RESEARCH ARTICLE

HPV Genotyping Linear Assay Test Comparison in Cervical Cancer Patients: Implications for HPV Prevalence and Molecular Epidemiology in a Limited-resource Area in Bandung, Indonesia

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Abstract

Background: Persistent infection with high risk human papillomavirus (hrHPV) is strongly associated with cervical cancer. Normal cervical cells may also harbor hrHPV, and detection of early hrHPV infection may minimize risk of cervical cancer development. This study aimed to compare two commercial HPV genotyping assays that may affordable for early screening in a limited-resource setting in Bandung, Indonesia. Materials and Methods: DNA from cervical biopsies with histologically confirmed as squamous cell cervical cacinoma were HPV genotyped by Linear Assay 1 (Roche Diagnostics, Mannheim, Germany) or Linear Assay 2 (Digene HPV Genotyping RH Test, Qiagen Gaithersburg, MD). In a subset of samples of each group, HPV genotype results were then compared. Results: Of 28 samples genotyped by linear assay 1, 22 (78.6%) demonstrated multiple infections with HPV-16 and other hrHPV types 18, 45 and/or 52. In another set of 38 samples genotyped by linear assay 2, 28 (68.4%) were mostly single infections by hrHPV type 16 or 18. Interestingly, 4 samples that had been tested by both kits showed discordant results. Conclusions: In a limited-resource area such as in Indonesia, country with a high prevalence of HPV infection a reliable cervical screening test in general population for early hrHPV detection is needed. Geographical variation in HPV genotyping result might have impacts for HPV prevalence and molecular epidemiology as the distribution in HPV genotypes should give clear information to assess the impact of HPV prophylactic vaccines.

Keywords: Cervical cancer - HPV genotypes - linear assay - Bandung Indonesia

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Introduction

High risk human papillomavirus (hrHPV) is known as the aetiological agent of cervical cancer, and persistent hrHPV might develop to cervical cancer later in the life time of women (Castellsagué, 2008). Cervical cancer is the second most prevalent female cancer after breast cancer worldwide, of which 80% of the patients reside in the developing countries in sub-Saharan Africa, Latin America, and South and South East Asia (Castellsagué, 2008). In Indonesia, cervical cancer ranks first among gynaecological cancers and becomes a major health problem (Aziz, 2009). The hrHPV genotypes, including HPV types 16, 18, 31, 52, and 58 that are most prevalent globally and also other hrHPV types such as HPV type 33, 35, 39, 45, 51, 56, 59, 68, 73, and 82, are considered as the most carcinogenic types; where as HPV types 26, 53, and 66 are considered as probably carcinogenic, and HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 are the low-risk types (de Sanjosé et al., 2007). hrHPV might infect transiently and causes a self-limiting disease, and thus, normal cyotological examination cervical tissue may harbor hrHPV (Bruni et al., 2010). Therefore, detection of early hrHPV infection will minimize the cervical cancer development risks.

For early diagnosis of epithelial neoplasia such as cervical dysplasia, anogenital lesions, and also oral and oropharyngeal squamous cell carcinomas, sensitive and specific detection of HPV in cervical samples might be a useful tool (Pannone et al., 2012). The most common used test for cervical abnormality screening in general population is conventional PAP smear, though some other alternatives in a limited-resource setting may exist such as liquid based cytology and visual inspection with acetic acid (VIA) (Bradford and Goodman, 2013). With increasing molecular technologies for HPV detection,

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several tests have been developed to detect HPV infection specifically to detect the genotype of the HPV (Ge et al., 2012). Recent studies compared HPV genotyping tests and support the feasibility of HPV DNA testing instead of cytology examination as a primary test in population screening for cervical cancer (Lindemann et al., 2012; Martinez et al., 2012; Song et al., 2012), though these methods are quite expensive and not affordable in the limited-resource area. However, this is likely to be an option in the near future in many countries, and it would increase the efficiency of screening for cervical abnormalities.

