

p16^{INK4a} immunohistochemistry is a promising biomarker to predict the outcome of low grade cervical intraepithelial neoplasia: comparison study with HPV genotyping

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Objective: In cervical intraepithelial neoplasia (CIN), p16^{INK4a} immunohistochemistry has been reported to be a useful diagnostic biomarker. However, limited information is available about the association between the p16^{INK4a} immunohistochemistry and the outcomes of CIN. Here, we report p16^{INK4a} immunohistochemistry as an effective biomarker to predict the outcomes of CIN.

Methods: p16^{INK4a} immunohistochemistry was performed in patients with CIN from January 2000 to August 2009. Among these patients, we have performed a retrospective analysis of the medical records to evaluate the outcome of CIN 1–2 and performed statistical analysis to determine the correlation between p16^{INK4a} expression and the outcomes. We also performed HPV genotyping and analyzed the relation between the infecting human papillomavirus (HPV) genotype and the outcomes.

Results: A total of 244 patients, including 82 with CIN 1, 60 with CIN 2, and 102 with CIN 3, were examined. The rate of p16^{INK4a} overexpression increased with increasing CIN grade, 20.7% for CIN 1, 80.0% for CIN 2, and 89.2% for CIN 3, with significant differences between CIN 1 and CIN 2–3 group. In the 131 CIN 1–2 patients, the progression rate was significantly higher for the patients showing p16^{INK4a} overexpression than for those not showing p16^{INK4a} overexpression (p=0.005); the regression rate was also found to be significantly lower for the patients showing p16^{INK4a} overexpression (p=0.003). High-risk HPV genotypes were detected in 73 patients (73.7%). Both progression and regression rates were not significantly different between the high-risk HPV-positive and HPV-negative groups (p=0.401 and p=0.381, respectively).

Conclusion: p16^{INK4a} overexpression was correlated with the outcome of CIN 1–2, and p16^{INK4a} is considered to be a superior biomarker for predicting the outcome of CIN 1–2 compared with HPV genotyping.

Keywords: Biomarker, Cervical intraepithelial neoplasia, Human papillomavirus, Immunohistochemistry, p16^{INK4a}

INTRODUCTION

Cervical cancer is the second most common malignancy in women worldwide [1]. The incidence of cervical cancer in

young women has been increasing recently. Cervical intraepithelial neoplasia (CIN) is a precancerous lesion that can be treated effectively to prevent progression to cervical cancer. CIN 1 lesions are usually followed up without treatment; however, 10% of CIN 1 lesions progress to CIN 3 or cervical cancer. In patients of CIN 2, 20% of CIN 2 lesions progress to CIN 3 or cervical cancer, and 40% of CIN 2 lesions regress spontaneously [2].

For many gynecologists, the management of patients with CIN 1–2 is controversial [2]: should they observe patients until spontaneous regression or treat patients with ablative or excisional procedures? Although it may be appropriate to

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treat only patients that are at high risk of progression, and to observe low risk patients that may regress spontaneously, it is not easy to predict the outcome of each patient. There is a growing need to establish an effective biomarker that would serve as a reliable predictor of the outcomes.

One of the important biomarkers for CIN and cervical cancer is the human papillomavirus (HPV) genotyping. HPV DNA is found positive in more than 90% of cervical cancer patients [3], and especially, high-risk genotypes of HPV are considered to be associated with development of CIN and cervical cancer [3-5]. It has been shown that HPV genotyping can detect women with cytological abnormalities and it has a potential role in identifying women at risk of residual or recurrent disease after treatment of CIN [6]. However, HPV genotyping is less specific than cytology, because many infections regress without progressing to high-grade lesions and a positive HPV test does not necessarily discriminate between transient and chronic infection [7,8].

Previous experimental and epidemiologic studies have shown that expression of E6 and E7 genes of the high-risk genotypes of HPV in the squamous epithelial cells of the uterine cervix may result in neoplastic growth, and that infection with high-risk HPV results in the expression of p16^{INK4a} [9,10]. p16^{INK4a} is one of the cyclin-dependent kinase inhibitors that prevents phosphorylation of retinoblastoma protein (Rb) and therefore plays an important role in the regulation of the mammalian cell cycle [11]. Although p16^{INK4a} protein is considered to be a tumor suppressor, paradoxical p16^{INK4a} overexpression has frequently been observed in CIN lesions associated with high-risk HPV infection [12]. p16^{INK4a} overexpression is associated with dysfunction of the pRb protein through naturally arising mutations, or its binding to the HPV 16 E7 protein and E7 protein might induce both abnormal cell cycle progression and p16^{INK4a} overexpression [9]. Previous studies have mentioned that p16^{INK4a} immunohistochemistry could be a biomarker to predict the outcomes of CIN lesions, although the predominance as a biomarker compared with HPV genotyping was not fully elucidated [13-16]. Therefore, we examined p16^{INK4a} immunohistochemistry of the uterine cervix as an effective biomarker compared with HPV genotyping in patients with CIN 1-2 by retrospective nested cohort study.

