Human papillomavirus genotypes among infected Thai women with different cytological findings by analysis of E1 genes

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SUMMARY

Cervical cytological data may not be sufficient for cervical cancer screening and prevention. In this project, we determined HPV genotype among infected Thai women with different cytological findings by characterization of E1 genes. Five hundred and thirty-five specimens were tested by PCR amplification of the E1 genes. HPV genotypes were determined by sequencing, comparison with the GenBank database and were analyzed in relation to different cytological findings. HPV-DNA by PCR were typed and revealed 32 different genotypes. HR-HPV (HPV16, 18 or 52) was detected in all samples with cervical cancer cytology. HPV16 was most prevalent irrespective of cervical cytology. Moreover, HPV31 and 52 were most prevalent in the HSIL and LSIL groups whereas HPV66 was found mostly in the LSIL group. The LSIL group displayed the highest variation of HPV genotypes. Moreover, HPV31 and 52 predominated in the HSIL and LSIL groups especially HPV52 which was found in cancer samples. We hoped that these data of HPV genotypes can be used as preliminary data of HPV in Thailand and can serve as basic data for future research into the HPV genotype in south-east Asia.

KEY WORDS: Human papillomavirus, Cervical cytology, Genotyping, E1 gene

INTRODUCTION

Cervical cancer is the most common cancer in women worldwide. Its cause is human papillomavirus (HPV) and thus, knowledge of the biology, epidemiology and HPV infection has increased. HPV has been identified as a causal agent for cervical squamous neoplasia. HPV is a highly variable small, non-enveloped, icosahedral DNA virus that replicates in the nucleus of squamous epithelial cells. The virion particles consist of a single molecule of double-stranded circular DNA about 8,000 bp in size. Genital HPV types were classified into high- and low-risk HPV types, depending on their potential to induce invasive cancer. In a recent study by Munoz *et al.* (Muñoz *et al.*, 2003), HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 have been regarded as high risk (HR-HPV) and types 26, 53, and 66 as potentially high risk (probably HR-HPV), whereas types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and candidate HPV89 are regarded as low risk (Muñoz *et al.*, 2003).

In contrast, at a recent meeting of the IARC (Lyon, France) monograph series, the working group concluded that sufficient evidence was available only for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 to define those types as class I carcinogens for humans, where-

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as HPV6 and 11 were defined as possible carcinogenic types (Crawford *et al*., 1963). In Northeast Thailand, HPV genotypes 16 and 18 have been reported to cause approximately two out of three cervical cancer cases (Sriamporn *et al*., 2006). However, Clifford *et al.* having studied the worldwide distribution of HPV including Thailand at Lampang and Songkla, found that heterogeneity was a significant finding in this part of Asia (Clifford *et al*., 2005). Many millions of women are diagnosed every year with such abnormal smear.

Detection of type-specific HPV DNA in abnormal smears could be used as a more specific predictor of high-grade cervical intraepithelial neoplasia (Cuzick *et al.*, 1994) and identification of high-risk HPV genotypes may permit selection of patients who are at increased risk for disease (Cuzick *et al.*, 1995). Several genotyping methods have been developed to identify high-risk HPV in liquid-base cytology (LBC) samples and tissue samples (Sherman *et al*., 1997; Depuydt *et al*., 2003; Arbyn *et al*., 2004). Molecular techniques applied for HPV DNA detection (Hubbard, 2003) include a direct probe method using Southern blotting and in situ hybridization, signal amplification methods such as the hybrid capture II (HCII) assay (Lorincz, 1996), and target amplification methods by PCR (Burd, 2003). They are time-consuming and require relatively large amounts of highly purified DNA. HCII has received FDA approval for use in cervical cancer screening.

