Abstract  Purpose: Inactivation of p16 gene by CpG methylation is a frequent event in oral epithelial dysplasia. To investigate the predictive value of p16 methylation on malignant potential in oral epithelial dysplasia, we carried out the prospective cohort study. 

Experimental Design: One hundred one patients with histologically confirmed mild or moderate oral epithelial dysplasia were included in the present cohort study. p16 Methylation status of the oral epithelial dysplasia lesions from 93 cases was obtained by methylation-specific PCR. Progression of the oral epithelial dysplasia lesions was examined in 78 cases histologically during a 45.8 months follow-up period. The association between p16 methylation and progression of oral epithelial dysplasia was analyzed.

Results: Of the 93 enrolled cases, 15 cases were lost during the follow-up because of changes of contact information, with a compliance of 83.9%. p16 Methylation was detectable in oral epithelial dysplasia lesions from 32 (41.0%) of 78 enrolled patients. Oral epithelial dysplasia–related squamous cell carcinomas were observed in 22 patients (28.2%) during the follow-up. Rate of progression to oral cancer in patients with the p16-methylated oral epithelial dysplasia was significantly higher than that with the p16-unmethylated oral epithelial dysplasia (43.8% versus 17.4%; adjusted odds ratio, 3.7; P = 0.013), especially for patients at the baseline age of ≥60 years (adjusted odds ratio, 12.0; P = 0.003) and patients with moderate oral epithelial dysplasia (adjusted odds ratio, 15.6; P = 0.022). The overall sensitivity and specificity of prediction of malignant transformation of oral epithelial dysplasia by p16 methylation were 63.6% and 67.9%, respectively.

Conclusion: p16 Methylation was correlated with malignant transformation of oral epithelial dysplasia and is a potential biomarker for prediction of prognosis of mild or moderate oral epithelial dysplasia. (Clin Cancer Res 2009;15(16):5178–83)

Imaging, Diagnosis, Prognosis

Methylation of p16 CpG Island Associated with Malignant Progression of Oral Epithelial Dysplasia: A Prospective Cohort Study
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Leukoplakia is the most common oral precancerous lesion (1). About 8% of oral leukoplakia will progress to oral squamous cell carcinoma because about 17% to 25% of the leukoplakia lesions contain oral epithelial dysplasia (2, 3). Current diagnosis of oral epithelial dysplasia is based primarily on morphologic criteria (4). It is virtually impossible to identify a person with oral epithelial dysplasia at high risk for developing oral cancer on histopathologic grounds alone. A practical biomarker is not presently available for clinical management of oral epithelial dysplasia (5). Inactivation of the p16

p16

INK4A (CDKN2A) gene is a frequent event in oral epithelial dysplasia and might play an important role in oral carcinogenesis (6).

P16 protein is a cell cycle regulator involved in the inhibition of G1 phase progression (7). Methylation of CpG islands of p16 gene silences its transcription (8). Abrupt inactivation of p16 gene by methylation is an early frequent event in multiple human cancers, including oral squamous cell carcinomas and its precancerous oral epithelial dysplasia lesions (9–13). In nested case-control studies, it was observed that p16 methylation was
correlated with increased risk for carcinomas of the stomach and lung (14, 15). However, the prediction value of p16 methylation for identification of malignant potential of epithelial dysplasia lesions has not yet been investigated. In the present prospective cohort study, we observed that p16 methylation increased the risk for malignant transformation of oral epithelial dysplasia significantly.

**Materials and Methods**

**Patients and study design.** One hundred one patients with mild or moderate oral epithelial dysplasia were selected from cases with oral leukoplakia, lichen planus, or chronic discoid erythenematous at Peking University School of Stomatolgy between 1995 and 2005. All of the patients with oral epithelial dysplasia had been diagnosed pathologically. The baseline oral epithelial dysplasia lesions were classified as mild, moderate, and severe grades by at least two senior pathologists at Department of Pathology at Peking University School and Hospital of Stomatolgy, using the criteria from 2005 WHO Classification System (16).

