Hypermethylation of \( p16^{INK4a} \) in Chinese lung cancer patients: biological and clinical implications

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Promoter hypermethylation of the \( p16^{INK4a} \) gene was investigated in 111 cases of tumor tissue, as well as in 136 circulating plasma and 95 sputum samples from Chinese patients with primary lung cancer, using a modified protocol of semi-nested methylation-specific-PCR (MSP). The results showed hypermethylated \( p16 \) sequence in 80.2% of tumor tissues and frequencies of 75.7 and 74.7% in plasma and sputum specimens, respectively. Among the patients, 50 cases of matched plasma, sputum and tumor tissue from the same individual were analyzed. Of these, hypermethylation of the \( p16 \) promoter was detected in 84.0% of the tumor tissues, with frequencies of 72.0 and 76.0% in the corresponding plasma and sputum, respectively. Notably, only patients whose tumor tissue showed hypermethylation of \( p16 \) exhibited the same aberrant methylation in their sputum and/or plasma. Hypermethylation of \( p16 \) in sputum and plasma samples may provide a more sensitive approach to molecular diagnosis of lung cancer than relying on conventional cytotological analysis. Our data show that a combination of cytotological analysis of sputum and examination of \( p16 \) hypermethylation in sputum and plasma identified 92.0% (46/50) of the lung cancer patients studied, offering an effective means of early detection of lung cancer.

Introduction

Lung cancer is now the leading cause of tumor-related death in China. Mortality from lung cancer could be greatly reduced by diagnosis and treatment at early stages of the disease. Effective screening tools for early detection of lung cancer remains a major challenge in the field, however. Biomarkers that detect lung cancer in early stages, and identify pre-tumor or early tumor lesions, enabling earlier therapeutic intervention, would be invaluable resources.

Previous studies have shown that gene mutations in \( K\text{-ras} \) and \( p53 \), and microsatellite instability, can be detected in sputum and plasma samples from lung cancer patients, implicating their potential value as molecular biomarkers for this malignancy (1–8). Recently, studies on epigenetic changes in lung cancer have demonstrated that a number of genes, including \( p16 \), \( APC \), \( FIHT \), \( RAR\beta \), \( MGMT \), \( DAPK \), \( CDH13 \), \( RASSF1A \), \( TIMP-3 \) and \( GSTP1 \) are hypermethylated in tumor tissues (9–19). DNA methylation plays an essential role in normal development and maintaining genomic stability (20–22). Alterations in methylation patterns frequently occur in tumor cells, and hypermethylation in the promoter region of tumor suppressor genes, associated with an epigenetically mediated gene silencing, is a common feature in human cancers (23,24). Aberrant hypermethylation of \( p16^{INK4a} \), a well-known tumor suppressor gene, has been reported to be an early event in lung carcinogenesis and a potential biomarker for early diagnosis (25): previous studies showed that hypermethylation of \( p16 \) could be detected in the serum and/or plasma of patients with a variety of malignancies, including lung cancer (16,26–28). This epigenetic alteration was reportedly detectable in sputum samples from patients prior to clinical evidence of malignancy (29,30). A nested-PCR approach permitting improvement of the methylation-specific PCR (MSP) procedure has made this amplification method more sensitive, facilitating the detection of aberrant methylation of \( p16 \) in nanogram-quantities of DNA from lung cancer patients (29,31).

We used previously a modified semi-nested MSP for analyzing the hypermethylation status of the \( p16 \) promoter in plasma DNA derived from non-small cell lung cancer (NSCLC) patients (31). Circulating plasma and sputum from lung cancer patients are specimens that can be more readily obtained by relatively non-invasive approaches. They, however, yield very limited quantities of DNA. In the present study, an MSP approach has been applied to an increased number and more diverse cases of plasma, as well as to sputum and tumor samples from Chinese primary lung cancer patients, with the aim of further evaluating aberrant methylation of the \( p16 \) promoter as an efficient biomarker for early detection of this malignancy.

Materials and methods

Human tissue samples

Circulating plasma, sputum and tumor tissue were collected from lung cancer patients in the Cancer Hospital, PUMC and CAMS. Sample numbers collected were 136, 95 and 111, respectively. Plasma and sputum samples were obtained before surgery. Among the 136 plasma samples, tumor tissues were obtained following surgical resection from 111 matched patients, and 50 of these 111 cases also provided sputum samples for analysis. Informed consent from the hospital was obtained from each patient before surgery. The histological type of the tumors investigated included squamous cell carcinoma (SCC), adenocarcinoma (ADC), adenosquamous carcinoma (ASC), large cell lung cancer (LCLC) and small cell lung cancer (SCLC), which were classified according to the WHO Histological Typing of Lung Tumors, and staged following the TNM classification of malignant tumors as defined by the International Union Against Cancer.

