ความชุกและการกระจายตัวของการติดเชื้อไวรัสฮิวแมนแปปปิโลม่าชนิดความเสี่ยงสูงในโรงพยาบาลมะเร็งลำปาง

Prevalence and genotypic distribution of high-risk human papillomavirus infection among women screened for cervical cancer at Lampang Cancer Hospital

ดุริยา ฟองมูล* มินตา นากอง สมเกียรติ ลลิตวงศา วรันกร เกยูรวงศ์

Duriya Fongmoon Minta Nakong Somkiat Lalitwongsa Warankorn Keyoonwong*

กลุ่มงานวิจัยและประเมินเทคโนโลยี โรงพยาบาลมะเร็งลำปาง กระทรวงสาธารณสุข จังหวัดลำปาง ประเทศไทย

Lampang Cancer Hospital, Department of Medical Services, Ministry of Public Health, Lampang, Thailand

** Corresponding author (Email: duriya19@hotmail.com)*

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บทคัดย่อ

บทนำ: การติดเชื้อไวรัสเอชพีวีชนิดความเสี่ยงสูง สามารถเหนี่ยวนำให้เกิดความผิดปกติเป็นรอยโรคก่อนมะเร็งบริเวณชั้นเยื่อเมือกหรือเยื่อบุผิว ทำให้เป็นสาเหตุของความผิดปกติของปากมดลูกและมะเร็งปากมดลูก ดังนั้น การตรวจหาเชื้อไวรัสเอชพีวีชนิดความเสี่ยงสูง จึงเป็นวิธีหนึ่งในการตรวจคัดกรองมะเร็งปากมดลูกในปัจจุบัน งานวิจัยครั้งนี้จึงมีวัตถุประสงค์ในการศึกษาความชุกและการกระจายตัวของการติดเชื้อไวรัสเอชพีวีความเสี่ยงสูง และหาความสัมพันธ์ของผลการตรวจคัดกรองมะเร็งปากมดลูก 2 วิธี คือ วิธี HR-HPV testing และ liquid-based cytology

วัสดุและวิธีการ: ตรวจหาเชื้อเอชพีวีชนิดความเสี่ยงสูงด้วยเทคนิค PCR และ DNA hybridization โดยใช้ชุดตรวจ GP5+/6+ และตรวจหาจีโนทัยป์ด้วยเทคนิค PCR และไฮบริไดเซชันด้วยชุดตรวจ Linear Array HPV genotyping test kit

ผลการศึกษา: จากสิ่งส่งตรวจซึ่งเป็นเซลล์ที่เก็บจากบริเวณปากมดลูก 2,435 ตัวอย่าง พบว่า 145 ตัวอย่าง (6%) ให้ผลบวกกับการตรวจหาการติดเชื้อเอชพีวีชนิดความเสี่ยงสูงในจำนวนนี้ 81 ตัวอย่าง เป็นการติดเชื้อจีโนทัยป์เดียว อีก 64 ตัวอย่าง เป็นการติดเชื้อแบบ 2 จีโนทัยป์ ขึ้นไป เชื้อเอชพีวีจีโนทัยป์ 52 พบมากที่สุด รองลงมาคือ 16 และ 18

สรุปผลการศึกษา: การติดเชื้อเอชพีวีชนิดความเสี่ยงสูง มีความสัมพันธ์กับความผิดปกติของการเจริญเติบโตของเซลล์บริเวณปากมดลูก ดังนั้นการตรวจหาการติดเชื้อเอชพีวีชนิดความเสี่ยงสูง จึงเหมาะที่จะนำมาใช้เป็นวิธีการตรวจคัดกรองมะเร็งปากมดลูก อย่างไรก็ตาม การตรวจหาเอชพีวีชนิดความเสี่ยงสูงร่วมกับการตรวจคัดกรองมะเร็งปากมดลูกวิธีอื่นๆ จะช่วยเพิ่มความไวและความจำเพาะในการตรวจค้นหาความผิดปกติที่ปากมดลูกและมะเร็งปากมดลูก

