RESEARCH COMMUNICATION

High-risk Human Papillomavirus Genotype Detection by Electrochemical DNA Chip Method

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Abstract

High-risk human papillomavirus (HPV) genotypes are the major cause of cervical cancer. Hence, HPV genotype detection is a helpful preventive measure to combat cervical cancer. Recently, several HPV detection methods have been developed, each with different sensitivities and specificities. The objective of this study was to compare HPV high risk genotype detection by an electrochemical DNA chip system, a line probe assay (INNO-LiPA) and sequencing of the L1, E1 regions. A total of 361 cervical smears with different cytological findings were subjected to polymerase chain reaction-sequencing and electrochemical DNA chip assessment. Multiple infections were found in 21.9% (79/361) of the specimens, most prevalently in 20-29-year olds while the highest prevalence of HPV infection was found in the 30-39-year age group. The most prevalent genotype was HPV 16 at 28.2% (138/489) followed by HPV 52 at 9.6% (47/489), with the other types occurring at less than 9.0%. The electrochemical DNA chip results were compared with INNO-LiPA and sequencing (E1 and L1 regions) based on random selection of 273 specimens. The results obtained by the three methods were in agreement except for three cases. Direct sequencing detected only one predominant genotype including low risk HPV genotypes. INNO-LiPA identified multiple infections with various specific genotypes including some unclassified-risk genotypes. The electrochemical DNA chip was highly accurate, suitable for detection of single and multiple infections, allowed rapid detection, was less time-consuming and was easier to perform when compared with the other methods. It is concluded that for clinical and epidemiological studies, all genotyping methods are perfectly suitable and provide comparable results.

Keywords: Genotyping - high-risk human papillomavirus - DNA chip

Asian Pacific J Cancer Prev, **13**, 1151-1158

Introduction

Human papillomavirus (HPV) is related to the development of cervical cancer and precancerous lesions. HPV is transmitted generally to the surface of the cervical epithelium by sexual skin-to-skin contact and can not be transferred by inanimate objects. Screening should be performed within 2-3 years after the first sexual relationship. HPV infection is predominant in younger age groups, will clear within 1-2 years and proceed to cancer rarely. Promiscuity may increase the risk of HPV infection (Koutsky, 1997; Vizcaino et al., 2000; Wright et al., 2002). Most infections are asymptomatic and transient. The host immune system may clear the HPV infection in low-grade squamous intraepithelial lesions (LSIL) spontaneously with the tissue reverting to normal in 47% of patients while 20% may develop high-grade squamous intraepithelial lesions (HSIL) within 2 years (Melnikow et al., 1998).

According to a cohort study, HPV cumulative incidence rates are associated with younger women, certain ethnic groups and high frequency of alcohol consumption. Forty-three percent of women, who had attended at least one follow-up visit, were HPV negative. The incidence tended to decrease by 20% in the first year, 14% and 9% in the second and third years, respectively. The median duration of HPV infection was 8 months. 70% of women were no longer infected one year after the incident and 9% continued to be infected for two years. The longest median duration of infection was established for HPV 16, 18, 61, 73 and AE7. In developed countries, the highest rates of HPV infection have been observed among 15-25-year old women who might reflect transmission during their first sexual intercourse whereas the infection rate