Moreover, HCII has proven reliable (Castle *et al*., 2004; Carozzi *et al*., 2005) and sensitive for detection of cervical precancer (Schneede *et al*., 2001; Schiffman *et al*., 2005). Nonetheless, it can only serve as a modestly specific predictor of cervical precancer and cancer risk (Castle *et al*., 2008). For HPV genotyping, target products amplified by PCR are subjected to sequence analysis (Asto *et al*., 2004), RFLP analysis (Yoshikawa *et al*., 1991; Bernard *et al*., 1994; Sasagawa *et al*., 2000), and hybridization with type-specific probes (Kleter *et al*., 1999; Han *et al*., 2006; Jiang *et al*., 2006). The most widely used PCR systems which target the L1 region of the virus are MY09-MY11 but the L1 region is subject to frequent multiple nucleotide variations. In contrast, the E1 gene contains a nucleotide region sufficiently conserved for primer design and a recently developed test kit focuses on E1 for HPV genotyping (Papillocheck, Frickenhausen, Germany). In addition, direct sequencing is considered the gold standard method as it yields the nucleotide sequence of HPV. Since HPV genotyping is critical for HPV vaccination trials and for monitoring the efficiency of HPV vaccines this project has focused on HPV genotyping based on the E1 region by using PCR analysis. This project aimed to investigate the prevalence of HR- HPV, probably HR-HPV and LR-HPV (HPV6 and 11) genotypes in Thai women with different cytological findings by direct sequencing of the E1 region. Determination and analysis of the most prevalent HPV genotypes can serve as preliminary data of HPV in Thailand. In addition, these results will also be useful to clinicians for diagnosis and prevention.

MATERIALS AND METHODS

The Ethics Committee of the hospital and faculty of Medicine, Chulalongkorn University, approved all study protocols. The HPV positive samples were chosen from among the specimens obtained during the patients' routine check up or investigation and treatment. All the studied specimens were anonymous with a coding number for analysis and permission was granted by the director of the hospital. Informed consents and IRB are approved under certification reference number is COA No. 559/2008 and IRB No. 206/51. In addition, all specimens were exclusively used for academic research and the patients were not remunerated. The HPV positive samples were randomly chosen from positive specimens' representative for each cytological category.

Sample collection
HPV samples of Thai women representing patients with different cytological data from Bangkok province were obtained from Samitivej Srinakharin Hospital and King Chulalongkorn Memorial Hospital, Thailand. The specimens originated from the patients' routine check up or investigation and treatment. All specimens were collected for cytology by LBC (ThinPrep®, Hologic, West Sussex, UK). The specimens were sent as anonymous with a coding number. All HPV samples were stored at -70 °C until used.

Cytological diagnosis

All 515 samples were initially screened by a cytotechnician and once more by a cytopathologist. Also, all 515 cases were confirmed by a gynecologist with clinical experience. Our cytopathologists have applied the following standards:
- Cytological Report System: The Bethesda System 2001.
- Recommendation for management: ASCCP guidelines.
- TQM-CQI in gynaecology and non-gynaecology: Mandated by Clinical laboratory improvement Amendments of 1988 (CLIA 88) applicable guide.

Normal cytological samples were selected from samples that were positive tested with Hybrid captures II (HCII). According to the HCII manufacturer's guidelines, a relative light unit/cut-off (RLU/CO) ratio ≥1 is interpreted as positive whereas a ratio RLU/CO below 1 is interpreted as negative.

Primer design

The following complete nucleotide sequences of HR-HPV genotypes were downloaded from the GenBank database: HPV6 (NC001355), HPV11 (M14119), HPV16 (HPV16R, EU118173), HPV18 (X05015, EF202155), HPV26 (NC001583), HPV31 (J04353), HPV33 (M12732), HPV35 (M74117), HPV39 (M62849), HPV45 (EF202159, EF202167), HPV51 (M62877), HPV52 (X74481), HPV53 (NC001593), HPV56 (EF177181, EF177178), HPV58 (D90400), HPV59 (X77858), HPV66 (EF177191, EF177185), HPV68 (DQ080079), HPV70 (HPU212941, HPU22461), HPV73 (X94165) and HPV82 (AB027021). Subsequently, alignments were performed using CLUSTAL X (Version 1.81 from ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) and BioEdit sequence alignment Software Version 5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Assay target regions were first identified by visual inspection of the sequence alignment. Primers were chosen from constant regions of all specific sequences. Primers and probes were analyzed using the primer design software OLIGOS Version 9.1 and FastPCR Version 3.8.20 (Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) to predict the percentage of G+C content, potential for dimerization, cross-linking and secondary structure.

