RESEARCH ARTICLE

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P16/Ki-67 Dual Staining in Positive Human Papillomavirus DNA Testing for Predictive Diagnosis of Abnormal Cervical Lesions in Northeastern Thai Women

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Abstract

Objective: Cervical cancer screening can effectively reduce new cervical cancer cases, including in Thailand. The abnormal results are subsequently referred for colposcopy. To avoid unnecessary colposcopy, an efficient triage is still needed for validation. This study aimed to investigate the overall positivity of cytology-based screening, HPV detection, and p16/Ki-67 dual staining and evaluate different triage strategies for predictive diagnosis of abnormal cervical lesions in northeastern Thailand. **Methods:** Cervical cells were collected from 191 women who came for cervical screening in the gynecological outpatient department during March 2019-February 2020. Pap smear samples were classified into 6 groups including 17 atypical glandular cells (AGC), 21 atypical squamous cells of undetermined significance (ASC-US), 7 atypical squamous cells - cannot exclude HSIL (ASC-H), 26 low-grade squamous intraepithelial lesions (LSILs), 19 high-grade SILs (HSILs) and 101 no squamous intraepithelial lesion (noSIL). Polymerase chain reaction (PCR) was performed for HPV DNA detection. HPV genotyping was determined by reverse line blot hybridization. P16/Ki-67 dual staining was performed by using CINtec PLUS Cytology kit. Biopsies from abnormal screening were collected for surgical pathology classification. **Results:** High-risk HPV (HR-HPV) infection was 2.97%, 29.41%, 38.10%, 57.14%, 46.15% and 84.21% in noSIL, AGC, ASC-US, ASC-H, LSIL and HSIL cytology respectively. P16/Ki-67 in noSIL, AGC, ASC-US, ASC-H, LSIL and HSIL was 0.99%, 5.88%, 9.52%, 42.86%, 26.92% and 63.16%, respectively (P-value < 0.001). Among p16/Ki-67 positive cases, 96.15% (25/26) were infected with HPV and 84.62% (22/26) were HR-HPV. The overall positivity of each and co-testing between cytology or HPV DNA testing or p16/Ki-67 dual staining was evaluated. In each cervical lesion, primary HPV DNA testing showed the highest sensitivity, but low specificity. The combined all HPV/HR-HPV with p16/Ki-67 detection increased the specificity of abnormal cervical lesions. **Conclusion:** P16/Ki-67 dual stain cytology in HPV-positive women performs well for diagnosis of abnormal cervical lesions and should be considered for management of HPV-positive women to avoid unnecessary colposcopy referrals.

Keywords: Cervical cancer screening- Human papillomavirus- High-risk HPV- P16/Ki-67 dual staining- and Pap smear

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Introduction

Cervical cancer, one of the most common gynecological malignancies, is the leading cancer in Thai women (Kengsakul et al., 2021). However, it has been acknowledged as the first cancer that can be effectively prevented as it can be detected at an early stage or pre-cancer stage thus, increasing the chances for successful treatment outcomes and improved survival of patients. The rates of cervical cancer have been greatly reduced in industrialized countries by regular cytological assessment through detecting, managing, and treating precancerous lesions (McGraw & Ferrante, 2014). Cervical cytology (Papanicolaou [Pap] smear) is the most frequently used screening tool. However, Pap smear has limitations of sensitivity and specificity profile and a high rate of equivocal results, thus, required for regular retesting. Still, approximately almost 30% of patients with cervical cancer had at least one previous false-negative Pap smear (Pirtea et al., 2019), indicating that more accurate screening tests are required.

High-risk human papillomavirus (HR-HPV) infection

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is the major cause of cervical cancer. Recently, several methods are available for testing HPV infection and HPV genotyping that are more sensitive than cervical cytology alone in detecting CIN2 or more severe diagnoses (CIN2+) and CIN3 or more severe diagnoses (CIN3+) (Arbyn et al., 2012; Naucler et al., 2007). However, highly sensitive HPV screening also detects transient HPV infections, which increases the number of positive screening results and the number of colposcopies performed (Arbyn et al., 2012; Castle et al., 2009). Particularly, transient HPV-positive women who are cytology negative for intraepithelial lesion or malignancy (NILM) or low-grade cytology, are referred to colposcopy. This screening program can lead to a larger number of negative results of colposcopy or biopsy for precancerous lesions or cancer (Wentzensen et al., 2016). To choose benefits from colposcopy referral, the optimal triage protocol for HPV-positive women to enhance colposcopy capacity.