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appears to have become stationary in the age groups of 40 years or older (Schiffman et al., 1993; Ho et al., 1998). Diagnosis of abnormal cervical epithelial cells is usually obtained by Papanicolaou (Pap) smears. Cytology based diagnosis has been found accurate in only 40-80% of high-grade cervical intraepithelial neoplasia and thus, can present a problem in terms of interpretation errors or sampling (Cuzick et al., 1995). The major problem is that abnormal cells are missed during collection or missed on microscopic examination (Cuzick, 2002). Papillomaviruses can neither be detected by serological assays nor can they be cultured. Several diagnostic tests based on molecular biology apply various techniques such as direct sequencing, hybridization with type-specific probes or restriction fragment length polymorphism analysis. Combination of several techniques should yield optimum performance in both research and clinical settings. The advantage of HPV DNA testing is its higher sensitivity so that infection can be detected prior to the onset of cervical disease. The common HPV genotypes in cervical tumors worldwide are high-risk HPV genotypes 16, 18, 31, 33 and 45 (Munoz et al., 2000). Globally, the predominant cause of invasive cervical cancers is HPV16 accounting for 52-58% followed by HPV 18 accounting for 13-22%. The remainder is due to other HPV types depending on geographical variations and ethnicity of the population (Smith et al., 2007). For example in Asian populations, HPV genotypes 52 and 58 are usually found while they are rare in Western countries (Lai et al., 1999). HPV typing provides important data for the distinction between high and low risk. Women positive for high-risk HPV genotypes within a short time proceed from atypical squamous cells of undetermined significance (ASCUS) to more severe lesions and the lesions are more extensive compared with low-risk HPV genotypes (Schlecht et al., 2003). Hence, cervical cancer prevention programs could integrate molecular HPV testing either as a screening tool, triage policies of women with borderline or mildly dyskaryotic (BMD) smears and post-treatment observation (Snijders et al., 2003).

The HPV DNA tests usually focus on a region of the L1 major capsid-forming gene (Gravitt et al., 2000). For example, Hybrid capture 2 test (HCII), (Digene, Gaithersburg, MD), INNO-LiPA, (Innogenetics, Belgium) and polymerase chain reaction (PCR) with used commonly primer systems have been used largely worldwide. The HCII assay is an available kit commercially which has been used widely for HPV detection and routine screening. According to the manufacturer's guidelines, relative light unit/cut-off (RLU/CO) ratios that are ≥ 1.00 indicate a positive result while a ratio below 1 is interpreted as a negative result. The Food and Drug Administration (FDA) approved the use of HPV DNA testing as an adjunct to cervical cytology screening in women aged over 30 years to manage ambiguous cytology results and with a cut-off for a positive test result of 1.0 pg HPV DNA/ml or about 5,000 HPV DNA copies, 13 high/probable high-risk and 5 low-risk genotypes (Wright et al., 2004) can be detected. Its main limitation is that the result does not show a specific genotype of HPV but that it provides a positive result when at least one genotype is present. Based on previous studies the results between borderline negative and positive (grey zone) are difficult to interpret. The range of the grey zone should encompass RLU/CO between 0.4 and 4. The HCII was recommended to be combined with another method such as PCR because this method is less sensitive than PCR (Cope et al. 1997; Federschneider et al., 2004; Seme et al., 2006). PCR based HPV detection usually relies on degenerate and/or consensus primers to identify the specific HPV types in the highly conserved region of the L1 gene (Resnick et al., 1990). The consensus primers have been developed to amplify the E1 regions for HPV identification (Klug et al., 2008). Yet, multiple infections cannot be detected by PCR and hence, oncogenic genotypes might not be identified as such (Karlsen et al., 1996). For epidemiological studies and patient management, the incidence of multiple infections and diversity of genotypes makes the development of reliable methods a necessity (Sotlar et al., 2004). Up to 25% of infections are due to multiple HPV genotypes as is shown by HPV typing regularly. Multiple genotypes have an effect on the probability of underlying pre-cancerous lesions and make clinical data interpretation very complex (Herrero et al., 2005; Plummer et al., 2007). Multiple HPV infections have been found among women with mild or moderately abnormal lesions but their persistence and progression are unclear (Rousseau et al., 2003).

A new HPV detection technique using an electrochemical DNA chip system has been developed. Applying this technique, single or multiple infections of 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) can be detected. In this system, all processes from reaction through measurement and analysis have been integrated into single compact equipment. It is based on Loop-mediated isothermal amplification (LAMP) and hybridization with a specific DNA probe on a gold electrode which high sensitivity. This method relies on a current-detection technique which measures the charge released by an electrochemical agent. With this system, HPV can be detected in a short time (Figure 1).

