Demographic characteristics, risk factors and immunocytochemistry of p16^{INK4a}, Ki-67, MCM5, and survivin as predictors for the progress of cervical precancer lesion

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Abstrak

Tujuan *Untuk mengidentifikasi faktor-faktor prediksi dan biomarker dalam perkembangan lesi prakanker leher rahim atau neoplasia serviks intraepitel (CIN).*

Metode *Penelitian dilakukan dari bulan Agustus 2007 hingga September 2008. Desain penelitian adalah kasus-kontrol dengan stratifikasi uji respons dosis. Kasus adalah penderita dengan CIN. Kontrol adalah pasien non CIN. Dilakukan analisis bivariat diikuti dengan analisis multivariat.*

Hasil *Ada 130 pasien, yang terdiri dari 124 pasien yaitu CIN 1, CIN 2 dan CIN 3, dengan jumlah masing-masing 30, 41, 33, dan 26 pasien non CIN. Analisis bivariat menunjukkan bahwa umur <41 tahun, pendidikan ≥ 13 tahun, mitra seksual ≥ 2, hubungan HPV DNA positif, ekspresi p16^{INK4a}, Ki-67, MCM5 dan Survivin tinggi merupakan variabel independen untuk terjadinya CIN dengan nilai P <0,05. Namun demikian, hasil analisis multivariat, menunjukkan bahwa variabel independen yang ditemukan adalah umur, pendidikan ≥ 13 tahun, ≥ 2 orang mitra seksual, HPV DNA positif, dan ekspresi berlebih p16^{INK4a}, Ki-67 dan Survivin yang menunjukkan nilai P <0,005.*

Kesimpulan *Usia muda, pendidikan usia ≥ 13 tahun, mitra seksual ≥ 2 orang, HPV DNA positif, ekspresi p16^{INK4}a, Ki-67 dan Survivin tinggi merupakan faktor risiko untuk terjadinya peningkatan CIN, dan digunakan dalam persamaan untuk memprediksi peningkatan lesi prakanker serviks.* ***(Med J Indones 2010; 19:147-53)***

Abstract

Aim To identify the predictive factors and biomarkers in the progression of cervical precancer lesion or Cervical Intraepithelial Neoplasia (CIN).

Methods The study was conducted from August 2007 to September 2008. Design of the study was case-control with stratifications of test dose response. The cases were patients with CIN. Control patients were non CIN patients. Bivariate analysis followed by multivariate analysis was conducted.

Results There were 130 patients, consisting of 124 CIN patients divided into CIN 1, CIN 2 and CIN 3, with the following numbers of patients: 30, 41, and 33, respectively and 26 patients without CIN (non CIN). Bivariate analysis showed that age < 41 years, education ≥ 13 years, sexual partner ≥ 2, first sexual relationship at age < 22 years, smoking, the presence of sexuallly transmitted infections, positive HPV DNA, high p16^{INK4a}, Ki-67, MCM5 and Survivin expression constituted independent variables for the occurrence of CIN with *P* value of < 0.05. However, on multivariate analysis, independent variables that emerged were age, education ≥ 13 years, sexual partner ≥ 2 persons, positive HPV DNA, and over expression of p16*INK4a*, Ki-67 and Survivin that showed a *P* value of < 0.005.

Conclusion Younger ages, education age ≥ 13 years, sexual partner ≥ 2 persons, positive HPV DNA, high p16^{INK4a}, Ki-67 and Survivin expression constituted the risk factors for the occurrence of the progress of CIN, and was used in the equation to predict the progress of cervical precancer lesion. ***(Med J Indones 2010; 19:147-53)***

Key words: *case control study, equation, HPV DNA*

The progress of cervical precancer lesions in patients with persistent HPV infection could be identified through the changes in the expression of biomolecular markers. Potential biomarkers that could predict the progress of cervical precancer lesions included p16^{INK4a}, Ki-67, MCM5 and Survivin. The higher degree of the lesion, the higher the expression of its biomolecular marker. The p16^{INK4a} was an inhibitor of the enzyme cyclin dependent kinase (CDK) that played a vital role in cell proliferation by inhibiting cyclin-cdk bindings. One of the result is the prevention of pRb phosphorylation.[1] According to Redman et al,[2] the presence of p16^{INK4a}

