RESEARCH ARTICLE

Comparison of p16INK4a Immunocytochemistry with the HPV Polymerase Chain Reaction in Predicting High Grade Cervical Squamous Intraepithelial Lesions

Junita Indarti*, Darrell Fernando

Abstract

<u>Aim</u>: To compare p16INK4a immunocytochemistry with the HPV polymerase chain reaction in predicting high grade cervical squamous intraepithelial lesions. <u>Materials and Methods</u>: This diagnostic case-control study was conducted from January 2010 until December 2010. We obtained 30 samples, classified according to the degree of cervical intraepithelial neoplasia (CIN): 11 samples for CIN 1, 9 samples for CIN 2, and 10 samples for CIN 3. HPV PCR, p16INK4a immunocytochemistry, and histopathological examination were performed on all samples. Statistical analysis was conducted using SPSS 20.0. <u>Results:</u> In predicting CIN 2-3, we found p16INK4a to have similar specificity and positive predictive value as HPV PCR (95%, 97.2% vs 96.7%), but better sensitivity (87.5% vs 72.5%) and negative predictive value (82.1% vs 67.6%). The most prevalent types of high-risk HPV in our study were HPV 33, 35, 58, 52, and 16. <u>Conclusions:</u> p16INK4a has better diagnostic values than HPV PCR and may be incorporated in the triage of ASCUS and LSIL to replace HPV PCR. Genotype distribution of HPV differs in each region, providing a challenge to develop HPV vaccines based on the epidemiology of HPV in that particular region.

Keywords: Human papillomavirus - genotyping - polymerase chain reaction - p16INK4a - precancerous cervical lesions

Introduction

World Health Organization (2005) found more than 500,000 new cases of cervical cancer, which over 90% are found in developing countries. Ninety-five percent of deaths by cervical cancer occur in developing countries. In Indonesia, cervical cancer is the most frequent malignancy found in women and accounts for the highest number of deaths. The incidence is 40.000 new cases/year, and 62% present in an advanced stage (Hacker, 2005; Suwiyoga, 2006).

Infection by high-risk human papillomavirus (HPV) is a well known risk factor for cervical cancer. High risk HPV include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82. HPV 26, 53, and 66 are also classified as probable high-risk types (Munoz, 2003). The natural history of cervical cancer carcinogenesis may take up to 10-20 years, therefore most cases can be prevented by early detection and prompt treatment of precancerous lesions (Wenham, 2002).

A good screening test should be accurate, reproducible, inexpensive, easy to perform and easy to follow up, acceptable, and safe. The tests that meet these criteria are cytology, both conventional cytology and liquid based cytology, HPV DNA test, and visual inspection with acetic acid or Lugol's iodine (WHO, 2005).

When used as a primary screening test, HPV DNA has a mean sensitivity of 85% and mean specificity of 84%. The combination of cytology and HPV DNA has a very high sensitivity and negative predictive value, approaching 100%. Nevertheless, HPV DNA tests are expensive and entail complex laboratory requirements and specimen transport (WHO, 2005).

Molecular changes in cervical carcinogenesis by HPV infections have lead to the discovery of several potential biomarkers that may help identify diseases related to HPV infections in epithelial cells (Wenham, 2002). Biomarkers have important roles in the ASCUS LGSIL Triage Study (ALTS) triage of atypical squamous cells of undetermined significance (ASCUS) and low grade squamous intraepithelial lesions (LGSIL). Biomarkers may also enhance the accurary of current screening tests. A numerous amount of studies have studied biomarker expression by immunohistochemistry, but not immunocytochemistry. One of the most frequently studied biomarkers is p16INK4a.

p16INK4a is a tumor suppressor protein, which indirectly inhibits phosphorylation of retinoblastoma protein (pRb) by inhibiting cyclin-dependent kinase 4 and 6 (von Knebel Doeberitz, 2002) .In dysplastic lesions and cervical cancer, increased production of p16INK4a correlates with severity of dysplasia. This may be seen

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in histological preparations of liquid samples with immunocytochemistry or immunohistochemistry staining. p16INK4a has also been proposed as a more sensitive biomarker compared to HPV DNA tests (Eleuterio, 2007).