The aim of this study has been to detect HPV genotypes using PCR within the L1 and E1 regions of the HPV genome using consensus primers followed by direct sequencing, Electrochemical DNA chip and INNO-LiPA in groups of patients with various cervical lesions to

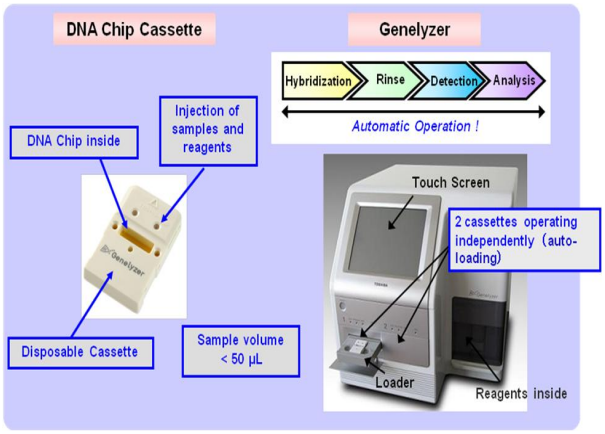

Figure 1. Principle of Electrochemical DNA Chip Technique and Materials

establish an effective procedure for virus genotyping in clinical samples.

Materials and Methods

All specimens were collected from cervical smears at the Department of Pathology, Samitivej Hospital, Bangkok, Thailand and Department of Gynecology, National Cancer Institute, Bangkok, Thailand between December 2010 and April 2011. Cytology results were assessed by a pathologist. All specimens were kept in LBC buffer (ThinPrep®, Hologic, West Sussex, UK) or phosphate buffered saline (PBS). The specimens were sent as anonymous with a coding number and patient's age. All specimens were stored at –70 °C until used. The protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB 148/53).

Study population

A total of 361 cervical smear samples were collected for confirmation of a positive diagnosis by PCR and Electrochemical DNA chip method. The ages of the patients enrolled in this study were between 19 and 88 years. The specimens were divided into 4 groups: Normal, 76 samples, low-grade squamous intraepithelial lesions, 69 samples, high-grade squamous intraepithelial lesions, 60 samples and invasive cervical cancer, 156 samples. The control group comprised specimens with normal cytology and HPV DNA negative by PCR. From this collection of samples, 273 were selected randomly for screening using the INNO-LiPA and 124 were selected randomly for screening using the Hybrid capture 2.

DNA isolation

DNA extraction and purification were performed using the Qiamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. After extraction, all specimens were subjected to PCR amplification of the β-globin gene to serve as an internal control as described previously (Shadrina et al., 2007) and stored subsequently at –20°C until tested.

HPV Detection and Genotyping

Polymerase Chain Reaction, the Polymerase Chain Reaction (PCR) amplification was performed within the L1 and E1 regions of the HPV genome with consensus primers and reaction conditions as described previously (Lurchachaiwong et al., 2009) in a thermal cycler (Eppendorf, Hamburg, Germany). A negative control was included in each assay. The PCR products were subjected to electrophoresis in a 2% agarose gel (FMC Bioproducts Rockland, ME) stained with ethidium bromide and HPV positive samples were detected subsequently by UV transillumination (Gel Doc 1000, BIO-RAD, CA) and purified with the HiYield Gel/PCR DNA Fragments Extraction kit (RBC Bioscience, New Taipei, Taiwan) according to the manufacturer's specifications. The purified products were subjected to direct sequencing by FirstBASE Laboratories SDNBHD (Selangor Darul Ehsan, Malaysia) and the HPV sequences were analyzed by the BLAST program available at the GenBank database (http://www.ncbi.nlm.gov/BLAST).