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in the biopsy specimens indicated that the lesion was progresing into a higher degree. Negri et al reported that CIN 1 lesion with diffuse immunoreactive of $p16^{INK4a}$ was statistically more progressive to become CIN 3, than CIN 1 with negative $p16^{INK4a}$. Most of CIN 1 lesions that were not reactive to $p16^{INK4a}$ antibody would regress at the follow-up.[3]

Recent studies showed that there was significant correlation between the proliferation activity, Ki-67 distribution in the positive cells, and CIN degree, so that it could be concluded that Ki-67 could be used to identify which women would carry the highest risk for the progress and/ or recurrence of cervical squamous precancer lesions.[4]

The staining of Ki-67 was also correlated with the degree of dysplasia and the presence of HPV 16, than with other high risk types of HPV.[5] Sarian et al found that the Ki-67 expression in the histopathological results of normal cervix or cervisitis was 20.4%, CIN 1: 23.6%, CIN 2: 35.1%, and CIN 3: 47.1%.[6]

The MCM5 is an important protein in the regulation of cell cycles, i.e., as a key to initiating DNA replication. The MCM5 marked all the degrees, and the intensity of statining showed that it was not dependent on the high-risk HPV. It was emphasized that MCM5 was a potential biomarker for both dysplasia that was dependent on HPV infection (HPV dependent) and dysplasia that was not dependent on HPV infection (HPV independent).[7] In the study conducted by Murphy et al, it was found that strong immunostaining of MCM5 in CIN 1 was 93.4%, CIN 2: 96%, and CIN 3: 91%. Based on the linear regression analysis, it was found that there was a significant correlation between MCM5 staining and CIN degree. However, the degree of staining intensity was not dependent on the status of high-risk HPV.[7]

Survivin was a protein family of inhibitor of apoptosis protein (IAP). Survivin was not found in the exfoliated epithelium; however, it was expressed in the cervical cancer and tumor originating in the epithelial cell.[8] The expression of survivin in the malignant tumor was associated with unfavorable prognosis.[9] Only a few studies have been performed on the immunocytochemistry expression of Survivin in cervical precancer lesion. In the study conducted by Zulham et al at the Faculty of Medicine University of Indonesia/ Dr. Cipto Mangunkusumo General Hospital (RSCM), Survivin staining, which was based on conventional Pap smear test, the sensitivity was found at 30.76%, specifity at 71.42%, positive predictive value at 15.38%, and negative predictive value at 85.93%.[10]

Although several authors have reported the risk factors for the progression in cervical precancer lesion, this study would make a scoring model which could be used to predict the probability for the occurrence of cervical precancer lesion progression, and to identify the risk factors for the progression.

METHODS

The study was conducted from August 2007 to September 2008. This is a case control study with stratification to estimate the magnitude of various risk factors that influence the progressivity of cervical pre-cancer lesions (CIN). Risks are stated and expressed in odds ratio (OR). The research population was patients referred for colposcopy examination in the Colposcopy Clinic of the Department of Obstetrics and Gynecology, Cipto Mangunkusumo Hospital from August 2007 until September 2008. Patients with CIN was used as the case of the study, and non-CIN was used as controls.

Inclusion criteria of cases

Women of reproductive age (18-50 years old) or > 50 years old with a cytology result without any signs of atrophy, has had sexual intercourse, result of targeted histophatological biopsy showed positive CIN, consented / agreed to participate in the study
Have signed the Informed Consent

Inclusion criteria of control group

Women of reproductive age (18-50 years old) or > 50 years old with a cytology result without any signs of atrophy, has had sexual intercourse, result of histophatological biopsy showed normal cervix without CIN, consented / agreed to participate in the study, have signed the Informed Consent

Exclusion criteria

The exclusion criteria for both case and control are pregnant women, and women who are menstruasing. Patients that fulfilled our study criteria were asked to sign an informed consent form after explanation, and to fill a questionnaire to get demographic data and history taking.