MATERIALS AND METHODS

1. Patient selection

This retrospective study was approved by the Ethics Committee of Keio University School of Medicine (No. 20080180).

Between January 2000 and August 2009, 244 consecutive patients with CIN were examined and treated at the Department of Obstetrics and Gynecology, Keio University Hospital, Tokyo, Japan. The number of patients with CIN was as follows: CIN 1, 82 patients; CIN 2, 60 patients; CIN 3, 102 patients. Conventional cytology, colposcopically directed biopsy, histological examination and p16^{INK4a} immunohistochemistry were conducted in all the patients for their initial visits. The initial cervical exfoliated cells for Pap smear also collected in PreservCyt (Hologic Inc., Bedford, MA, USA) were utilized for HPV genotyping. Patients diagnosed with CIN 1-2 were followed up at three months intervals and received conventional Pap smear and colposcopic examinations. At each visit, if any abnormal lesions were suspected, colposcopically directed biopsy was performed. Patients with CIN 3 were immediately treated by laser vaporization or cone resection without follow-up. While reviewing the medical records, we collected data including the results of subsequent cytological or histological examination results and subsequent treatments if the patient underwent any procedures. Among CIN 1 and CIN 2 patients, eleven patients with past history of treatments such as laser vaporization or laser conization were excluded and we evaluated the outcomes of the CIN 1 and CIN 2 lesions after the initial diagnosis. Consequently, a total of 131 patients were enrolled, including 76 patients with CIN 1 and 55 patients with CIN 2 lesions (Fig. 1).

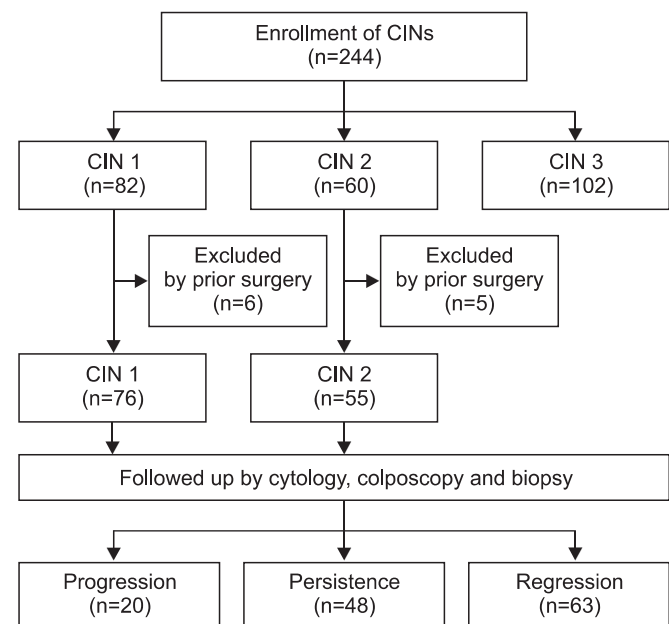


Fig. 1. Patients. CIN, cervical intraepithelial neoplasia.

2. Definition of CIN progression and regression

Progression was defined as the appearance of histologically confirmed CIN 3 or more during the follow-up period. We defined regression as at least two consecutive conventional cytology with normal colposcopic findings. Women were regarded as having persistent lesions when they did not have either regression or progression over the follow-up period [17].

3. p16^{INK4a} immunohistochemistry and interpretation of results

All samples were prepared from colposcopically directed punch biopsy specimens. The immunohistochemistry was performed as follows. Formalin-fixed and paraffin-embedded specimens were stained on a Dako Autostainer (Dako Norden A/S, Glostrup, Denmark) using a CINtec p16^{INK4a} Histology Kit for the DakoCytomation Autostainer according to the manufacturer’s instructions. Briefly, antigen retrieval was performed by heating in a water bath at 95°C for 20 minutes. After blocking endogenous peroxidase activity, the slides were incubated with primary antibody (E6H4 clone). Then, a secondary antibody reagent for visualization was employed from the above-mentioned kit. The slides were incubated with DAB (3,3'-diaminobenzidine). Substrate-chromogen solution

and counterstaining was performed with Mayer’s hematoxylin before coverslipping.

