Comparison of \textit{p}^{16\text{INK4a}} Expression between Low Grade Squamous Intraepithelial Lesion and High Grade Squamous Intraepithelial Lesion from LEEP/Biopsy Specimens

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\textbf{ABSTRACT}

\textit{p}^{16\text{INK4a}} protein is associated with the progression of pre-cancerous lesions. \textit{p}^{16\text{INK4a}} is important because it illustrates the extent of DNA of HPV that have been integrated into the host DNA and has damaged the function of PRB. The examination of \textit{p}^{16\text{INK4a}} be important as additional checks on pre-cancerous lesions because it can assess the progression of pre-cancerous lesions. The purpose of this study was to investigate the differentiation of \textit{p}^{16\text{INK4a}} expression between HSIL and LSIL histological specimen. This was an observational study with cross sectional approach in Gynecology Oncology Clinic and Pathology Anatomy Department of Dr Soetomo General Hospital, Surabaya. The samples are histological specimens collected from storage of Pathology Anatomy Department of Dr. Soetomo General Hospital, Surabaya Mei 1st, 2011 to August 31st, 2011. Samples were collected consecutively for each group (HSIL,LSIL), thus 32 samples totally. All samples were processed by immunocitochemistry using monoclonal mouse anti-human JCS, by Dako, Glostrup Denmark and expression \textit{p}^{16\text{INK4a}} were evaluated by cytopatologist. \textit{p}^{16\text{INK4a}} expression were counted semiquantitavely with Klaes criteria. The differentiation of \textit{p}^{16\text{INK4a}} expression between HSIL and LSIL smear was analyzed by Mann Whitney test. It was found that the \textit{p}^{16\text{INK4a}} expression in LSIL; 12.5% were negative and 31.25% were 1, 25% were 2 and 31.25% were 3 score from Klaes criteria. The differentiation of \textit{p}^{16\text{INK4a}} expression between HSIL and LSIL smear were significant \( p = 0.001 \) (\( p < 0.05 \)). In conclusion, \textit{p}^{16\text{INK4a}} expression is higher in HSIL (MOG 2012;20:117-121)

\textbf{Keywords:} \textit{p}^{16\text{INK4a}} expression, LSIL, HSIL, Cervical precancerous lesion, LEEP/Biopsy.

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\textbf{INTRODUCTION}

Disease course of cervical squamous cell carcinoma is one model of carcinogenesis is through the stages or multistep, starting from the beginning to the carcinogenesis process of morphological changes to grow into invasive cancer.\textsuperscript{1} CIN terminology was first introduced by Richart. To link between cytology and histology findings, was made of The Bethesda System is prepared to be an international standard. The Bethesda system is more instrumental complement what has been described by the CIN system. In epidemiological and laboratory has demonstrated that HPV infection is a major cause pre-malignant and malignant lesions of cervical epithelium, namely the discovery of DNA HPV in 95% to 100% of cases.\textsuperscript{2,3} HPV is the initiator of the factors
that cause cervical cancer cell disruption cervix. Integration of HPV DNA in the genome of cells of the body is the beginning of the process that leads to transformation. Oncoprotein early 6 and 7 (E6, E7) from HPV is the cause of the occurrence of malignant degeneration. Oncoprotein E6 to bind p53 Tumor Suppressor Gene so (TSG) p53 would lose its function. While the E7 oncoprotein to bind TSG Retinoblastoma protein (PRB), this bond would cause the release of E2F from the PRB, and E2F is a transcription factor that goes without cell cycle control, DNA repair does not occur, and apoptosis does not occur.1-4

Protein \( p^{16\text{INK4a}} \), a tumor suppressor protein that inhibits cyclin D1 complex workphosphorylate cdk4 and cdk6 in the PRB, so the PRB will be detached from the bonds of PRB/E2F. Accumulation of E2F will induce activity of \( p^{16\text{INK4a}} \). Overexpression of \( p^{16\text{INK4a}} \) in cervical cancer that occur as a result of functional inactivation of PRB by HPV E7 proteins. Many studies show a linear relationship between the expressions of \( p^{16\text{INK4a}} \) with degree of dysplasia.5,6 In epidemiology, it is evident that HPV infection is a factor that plays an important role in the incidence of cervical cancer, but it does not mean that the presence of HPV infection will develop into cervical cancer. From the study mentioned that only 30-40% of patients who Risk Papillomavirus High infection (HR HPV) which will develop into cervical cancer.7,8,9

\( p^{16\text{INK4a}} \) protein is associated with the progression of precanceros lesions, as shown by a study conducted by Negri et al, who showed that a pre-cancero us lesions LSIL with a diffuse \( p^{16\text{INK4a}} \) expression (high) will be more progress to HSIL compared with LSIL who have low expression of \( p^{16\text{INK4a}} \). Hariri and Oster reported a negative predictive value (NPV) of \( p^{16\text{INK4a}} \) by 96%. Low Risk Human Papillomavirus infection has the ability to integrate with host DNA is low, so PRB hosts are not disturbed, it causes the expression of \( p^{16\text{INK4a}} \) in low-risk HPV infection is low.10,11