In Indonesia, HPV genotyping tests have hampered with limited resources and costs that makes the test not affordable for population screening, however, study on HPV genotype prevalence using an INNO-line probe assay prototype research genotyping assay (Innogenetics, Ghent, Belgium) had been conducted in Indonesia by Vet et al, and showed that the prevalence of HPV genotypes in general population in three area in Indonesia were dominated by HPV-52, 16, 18, and 39 respectively (Vet et al., 2008). The same group has identified that the HPV-18 in Jakarta, capital city of Indonesia was especially found in adenosquamous carcinoma (Schellekens et al., 2004). HPV genotyping tests from several companies have been recently introduced in Indonesia. The aim of this study was to compare two commercial linear assays available, that might be useful for screening programs and for monitoring the effectiveness of HPV vaccination in Bandung, Indonesia.

Materials and Methods

Study subjects

Consecutively new cervical cancer patients with FIGO criteria IIA/B consented to take part in the study. This study was part of the HPV prevalence survey in Bandung and surrounding areas, conducted at the Department of Obstetrics and Gynaecology, Hasan Sadikin Hospital from July 2010 to November 2010. Ethical clearance was granted from the Faculty of Medicine Universitas Padjadjaran, Bandung.

Biopsies from the cervical tissue were diagnosed by the hospital pathologists and classified according to the WHO Classification. Histologically confirmed moderate differentiated squamous cell cervical cancer was isolated for DNA material according to manufacturer protocol, and then HPV genotype tests were performed using Linear Assay 1 (Roche Diagnostics, Mannheim, Germany) or Linear Assay 2 (Digene HPV Genotyping RH Test, Qiagen Gaithersburg, MD).

Linear assay 1

Linear assay 1 can detect 37 high- and low-risk HPV genotypes, i.e. 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108. In brief, PCR amplicons were denatured by denaturation solution and subjected to hybridization with a single HPV genotyping strip that was coated with HPV type-specific and human β-globin probes. The biotin-labeled amplicons were hybridized to the probes only if the type-specific sequence matched the amplicons. The biotin-labeled amplicons were detected by colorimetric development and the results were read visually by comparing the pattern of colored lines to the provided reference guide. Gold plated PCR machine has been used to perform linear assay 1.

Linear assay 2

The second linear assay HPV genotyping test was determined in other set of DNA isolated biopsies. This assay detects only high risk HPV 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, and 72. Gradient PCR machine has been used to perform linear assay 2.

Comparison linear assay 1 and 2

In a subset of the samples tested with linear assay 1 (4 samples) were tested again with linear assay 2 to compare the HPV genotypes results.

Results

From June 2010 to November 2010 biopsy samples classified as non-keratinizing and moderately differentiated squamous cell carcinoma (SCC) cases were randomly genotyped (n 66) using two different commercial kits i.e Linear Assay 1 or Linear Assay 2. Negative results for HPV can only be detected in one of 66 samples, and the human control β-globin was positive in that sample, showing that the human DNA was well isolated.

Single or multiple HPV infections in cervical cancer biopsies

The result of HPV genotyping test from 66 samples has shown that single infection (47%) was detected in equal percentage compared to multiple infections (51.5%), with HPV 16 (22.7%) and HPV 18 (19.7%) dominated the single infection (Table 1). Interestingly, when we observed more into the detail and compared the HPV genotypes between both Linear Assay tests, Linear Assay 1 was mostly multiple infections and Linear Assay 2 showed more single infection.

As a total samples, the prevalence of the HPV genotyping in the squamous cell carcinoma was dominantly infected by high-risk type HPV-16 (74.2%), followed by HPV 18, 45 and 52, respectively in decreasing percentage (Table 2). Interestingly, HPV 52 that was detected by Linear Assay 1 in a high percentage, did not come in single infection, but as co-infection with HPV 16 or 18 (Table 1). Moreover, HPV-52 that was detected by Linear Assay 1 cannot be distinguished by other HPV 33, 38 and 58 as stated in the manufacturer guidelines (data not shown), in opposite to Linear Assay 2 that can detect HPV 52 individually.