Data of demographic factors i.e.: age, parity, education, and data of estabilished risk factors i.e.: the number of sexual partners, first sexual relationship, smoking, oral contraception, and sexually transmitted infection were noted. After that, specimens were obtained from

cervical swabs using cervex brush, and submersed in Liqui-prep solution followed by slide processing and immunocytochemical staining with $p16^{INK4a}$, Ki-67, MCM 5 and Survivin. A second swab was taken for HPV DNA examination. During colposcopy whenever a lesion was discovered (abnormal colposcopy), a targeted biopsy was taken. The biopsy tissue was then sent and examined by a pathologist at the Anatomic Pathology department, Cipto Mangunkusumo Hospital, Jakarta or at Hasan Sadikin Hospital, Bandung. If the cervical histopathological results (gold standard) were CIN, the patient would then be grouped in the case group, and further grouped into CIN I, CIN II, and CIN III. If the cervical histopathological results showed normal cervix or non-CIN, the patient would then be grouped as control.

HPV DNA examination

This examination was carried out by HPV DNA hybrid capture 2 (HPV DNA HC2) that included 5 stages, which were DNA denaturation, probe mixing and hybridization, hybrid capture, hybrid DNA-RNA detection, and finally detection, validation, and interpretation. This examination included both positive and negative control that were provided inside the kit to determine the cut off value.

Immunocytochemical staining (ICC) of the slides

Immunocytochemical staining used the labelling streptavidin biotin complex (LSAB) method. The ICC staining of slides for $p16^{INK4a}$, Ki-67, and MCM5 was conducted in the Departement of Anatomic Pathology of the Hasan Sadikin Hospital, and the ICC staining of slides for Survivin was conducted at the Department of Histology, Faculty of Medicine University of Indonesia. As a positive control, a tissue biopsy of cervical cancer was used. On every staining, a comparison was always made with positive and negative controls.

In ICC staining, $p16^{INK4a}$, Ki-67, MCM5, and Survivin were identified using mouse monoclonal anti-$p16^{INK4a}$ antibody [2D9A12], Abcam ab54210 (Abcam Cambridge UK, dilution 1:100); mouse monoclonal anti-Ki-67 antibody (cloneBGX-Ki-67, Biogenex, England, dilution 1:50), mouse monoclonal anti-MCM5 antibody (CRCT.1 [A2.7A3], dilution 1:100) and policlonal rabbit anti-survivin antibody (Sigma SNA S 8191, dilution 1:100), respectively as the primary antibodies. Further, biotinylated universal secondary antibody were used for $p16^{INK4a}$, Ki-67, and MCM5, followed by peroxidase conjugated–streptavidin, and peroxidase conjugated goat anti rabbit antibody (dilution 1:100) were used for survivin. Finally, visualization was done using DAB as substrate.

Data analysis

Statistical analysis was conducted using the STATA version 9.2 program. The data of demographic characteristic and established risk factors were first noted, tabulated and descriptive analysis was done to see the distribution of frequencies. Further, continuous data were presented as mean and standard deviation, and minimal and maximal value.

The cut-off point (COP) was determined for age, education, parity, established risk factors, HPV DNA, and immunocytochemical expression of $p16^{INK4a}$, Ki-67, MCM5, and Survivin.

Bivariate analysis was used to identify the association of each independent variable with the occurrence of CIN and its progressivity, and represented as Odds ratio (OR). The next step of the analysis was to apply multinominal logistic regression, using the stepwise multivariate model. The data included in the multivariate analysis were demographic data, the risk factors, HPV DNA and results of immunocytochemistry stainings.

Finally, a model for predicting the risk of the progress of CIN and the equation to calculate the probality of the progression was developed.

