Research Report

Expression of p16^{INK4a} Biomarker has a Diagnostic Value in Predicting the Progressivity of Precancerous Cervical Lesion

Ekspresi biomarker p16^{INK4a} sebagai prediktor progresivitas lesi prakanker serviks

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Abstract

Objective: To evaluate clinical value of p16^{INK4a} biomarker level, by doing p16^{INK4a} immunocytochemistry staining, as a predictor of progressivity of precancerous cervical lesion.

Method: Design of this research is case-control study which will be stratified. Research was conducted in Cytology Laboratorium, Gynecology Specialistic Division, Department of Obstetrics and Gynecology, Dr. Cipto Mangunkusumo Hospital from August 2007 to September 2008. Immunocytochemistry examination was conducted in Anatomic Pathology Department, Dr. Hasan Sadikin Hospital. We divided the sample into two categories; patients with and without cervical intraepithelial neoplasia (CIN). This research will evaluate HPV infection and p16^{INK4a} biomarker by doing p16^{INK4a} immunocytochemistry (ICC) staining.

Results: We have done immunocytochemistry examination in 130 patients, 26 without CIN and 104 with CIN. Immunocytochemistry cut-off point level is 50, which means value < 50 means low (L) p16^{INK4a} expression and in reverse, value ≥ 50 means high (H) p16^{INK4a} expression. The bivariate analysis of our study were p16^{INK4a} (H) expression has the risk of CIN 1 with OR 8.4, for CIN 2 with OR 13 and for CIN 3 with OR 21, greater than p16^{INK4a} (L) with all p values are significant. ICC p16^{INK4a} (H) expression, age, number of sexual partners, and education are contributory to the risk of progressivity in CIN. The multivariate analysis demonstrates, ICC expression of p16^{INK4a} (H) has a risk (OR) of CIN 1 17.19 times greater than p16^{INK4a} (L); p16^{INK4a} (H) expression's OR for CIN 2 is 25.56; and for OR CIN 3 is 37.32. The expression of p16^{INK4a} has a significant p value and high OR, thus ICC expression of p16^{INK4a} is suggested to be included in the algorithm of precancerous cervical lesion guidelines and scoring of probability of CIN as an alternative to HPV DNA considering less cost of the test. In this research, we found a scoring model to determine probability of progressivity in precancerous cervical lesion.

Conclusion: The expression of p16^{INK4a} has a diagnostic value in predicting the progressivity of precancerous cervical lesion.

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Keywords: p16^{INK4a} expression, immunocytochemistry, predictor of progressivity, precancerous cervical lesion

Abstrak

***Tujuan**: Untuk mengevaluasi nilai klinis ekspresi biomarker p16^{INK4a} dengan pemeriksaan imunositokimia (ISK) sebagai faktor prediktor progresivitas lesi prakanker serviks.*

***Metode**: Desain penelitian ini adalah studi kasus-kontrol yang akan dilakukan stratifikasi / tes dosis respon. Penelitian dilakukan di Poliklinik Kolposkopi dan Laboratorium Sitologi, Divisi Ginekologi Spesialistik, Departemen Obstetri dan Ginekologi, RSUPN Dr. Cipto Mangunkusumo, Jakarta. Penelitian berlangsung Agustus 2007 sampai September 2008. Pemeriksaan ISK dilakukan di Laboratorium Patologi Anatomi RS Dr. Hasan Sadikin, Bandung. Kami mengkategorikan pasien menjadi 2 kelompok yaitu kelompok kasus, yaitu pasien dengan NIS dan kelompok kontrol, yaitu pasien dengan NIS negatif (non NIS). Penelitian ini akan mengevaluasi biomarker p16^{INK4a} dengan cara pemeriksaan imunositokimia p16^{INK4a}.*