All cases involved primary lesions without any treatment history of laser, radiation therapy, or chemotherapy. Information on clinical variables, lesion site, habits, and medical history was collected. Follow-up examination was made regularly every 3 mo. If there was observable appearance of malignant development or recurrence, an additional examination and biopsy were carried out. From July 2007 to October 2008, careful examination was made for 27 cases who did not get a biopsy during the follow-up examination because of obvious disappearance/regression of baseline lesion (Fig. 1). The study was approved by the Institutional Review Boards of Peking University School of Stomatolgy and School of Oncology, and all patients gave written informed consent. This trial is registered in the U.S. NIH ClinicalTrials Protocol Registration System in accordance with the criteria outlined by the International Committee of Medical Journal Editors.  

**Detection of p16 methylation by methylation-specific PCR.** As described previously (14), genomic DNA samples were extracted from paraffin blocks of biopsies or resected specimens with oral epithelial dysplasia. Methylation status of p16 CpG island was detected by methylation-specific PCR (Supplementary Fig. S1; 150 bp for methylated copy and 151 bp for unmethylated copy) and analyzed using denatured high performance of liquid chromatography at Peking University School of Oncology. Paraffin blocks of xenografted p16-methylated RKO and p16-unmethylated MGC803 human cancer cell lines were used as positive and negative control for each experiment throughout the analysis procedure (including DNA extraction, bisulfite modification, methylation-specific PCR, and denatured high performance of liquid chromatography detection), respectively (17).

**Quantification of p16 methylation by Methylight.** Results of p16 methylation in oral epithelial dysplasia by methylation-specific PCR were further confirmed by modified Methylight (Supplementary Methods; Supplementary Fig. S3) at Peking University School of Oncology, as described (18, 19). When a sample had \( C_{h_ii\tiny{methylated}-p16} > C_{h_ii\tiny{unmethylated}} \), it was considered as strong p16 methylated.

**Follow-up examination and histopathology.** The follow-up examination was carried out in a double-blind study with a 3-mo interval. Rebiopsy was done as clinically indicated, for example, the lesion recurs or has tendency for malignant development. Pathologic diagnosis was made by at least two pathologists without the knowledge of baseline p16 methylation, based on the WHO criteria, at Peking University School of Stomatolgy (Fig. 2; ref. 16). The pathologic changes of the lesions were subclassified as malignant transformation, progression/persistent, and regression, as listed (Supplementary Methods).

**Statistical analysis.** Results were displayed by constituent ratios of enumeration or ranked data. SPSS13.0 software was used to do univariate and multivariate analysis with \( \chi^2 \) test and forward binary logistic regression analysis. \( \Delta C \) values were analyzed with Student’s t test. Cancer-free survival was analyzed with Kaplan-Meier test. All \( P \) values were two sided, and \( P < 0.05 \) was considered statistically significant. Each biopsy at baseline and follow-up analysis was assigned a severity score according to its histopathologic diagnosis: 1 for carcinoma, 2 for severe-grade oral epithelial dysplasia, 3 for moderate-grade oral epithelial dysplasia, 4 for mild oral epithelial dysplasia, 5 for hyperplasia, and 6 for normal.

**Results**

**Patients’ basic information.** p16 Methylation status was obtained for 93 of 101 cases with mild or moderate oral epithelial dysplasia by methylation-specific PCR–denatured high performance of liquid chromatography (Supplementary Fig. S2). These results were consistent with the results of Methylight significantly (Supplementary Table S1). Fifteen cases were lost during follow-up because of changes of contact information (three cases with p16-methylated oral epithelial dysplasia and 12 cases with p16-unmethylated oral epithelial dysplasia). Thus, 78 cases with follow-up information were enrolled into the final cohort analysis, with the compliance of 83.9%. The age and gender proportion between p16-methylated and -unmethylated groups were similar (Table 1; \( P > 0.05 \)). The follow-up time ranged from 3 months to 124 months, with an average of 45.8 months.

Among 60 patients for whom information of cigarette smoking status was available, the p16 methylation rate was similar between 19 smoking patients and 41 nonsmoking patients (36.8% versus 39.0%). However, the prevalence of the smoking in males was significantly higher than that in females (17 of 24 versus 2 of 36; \( P = 0.000 \)).

p16 Methylation is an independent predictor for malignant progression of oral epithelial dysplasia. Malignant progression of oral epithelial dysplasia to oral squamous cell carcinoma was observed in 22 of 78 patients. The average baseline age of these
cancer patients was slightly higher than that of patients without cancer development (59.6 versus 55.8 years; \( P = 0.174 \)).