DNA extraction

DNA was obtained by organic extraction (phenol-chloroform) of the samples. Briefly, cellular pellets were re-suspended in 400 µl lysis buffer. Samples were incubated at 95°C for 30 min, mixed for 2 min, and digested with 50 µl of proteinase K (20 g/l). After overnight incubation at 50°C, samples were heated to 95°C for 10 min to inactivate the proteinase K. Phenol-chloroform extraction followed by high-salt isopropanol precipitation was performed as described previously (Broccolo *et al.*, 2005) and purified material was re-suspended in a final volume of 30 µl deionized water, respectively.

Human papillomavirus (HPV) detection by amplification and HPV sequencing

Polymerase chain reaction was performed to amplify the E1 region of HPV. The reaction mixture comprised 2 µl DNA, 0.5 µM of each primer, 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions for the first round: initial denaturation at 94ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 45 s, and extension at 72°C for 1.00 min, and concluded by a final extension at 72 °C for 7 min. The primers employed were HPV-E1F1_1219 (nt 1160-1183); 5'- AGTACAGGTTCTAAAACGAAAGT-3' and HPV-E1R1_2119 (nt 2076-2100) 5'- CATTATCAAATGCCCAYTGYACCAT-3'. These primers yield a PCR product of 940 bp. Second round PCR was performed under the following conditions: Denaturation at 94ºC for 3 min, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, and concluded by a final extension at 72°C for 7 min. The primers employed were HPV-E1F2_1383 (nt 1315-1335) 5'-GCGAAGACAGCGGNTATGGC-3') and HPV-E1R1_2119. These primers yield a PCR product of 785 bp. The nucleotide positions of each primer were based on HPV16R (Myers *et al.*, 1997) and X05015.

Housekeeping gene detection

The housekeeping gene β-globin was selected to

serve as an internal control for DNA extraction, using conventional PCR as a detection method. Primer sequences for the β-globin gene have been previously described (Shadrina *et al.*, 2007)

Agarose gel electrophoresis
The PCR products were mixed with loading buffer and run on a 2% agarose gel (FMC Bioproducts, Rockland, ME) at 100 Volts for 60 minutes. After electrophoresis, the DNA bands were stained with ethidium bromide and visualized by UV transillumination (Gel Doc 1000, BIO-RAD, CA). Positive specimens were confirmed by direct sequencing serving as the gold standard and the resulting sequences were submitted to the Genbank database under accession numbers FJ610146-52 and GQ 161244-751.

Preparation of positive controls for HPV
The HPV genotypes were confirmed by direct sequencing. Subsequently, the purified products were inserted into the pGEM-T Easy Vector System (Promega, Medison, WI) as described previously (Lurchachaiwong *et al.*, 2008). The plasmids were extracted and purified using the FastPlasmid Mini kit (Eppendorf, Hamburg, Germany). These DNA preparations were used as positive controls and for the sensitivity test. The plasmids contained the E1 region of HPV 6, 16, 18, 30, 31, 33, 35, 39, 42, 51, 52, 58, 66, 68.

Sensitivity and specificity
DNA cloned as described above served as positive control for HPV detection and determination of test sensitivity. DNA concentration was determined by measuring OD_{260}. DNA samples with known concentrations were 10-fold serially diluted from 10^7-10 copies/µl and then used as templates for sensitivity tests. The specificity of the PCR assay was evaluated by cross reaction tests

with DNA and RNA viruses which might be present in vaginal secretions. We selected Hepatitis B virus (HBV), Parvovirus 4 (PARV4), Parvovirus B19, Human immunodeficiency virus (HIV), Hepatitis C virus (HCV) and Herpes Simplex virus (HSV-2).