RESULTS

As many as 130 patients were found to meet the inclusion criteria, which consisted of 124 CIN patients as the cases that were divided into groups of CIN 1, CIN 2, and CIN 3, of 33, 41 and 30 patients, respectively, and control patients of 26 subjects.

Data of demographic factors were shown in Table 1, and distribution of some risk factors showed that the number of sexual partners had a range of 1-20 with a mean of 1.88 (standard deviation 2.35) (Table 2).

Table 1. Demographic profile of the patients (cases and controls)

Demographic profile	n=130	
	N	%
Age		
<20 years	10	7.69
20-29 years	31	23.85
30-39 years	42	32.31
40-49 years	42	32.31
>50 years	5	3.85
Range= 18-51 years		
Mean (SD)= 34.65 (9.59)		
Length of education		
0-9 years	61	46.92
10-12 years	46	35.38
≥13 years	23	17.69
Range= 0-22 th years		
Mean (SD)= 10.21 (4.20)		
Parity		
0	39	30
1	21	16.15
1-3	56	43.08
4-6	14	10.77
Range= 0-6		
Mean (SD)= 1.72 (1.51)		

Bivariate analysis on demographic data and established risk factors identified the risk factors for the occurrence of CIN 1, CIN 2, and CIN 3 included age < 41 years (P< 0.0001), sexual partner ≥ 2 persons for CIN 2 and CIN 3 (P= 0.003 and 0.012), first sexual relationship < 22 years for CIN 3 (P= 0.021), and the presence of sexual transmitted infection for CIN 1, 2, and 3 (P= 0.028, 0.007 and 0.014). The result of bivariate analysis for various ICC expression and HPV DNA is shown Table 3, and the result of multivariate analysis in Table 4.

The equation to calculate the probability of the progression of CIN is:

$$\Pr(\text{progress\_CIN0}) = \frac{1}{\text{Total score } 1+e}$$

The total score for each individual can be calculated using the scores in Table 5. The result of using this equation were in accordance with the individual result examination.

Table 2. Demographic risk factors for the occurrence of CIN

Characteristics of risk factors	N=130	
	N	(%)
Number of sexual partner		
1 person	89	68.46
2-4 persons	33	25.38
≥ 5 persons	8	6.15
Range= 1-20		
Mean (SD)= 1.88 (2.35)		
First sexual relationship		
<17 years	26	20
17-20 years	51	39.23
≥ 21 years	53	40.77
Range= 10-36 years		
Mean (SD)= 20.58 (5.00)		
Oral contraception		
No	119	91.54
<5 years	4	3.08
≥5 years	7	5.38
Smoking		
No smoking	112	86.15
<10 cigarettes/day	7	5.38
10-20 cigarettes/day	8	6.15
≥21 cigarettes/day	3	2.32
Sexually Transmitted Infection		
No laboratory results	92	70.77
Laboratory results were present	38	29.33

Table 3. Odd's Ratio and P values for the progression to CIN according to the various immunocytochemistry expression and HPV DNA

Characteristics of ICC expression	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
$p16^{INK4a}$							
Low (< 50)	21(80.77)	11(33.33)	1	10(24.39)	1	5(16.67)	1
High (≥ 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)
			0.001		0.000		0.000
Test for trend (X^2; *P* value)= (29.93; 0.000)							
Ki-67							
Low (< 7.5)	24(92.31)	22(66.67)	1	26(63.41)	1	18(60.00)	1
High (≥ 7.5)	2 (7.69)	11(33.33)	6(1.19;30.1)	15(36.59)	6.9(1.43;33.49)	12(40.00)	8(1.59;40.30)
			0.030		0.016		0.012
Test for trend (X^2; *P* value)= (8.45; 0.038)							
MCM5							
Low (< 15)	21(80.77)	21(63.64)	1	21(51.22)	1	15(50.00)	1
High (≥15)	5(9.23)	12(36.36)	2.4(0.71;8.01)	20 (48.78)	4(1.26;12.65)	15(50.00)	4.2(1.25;14.08)
			0.155		0.018		0.020
Test for trend (X^2; *P* value)= (7.42l; 0.060)							
Survivin							
Low (< 70)	21(80.77)	19 (57.58)	1	6 (14.63)	1	4(13.33)	1
High (≥ 70)	5 (19.23)	14 (42.42)	3.1(0.94;10.22)	35 (85.37)	24.5(6.64;90.29)	26(86.67)	27.3(6.50;114.65)
			0.064		0.000		0.000
Test for trend (X^2; *P* value)= (42.59; 0.000)							
HPV DNA							
Negative	26(100)	26(78.79)	1	18(43.90)	1	9(30)	1
Positive	0(0.00)	7(21.21)	6.73(0.77;58.72)	23(56.10)	31.94(3.94;258.73)	21(70)	50(5.89;424.15)
			0.000		0.000		0.000
Test for trend (Fisher exact. *P* value)= (20.3125; 0.000)							