The HPV E6 and E7 oncoproteins were commonly associated with the pathogenesis of cervical neoplasia. They bind to regulatory tumor suppressor proteins p53 and Rb and cause their degradation and functional inactivation, respectively. The binding of the E7 oncoprotein to Rb releases the E2F from the complex with hypophosphorylated Rb and causes the progression of the cell cycle. The functional inactivation of Rb thus causes overexpression of the CDK inhibitor p16/INK4a through negative feedback control to check the cell proliferation through regulation of CDK4 and 6. Therefore, HPV-mediated cervical neoplasia and dysplasia overexpress p16/INK4a. In addition, Ki-67 which is a reliable marker of DNA replication, and cell proliferation is often expressed in cervical neoplasia and dysplasia (Agoff et al., 2003; Izadi-Mood et al., 2012; Klaes et al., 2001). P16INK4a and Ki-67 are important biomarkers for the detection of HR-HPV-associated dysplastic changes in cervical biopsy samples. They can be used as complementary tests to differentiate dysplastic and nondysplastic lesions and help to confirm the histopathological diagnosis (Hebbar and Murthy, 2017).

This study aimed to evaluate a predictive diagnosis of abnormal low-grade cervical lesion screening in northeastern Thailand by investigating the overall positivity of cytology-based screening (Pap test), HPV DNA detection, or p16/Ki-67 dual staining and comparison with surgical pathology results.

Materials and Methods

Study design and description of participants

We carried out a cross-sectional diagnostic study at Srinagarind Hospital, Khon Kaen, Thailand. Women aged ≥18 years old who came for cervical screening in the gynecological outpatient department were recruited. A total of 191 women were consecutively enrolled in this study between March 2019-February 2020. Eligibility included an intact uterus, the ability to undergo informed consent, an interview procedure, and a pelvic examination. Exclusion criteria encompassed the previous operation for cervical disease (including the loop electrosurgical excision procedure (LEEP), cold-knife conization, hysterectomy, laser therapy, chemotherapy/radiation treatment, pregnancy, and malignant diseases outside the cervix. Nurses in the outpatient department identified potential participants who attended the clinic and explained the study in detail. Written informed consent was obtained from all participants before study enrollment.

Specimens and nucleic acid extraction

Cervical cells were collected using the Cervex-Brush® (Rovers Medical Devices, Oss, the Netherlands) for conventional Pap smear and kept in 95% ethanol, then stored at -80ºC until used for nucleic extraction and p16/Ki-67 dual-staining. Results of the Pap smear were classified according to the 2001 Bethesda Classification System by experienced cytologists. The biopsy was collected during colposcopy procedures for surgical pathology classification by pathologists with experience in gynecological pathology. The specimens were classified into 6 groups including 17 atypical glandular cells (AGC), 21 atypical squamous cells of undetermined significance (ASC-US), 7 atypical squamous cells – cannot exclude HSIL (ASC-H), 26 low-grade squamous intraepithelial lesions (LSILs), 19 high-grade SILs (HSILs) and 101 no squamous intraepithelial lesion (noSIL).

HPV detection and genotyping

Polymerase chain reactions (PCR) and reverse line blot hybridization (RLBH) were performed for HPV detection and genotyping, respectively. DNA was extracted from cervical scrape cells using TRIzol® Reagent (Life Technologies, California, USA) according to the manufacturer's instructions. The quality of the DNA was confirmed by amplifying fragments of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes, respectively (Teeratakulpisarn et al., 2007). The extracted DNA samples were subjected to PCR using GP5+/GP6+ primers for HPV detection (van den Brule et al., 2002). HPV genotyping was performed by RLBH as described previously (van den Brule et al., 2002) which can be used for the detection of 37 different HPV genotypes.

p16/Ki-67 Dual-Stained Cytology

A commercial kit for simultaneous detection of p16 and Ki-67 in cervical cytology samples was used (CINtecPlus, Roche mtm Laboratories, Germany) according to the manufacturer's instructions. The staining was performed by using BenchMark XT automated slide-staining system (Roche Diagnostics, Basel, Switzerland). All slides were evaluated by virologists and a specialized gynecopathologist. Samples were considered p16/Ki-67 dual-stain positive when immunoreactivity for both p16 and Ki-67 was detected within the same cell in at least one cell per slide.

Statistical analysis

The sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) with a 95% confidence interval (CI) were used to evaluate the triage value of p16/Ki-67 dual-stained cytology in cervical cancer screening. All statistical analyses were performed using the Stata 10.0 (StataCorp LP, Texas) software.

P < 0.05 was assumed to be statistically significant.

Results

Prevalence and Type Distribution of HPV Infection

Of the 191 enrolled women, the information of study population is shown in Supplementary Table S1.The HPV prevalence was 8.91% (9/101), 47.06% (8/17), 66.67% (14/21), 71.43% (5/7), 88.46% (23/26) and 100.0% (19/19) in noSIL, AGC, ASC-US, ASC-H, LSIL and HSIL cytology, respectively (Figure 1A). HPV genotypes were determined in HPV positive samples. HR-HPV infection was 2.97% (3/101), 29.41% (5/17), 38.10% (8/21), 57.14% (4/7), 46.15% (12/26) and 84.21% (16/19) in noSIL, AGC, ASC-US, ASC-H, LSIL and HSIL cytology respectively (Figure 1B). Ten single HR-HPVs as HPV 16, 33, 39, 45, 51, 52, 56, 58, 68, and 82 subtype IS39 accounted for 42.31% (33/78) and multiple type infection with HPV 16, 18, 33, 39, 52, 68 accounted for 19.23% (15/78) (Figure 2). Six low-risk (LR)-HPVs as HPV 11, 40, 55, 66, 70 and CP8304 were observed in 15.38% (12/78). HPV 16 was found to be the most common genotype among HPV positive samples. Eighteen samples (23.08%) of HPV positive cases were unidentified HPV genotypes.