Branca et al. and Ozgul et al. reported that expression of \( p^{16\text{INK4a}} \) in touch directly with the increasing degree of dysplasia.12 The role of \( p^{16\text{INK4a}} \) is important because it may illustrate how large DNA of HPV that have been integrated into the host DNA and has damaged the function of PRB. So the examination of \( p^{16\text{INK4a}} \) be important as additional checks on pre-cancerous lesions because it can assess the progression of precancerous lesions of how big that HPV DNA has been integrated into the host DNA with observing the color intensity produced by this immuno-histochemical staining.10,11

MATERIALS AND METHODS

The design of the study is a double blind cross sectional study. It was performed on May to July 2011. Subject of the research was taken from preparations of LEEP/Biopsy which was stored in histopathological laboratory and was read with result of LSIL and HSIL. They were re-evaluated by the cytopathologist to see its proper and then and 32 readable-preparations were taken, then they were performed discoloration and then they were given new staining by immunocytochemistry p16 by antibody monoclonal JC8, which was produced by DAKO, Glostrup, Denmark, and then their \( p^{16\text{INK4a}} \) expression was evaluated.

The LEEP/Biopsy preparations were performed discoloration by being soaked in acid alcohol 1% for 30 minutes, rinsed by aquades, and cooled for 20 minutes. We performed procedure of immunocytochemistry staining, by primary antibody JC8 about one night in temperature as low as 4°C and secondary antibody labelled biotin (Anti Rabbit IgG Biotin labelled) about one hour in room temperature, brown dye by Cromogen DAB (3,3-diaminobenzidine tetratetrahydrochloride) about 10-20 minutes in room temperature, and Counterstain by Hematoxylin Eosin about 5 minutes in temperature room. \( p^{16\text{INK4a}} \) expression was marked by the brown colour absorption on the positive cells, either on the nucleus and the cytoplasm. \( p^{16\text{INK4a}} \) expression assessed by a pathologist using the criteria of Klaes to see a picture of brown on the nucleus and cytoplasm, which is divided into semiquantitative: Score 0 (Negative): <1% of all cells positive, Score 1 (sporadic): only a few cells are positive but not more than 5% of all cells, Score 2 (Focal): a few small groups of cells are positive but less than 25% of all cells, with increased intensity of brown color, and Score 3 (Diffuse):> 25% positive cells, with the intensity of the color brown are greatly increased.14 To see the Comparison of \( p^{16\text{INK4a}} \) between LSIL and HSIL using Mann Whitney test.

RESULTS

The result that we got was tabulated as Table 1 and it was figured out on Figure 1. On LSIL, we found 2 samples (12.5%) of negative \( p^{16\text{INK4a}} \) expression/score 0, 5 samples of expression with score 1, 4 samples with score 2 and 5 samples with score 3 from samples evaluated. While on HSIL, we got only (6,25%) of \( p^{16\text{INK4a}} \) expression/score sample (93,75%) of expression with score 5 samples (31.5%) of score 2 from total 16 HSIL samples examined.
Table 1. \( p_{16}^{\text{INK4a}} \) expression

<table>
<thead>
<tr>
<th>Reading</th>
<th>( p_{16}^{\text{INK4a}} ) Score</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score 0</td>
<td>Score 1</td>
<td>Score 2</td>
<td>Score 3</td>
</tr>
<tr>
<td>LSIL</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(12.5%)</td>
<td>(31.25%)</td>
<td>(25%)</td>
<td>(31.25%)</td>
</tr>
<tr>
<td>HSIL</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>(6.25%)</td>
<td>(93.75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

For the positive result, LSIL group while most of HSIL group has reached score though the result showed that the score of \( p_{16}^{\text{INK4a}} \) expression on HSIL pap smears are higher than LSIL, but the statistic analysis by Mann-Whitney show the significantly different result (\( p = 0.001 \)).

We can see the feature which was resulted from the procedure of immunocytochemistry staining procedure. Hematoxilin eosin counterstain on the staining was intended to keep the epithelial cells morphology as seen on Papaniculaou staining. The integration feature between HPV DNA and host cell DNA could be evaluated by seeing the \( p_{16}^{\text{INK4a}} \) expression which was resulted by reaction between JC8 primary antibody and DAB chromogen, showed by brownish intensity on cytoplasm and nucleus.

**DISCUSSION**

Cervical cancer remains the second ranked cause of cancer death among women in developing countries. As already known that HPV infection was detected in 99.7% of patients with cervical cancer, HPV infection is an infection that is very important in cervical cancer journey. In case-control study, the prevalence of HPV infection in cervical cancer squamous cell carcinoma types encountered a number from 78.4 to 98.1%. In addition to a simple infection, HPV particle interaction with specific host cell is also required for the initiation and progression of HPV that cause dysplasia. This virus will infect epithelial stem cells in place that are structurally and anatomically easily accessible, namely the squamous columnar junction in the transformation zone. This virus will utilize the host cell's DNA replication machinery to replicate episomal genome.