***Hasil**: Dilakukan pemeriksaan ISK pada 130 pasien, 26 tanpa NIS dan 104 dengan NIS. Nilai titik potong ekspresi ISK p16^{INK4a} yang didapatkan adalah 50, nilai ekspresi ISK p16^{INK4a} < 50 berarti ekspresi rendah dan sebaliknya ≥ 50, tinggi. Analisis bivariat menunjukkan hasil yang serupa dengan analisis multivariat. Di mana ekspresi ISK p16^{INK4a} tinggi memiliki odds ratio (OR) 8,4 pada NIS 1, OR 13 pada NIS 2, dan OR 21 pada NIS 3. Analisis multivariat menunjukkan temuan serupa, ekspresi p16^{INK4a} tinggi memiliki OR 17,19 pada NIS 1, 25,56 pada NIS 2 dan OR 37,32 pada NIS 3, dengan nilai p yang bermakna. Ekspresi ISK p16^{INK4a} tinggi, usia, jumlah pasangan seksual, dan pendidikan memiliki kontribusi terhadap risiko progresivitas NIS, dan dibuat suatu model skoring untuk menilai probabilitas progresivitas pada lesi prakanker serviks. Pemeriksaan ISK p16^{INK4a} disarankan masuk ke dalam triase algoritma penatalaksanaan lesi prakanker serviks (Atypical Squamous Cells of Undertemined Significance dan Lesi Intraepitelial Skuamosa Derajat Rendah) sebagai alternatif pilihan selain HPV DNA dengan pertimbangan biaya yang lebih murah.*

***Kesimpulan**: Perubahan ekspresi p16^{INK4a} mempunyai kemampuan memprediksi progresivitas lesi prakanker serviks (NIS).*

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***Kata kunci**: ekspresi imunositokimia p16^{INK4a}, prediktor progresivitas, lesi prakanker serviks*

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INTRODUCTION

Cervical cancer is the second most common cancer found in women worldwide, and contributes to the highest mortality rate among gynecological cancer.[1] In 2002, approximately 493.000 new cases were diagnosed, and 83% of them were found in developing countries.[2] The prevalence of cervical cancer in Indonesia is 100 - 190 per 100.000 citizen.[3] Pathological and surgical data in 2002 from 11 pathology centers in Indonesia showed that cervical cancer is the most

common cancer, consisting of 23.54% among all cancers in men and women and 30.63% among ten most prevalent cancers in women.[4]

Cervical cancer is a significant health problem in Indonesia due to the fact that most cervical cancer patient (62%) are presented with late stages.[5] Cervical cancer is a slow growing disease, which takes 5 - 20 years from carcinoma in situ to develop into cancer.[6]

Pap smear has dramatically reduced the incidence of cervical cancer in countries where women has access to cervical cytology examination facilities and further treatment of malignant diseases. Around 50% of cervical cancer cytological findings were undetected in the first specimen evaluation and 40% of cervical cancer was diagnosed in women who has previously received a negative test result.[7] In developing countries, it was estimated that only 5% of women have had Pap smear evaluation, including in Indonesia.[1,8] These women expect an evaluation with high accuracy to avoid the relatively high rate of false negatives in Pap smear result (10 - 50%)[9], thus a new examination to increase accuracy is needed.

Worldwide, 50 - 60% women have had HPV infection in her lifetime, however only 10 - 20% of the infection became persistent and contributed to the development of high degree precancerous cervical lesion or cervical cancer.[8]

The advance of cervical carcinogenetic detection as molecular changes due to HPV infection has opened a path for potential biomarkers to use as a mediator in identifying diseases associated by HPV infection in epithelial cells.[10] These biomarkers could one day have an important role in detection lesions with potential progressivity and also contributes in increasing the sensitivity of diagnostic procedures available today.

Using liquid-based cytology specimens, $p16^{INK4a}$ immunocytochemistry analysis has a higher Positive Predictive Value than reflex HC2 HPV testing for identifying CIN2/3 among patients with LSIL and might be useful for selecting patients with LSIL for colposcopy.[11]

It is desired that the examination of this biomarker profile could improve the accuracy of Pap smear screening, especially in doubtful cases such as Atypical Squamous Cell Undetermined Significant (ASCUS) or low-grade squamous intraepithelial lesion (LGSIL), in determining the most appropriate treatment based on the triage or as a consideration for CIN 1 cases, whether a more conservative or more aggressive treatment. Aditionally, this biomarker test could improve the accuracy of cytology specimen screening or HPV DNA test. A potential biomarker currently studied is the $p16^{INK4a}$.