Determination of p16/Ki-67 positivity by cytological analysis

P16/Ki-67 positivity was determined by p16/Ki-67 dual-stained cytology. The dual positive staining was shown in Figure 3. The upward trend of p16/Ki-67 positivity with the severity of cytology was observed. As shown in Table 1, the positivity rate for p16/Ki-67 in NoSIL, AGC, ASC-US, ASC-H, LSIL and HSIL was 0.99% (1/101), 5.88% (1/17), 9.52% (2/21), 42.86% (3/7), 26.92% (7/26) and 63.16% (12/19), respectively (P-value < 0.001). Notably, p16/Ki-67 positivity with HR-HPV infection AGC, ASC-US, ASC-H, LSIL and HSIL were 20.0% (1/5), 25.0% (2/8), 75.0% (3/4), 33.33% (4/12), 75.0% (12/16), respectively (P-value < 0.05). The correlation between p16/Ki-67 and HPV genotypes demonstrated that almost all p16/Ki-67 cases were HR-HPV positive. Interestingly, a p16/Ki-67 positive sample in the noSIL population had an unidentified HPV genotype. Two p16/Ki-67 positive samples in LSIL cytology were LR-HPV positive and 1 sample was HPV undetected (Table 1 and Supplementary Table S2).

Correlation between HPV and p16/Ki-67 by cytological and surgical pathology stratification

Ninety-nine samples with abnormal cervical cytology by Pap smear or HR-HPV infection were referred to the colposcopy clinic. The overview of Pap cytology and surgical pathology results are shown in Supplementary Table S3 which showed the equivocal interpretation between cytology and surgical pathology that commonly occurs in the classified levels lower than HSIL. The distribution of HPV and p16/Ki-67 by cytological and surgical pathology stratification is shown in Table 2. In the group of noSIL, AGC, and ASC-US cytology which

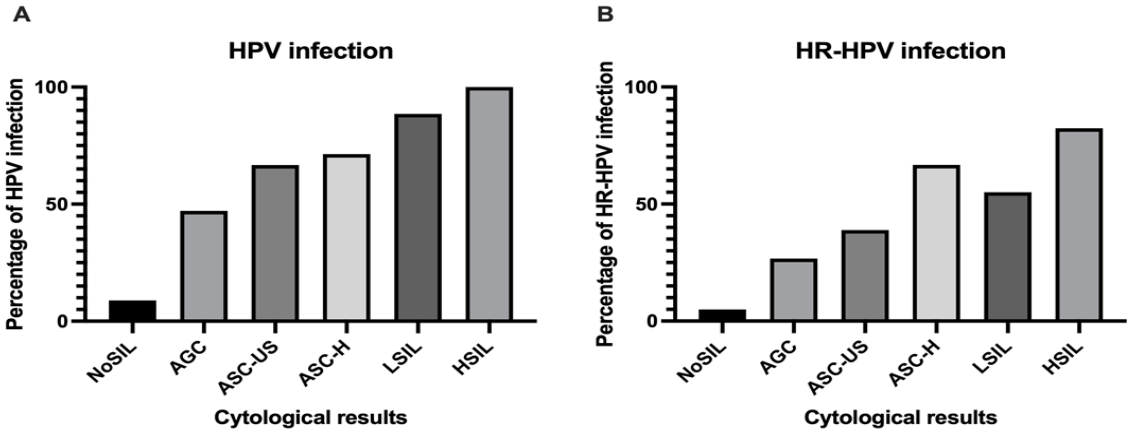

Figure 1. (A) Prevalence of HPV Infection and (B) HR-HPV Infection in Cervical Lesions During March 2019-February 2020.

Table 1. Distribution of p16/Ki-67 Positivity by Cytological Report and HPV Infection

Cervical cytology	P16/ki67 positive (%)			
	HPV negative	LR/Un-HPV positive	HR-HPV positive	Total
NoSIL	0/92 (0)	1/6 (16.67)	0/3 (0)	1/101 (0.99)
AGC	0/9 (0)	0/3 (0)	1/5 (20.0)	1/17 (5.88)
ASC-US	0/7 (0)	0/6 (0)	2/8 (25.0)	2/21 (9.52)
ASC-H	0/2 (0)	0/1 (0)	3/4 (75.0)	3/7 (42.86)
LSIL	1/3 (33.33)	2/11 (18.18)	4/12 (33.33)	7/26 (26.92)
HSIL	0/0 (0)	0/3 (0)	12/16 (75.0)	12/19 (63.16)

