RESEARCH ARTICLE

Clinical Prediction Based on HPV DNA Testing by Hybrid Capture 2 (HC2) in Combination with Liquid-based Cytology (LBC)

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Abstract

Primary screening by HPV DNA testing is an effective method for reducing cervical cancer and has proven more sensitive than cytology. To advance this approach, many molecular methods have been developed. Hybrid capture 2 provides semi-quantitative results in ratios of relative light units and positive cutoff values (RLU/PC). Twenty-five thousand and five patients were included in this study to analyze the correlation between the ratio of RLU/PC and stage of cervical dysplasia. The results show that the RLU/PC ratios ranged from 0-3500 while almost normal cases, ASC-US and ASC-H, had values below 200. Of those samples negative for cytology markers, 94.6% were normal and their RLU/PC ratios were less than 4. With an RLU/PC ratio greater than 4 and less than or equal to 300, the percentages in all age groups were normal 53.6%, LSIL 20.2%, ASC-US 17.2%, HSIL 6.13%, ASC-H 2.72%, and AGC 0.11%, respectively. In contrast, 64.0% of samples with a RLU/PC ratio greater than 300 and less than or equal to 3500 were LSIL. These results should contribute to cost effective cervical cancer management strategies. Further studies of associations with particular HPV genotypes would be useful to predict the risk of progression to cancer.

Keywords: Hybrid capture 2 - human papillomavirus - LSIL - HSIL - Thailand

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Introduction

Human papillomavirus (HPV) is highly prevalent in genital tract infections of the population worldwide. Most are cleared without consequences while some proportion can persist in severe lesions and eventually progress to cervical dysplasia and invasive malignancies (Menzo et al., 2008). The persistence of HPV infection is associated with high-risk HPV types and encountered more frequently in women above the age of 30 (Forslund et al., 2002). A broader range of HPV genotype distribution has been detected in women with normal cytology than invasive cervical carcinoma among populations in various geographical areas (Clifford et al., 2006). Based on their inherent risk to cause cancer, more than 40 HPV genotypes have been identified in the genital tract's mucosal epithelium and accordingly, have been classified into high, potentially high and low-risk types (Munoz et al., 2003). High-risk HPV genotypes are mainly associated with progression and persistence of cervical cancer while some co-factors such as weak immune response, HPV persistence and high parity represent increased risks for cancer progression (Bosch et al., 2002).

Pap cytology screening is applied as an early detection method of cervical neoplasia. This method detects cellular changes during late stages of infection (Nanda et al., 2000) but is of low sensitivity and poor reliability. A high false negative rate has increased the problems inherent in cytological screening. Molecular techniques have been required to reduce the false negative rate, identify the earlier stages of infection and thus, improve follow-up because most infections are not microscopically evident (Schiffman et al., 2003; Molijn et al., 2005). Various molecular assays for HPV testing have been developed based on several techniques such as serology and molecular biology (Bhatla et al., 2009). Moreover, clinical studies have demonstrated that HPV DNA testing is more sensitive than repeated cytology to detect severe dysplasia especially in atypical squamous cells of undetermined significance (ASC-US) (ALTS, 2003). The widely used techniques for HPV DNA detection are polymerase chain reaction (PCR) with specific or

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consensus primers and other assays such as those based on liquid-phase hybridization as for example, Hybrid Capture 2 (Qiagen/Digene Diagnostics, Gaithersburg, MD), Cervista (Hologic, Madison, WI) and INNO-LiPA (Innogenetics N.V., Ghent, Belgium). All techniques except for HC2 which provides semi-quantitative results are solely qualitative analyses. Recently, the US Food and Drug Administration (FDA) has approved some assays' application for high-risk HPV testing such as Hybrid capture 2 (Belinson et al., 2011). The Hybrid capture 2 (HC2) assay is a commercially available kit which has been considered the gold standard for HPV testing in the medical field worldwide. HC2 can detect the most common carcinogenic HPV genotypes. The FDA has recommended 1.0 relative light unit for positive result (1 pg HPV DNA per 1 ml of sampling buffer) (Iftner et al., 2003) and use in patients with equivocal cervical cytology results in conjunction with primary routine Pap testing for women over the age of 30 (Nishino et al., 2011). The main limitations are potential cross-reaction with untargeted non-carcinogenic HPV genotypes such as 11, 54 and 66 which would reduce the positive predictive value of HPV testing and clinical specificity, inability to identify specific types, discriminate multiple infections and lack of an internal control to evaluate specimen adequacy (Seme et al., 2006; Castle et al., 2008). The cross-reactivity is usually found in women with cytologic changes due to often multiple, non-carcinogenic HPV genotypes and higher viral loads (Castle et al., 2002).

The aim of this study was to determine the correlation between the RLU/PC by HC2 and the degree of cytological abnormalities and dysplasia lesions.

