

Expression of geminin, p16, and Ki67 in cervical intraepithelial neoplasm and normal tissues

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Abstract

Geminin is a protein involved in cell cycle progression. We aimed to evaluate the diagnostic value of geminin expression in cervical intraepithelial neoplasm (CIN).

The expression of geminin, p16, and Ki67 was examined in 95 samples, including CIN1 (n=45), CIN2/3 (n=40), and normal cervical tissues (n=10) by immunohistochemistry. The correlation between geminin or p16 expression and human papillomavirus (HPV) status was also evaluated.

Geminin expression was negative in all normal tissues and expressed in 13.3% of CIN1 and 90.0% of CIN2/3. P16 expression was demonstrated in 24.4% of CIN1 and 87.5% of CIN2/3. The corresponding Ki67 expression was 35.6% and 95.0%. The specificity of geminin for differentiating between CIN1 and CIN2/3 was 86.7%, while for p16 and Ki67 the corresponding values were 75.6% and 64.4%. The sensitivity of geminin, p16, and Ki67 was 90.0%, 87.5%, and 95.0%, respectively. The positive predictive value (PPV) and accuracy of geminin were higher than p16 and Ki67. In addition, geminin expression showed a weak correlation with HPV status, but there was no association between p16 expression and HPV status.

These results suggested that geminin had a high degree of sensitivity and specificity in determining CIN2/3. In addition to p16 and Ki67, geminin might be used as a new biomarker to distinguish between CIN1 and CIN2/3.

Abbreviations: CIN = cervical intraepithelial neoplasm, HPV = human papillomavirus, HSIL = high-grade squamous intraepithelial lesion, LSIL = low-grade squamous intraepithelial lesion, MCM = minichromosome maintenance complex component, NPV = negative predictive value, PPV = positive predictive value.

Keywords: cervical intraepithelial neoplasm, diagnosis, geminin, Ki67, p16

1. Introduction

Invasive cervical carcinoma is preceded by preinvasive cervical intraepithelial neoplasm (CIN). The most recent classification system of World Health Organization (WHO) applied low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL). However, CIN is previously classified into CIN1, CIN2, and CIN3 on the basis of the extent of epithelial involvement.^[1] CIN1 is usually not precancerous and does not require treatment. The progression rate of CIN2/3 to invasive cervical cancer is 10% to 40%.^[2,3] Given the

malignant potential of CIN2/3, it is important to have an accurate grading of CIN and to treat these patients properly. High-risk human papillomavirus (HPV) types, including HPV-16 and HPV-18, have been identified as the major cause of cervical carcinomas and precancerous lesions. Currently, the incidence rate of cervical cancer has decreased through screening by cytological Pap smear testing and HPV testing.^[4] HPV-18, one common reason for CIN2/3, is poorly detected by cytology and colposcopy.^[5] Furthermore, HPV testing is more sensitive than cytology for predicting CIN2/3, but is of less specificity.^[6] Histological diagnosis of cervical biopsies is regarded as “gold standard.” However, the interobserver and intraobserver variabilities are high.^[1] For these reasons, it is important to have an accurate diagnosis and prediction of progression risk for clinical management of patients with CIN.

P16 (also known as p16INK4a), a cyclin-dependent kinase inhibitor, is a cell-cycle regulatory protein. The high-risk HPV E7 oncoproteins bind and inactivate pRb, leading to abnormal cell proliferation. P16 is an accurate marker for this event. However, p16 is a less specific marker because CIN1 and CIN2/3 sometimes show similar p16 expression, and p16 also can be found in inflammatory cervical lesions.^[7,8] Ki67 is a marker of cell proliferation. A number of studies have shown that an increased expression of Ki67 is correlated with higher cervical CIN grade and is a highly sensitive biomarker for differentiating between CIN1 and CIN2/3, but Ki67 immunostaining is variable and less specific in many cases.^[9,10] Currently, p16 and Ki67 have been proposed to identify persistent infections with high-risk HPV types of cervical precursor lesions. Despite the value of these 2 complementary alternative biomarkers, staining of p16 and Ki67 is not sufficient to give a definite diagnosis and an accurate differentiation in some cases.

