Correction between p16ink4a expression and human papillomavirus in atypical glandular cells of undetermined significance-categorized pap smears with follow-up biopsies

Su-Feng Chen, M.T., M.S., Shih-Fang Yang, D.M.D., Ph.D., Tang-Yuan Chu, M.D., Ph.D., Hung-Cheng Lai, M.D., Ph.D., Ya-Wen Lin, Ph.D., Chien-Yu Bai, M.D., Ya-Wen Lin, Ph.D., and Shin Nieh*, D.M.D., M.S., M.I.A.C.

1 Institute of Clinical Dentistry, National Yang-Ming University, 2Department of Obstetrics and Gynecology, 3Department of Graduate Institute of Medical Sciences, 4Department of Pathology, National Defense Medical Center & Tri-Service General Hospital, Taipei, Taiwan, Republic of China

*Corresponding author by whom the reprint requests should be addressed to Professor Shin Nieh at Department of Pathology, School of Medicine, National Defense Medical Center and Tri-Service General Hospital,

No. 325, Cheng-Kung Rd., Sec.2, Neihu 114, Taipei, Taiwan, R.O.C.

Tel: +886-2-87927155

Fax: +886-2-87927159

Email: niehshin1014@yahoo.com.tw
ABSTRACT

Objective. This study was to correlate high-risk human papillomavirus (HR-HPV) viral load to p16INK4A (p16) expression in atypical glandular cells (AGC)-categorized Pap smears with follow-up biopsies for elucidating their relationships.

Methods. We enrolled 36 AGC-categorized Pap smears with subsequent follow-up biopsies. HR-HPV viral load was determined by Hybrid Capture II assay in each AGC-diagnosed Pap smear. Both smears and biopsies were immunostained with a primary anti-p16 antibody, clone E6H4. Correlations between HR-HPV viral load in each AGC-diagnosed Pap smear and p16 expression of smears with follow-up biopsies were performed.

Results. Comparative analysis of two tests disclosed both consistencies and discrepancies. There were significant differences (p = 0.02) between negative or weak p16 expression of Pap smears with the presence of reactive lesion or LSILs/CIN1s in follow-up biopsies and negative HR-HPV viral load. However, no significant difference (p = 0.317) was found between p16 expression of Pap smears with the presence of HSIL/CIN2, 3 and AIS or adenocarcinoma in follow-up biopsies and high HR-HPV viral load. In addition, there were significant differences (p = 0.012) in specificity, but no significant differences were found in sensitivity (p = 0.604), positive and negative predictive value (p = 0.066, and 0.264) between p16 immunoexpression and HR-HPV viral load.

Conclusions. Pathogenic activity of HR-HPV was indicated by p16 expression on smears and tissue sections, which appears to be a better strategy than HR-HPV viral load test for the detection of clinically insignificant lesions from AGC-categorized Pap smears.

Keywords: atypical glandular cells; immunocytochemistry; p16INK4A; human papillomavirus
INTRODUCTION