Abbreviations: ADC, adenocarcinoma; ASC, adenosquamous carcinoma; LCLC, large cell lung cancer; MSP, methylation-specific PCR; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; TNM, tumor-node-metastasis; TSG, tumor suppressor gene.

\(^*\)These authors contributed equally to the work.
DNA from a prostate cancer cell line, TSU-PR1, which contains methylated p16 (33), was used as a positive control for the MSP. Each of the PCR amplifications was repeated at least once to confirm the result.

### Results

#### Efficiency test of semi-nested MSP

In this study, we evaluated efficiency of the modified semi-nested MSP used in examining plasma as well as sputum samples from cases of SCC and ADC, which represented clear clinical disease stages. The data derived from analyzing the plasma samples have proved our previous assumption that this modification significantly increased the sensitivity of the technique for all clinical stages (31). In the present investigation, the detectable frequency of hypermethylated p16 was increased from 50.7% (the first PCR) to 79.1% (the second PCR) in 67 cases of SCC ($P = 0.0006$), and from 36.6% (the first PCR) to 63.4% (the second PCR) in 41 cases of ADC ($P = 0.0151$) (data not shown). With sputum from 42 cases of SCC and 30 of ADC, the frequency was increased from 66.7% (the first PCR) to 81.0% (the second PCR) in the SCC ($P = 0.1365$), and from 43.3% (the first PCR) to 66.7% (the second PCR) in the ADC ($P = 0.0693$), as given in Tables I and II. By cytological analysis for sputum samples, only 54.8% of the SCC ($P = 0.0102$) and 33.3% of the ADC ($P = 0.0098$) had been detected, respectively.

Sequencing of the semi-nested PCR product from the p16 methylating-positive plasma samples confirmed the presence of 11 methylated CpG dinucleotides in the DNA lying between the sense and anti-sense primers (data not shown).

#### Table I. p16 methylation and routine cytological analyses of sputum samples from SCC patients

<table>
<thead>
<tr>
<th>Stages (n)</th>
<th>1st PCR</th>
<th></th>
<th></th>
<th></th>
<th>2nd PCR</th>
<th></th>
<th></th>
<th></th>
<th>Cytology</th>
</tr>
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<tr>
<td></td>
<td>M*</td>
<td>U*</td>
<td>Total (%)</td>
<td>M*</td>
<td>U*</td>
<td>Total (%)</td>
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</tr>
<tr>
<td>I (9)</td>
<td>7</td>
<td>2</td>
<td>7/9 (77.8)</td>
<td>7</td>
<td>2</td>
<td>7/9 (77.8)</td>
<td>5</td>
<td>4</td>
<td>5/9 (55.6)</td>
</tr>
<tr>
<td>II (22)</td>
<td>13</td>
<td>9</td>
<td>13/22 (59.1)</td>
<td>18</td>
<td>4</td>
<td>18/22 (81.8)</td>
<td>9</td>
<td>13</td>
<td>9/22 (40.9)</td>
</tr>
<tr>
<td>III/IV (11)</td>
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<td>3</td>
<td>8/11 (72.7)</td>
<td>9</td>
<td>2</td>
<td>9/11 (81.8)</td>
<td>9</td>
<td>2</td>
<td>9/11 (81.8)</td>
</tr>
<tr>
<td>Total (42)</td>
<td>28</td>
<td>14</td>
<td>28/42 (66.7)</td>
<td>34</td>
<td>8</td>
<td>34/42 (81.0)</td>
<td>23</td>
<td>19</td>
<td>23/42 (54.8)</td>
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</table>

*Total (%)

### Table II. p16 methylation and routine cytological analyses of sputum samples from ADC patients

<table>
<thead>
<tr>
<th>Stages (n)</th>
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<th></th>
<th></th>
<th></th>
<th>2nd PCR</th>
<th></th>
<th></th>
<th></th>
<th>Cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M*</td>
<td>U*</td>
<td>Total (%)</td>
<td>M*</td>
<td>U*</td>
<td>Total (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (9)</td>
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<td>8</td>
<td>6/14 (42.9)</td>
</tr>
<tr>
<td>III/IV (7)</td>
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<td>5</td>
<td>2/7 (28.6)</td>
<td>4</td>
<td>3</td>
<td>4/7 (57.1)</td>
<td>2</td>
<td>5</td>
<td>2/7 (28.6)</td>
</tr>
<tr>
<td>Total (30)</td>
<td>13</td>
<td>17</td>
<td>13/30 (43.3)</td>
<td>20</td>
<td>10</td>
<td>20/30 (66.7)</td>
<td>10</td>
<td>20</td>
<td>10/30 (33.3)</td>
</tr>
</tbody>
</table>

*Total (%)

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### Specimen preparation and DNA isolation

To enrich for the tumor cells from sections of paraffin-embedded tumor tissue, microdissection was carried out according to the protocol described previously (31). The tumor cells collected were digested with SDS/proteinase K, and the aqueous solution was extracted with phenol–chloroform. DNA was isolated by ethanol precipitation using standard protocols.

