Abstract. Background: The distribution of promoter methylation throughout the lungs of patients with non-small cell lung cancer (NSCLC) is unknown. In this explorative study, we assessed the methylation status of the promoter region of 11 genes in brush samples of 3 well-defined endobronchial locations in patients with NSCLC and in brushes of former and current smokers without NSCLC.

Materials and Methods: The methylation status of RASSF1A, GATA4, GATA5, SFRP1, RARβ2, DAPK, MGMT, p16, p14, CHFR and APC2 was determined in all samples using real-time methylation-specific PCR. Results: Ten patients with NSCLC and 18 non-NSCLC controls were included. Eight patients had one or more methylated genes in their tumor brush. Promoter methylation of genes in proximal or contralateral locations was much less frequent than in tumor brushes, and almost exclusively occurred in normal tissue if the same gene was also methylated in the tumor brush. Conclusion: Promoter methylation almost exclusively occurred in tumor cells of patients with NSCLC.

DNA-methylation is one of the most well-known epigenetic processes (1) and refers to the binding of a methyl group to cytosine nucleotides in the DNA sequence. Methylated cytosines are found in relatively high contents in CpG islands. These CpG islands are distributed in a non-random manner across the humane genome, including the promoter regions of many tumor suppressor genes. Aberrant DNA-methylation of CpG islands may lead to the transcriptional silencing of tumor suppressor genes, and is among the earliest and most frequent alterations in cancer. The varying levels of tumor suppressor gene methylations among different malignancies can be measured with a sensitive methylation-specific polymerase chain reaction (MSP), which seems a promising tool for early cancer detection. Aberrant methylation is common in non-small cell lung cancer (NSCLC) (2).

Promoter methylation of multiple genes has not only been observed in NSCLC tumor tissue and NSCLC cell lines (3, 4), but also in histologically tumor-free lung tissue of patients with resected NSCLC (5, 6), lymph nodes (7, 8), tumor brushes (9), serum (10), bronchoalveolar lavage (BAL) (11-14) and sputum (15, 16) of patients with NSCLC. In addition, promoter methylation was also detected in sputum and bronchial brushes of heavy smokers (9, 17, 18). The detection of promoter methylation in sputum or brushes of cancer-free heavy smokers proved to be a risk factor for the subsequent development of cancer (9, 15, 19), highlighting the potential of methylation assays as lung cancer screening tool. Evidence for a possible association between the presence of promoter methylation and smoking status and/or number of packyears is conflicting (12, 13, 17, 18, 20-23). Altogether, methylation assays offer new possibilities in studying the pathogenesis, diagnosis, and eventually the treatment of NSCLC.

One could hypothesize that aberrant promoter methylation occurs randomly in bronchial epithelium that is exposed to tobacco smoke. Local recurrences or second primary tumors after first-line treatment may develop from premalignant lesions other than the lesion responsible for the first tumor. The presence of random promoter methylation throughout the lungs could therefore be a sign of the presence of random, multiple lesions with genetic damage caused by tobacco smoke.

In contrast with the occurrence of multiple, random lesions, one could as well hypothesize that molecular and subsequent histological abnormalities first arise at one,
unique location. Synchronous abnormalities observed in other locations, and also subsequent local recurrences and second primary tumors, could all originate from this primary tumor by means of clonal expansion (24). This concurs with the field cancerization theory, originally proposed by Slaughter in 1953 (25). Obviously, the field cancerization theory was based on histological parameters and not on methylation events. According to the field cancerization theory, promoter methylation detected in tissue sampled from locations other than the primary tumor originates from the primary tumor by either clonal expansion or cellular contamination. The clonal expansion theory predicts a decreasing number of promoter methylation events with increasing distance from the primary tumor.