Materials and Methods

This is a diagnostic case-control study aiming to compare the diagnostic value of p16INK4a immunocytochemistry and HPV PCR tests with histopathological examination as the gold standard. This study was conducted in the Colposcopy Clinic, Departement of Obstetrics and Gynecology, Cipto Mangunkusumo National Referral Hospital from January 2010 until December 2010. Women eligible for this study were aged 18-50 years, or above 50 years without cytological findings of atrophy, and were not pregnant nor menstruating during the visit. We obtained 30 samples, further classified according to the degree of cervical intraepithelial neoplasia (CIN): 11 samples for CIN 1, 9 samples for CIN 2, and 10 samples for CIN 3. Samples without CIN in histopathological examination were also excluded from this study.

Samples were then examined for HPV PCR, p16INK4a immunocytochemistry (ICC), and histopathological examination. Speciments for PCR and ICC were obtained from liquid based cytology (Liqui-prepTM), taken before colposcopy examination. Speciments for histopathological examination were obtained from target biopsy during colposcopy.

HPV PCR was performed using Linear Array® HPV Genotyping Test (Roche), detecting 37 types of HPV, consisting of 15 high risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82), and 22 low risk types (HPV 6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 67, 66, 69, 70, 71, 72, 81, 83, 84 IS39, CP6108).

For p16INK4a immunocytochemistry, we used mouse monoclonal anti-p16INK4a antibody [2D9A12] (Abcam® ab54210) diluted 100 times as primary antibody and biotinylated universal secondary antibody as secondary antibody. Slides were then evaluated by an expert pathologist, assessing the intensity and distribution of stainings. A sample is positive if the nucleus with/ without cytoplasm is stained dark brown. Intensity is graded on a scale of 0-3: 0: no staining, 1: low intensity, 2: moderate intensity, and 3: high intensity. Distribution is the percentage of stained cells. Expression is calculated by multiplying intensity and distribution. Detailed information on specimen processing can be acquired by contacting our corresponding author.

Statistical analysis was performed with SPSS 20.0.

Results

The mean age in CIN 1, CIN 2, and CIN 3 is 38.6 years (23-54 years), 40.9 years (30-53 years), and 44.9 years (37-48 years), respectively.

From HPV PCR testing, we found 9 samples (30%) had mixed infections, 5 samples (16.7%) with single high-risk type infection, 1 sample (3.3%) with low-risk type infection, and 14 negative samples (46.7%). Types of HPV found in all samples are shown in Table 1.

We found the cut-off point of p16INK4a ICC expression was 110. Expression of this marker was low if the score was <110, and high if ≥110. We found 17 samples with high p16INK4a expression and 13 samples with low p16INK4a expression. Expression of p16INK4a was low in all samples with CIN 1. In CIN 2, high expression was found in 7 (77.8%) samples. Expression of p16INK4a was high in all samples with CIN 3. The correlation between degree of CIN with HPV PCR test and p16INK4a expression is shown in Table 2.

By Chi-square analysis, we found a significant correlation between HPV PCR test results and p16INK4a expression with degree of CIN, with p<0.001 for both tests. A HPV PCR test positive for high-risk type also significantly correlates with a high p16INK4a expression (p<0.001).

Using a 2×2 table, we calculated the sensitivity, specificity, positive predictive value, and negative predictive value of HPV PCR and p16INK4a expression. The standard examination was histopathological

Table 1. Types of HPV Found in All Samples

HPV type		Amount (%)
High risk	HPV 33	7
	HPV 35	6
	HPV 58	6
	HPV 52	5
	HPV 16	4
	HPV 51	3
	HPV 59	2
	HPV 68	2
	HPV 18	1
	HPV 39	1
	HPV 45	1
Low risk	HPV 6	1
	HPV 11	1
	HPV 26	1
	HPV 62	1
	HPV 71	1

*HPV: human papillomavirus

Table 2. Correlation between Degree of CIN with HPV PCR Test and p16INK4a Expression

	CIN 1 (n=11)	CIN 2 (n=9)	CIN 3 (n=10)
High-risk HPV	0 (0%)*	5 (55.6%)	9 (90%)**
High p16INK4a expression	0 (0%)	7 (77.8%)	10 (100%)

*Ten samples were negative, one sample was positive for HPV 6 (low-risk type); **DNA was undetected in the tenth speciment; HPV PCR: Human Papillomavirus polymerase chain reaction, ICC: immunocytochemistry, CIN: cervical intraepithelial neoplasia