In cervical dysplasia and cancer, increased production of $p16^{INK4a}$ protein may be associated with the degree of dysplasia demonstrated on a liquid-based specimen with immunocytochemical smear. The $p16^{INK4a}$ is also suggested to be a more sensitive marker than HPV DNA and $p16^{INK4a}$ is currently the most promising mediator biomarker of cervical dysplasia.[12]

Protein $p16^{INK4a}$ is a tumor suppressor protein which acts as a CDK inhibitor. It is encoded by CDKN2A located in chromosome 9 p 21. In dysplasia and cervical cancer, increased production in protein $p16^{INK4a}$ is related with the dysplastic degree that can be seen in immunohistochemistry (IHC) staining or in liquid-based cytology. Protein $p16^{INK4a}$ is now considered as a more sensitive screening method compared to HPV DNA test. The overexpression of $p16^{INK4a}$ in epithelial cells applies to all degree of dysplasia.[13]

HPV infection are characterized by expression of the viral oncogen E6 and E7. Interference of the viral oncogenes E6 and E7 with the apoptosis and cell cycle regulator p53 and pRb in proliferating cells, result in the induction of genomic instability. The inactivation of Rb as a result of deregulated HR-HPV E7 expression and the interaction of the E7 oncoprotein with pRb pathway uniformly result in premature degradation of the pRb complex.[12]

The pRb-E2F complex mediates the negative feedback for $p16^{INK4a}$ transcription. Alteration in pRb-E2F complex by E7 releases $p16^{INK4a}$ gene from transcription inhibition and results in over-expression of $p16^{INK4a}$ gene.[12]

Some research have proven that low grade lesion with a $p16^{INK4a}$ positive has progression risk higher than lesion with $p16^{INK4a}$ negative. These research show that $p16^{INK4a}$ can be used as a marker to differentiate between a more progressive lesion and lesion that will spontaneously regress.[14]

The prognosis of a precancerous cervical lesion or cervical cancer is generally differentiated in clinical profile and histopathological profile. Although those factors are considered or contributed in the disease's progressivity and prognosis, there are still other factors that couldn't be differentiated clearly. Thus, a biomarker profile is considered to make the distinction.[15]

The objective of this study is to determine the role of biomarker $p16^{INK4a}$ by immunocytochemistry on the progressivity of precancerous cervical lesion in patients with HPV infections.

METHOD

The study design is case-control. There were 130 patients presented to Colposcopy Clinic Obstetrics and Gynecology Department in Dr. Cipto Mangunkusumo National Hospital, Jakarta who met the inclusion criteria, admitted to the study. The CIN case group consists of 104 patients and the non-CIN control group consists of 26 patients.

The inclusion criteria for the case group includes: 18 - 50 years of age; have had sexual intercourse; and histological CIN (+) from biopsy. The inclusion criteria for the control group includes: 18 - 50 years of age; have had sexual intercourse; and histological CIN (–) from biopsy. The exclusion criteria for both groups are pregnancy. These patients were selected by Pap smear and target biopsy result (in accordance to study guidelines).

ICC smear for $p16^{INK4a}$ was conducted in the Laboratorium of Pathology Anatomy, Dr. Hasan Sadikin Hospital, Bandung. There were 123 patients who were eligible to undertake this examination. Seven patients were excluded because 4 patients had inconclusive results and 3 patients had less or too few cells

to examine. The $p16^{INK4a}$ expression was detected by using a primer for mouse monoclonal anti-$p16^{INK4a}$ antibody 2D9A12, Abcam ab54210 (Abcam Cambridge UK). Semiquantitative ICC assessment was conducted by 2 anatomy pathologists specializing in this field. The $p16^{INK4a}$ expression is positive if a dark brown stain was present in the nucleus with or without cytoplasmic staining, with subsequent assessment for intensity and distribution.

Statistical analysis was conducted using the software Stata version 9.2. Analysis started as univariate to determine the distribution of demographic, risk factor, and $p16^{INK4a}$ expression frequencies. Bivariate analysis was used to determine the distribution of free variables frequency and was associated with CIN progressivity, which was represented by Odds Ratio (OR). The next step is using the multinomial logistic regression analysis to design the best stepwise multivariate logistic regression model by including the variable which has p value < 0.25.

Categorization is based on value limit, by calculating the cut-off points (COP) for demographic factors (such as age, educational status, parity), risk factors (number of sexual partners, first sexual intercourse, oral contraception, smoking, and sexually transmitted infection), and $p16^{INK4a}$ expression in ICC examinations. The $p16^{INK4a}$ expression is considered low if <COP and likewise ≥ COP is considered high. Before calculating the scoring system, data input was performed beforehand, due to several incomplete data in some variables.