Electrochemical DNA chip, samples positive for HPV DNA by direct sequencing were selected for Electrochemical DNA chip. The Loop mediated isothermal amplification (LAMP) reaction and detection were performed using Electrochemical DNA chip (Toshiba, Tokyo, Japan) according to the manufacturer's instructions. Specimens obtained from the control groups served as negative controls. Genotyping for HPV was accomplished by hybridization and subsequent detection by Electrochemical DNA chip. The Electrochemical DNA chip contained specific DNA probes in the L1 region of 13 high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The LAMP conditions were denaturation at 95 °C for 5 min, followed by 90 min at 65 °C and 5 min at 80 °C. The automated hybridization was performed using a GenelyzerTM for Medical Field GLH-2C601 (Toshiba, Tokyo, Japan). The principle of electrochemical DNA chip and the instrument are shown in Figure 1. The results are shown in the bar chart between electric current peak values on the Y-axis and HR-HPV genotypes on the X-axis. The cross reaction between LAMP primers using PCR amplicon and the target for LAMP was checked. The specific primer amplified only for the respective target and unamplified for other unrelated targets was chosen. The cross hybridization of the electrochemical DNA chip was not observed. The sensitivity of the DNA chip was validated by using 3DNA chips against different sample concentrations ranging from 250 copies/reaction to 50 copies /reaction for 13 HPV types. The DNA chips could detect all 13 HPV types at the lowest concentration of 100 copies/reaction.

INNO-LiPA of the samples confirmed positive by the Electrochemical DNA chip, 273 samples were selected randomly for testing with the INNO-LiPA method (INNO-LiPA Genotyping Extra, Innogenetics, Belgium). HPV 6 from the INNO-LiPA kit was used as positive control. HPV negative samples were used as negative control. Amplification and Hybridization steps were performed in the L1 region using INNO-LiPA HPV genotyping Extra Amp and INNO-LiPA HPV genotyping Extra according to the manufacturer's instructions. Applying this assay, 28 HPV genotypes could be identified covering high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), probably high-risk (HPV 26, 53 and 66), low-risk (HPV 6, 11, 40, 43, 44, 54 and 70), some unclassified-risk HPV genotypes (HPV 69, 71 and 74) and HPV X, respectively. The AutoBlot 3000H machine was used for the hybridization process. For fully automated interpretation, the scanner and LiRAS (Line Reader & Analysis Software, Innogenetics, Belgium) for LiPA HPV software was used. The results were considered concordant when at least one genotype was identical by all assays. Data were considered discordant when one or more genotypes were different in all assays.

Hybrid Capture 2 (HCII), 124 samples were selected randomly for testing by HCII (Digene, Gaithersburg, MD). This test is a method used widely based on amplification and hybridization of the HPV DNA. It has been calibrated to detect 13 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 low-risk

Table 1. Comparison of Three HPV Genotyping Methods

A. Electrochemical DNA chip – PCR (Total = 361)

Result	PCR			
	L1 (%)		E1 (%)	
	Ve+	Ve-	Ve+	Ve-
Electrochemical DNA chip:				
Positive;		16 (4)		13 (3.6)
Same result	184 (51)		190 (53)	
More types detected	71 (20)		67 (19)	
Discordance (types included)	2 (0.6)		12 (3.3)	
Discordance (types not included)	26 (7.2)		16 (4.4)	
Negative;		46 (13)		38 (11)
Included	1 (0.3)		3 (0.9)	
Not included	15 (4.2)		22 (6.1)	
Total	299 (83)	62 (17)	310 (86)	51 (14)

B. Electrochemical DNA chip – INNO-LiPA (Total = 273)

Result	INNO-LiPA (%)	
	Ve+	Ve-
Electrochemical DNA chip: Positive;		
Same result	165 (60.4)	4(1.5)
More types detected	36 (13.2)	
Discordance (types included)	2 (0.7)	
Less types detected (types included)	19 (7.0)	
Less types detected (types not included)	21 (7.7)	
Negative;		11 (4.0)
Included	3 (1.1)	
Not included	12 (4.4)	
Total	258 (94.5)	15 (5.5)