A bronchial brush is an excellent tool for obtaining a large sample of bronchial epithelial cells of well-defined locations in the bronchial tree. These brushes are widely used for the cytological diagnosis of pulmonary abnormalities, such as NSCLC. Bronchial brushes generally contain more than one million cells, 80-90% of which are epithelial cells (26, 27). One study reported that 32% of former smokers had one or more genes with promoter methylation in epithelial cells obtained with a carinal bronchial brush (18). Zochbauer-Müller et al. observed promoter methylation in up to 20% of bronchial brushes of smokers (17). In another study, promoter methylation of p16 was observed in 16% and 8% in bronchial brushes of patients with lung cancer versus smokers without lung cancer, respectively (9).

To evaluate the distribution of promoter methylation, bronchial epithelial cells of three well-defined endobronchial locations in patients with NSCLC were collected and the presence of promoter methylation of eleven genes [RAS-association domain family 1 isoform A (RASSF1A), GATA-binding protein 4 (GATA4), GATA-binding protein 5 (GATA5), secreted frizzled-related protein 1 (SFRP1), retinoic acid receptor beta-2 (RARβ2), death-associated protein kinase (DAPK), O6-methylguanine-DNA-methyltransferase (MGMT), cyclin-dependent kinase inhibitor 2A (CDKN2A/p16 and p14), checkpoint with forhead and ring finger domains (CHFR), and adenomatous polyposis coli 2 (APC2)] at those three locations in the bronchial tree was quantitatively determined.

**Materials and Methods**

**Patients.** Patients who were scheduled for a diagnostic bronchoscopy during the work-up for suspected lung cancer were asked to participate in the study. Ten patients were included (6 females, 4 males), with a median age of 54 years (range, 40 to 70 years). Six patients were current smokers; four patients were former smokers, 1 to 12 years since quitting. The median number of packyears was 33 (range, 20 to 60). Five patients had adenocarcinoma, four had large cell carcinoma and one patient had a neuroendocrine carcinoma. At diagnosis, one patient had stage IA disease, two had stage IIIB and seven patients had stage IV disease.

Controls were former and current smokers who had no history of lung cancer, in order to match patients and controls as well as possible. Eighteen controls (15 males) were included, with a median age of 59 (range, 23 to 77 years). Eight controls were former smokers (1 to 39 years since quitting) and 10 were current smokers. The median number of packyears was 23 (range, 1 to 70).

The study protocol was approved by the Medical Ethics Committee of the University Medical Center Groningen. All patients and controls gave informed consent.

**Sample collection.** Flexible fiberoptic bronchoscopy (Olympus, Tokyo, Japan) via nasal introduction was performed under local or general anesthesia. In controls, one bronchial brush was obtained from the normal-appearing main bronchi. In patients, brushes were sampled of three well-defined endobronchial locations. One brush was collected from the tumor, a second brush from the normal-appearing main bronchi 2-3 cm proximal from the tumor, and a third from the normal appearing main bronchus of the contralateral lung. To avoid contamination of the bronchoscope with tumor cells, the contralateral brush was collected first, followed by the proximal brush and finally the tumor brush. Moreover, all brushes were sheathed cytology brushes (Cellebrity Endoscopic Cytology Brush; Boston Scientific, Boston, MA, USA).

After sampling, brushes were stored in a solution of 50% ethanol and 2% polyethylene glycol at room temperature until further processing.

**DNA isolation and modification.** Cells attached on the bronchial brush were collected by centrifugation at 960 relative centrifugal forces for 10 minutes and by an additional washing with phosphate buffered saline (PBS) and a centrifugation step. Genomic DNA was then extracted using the classic SDS/protein K digestion and phenol-chloroform extraction, and resuspended in 50 μl TE (3 mM Tris, 0.2 mM EDTA; pH 8.0). The DNA was quantified using the Picogreen dsDNA quantification kit (Molecular Probes, Invitrogen, Breda, the Netherlands) following the manufacturer’s recommendations. The average yield of DNA was 19.2 μg (range, 0.5 to 87.7 μg). Up to 1.5 μg of genomic DNA were modified using sodium bisulfite to deaminate selectively unmethylated cytosine residues to uracil, while 5-methyl cytosine residues were not modified. The bisulfite modification was performed using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA), which includes successive steps of conversion, desalting and desulfonation. At the end of the procedure, the modified DNA was eluted in 50 μl of 1mM Tris-HCl (pH 8.0), and then stored at −80°C until used for real-time MSP.