RESULTS

The data for demographic and risk factors were collected compeletely from 130 patients. Subsequently, data input for $p16^{INK4a}$ expression by ICC was performed. The number of patients with CIN 1 was 33, CIN 2 was 41, and CIN 3 was 30, with 26 patients as control. Immunocytochemical evaluation of $p16^{INK4a}$ was performed for these patients.

Based on the calculation of COP, the COP value for each variable is as follows:

1. Age < 41 years old
2. Education ≥ 13 years
3. Parity ≥ 2
4. Number of sexual partners ≥ 2
5. First sexual intercourse < 22 years of age
6. $p16^{INK4a}$ expression ≥ 50

Table 1 demonstrates the data distribution of the subject of this study based on the characteristic of ICC $p16^{INK4a}$ expression, in which 47 patients were categorized as low (L) $p16^{INK4a}$ expression, whereas 83 were high (H).

Table 1. Subjects Distribution Based on Immunocytochemistry

Immunocytochemistry Expression Characteristics	n = 130	
	n	%
$p16^{INK4a}$		
L (< 50)	47	36.15
H (≥ 50)	83	63.85

Note: H = high expression, L = low expression.
$p16^{INK4a}$ expression value = intensity x distribution

Table 2 shows that immunocytochemistry expression of $p16^{INK4a}$ (H) has the risk (OR) of CIN 1 8.4 times compared to $p16^{INK4a}$ (L) [95% CI, OR 2.49; 28.3 and p value = 0.001]. Whereas the risk of CIN 2, ICC expression of $p16^{INK4a}$ (H) has 13 times than the risk of $p16^{INK4a}$ (L) [95% CI OR: 3.89; 43.56 and p value = 0.0001] and for CIN 3 is 21 times [95% CI OR: 5.34; 82.53, p value < 0.0001].

Table 3 demonstrates the progressivity prediction model (fit model). For CIN 1, ICC $p16^{INK4a}$ (H) expression OR 15.91, p = 0.001; age OR 19.23, p = 0.0001; sexual partners OR 2.34, p = 0.394; education OR 3.01, p = 0.208 are contributory to the risk of progressivity in (CIN). In CIN 2 ICC expression of $p16^{INK4a}$ (H) OR 30.82, p = 0.0001; age OR 17.81, p = 0.001; sexual partners OR 7.92, p = 0.037, education OR 6.25, p = 0.066 are at risk for CIN progressivity. In CIN 3 ICC expression of $p16^{INK4a}$ (H) OR 40.34, p = 0.0001; age OR 12.87, p = 0.003; sexual partners OR 5.57, p = 0.089, education OR 2.39, p = 0.415 are all contributory to the risk of CIN.

Table 2. Odds Ratio and P value for CIN according to ICC $p16^{INK4a}$ expression characteristics.

ICC Expression Characteristics	Non-CIN n (%)	CIN 1 n (%)	OR (95%CI) p value	CIN 2 n (%)	OR (95%CI) p value	CIN 3 n (%)	OR (95%CI) p value
$p16^{INK4a}$							
L (< 50)	21 (80.77)	11 (33.33)	1	10 (24.39)	1	5 (16.67)	1
H (≥ 50)	5 (19.23)	22 (66.67)	8.4 (2.49; 28.29)	31 (75.61)	13 (3.89; 43.57)	25 (83.30)	21 (5.34; 82.53)
Test for trend (Chi^2, p Value)	29.93	0.000	0.001		0.000		0.000

Table 3. The prediction model of CIN with the evaluation of ICC $p16^{INK4a}$ expression.

Variable	coef	SE coef	coef/SE coef	OR	(95%CI OR)	p value	Score
CIN 1							
$p16^{INK4a}$	2.767	0.800	3.460	15.91	(3.32; 76.27)	0.001	4
Age	2.957	0.813	3.637	19.23	(3.91; 94.63)	0.000	4
Sexual Partner	0.851	1.000	0.852	2.34	(0.33; 16.62)	0.394	1
Education	1.100	0.994	1.107	3.01	(0.43; 21.07)	0.268	1
Constant	-2.824	0.761	-3.710			0.000	
CIN 2							
$p16^{INK4a}$	3.428	0.834	4.110	30.82	(6.01; 158.05)	0.000	5
Age	2.880	0.841	3.426	17.81	(3.43; 92.55)	0.001	4
Sexual Partner	2.069	0.990	2.090	7.92	(1.14; 55.13)	0.037	3
Education	1.833	0.997	1.839	6.25	(0.89; 44.12)	0.066	2
Constant	-3.616	0.850	-4.253			0.000	
CIN 3							
$p16^{INK4a}$	3.697	0.870	4.247	40.34	(7.32; 222.18)	0.000	5
Age	2.555	0.848	3.014	12.87	(2.44; 67.75)	0.003	4
Sexual Partner	1.718	1.011	1.698	5.57	(0.77; 40.45)	0.089	2
Education	0.872	1.069	0.816	2.39	(0.29; 19.42)	0.415	1
Constant	-3.543	0.867	-4.085			0.000	