C. INNO-LiPA – PCR (Total = 273)

Result	PCR			
	L1 (%)		E1 (%)	
	Ve+	Ve-	Ve+	Ve-
INNO-LiPA: Positive;		14 (5.1)		14 (5.1)
Same result	166 (61)		167 (61)	
More types detected	51 (18.7)		52 (19.0)	
Discordance (types included in)	14 (5.1)		17 (6.2)	
Discordance (types not included)	13 (4.8)		8 (2.9)	
Negative;		11 (4.0)		5 (1.9)
Included	1 (0.4)		9 (3.3)	
Not included	3 (1.1)		1 (0.4)	
Total	248 (91)	25 (9.1)	254 (93)	19 (7)

genotypes (6, 11, 42, 43 and 44). The HCII test was performed by following the manufacturer's instructions with the automated HCII system (Terry et al., 2001). The results are shown as relative light unit/cutoff (RLU/CO), the ratio related to the luminescence of the 1.0 pg/ml HPV 16 standard from the kit. An RLU/CO ratio < 0.4 is considered a negative result, a ratio > 4 is positive while a ratio between 0.4 and 4 is a grey zone. However, with this test specific HPV genotypes cannot be identified (Federschneider et al., 2004; Seme et al., 2006).

Table 2. Percentage of the Comparison of Genotyping by Three Assays

Method/genotyping result	% Concordance	% Discordance
Sequencing L1 region & Electrochemical DNA Chip	83.3	16.7
Sequencing E1 region & Electrochemical DNA Chip	81.7	18.3
Electrochemical DNA Chip & INNO-LiPA	84.6	15.4
INNO-LiPA & Sequencing L1 region	83.5	16.5
INNO-LiPA & Sequencing E1 region	82.1	17.9

Results

The L1 and E1 regions of the HPV genome was selected for amplification and those specimens negative for the internal control (β-globin) were excluded. Some results showed different genotypes in each region especially in the low-grade squamous intraepithelial lesions group, which may be due to multiple infections. To solve this problem, the Electrochemical DNA chip was used and compared the results with INNO-LiPA and HCII. The GenelyzerTM for Medical Field GLH-2C601 (Toshiba, Tokyo, Japan) was used to interpret "Positive" if the difference of average current peak values between each type and the negative control was 10 nA or more and "Negative" if the difference of average current peak values between each type and the negative control was less than 10 nA.

Comparison of three HPV genotyping methods

More HPV genotypes can be detected by the Electrochemical DNA chip than by the INNO-LiPA method 13.2% (36/273) (Table 1). When comparing the Electrochemical DNA chip and INNO-LiPA methods with direct sequencing, the results showed nearly 20% of the sequenced samples had multiple infections. In addition, the Electrochemical DNA chip provided more discordant results than INNO-LiPA because the Electrochemical DNA chip cannot detect uncommon HPV genotypes. A comparison between genotyping results is shown in Table 2. The results are most concordant between Electrochemical DNA chip and INNO-LiPA (84.6%) while they are most discordant between Electrochemical DNA chip and sequencing in the E1 region (18.3%). The results most concordant with Electrochemical DNA chip are obtained by INNO-LiPA while the more uncommon HPV genotypes can be detected by direct sequencing.

Interestingly, some HPV genotypes which are high-risk, low-risk and unclassified-risk such as HPV 53, HPV 82, HPV 34, HPV 44, HPV 67 and HPV 84 were detected in CA samples by this method, while the probably high, low and unclassified-risk genotypes such as HPV 66, HPV 11 and HPV 74 were found in other groups especially in the normal group by direct sequencing. Upon comparing between Electrochemical DNA chip and INNO-LiPA, 15 cases showed discordant results. In three cases, Electrochemical DNA chip results were negative, whereas the INNO-LiPA detected HR HPV genotypes. The remaining cases were HR, pHR and LR and unclassified HPV.