Rate of progression to oral squamous cell carcinoma in the patients with the p16-methylated oral epithelial dysplasia were always higher than that with the p16-unmethylated oral epithelial dysplasia among different subgroups such as sex, age, baseline oral epithelial dysplasia grade, lesion site, surgical resection history, and length of follow-up time (Table 2). Multivariate analysis showed that the risk for malignant transformation for patients with oral epithelial dysplasia was increased significantly by p16 methylation (adjusted odds ratio, 3.7; \( P = 0.013 \)), especially among patients at the baseline age of \( \geq 60 \) years (adjusted odds ratio, 15.6; \( P = 0.022 \)). Among patients with the p16-methylated oral epithelial dysplasia, the rate of malignant transformation in the subgroup at the baseline age of \( \geq 60 \) years was significantly higher than that of \( <60 \) years (\( P = 0.036 \)). For these older patients, the sensitivity and specificity for prediction of malignant transformation of oral epithelial dysplasia lesion by p16 methylation were up to 76.9% (10 of 13) and 78.3% (18 of 23), respectively.

Among the 56 patients without malignant transformation of oral epithelial dysplasia, the rate of noncancerous progression or persistence of oral epithelial dysplasia lesions with p16 methylation was also slightly higher than that without p16 methylation (55.6% versus 39.5%; \( P = 0.258 \)). Comparing the prevalence of p16 methylation among oral epithelial dysplasia lesions with different prognosis, we observed that p16 methylation correlated with the severity of progression/regression of oral epithelial dysplasia significantly (Pearson \( r = 0.341; P = 0.011 \)): 63.6% (14 of 22) for oral epithelial dysplasia progressed to oral squamous cell carcinoma, 44% (11 of 25) for oral epithelial dysplasia progressed to high grades or persistent, and 22.6% (7 of 31) for oral epithelial dysplasia regressed. Moreover, the follow-up status of p16 methylation in rebiopsy samples by MethyLight was consistent with the baseline status by methylation-specific PCR and MethyLight. MethyLight strong p16-methylation rate (11 of 24) in the tested rebiopsy samples from patients with the methylation-specific PCR–p16-methylated oral epithelial dysplasia was significantly higher than that (2 of 25) from patients with the methylation-specific PCR–p16-unmethylated oral epithelial dysplasia (\( P = 0.007 \)).

Patients with p16-methylated oral epithelial dysplasia had a shorter cancer-free survival. To investigate the difference of the onset time for malignant transformation of oral epithelial dysplasia with and without p16 methylation during the follow-up, cancer-free survival was first analyzed by the Kaplan-Meier method. Results showed that cancer-free survival (median, 75.0 months) in the patients with the p16-methylated oral epithelial dysplasia was significantly shorter than that (median, 115.0 months) in the cases without the p16-methylated oral epithelial dysplasia (Mentel-Cox log rank test; \( P = 0.028 \), two-sides; Fig. 3). However, such difference was likely not significant after other factors were adjusted with Cox regression analysis (\( P = 0.056 \)). But the baseline age of patients was still a significant independent predictor for the cancer-free survival (\( P = 0.029 \)). The average onset age at which oral squamous cell carcinoma diagnosed for patients with p16-methylated oral epithelial dysplasia was slightly higher than that without p16 methylation (median, 70.0 versus 60.4 years; \( P = 0.149 \)).

### Discussion

Although it was reported that about 8% of oral epithelial dysplasia progressed to oral squamous cell carcinoma (2, 3), the exact initial cause of malignant transformation of leukoplakia is not clear. Early prediction of malignant potential of oral epithelial dysplasia is crucial for clinical management of patients with the disease. Because it is impossible to identify the malignant potential of oral epithelial dysplasia lesions on histopathologic grounds alone, it attracts lot of attention to characterize molecular biomarkers for prediction of prognosis of oral epithelial dysplasia (20, 21). In the present study, we observed that p16 methylation was a significant independent predictor of malignant prognosis of oral epithelial dysplasia with sensitivity of 63.6% and specificity of 67.9% (adjusted odds ratio, 3.7; \( P = 0.013 \)). Among patients at the baseline age of \( \geq 60 \) years, the sensitivity and specificity were 76.9% and 78.3%, respectively. To best of our knowledge, this is the first report that p16 methylation increases the risk for malignant transformation of oral epithelial dysplasia significantly.