Tabel 4. Models for predicting the progression to CIN by various variables

Characteristics	Coef	SE Coef	*OR*	(95% *CI*)	*P*	Score
CIN 1						
$p16^{INK4a}$	2.251	0.814	9.49	1.92-46.83	0.006	21
Ki-67	1.545	1.043	4.69	0.61-36.19	0.138	11
Survivin	0.015	0.806	1.02	0.21-4.93	0.985	0
High risk HPV	0.164	1.222	1.18	0.11-2.94	0.893	1
Age	2.754	0.806	15.71	3.24-76.23	0.001	25
Sexual partner	0.990	1.055	2.69	0.34-21.28	0.348	7
Length of education	1.383	1.024	3.99	0.54-29.64	0.177	10
Constant	-2.878	0.797				
CIN 2						
$p16^{INK4a}$	2.316	0.903	10.14	1.73-59.46	0.010	19
Ki-67	1.168	1.107	3.22	0.37-28.18	0.292	8
Survivin	1.921	0.869	6.83	1.24-37.51	0.027	16
High risk HPV	1.804	1.201	6.07	0.58-63.88	0.133	11
Age	2.239	0.905	9.38	1.59-55.29	0.013	18
Sexual partner	2.037	1.075	7.66	0.93-63.08	0.058	14
Length of education	2.227	1.082	9.27	1.11-77.34	0.040	15
Constant	-4.634	1.046				
CIN 3						
$p16^{INK4a}$	2.559	0.953	12.92	2.00-83.62	0.007	20
Ki-67	1.038	1.122	2.82	0.31-25.43	0.355	7
Survivin	1.976	0.916	7.21	12.0-43.0	0.031	16
High risk HPV	2..071	1.219	7.94	0.73-86.34	0.089	13
Age	1.886	0.924	6.59	1.08-40.30	0.041	15
Sexual partner	1.695	1.100	5.44	0.63-46.98	0.123	11
Length of education	1.333	1.157	3.79	0.39-36.63	0.250	9
Constant	-4.697	1.109				

Coef= coefficient, SE= standard error, OR= Odds ratio, CI= confidence interval

Table 5. Probability for the progression to CIN1, CIN2, and CIN3 by various variables

Variable	CIN 1 Score (ICxI)	CIN 2 Score (ICxI)	CIN 3 Score (ICxI)
$P16^{INK4a}$ ICC			
High	2.251	2.316	2.559
Low	0	0	0
Ki-67 ICC			
High	1.545	1.168	1.038
Low	0	0	0
Survivin ICC			
High	0.015	1.921	1.976
Low	0	0	0
High-risk HPV DNA			
Positive	0.164	1.804	2.071
Negative	0	0	0
Age (years)			
< 41	0.990	2.239	1.886
≥ 41	0	0	0
Sexual partner			
< 2	0.990	2.037	1.695
≥ 2	0	0	0
Education			
≥ 13 years	1.383	2.227	1.333
< 13 years	0	0	0
Constant	-2.878	-4.634	-4.697
Total score			

IC= individual characteristic, I= index, ICC= immunocytochemistry

DISCUSSION

The study conducted by Bibbo et al[11] in the cervical smear using liquid-based cytology found strong staining in the cytoplasm in low grade intraepithelial lesion to be as high as 73.68%, while in high grade intraepithelial lesion 96.15%. Our study found almost similar results, i.e. high $p16^{INK4a}$ expression in CIN 1, CIN 2 and CIN 3 was 66.67%, 75.61% and 83.30%, respectively (Table 3).