The evaluation of immunoreactivity was reported previously. Briefly, immunoreactivity was evaluated on the basis of nuclear and/or cytoplasmic staining, 1) evaluation of the staining intensity: –, no staining; +, weak staining; ++, moderate staining; +++, strong staining, and 2) evaluation of the percentage of positively stained cells in each section. Immunoreactivity for p16^{INK4a} was then classified as negative, weakly positive, moderately positive, and strongly positive (Table 1, Fig. 2). The p16^{INK4a} overexpression rate was calculated by adding the number of moderately and strongly positive specimen.

4. HPV genotyping by polymerase chain reaction

The procedure of HPV genotyping has been described previously [18,19]. Briefly, genomic DNA was extracted from exfoliated cells, using proteinase K and phenol-chloroform treatment. The specimens were tested for the presence of HPV DNA by polymerase chain reaction (PCR) analysis with consensus primer pairs (L1C1 and L1C2) designed to amplify an approximately 250-bp segment of the viral DNA. The consensus primer pairs target the HPV L1 open reading frame and detect a broad range of genital HPVs. The HPV genotyp-

Table 1. Evaluation of p16^{INK4a} immunohistochemistry

| Intensity | Evaluation of the percentage of positively stained cells | | |
|---------------|--|----------------------|----------------------|
| | <5 | 5 to <50 | ≥50 |
| Negative(-) | Negative | | |
| Weak (+) | Weakly positive | | |
| Moderate (++) | Weakly positive | Moderately positive* | Moderately positive* |
| Strong (+++) | Weakly positive | Moderately positive* | Strongly positive* |

*Indicates p16^{INK4a} overexpression.

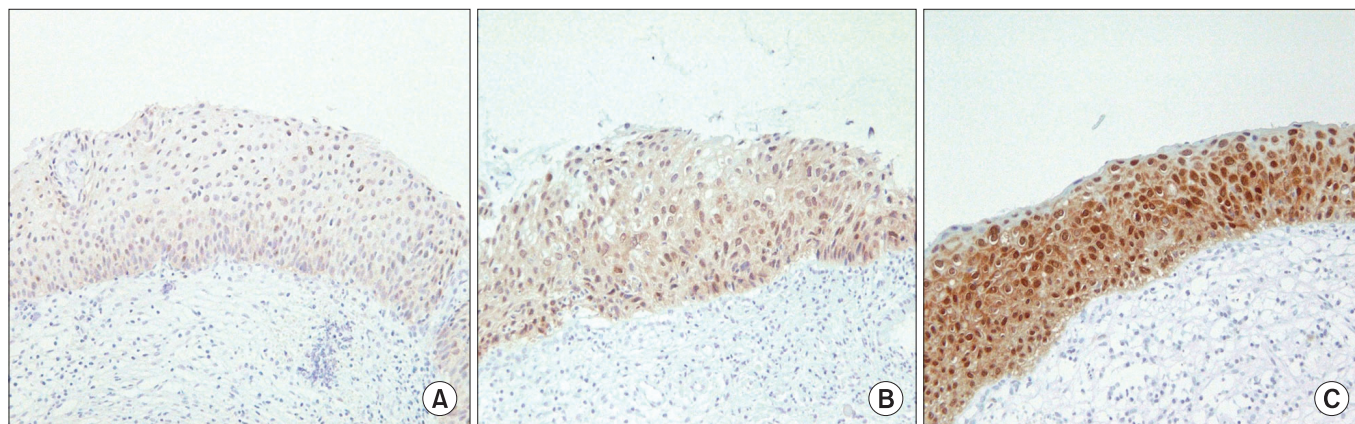


Fig. 2. p16^{INK4a} immunohistochemistry of cervical biopsy specimens. Immunoreactivity for p16^{INK4a} was classified as negative, weakly positive (A), moderately positive (B), or strongly positive (C) (microscope objective: × 10).

ing was determined by the direct sequencing method [19], which was determined based on an approximately 200 bases of L1 sequence and a search of the NCBI database (GenBank sequences; <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) using Sequencing Analysis ver. 3.3 (The Perkin-Elmer Co., Norwalk, CT, USA) [18].