Papanicolaou (Pap) smear is reported to play a key role in the screening of the cervical lesions, which are closely associated with high-risk human papillomavirus (HR-HPV) infection [1-3]. Nevertheless, the Pap test inevitably has its value as well as its limitations with respect to the sensitivity and specificity [4, 5]. This is particularly relevant to one of the special diagnostic problems concerning atypical glandular cells (AGC). In fact, AGC-containing smears occupy only a small fraction (less than 1%) in total Pap smears. Nevertheless, up to 40% of AGC-containing smear diagnoses may represent corresponding significant lesions in follow-up biopsies, which need further treatment. Those clinically significant lesions consist of high-grade intraepithelial lesions, and glandular neoplasia, either in situ or invasive adenocarcinoma. The remaining 60% of AGC-containing smears do not reflect such serious lesions at all [6]. Predictions of AGC-categorized smears are difficult so that the outcomes of the smears have become controversial. There may be substantial inter-observer variability and false-negative or false-positive results resulting in repeated cytological testing, and unnecessary investigations or treatments. These limitations have created a strong demand for a reliable marker that may objectively resolve the so-called “diagnostic dilemma associated with A-containing smears. Substantial clinical and epidemiological data have indicated that persistence of HR-HPV in cervical scrapes is associated with the development, maintenance and progression of squamous intraepithelial lesions (SILs), as well as glandular abnormalities [1-3, 7-10]. The development has prompted consideration of its use as an adjuvant and as a primary screening tool for cervical lesions. The use of molecular techniques of an affordable FDA-approved HPV test [11, 12] has been proposed as a way to improve the results of conventional diagnostic strategies. Based on our previous study data [13,14], retrospective immunocytochemical p16INK4A (p16) staining was successfully applied to conventional Pap smears and might as well serve as an effective biomarker.
for cytological diagnoses reliably distinguishing benign from malignant lesions in atypical squamous cells of undetermined significance (ASCUS)- and AGC-categorized Pap smears when correlated with follow-up biopsies. Overexpression of p16 was reported to be an indicator of the pathogenic activity of HR-HPV [15]. The pathogenesis between p16 expression and high-risk HPV appears not substantially clear, due to lack of clinico-pathological data to clarify their relationships. Our another recent study [16] fortunately demonstrated that directly visualized p16 immunostaining on smears appeared to be a more effective method than HR-HPV viral load for the detection of reactive change and LSIL/CIN1 from ASCUS-categorized Pap smears by comparative analysis. To the best of our knowledge, the correlations between cytology, histology, p16 expression and HPV tests in association with AGC-categorized Pap smears have not yet been reported in the literature. They are indeed worthy of detailed investigation. Therefore, we aimed to correlate HR-HPV viral load to p16 expression in AGC-categorized Pap smears with follow-up biopsies in order to elucidate their relationships in gynecological pathology.
MATERIALS AND METHODS

Cytology and Tissue Specimen Preparation with Immunostaining of p16

Individual Pap smears from 116 patients with cytological diagnoses of AGC were retrieved from 18,795 Pap smears examined by our cytopathology and histopathology laboratory, in the Department of Pathology, Tri-Service General Hospital, Taipei, in recent two years. Of these 116 patients, 65 were either lost to follow-up or were not available for biopsies. Follow-up histological confirmation was made for 51 Pap smears. Of these 51 follow-up biopsies, 15 were excluded because the specimen was inadequate for a definite diagnosis. Therefore, the current study enrolled 36 AGC-diagnostic Pap smears. Generally speaking, the diagnosis of AGC-categorized smears should be qualified, if possible, to indicate whether the cells are thought to be of endocervical or endometrial origin or not otherwise specified on the basis of the concept of 2001 Bethesda System consensus. Furthermore, the diagnosis of AGC should be subclassified according to whether an undetermined (general AGC) or neoplastic (AGC- favor neoplasia, AGC-FN) process is favored[17]. All studied smears were initially rescreened by three qualified cytotechnicians and then reviewed by three certified cytopathologists. Under a multihead microscope, a total agreement by all participating evaluators was required for each individual case. Three groups of results of follow-up biopsies were classified as group 1, clinically insignificant lesions (reactive lesion or LSIL/CIN1), groups 2 and 3, clinically significant lesions (HSIL/CIN2 or CIN3, and AIS or adenocarcinoma). Cases of both weakly and strongly positive expressions were all included in the total number of immunoreactive samples to represent varieties of pathogenic activity of HR-HPVs. The cytological and follow-up histological data were correlated. Further immunoreactivity for p16 was interpreted in a blind fashion, and the results were then correlated with the histological data.
Immunocytochemical and Immunohistochemical Analyses

Both smears and follow-up biopsies were subsequently immunostained with a primary anti-p16 antibody, clone E6H4 (MTM Laboratories, Heidelberg, Germany). Immunoreactivity for p16 was interpreted in a blind fashion, and the results were then correlated with the histological data. Known positive and negative controls were included in each run. For the negative control, normal serum IgG (Vector) was substituted for primary antibody. The positive control was prepared from a well-established case of squamous cell carcinoma of the cervix. The expression of p16 in smears and corresponding biopsies was also scored by three cytopathologists. Samples were considered positive if a brown reaction product was present, predominantly in the nucleus and cytoplasm. The intensity of positive staining in the smears was graded as strongly positive (++): more than 50% of cell clusters or more than 50 AGC cells presenting immunoreactivity; weakly or sporadically positive (+): less than 50% of cell clusters or less than 50 AGC cells presenting immunoreactivity; negative (−): no stain. The corresponding biopsies were scored as exhibiting negative, sporadic, focal, or diffuse staining. Strong nuclear and cytoplasmic staining was considered a positive reaction, similar to the criteria for smears, and distribution was scored on a semi-quantitative scale: negative, <1% of the cells were positive; sporadic, isolated cells were positive, but constituted <5% of cells; focal, small clusters of positive cells, constituting <25% of cells; and diffuse, >25% of cells were positive. The atypical cells or cell clusters diagnosed as AGC were dotted and counted at 4X scanning power in both the conventional Pap smears and the immunostained smears, in order to easily identify the atypical cells. All cells and cell clusters that stained positively were evaluated under high-power magnification. Detailed information and analyses were provided in our previous article [14].