NoSIL, no squamous intraepithelial lesion; AGC, atypical glandular cells; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; LR-HPV, low risk-human papillomavirus; HR-HPV, high risk-human papillomavirus

Table 2. Distribution of HPV and p16/Ki-67 by Cytological and Surgical Pathology Stratification

Pap cytological results	Surgical pathology results	n	HPV (+)/ P16/Ki67 (-) % (n)	HPV (-)/ P16/Ki67 (+) % (n)	HPV (+)/ P16/Ki67 (+) % (n)
NoSIL (n = 101)	Not determined	92	0 (0/92)	0.00 (0/92)	0 (0/92)
	Normal colposcopic finding	5	100.00 (5/5)	0.00 (0/5)	0.00 (0/5)
	LSIL	4	75.00 (3/4)	0.00 (0/4)	25.00 (1/4)
AGC (n = 17)	AGC	11	36.36 (4/11)	0.00 (0/11)	0.00 (0/11)
	LSIL	5	60.00 (3/5)	0.00 (0/5)	0.00 (0/5)
	HSIL	1	0.00 (0/1)	0.00 (0/1)	100.00 (1/1)
ASC-US (n = 21)	ASC-US	8	87.50 (7/8)	0.00 (0/8)	0.00 (0/8)
	LSIL	13	38.46 (5/13)	0 (0/13)	15.38 (2/13)
ASC-H (n = 7)	ASC-H	4	25.00 (1/4)	0.00 (0/4)	50.00 (2/4)
	LSIL	3	33.33 (1/3)	0.00 (0/3)	33.33 (1/3)
LSIL (n = 26)	LSIL	24	62.5 (15/24)	4.17 (1/24)	25.00 (6/24)
	Normal colposcopic finding	2	100.00 (2/2)	0.00 (0/2)	0.00 (0/2)
HSIL (n = 19)	LSIL	2	50.00 (1/2)	0.00 (0/2)	50.00 (1/2)
	HSIL	16	37.50 (6/16)	0.00 (0/16)	62.50 (10/16)
	Suspected invasion	1	0.00 (0/1)	0.00 (0/1)	100.00 (1/1)

NoSIL, no squamous intraepithelial lesion; AGC, atypical glandular cells; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; HR-HPV, high risk-human papillomavirus; LR-HPV, low risk-human papillomavirus; Un-HPV, undetermined genotype-human papillomavirus

corresponded with surgical pathology results, no p16/Ki-67 positivity was observed. Interestingly, one noSIL cytology case (1/4, 25%) had p16/Ki-67 positive with HPV and was consequently diagnosed as LSIL surgical pathology. One case of AGC cytology was surgical pathologically classified as HSIL and this sample was positive with HPV and p16/Ki-67 dual staining (1/1, 100%). Two cases of ASC-US cytology and consequently diagnosed as LSIL surgical pathology (2/13, 15.38%) were p16/Ki-67 positive with HPV infection. In samples diagnosed with ASC-H cytology and surgical pathology, 50% (2/4) of samples were positive with HPV+p16/Ki-67. One of 3 samples (33.33%) consequently diagnosed as LSIL was positive with HR-HPV+p16/Ki-67. Among LSIL cytology and surgical pathology samples, 25.00% (6/24) were positive for HPV+p16/Ki-67 and 4.17% (1/24) were positive for p16/Ki-67 alone. Among HSIL cytology samples, 50% (1/2) of samples diagnosed as LSIL surgical pathology, 62.50% (10/16) of samples diagnosed as HSIL surgical pathology, and 100% (1/1) of samples diagnosed as suspected invasion surgical pathology were positive with HPV+p16/Ki-67. These results suggested the upward trend of p16/Ki-67 positivity with the severity of surgical pathology and the positive rates of p16/Ki-67 correlated with HPV infection.

Evaluation of triage strategies for detection of abnormal cervical lesions.

Detailed results of clinical performance of 5 different triage strategies for detection of abnormal cervical lesions

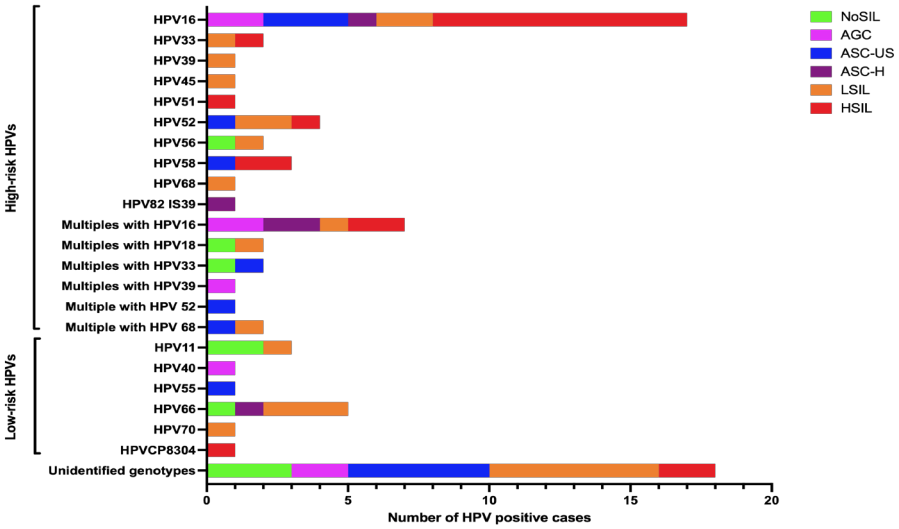

Figure 2. HPV Genotype Distribution in Different Cervical Lesions During March 2019-February 2020