Sputum specimens, treated with CytoLyt solution and PreservCyt solution (CYTYC, Boxborough, MA, USA) according to the manufacturer’s recommendation, were centrifuged (2000 r.p.m. for 10 min at 4°C) to collect cell pellets, which were then washed twice with PBS. DNA from sputum cells was obtained by the standard method noted above.

All the circulating plasma samples collected were re-centrifuged to remove contaminating lymphocytes. DNA was isolated from the supernatant (400 μl) obtained by the standard method noted above.

#### p16 hypermethylation analysis

**Bisulfite conversion.** Conversion was carried out following the protocol of Herman et al. (32). Briefly, plasma DNA (0.5–1.0 μg), or sputum or tumor tissue DNA (1.0–2.0 μg), was treated with sodium bisulfite (Sigma, MO, USA). After purification, 0.3 M NaOH incubation and ethanol precipitation, the DNA was resuspended in 20 μl of TE, as described previously (31). All bisulfite-converted DNA samples were stored at −20°C until subsequent PCR was performed.

**MSP.** A modified semi-nested MSP (31) was performed in a 25 μl reaction volume to detect hypermethylation of the p16 gene. In the first PCR, 5 μl of the bisulfite-treated DNA solution (that is, ∼60–120 ng for plasma DNA, and 120–250 ng for sputum and tumor tissue DNA) was added to a mixture of 200 μM dNTPs, 0.5 μM each of sense and antisense primers (p16MS and p16MAS2; TTATTAGGCGGGCGCGGATCGC and CCACCTAAATC-GACCTCCGACCCG, respectively), 1× PCR buffer and 2 U of Taq polymerase (Promega, WI, USA). The second PCR used 1 μl of 10-fold diluted products from the first PCR as the template, and the PCR reagents described above together with the specific antisense primer p16MAS1 (GACCCCGAACCGCGC-GACCGTAA). Both PCR reactions consisted of 40 cycles at 95°C (30 s), 64/67°C (60 s), 72°C (2 min), and an initial denaturation at 95°C for 3 min and a final elongation at 72°C for 10 min.

To control for bisulfite conversion, all modified DNA was also amplified using the primers (p16US, p16USAS2 and p16USAS1) for unmethylated p16 sequence, under the same conditions described in reference (31).

#### Statistical analysis

χ² test or Fisher’s exact test was applied in statistical comparisons, and the statistic software SPSS 1.0 was run for analyzing the correlations between p16 hypermethylation and clinicopathological parameters.
Methylation of p16 in different specimens of lung cancer patients

In order to examine the methylation status of the p16 promoter region in patient materials, a total of 111, 136 and 95 samples of tumor tissue, plasma and sputum, respectively, from primary lung cancer patients were analyzed. Hypermethylated p16 was detected in 80.2% of the tumor tissues, and in 75.7 and 74.7% in the plasma and sputum specimens, respectively. For each kind of specimen, there was no obvious difference in frequencies among different histological tumor types ($P$ $\hat{=} 0.3857, 0.1244$ and 0.3801 for the tumor tissue, plasma and sputum, respectively), as shown in Table III.

Methylation of p16 in the set of specimens derived from 50 lung cancer patients

We examined p16 methylation in 50 cases where tumor tissue, plasma and sputum samples were all available from the same patient. Among them, the frequencies for hypermethylation of p16 were 84.0% in the tumor tissue, and 72.0% and 76.0% in the corresponding plasma and sputum specimens, respectively. A lower frequency was observed in samples from ADC patients. With regard to SCC, one of the major histological types of NSCLC, hypermethylation of p16 was more frequently identified in sputum samples than in the corresponding plasma (81.8 and 65.2%, respectively), but with no statistical significance ($P = 0.2963$). The data are summarized in Table IV, and representative pictures of agarose gel electrophoresis of the semi-nested MSP products for p16 are shown in Figure 1.