Data analysis
All samples were genotyped by using direct sequencing as the gold standard. Sequences were analyzed with the BLAST analysis tool (http://www.ncbi.nlm.gov/BLAST) and compared with the GenBank database.
Positive specimens were confirmed by direct sequencing serving as the gold standard and the resulting sequences were submitted to the Genbank database under accession numbers FJ610146-52 and GQ 161244-751.

RESULTS

This project has been aimed at establishing the correlation between cytological data and HR-HPV genotypes based on PCR amplification of the E1gene.
Specificity and sensitivity of PCR amplification were analyzed and the results confirmed that none of our E1 primers (Table1) cross-reacted with HIV, Parv4, Parvovirus B19, HCV, HBV and HSV-2. Sensitivity of the E1 primers of HPV 6, 16, 18, 30, 31, 33, 35, 39, 42, 51, 52, 58, 66, 68 was determined at 10^2 copies/µl. Samples with PCR negative for the house keeping gene (β-globin) were excluded from this study.

Genotype distribution in relation to cervical cytology
All 515 samples originated from women between 19 and 83 years of age and tested positive for HPV

TABLE 1 - *Conserved E1 primers for amplification and sequencing of HPV.*

Gene	Round	Primer name	Sequence (5'-3')	[a]Position	Product (bp)
E1	1	HPV-E1F1_1219 HPV-E1R1_2119	AGT ACA GGT TCT AAA ACG AAA GT CAT TAT CAA ATG CCC AYT GYA CCA T	1110-1132 1941-1965	855
	2	HPV-E1F2_1383 HPV-E1R1_2119	GCGAAGACAGCGGNTATGGC CAT TAT CAA ATG CCC AYT GYA CCA T	1249-1268 1941-1965	716

[a]Position based on reference sequence HPV16R (32).

DNA by PCR amplification of the E1 gene. More specifically, 16 samples (3.11%) could be categorized as CA (cervical cancer), 98 samples (19.03%) as HSIL (high-grade squamous intraepithelial lesion), 136 samples (26.41%) as LSIL (low-grade squamous intraepithelial lesion), 79 samples (15.34%) as ASC-US (Atypical squamous cells of undetermined significance) and 186 samples (36.12%) as normal.

Cervical cancer cytology

The 16 cases positive for squamous cell carcinoma (15 samples) and adenocarcinoma (1 sample) contained HR-HPV of three different genotypes which were HPV 16 (11/16; 68.75%), 18 (3/16; 18.75%) and 52 (2/16; 12.5%) (Table 2). Based on our results, HR-HPV genotypes were found in 100% of the CA group.

HSIL cytology

These 98 samples mostly contained the HR-HPV genotypes 16 (40/98; 40.82%), 52 (15/98; 15.31%), 31 (13/98; 13.26%), 33 (4/98; 4.08%) and 18 (4/98; 4.08%) and other less prevalent HPV genotypes (miscellaneous) such as HPV 6, 30, 35, 39, 42, 44, 53, 55, 56, 58, 68, 70, 71, 73, 74 and 90. All mis-

TABLE 2 - *Amount of HPV genotype distribution in each categories cervical cytopathology (CA, HSIL, LSIL, ASC-US and Normal).*