Detection of HR-HPV DNA using the Hybrid Capture II Assay

Following preparation of Pap smears collected by using a cervical cytobrush, the viral load of
HR-HPVs was tested by HC II assay from the cervical swab specimens and then transported in Digene Specimen Transport Medium (Digene Diag, MD, USA). The assay kit detects high-risk types HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The low-risk group detects the types most commonly associated with condyloma acuminatum: HPV 6, 11, 42, 43 and 44 (Detailed procedure as described on http://www.digene.com). Comparative analysis of p16 immunoexpression in smears and follow-up biopsies with the corresponding HR-HPV viral load was performed.

Data Analysis

The sensitivity, specificity, positive predictive value, and negative predictive value [18] of both p16 immunostaining and HR-HPV viral load were determined by subjecting the data to a true diagnosis for the immunopositive cells and the quantitatively measured copy numbers of HR-HPV. Both datasets were also examined for correlations. Statistical significance was determined on the basis of Pearson Chi-Square Test, Yates-Corrected Chi-Square test and Fisher’s exact test.
RESULTS

Correlation between Cytology and Histology

All 36 Pap smears were reclassified into 22 (61%) as general AGC and 14 (39%) as AGC-favor neoplasia. Follow-up biopsies revealed that 15 (42%) cervices had no obvious abnormalities but only reactive changes and 2 cases (6%) of LSIL/CIN1. More than half cases (19/36, 52%) of follow-up biopsies concerning AGC-containing smears represent significant lesions. Above data and detailed information regarding the 2001 Bethesda System classification of AGC were presented in Table 1.

Correlation of HR-HPV Viral Load with p16 Immunoreactivity

Of the 36 cases studied, 10 (28%) cases were negative for HR-HPV viral load and 26 (72%) cases were positive for HR-HPV viral load. Immunostaining showed that 14 cases (39%) were negative and 22 (61%) were positive for p16 expression (Table 2). Outcomes of AGC-diagnosed Pap smears with follow-up biopsies appeared to correspond well with the immunoreactivity of p16 (Fig. 1). Comparative studies of HR-HPV viral load with p16 expression were conducted and these two tests indicated consistencies as well as discrepancies. The analytical data from the 36 cases revealed that 8 (22%) cases (7 reactive lesions or LSIL and 1 HSIL) were negative for both HR-HPV viral load and p16 expression and 20 (56%) (4 reactive lesions or LSILs/CIN1s, 9 HSILs/CIN2s or CIN3s and 7 AIS or adenocarcinomas) cases were positive for both HR-HPV viral load and p16 expression. However, discrepancies existed and consisted of 6 (17%) cases positive for HR-HPV viral load and negative for p16 expression and 2 (6%) cases negative for HR-HPV viral load and positive for p16 expression.
Statistical Analysis

Strong immunopositive smears for p16 predicting the presence of clinically significant lesions (HSILs/CIN2s or CIN3s and AIS or adenocarcinomas) and positive for HPV-DNA were subjected to analysis for true test results. The association of immunostained atypical cells with a significant lesion in the corresponding biopsy was considered to have a positive predictive value. Conversely, the correlation with negative or weak immunostaining without a significant lesion (reactive lesion or LSIL/CIN1) was considered to have a negative predictive value. For the interpretation of HR-HPV viral load, a ratio of 1.0 or greater was regarded as positive for HPV DNA, and a ratio of less than 1.0 was regarded as negative. The results of the Pearson Chi-Square Test, Yates-Corrected Chi-Square test and Fisher's exact test (Table 2) clearly indicated that there were statistical significances between negative or weak p16 expressions of AGC-categorized Pap smears with the presence of reactive lesions or LSILs/CIN1s in follow-up biopsies and the data of HR-HPV viral load (p = 0.02). No statistical significance was found in p16 expression of AGC-categorized Pap smears with the presence of HSIL, and AIS or adenocarcinoma in follow-up biopsies and positive HR-HPV viral load (p = 0.317).