5. Statistical analysis

Statistical analysis was performed using SPSS ver. 21.0 (IBM Co., Armonk, NY, USA). The χ^2 test was used to assess the association among the p16^{INK4a} expression status, HPV genotyping and the histological diagnosis. Kaplan-Meier analysis was used to estimate the rates of progression and regression of the disease. A log-rank test was used to determine the statistical significance. A p-value < 0.05 was considered to denote statistical significance.

RESULTS

A total of 244 patients ranged in age from 22 to 83 years, with a median age of 37.0 years. Among the patients, we evaluated the outcomes of those with CIN 1–2, including 76 patients with CIN 1 and 55 patients with CIN 2. The median follow-up period of the patients with CIN 1–2 was 40 months (range, two to 104 months). The rate of p16^{INK4a} overexpression increased with increasing CIN grade: 17 patients with CIN 1 (20.7%), 48 patients with CIN 2 (80.0%), and 91 patients with CIN 3 (89.2%). The rates of p16^{INK4a} overexpression were significantly higher in the CIN 2–3 patients than in the CIN 1

patients (p < 0.001).

Of all the patients examined, there were 61 patients (46.6%) showing p16^{INK4a} overexpression. During the follow-up period, 14 patients (23.0%) with p16^{INK4a} overexpression showed progression to higher-grade lesions, whereas only 6 (8.6%) among the patients without p16^{INK4a} overexpression showed progression. Two CIN 1 cases and 12 CIN 2 cases with p16 overexpression showed progression to higher-grade lesions. According to the results of the Kaplan-Meier analysis, there was a significant difference in the progression rate between the patients with p16^{INK4a} overexpression and those not showing p16^{INK4a} overexpression (p = 0.005) (Fig. 3). A total of 17 (27.9%) patients with p16^{INK4a} overexpression showed regression, whereas 46 patients (65.7%) not showing p16^{INK4a} overexpression showed regression. The difference in the regression rate between these two groups was also significant (p = 0.003) (Fig. 4).

The HPV genotyping of 131 patients with CIN 1–2 revealed infection by the following sixteen HPV genotypes; 6, 16, 18, 31, 33, 35, 51, 52, 56, 58, 59, 68, 70, 71, 84, and 90. In this study, we defined HPV16, 18, 31, 33, 35, 51, 52, 56, and 58 as high-risk HPVs prevalent in Japanese women [18,20,21]. Among 201 patients who were tested with HPV genotyping, 162 patients (80.0%) were identified to have infection with high-risk HPV genotypes. The rate of infection with high-risk HPV also increased with increasing CIN grade, with significant differences between CIN 1 (65.1%) and CIN 2–3 lesions (87.7%) (p < 0.001).

Among the patients, 73 had infection with prevalent high-risk HPV, 10 had infection by other genotypes of HPV (6, 59, 68, 70, 71, 84), 26 were negative for HPV infection, and 32 were not tested for the HPV infection status. Although