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Geminin is located on the sixth chromosome, and the protein has about 209 amino acids. Geminin is an important component of the licensing system that is involved in cell cycle progression.^[11] Alterations in geminin expression are associated with cell proliferation, differentiation, and development. The expression of geminin is increased as the cell cycle progresses and downregulated when the cells exit the cell cycle.^[12] A great number of studies have reported that geminin plays a vital role in cancer pathophysiology and development. Differential geminin expression is associated with various cancers, including breast cancer, colorectal carcinomas, and small lung adenocarcinoma.^[13–15] Suppression of geminin can inhibit cancer cell proliferation without affecting the normal cells.^[16] Using high-density microarrays, Martin et al^[17] have identified several genes involved in cell cycle regulation that are differentially expressed in premalignant and malignant cervical disease, including geminin, p16, minichromosome maintenance complex component (MCM) 3, and MCM5. Real-time polymerase chain reaction (PCR) and immunohistochemistry were used to confirm microarray results. This study suggested that geminin might play a role in prediction of progression of cervical precursor lesions.

In this study, we aimed to evaluate and compare staining pattern for geminin, p16, and Ki67 expression in normal cervical tissues, CIN1 and CIN2/3 to determine whether geminin can serve as an additional marker for the diagnosis of CIN2/3. In addition, we investigated whether geminin could discriminate between HPV-positive and HPV-negative precursor lesions.

2. Methods

2.1. Patients and controls

A total of 95 cervical samples were recruited from the First Affiliated Hospital of Nanjing Medical University between January 2015 and January 2016, including 10 normal cervical tissues, 45 CIN1, and 40 CIN2/3. The diagnosis of CIN was peer-reviewed according to the International Federation of Gynecology and Obstetrics criteria. All hematoxylin-eosin stained slides were reviewed by 2 independent pathologists. Exclusion criteria are patients who had a clinical record of radiotherapy or chemotherapy. The clinical characteristics of the subjects are summarized in Table 1. This research project was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Informed written consent was obtained from all subjects.

2.2. HPV detection

HPV DNA was detected by the Hybrid Capture II System. The scrapes were tested for the presence of high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

Table 1
Patient clinicopathologic characteristics.

	Control group	CIN1	CIN2/3
Number of enrolled subjects	10	45	40
Age	46.1 ± 5.9	48.1 ± 7.9	47.8 ± 8.3
HPV-positive tests		37 (82.2%)	39 (97.5%)*

CIN=cervical intraepithelial neoplasm, HPV=human papillomavirus.

* $P < .05$ compared with patients with CIN1; 1-way analysis of variance (ANOVA) for patients demographical data (age).

2.3. Immunohistochemistry analysis

All formalin-fixed paraffin-embedded sections were cut at 5 μ m thickness and then dewaxed through xylene and dehydration with graded ethanols. The tissue sections were treated with 3% hydrogen peroxidase solution for 20 minutes to block endogenous peroxidase activity. Antigen retrieval was performed in 10 mmol/L of citrate buffer (Dako, Carpinteria, CA). The sections were then incubated overnight with a monoclonal mouse antibody to geminin (Abcam, Cambridge, MA) at 1:100 dilution, a monoclonal rabbit antibody to p16 (Abcam, Cambridge, MA) at 1:100 dilution, and a monoclonal rabbit antibody to Ki67 (Abcam, Cambridge, MA) at 1:500 dilution, respectively. Subsequently, tissue sections were counterstained with hematoxylin and then examined by light microscopy. For negative controls, substitution of primary antibody with TBS was run simultaneously.