	Cytological data (Number of samples)						
	CA	HSIL	LSIL	ASC-US	Normal	Total	Percentage
HPV6	-	1	1	2	1	5	0.97%
HPV11	-	-	-	-	1	1	0.19%
HPV16	11	40	19	12	42	124	24.08%
HPV18	3	4	1	2	2	12	2.33%
HPV30	-	1	2	1	1	5	0.97%
HPV31	-	13	19	12	20	64	12.43%
HPV32	-	-	1	1	4	6	1.17%
HPV33	-	4	1	1	-	6	1.17%
HPV34	-	-	4	6	6	16	3.11%
HPV35	-	1	6	1	7	15	2.91%
HPV39	-	2	9	3	7	21	4.08%
HPV40	-	-	1	-	-	1	0.19%
HPV42	-	1	4	1	8	14	2.72%
HPV44	-	1	-	-	-	1	0.19%
HPV51	-	-	9	2	3	14	2.72%
HPV52	2	15	12	5	10	44	8.54%
HPV53	-	1	3	-	3	7	1.36%
HPV55	-	1	1	1	1	4	0.78%
HPV56	-	3	7	2	2	14	2.72%
HPV58	-	3	1	1	-	5	0.97%
HPV59	-	-	2	5	8	15	2.91%
HPV66	-	-	13	7	13	33	6.41%
HPV68	-	1	6	5	9	21	4.08%
HPV70	-	1	2	-	1	4	0.78%
HPV71	-	1	2	3	10	16	3.11%
HPV73	-	2	1	1	-	4	0.78%
HPV74	-	1	2	2	2	7	1.36%
HPV81	-	-	1	-	-	1	0.19%
HPV82	-	-	1	1	-	2	0.39%
HPV85	-	-	-	-	1	1	0.19%
HPV90	-	1	5	2	23	31	6.02%
HPV91	-	-	-	-	1	1	0.19%
Total	16	98	136	79	186	515	100%

cellaneous genotypes were found in 22 samples (22/98; 22.45%) (Table 2). Based on our results, HR-HPV genotypes were found in 89.8% (88/98) of the HSIL group.

LSIL cytological data
These 136 samples mostly contained genotypes16 (19/136; 13.97%), 31 (19/136; 13.97%), 66 (13/136; 9.84%), 52 (12/136; 8.82%), 39 (9/136; 6.62%) and 51 (9/136; 6.62%) as well as the less prevalent miscellaneous HPV genotypes 6, 18, 30, 32, 33, 34, 35, 40, 42, 53, 55, 56, 58, 59, 68, 70, 71, 73, 74, 81, 82 and 90. Miscellaneous genotypes were found in 55 samples (55/136; 40.44%) (Table 2). HR-HPV genotypes were found in 69.12% (94/136) of the LSIL group.

ASC-US cytology
In the 79 samples with ASC-US cytology, we detected mostly HPV genotypes 16 (12/79; 15.19%), 31 (12/79; 15.19%), 66 (7/79; 8.86%), 52 (5/79; 6.33%), 59 (5/79; 6.33%) and 68 (5/79; 6.33%) along with less prevalent (miscellaneous) genotypes 6, 18, 30, 32, 33, 34, 35, 39, 42, 51, 55, 56, 58, 71, 73, 74, 82 and 90. Miscellaneous genotypes were found in 33 samples (33/79; 41.77%) (Table 2). HR-HPV genotypes were found in 67.09% (53/79) of the ASC-US group.

Normal cytology
The 186 samples with normal cytology mostly contained HPV genotypes 16 (42/186; 22.58%), 90 (23/186; 12.37%), 31 (20/186; 10.75%), 66 (13/186; 6.99%), 52 (10/186; 5.38%) and 71 (10/186; 5.38%) as well as less prevalent (miscellaneous) genotypes 6, 11, 18, 30, 32, 34, 35, 39, 42, 51, 53, 55, 56, 59, 68, 70, 74, 85 and 91. We detected miscellaneous genotypes in altogether 68 samples (68/186; 36.56%) (Table 2). HR-HPV genotypes were found in 59.14 % (110/186) of samples with normal cytology.

