Promoter Hypermethylation of Resected Bronchial Margins: A Field Defect of Changes?

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ABSTRACT

Purpose: Histologically positive bronchial margins after resection for non-small cell lung cancer are associated with shortened patient survival due to local recurrence. We hypothesized that DNA promoter hypermethylation changes at bronchial margins could be detected in patients with no histological evidence of malignancy and that they would reflect epigenetic events in the primary tumor.

Experimental Design: Bronchial margins, primary tumor, bronchoalveolar fluid, and paired nonmalignant lung were obtained from 20 non-small cell lung cancer patients who underwent a lobectomy or greater resection. Disease-specific recurrence was the primary end point. The methylation status of p16, MGMT, DAPK, SOCS1, RASSF1A, COX2, and RARβ was examined using methylation-specific polymerase chain reaction.

Results: All malignancies had methylation in at least one locus. Concordance of one gene with an identical epigenetic change in the tumor or bronchial margin was observed in 85% of patients. Only one patient had methylation at the bronchial margin for a gene that was not methylated in the corresponding tumor. Median time to recurrence was 37 months (range, 5–71 months). There were two local recurrences and five metastases. There were no significant correlations between DNA methylation in tumor, margins, or bronchoalveolar fluid specimens and either regional recurrence or distant metastases.

Conclusions: Histologically negative bronchial margins of resected non-small cell lung cancer exhibit frequent hypermethylation changes in multiple genes. These hypermethylation abnormalities are also present in the primary tumor and thus may represent a field defect ofpreneoplastic changes that occurs early in carcinogenesis.

INTRODUCTION

It is widely accepted among clinicians that macroscopic or microscopically positive bronchial margins after pulmonary resection for lung cancer curtail patient survival due to local lung cancer recurrence. The observation that incomplete tumor resection with resulting residual macroscopic (R2) disease leads to recurrent local cancer has long been an important clinical dictum (1). Recently, Hofmann et al. (2) reported a small series with 12 patients who had R2 disease. Of these, all patients experienced local recurrence and had poor long-term survival. However, the evidence that microscopically positive bronchial margins (R1) lead to recurrent cancer has been less compelling, and there have been numerous reports over the last three decades documenting long-term survivors despite microscopic residual disease at the bronchial margin (3–6).

Despite the equivocal evidence regarding microscopically positive bronchial margins of lung tumors, a large survey conducted by the College of American Pathologists of 464 institutions found that more than 90% of pathologists routinely report the presence or absence of neoplasm at the bronchial margin (7). The incidence of residual macroscopic or microscopic disease at the bronchial margins ranges from 1.6% for the former (2) and up to 17% for the latter (8–14). Based on the pathological documentation of residual positive bronchial margins after surgery, adjuvant therapy, often as radiotherapy, is initiated in an attempt to prevent local recurrence (15).

Aberrant promoter hypermethylation has been shown to be a common event in human cancer mainly due to loss of function of tumor suppressor genes (16). This neoplastic related event is thought to occur early in carcinogenesis, and hence, promoter hypermethylation is being widely studied as a biomarker system for the diagnosis and detection of early cancer (17). In lung cancer, promoter hypermethylation has been detected in the blood (18), bronchial lavages (19), induced sputa (20), and even in pleural fluid (21) of patients with primary pulmonary malignancy. The present study hypothesizes that DNA promoter hypermethylation changes could be detected at the bronchial margins of patients who had no evidence of malignancy by histopathological criteria. As a secondary question, we asked whether epigenetic changes in the bronchoalveolar fluid would also reflect hypermethylation at the tumor or bronchial margin.

MATERIALS AND METHODS

Study Design. We examined the primary tumor, bronchial margins, paired nonmalignant pulmonary parenchyma, and bronchoalveolar fluid from 20 patients who had surgical resection for lung cancer with curative intent at the Johns Hopkins Medical Institutions between 1996 and 1999. All patients had no evidence of macroscopic or microscopic disease at the resected bronchial margins. Only patients who had undergone lobectomy or greater resections were considered because there is strong evidence from a prospective, multi-institutional randomized trial.
that surgical resections less extensive than a standard lobectomy are at a significant increased risk of local recurrence (22). Local recurrence was defined as cancer of the same histology recurring in the ipsilateral chest within 5 years. Patient tissue and bronchoalveolar fluid samples were collected prospectively at time of resection. Informed consent was obtained from all patients for harvesting of their tissue fresh for subsequent molecular marker studies. Cancer-specific recurrence was the primary end point of the study. Outcome data were derived from a comprehensive database that is maintained using the institution’s cancer registry, patient charts, and telephone calls to local primary physicians. There was 95% complete follow-up in this study. The research protocol of this study was approved by the Johns Hopkins Hospital institutional review board.