2.4. Evaluation of geminin, p16, and Ki67 expression

Two independent authors (YX and WC) scored the expression of immunostaining slides. First, 10 normal cervical tissues were evaluated for geminin expression because there is not yet a validated cutoff value for positivity of geminin staining. We investigated that geminin expression was absent in most normal tissues. Therefore, immunostaining with Ki67 and geminin was considered positive when more than 5% of the cells showed strong positive nuclear staining. The immunoreactivity of p16 was judged as positive when there was a diffuse staining in both nuclear and cytoplasm of basal or parabasal cells.^[18] The cutoff value for interpretation of p16 staining is 5%. All the markers were classified into 4 groups: 0 (all cells negative), 1+ (positive staining in 6–25% of cells), 2+ (positive staining in 26–50% of cells), or 3+ (positive staining in more than 50% of cells). For the statistical analysis and evaluation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy, we subdivided the staining results into 2 groups: nuclear or cytoplasmic immunoreactivity in less than 25% (all samples that were scored 0 and 1+) or more than 25% (all samples that were scored 2+ and 3+).

2.5. Statistical analysis

Statistical analysis was performed using SPSS software package, version 21.0 (IBM Corp, Armonk, NY). Data in this study are expressed as means \pm standard deviation (SD). Chi-squared tests were used to differentiate between CIN1 and CIN2/3 and 1-way analysis of variance (ANOVA) was used to analyze the age among groups. Spearman rank correlation coefficients were analyzed to investigate the possible correlations between HPV status and geminin/p16 expression. Sensitivity, specificity, PPV, NPV, and accuracy were calculated on the basis of geminin, p16, and Ki67 staining results by the formulas of Galen and Gambino: Sensitivity=True Positive/(True Positive+False Negative); Specificity=True Negative/(False Positive+True Negative); PPV=True Positive/(True Positive+False Positive); NPV=True Negative/(False Negative+True Negative); Accuracy=True Positive+True Negative/(True Positive+False Positive+False Negative+True Negative). All statistical tests were 2-tailed and a P value of less than .05 was considered to be significant.

3. Results

Table 1 gives an overview of the clinicopathological characteristics of all 95 cases. All slides were re-reviewed and the

Table 2

Results for geminin, p16, and Ki67 expression according to histopathology; the number of samples that were scored as 2+ or 3+.

Diagnosis	Geminin	p16	Ki67
Control group	0/10 (0%)	0/10 (0%)	0/10 (0%)
CIN1	6/45 (13.3%)	11/45 (24.4%)	16/45 (35.6%)
CIN2/3	36/40 (90.0%)*	35/40 (87.5%)*	38/40 (95.0%)*

CIN=cervical intraepithelial neoplasm.
* $P < .05$ compared with patients with CIN1.

histopathologic results were consistent with the initial diagnosis. There were no differences in patient age among the groups ($P > .05$). The HPV in patients with CIN1 was more frequently detected compared with the patients with CIN2/3 ($P < .05$).

The immunohistochemical staining results for geminin, p16, and Ki67 are summarized in Table 2. Geminin expression was absent in all of the normal cervical samples. In 1 (10%) normal cervical sample, geminin was immunoreactive in the cytoplasm of some cells, but it was not present in more than 5% of the cells. Geminin staining was present in less than 25% of the cells in 39 (86.7%) of 45 CIN1 and scored as 2+ in the other 6 (13.3%) CIN1. Our study demonstrated that the expression of p16 and Ki67 was completely negative in normal tissues. Staining with p16 was positive in more than 25% of the cells in 11 (24.4%) of the CIN1 and 35 (87.5%) of the CIN2/3, while Ki67 expression was found in 16 (35.6%) CIN1 and 38 (95.0%) CIN2/3, respectively. The expression of geminin, p16, and Ki67 was all significantly different between CIN1 and CIN2/3 ($P < .05$).

Then, we studied the performance of geminin, p16, and Ki67 in determining the clinical significant lesions and the results are summarized in Table 3. Geminin showed higher specificity (86.7%) than p16 (75.6%) and Ki67 (64.4%). The sensitivity of geminin, p16, and Ki67 was 90%, 87.5%, and 95%, respectively. The PPV was higher for geminin (85.7%) than for p16 (76.1%) and Ki67 (70.3%). The NPV was comparable for all these 3 markers. In addition, the accuracy of geminin was higher (88.2%) than p16 (81.2%) and Ki67 (78.8%). Among the 85 Chinese patients with CIN, 76 (89.4%) were found HPV-positive and 9 (10.6%) HPV-negative. The relationship between HPV status and geminin or p16 expression was analyzed as summarized in Table 4. Geminin expression pattern exhibited a weak correlation with HPV status (correlation coefficient = 0.264, $P = .015$). However, no correlation was found between p16 expression and HPV status (correlation coefficient = 0.144, $P = .190$).