Materials and Methods

This protocol was approved by the Ethics Committee of the Samitivej Srinakharin Hospital. All specimens have been obtained during the patients' routine screening and treatment at Samitivej Srinakharin hospital from 2004 up to the present. In order to protect patient confidentiality, all relevant personal information of each patient, such as names, addresses, and hospitalization numbers, were removed before the analysis. Only the information concerning the ratios of relative light units and positive cutoff values (RLU/PC) obtained by Hybrid Capture 2, age, and cytology data were used in this study.

Study population

Twenty-five thousand and five liquid-base (ThinPrep®, Hologic, West Sussex, UK) specimens were used in this study. These comprised 21,995 specimens with normal cytology, 1,207 specimens with atypical squamous cells of undetermined significance (ASC-US), 194 specimens of atypical squamous cells-cannot exclude HSIL (ASC-H), 1,314 specimens of low-grade squamous intraepithelial lesion (LSIL), 270 specimens of high-grade squamous intraepithelial lesion (HSIL), and 25 specimens of atypical glandular cells (AGC). HPV DNA was detected by Hybrid Capture 2 (HC2) with the interpretation of cervical cytology by a pathologist and cytologist. The combined tests of LBC and HC2, were used to find out the relationship between the Relative Light Unit (RLU) and clinical outcome by cytology data (LBC). Based on the different age intervals, the Pap result (Bethesda system) of LBC and RLU from HC2 were analyzed.

Hybrid Capture 2 (HC2)

All collected swab samples were kept in a collection tube at room temperature (25ºC) for testing by HC2 (Digene, Gaithersburg, MD). This test is based on amplification and hybridization to detect HPV DNA. Applying this assay, the denatured single strand DNA was hybridized with 2 RNA probe mixtures complementary to the 13 high-risk HPV genotype sequences (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 low-risk genotypes (6, 11, 42, 43 and 44). It was performed by following the manufacturer's instructions using an automated HC2 system (Terry et al., 2001; Lee et al., 2005). The emitted light was measured as RLU/PC, the luminescence of the 1.0 pg/ml HPV 16 standard from the kit (Cañadas MP et al., 2012). An RLU/PC 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 (Federschneider et al., 2004; Seme et al., 2006).

Data analysis

In order to discover preliminary relationship between the ratios of relative light units and positive cutoff values (RLU/PC), age groups, and cytological grade results, the data from 25,005 patients in this study were analyzed in three different manners. The details of each analysis are the following.

Distribution of RLU/PC ratios based on cytological grades of patients: The RLU/PC ratios were distinguished into 6 risk types according to patients' cytological grade results as follows: the risk type no.1 corresponds to normal cytology; the risk type no.2 corresponds to ASC-US; the risk type no.3 corresponds to ASC-H, the risk type no.4 corresponds to LSIL, the risk type no.5 corresponds to HSIL, and the risk type no.6 corresponds to AGC. The patients' RLU/PC ratios and their corresponding cytological grade results were subsequently plotted, as shown in Figure 1.

Percentage of each cytological grade along RLU/PC ratio ranges: The percentage of patients in each of six cytological grades along RLU/PC ratios ranging from 0-3500 with interested intervals were counted and

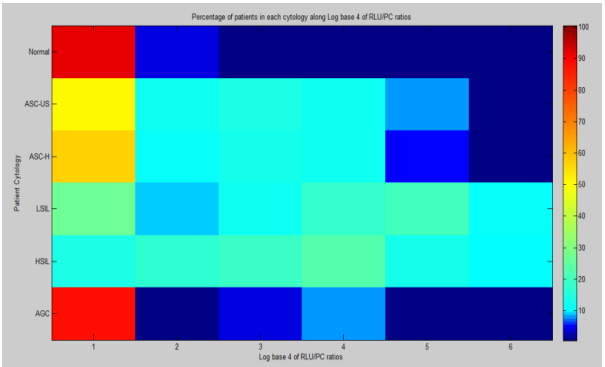

Figure 1. The Distribution of RLU/PC Ratios on Cytological Grades of Patients

illustrated in Table 1. Each percentage was computed by using this equation.

Percentage of patients in each cytological grade within the ratio range=(total number of patients in each cytological grade within the ratio range*100)/total number of patients in the corresponding cytological grade

Percentage of patients' cytological grades in relation to RLU/PC ratio and age ranges: The patient data were further analyzed to reveal the relation between their cytological grades and their corresponding RLU/PC ratio and age ranges. In this analysis, three levels of RLU/PC ratios ranging from 0-4, >4-300, and >300-3500 were separately summarized to clarify the low, high, and very high levels of RLU/PC ratios in the patient population, and the patients' age was allocated to 5 distinctive groups: 0-20, 21-30, 31-40, 41-50, and older than 51 years. Figure 2, 3, and 4 show the percentage of patients in each of the 6 cytological grades in relation to 0-4, >4-300, and >300-3500 RLU/PC ratio ranges according to each age group, respectively. The percentages were computed by the following equation.

