31**, 235-42.

Sano T, Oyama T, Kashiwabara K, et al(1998). Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. *Am J Pathol*, **153**, 1741-8.

Saqi A, Pasha TL, McGrath CM, et al (2002). Overexpression of p16INK4A in liquid-based specimens (SurePath) as marker of cervical dysplasia and neoplasia. *Diagn Cytopathol*, **27**, 365-70.

Sherman ME, Schiffman MH, Lorincz AT, et al(1994). Toward objective quality assurance in cervical cytopathology. Correlation of cytopathologic diagnoses with detection of high-risk human papillomavirus types. *Am J Clin Pathol*, **102**, 182-7.

Slawson DC, Bennett JH, Simon LJ, et al (1994). Should all women with cervical atypia be referred for colposcopy: a HARNET study. Harrisburgh Area Research Network. *J Fam Pract*, **38**, 387-92.

Solomon D, Schiffman M, Tarone R (2001). Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: baseline results from a randomized trial. *J Natl Cancer Inst*, **93**, 293-9.

Sung CO, Kim SR, Oh YL, et al(2009). The Use of p16INK4a Immunocytochemistry in ''Atypical Squamous Cells Which Cannot Exclude HSIL" Compared With ''Atypical Squamous Cells of Undetermined Significance" in Liquid-Based Cervical Smears. *Diagnostic Cytopathology*, **38**, 168-71.

Tarkkanen J, Auvinen E, Nieminen P, et al (2007). HPV DNA testing as an adjunct in the management of patients with low grade cytological lesions in Finland. *Acta Obstet Gynecol Scand*, **86**, 367-72.

Tsoumpou I, Arbyn M, Kyrgiou M (2009). p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. *Cancer Treat Rev*, **35**, 210-20.

van den Brule AJ, Pol R, Fransen-Daalmeijer N, et al (2002). GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol*, **40**, 779-87.

Virmani AK, Muller C, Rathi A, et al (2001). Aberrant methylation during cervical carcinogenesis. *Clin Cancer Res*, **7**, 584-9.

Volgareva G, Zavalishina L, Andreeva Y (2004). Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. *BMC Cancer*, **4**, 58.

Volgareva GM, Zavalishina LE, Frank GA, et al (2002). Expression of the protein marker p16INK4a in the cervix uteri cancer. *Arkh Patol*, **64**, 22-4.

Wright TCJr, Cox JT, et al (2002). 2001 Consensus Guidelines for the management of women with cervical cytological abnormalities. *Jama*, **287**, 2120-9.

Yarandi F, Izadi Mood N, Mirashrafi F, et al(2004). Colposcopic and histologic findings in women with a cytologic diagnosis of atypical squamous cells of undetermined significance. *Aust N Z J Obstet Gynaecol*, **44**, 514-6.

Zuna RE, Wang SS, Schiffman M, et al(2006). Comparison of human papillomavirus distribution in cytologic subgroups of low-grade squamous intraepithelial lesion. *Cancer*, **108**, 288-97.

zur Hausen H (2002). Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer*, **2**, 342-50.