Table 3. Clinical Performance of Different Triage Strategies for Detection of Abnormal Cervical Lesions

Cytology	Strategies	TP	FP	TN	FN	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
AGC (n = 17)	All HPV alone	4	4	2	7	36.4 (10.9 – 69.2)	33.3 (4.33 – 77.7)	50.0 (15.7 – 84.3)	22.2 (2.81 – 60.0)
	HR-HPV alone	2	3	3	9	18.2 (2.28 - 51.8)	50 (11.8 - 88.2)	40 (5.27 - 85.3)	25 (5.49 - 57.2)
	P16/ki67 alone	0	1	5	11	0 (0 - 28.5)	83.3 (35.9 - 99.6)	0 (0 - 97.5)	31.3 (11.0 - 58.7)
	All HPV + p16/ki67	0	1	5	11	0 (0 – 28.5)	83.3 (35.9 – 99.6)	0 (0 – 97.5)	31.3 (11.0 - 58.7)
	HR-HPV + p16/ki67	0	1	5	11	0 (0 - 28.5)	83.3 (35.9 - 99.6)	0 (0 - 97.5)	31.3 (11.0 - 58.7)
ASC-US (n = 21)	All HPV alone	7	7	6	1	87.5 (47.3 – 99.7)	46.2 (19.2 – 74.9)	50.0 (23.0 – 77.0)	85.7 (42.1 – 99.6)
	HR-HPV alone	4	4	9	4	50 (15.7 - 84.3)	69.2 (38.6 - 90.9)	50 (15.7 - 84.3)	69.2 (38.6 - 90.9)
	P16/ki67 alone	0	2	11	8	0 (0 - 36.9)	84.6 (54.6 - 98.1)	0 (0-84.2)	57.9 (33.5 - 79.7)
	All HPV + p16/ki67	0	2	11	8	0 (0 - 36.9)	84.6 (54.6 - 98.1)	0 (0-84.2)	57.9 (33.5 - 79.7)
	HR-HPV + p16/ki67	0	2	11	8	0 (0 - 36.9)	84.6 (54.6 - 98.1)	0 (0-84.2)	57 (33.5 - 79.7)
ASC-H (n = 7)	All HPV alone	3	2	1	1	75 (19.4 - 99.4)	33.3 (0.84 – 90.6)	60.0 (14.7 – 94.7)	50 (1.26 – 98.7)
	HR-HPV alone	3	1	2	1	75 (19.4 - 99.4)	66.7 (9.43 - 99.2)	75 (19.4 - 99.4)	66.7 (9.43 - 99.2)
	P16/ki67 alone	2	1	2	2	50 (6.76 - 93.2)	66.7 (9.43 - 99.2)	66.7 (9.43 - 99.2)	50 (6.76 - 93.2)
	All HPV + p16/ki67	2	1	2	2	50 (6.76 - 93.2)	66.7 (9.43 - 99.2)	66.7 (9.43 - 99.2)	50 (6.76 - 93.2)
	HR-HPV + p16/ki67	2	1	2	2	50 (6.76 - 93.2)	66.7 (9.43 - 99.2)	66.7 (9.43 - 99.2)	50 (6.76 - 93.2)
LSIL (n = 26)	All HPV alone	21	2	0	3	87.5 (67.6 – 97.3)	0 (0 - 84.2)	91.3 (72.0 – 98.9)	0 (0 – 70.8)
	HR-HPV alone	12	0	2	12	50 (29.1 - 70.9)	100.0 (15.8 - 100.0)	100.0 (73.5 - 100.0)	14.3 (1.78 - 42.8)
	P16/ki67 alone	7	0	2	17	29.2 (12.6 - 51.1)	100.0 (15.8 - 100.0)	100.0 (59.0 - 100.0)	10.5 (1.3 - 33.1)
	All HPV + p16/ki67	6	0	2	18	25.0 (9.77 – 46.7)	100.0 (15.8 - 100.0)	100.0 (54.1 – 100.0)	10.0 (1.23 – 31.7)
	HR-HPV + p16/ki67	4	0	2	20	16.7 (4.74 - 37.4)	100.0 (15.8 - 100.0)	100.0 (39.8 - 100.0)	9.09 (1.12 - 29.2)
HSIL (n = 19)	All HPV alone	16	3	0	0	100.0 (79.4 – 100.0)	0 (0 – 70.8)	84.2 (60.4 – 96.6)	N/A
	HR-HPV alone	14	2	1	2	87.5 (61.7 - 98.4)	33.3 (0.84 - 90.6)	87.5 (61.7 - 98.4)	33.3 (0.84 - 90.6)
	P16/ki67 alone	10	2	1	6	62.5 (35.4 - 84.8)	33.3 (0.84 - 90.6)	83.3 (51.6 - 97.9)	14.3 (0.361 - 57.9)
	All HPV + p16/ki67	10	2	1	6	62.5 (35.4 - 84.8)	33.3 (0.84 - 90.6)	83.3 (51.6 - 97.9)	14.3 (0.361 - 57.9)
	HR-HPV + p16/ki67	10	2	1	6	62.5 (35.4 - 84.8)	33.3 (0.84 - 90.6)	83.3 (51.6 - 97.9)	14.3 (0.361 - 57.9)