Clinical and Pathological Features. The mean age of the study population was 66.1 ± 10.9 years (median age, 68 years; range, 42–83 years), and there was a slight predominance of females. Because all patients were selected preoperatively to undergo resections with curative intent, the pathological staging of disease in this study consists predominantly of stage I and stage II disease. Pathological subtypes consisted of 18 patients with adenocarcinoma and 1 patient each with squamous and large cell cancer. There was one patient who, after stereotactic cranial irradiation of her isolated brain metastases, underwent a lobectomy for a T1N0 primary lung cancer despite her stage IV disease. There were 19 lobectomies and 1 pneumonectomy.

Tissue Samples. All lung cancer specimens were procured at the time of resection, subjected to an initial gross pathological examination, frozen in liquid nitrogen, and then stored at −80°C. Tumor staging was codified according to the tumor-node-metastasis (TNM) classification of the American Board of Cancer. For each patient, fresh frozen neoplastic tissue and sections of the corresponding resected bronchial margins were available for H&E staining as well as hypermethylation studies. Histologically paired malignant and nonmalignant lung tissue were available in 14 of 20 (70%) patients. Corresponding paired nonmalignant tissue sections were procured at the most distant site from the resected specimen. H&E staining confirmed the presence of cancer or the absence of malignancy in the bronchial margins and normal tissues.

Bronchoalveolar Lavage Fluid. Bronchoalveolar fluid was collected during flexible bronchoscopy performed at the time of surgical resection. The bronchoscope was guided into the segmental bronchus that had been identified radiographically to contain the tumor. Aliquots of 30 cc of warm saline were injected into the segmental bronchus until a volume of 30 cc was collected in a specimen trap. Bronchoalveolar fluid was centrifuged at 1800 × g for 10 min at 4°C, and the cell pellet was stored at −80°C until it was used for DNA extraction.

DNA Extraction. Genomic DNA from all lung cancers, the nonmalignant sections, the bronchial margins, and the bronchoalveolar fluid was isolated from fresh frozen, paraffin-embedded tissue or from fluid samples using a standard proteinase K digestion method. The bronchial margins obtained for this study were not the “true” surgical margins submitted for permanent section and clinical use. The bronchial margins used in this study were taken from the lobectomy specimens as the next section immediately after the clinical margin was procured. No microdissection of the section was done, so the section included mucosa, extramucosa, the entire bronchial wall, and the surrounding blood vessels and lymphatics.

Methylation-Specific Polymerase Chain Reaction. DNA methylation patterns for all lung cancers, bronchial margins, and nonmalignant lung tissue were determined by methylation-specific polymerase chain reaction (23). A multiplex-nested methylation-specific polymerase chain reaction assay as described previously (24) was used for all samples. The nested approach amplifies bisulfite-modified DNA initially with flanking polymerase chain reaction primers without preferentially amplifying methylated or unmethylated DNA. The resulting fragment is then used as the template for methylation-specific polymerase chain reaction. Primer sequences of p16, MGMT, DAPK, SOCS1, RASSF1A, COX2, and RARβ have all been described previously (17, 25–28). Cell lines used as positive controls have also been described (RKO methylated for p16, MDA 231 methylated for MGMT, RKO methylated for DAPK; Ref. 17). Placental DNA treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA) was used as a positive control for the remaining genes. DNA from normal lymphocytes was used as a negative control for methylated genes. The annealing temperature for all reactions was 60°C. Polymerase chain reaction products were analyzed as described previously (17). All investigators performing the methylation-specific polymerase chain reaction assays were blinded to patient identifiers and outcomes.

Statistical Analysis. Non-cancer-related deaths and any patients lost to follow-up were treated as censored observations. Survival and time to recurrence were calculated from the date of surgery to the time of death or censor and modeled using the Kaplan-Meier plot of estimated probability of survival. The association of risk factors to time-to-event or time-to-censor end points was analyzed using the log-rank test for univariate analysis and the Cox proportional hazards model for multivariate analysis. Results from this model are reported as relative risks with 95% confidence intervals (Stata Statistical Software, College Station, TX). Correlation between variables was estimated using the Fisher’s exact test or Student’s t test when appropriate; all reported P values are two-sided, and all associations were considered significant when P was ≤0.05.