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คำรหัส: ไวรัสฮิวแมนแปปปิโลม่า ไวรัสเอชพีวี มะเร็งปากมดลูก เอชพีวีความเสี่ยงสูง ความชุกของการติดเชื้อเอชพีวี

Abstract

Introduction: The persistent infection of high risk (HR) human papillomaviruses (HPV) induces precancerous lesions of human mucosal, cutaneous epithelia and further cause cervix abnormalities and developing of cervical cancer. Therefore, HR-HPV testing is recently used for cervical cancer screening. In this study, we aimed to determine the prevalence and genotypic distribution of HR-HPV and also to demonstrate the correlation between two methods of cervical cancer screening which are HR-HPV testing and liquid-based cytology.

Materials and Methods: HR-HPV testing was conducted employing the polymerase chain reaction (PCR) and DNA hybridization techniques with the utilization of the Diassay EIA HPV GP GP5+/6+ HR kit - a diagnostic kit for high risk human papillomavirus detection for cervical cancer. Genotyping was performed using Roche Linear Array HPV genotyping test kit. Cervical smears were interpreted using the 2001 Bethesda System.

Results: Cervical samples from a total number of 2,435 women were collected in this study. Out of these, 145 (6%) were found HR HPV positive. Eighty one of these HR-HPV positive cases were of single-genotype infection, while the remaining cases (64) were of double- and multiple-genotype infections. HPV genotype 52 was the most prevalent genotype, followed by genotype 16 and 58. Of 145 HR-HPV-positive cases, 46 (32%) showed abnormal cervical cytological results.

Conclusion: The HR-HPV test is an appropriate tool for screening of cervical cancer in women. However, the combination of HR-HPV test with other approved medical screening methods will enhance a higher level of sensitivity and specificity for detecting cervix lesions and cervical cancer.

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Keywords: Human papillomavirus, HPV, cervical cancer, high risk HPV, prevalence of HPV infection

Introduction

The incidence of cervical cancer in Thailand is ranked second highest among all cancer types in women age-standardized rate (ASR) = 16.7.[1] Cervical cancer has been ranked among the top 10 cancer types at Lampang Cancer Hospital located in the north region of Thailand. In 2013, 227 new cases of cervical cancer patients, (about 20%) were identified[2] Several validated and proven studies have shown that persistence of high-risk type of human papillomavirus (HR-HPV) (e.g. type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and a candidate type 67) infections are the main causes of cervix abnormalities which leading to cervical cancer.[3,4] In most developed countries, the incidence for cervical cancer is much lower than in Thailand. This could be due to the effectiveness of their respective 'Call and Recall' system of cervical cancer screening program. Such a program with regulated frequency of screening has evidence of benefits because early stages of cancer could be detected and the discovered lesions being timely treated and terminated. Screening program for cervical cancer can be conducted by various medically approved methods including HR-HPV test. Previous studies have shown that a combination of liquid based cytology Pap smear and HR-HPV test have yielded 99% sensitivity to detect cervical abnormalities and cancer.[5]

We report herein the prevalence of HR-HPV infection among women who participated in the organized cervical cancer screening program conducted in Lampang Cancer Hospital. HPV genotype distribution and a number of cervical abnormalities and cervical cancer in HR-HPV positive cases are also shown in this report.

Materials and methods

Study population

Cervical cells in liquid based brush cytology system obtained from 2,435 women who came to Lampang Cancer Hospital for cervical cancer screening during October 2013 - September 2014 were recruited in the study.

DNA extraction

The extraction of DNA from the 2,435 samples collected in this study was done by the viral nucleic acid kit (SpinStar™, Malaysia). Briefly, 500 µL of liquid-based brush cytology samples were centrifuged at 6200 xg for 10 minutes. Cell pellet was collected and proteinase K was added. Lysis buffer was added-in and pulse-vortex. Reaction was incubated at 65 ºC for 10 minutes. DNA was precipitated by absolute ethanol. The mixtures were subsequently transferred to the SpinStar™ column, centrifuged at 6200 xg for 2 minutes. The column was then placed in a clean collection tube and washed twice with washing buffer with centrifugation at 6200 xg for 2 minutes. After washing, membrane was dried by centrifugation at 17,000 xg for 10 minutes. Finally, the column was transferring to a clean 1.5 mL microcentrifuge tube and elution buffer was then added on to the center of the membrane, incubated at room temperature for 5 minutes and centrifuged at 9,600 xg for 2 minutes. Eluate containing DNA was stored at –20 ºC until use.