4. Discussion

The aim of this study was to assess whether geminin could be a biomarker to discriminate cervical high-grade lesions. Our results

Table 3

Sensitivity, specificity, PPV, NPV, and accuracy for differentiation between CIN1 and CIN2/3.

	Geminin (95% CI)	p16 (95% CI)	Ki67 (95% CI)
Sensitivity (%)	90.0 (77.0–96.0)	87.5 (73.9–94.5)	95.0 (83.5–98.6)
Specificity (%)	86.7 (73.8–93.7)	75.6 (61.3–85.8)	64.4 (49.8–76.8)
PPV (%)	85.7 (72.2–93.3)	76.1 (62.1–86.1)	70.3 (57.2–80.9)
NPV (%)	90.7 (78.4–96.3)	87.2 (73.3–94.4)	93.5 (79.3–98.2)
Accuracy (%)	88.2 (79.7–93.5)	81.2 (71.6–88.1)	78.8 (69.0–86.2)

95% CI=95% confidence interval, CIN=cervical intraepithelial neoplasm, NPV=negative predictive value, PPV=positive predictive value.

Table 4

Correlation between geminin or p16 expression and HPV status.

	HPV status		Correlation coefficient	P
	Negative	Positive		
Geminin				
Geminin ⁻	8	35	0.264	.015
Geminin ⁺	1	41		
p16				
p16 ⁻	6	33	0.144	.190
p16 ⁺	3	43		

HPV=human papillomavirus.

showed that geminin had a high degree of sensitivity and specificity in determining CIN2/3. The specificity of geminin (86.7%) exceeded that of the commonly used biomarkers p16 (75.6%) and Ki67 (64.4%). The sensitivity of geminin (90.0%) was higher than that of p16 (87.5%), but lower than that of Ki67 (95.0%).

Accurate histological grading of CIN was clinically important, because CIN2 and CIN3 were regarded as precursors of invasive cervical carcinomas and therapy was indicated. Histopathology is a gold standard for diagnosis of CIN. However, the interobserver and intraobserver variabilities in interpreting cervical biopsy specimen are relatively high.^[11] Therefore, it is still challenging to discriminate between CIN1 and CIN2/3.

The commonly used immunohistochemical markers p16 and Ki67 were not very accurate to help for the distinction between CIN1 and CIN2/3.^[18] Furthermore, these 2 markers were highly sensitive to detect the presence of CIN2/3, but their specificity was relatively low in our study. This is in accordance with many previous studies.^[19,20] Our results demonstrated that the specificity, PPV, and accuracy of geminin were highest among these biomarkers. In addition, geminin expression is significantly increased in CIN2/3 compared with CIN1. This implied that the use of geminin immunohistochemical analysis might be a surrogate marker of high-grade cervical intraepithelial neoplasia.

High-risk HPV persistent infection is significantly associated with CIN2/3 and invasive cervical carcinomas.^[21] In the present study, HPV positivity was detected in 82.2% of CIN1 and 97.5% of CIN2/3. Our results were higher than those in a recent study.^[22] A 4-year surveillance study by Zhang et al^[22] have reported that the prevalence of HPV increased with cervical lesions severity and the HPV positivity rates were 72.4% for CIN1, 81.4% for CIN2, and 88.1% for CIN3. The discrepancy might be caused by our small sample size. Moreover, the average age of patients in our study was 47.8 ± 7.9 years, which might be another reason for the higher HPV positivity rates because age was an important factor for high-risk HPV infection clearance.^[23] In the present study, p16 expression was not associated with HPV status. The finding is in accordance with previous studies.^[24,25] No correlation was found between p16 and HPV status in esophageal squamous cell carcinoma in Kazakh population by Wang et al,^[25] thus suggesting that p16 might be an unreliable surrogate marker for HPV status. Nevertheless, p16 expression was previously reported to exhibit a correlation with HPV status.^[26] The inconsistency might be attributed to absence of uniformity in cutoff value and variation in HPV status among patients from different geographic origins.^[27] Interestingly, a weak correlation between geminin and HPV status was observed in our study.