Percentage of patients in each cytological grade within the ratio and age ranges=(total number of patients in each cytological grade within the ratio and age ranges*100)/ total number of patients in the corresponding cytological grade

Results

As depicted in Figure 1, the result showed that the RLU/PC ratios of normal patients are distributed in a manner similar to those of ASC-US, while the ratios of ASC-H are assembled in the range below approximately 200. In addition, it further showed that the ratio values are continuously distributed ranging from 0-3000 in LSIL, while being periodically assembled in HSIL, and the majority of AGC assembled in the very low value range.

Table 1 illustrates that the percentages of patients in all cytological grades are very high in the range of 0-25 RLU/PC ratio, and that the percentages subsequently decline. Even though the percentages of LSIL and HSIL were also high at the range 0-25 RLU/PC ratio, the result further showed that, after this range, the percentage of LSIL is continuously distributed, while the percentage of HSIL is periodically assembled at the range of 25-75, 100-200 and 500-1500. This result not only showed the data distribution of each cytological grade along the ratio ranges as illustrated in Figure 1, but also revealed the percentage of patients of each cytological grade along the ratio ranges intervals for plotting the color graph. Besides, the result has proven consistent with Figure 1. Since all RLU/PC ratios in this data set have values ranging from 0-3300, these ratios were plotted into log base 4 of RLU/PC ratios scale intervals. This graph contained 6 intervals. (For instance, in horizontal line, a first rectangular in an above color graph represents 0-4 RLU/PC ratios, a second represents 5-16 ratios, a third represents 17-64, a fourth represents 65-256, a fifth represents 257-1024 and a sixth represents 1025-3300).

As for normal cytology, Figure 2A depicts that the percentage of normal patients can reach the highest value, which was 92.96%, for all age groups. This result corresponds to the fact that normal patients may have negative results by Hybrid Capture 2. Thus, the RLU/PC ratio value of these normal patients was lower value less than 4 while low percentage of patients, which were 27.70% and 14.81%, were found in LSIL and HSIL, respectively.

As illustrated in Figure 2B, the result showed the overall percentage of patients for all age groups in descending order were HSIL, LSIL, normal, respectively. This result indicated that, irrespective of age group, patients with an RLU/PC ratio greater than 4 and less than or equal to 300, might display HSIL (63.33%), LSIL (42.92%) and normal (6.80%), respectively.

According to Figure 2C, the results indicated that the

Table 1. The Percentage of Patients in all Cytological Grades

Ratio Range	Normal	ASC-US	ASC-H	LSIL	HSIL	AGC
0-4	92.96	51.95	57.22	27.7	14.81	88
>4-25	4.89	15.74	18.04	13.24	22.96	0
>25-50	0.87	7.13	5.67	6.32	9.26	4
>50-75	0.29	4.81	2.06	4.87	7.41	0
>75-100	0.27	2.57	2.58	4.03	5.93	0
>100-200	0.36	6.38	7.22	8.22	10.74	8
>200-300	0.12	3.23	3.61	6.24	7.04	0
>300-400	0.07	2.24	1.03	4.79	2.96	0
>400-500	0.04	1.66	1.03	2.89	1.48	0
>500-1000	0.07	3.23	1.55	9.82	6.3	0
>1000-1500	0.04	0.75	0	6.01	5.56	0
>1500-2000	0.01	0.25	0	3.27	2.96	0
>2000-2500	0	0.08	0	1.98	1.48	0
>2500-3000	0	0	0	0.61	0.37	0
>3000-3500	0	0	0	0	0.74	0
Total	100	100	100	100	100	100

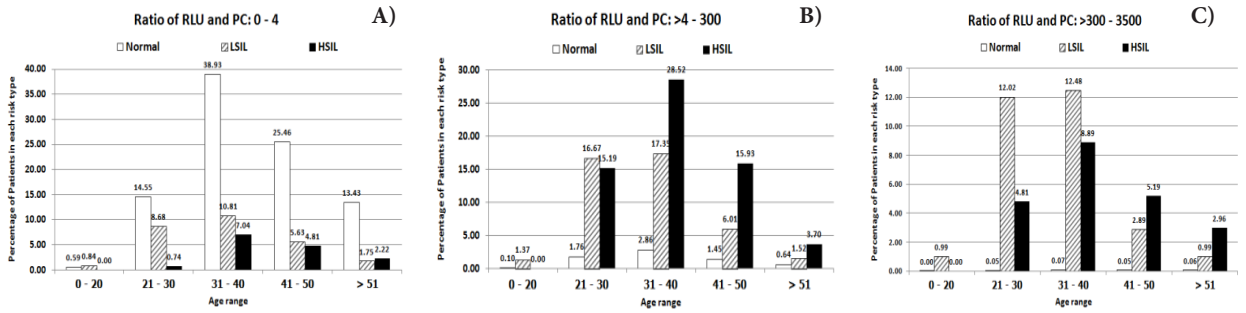

Figure 2. The Percentage of Patients' Cytological Grades. A) 0-4 RLU/PC Ratio and Age Ranges, B) >4-300 RLU/PC Ratio and Age Ranges and C) >300-3,500 RLU/PC Ratio and Age Ranges