Although 15 cases were lost during the follow-up, this did not overvalue the oral squamous cell carcinoma risk for the p16-methylated oral epithelial dysplasia because 12 of them (80%) were p16-unmethylated with low risk for malignant transformation. Thus, the loss of these 12 cases might lead to
a higher oral squamous cell carcinoma rate among patients with the \(p16\)-unmethylated oral epithelial dysplasia and thus lower oral squamous cell carcinoma risk for the \(p16\)-methylated oral epithelial dysplasia.

Dysfunction of \(p16\) gene is a frequent event in multiple human cancers and precancerous lesions at various organs, including the oral cavity (9–13). The \(p16/ARF\) inactivation rate was up to 70% to 85% in end-stage head and neck carcinomas (22–25).

Fig. 2. Photos of oral epithelial dysplasia and squamous cell carcinoma stained with H&E. Black bar, 50 \(\mu\)m of length. A–E, oral epithelial dysplasia without cancer development; F–J, oral epithelial dysplasia progressed to oral squamous cell carcinoma; K–O, oral squamous cell carcinoma progressed from (F to J), respectively.
It is well recognized that methylation of CpG islands around transcription starting sites silences p16 transcription epigenetically (8). Kresty et al. (11) reported that p16 methylation rate (57.7%) was much higher than the p16 mutation rate (15.4%) in 28 severe oral epithelial dysplasia lesions from 26 patients. We reported previously that p16 methylation was associated with malignant transformation of low-grade gastric dysplasia in a population-based nested case control study (14). In the present study, we found the total p16 methylation rate by methylation-specific PCR was 41% in 78 mild or moderate oral epithelial dysplasia lesions at baseline, which was consistent with results of the quantitative MethyLight assay. We also found the prevalence of p16 methylation at baseline significantly correlated with prognosis of oral epithelial dysplasia (Pearson $r = 0.341$; $P = 0.011$). This is consistent with the report of Hall et al. (26) that p16 methylation rate in oral epithelial dysplasia progressed to oral squamous cell carcinoma was also significantly higher than that in oral epithelial dysplasia without progression (8 of 14 versus 2 of 24). Taken together, these results indicate that p16 methylation is associated with prognosis of oral epithelial dysplasia.

In the present study, we found that among patients with the p16-methylated oral epithelial dysplasia, the rate of malignant transformation in the subgroup with the baseline age of $\geq 60$ years was significantly higher than that <60 years and that the baseline age was an independent predictor of cancer-free survival. This might account partially for the well-recognized phenomenon that age is the major risk factor for malignant transformation of oral epithelial dysplasia.

Generally, quantitative assays provide better information than qualitative assays. Although the quantitative MethyLight results in both initial biopsy and follow-up rebiopsy samples were consistent with those of the methylation-specific PCR–denatured high performance of liquid chromatography assay in the present study, we did not observe a significant increase of oral cancer risk in patients with oral epithelial dysplasia with the strong p16 methylation by MethyLight. Unlike in cultured cell lines, we reported that methylation status of CpG sites within the p16 CpG island in human tissue samples was not always homogeneous (17). Thus, different primer sets used in two kinds of assays might be the main reason accounting for detection difference of p16 methylation in these samples.

### Table 1. Basic information of patients with oral epithelial dysplasia enrolled into the final follow-up analysis

<table>
<thead>
<tr>
<th>Status of p16 M</th>
<th>Age (y)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Methylated</td>
<td>59</td>
<td>32-77</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>57</td>
<td>33-72</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>32-77</td>
</tr>
</tbody>
</table>

*Methylated group versus unmethylated group; $P > 0.05$.