Distribution of HPV genotypes based on E1 amplification
Amplification with primers HPV-E1F2_1383 and HPV-E1R1_2119 yielded a 716-bp product which upon direct sequencing allowed for HPV genotype determination. Direct sequencing is the gold standard method for confirming PCR results. Our group classified HPV based on Munoz *et al.* into HR-HPV (high-risk HPV), probably HR-HPV, LR-HPV (low-risk HPV) and UR-HPV (undetermined risk). All 515 HPV DNA positive samples were typed and revealed 32 different genotypes: 70.09% (n=361) were determined as HR-HPV, 7.76 % (n=40) as probably HR-HPV, 5.24% (n=27) as LR-HPV and 16.89% (n=87) as UR. HPV16 was the most prevalent genotype at 24.08% (124/515), followed by HPV31 (12.43%; 64/515), HPV52 (8.54%; 44/515), HPV66 (6.41%; 33/515) and HPV90 (6.02%; 31/515). HPV genotypes 18, 32, 33, 34, 35, 39, 42, 51, 53, 56, 59, 68, 71 and 74 were less frequent (from 1.0% to 4.0%) and others were rare (below 1.0%). The percentage of frequency was arrived at based on previously published criteria (Pannier-Stockman *et al.,* 2008).

HPV genotype 16 appeared to be most prevalent, irrespective of cervical cytology whereas genotype 18 was mainly found in the HSIL and CA groups. Furthermore, HPV genotypes 52 and 31 were detected in the LSIL, HSIL and CA groups whereas HPV genotype 33 was found only in HSIL. HPV genotypes 31, 33 and 52 were mostly found in abnormal cytological samples. In addition, HR-HPV genotypes 66 and 68 were usually found in LSIL to normal groups (Table 2). Our results demonstrated that using the E1 primers facilitated PCR amplification not only of HR-HPV genotypes but also of probably HR-HPV, LR-HPV and others.

DISCUSSION

In this project, our group focused on HPV genotype distribution among different categories of cervical cytology by PCR amplification of the E1 gene. The E1 gene encodes a function essential for virus replication and has until recently served to classify HPV genotype (Papillomacheck, Frickenhausen, Germany). Alignment of the E1 genes of most HPV types showed high variation in nucleotide sequence (data not shown). Furthermore, parts of the E1 gene are conserved and can be used for primer design. Upon amplification, the resulting products were confirmed by direct sequencing. PCR amplification of the E1 gene can help detect a broad panel of genotypes and provides sequence information on HPV. With our E1 primers, we could detect 32 different genotypes including HR-HPV, probably HR-HPV, LR-HPV and others. Distribution of HPV geno-

types showed increased prevalence of HR-HPV in direct relation to the severity of cervical cytopathology (normal cytology (59.14%), ASC-US (67.09%), LSIL (69.12%), HSIL up to 89.8% and CA 100%). The results obtained in the course of this project correlate with those published in a previous report (Pannier-Stockman *et al.*, 2008) in that HR-HPV genotypes are mainly detected in HSIL and to a lesser extent in LSIL or samples displaying normal cytology. Thus, the presence of HR-HPV genotypes is associated with a risk of progression to cervical cancer.

Irrespective of cervical cytology, HPV16 was the most prevalent genotype detected. This finding correlates with a previous work focusing on L1 gene detection (Clifford *et al.*, 2005; Sriamporn *et al.*, 2006; Bae *et al.*, 2008; Paanier-Stockman *et al.*, 2008), whereas HPV18 was the second most prevalent in the CA group. HPV16 is associated with both squamous cell carcinoma (SCC) and adenocarcinoma whereas HPV18 is mainly a risk factor for development of adenocarcinoma (Bulk *et al.*, 2006). Previous reports have revealed that HPV18 is more predominant in adeno/adenosquamous carcinoma than in SCC (Smith *et al.*, 2007). The CA samples available for this study mostly comprised squamous cell carcinoma. Unfortunately, a single sample diagnosed as adenocarcinoma was found to contain HPV18. In the CA group, HPV18 was found to be less prevalent than HPV16, supporting the finding that, HPV 52 is the 7th most common cervical HR-HPV type worldwide, but the 5th most common in Asia (Smith *et al.*, 2007). However, due to small sample size, this finding should be verified by larger studies.