Table 3. Concordance Between HCII and PCR HPV Genotyping and Between HCII and Electrochemical DNA Chip Test (Total = 124)

HCII results	PCR results (%)		Electrochemical DNA chip results (%)		Total (%)
	Ve+	Ve-	Ve+	Ve-	
Positive (>4)	85 (69)	1 (0.8)	82 (66)	4 (3.2)	86 (69)
Grey zone (0.4-4)	5 (4.0)	3 (2.4)	2 (1.6)	6 (4.8)	8 (6.4)
Negative (<0.4)	7 (5.7)	23 (19)	5 (4.0)	25 (20)	30 (24)
Total	97 (78)	27 (22)	89 (72)	35 (28)	124 (100)

Table 4. Prevalence of HPV DNA in Each Age Group by Electrochemical DNA Chip (10 Years Interval).

Age group	Normal		Squamous intraepithelial lesions				Cervical cancer		Total (%)
			Low-grade		Hight-grade				
	S[a]	M[b]	S[a]	M[b]	S[a]	M[b]	S[a]	M[b]	
<20	-	-	-	1	-	-	-	-	1 (0.3)
20-29	5	6	8	15	2	7	3	2	48 (13.6)
30-39	13	2	15	11	13	7	22	2	85 (24.1)
40-49	4	-	4	1	17	3	37	8	74 (21.0)
50-59	1	1	-	-	4	1	41	4	52 (14.8)
≥60	1	-	-	-	2	-	20	6	29 (8.2)
Total	24	9	27	28	38	18	123	22	289 (82.1)
Negative	41	-	8	-	2	-	11	-	62 (17.2)
No age	2	-	6	-	2	-	-	-	10 (2.8)

[a]Single, [b]Multiple

Comparison of HPV genotyping between and HCII

As shown in Table 3, 86/124 (69.4%) of specimens was positive by HCII. The percentage of positives by Electrochemical DNA chip was less than by PCR-sequencing because uncommon HPV genotypes were found such as HPV 66, HPV 42, HPV 71, HPV 81 and HPV cand 62 which cannot be detected by Electrochemical DNA chip.

Prevalence of HPV genotype by Electrochemical DNA chip

As shown in Table 4, the cytology grade was mild among younger age groups as opposed to severe among older ones. Unfortunately, information on the age of 10 samples could not be obtained. The average age of all samples is 41.6 years. Analysis of HPV genotypes by Electrochemical DNA chip showed that the highest prevalence of single infection was found in the CA group and decreases slightly in the high-grade squamous intraepithelial lesions, low-grade squamous intraepithelial lesions and normal group, respectively. Multiple infections are predominant in the low-grade squamous intraepithelial lesions group. High prevalence of HPV infection was found in the 30-39-year age group and decreased slowly in older age groups. In addition, multiple infections are predominant in the 20-29-year age group. Single, double and triple or more infections were found in 60.7% (219/361), 14.4% (52/361) and 7.5% (27/361), respectively. 17.5% (63/361) of the samples were HPV negative. The highest incidence of multiple HPV genotype infections in this study was six. The frequency of HPV genotypes found in this study was as follows: HPV 16 28.2% (138/489), HPV 52 9.6% (47/489) with the other types amounting to less than 9.0%. When classified by cytology, the highest frequency of HR-HPV genotype in all groups was HPV 16 except for low-grade squamous intraepithelial lesions (HPV 52). HPV 18 was the second most frequent genotype in the cervical cancer group only (Figure 2).