Table 4 shows the probability of CIN with ICC evaluation. The probability of CIN for patients with scores 1 - 8 is the highest, which is 62.50%. For patients wih scores ≥ 9, the probability of CIN 2/CIN 3 is 91.67%.

Table 4. Probability of CIN with Evaluation of ICC $p16^{INK4a}$ expression.

Score	Non CIN	CIN 1	CIN 2+3
0	100	0	0
1 - 8	1.79	62.50	35.71
≥ 9	2.08	6.25	91.67

Form 1. Scoring for the Prediction of CIN with Evaluation of ICC $p16^{INK4a}$ expression.

Variable	(KI)	CIN 1 (I)	Score (KI x I)	(KI)	CIN 2 (I)	Score (KI x I)	(KI)	CIN 3 (I)	Score (KI x I)
ICC $p16^{INK4a}$									
Expression (L)	0			0			0		
Expression (H)	1	4		1	5		1	5	
Age									
≥ 41 y.o	0			0			0		
< 41 y.o	1	4		1	4		1	4	
Sexual Partners									
< 2	0			0			0		
≥ 2	1	1		1	3		1	2	
Education									
< 13 years	0			0			0		
≥ 13 years	1	1		1	2		1	1	
Total Score									

DISCUSSION

The standard practice guidelines states that, ideally an abnormal Pap smear result of a patient is followed by a colposcopic examination for target biopsy, due to the fact that definitive therapy is undertaken after a histopathological result is obtained.

The treatment is based on ASCUS LG-SIL Triase Study (ALTS) for cases with the Pap smear results including ASCUS and LGSIL based on the American Society of Colposcopy and Cervical Pathology (ASCCP) guidelines is either conducting a follow-up test 4 - 6 months later, HPV DNA detection, or colposcopic evaluation.

Thus, by conducting immunocytochemistry evaluation of $p16^{INK4a}$ biomarker after the histopathological result came out, a more precise decision for the next treatment can be made, whether it is a conservative treatment, which is follow-up, or colposcopy. If the result of $p16^{INK4a}$ biomarker is known for CIN 1 patient, the score and probability of lesion progressivity could be determined, which could lead to a more accurate treatment decision. Although the cost is relatively higher for conducting this test, the treatment decision is more accurate either before or after a biopsy result is obtained. Moreover, this biomarker evaluation is expected to replace HPV DNA evaluation with a higher specificity.

A study by Bibbo et al on cervical swab used liquid-based cytology suggested strong cytoplasmic staining on low degree squamous intraepithelial lesion is 73.68%, whereas for high degree squamous intraepithelial lesion is 96.15%. This is consistent with our study, which shows the ICC expression of $p16^{INK4a}$ (H) in CIN 1 is 66.67%, 75.61% for CIN 2, and 83.30% for CIN 3.[16]