NoSIL, no squamous intraepithelial lesion; AGC, atypical glandular cells; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells – cannot exclude HSIL; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; HR-HPV, high risk-human papillomavirus; TP, true positive cases; FP, false positive cases; TN, true negative cases; FN, false negative cases; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio; CI, confidence interval

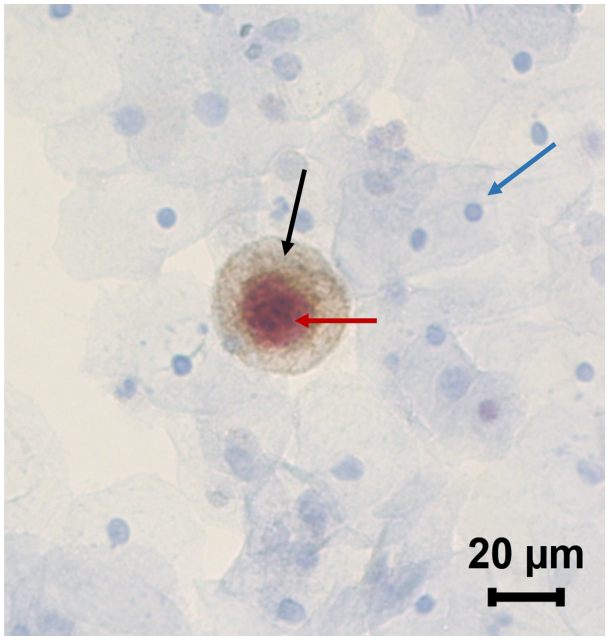

Figure 3. The p16/Ki-67 Dual-Staining Assay, Positive Reaction with LSIL Cytology. Brown cytoplasmic chromogen corresponds to p16 overload (black arrow); nuclear compartment red chromogen is a proof of high Ki-67 protein accumulation and proliferative activity (red arrow); blue arrow depicts negative cells, magnification 400x.

are shown in Table 3 including all HPV alone, HR-HPV alone, p16/ki67 alone, combined all HPV + p16/ki67, and combined HR-HPV + p16/Ki-67. In each lesion group, all HPV alone detection showed the highest sensitivity but had the lowest specificity for detection of abnormal cervical cells when compared to p16/ki67 alone and the two combination strategies. The sensitivity, specificity, PPV, and NPV of the combination strategies in the detection of ASC-H were the same (50%, 66.7%, 66.7%, and 50%, respectively). The two combination strategies showed 100.0% specificity and 100% PPV in the detection of LSIL but low NPV (10.0%, and 9.09%). However, the two combination strategies showed low sensitivity in the detection of LSIL (25.0%, and 16.7 respectively). In the detection of HSIL, HR-HPV alone detection showed 87.5 sensitivity and 87.5 % PPV while the two combination strategies had similar sensitivity (62.5%). The two combination strategies in HSIL cytology showed similar low specificity (33.3%) high PPV (83.3%) and low NPV (14.3%) as shown in Table 3. These results suggest that p16/Ki-67 dual stain cytology combined with HPV detection is effective and should be considered as a triage test for HPV-positive women to increase the specificity in the diagnosis of cervical lesions classified levels lower than HSIL.

Discussion

Cervical cytology is the most used triage test for cervical cancer screening and is recommended by national and international guidelines. Nonetheless, underdiagnoses and overdiagnoses of cervical precancerous lesions are common according to the high interobserver variability of cytopathologic detection and categorization. In this study, we evaluated the overall positivity of cytology-based screening (Pap test), HPV detection, or p16/Ki-67 dual staining as an efficient strategy for predictive diagnosis of abnormal cervical lesions in northeastern Thailand.