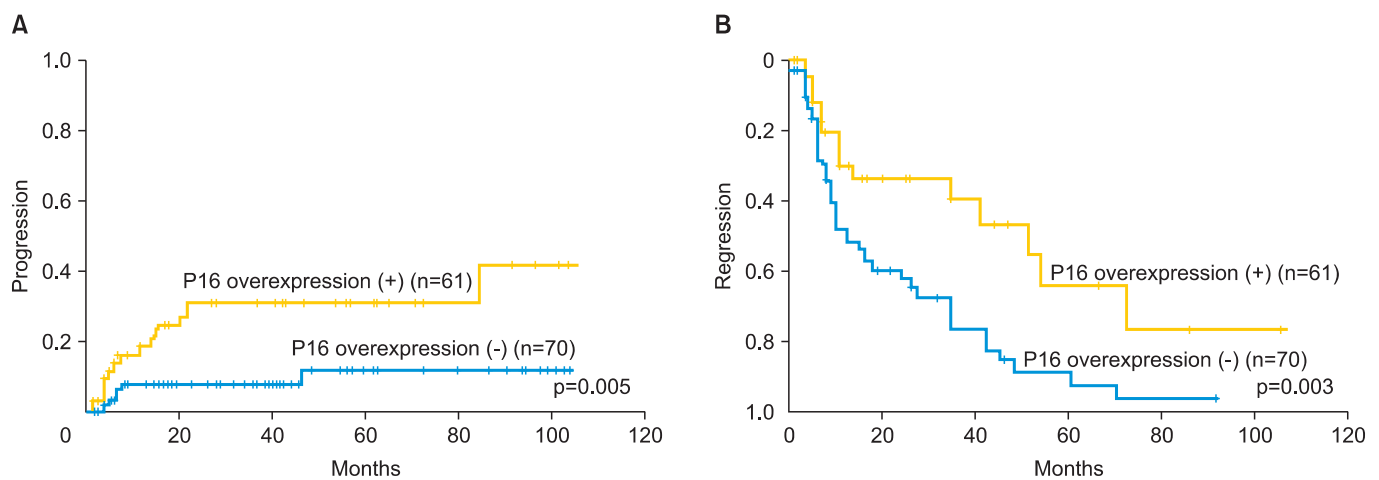


Fig. 3. (A) Kaplan-Meier analysis of the cumulative progression rate and follow-up periods in the patients with cervical intraepithelial neoplasia (CIN) 1–2. The progression rate for the patients showing p16^{INK4a} overexpression was significantly higher than that for patients showing no p16^{INK4a} overexpression (p < 0.05). (B) The cumulative regression rate and follow-up periods in patients with CIN 1–2. The regression rate for the patients showing p16^{INK4a} overexpression was significantly lower than that for the patients showing no p16^{INK4a} overexpression (p < 0.05).

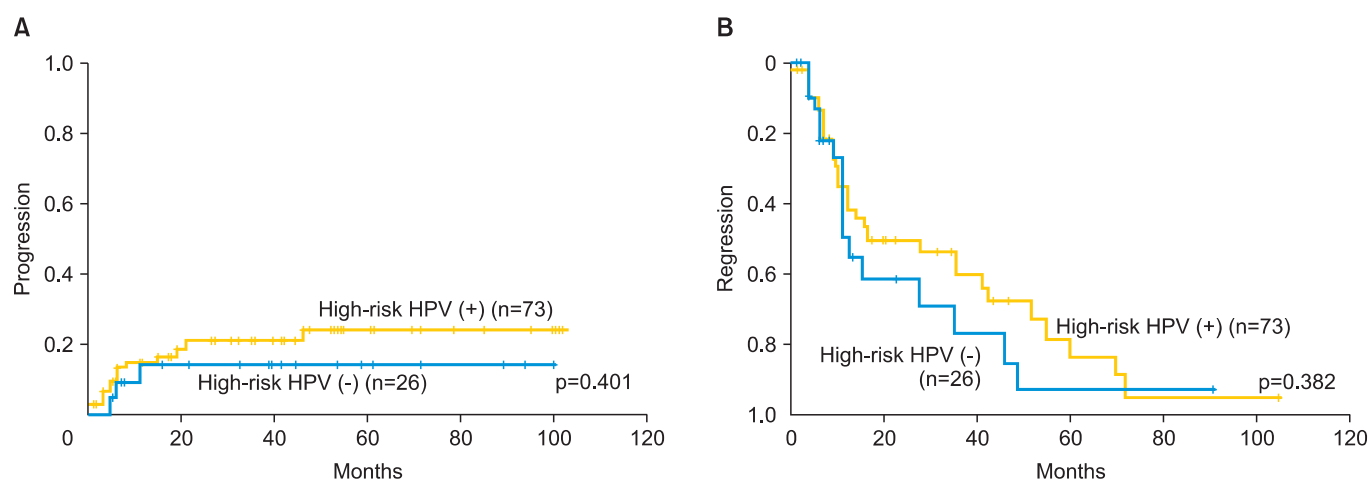


Fig. 4. (A) Kaplan-Meier analysis of the cumulative progression rate and follow-up periods (mo) for patients with CIN 1-2 lesions with prevalent high-risk human papillomavirus (HPV) infection. The progression rate in patients with prevalent high-risk HPV was not significantly different from that in patients who were negative for high-risk HPV. (B) The cumulative regression rate and follow-up periods (mo) for patients with CIN 1-2 with high-risk HPV infection. The regression rate in the patients with prevalent high-risk HPV was not significantly different from that in patients who were negative for high-risk HPV infection.