The HPV genotypes detected in the CA group were HPV16, 18 and 52, whereas the greatest diversity of HPV genotypes in the HSIL group was found HPV16, 52, 31, 18 and 33. More diversity of HPV in HSIL group is concerned with amount of samples in this study which was higher than CA samples. HPV16 and 18 are the most prevalent HPV types in cervical cancer worldwide (Bao *et al.*, 2003; Smith *et al.*, 2007), including Italy (Zappacosta *et al.*, 2009), the next most common HPV genotypes being 33, 45, 31, 58, 52 and 35 (Smith *et al.*, 2007). Meta-analysis of HPV distribution in HSIL revealed that HSIL infected with HPV16, 18 and 45 were progress to SCC (Clifford *et al.*, 2003) whereas our result showed

that HPV16, 52 and 31 were the most found in HSIL, respectively (Table 2). Smith *et al.* suggested that, among HSIL samples, certain HPV types have a specific risk for progression to cancer, dependent on the geographical region in question (Smith *et al.*, 2007). According to a recent study conducted on Korean women (Bae *et al.*, 2008), HPV 58 was the most prevalent genotype in HSIL and CA group after HPV16 and HPV18. In Japan HPV52 and HPV58 were more prevalent than HPV18 in HSIL but less prevalent than HPV18 in SCC (Miura *et al.*, 2006). Based on the results of our study, we detected HPV31, 33 and 52 more frequently among our samples than HPV58 and HPV18 in HSIL. Result of a previous study (Miura *et al.*, 2006) showed that the six most prevalent genotype in HSIL, HPV16, 18, 31, 33, 52, 58 in Japan, covered HPV genotypes found in our study. Moreover, genotype 66 (probably HR-HPV) was mostly found in LSIL, ASC-US and normal samples. HPV66 and 90 were most prevalent in samples with less abnormal cytology and were absent in HSIL and CA, whereas HPV16 can be found in all categories from normal to CA. In contrast, HPV90 is mainly found in Thai women with normal cytology but it could not be detected in any other category and hence, represents a virus of undetermined risk. LR-HPV is an important group because the genetic variation of it may concern with pathogenecity such as HPV81 (Minosse *et al.*, 2010). According to our and the above data (Bulk *et al.*, 2006; Smith *et al.*, 2007; Bae *et al.*, 2008; Bao *et al.*, 2008), a second generation HPV vaccine should focus on HPV 31, 33, 35, 45, 52 and 58 for prevention of HPV infection in all regions of the world, including Thailand.

Genotyping could be a useful tool for classification of HPV-positive women according to oncogenic potential and thus, relative risk of progression to CA as well as for evaluating the efficiency and epidemiological impact of vaccination programs. PCR and direct sequencing used for HPV genotyping in this project did not allow the detection of co-infection or multiple infections because only one major population of HPV can be detected by these methods. However, the advantage of applying these methods lies in their capacity to detect not only HR-HPV but also probably HR-HPV, LR-HPV and others up to 32 different genotypes. Furthermore, these molecular

methods have disclosed the various HPV nucleotide sequences, which can be subjected to further studies in the future. Sequencing is still a key element because this assay can identify HPV genotype. Even though it cannot identify co-infection genotype (*Carvalho et al., 2010*). In addition, our results are validated by L1-based assays by using MY09/MY11 and GP5+/GP6+ primers (Evander *et al*. 1992; Jacobs *et al*. 1995). We collected 243 samples on which Hybrid capture II (HCII) was performed and cytology data were available. Moreover, we examined the relationship between HCII relative light unit/cut-off (RLU/CO) ratios and PCR amplification results. However, we observed that the ratio (relative light unit of sample divided by positive cut off of HCII) may be correlated with the genotyping results of E1 and L1 detection (Lurchachaiwong *et al.*, 2009). These findings should be verified by larger studies, but may be used as preliminary data of HPV type distribution in Thailand. Future research of HPV genotype distribution in southeast Asia is required prior to the development of a new generation vaccine, to take into account the geographical differences that occur.

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