**Real-time MSP.** Briefly, for each gene of interest and beta-actin (ACTB), methylation was determined in real-time MSP using bisulfite-modified DNA as previously described by Herman et al. (28). ACTB was used as an independent reference gene. The promoter methylation status of RASSF1A, GATA4, GATA5, SFRP1, RARβ2, DAPK, MGMT, p16, p14, CHFR, and APC2 was calculated as the ratio of the gene of interest and ACTB according to the following equation: (copies methylated gene/copies ACTB) ×1,000. This ratio is a measure of the relative level of methylation in an individual sample.

**Statistical analysis and cut-off value.** Promoter methylation was considered present if the ratio was above a certain cut-off value. The relative level of methylation varied significantly among the 11 genes and therefore cut-off points were studied for each gene individually.
The gene-specific cut-off value was defined as the highest ratio observed in controls (visualized in scatter plots). In this way, the specificity of a single gene was set at 100%.

The presence of promoter methylation at the tumor, proximal, and contralateral locations was visualized. In addition, the overall level of methylation was determined using the methylation index (MI) (29). The MI is defined as the ratio of number of genes with promoter methylation to the number of genes tested ×100%.

Associations between promoter methylation and demographic and clinical characteristics of patients and controls were calculated using chi-square and Mann-Whitney tests, where appropriate. A p-value of <0.05 was considered statistically significant.

**Results**

**Exploring cut-off values using normal controls and tumor brushes.** Patients and controls were well-matched regarding smoking status, number of packyears and age. The control group consisted of fewer females than the patient group.

In epithelial cells from bronchial brushes of 18 controls and 10 patients with NSCLC, the ratios of expression of 11 methylated genes as compared to the expression of ACTB ranged from 0 to 71 (Table I). The mean ratio of methylated genes versus ACTB was higher in patients than in controls for all eleven genes except p14 (Table I). For DAPK and p14, the highest ratio observed in controls was higher than in patients with NSCLC; for the other nine genes at least one ratio in patients was higher than the highest ratio observed in controls (Figure 1). The percentage of controls with a ratio >0 but below our gene-specific cut-off value ranged from 94% (DAPK) to 0% (Table I).

**Distribution of promoter methylation in bronchial epithelium.** Eight out of ten patients with NSCLC had at least one gene with promoter methylation in the tumor brush and none of the 18 controls using our cut-off value, resulting in an overall sensitivity of 80% and an accuracy of 93% (Figure 2). One patient had 5 genes with promoter methylation, one patient 4, one patient 3, three patients 2, and two patients only one. Promoter methylation was most frequent in APC2 and RASSF1A (in 5 and 4 patients, respectively). For DAPK and p14, no promoter methylation was observed in these 10 patients.

Overall, in 10 NSCLC patients using a panel of 11 different genes we identified an MI of only 18% (20/110) (Figure 2) in tumor cells and 2.7% in normal epithelial cells. Epithelial cells obtained from the proximal and contralateral brush did not differ in the number of genes with promoter methylation. No random pattern of promoter methylation in normal bronchial epithelium at the proximal and contralateral localizations was observed. The highest overall number of methylated genes was observed in patient 8. His p16/ACTB ×1,000 for tumor, proximal and contralateral brushes were 4.6, 6.0 and 1.3, respectively, above the cut-off value of 1. Patient 8’s APC2/ACTB ratios ×1,000 were 11.2, 12.0, and 3.7, respectively, with a cut-off value of 2.6. APC2 was also methylated in the proximal brush of patient 1 (3.4) and in the contralateral brush of patient 6 (3.2).

**Correlation between promoter methylation and clinical characteristics.** The presence of promoter methylation in tumor brushes was not associated with exposure to smoking (p=0.53 for number of packyears) or acute smoking effects (p=0.2 for current versus former smokers). In this study, age and gender were also not associated with the presence of methylation (p=0.53 and p=0.75, respectively). Finally, the histological subtype of NSCLC was not associated with methylation (p=0.29). The two patients without any promoter methylation were a 51-year-old female, a current smoker with 35 packyears, and a 69-year-old male, a current smoker with 40 packyears, both with adenocarcinoma of the lung.