We showed that the overall prevalence of HPV infection was increased according to the severity of cervical precancerous lesions. We observed that the most common genotype among HPV positive samples was HPV 16, followed by HPV 52 and HPV 58, respectively which was broadly comparable to information previously reported in the Thai population. HPV 52, 16, and 58 were previously reported to be the most common genotypes in women attending a cervical cancer screening program (Kantathavorn et al., 2015; Paengchit et al., 2014). The three most common HR-HPV genotypes noted among Thai women with cervical intraepithelial neoplasia (CIN) 2-3 were HPV 16 (38.5%), HPV 58 (20.0%), and HPV 18 (5.5%) suggesting a high oncogenic potential of these genotypes for women in Thailand region (Aromseree et al., 2014). The information from our study supports as genotypic evidence that is essential for developing an appropriate HPV vaccination program and guiding the clinical application of newly HPV-based interventions as part of Thailand's cervical cancer prevention strategies.

P16/Ki-67 co-expression was strongly associated with HR-HPV persistent infection (Yu et al., 2016), leading to an increased risk of cancer development, and is recommended to increase the specificity for CIN2+ diagnosis, compared to individual assessment of other immunostaining markers (Lim et al., 2016). In this study, we demonstrated the upward trend of p16/Ki-67 positivity with the cervical lesion severity of cytology which is also associated with HR-HPV infection. This is consistent with the findings of other studies. The use of p16/Ki-67 dual-stained cytology was first examined in HPV-positive women with normal cytology by Petry et al. (Petry et al., 2011) and demonstrated that dual-stained cytology could well identify women who were at risk for developing high-grade CIN lesions. A study from Uijterwaal et al., (2015) indicated well performance of p16/Ki-67 dual-stained cytology in triaging HPV-positive women with normal cytology. In our cohort, a minority of noSIL and LSIL cytology cases were p16/Ki-67 positive with LR-HPV or HPV negative. In the previous study, the authors reported the p16/Ki-67 positivity (11.16%) in HPV negative cases (Zhang et al., 2019). However, this might be due to the undetectable level of HR-HPV copy in the samples as well.

Although the Pap test is the primary screening procedure to detect abnormal cervical cells, colposcopy is the most common procedure performed in patients referred for cervical cytologic abnormalities. Our results demonstrated that the equivocal interpretation between cytopathology and surgical pathology occurred in the classified levels lower than HSIL. According to the lack of ability of HPV DNA test to detect precancerous lesions (Okunade, 2020), p16/Ki-67 dual-stained cytology in combination with primary HPV testing is necessary to complete cervical screening by objective, non-morphological molecular methods, which may be particularly important in developing countries. Surprisingly, our study showed a significant improvement in specificity when using a p16/Ki-67 dual stain cytology as a triage test for HPV-infected women. The combination of p16/Ki-67 dual stain cytology and all HPV detection as triage maintains similar specificity to a combination with extended HR-HPV genotyping. We furtherly found that the combination of p16/Ki-67 dual stain cytology and all HPV detection had higher specificity than either all HPV detection alone or HR-HPV detection. This may be due to genotyping alone does not represent what is occurring within the cell (Stoler et al., 2020). It's likely to have a possibility of misdiagnosis or no follow-up for women who have an infection with a certain genotype associated with lower risk but that is already transforming into higher grade lesions; notably, p16/Ki-67 immunostaining was positively correlated with the degree of cervical lesions (Shi et al., 2019). In addition, the high specificity of the combination of p16/Ki-67 dual stain cytology and all HPV detection strategy would allow less frequent screening repetitions for low-risk women and avoid unnecessary colposcopies, therefore improving the workload of healthcare services and the psychological burden of the women involved.

Despite the use of p16/Ki-67 dual stain cytology may increase the costs of screening tests when compared to pure HPV detection and genotyping as the triage, it could improve the colposcopy referral rate, reduce the follow-up burden, and improve the early detection of cervical precancerous lesions, thus this approach may be a cost-effective and efficient strategy (Song et al., 2021). According to the high-throughput and easy-to-interpret results of p16/Ki-67 dual stain cytology utilized recently, they are affordable and likely to serve as a potential candidate for large-scale cervical screening programs.

There are the main strengths of our current study. It included several groups of cervical precancerous lesions according to Bethesda classification with the ascertainment due to the colposcopy and surgical pathology confirmation. The p16/ Ki-67 dual staining was performed on all HPV-positive and HPV-negative women. The cytologic and surgical pathologic correlation was immediate results since the interval between colposcopy and abnormal screening test results were not exceeding 3 months. The limitation of our study is that the large sample size is required for further study to permit a strong statistical power.

In conclusion, p16/Ki-67 dual stain cytology combined with HPV detection is more effective than HR-HPV genotypes alone in the triage of HPV-positive women. Hopefully, these findings provide new insights for the management of HPV-positive women and increase the body of evidence supporting dual-staining as a triage tool

for colposcopy.

Author Contribution Statement

Conceptualization: SA CP TE. Funding acquisition: SA CP. Investigation: SA WP SS PT. Project administration: SA CP. Supervision: SA AT PK TE CP. Writing ± original draft: SA Review and editing: All authors.

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Ethics Approval

This study was conducted with approval from Khon Kaen University Ethics Committee for Human Research (HE611591).