overall percentages of patients in each cytological grade, irrespective of age group, with an RLU/PC ratio greater than 300 and less than or equal to 3500 in descending order were LSIL (29.38%), HSIL (21.85%), normal (0.24%), respectively.

Discussion

Papanicolaou (Pap) smear screening has in the past helped reduce the incidence of cervical cancer. Conventional cytology is imperfect for cervical screening and failed to reduce cervical cancer rates in developing countries. The new methods, the LBC and HPV testing are applied to reduce the number of false negatives impacting conventional cytology. HPV DNA detection was developed as a strategy to prevent cervical cancer. HPV testing is more sensitive than cytology at detecting high-grade squamous intraepithelial lesions (HSIL) but less specific (Núñez-Troconis J et al., 2009). HPV testing has been approved as a triage test for women with ASCUS cytology who need additional clinical management. HC2 represents a second generation commercially available HPV detection kit which has been designed for HPV DNA sequence detection by type-specific or consensus PCR. The percentage of HPV DNA detection by HC2 as reported for European countries is usually close to 10% (Giorgi et al., 2010; Ogilvie et al., 2013) while it amounts to 44.9% in Brazil (Carestiato et al., 2006). Previous studies on human cervical samples have shown similar HPV detection results by PCR using L1 consensus primers (MY09/MY11 or GP5+/GP6+) (Peyton et al., 1998; Bozzetti et al., 2001). The HC2 had greater positive likelihood ratios than PCR while PCR had negative likelihood ratios than HC2 in cytological grade severity than LSIL (Tsiodras et al., 2010; Comar et al., 2012).

Awareness of HPV genotype distribution in the general public is important for both primary screening of cervical cancer and prophylactic vaccination (Tsao et al., 2010). The distribution of RLU/PC ratios are similar in all groups may due to an imbalance of each patients group. According to Figure 2, even though there exists a low percentage of patients in the remaining cytological grades, this can be considered false positives due to the fact that the HC2 testing related with the number of HPV genotypes present and cross-reactivity with the untargetd HPV genotypes which phylogenetically related with the targeted genotypes was not absolute. The untargeted HPV genotypes, such as in multiple infections, can lead to a high positive results of HC2 while decrease the specificity. In previous study, 8% of HC2 positive results is cross-reactivity (Castle et al., 2008). In addition, some occasional error may be due to an inadequate volume for detection, since the HC2 automatic technical platforms requires at least 4 ml (Kurian et al., 2011).

Of those patients with an RLU/PC ratio greater than 4 and less than or equal to 300 (Figure 3), the normal patients can represent the highest overall percentage, which is due to the large number of normal patients in comparison with the other groups of patients. Regardless of the normal group of patients and imbalance of patients in each group, this figure further reveals that the percentages of LSIL and HSIL were found to be quite high in this ratio range in the 21-50 year age group. Especially in the 41-50 year age group, HSIL has a highly percentage than LSIL 2.6 times and normal 11 times. This result is important to help patients age over 40 years which have RLU/PC ratio in this range for more risk to be a HSIL and must more concern in cervical cancer management strategies.

With regard to patients with an RLU/PC ratio greater than 300 and less than or equal to 3500 (Figure 4), despite the fact that the number of normal patients was very high and by far exceeded that of the other groups, this result illustrates that the percentages of patients in LSIL and HSIL were higher than that of the normal group for this ratio range. Especially, it further reveales the very high likelihood of patients in the 21-40 year age group with an RLU/PC ratio ranging from >300-3500 to have LSIL cytology. Based on previous studies, high-risk HPV was detected in 83% of women with LSIL cytology and a mean age of 24.9 years. Our result is in agreement with other studies which implies that it might be useful to triage women in the 21-40 year age group with LSIL (Ronco et al., 2007; Thrall et al., 2009).

The results of this study demonstrate that HPV infection is a major risk factor for cervical cancer development and show the relation between RLU/PC and the degree of dysplasia with patients with a high RLU/PC ratio more likely to have abnormal lesions, irrespective of age group. This study may help clinicians to predict the outcome of each HPV infected case. These results may represent essential information for cervical cancer management strategies and cost effective HPV vaccination. In addition, various co-factors such as smoking and sexual behavior can increase the accuracy of predicting the risk of HPV infection and progression to cancer.

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