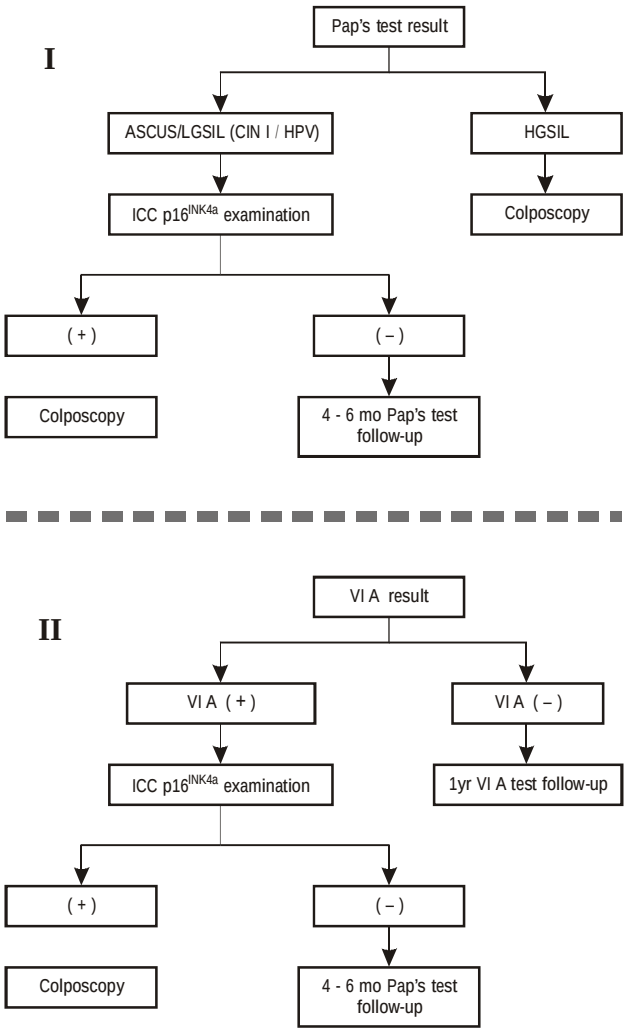
In bivariate analysis of our study, ICC expression of $p16^{INK4a}$ (H) compared with (L) $p16^{INK4a}$ had a consistently increasing risk (OR) for the occurence of CIN 1, CIN 2, and CIN 3 with p value < 0.01. This was in line with the study by Branca et al[17], which in bivariate analysis found OR of IHC expression $p16^{INK4a}$ on CIN 2 at 10.0 and on CIN 3 at 8.1, with p value < 0.01.

Similarly, in multivariate analysis, we found the ICC expression of (H) $p16^{INK4a}$ compared with (L) $p16^{INK4a}$ had a consistently increasing risk for occurence of CIN 1, CIN 2, and CIN 3. This is also in line with the study conducted by Branca et al[17], which in multivariate analysis of IHC expression of $p16^{INK4a}$ found adjusted OR for High Grade Squamous Intraepithelial Lesion (HGSIL) at 4, with p value 0.001.

The higher the grade of CIN, the higher the ICC expression of $p16^{INK4a}$. This was consistent with high-risk (HR) HPV infection in our study which found an increase corresponding with the increase of CIN grade. The role of E7 HR-HPV oncoprotein in relation to the progression of CIN could be explain in our study. Protein $p16^{INK4a}$ was a cdk inhibitor protein which would inhibit the binding of cyclin and cdk in which binding was necessary for phosphorilating pRB binding with E2F, such that E2F as the transcription factor was released and would active cell cycle. In condition in which there was HR-HPV infection, there would be deregulation of cell cycle because E7 HR-HPV oncoprotein would bind pRb such that E2F released and uncontrolled proliferation would occur. On the other hand, in the condition in which pRb is not active, there would be an increase of $p16^{INK4a}$ through the negative feedback mechanism, and the inhibition of promoter $p16^{INK4a}$ would dissapear such that an overexpression of $p16^{INK4a}$ would occur.

We found a predictive model for the progression, there were 4 variables included into this model. If a score 1 – 8 was found, the probability of CIN 1 would be the highest, i.e., 62.50%. If the score was ≥ 9, the probability for the occurence of CIN 2/CIN 3 was 91.67%.

The expression of $p16^{INK4a}$ has a significant p value and high OR, thus ICC expression of $p16^{INK4a}$ is included in the algorithm of cervical precancerous cervical lesion guideline.



Note:
1. *LGSIL: Low Grade Squamous Intraephitelial Lesion*
2. *HGSIL: High Grade Squamous Intraephitelial Lesion*

Figure 1. The Algorithm of $p16^{INK4a}$ Role in CIN Guideline Based on Abnormal Pap Smear Result (I) and Positive Visual Inspection with Acetic Acid (VIA) (II)

RESULT

Patients aged < 41 years, ≥ 13 years length of education, first time sexual intercourse < 22 years, ≥ 2 sexual partners and ICC $p16^{INK4a}$ high expression are risk factors for CIN progressivity and they can be presumed as predictors.

There is a correlation between the level of $p16^{INK4a}$ expression with the progression of cervical precancer lesions (CIN). The higher the CIN grade, the higher the $p16^{INK4a}$ expression presentation and the OR increases.

GRATITUTION

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