Human papilloma virus high risk type testing

DNA samples were tested for HR-HPV infection by GP5+/6+ PCR kit (Diassay, Netherlands). Forty µL of HPV GP PCR master mix containing polymerase enzyme and 10 µL of DNA sample were mixed. The reaction mixture was run in a real-time PCR machine (Bio-Rad CFX 96) for the thermal cycle run with 1 cycle at 95 ºC for 4 minutes, 40 cycles of 3 steps including 1) 94 ºC for 20 seconds 2) 38 ºC for 30 seconds 3) 71 ºC for 80 seconds followed by a final extension at 71 ºC for 4 minutes. The PCR products were stored at 4 ºC if DNA hybridization was performed on the next day.

PCR products were further detected by EIA started from the first step by immobilizing PCR products, negative control and positive control into the said ‘wells’. After 60 minutes of incubation at 37 ºC, washing step thrice was done. Denaturation solution was then added following by washing step following with pre-heated probe solution adding. After 5 times of washing, the excess probe conjugate was added and incubated in dark. Finally, stop solution was added and absorbance at 450 nm was observed within 10 minutes.

Genotyping assay

All positive samples detected by HR-HPV testing were sent to the AMS Clinical Service Center, Chiang Mai University for genotyping. Genotyping was conducted by Linear Array HPV Genotyping Test kit (Roche Diagnostics, Germany) and run on GeneAmp PCR System 9700 (Applied Biosystems®, USA). This method consists of four steps Firstly, specimen preparation; Lysis of cervical cells in the collected specimens, Isolation and Purification steps to obtain HPV DNA. The second step of PCR amplification is running the thermal cycler with one cycler of 50 ºC for 2 minutes, followed by a cycle of 95 ºC for 9 minutes and then a 40 cycle systemized in three steps of 1) 95 ºC in 30 seconds 2) 55 ºC in 1 minute 3) and 72 ºC in 1 minute. Upon completion, the reaction was run on 72 ºC for 5 minutes and held Amplicon at 72 ºC indefinitely. This was next followed by third step which is the hybridization of the amplified products. The last step was the colorimetric determination of probe-bound amplified products. This was done by adding streptavidin-HRP conjugate to bind with the biotin-labeled hybridization of amplicon and oligonucleotide probes and then, adding of substrate (TMB). Blue line (s) can be visually read and referred to the LINEAR ARRAY HPV Genotyping Test Reference Guide.

Cytology testing

Cervical samples were collected and preserved in liquid-based cytology medium (PathTezt, Biocytech Corp, Perak, Malaysia). Cervical smears were interpreted using the 2001 Bethesda System for reporting cervical cytology results.

Statistical analysis

Descriptive statistic and percentage of agreement were used in this study.

Results

A total of 2,435 women were attended in the HPV testing conducted at Lampang Cancer Hospital. Age ranged from 20-80 years old and 1,211 out of 2,435 (49.74%) women were menopause. The results revealed that 145 (6%) of the 2,435 women were found 'positive' for HR-HPV (Figure 1). Eighty-one of these 145 cases (56%) were single genotype infection, 30 samples (21%) were double genotype infections, and 6 samples (4%) were triple or more genotypes infections. There were 28 samples (19%) was untypable (Figure 2). Untypable of HPV in HR-HPV positive samples is still unclear to explain. However, it may be caused from the limitation of detection by the genotyping assay or false positive of HR-HPV Diassay test.