Integration of HPV DNA in the genome of cells of the body is the beginning of the process that leads to transformation. HR HPV infection often found in young women and several cross-sectional studies mention the highest prevalence was found in the average age of 20-35 years, ie more than 30%. In the longitudinal cohort study mentioned that the acute period of infection is about 8-10 months. Just a few of these infections are persistent and cause epithelial dysplasia in the cervix. With reference to these epidemiological studies, the use of virological examination as the primary parameter screening still debated because of the high prevalence of infected women without pathological changes in the cervical epithelium, so the discovery of HR HPV infections have low positive predictive value of the occurrence of dysplasia and HR HPV negative has a negative predictive value is very high because of the close relationship between persistent infection with HR HPV cervical dysplasia and cancer.

Although epidemiology is said clearly that HPV infection is important in the incidence of cervical cancer but not necessarily in the presence of HPV infection will develop into cervical cancer. It says only 30-40% of patients who are infected High Risk Papillomavirus (HR HPV) which will develop into cervical cancer. Metaplasia and reactive changes are not associated with HPV infection can give a LSIL or CIN I.

Protein \( p_{16}^{\text{INK4a}} \), a tumor suppressor protein that inhibits cyclin D1 complex work phosphorylate cdk4 and cdk6 in the PRB, so the PRB will be detached from the bonds of PRBE2F. Accumulation of E2F will induce activity of \( p_{16}^{\text{INK4a}} \). Overexpression of \( p_{16}^{\text{INK4a}} \) in cervical cancer that occur as a result of functional inactivation of PRB by HPV E7 proteins.

In this study, the results obtained in the LSIL group: 12.5% of samples did not give the expression of \( p_{16}^{\text{INK4a}} \), 31.25% of samples gave sporadic expression, focal 25% sample and 31.25% of the sample diffuse. It is in accordance with those reported by Doeberitz that...
obtained 60% of LSIL cases showed increased expression of $p^{16INK4a}$, while the other 40% have not improved despite acquired HPV infection and provide an overview koilocytosis, so 12.5% of samples that do not provide $p^{16INK4a}$ expression can be derived from LSIL samples caused by infection with LR HPV E7 with a lower affinity of HR HPV. In addition, metaplasia and reactive changes are not associated with HPV infection can give a LSIL or CIN I. Some histological findings that could also provide an overview LSIL include: squamous metaplasia, basal cell hyperplasia, hyperplasia microglandular, and inflammation. In 5 cases of LSIL or 31.25% of the results obtained are diffuse, this suggests that HPV DNA has been integrated with the host cell's DNA, but the picture still LSIL cell morphological changes due to the nature of the disease course of cervical cancer through a multistep itself and takes time about 5 years, so the samples are examined LSIL may originate from LSIL patients who are being developed into HSIL. Negri et al. reported that a precancerous lesions LSIL with a diffuse $p^{16INK4a}$ expression (high) will be more progress to HSIL compared with LSIL who have low expression of $p^{16INK4a}$. By looking at these results, it is for patients with LSIL with a picture of this increased expression of $p^{16INK4a}$ should consider more aggressive therapy, given the possibility of evolving into a higher lesion is very large. While in preparation biopsy or LEEP with reading the results obtained 93.75% HSIL HSIL preparations had a score of 3 means that a very strong expression of $p^{16INK4a}$ protein in HSIL and preparations are consistent with research conducted by Nieh et al. who found that the majority of LSIL only weakly positive and the majority of HSIL is a strong positive.

Branca et al. and Ozgul et al. reported that expression of $p^{16INK4a}$ in touch directly with the increasing degree of dysplasia. The study by Bibbo and Klaes found that $p^{16INK4a}$ positive in all samples of HSIL and squamous carcinoma. Pientong et.al found that in all cases examined HR HPV positive by PCR also obtained positive results on examination and concluded that the $p^{16INK4a}$ $p^{16INK4a}$ could be used as a diagnostic method to examine the HR HPVinfectedcells. Statistically $p^{16INK4a}$ expression differences obtained from both groups. From the statistical analysis significant differences were found, with $p = 0.001$. ($P < 0.05$). This difference results reinforce the hypothesis that the expression of $p^{16INK4a}$ protein in the preparations was higher than LSIL HSIL. Because the more HSIL HPV DNA has been integrated with the host cell genome. The more protein E7 of HR HPV virus that inactivates the function of PRB, the more independent of E2F by PRB bond and free, so it will cause uncontrolled transformation of cervical cells and induce protein $p^{16INK4a}$ as an inhibitor for inhibiting the release of E2F from the PRB, so that the dosage appears that increased expression of $p^{16INK4a}$.

**CONCLUSION**

$p^{16INK4a}$ expression is higher in HSIL.

**REFERENCES**