The sensitivity, specificity, positive and negative predictive value of both p16 immunostaining and HR-HPV viral load were also calculated. The p16 immunostaining had 95% sensitivity, 88% specificity, 90% positive predictive value and 94% negative predictive value, compared with the results of HR-HPV viral load in the presence of significant lesions in follow-up biopsies, which had 84% sensitivity, 41% specificity, 62% positive predictive value and 70% negative predictive value.

There were statistical significances in specificity (p = 0.012) between p16 immunostaining and HR-HPV viral load. No statistical significances were found in sensitivity (p = 0.604), positive and negative predictive value (p = 0.066, and p = 0.264).
DISCUSSION

As seen in the result, our study disclosed that more than half cases (19/36, 53%) of follow-up biopsies concerning AGC-containing smears represented significant lesions. However, most of them were HSIL/CIN2 or CIN3 (12/19, 63%) rather than AIS or adenocarcinomas, identical to other previous reports [6,9,19]. Since p16 is directly or indirectly associated with HR-HPV via the production of the viral oncoprotein, E7. It binds and inactivates the host cellular tumor-suppressor protein pRB, a negative regulator of p16 transcription, resulting in disruption of the Rb pathway at the G1 checkpoint [6,8-10,13-16,19,23,25-28]. Overexpression of p16 may represent an indicator of pathogenic activity of HR-HPV, which is supported by the strong p16 immunopositivity in more severe significant lesions and weak immunopositivity in LSILs/CIN1s, as well as negative staining in reactive lesions in both smears and biopsies. Although HR-HPVs are known to play a major role in the carcinogenesis of most cervical cancer, the exact nature of cervical carcinogenesis has still waited to be elucidated. The association between p16 immunoexpression and HR-HPV varies considerably between reports [15,16,19-23]. These two ancillary diagnostic tools both appeared to be effective and needed to be clarified by correlation. The relationships between p16 expression in AGC-diagnosed Pap smears with follow-up biopsies and HR-HPV viral load were reevaluated and hoped to be clarified by correlations and analyses cases by cases in the current study. Based on our previously published data [13,14,16], discrepancies between immunoreactivities of p16 on abnormal Pap smears with follow-up biopsies, however, did occur from case to case. Possible explanations are disease progression and regression. If we included HR-HPV viral load as a main contributing factor to cervical carcinogenesis, latent or subclinical HPV infection presenting high viral load before p16 expression was found in some reactive cervical lesions and LSILs/CIN1s. p16 immunoexpression of the cervical lesions appears to be late event following HPV infection. In our study, there were 6 (17%) cases that were positive for HR-HPV viral load and negative for p16 expression. However, reactive cervical lesions may transiently contain HR-HPV viral load, of which many may not necessarily promote clinically significant lesions unless a high HR-HPV viral load is persistent and the immune system of the host is somewhat altered [3,12,24,29-31].
Overexpression of p16 was observed only in two (6%) cases with negative HPV viral loads, suggesting that HPV-independent mechanisms might lead to overexpression of p16 in such lesions. In other words, immunoexpression for p16 might be evoked via pathways other than HPV infection through gene mutation, hypomethylation or hypermethylation, which suggested that some of the observed cervical preneoplastic and neoplastic lesions could be induced in a heterogeneous way. However, although it only occupies a small portion of tested cases, we cannot completely exclude the possibility that the technical sensitivity of HPV detection was a limiting factor. In addition, we found only one of 36 (3%) case ending up HSIL in follow-up biopsy neither showing evidence of HPV infection or overexpression of p16, which allowed us to speculate that a p16 gene deficiency, might contribute to carcinogenesis in some of the SILs or even cervical cancers. Associated details by Hybrid Capture II assay, which may although cover most HR-HPV types, also deserve further investigation to avoid exceptional ones.