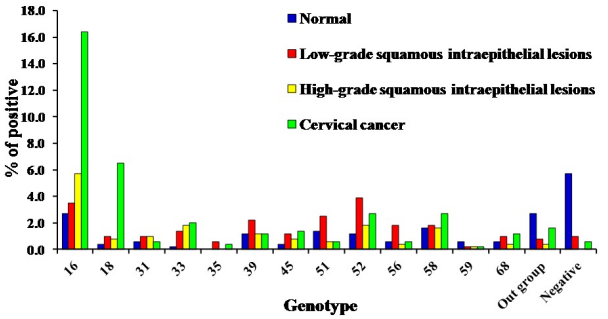

Figure 2. Distribution of HPV Genotypes by Electrochemical DNA Chip

Discussion

In young women, about 50% of new infections of most low-grade squamous intraepithelial lesions may disappear spontaneously within 12 months whereas the clearance rate in older women is lower (Chumworathayi et al., 2010). Approximately 1% of low-grade squamous intraepithelial lesions and 12% of high-grade squamous intraepithelial lesions may progress to invasive cervical cancer (Clifford et al., 2005) and more than 90% of HR-HPV genotypes pose an increased risk for progression to cervical cancer. Thus, identification of HR-HPV genotypes may help prevent development of cervical cancer and is important for developing HPV vaccines suitable for each area. According to other studies, diagnosis based on cytology showed various multiple infections in 7% to 23% of patient samples (Rousseau et al., 2003). The infection shows a peak in young women aged below 25 and decreases slightly to less than 4% above the age of 40 (Peto et al., 2004). The variations depend on characteristics of the population studied and detection methods. Geographical, clinical factors and demographics contribute to various extents to multiple HPV infections (Trottier et al., 2006). Many assays available for clinical and research use have shown different results depending on PCR efficiencies for each genotype. HPV assays should be standardized, reliable and accurate. To identify multiple genotypes most efficiently, a combination of typing methods and cytology is more suitable for risk assessment but expensive for routine diagnosis (Galan-Sanchez et al., 2009). Multiple infections, which increase the risk of invasive cervical cancer compared with single infection, are usually found in low-grade squamous intraepithelial lesions patients (Tucker et al., 1993).

In this study, a new, highly sensitive and time saving Electrochemical DNA chip method was used and compared with other HPV testing methods (PCR direct sequencing, HCII, INNO-LiPA). Upon testing with Electrochemical DNA chip, the highest prevalence of HPV infection was found in the 30-39-year age groups

(24.1%) and decreased slightly. Single HPV infection was more prevalent than multiple HPV infections. HPV 16 is the most prevalent genotype in high-grade squamous intraepithelial lesions (33.7%) and cervical cancer (42.1%) and the second most predominant genotypes HPV 33, HPV 52 (10.8%) and HPV 18 (16.8%), respectively which correlated with other findings that HPV 16 is the most prevalent genotype and HPV 18 is the second in the CA group, nearly the world average (15.0%) (Muñoz et al., 2003; Clifford et al., 2005). In addition, the third most prevalent genotype in both groups is HPV58 (9.6% and 6.8% respectively) and the remaining genotypes amount to 35.1% and 34.3%. Interestingly, a high incidence of HPV 58 has been found in Asia, Africa and other areas while it is not common worldwide especially in Europe and North America. According to a study conducted in Korea and Japan, HPV 58 is highly prevalent in high-grade squamous intraepithelial lesions and squamous cell carcinoma (Miura et al., 2006). A previous study of HPV 58 described its evolution and spread. The original source of ancient HPV58 may have been in West Africa and Southeast Asia may be a subsequent "relay center" (Chan et al., 2002; Li et al., 2009). These results have shown the diversity of HPV genotypes which provide information with regard to the design of multivalent prophylactic vaccines suitable for each geographical area.