**Table I. Promoter methylation status of eleven genes in endobronchial epithelial cells from 10 patients with NSCLC and 18 controls.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean ratio gene/ACTB ×1,000 in patients (range)</th>
<th>Mean ratio gene/ACTB ×1,000 in controls (range)</th>
<th>Percentage of controls with a ratio gene/ACTB ×1,000 &gt; 0</th>
<th>Cut-off value (based on 100% specificity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF1A</td>
<td>70.8 (0-393)</td>
<td>0.18 (0-2.5)</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>GATA4</td>
<td>21.9 (0-217)</td>
<td>0.03 (0-0.2)</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>GATA5</td>
<td>1.1 (0-11)</td>
<td>0.06 (0-0.3)</td>
<td>28</td>
<td>0.5</td>
</tr>
<tr>
<td>SFRP1</td>
<td>1.7 (0-17)</td>
<td>0.10 (0-0.8)</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>RARβ2</td>
<td>1.1 (0-6)</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>DAPK</td>
<td>0.08 (0-0.5)</td>
<td>0.07 (0-0.5)</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>MGMT</td>
<td>13.9 (0-138)</td>
<td>0.02 (0-0.2)</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>p16</td>
<td>0.63 (0-4.6)</td>
<td>0.01 (0-0.1)</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>p14</td>
<td>0.02 (0-0.2)</td>
<td>0.05 (0-0.6)</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>CHFR</td>
<td>55.0 (0-549)</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>APC2</td>
<td>5.4 (0-30)</td>
<td>0.77 (0-2.57)</td>
<td>94</td>
<td>2.6</td>
</tr>
</tbody>
</table>
Discussion

The present exploratory study is the first study to evaluate the presence of promoter methylation at different endobronchial locations in patients with NSCLC. Promoter methylation was observed in tumor brushes of up to 80% of patients with NSCLC, whereas it was uncommon in proximal and contralateral brushes, and almost exclusively present if the same gene was also methylated in the tumor brush.

The overall frequencies of promoter methylation in proximal and contralateral brushes were substantially lower than in tumor brushes (Figure 2). In the present study, no random pattern of distribution of promoter methylation in the proximal and contralateral endobronchial locations was observed; methylation at these locations was, in all but one case, only present if the tumor brush also contained promoter methylation of the same gene. This observation favors the idea that promoter methylation is primarily
present in tumor cells, and subsequently may spread from the primary tumor to adjacent and even further locations in the bronchial epithelium, as was predicted with the theory of clonal field expansion (24). Exposure to tobacco smoke causes genetic damage to the entire bronchial epithelium, as was recently demonstrated by the construction of a biomarker panel that was able to predict the presence of lung cancer in normal airway epithelium of smokers with suspected lung cancer (30). This panel has no overlap with the genes we tested. In our study, smoking did not result in a random development of promoter methylation in the entire bronchial epithelium in patients with lung cancer. The absence of methylated genes in our controls, who all had a long history of exposure to tobacco smoke, is in line with this finding.

In contrast, several other studies reported the detection of promoter methylation in exfoliative bronchial material in 5-15% of heavy smokers without NSCLC (6, 9, 11-15, 18-20, 31-37). Some of these studies applied quantitative methylation-specific PCR (11, 13, 14, 35, 37). For most of the genes in our panel, several of our controls had detectable ratios (Table I), but these levels were generally well below our chosen cut-off values. Our findings with real-time PCR are different from the reported results using quantitative PCR. These quantitative (nested) PCRs are very sensitive; it is claimed that 1 positive marker in 50,000 negative markers can be detected (15). The prognostic and clinical value of one positive signal among many negative signals is difficult to interpret; we think that ratios are much more clinically meaningful. Apart from technical differences between our study and other studies, the reported methylation frequencies in other studies may be due to patient selection, for instance presence of sputum atypia (17), or use of controls with a history of cancer (18). In addition, our strategy differed from others in that we set our design and test condition in such a way as to have the highest specificity for NSCLC.