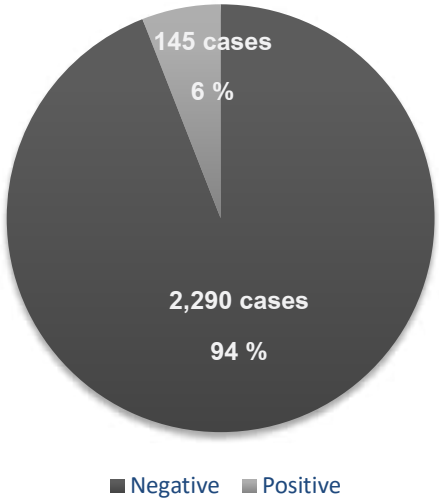

Figure 1 *The result of HPV GP5+/6+ Diassay test in Lampang Cancer Hospital during 1st October 2013 – 30th September 2014.*

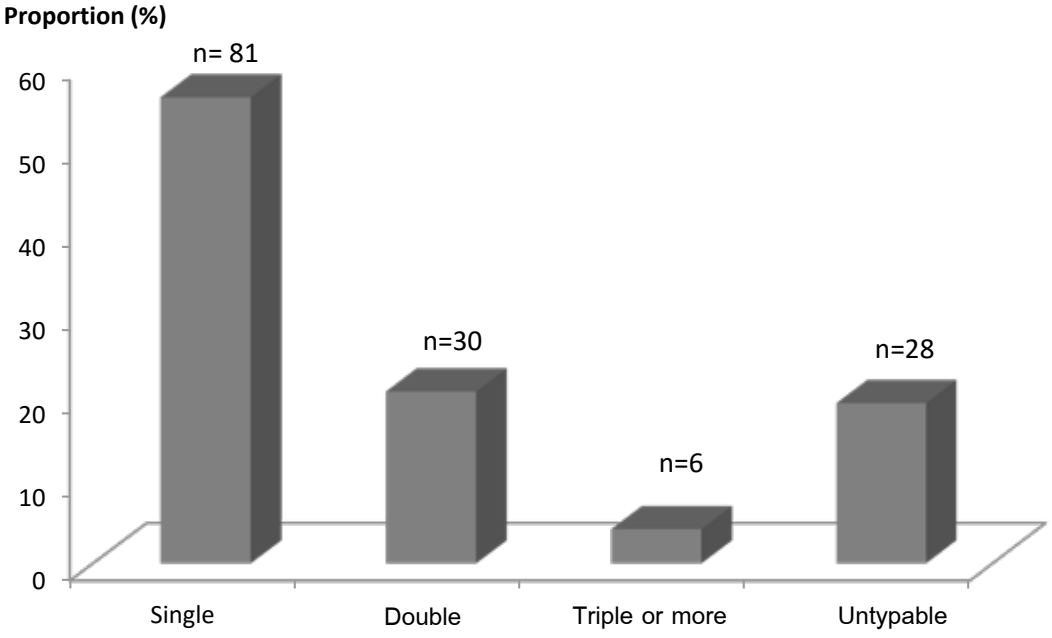

Figure 2 *Pattern of HPV infection among 145 samples positive by HR-HPV Diassay test.*

HPV genotype 52 was the most frequently or common genotype in this population. This was followed by genotypes 16 and 58, respectively, in both single and multiple infections. Surprisingly, HPV 18 was ranked at the fourth in this report (Figure 3). HPV genotype patterns of multiple infections were shown in (Table 1).