The sample size required to detect HPV DNA with an α of 0.05, a power of 80%, OR of 9, and the proportion of high risk HPV detection with a normal biopsy of 0.10 was calculated to be 20 for each group. With the intention of conducting a stratification/dose response test for CIN I, CIN II, CIN III, and control, we concluded that the minimal number of samples in each group should be 20.

The sample size to evaluate the expression of $p16^{INK4a}$ with an α of 0.05, a power of 80%, the proportion of $p16^{INK4a}$ in the CIN group of 0.83, and an OR of 6 was calculated to be 21 for each group. The sample size to evaluate the expression of Ki-67 with an α of 0.05, a power of 80%, the proportion of Ki-67 in the Non-CIN group of 0.91, and an OR of 6 was calculated to be 34 for each group. The sample size to evaluate the expression of MCM5 with an α of 0.05, a power of 80%, the proportion of MCM5 in the CIN group of 0.86 and an OR of 6 was calculated to be 14 for each group.[12]

The sample size to evaluate the expression of Survivin with an α of 0.05, a power of 80%, of and the proportion of survivin in the CIN group of 0.86[12] and an OR of 6 was calculated to be 14 for each group. According to the sample size calculations above and based on the Odds ratio in genetic or biomolecular studies that usually is between 6-7, an OR of 6 was chosen. With an OR of 6, the order of sample size according to the size for $p16^{INK4a}$, Ki-67, MCM5, and Survivin are 21, 34, 14, and 14, respectively, and for HPV DNA with an OR of 9, the sample size is 20. From the data above, the largest number of sample is 34, and was determined to be the sample size for each group: i.e. CIN I = CIN II = CIN III = Control.

In the current study, we found that high Ki-67 ICC expression had a higher risk for the occurrence of CIN 1, CIN 2 and CIN 3 than low Ki-67 expression (Table 3). However, multivariate analysis showed that it was not statistically significant (Table 4).

Bivariate analysis of the MCM5 ICC expression in our study showed that high MCM5 had a higher risk for the occurrence of CIN 1 than low MCM5 expression. However, this risk was not statistically significant, while high MCM5 expression had a risk for the occurrence of CIN 2 and CIN 3, which was statistically significant (Table 3). Although statistically significant on bivariate analysis, in multivariate analysis MCM5 ICC expression were ruled out.

On univariate analysis, we found that high Survivin ICC expression to be increasingly on the rise with the increase of CIN degree. Bivariate analysis showed that high Survivin ICC expression had a higher risk for the occurrence of CIN 1 than low Survivin, although it was statistically not significant, yet it was statistically significant for the occurrence of CIN 2 and CIN 3 (Table 3), the same as in multivariate analysis (Table 4).

In our study, multivariate analysis showed that the independent risk factors to the progression to CIN, with *P* value of <0.25 were younger ages, sexual partner ≥ 2 persons, length of education ≥ 13 years, positive high HPV DNA, and high $p16^{INK4a}$, Ki-67, and Survivin ICC expression. Based on these findings, the probability of the progression to CIN for each individuals can be predicted using the equation and the values in Table 5.

In conclusion, on multivariate analysis, age < 41 years, and high ICC expression of $p16^{INK4a}$ and Survivin were the most important independent risk factors for the occurrence of the progression of CIN. The model to calculate the probability for the progression to CIN was developed using age, number of sexual partners, length of education, positivity for HPV DNA and high ICC expression of $p16^{INK4a}$, Ki-67 and Survivin as predictors, and the results were in accordance with the individual's examination results. The model is easy to use in the routine clinical applications.

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