Discordance distribution of linear assay 1 vs. linear assay 2

Since there was a significantly difference distribution of HPV genotype between Linear Assay 1 and 2, we proceed to test another 4 samples DNA using both tests

Table 1. HPV Genotypes in Non-keratinizing Moderately Differentiated Squamous Cell Carcinoma from Bandung from July to November 2010

	Total N=66	Linear Assay 1* N=28	Linear Assay 2* N=38
No Infection	1	1	--
Single infection			
HPV 16	15 (22.7%)	5 (17.9%)	10 (26.3%)
HPV 18	13 (19.7%)	--	13 (34.2%)
HPV 35	1	--	1
HPV 45	2	--	2
HPV 52	--	--	--
Total	**31 (47 %)**	**5 (17.9%)**	**26 (68.4%)**
Multiple infections			
HPV 16, 18	12	6	6
HPV 16, 45	1	--	1
HPV 16, 51	1	1	--
HPV 16, 61###	1	1	--
HPV 16, 18, 45	5	1	4
HPV 16, 18, 45 52	6	5#	1##
HPV 16, 18, 51	1	1	--
HPV 16, 18, 52	2	2#	--
HPV 16, 33, 45, 52, 55	1	1#	--
HPV 16, 45, 52	4	4#	--
Total	**34 (51.5 %)**	**22 (78.6%)**	**12 (31.6%)**

*Linear Assay 1 from Roche, **Linear Assay 2 from Qiagen, #HPV-52 in Linear Assay 1 is destined for HPV -52/33/38/58 , ##HPV-52 in Linear Assay 2 is single HPV-52, ###HPV-61 belongs to low-risk HPV genotypes

Table 2. Incidence of HPV Genotyping of Non-Keratinizing Moderately Differentiated Squamous Cell Carcinoma from Bandung from July to November 2010

	Total n=66	Linear Assay 1* n=28	Linear Assay 2* n=38
HPV-16	49 (74.2%)	27 (96.4%)	22 (57.9%)
HPV-18	39 (59.1%)	15 (53.6%)	24 (63.2%)
HPV-33	1	1	--
HPV-35	1	--	1
HPV-45	19 (28.8%)	11 (39.3%)	8 (21.1%)
HPV-51	2	2	--
HPV-52#,##	13 (19.7%)	12 (42.9%)#	1 (2.6%)##
HPV-55	1	1	-
HPV-61###	1	1	-

*Linear Assay 1 from Roche; **Linear Assay 2 from Qiagen; #HPV-52 in Linear Assay 1 is destined for HPV -52/33/38/58; ##HPV-52 in Linear Assay 2 is single HPV-52; ###HPV-61 belongs to low-risk HPV genotypes

Table 3. Concordant Result of HPV Genotype in Two Different Linear Assay Tests

HPV type	Linear Assay 1*	Linear Assay 2*	Agreement
Sample 1	16, 18	18	50%
Sample 2	16, 18, 45, 52#	16, 18, 45	75%
Sample 3	16, 45, 52#	16, 18, 45, 52##	75%
Sample 4	16, 61###	16	100%

*Linear Assay 1 from Roche; **Linear Assay 2 from Qiagen; #HPV-52 in Linear Assay 1 is destined for HPV-52/33/38/58; ##HPV-52 in Linear Assay 2 is single HPV-52; ###HPV-61 belongs to low-risk HPV genotypes, and can only be detected in Linear Asssay 1. Linear Assay 2 can not detect low-risk HPV genotype

Linear Assay 1 and Linear Assay 2 and compared. The result depicted in table 3 has indicated that there was a disagreement result between two tests.

Discussion

Human papillomavirus (HPV) has been identified as the major pathogen that cause cervical cancer worldwide, and HPV genotyping test is recommended in primary cervical cancer screening in many countries, especially in developed countries (Donna et al., 2012; Hoppenot et al., 2012). There may be some geographical variation in prevalence of carcinogenic types, and therefore HPV genotype distribution in cervical cancer is essential to assess the impact of HPV prophylactic vaccines (Munoz et al., 2003). Furthermore, HPV genotyping detection become very important and must be considered for designing diagnostic tests.