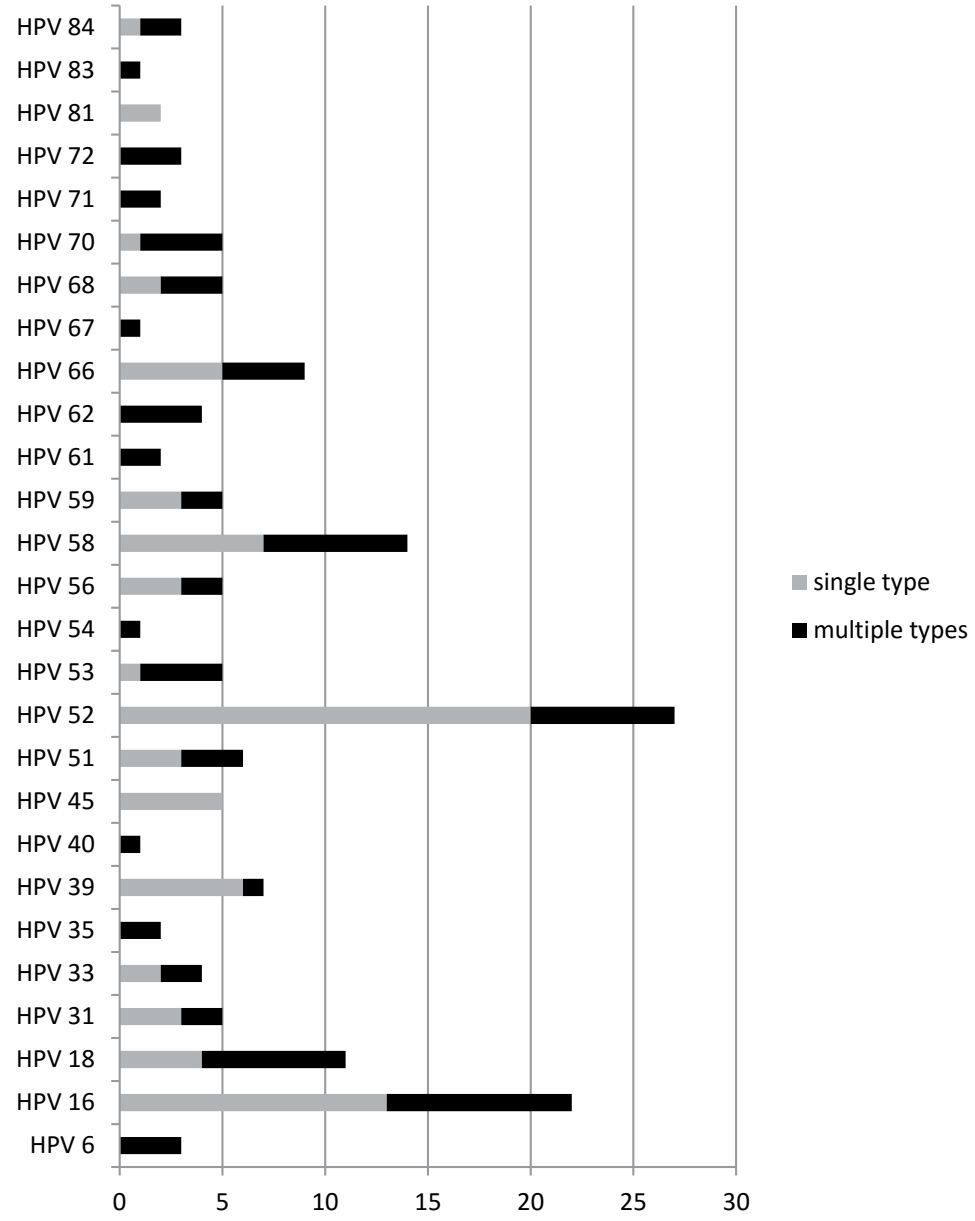

Figure 3 *Absolute frequency of HPV genotypes detected as single and multiple HPV infections.*

Table 1 HPV genotypes in double, triple and quadruple infection cases. (Each pattern n=1).

HPV Double Infection	HPV Triple Infection	HPV Quadruple Infection
18,71	18,56,70	52,68,70,72
18,62	66,68,83	
58,71	58,72,84	
67,70	18,52,66	
40,58	31,35,52	
18,52		
16,18		
58,61		
35,52		
51,52		
53,58		
16,72		
59,62		
16,70		
18,84		
16,54		
31,61		
16,66		
16,68		
6,56		
16,62		
62,66		
6,59		
53,58		
6,51		
16,51		
16,52		
53,58		
33,53		
33,39		

Prevalence of HPV infection in the age group of >70 years (13.34%) and <30 years (12.25%) showed the highest percentage among others. However, total case number of these 2 age groups was much less than other groups. Therefore, this part of result should be re-analyzed when the number of cases is increased in the future (Table 2).