In summary, the current report appears to be the pilot study to correlate between HR-HPV viral load and p16 expression in AGC-categorized Pap smears with follow-up biopsies which display certain consistencies and discrepancies. There is a close association between strong p16 expressions in AGC-categorized smears with the presence of HSIL/CIN2 or CIN3 and AIS or adenocarcinoma in follow-up biopsies and positive HR-HPV viral loads. Conversely, there is also a clear association between the lack of p16 expression and the absence of significant lesions in follow-up biopsies with high specificity, though not consistent with a negative HR-HPV viral load. In terms of AGC-categorized Pap smears ending up with reactive changes or LSIL/CIN1 in follow-up biopsies, negative (reactive change) or weak (LSIL/CIN1) immunoreactivity of p16 appears to be more reliable for predicting outcomes than the results of HR-HPV testing when compared with both specificities. However, in terms of AGC-categorized Pap smears with HSIL/CIN2 or CIN3 and AIS or adenocarcinoma in corresponding follow-up biopsies, testing of HR-HPV viral load and immunoreactivity of p16 appears to have similar reliability for predicting outcomes when compared with both sensitivities. We conclude that, through comparative analysis,
directly visualized p16 immunoexpression not only offers an objective parameter for the clarification of the ambiguous areas in gynecological pathology, but also represents a more effective method than the test of HR-HPV viral load to determine the outcome of clinically insignificant lesions including reactive change or LSIL/CIN1 from AGC-categorized smears.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Three comparative features of the representative AGC-categorized smears (upper and middle panels) and corresponding follow-up biopsies (lower panels) with following immunocytochemistry (ICC) and immunohistochemistry (IHC) for p16 expression. Note: Panel D and G (ISM) represent panel A negatively immunostained for p16. Panel E and H (CIS) represent panel B positively immunostained for p16. Panel F and I (Ad-Ca) represent panel C positively immunostained for p16. AGC-Endocx: AGC-of endocervical origin, AGC-NOS: AGC-not otherwise specified, AGC-FN-Endocx: AGC-favor neoplasia-of endocervical origin. ISM: Immature squamous metaplasia, CIS: carcinoma in situ, and Ad-Ca: adenocarcinoma. (Original magnification: A, B, C, D, E, and F X200; G, H, and I X100)

![Images of smears and biopsies with immunostaining for p16]

Table 1  Correlations between 36 AGC-categorized Cytological Diagnosis and Histological Diagnosis

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>AGC Total cases (n=36)</th>
<th>General AGC n=22 (61%)</th>
<th>AGC-favor Neoplasia n=14 (39%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>AGC Endocx (n=11)</td>
</tr>
<tr>
<td>Reactive change</td>
<td>15 (42)</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>LSIL/CIN 1</td>
<td>2 (6)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>HSIL/CIN 2, 3</td>
<td>12 (33)</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>AIS</td>
<td>4 (11)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>CA</td>
<td>3 (8)</td>
<td></td>
<td>0</td>
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</table>

Table 2  Distribution and true diagnosis analysis for significant lesions regarding HPV-DNA viral load and p16 expression in 36 AGC categorized smears

<table>
<thead>
<tr>
<th>Follow-up biopsies</th>
<th>Test used</th>
<th>HPV n = 36</th>
<th>p16 n = 36</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(+) - 26</td>
<td>( ) - 10</td>
<td>(+) - 22</td>
</tr>
<tr>
<td>Reactive lesion or CIN1 (n = 17)</td>
<td>†</td>
<td>10</td>
<td>4</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>CIN2 or CIN3 (n = 12)</td>
<td>†</td>
<td>9</td>
<td>11</td>
<td>0.317&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>AIS or Ad-Ca (n = 12)</td>
<td>†</td>
<td>7</td>
<td>7</td>
<td></td>
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<td></td>
<td></td>
<td>0</td>
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Sensitivity: 84% 95% 0.604<sup>c</sup>
Specificity: 41% 88% 0.012<sup>b</sup>
Positive predictive value: 62% 90% 0.066<sup>b</sup>
Negative predictive value: 70% 94% 0.264<sup>c</sup>

<sup>a</sup> Pearson Chi-Square Test
<sup>b</sup> Yates-Corrected Chi-Square Test
<sup>c</sup> Fisher’s exact test
CIN, cervical intraepithelial neoplasia, AIS, adenocarcinoma in situ, Ad-Ca, adenocarcinoma