RESULTS

Patient Outcome. Median time to recurrence for the entire cohort was 37 months (range, 5–71 months). There were seven recurrences (two local to the ipsilateral lung and five distant metastases). Distant metastases involved the skeletal bone in three patients and brain and contralateral lung lesions in one patient each. An additional patient suffered a second primary lung cancer in the contralateral lung 13 months after surgical resection. Median survival for all patients in this series was only 46 months, with approximately 7 of 20 (35%) patients succumbing to disease. Only one patient was lost to follow-up, and length of follow-up ranged from 5 months to 6 years. In this cohort treated with surgery alone, the 5-year disease-specific survival was 58 ± 11%. By univariate and multivariate analysis, when patient demographics are considered, only tumor stage was a strong predictor of overall survival. Patients with advanced-stage disease (≥stage 2; n = 11) had a relative risk
of dying that was twice that of patients with stage 1 disease (n = 9). No demographic variable predicted tumor recurrence.

Methylation Analysis of Tumor and Bronchial Margins. Using p16, MGMT, DAPK, SOCS1, RASSF1A, COX2, and RARβ genes, 20 of 20 (100%) malignancies demonstrated methylation in at least one locus (Fig. 1). The methylation frequencies were as follows: p16 and RARβ, 45%; SOCS1 and COX2, 55%; DAPK, 25%; RASSF1A, 60%; and MGMT, 70%. Fifteen percent of the tumors had only one gene methylated, 15% of the tumors had two genes methylated, 5% of the tumors had three genes methylated, 40% of the tumors had four genes methylated, and 25% of the tumors had five or more genes methylated. The methylation status of all genes analyzed was independent of each other. There was significant concordance among genes with regard to epigenetic changes between tumors, margins, and washings (Table 1). Only p16 and RASSF1A failed to show statistically significantly concordance between tumors and bronchial margins or bronchial margins and washings, respectively. There was strong concordance in the methylation status of all seven genes between the bronchial washings and tumors. If the tumor and bronchial margins are considered, 17 of 20 (85%) patients had at least one gene with an identical epigenetic change in both. In fact, identical methylation events in both the tumor and the bronchial margin (T/M) outnumbered methylation in the tumor alone (T+M–) by 4:3 (Fig. 1; Table 2). Methylation was observed at the bronchial margin but not in the primary tumor in only one patient and a single gene (DAPK; Table 2). In addition, methylation-specific polymerase

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**Table 1** Concordance between methylated genes and tumor, bronchial margins, and bronchoalveolar fluid

<table>
<thead>
<tr>
<th>Genes</th>
<th>% Concordance*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL of margins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cox2</td>
<td>43.2</td>
<td>60.0–80.1</td>
</tr>
<tr>
<td>Soc3</td>
<td>47.9</td>
<td>40.0–82.0</td>
</tr>
<tr>
<td>DAPK</td>
<td>58.3</td>
<td>50.9–84.8</td>
</tr>
<tr>
<td>MGMT</td>
<td>70.6</td>
<td>48.3–92.9</td>
</tr>
<tr>
<td>RASSF1</td>
<td>37.5</td>
<td>−19.7–76.9</td>
</tr>
<tr>
<td>RARβ</td>
<td>48.3</td>
<td>13.4–83.1</td>
</tr>
<tr>
<td>p16</td>
<td>61.5</td>
<td>35.6–87.5</td>
</tr>
</tbody>
</table>

| Margins and tumors | | |
| Cox2 | 51.9 | 22.0–81.8 |
| Soc3 | 51.9 | 22.0–81.8 |
| DAPK | 38.5 | 1.0–75.9 |
| MGMT | 68.7 | 45.5–92.0 |
| RASSF1 | 44.4 | 13.2–75.7 |
| RARβ | 35.5 | 4.4–66.6 |
| p16 | 46.8 | 16.1–77.6 |

| Abbreviations: T, tumor; M, margin. |

* Based on Lin’s concordance coefficient for agreement on a continuous measure obtained by two persons or methods (Ref. 32).

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**Table 2** Methylation status of tumor and margins

<table>
<thead>
<tr>
<th>Gene</th>
<th>T+</th>
<th>M+</th>
<th>T−</th>
<th>M−</th>
<th>T+</th>
<th>M−</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPK</td>
<td>5</td>
<td>1</td>
<td>14</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>SOCS1</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>COX</td>
<td>7</td>
<td>0</td>
<td>9</td>
<td>4</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>MGMT</td>
<td>8</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>RASSF1</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>p16</td>
<td>2</td>
<td>0</td>
<td>11</td>
<td>7</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>RARβ</td>
<td>4</td>
<td>0</td>
<td>11</td>
<td>5</td>
<td>11</td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviations: T, tumor; M, margin.
chain reaction was used to analyze paired, nonmalignant pulmonary parenchyma that had two genes methylated by methylation-specific polymerase chain reaction (SOCS1 and MGMT), and these methylated genes corresponded identically to the methylated genes in the primary malignancy itself (Fig. 1).