The general population screening may hampered to the fact that the HPV genotyping test is too expensive and laborious. An alternative for a cheaper method, hence reliable result is needed. In Indonesia, with a limited-resource setting, and where the cervical screening is not a national program, cytomorphological examination still play a major role as the performance of this cytomorphological examination has a high specificity compared to INNO-LiPA assay (Rachmadi et al., 2012). Recently, several molecular tests for detection HPV genotypes are commercially available ranging from the test to detect only the high risk HPV genotype to both high and low risk genotypes. Many studies compared the sensitivity and concordant results and many technologies are introduced (Lindemann et al., 2012; Martinez et al., 2012; Song et al., 2012). The high risk human papillomaviruses (hrHPV), particularly HPV 16 and 18, are consistently identified in cervical cancer cases regardless of geographical region (de San Jose et al., 2010). Our study has been detected the HPV genotypes infected the cervical cancer especially in the squamous cell carcinoma. The HPV-16 was dominantly infected the cervical cancer, followed by HPV 18, 45 and 52, concordant the global result. Furthermore, study in three regions in Indonesia has reported that though HPV-16 and HPV-18 were equally common in the general population, HPV-52 was the most prevalent type and this HPV-52 is recommended to be included for prophylactic HPV vaccination (de Vet et al., 2008). In our study, HPV 52 did not come as a single infection, but as a co-infection with other hrHPV-16 or 18 suggesting that HPV 52 is a transient infection, in opposite to HPV 16 and HPV 18 that contributed greatly in the cervical cancer development. This difference might occur due to different in HPV genotyping tests used.

The limitation of this study was that in our limited-resource setting we were unable to test every single samples test twice to compare the agreement between two tests. The commercial tests are too expensive though those two kits have plus and minus points. Several studies around the world has showed that linear assay 1 we used has a good performance and is sensitive (Wong et al., 2010; Steinau et al., 2012), hence this test is costly and need a especial PCR machine which is gold plated to get a good reliable result. In low- and medium-resourced countries where the PCR machines are scarce this assay cannot be performed. The good point for linear assay 1 is that the HPV genotypes detected are both high and low risk HPV. In contrary, linear assay 2 uses simple gradient PCR machine, hence the genotypes detected are only the high risk HPV. Though reports compared both test that

the concordant and agreement of the test is high (Smith et al., 2012), we cannot show in our study since we can only allow limited samples to be tested for both tests. Since the assay 2 use simple gradient PCR machine that more available in laboratories, this assay might give a good alternative to detect HPVinfection, allowing simple and rapid HPV genotyping and detecting multiple hrHPV infections.

HPV 52 that was detected by Linear Assay 1 in a high percentage, did not come in single infection, but as co-infection with HPV 16 or 18 (Table 1). HPV-52 that was detected by Linear Assay 1 cannot be distinguished by other HPV 33, 38 and 58 as stated in the manufacturer guidelines (data not shown), in opposite to Linear Assay 2 that can detect HPV 52 individually. In Indonesian population HPV 52 is prevalent (de Vet et al., 2008), alternative is given replace with available using genoflow technique since linear assay 1 cannot distinguish between 52/31. GenoFlow (GF) HPV Array-Test (Diagcor Bioscience Inc., Hong Kong) was recently developed which can detect 33 HPV genotypes by a "flow-through" hybridization technology (Wong et al., 2012). GF and LA showed significant discrepancy in detecting HPVgenotypes 39, 52, 26, 66, and 11. More sensitive detection of HPV52 by GF offers an advantage in regions where HPV52 is more prevalent (Wong et al., 2012), hence, linear assay 1 has an advantage for internationally comparable genotyping studies (Steinau et al., 2012).

Since HPV genotyping test is costly, especially in Indonesia with limited-resource facility, choosing one or another test might give a different distribution that may impact the molecular epidemiology in the area. For limited-resource, manual liquid based cytologi in primary screening is still preferable (Nandini et al., 2012). In Indonesia, cervical cancer screening programs consists of Pap smears and now visual inspection with acetic acid is also increasing, however, the uptake of screening remains low (Domingo et al., 2008). Furthermore, the genotyping result might not give a difference in therapy management from the clinical point of view, thus, with lots of HPV genotyping detection available in the market one should be aware of the advantage and disadvantage of the tests and has the possibility to compare the techniques to get higher agreement between tests.

In a poor resource area, HPV detection especially high risk HPV that mostly infect the population, is of clinical important. It has to be taken in account that when expensive diagnostic tool is used, sensitivity and specificity of the test need to be compared, as the differences in results might give a different genotyping distribution in the population. Study on HPV type distribution is necessary for use of vaccine that can protect against HPV types circulated in the area. Knowledge of the epidemiological distribution is necessary to predict the effect of vaccines on incidence of infection and evaluate cross-protection from current vaccines against infection with other types. Continued HPV surveillance national wide using the same method would be useful, to assess the potential for changing type-specific HPV prevalence in the post-vaccination era in Indonesia. These data are essential for local decision makers regarding HPV screening and vaccination policies.

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