To our knowledge, only one study reported on promoter methylation assays (using quantitative MSP) on brushes of patients with lung cancer (9). In brushes from either the main bronchus or tumor of patients, a 16% methylation frequency was observed for p16, dropping to 8% for chronic smokers without lung cancer. Real-time MSP has not been performed on bronchial epithelial cells collected by sheathed brushes before. In our study, single gene methylation frequencies of tumor brushes ranged from 0-50%, with an overall methylation index of 18%.

The quantitative results of a methylation-specific real-time PCR are often dichotomized for comparative purposes (11, 35, 37-39). Different methods for defining the optimal cut-off values for dichotomization exist. One could define

![Figure 2. Distribution of gene promoter methylation at three endobronchial locations (tumor, 2-3 cm proximal of tumor, contralateral lung) in 10 patients using a cut-off value based on 100% specificity in 18 controls. Grey denotes unmethylated genes, black methylated genes. The right column provides a summary with the number of methylated genes at a single location.](image-url)
cut-off values based on methylation frequencies of genes in tumor tissue. These frequencies can be derived from previously published studies (38) and the cut-off values are adjusted to match the expected methylation frequencies. It is essential for this method that consistency is observed in independently established promoter methylation frequencies of tumor tissues for individual genes (38). Yet the methylation frequencies in exfoliative samples are generally lower than those in tumor tissue (17). Moreover, it is known that median methylation ratios were observed to be higher in tumor tissue than in other tissues (11, 37). This is partially explained by the fact that brushes and sputum contain relatively fewer bronchial epithelial tumor cells than tumor tissue due to contamination with other cells. Therefore, we chose to define a specific cut-off value for brushes based on the ratios observed in controls, with specificity set at 100%. This means that cut-off points specifically determined for brushes are more appropriate for the use of methylation assays in the early detection of cancer in exfoliative samples than tissue-based cut-off values.

In our study, methylation was much more pronounced in tumor cells as compared to non-tumor cells from the same individual. This finding suggests that the tumor field effect may be limited in terms of distance from the tumor and/or percentage of affected cells in the vicinity of the tumor. In addition, if methylation tests are to be used as a screening tool for lung cancer, our results suggest that the value of such a test is highly dependent on the presence of tumor cells in the samples to be screened. Whether or not the presence of promoter methylation was correlated to the cytological features of the samples (e.g. normal epithelium, dysplasia) was not determined in our study.

We did not observe any association between smoking history, age, gender, or even tumor type, in agreement with most studies (11-13, 17, 18, 20, 23, 35, 40). In contrast, some studies reported that the frequency of methylation is higher in current smokers versus former or never smokers (12, 14, 21, 41), in males versus females (6), or is dependent on tumor histology (41, 42). Our results are not necessarily discrepant with results of other groups observing methylation in smokers without lung cancer and in cancer-free tissue. The difference may be due to the use of cut-off values, as described earlier, differences in specimens, our sample size, or other undefined factors.

In conclusion, promoter methylation was almost exclusively observed in endobronchial tumor cells and was very infrequent or absent in cancer-free samples. Whether the promoter methylation observed by other groups in cancer-free tissue and in controls without lung cancer, in contrast with our results, is a result of contamination with tumor cells or is due to methodological issues such as the high specificity of real-time MSP is not known.

**Conflict of Interest**

No financial or other potential conflicts of interest are declared for Drs de Jong and Kramer. Drs Verpoorten and Louwagie are employees of Oncomethylome Sciences SA. Dr Groen is consultant to Oncomethylome Sciences SA and received a research grant of Oncomethylome Sciences SA.

**Acknowledgements**

The authors thank Alexandre Meunier for his technical assistance.

**References**

De Jong et al: Promoter Methylation in NSCLC


Received June 26, 2008
Revised November 25, 2008
Accepted December 2, 2008