Table 2 Percentage of HR-HPV positive in each age group.

Age group	HR-HPV positive (%, n/N)
< 30	12.25% (6/49)
31-40	7.86% (26/331)
41-50	7.15% (58/812)
51-60	4.63% (46/994)
61-70	3.0% (7/234)
>70	13.34% (2/15)

Note: *n = number of HR-HPV positive cases, N = total number of women in each age group.*

The report reveals only 46 out of 145 cases (31.7%) showed abnormal results by cytology test as shown in Table 3. The remaining balance cases were diagnosed as normal cervical cells by cytology test (Table 3). Percentage of agreement between HR-HPV and liquid-based cytology tests was 95.89% and kappa value was 0.4635. Kappa of 0.41-0.60 considered as moderate agreement, any kappa above 0 will become statistically significant (Table 3). Among 46 HR-HPV positive cases with an abnormal cytological result, 30 (65%) were identified as atypical squamous cells of undetermined significance (ASC-US), 5 (11%) were low grade squamous intraepithelial lesion (LSIL) and 8 (17%) were high grade squamous intraepithelial lesion (HSIL). Two cancer cases were diagnosed as one squamous cell carcinoma and one adenocarcinoma. One case presented atypical glandular cells (Table 4).

Table 3 Liquid-based cytology and Diassay results from 2,435 samples.

		Cytology		
		Normal	**Abnormal**	**Total**
Diassay (HR-HPV)	Positive	99 (4.07%)	46 (1.89%)	145 (5.96%)
	Negative	2289 (94.00%)	1 (0.05%)	2290 (94.05%)
	Total	2388 (98.07%)	47 (1.93%)	2435 (100%)

Table 4 Number of HR-HPV positive cases which found abnormalities of cervical cells from cytology report.

Cytologic Results	Number
ASC-US	30
LSIL	5
HSIL	8
Squamous cell carcinoma	1
Adenocarcinoma	1
Atypical glandular cell	1
Total	46

Note: *ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL high-grade squamous intraepithelial lesion.*

Discussion and Conclusion

The prevalence of HR-HPV infection among the women attended for cervical cancer screening at Lampang Cancer Hospital was 6%. Chansaenroj *et. al.* studied in a women health check at the King Chulalongkorn Memorial Hospital, found 8.7% prevalence of high risk HPV infections.[6] In our study, HPV genotype 52 was the most common and followed in ranking of genotypes 16, 58, 18 and 66, respectively. However, study of Chansaenroj *et.al*. showed that HPV 16 was the most common following with types 90, 71, 66 and 52.[6] The study was performed during 2008-2009 by conventional PC for HPV testing and sequence analysis for HPV genotyping. In contrast to the present work, PCR and EIA were used for HR-HPV testing and PCR with hybridization of amplified products were used for genotyping. Many evidences have shown that HPV 16 and 18 can lead to pre-neoplastic lesions in a shorter period of time compared to other types.[7, 8] In addition, HPV 58 was also the third most common type in cervical cancer for Asian[9] and related to a progression of high-grade squamous intraepithelial lesion (HSIL).[10] These finding affirmed that the most common types of high risk HPV infections were geographically varied from region to region.[11]

From this study, single HPV infection was found in 81 cases (56%) less than that of another which showed 89% of single infections[12] since attended population was aimed at only women coming for cervical cancer screening not cervix cancer focused group. Another study in cancerous lesion samples also found 30-37% of HPV single infection and were dependent on staging of the disease.[13] Totally, 64 cases (44%) of multiple HPV infections were uncovered in our study whereas other studies showed 11.6%.[12] Moreover, 29 (38%) from another report were dependent on the stage of cancer lesion tissues.[13]

The cause of cervical cancer is not confined only to the presence of HR-HPV infections but also viral genome status and host genetic factors.[14] Our results have also uncovered 46 cases (31.7%) of cervix abnormalities out of a total 145 HR-HPV positive cases. There was only one case demonstrated abnormalities of cervix by cytology test with HR-HPV negative. It revealed that almost of cervical abnormalities cases had positive for HR-HPV testing. From this study, HR-HPV testing could be a reliable screening test for cervical cancer because different types of high risk HPV viruses can be detected. For positive high risk HPV cases, the screening test will be further enhanced by a medically validated DNA methylation test where as a higher efficacy of cervical cancer screening program can be achieved.[15]