### Table 2. Comparison of prevalence of p16 methylation and malignant transformation of oral epithelial dysplasia between different subgroups with multivariate analysis

<table>
<thead>
<tr>
<th>Items</th>
<th>Subgroups</th>
<th>Total</th>
<th>p16 M</th>
<th>Cancer</th>
<th>p16 M</th>
<th>Cancer</th>
<th>p16 U</th>
<th>Cancer</th>
<th>Adjusted odds ratio (95% confidence interval); $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>p16 M</td>
<td>rate (%)</td>
<td>n</td>
<td>Cancer cases (%)</td>
<td>n</td>
<td>Cancer cases (%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>31</td>
<td>45.2</td>
<td>32.3</td>
<td>14</td>
<td>7 (50.0)</td>
<td>17</td>
<td>3 (17.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>47</td>
<td>38.3</td>
<td>25.5</td>
<td>18</td>
<td>7 (38.9)</td>
<td>29</td>
<td>5 (17.2)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>&lt;60</td>
<td>42</td>
<td>40.5</td>
<td>21.4</td>
<td>17</td>
<td>4 (23.5)*</td>
<td>25</td>
<td>5 (20.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\geq 60$</td>
<td>36</td>
<td>41.7</td>
<td>36.1</td>
<td>15</td>
<td>10 (66.7)</td>
<td>21</td>
<td>3 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Baseline grade</td>
<td>Mild</td>
<td>53</td>
<td>39.6</td>
<td>28.3</td>
<td>21</td>
<td>8 (38.1)</td>
<td>32</td>
<td>7 (21.9)</td>
<td>15.60 (1.48-164.38); 0.022</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>25</td>
<td>44.0</td>
<td>28.0</td>
<td>11</td>
<td>6 (54.5)</td>
<td>14</td>
<td>1 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Surgical excision</td>
<td>Yes</td>
<td>53</td>
<td>41.5</td>
<td>32.1</td>
<td>22</td>
<td>11 (50.0)</td>
<td>31</td>
<td>6 (19.4)</td>
<td>4.17 (1.23-14.14); 0.022</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>25</td>
<td>40.0</td>
<td>20.0</td>
<td>10</td>
<td>3 (30.0)</td>
<td>15</td>
<td>2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Lesion site</td>
<td>Tongue or floor</td>
<td>39</td>
<td>41.0</td>
<td>33.3</td>
<td>16</td>
<td>7 (43.8)</td>
<td>23</td>
<td>6 (26.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>39</td>
<td>41.0</td>
<td>23.1</td>
<td>16</td>
<td>7 (43.8)</td>
<td>23</td>
<td>2 (8.7)</td>
<td>8.17 (1.41-47.22); 0.019</td>
</tr>
<tr>
<td>Follow-up (mo)</td>
<td>&lt;37</td>
<td>35</td>
<td>31.4</td>
<td>22.9</td>
<td>11</td>
<td>4 (36.4)</td>
<td>24</td>
<td>4 (16.7)</td>
<td>4.09 (1.03-16.28); 0.046</td>
</tr>
<tr>
<td></td>
<td>$\geq 37$</td>
<td>43</td>
<td>48.8</td>
<td>32.6</td>
<td>21</td>
<td>10 (47.6)</td>
<td>22</td>
<td>4 (18.2)</td>
<td>3.69 (1.31-10.39); 0.013</td>
</tr>
</tbody>
</table>

*Among patients with p16-methylated oral epithelial dysplasia; <60 years versus $\geq 60$ years; $P = 0.036$, two sides.
oral epithelial dysplasias. CpG sites located within 3′-end of primers are important for detection of methylation of CpG islands by assays using methylation-specific primers such as methylation-specific PCR and MethyLight. Although a methylation-specific probe was used in MethyLight, no CpG sites were included at the 3′-end of two primers used in the MethyLight assay. A novel MethyLight method using the methylation-specific PCR primers and methylation-specific probe related to other CpG sites might be useful to set cutoff value for clinical application of p16 methylation. Based on our experiences, it is difficult to design an optimal methylation-specific probe within the 150-bp methylated-p16 fragment amplified by the current methylation-specific PCR assay. Our previous and present studies suggested that methylation-specific PCR–denatured high performance of liquid chromatography assay might be a feasible method for prediction of prognosis of precancerous lesions.

In conclusion, p16 methylation increases the risk for malignant transformation of epithelial dysplastic lesions and can be used to predict malignant potential of oral epithelial dysplasia. Detection of p16 methylation by methylation-specific PCR–denatured high performance of liquid chromatography is convenient and should be carried out as a regular test for patients with oral epithelial dysplasia.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Methylation of $p16$ CpG Island Associated with Malignant Progression of Oral Epithelial Dysplasia: A Prospective Cohort Study

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