Discussion

As an important tumor suppressor gene, p16 inactivation has been observed in over 50% of primary lung cancers (34–36).
aberrant methylation of p16 were the main mechanisms of p16 inactivation, while homozygous deletion accounted for only 7.7% of the cases with negative p16 expression (44). In that investigation, we also used immunohistochemical staining to correlate the MSP results with protein data. We found that most of those tumor tissues expressed no or negligible P16 protein. The data implicated a significant role for hypermethylated p16 in lung carcinogenesis. Further, several studies, including ours, have shown that this epigenetic alteration can be detected in circulating plasma/serum and sputum specimens of lung cancer patients (16,29–31). Hypermethylation of p16 in sputum samples may occur before any clinical evidence of tumor, and serve as an indicator of pre-cancerous lesion or of an increased risk of lung cancer developing (29,30).

Here, the modified semi-nested MSP developed was used to identify hypermethylation within the p16 promoter region in a large number and diverse specimens, including samples from tumor tissue, circulating plasma and sputum, collected from primary lung cancer patients. We compared results from a first PCR to those obtained from a second PCR to test the efficiency of this technique. Our data further confirmed that semi-nested MSP is a highly sensitive method for detecting this epigenetic alteration even with nanogram-quantities of DNA. The results from samples of plasma paired with tumor tissue are similar to those obtained in our previous study (31), showing that this approach is not only sensitive, but also reproducible.

Notably, in 42 cases where p16 hypermethylation was observed in tumor tissues and the corresponding sputum and/or plasma samples, only 16 (38.1%, \( P < 0.0001 \)) cases were identified with cancer cells by conventional cytological examination on sputum samples (data not shown). The results suggest that compared with cytological analysis, detection of p16 hypermethylation in both sputum and plasma could prove a more sensitive and specific method for molecular diagnosis of the disease. It must be noted, however, that in eight cases where no detectable p16 hypermethylation was observed in any sample, four of them were identified as positive by sputum cytological analysis. This implicates a role for a combination of conventional cytological examination and the molecular (MSP) analysis as being a preferred approach for early detection of lung cancer.

In our study, from 50 patients, tumor tissue, plasma and sputum were all available for the investigation. Notably, in all the cases where tumor tissue had hypermethylated p16, aberrant methylation was also observed in their sputum and/or plasma specimens, whereas no hypermethylation of p16 was detected in the sputum and plasma from the patients whose tumor sample lacked this epigenetic alteration (data not shown). The results indicate that the semi-nested MSP is not only a sensitive, but also a specific approach, having a potential predictive role for lung cancer. Taken it as a whole, there was no obvious difference (\( P = 0.3448 \)) for the frequencies of hypermethylated p16 gene, among those three kinds of samples from the 50 cases investigated.

SCC and ADC, which are the major histological types of NSCLC, accounted for 78.0% (39/50) of the 50 cases where all three kinds of specimen were analyzed in this work (see Table IV). In the SCC cases, hypermethylation was more frequently detected in the sputum than in the plasma (81.8 and 68.2%, respectively), whereas with ADC, the frequencies were both 58.8% in sputum and plasma samples, but the correlation between the results was not perfect, implying that detection of hypermethylation p16 in combined clinical specimens may be needed to increase the efficiency of this molecular approach, particularly in cases where the levels of DNA are low. Aberrant methylation of p16 was detected in all of the tumor and plasma samples from the 11 patients with other histological types (including four ASC, one LCLC and six SCLC). However, with such limited case numbers, we cannot state with confidence that detecting this marker in plasma and sputum is diagnostic for these histological types.

Our data from both the present study (see Table III) and previous investigation (31) demonstrated that in the tumor tissues, no statistically significant differences are observed among histological types or clinical stages, indicating that p16 hypermethylation is a common and early event during lung carcinogenesis in general. The frequency of p16 hypermethylation, in this study, was high as compared with reports involving Western cases (9,16,29,30). In addition to etiological factors, racial differences might play a factor in the high rate of p16 hypermethylation observed in the Chinese lung cancers.

As a control for our work, we also analyzed 25 plasma samples from healthy individuals using the same semi-nested MSP protocol. There was no PCR product obtained in any case. When wild-type p16 primers were used, PCR products were still not observed (data not shown). The results suggest that the quantity of DNA in circulating plasma from healthy individuals is below the level detectable by the semi-nested MSP protocol we used.

The work reported in this paper is the first part of a series of studies we hope to perform. We propose in future to analyze plasma and sputum samples derived from age/sex matched, smoking/non-smoking non-cancer individuals and non-lung-cancer patients, to evaluate the utility of the methods now developed for screening lung cancers. Subject to the validation, p16 hypermethylation may prove an effective biomarker for early detection of lung cancers. Molecular analysis of sputum and circulating plasma for this marker, assuming the next stage studies support this hypothesis, would provide a useful approach for screening high-risk populations for earlier diagnosis and therapeutic intervention of this important malignant disease.

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