In conclusion, this report was performed using hospital liquid based cytology samples to do Pap smear and HR-HPV testing. The results revealed that HR-HPV infections can be found in women ages range from 24 to 75

years old. Approximately 32% of HR-HPV positive cases showed abnormalities of cytology reports. Single HPV infection was the most common (56%) and HPV 52 was the first rank or most common for both single and multiple type infections.

References

1. Khuhaprema T, Attasara P, Sriplung H, Wiangnon S, Sangrajrang S. Cancer in Thailand. Volume VII, 2007-2009, Bangkok; 2013.
2. Daoprasert K, Pagowong R, Wachirasoponkit S, Wongyai P, wongchai W, Chainam Y, et al. Hospital-Based Cancer Registry, 2003 (in Thai).
3. Schlecht NF, Platt RW, Duarte-Franco E, Costa MC, Sobrinho JP, Franco EL, et al. Human Paillomavirus Infection and Time to Progression and Regression of Cervical Intraepithelial Neoplasm. J Natl Cancer Inst 2003; 95(17): 1336-43.
4. Baseman JG, Koutsky LA. The epidemiology of human papillomavirus infection. J Clin Virol. 2005 Mar, 32 Suppl1: 516-24.
5. Srisomboon J. Genotyping and HPV Testing application in clinical hallmarks. 2010 Nov. Available from: http://www.med.cmu.ac.th/dept/obgyn/2011/index.php?Option=com_content&view=article&id=378:hpv-test&catid=40&Itemid=482 (in Thai).
6. Chansaenroj J, Lurchachaiwong W, Termrungruanglert W, Tresukosol D, Niruthisard S, Poovorawan Y, et al. Prevalence and Genotypes of Human Papillomavirus among Thai Women. Asian Pacific J Cancer Prev 2012; 13(4): 1151-8.
7. Castle PE, Solomon D, Schiffman M, Wheeler CM. Human papillomavirus type 16 infections and 2-years absolute risk of cervical precancer in woman with equivocal or mild cytologic abnormalities. J Natl Cancer Inst 2005; 97: 1066-71.
8. Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Schiffman M, et al. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst 2005; 97: 1072-79.
9. Clifford G, Franceschi S, Diaz M, Munoz N, Villa LL. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine 2006; 24(53): 26-34.
10. Song JS, Kim EJ, Choi J, Gong G and Sung CO. Significance of HPV-58 infection in women who are HPV-positive, cytology-negative and living in a country with a high prevalence of HPV-58 infection. PLoS One 2013; 8: 7.
11. Aromseree S, Chaiwongkot A, Ekalaksananan T, Kongyingyoes B, Patarapadungkit N, Pientong C. The Three Most Common Human Papillomavirus oncogenic types and their integration state in Thai women with cervical Precancerous lesions and carcinomas. J Med virol 2014; 86: 1911-9.
12. Chinchai T, Chansaenroj J, Swangvaree S, Junyangdikul P, Poovorawan Y. Prevalence of Human Papillomavirus genotypes in cervical cancer. Int J Gynecol Cancer 2012; 22: 1063-8.
13. Suthipintawong C, Siriaunkgul S, Tungsinmunkong K, Pientong C, Ekalaksananan T, Chongsuwanich T, et al. Human Papillomavirus preva lence, genotype distribution and pattern of infection in Thai women. Asian Pacific J Cancer Prev 2011; 12(14): 853-6

14. Hausen HZ. Papillomaviruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000 May 3; 92(9): 690-8

15. De Strooper LM, vanzummeren M, Steenberger RD, Bleeker MC, HesselinkAT, Meijer CJ, et al. CADM1,MAL and miR124-2 methylation analysis in cervical scrapes to detect cervical and endometrial cancer. J Clin Pathol 2014; 62(12): 1067-71