Table 2. Outcomes of patients with HPV 16 and 18 infection

| | Follow up of CIN 1-2, n (%) | | P-value |
|-----------------|-----------------------------|------------|---------|
| | Progression/persistence | Regression | |
| HPV 16 or 18 | 13 (52.0) | 12 (48.0) | 0.600 |
| Other genotypes | 34 (45.9) | 40 (54.1) | |

The definition of progression/persistence and regression was described in the manuscript.
CIN, cervical intraepithelial neoplasia.

patients infected with prevalent high-risk HPV showed a higher tendency towards progression of the CIN lesions, there was no significant difference in the rate of progression of the lesions between high-risk HPV-positive and negative groups (Fig. 4). There were also no significant differences in the rate of progression or regression of the lesions between patients infected with HPV 16 or 18 and those infected with neither of these HPV genotypes (p=0.60) (Table 2).

Among 76 patients with CIN 1, 58 patients were evaluated for HPV genotyping. The associated HPV genotypes with CIN 1 patients were 16, 18, 31, 33, 35, 51, 52, 56, 58, 59, 68, 70, 84, and 90. There was also no significant difference in the rate of progression and regression between high-risk HPV-positive and negative groups among the CIN 1 patients according to the Kaplan-Meier analysis (p=0.951, p=0.652, respectively).

DISCUSSION

In this study, the rate of p16^{INK4a} overexpression increased

with increasing CIN grade, indicating that p16^{INK4a} overexpression could reflect the potential of malignant transformation of cervical epithelial cells. In our previous report, increase in the rate of p16^{INK4a} overexpression may suggest more severe inactivation of pRb by high-risk HPVs which frequently cause progression from CIN 1 to higher grade, and p16^{INK4a} overexpression may be a reliable diagnostic marker for high-risk CIN lesions [22]. We also have shown here in this study there was a significant difference in the progression rate between patients showing p16^{INK4a} overexpression and those not showing p16^{INK4a} overexpression, and a significant difference in the regression rate between these two groups. These results suggest that p16^{INK4a} overexpression was correlated with the outcome of the CIN 1-2 lesions, and that p16^{INK4a} immunohistochemistry may be a promising biomarker for predicting the outcome of CIN 1-2.

Recent publications have demonstrated a correlation between p16^{INK4a} overexpression and the risk of progression or regression of CIN and cervical cancer [13-15,23]. Among these reports, Omori et al. [13] indicated that the p16^{INK4a} expression level was associated with CIN 2 progression, while other studies demonstrated that the p16^{INK4a} expression level was only correlated with the CIN 1 outcomes. Negri et al. [23] mentioned that CIN 1 patients with p16^{INK4a} overexpression showed a significantly higher tendency towards progression to CIN 3 than those that did not show p16^{INK4a} expression. However, clinical information was limited because they only selected CIN 1 patients that showed regression or progression to CIN 3, not including persistent patients. Here in this study, we evaluated both CIN 1 and CIN 2 patients for their outcomes

which could be predicted by p16^{INK4a} immunohistochemistry.

From the results of our study, p16^{INK4a} overexpression could be superior biomarker to the HPV genotyping for predicting the outcomes of CIN 1–2; however, we need to consider several aspects of this result. One point is that this study is a retrospective cohort study which has relatively small number of patients and 32 patients were not tested for HPV genotyping. Also, there is a possibility that the direct PCR sequencing for HPV genotyping could not fully cover the entire HPV infections. Therefore, we may have failed to identify all the cases with high-risk HPV infection. Considering more definitive and reliable method of HPV genotyping is available, we could reevaluate the correlation between infecting HPV genotypes and the outcomes CIN 1–2. The last point is that, during the course of the follow-up, the progression and regression rates reached plateaus which were shown in Figs. 3, 4. This partly reflects the surgical interventions, such as laser conization or ablation, and that both progression and regression rates could be different if these procedures were not undertaken. These aspects may lead to the lack of significant differences between the patients infected with high-risk HPV and those with other HPV genotypes.

The management of CIN still poses a dilemma for gynecologists; therefore, there could be unnecessarily prolonged management, overtreatment, and patients' anxiety. In addition, young patients with CIN tend to be lost to be followed up. If we perform p16^{INK4a} immunohistochemistry as a routine laboratory test for identifying the high-risk patients, especially among young patients, we may reduce the potential rate of progression of CIN to cervical cancer in the future. This is the first single-institution study, until date, to demonstrate that p16^{INK4a} immunohistochemistry may be an more appropriate biomarker than HPV genotyping for predicting the outcomes of CIN 1–2 patients.

CONFLICT OF INTEREST

The corresponding author Takuma Fujii received lecture fee from MSD Co. (Tokyo, JAPAN).

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