Table 3. Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value of HPV PCR and p16INK4a Expression Compared to Histopathological Examination*

	HPV PCR	ICC
Sensitivity	72.50%	87.50%
Specificity	95.80%	95.80%
Positive predictive value	96.70%	97.20%
Negative predictive value	67.60%	82.10%

* A positive histopathological examination implicates CIN 2-3 (high grade lesions); HPV PCR: Human Papillomavirus polymerase chain reaction, ICC: immunocytochemistry, CIN: cervical intraepithelial neoplasia

Table 4. Comparison of HPV Genotype Distribution among Other Studies

Study	Our study	Vet JNI (2008)	Schmeink (2012) Leslink (2008)	Shen (2013)		Kim (2012)		Rositch (2012)
Location	Jakarta, ID	Jakarta, ID	National Nijmegen, NL	Henan, CN		Korea		Worldwide meta-analysis
				CIN 2-3	Ca	CIN	Ca	Persistent HPV infection
Most prevalent	HPV 33	HPV 52	HPV 52	HPV 16	HPV 16	HPV 16	HPV 16	HPV 16
HPV types	HPV 35	HPV 16	HPV 16	HPV 16	HPV 18	HPV 58	HPV 18	HPV 31
	HPV 58	HPV 39	HPV 18	HPV 51	HPV 33	HPV 18	HPV 31	HPV 33
	HPV 52	HPV 51		HPV 56	HPV 18	HPV 33	HPV 33	HPV 52
	HPV 16	HPV 18		HPV 31	HPV 51	HPV 66		

*ID: Indonesia; NL: Netherlands: CN: China

examination, a positive result implicates CIN 2-3 (high grade lesions), whereas CIN 1 (low grade lesions) is considered negative. As some cells had a value of "0", we added 0.5 to all cells. The results are shown in Table 3.

Discussion

We found 30% of the samples were infected with multiple types of high-risk HPV, and the high-risk types most commonly found are HPV 33, 35, 58, 52, and 16. Comparison between the findings in our study and previous studies is shown in Table 4.

From these data, we can see that the most prevalent types of high risk HPV in Asia are HPV 33, HPV 16, HPV 58, and HPV 52. According to HPV phylogenicity, these four types belong to the same species, alpha-papillomavirus species 9 (de Villiers, 2004). This relates with HPV vaccination, as current vaccines only offer protection to high risk types 16 and 18. Some studies have proven cross protection of HPV vaccine; there may be some degree of protection towards HPV 31 and HPV 45 in bivalent vaccines and HPV 31 in quadrivalent vaccines (Bonanni, 2009). We realize that data on HPV types in our study may not be applied directly to the general population because of the small sample size. Further studies with larger samples should be conducted to provide endetailed information on HPV distribution, so vaccines can be developed according to the types of HPV prevalent in that particular area.

In our study, we found the diagnostic vales of p16INK4a immunocytochemistry are better than HPV PCR, especially for the specificity and negative predictive value. Our findings are consistant with previous studies comparing HPV DNA tests with p16INK4a expression. Ekalaksananan (2011) found the sensitivity and specificity of p16INK4a expression to detect CIN 2-3 is higher than HPV DNA tests, 89.5% vs 84% and 56.2% vs 49%, respesctively. Nasioutziki (2011) reported HPV DNA tests have better sensitivity, but have lower positive predictive value and specificity compared to p16INK4A (44% vs 71%, 78% vs 92%).

Roelens (2012) conducted a meta-analysis on p16INK4a immunocytochemistry to detect CIN 2+, and found the sensitivity is 83.2% (95%CI 76.8%-88.2%) in patients with ASCUS cytology and 83.8% (95% CI, 73.5%-90.6%) in patients with LSIL. The specificity in ASCUS and LDR is 71% (95% CI, 65%-76.4%) and

65.7% in CIN 1 (61.7%-70%), respectively. In the triage of patients with ASCUS, p16INK4a has a similar sensitivity as HPV PCR but has a significantly higher specificity in the detection of patients with LSIL, p16INK4a has a lower sensitivity but has a higher specitivity when compared to HPV PCR.

From these studies, we can conclude that p16INK4a has better diagnostic values than HPV PCR and may be incorporated in the triage of ASCUS and LSIL to replace HPV PCR. The genotype distribution of HPV differs in each region, providing a challenge to develop HPV vaccines based on the epidemiology of HPV in that particular region.

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