The negative results in Electrochemical DNA chip and "X" results in INNO-LiPA were compared with the direct sequencing results and showed some unclassified-risk genotypes such as HPV 34 and HPV 67. Among phylogenetic species, HPV 34 and HPV 67 were found within α11 and α9, respectively with carcinogenic genotypes and thus, had been found in the cancer group (Matsukura et al., 2004; Castle et al., 2008). Furthermore, the genotyping method in this study (Electrochemical DNA chip, Toshiba) was designed to detect only 13 HR-HPV genotypes and the INNO-LiPA kit does not include either HPV 34 or HPV 67 and thus, some uncommon genotype may have been missed. However, the sequencing method applied has the capacity to detect these genotypes and others which are uncommon such as HPV 67, HPV 70 and HPV 84. HR-HPV testing can be applied to primary screening and management. For this reason, clinical laboratories should determine advantages and disadvantages of each method for HPV typing. All methods produced highly similar results. HCII is used widely for HPV detection but it cannot identify specific genotypes. The HCII ratios within the "grey zone" are difficult to interpret. Direct sequencing may only show the predominant genotype, is time consuming and multiple infections are difficult to detect. However, it provides nucleotide sequence information with regard to mutation or evolution of HPV. INNO-LiPA can detect multiple infections and found some uncommon genotypes. In the previous study, the prevalence of probably high-risk, low-risk and unclassified-risk was high in low-grade squamous intraepithelial lesions grade and normal cytology such as HPV 66, HPV 11 and HPV 71, respectively (Chansaenroj et al., 2010). Therefore, the INNO-LiPA technique may be useful for detecting HPV genotypes in patients with cytology under the low-grade squamous intraepithelial lesions grade to prevent progression to cervical cancer and for follow-up of women who are at risk. With some genotypes, one probe was sufficient for genotyping while multiple probes were required for other genotypes. Thus, some genotypes for which the same probes were used could not be discriminated. Although, multiple HPV infections were found in 10-40% of HPV positive cases (Tsao et al., 2010), the relation between multiple HPV infections and development of cervical carcinoma has remained inconclusive. Still, various results have contributed to the notion that this may be possible. A study on the odds ratio of risk factors showed that multiple infections were associated with low-grade squamous intraepithelial lesions (OR=24), high-grade squamous intraepithelial lesions (OR=16) and cervical cancer (OR=8.3) (Sasagawa et al., 2001). Thus, multiple HPV infections still need to be identified. Electrochemical DNA chip is more suitable for identification of both single and multiple infections of HR-HPV genotypes than direct sequencing. The size of the overall system and the time required for examination is less in comparison to sequencing and the other commercial kits. In the comparative study, the false negative results by the Electrochemical DNA chip were approximately 10% which almost certainly were uncommon HPV genotypes.

The limitation of Electrochemical DNA chip is that it has specific probes for only 13 HR-HPV genotypes. Hence, approximately 5% false negative results were found when compared with the INNO-LiPA or direct sequencing. Finally, the operation of HPV genotyping assays depends on their primer sets. The differences in HPV prevalence depend on many factors such as sample size, measurement and detection method. Combinations of highly concordant HPV genotyping methods for primary screening are recommended. Significance of HPV prevalence in any area should be considered for vaccination program.

In conclusion, the Electrochemical DNA chip is more suitable to identify both single and multiple infections of HR-HPV genotypes than direct sequencing. It can reduce size of the overall system and time required for examination compared to sequencing and the other commercial kits. In comparison with sequencing (L1 region), the Electrochemical DNA chip detected more genotypes in 24.4% of samples. This study showed that the significance of viral genotypes of HR-HPV and others should be considered for vaccination programs. Finally, combinations of highly concordant HPV genotyping methods are recommended for primary screening.

Acknowledgements

This work was partially supported by Higher Education Research promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR 1155A), Thailand Research Fund (DPG5480002), Center of Excellence in Clinical Virology, CU Centenary Academic Development Project and King Chulalongkorn Memorial Hospital for their generous support and the staff of the Department of

Pathology, Samitivej Srinakharin hospital, Thailand and Department of Obstetrics and Gynecology, National Cancer Institute for providing the samples. Finally we would like to thank Ms. Petra Hirsch for reviewing the manuscript.

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