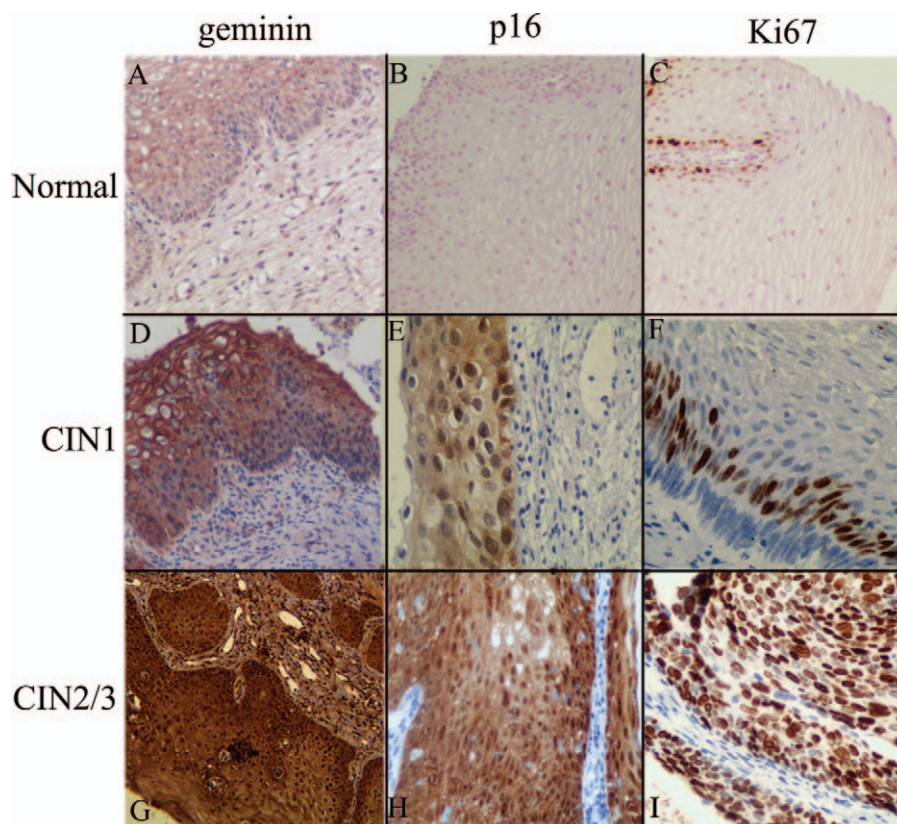


Figure 1. Representative immunohistochemical images of geminin (A, D, and G, $\times 100$), p16 (B, $\times 100$, E and H, $\times 200$), and Ki67 (C, $\times 100$, F and I, $\times 200$) expression in normal tissues, CIN1, and CIN2/3. All pictures were taken at original magnification.

There are some limitations in our study. First, the sample size of our study is relatively small. In addition, the cutoff value for increased geminin expression used in our study needs to be validated in an independent set of cervical samples. Another limitation in this study is that we focused on the expression of geminin in CIN, but did not evaluate geminin expression in cervical carcinomas. Our results suggested that geminin might serve as a diagnostic biomarker for identifying CIN2/3; however, we are not informed about its clinical use as a prognostic variable in cervical carcinomas. Furthermore, additional mechanistic studies to investigate the role of geminin in the pathogenesis of CIN progress are needed.

In conclusion, geminin is a highly sensitive and specific biomarker for CIN diagnosis. It can be used in addition to p16 and Ki67 staining in routine pathology practice when discriminating between CIN1 and CIN2/3 is difficult.

5. Conclusion

Our study showed that the immunoquantification of geminin appeared to be a new marker for the differentiation between CIN1 and CIN2/3 when there is doubt about the grading of CIN. Nonetheless, further studies are necessary before implementing geminin in clinical practice (Fig. 1).

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