Conflict of Interest

The authors have declared that no conflict of interests exists.

References

Agoff SN, Lin P, Morihara J, et al (2003). p16(INK4a) expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. *Mod Pathol*, **16**, 665–73.

Arbyn M, Ronco G, Anttila A, et al (2012). Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. *Vaccine*, **30**, 88-99.

Aromseree S, Chaiwongkot A, Ekalaksananan T, et al (2014). The three most common human papillomavirus oncogenic types and their integration state in Thai women with cervical precancerous lesions and carcinomas. *J Med Virol*, **86**, 1911–9.

Castle PE, Rodríguez AC, Burk RD, et al (2009). Short term persistence of human papillomavirus and risk of cervical precancer and cancer: population based cohort study. *BMJ (Clinical Research Ed.)*, **339**, b2569.

Hebbar A, Murthy VS (2017). Role of p16/INK4a and Ki-67 as specific biomarkers for cervical intraepithelial neoplasia: An institutional study. *J Lab Physicians*, **9**, 104–10.

Izadi-Mood N, Asadi K, Shojaei H, et al (2012). Potential diagnostic value of P16 expression in premalignant and malignant cervical lesions. *J Res Med Sci*, **17**, 428–33.

Kantathavorn N, Mahidol C, Sritana N, et al (2015). Genotypic distribution of human papillomavirus (HPV) and cervical cytology findings in 5906 Thai women undergoing cervical cancer screening programs. *Infect Agents Cancer*, **10**, 7.

Kengsakul M, Laowahutanont P, Wilailak S (2021). Experiences in the prevention and screening of cervical cancer within Thailand. *Int J Gynaecol Obstet*, **152**, 48–52.

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.

Lim S, Lee MJ, Cho I, Hong R, Lim SC (2016). Efficacy of p16 and Ki-67 immunostaining in the detection of squamous intraepithelial lesions in a high-risk HPV group. *Oncol Lett*, **11**, 1447–52.

McGraw SL and Ferrante JM (2014). Update on prevention and screening of cervical cancer. *World J Clin Oncol*, **5**, 744–52.

Naucler P, Ryd W, Törnberg S, et al (2007). Human papillomavirus and Papanicolaou tests to screen for cervical cancer. *N Engl J Med*, **357**, 1589–97.

Okunade KS (2020). Human papillomavirus and cervical cancer. *J Obstet Gynaecol*, **40**, 602–8.

Paengchit K, Kietpeerakool C, Lalitwongsa S (2014). Prevalence and genotype distribution of HPV among women attending a cervical cancer screening mobile unit in Lampang, Thailand. *Asian Pac J Cancer Prev*, **15**, 6151–4.

Petry KU, Schmidt D, Scherbring S, et al (2011). Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology. *Gynecol Oncol*, **121**, 505–9.

Pirtea L, Secosan C, Margan M, et al (2019). p16/Ki-67 dual staining has a better accuracy than human papillomavirus (HPV) testing in women with abnormal cytology under 30 years old. *Bosn J Basic Med Sci*, **19**, 336–41.

Shi Q, Xu L, Yang R, Meng Y, Qiu L (2019). Ki-67 and P16 proteins in cervical cancer and precancerous lesions of young women and the diagnostic value for cervical cancer and precancerous lesions. *Oncol Lett*, **18**, 1351–5.

Song F, Yan P, Huang X, et al (2021). Triaging HPV-positive, cytology-negative cervical cancer screening results with extended HPV genotyping and p16(INK4a) immunostaining in China. *BMC Infect Dis*, **21**, 400.

Stoler MH, Baker E, Boyle S, et al (2020). Approaches to triage optimization in HPV primary screening: Extended genotyping and p16/Ki-67 dual-stained cytology-Retrospective insights from ATHENA. *Int J Cancer*, **146**, 2599–2607.

Teeratakulpisarn J, Ekalaksananan T, Pientong C, Limwattananon C (2007). Human metapneumovirus and respiratory syncytial virus detection in young children with acute bronchiolitis. *Asian Pac J Allergy Immunol*, **25**, 139–45.

Uijterwaal MH, Polman NJ, Witte BI, et al (2015). Triaging HPV-positive women with normal cytology by p16/Ki-67 dual-stained cytology testing: baseline and longitudinal data. *Int J Cancer*, **136**, 2361–8.

van den Brule AJC, 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.

Wentzensen N, Schiffman M, Palmer T, Arbyn M (2016). Triage of HPV positive women in cervical cancer screening. *J Clin Virol*, **76**, 49–55.

Yu LL, Guo HQ, Lei XQ, et al (2016). p16/Ki-67 co-expression associates high risk human papillomavirus persistence and cervical histopathology: a 3-year cohort study in China. *Oncotarget*, **7**, 64810–9.

Zhang SK, Jia MM, Zhao DM, et al (2019). Evaluation of p16/Ki-67 dual staining in the detection of cervical precancer and cancer in China. *Cancer Epidemiol*, **59**, 123–8.