In this series, lung tumors larger than 3 cm (T2 lesions) were significantly more likely to be methylated for DAPK (P = 0.008). The methylation status did not correlate, however, with any other clinicopathological parameter such as tumor stage, tumor location, or tumor histology.

Median time to recurrence was 36.9 months. Table 3 shows the median interval to recurrence according to each individual gene that is methylated at the bronchial margin. There was no statistically significant correlation between the methylation status of any gene or combination of multiple genes in the tumor or bronchial margin that predicted either regional or distant recurrence. However, recurrences only occurred in patients with methylation-positive bronchial margins. The three patients in the cohort who had methylation-negative bronchial margins had neither local recurrences nor distant metastases (Fig. 2). These data suggest that hypermethylation changes at the resected bronchial margins are not indicative of tumor micrometastases but may rather represent a field defect of preneoplastic epigenetic changes at these margins.

**Table 3** Median survival by methylation status of bronchial margins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Median months to recurrence</th>
<th>Patients with margins methylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>36.9</td>
<td>17</td>
</tr>
<tr>
<td>SOCS1</td>
<td>48.9</td>
<td>9</td>
</tr>
<tr>
<td>MGMT</td>
<td>50.4</td>
<td>8</td>
</tr>
<tr>
<td>COX2</td>
<td>18.2</td>
<td>7</td>
</tr>
<tr>
<td>DAPK</td>
<td>55.6</td>
<td>6</td>
</tr>
<tr>
<td>RASSF1</td>
<td>48.9</td>
<td>5</td>
</tr>
<tr>
<td>RARβ</td>
<td>36.4</td>
<td>4</td>
</tr>
<tr>
<td>p16</td>
<td>46.9</td>
<td>2</td>
</tr>
</tbody>
</table>

**Methylation Analysis of Bronchoalveolar Fluid.** Bronchoalveolar fluid was positive for hypermethylation in 15 of 20 (75%) of the patients. In many cases, such as patient 1, the primary malignancy and the corresponding bronchoalveolar fluid had identical epigenetic events. However, it was often observed (patients 2–6) that methylation in the bronchoalveolar fluid, although identical to those of the tumor, represented only a few of the epigenetic changes observed in the tumor. Frequently, these events were also mirrored by aberrant methylation at the bronchial margins (Fig. 1). In the five cases in which the bronchoalveolar fluid demonstrated no DNA methylation despite at least one positive methylation event in the tumor, the tumors were all peripheral and small (Fig. 1). Only a single patient (patient 14) had methylation present in one gene in the bronchoalveolar fluid that was not in the primary tumor. Positive methylation of this gene in one patient was detected not only in the bronchoalveolar fluid but also at the bronchial margin (Fig. 1), suggesting that the source of the finding in the bronchoalveolar fluid was the normal-appearing bronchus.

**DISCUSSION**

Epigenetic changes in the lung parenchyma and bronchial epithelia are frequent events that occur early in tumorigenesis, most likely as a result of overwhelming damage from tobacco carcinogens in cigarette smoke (20, 29). Far from being limited only to invasive cancer, it is becoming evident that promoter hypermethylation may also represent preneoplastic/preinvasive epigenetic changes even in histologically normal-appearing, smoking-damaged lung epithelium (20). The high degree of concordance between primary tumor and bronchial margins in this study suggests that these epigenetic events represent a field defect of widespread epigenetic change in bronchial epithelia that can occur both in the primary lung neoplasm and at the resected bronchial margins. The strong concordance between the genes methylated at the bronchial margin and in the corresponding tumor also supports the hypothesis proposed by Belsky et al. (20) that although not transforming themselves, these individual early changes permit the future acquisition and accumulation of other genetic and epigenetic changes that do, in time, lead to malignancy. The fact that the bronchial margins lack microscopic evidence of malignancy suggests that these epigenetic changes are early. However, p16 hypermethylation may reflect progression to transformed cells.

Despite sampling of the nonmalignant tissues at the site most distant from the primary tumor in the lobectomy specimen, (distances from the tumor were not routinely collected in this study), one patient did exhibit positive methylation in both SOCS1 and MGMT in the nonmalignant lung. The methylation of the CpG islands of even histologically normal pulmonary parenchyma in the same lobar distribution as the malignancy further supports a large field of early methylation changes perhaps representing a clonal expansion of cells with abnormal hypermethylation.

The lack of correlation between microscopically positive
tumor foci at the bronchial margin (R1) and clinical outcome has been a source of confusion for many years. Recently, there is evidence that there may exist subgroups of R1 patients that differ in their long-term prognosis due to the exact nature of the minimal residual disease on the bronchial stump (14, 15). Passlick et al. (15) suggest that it is the location of the residual disease at the bronchial stump that is of critical importance and that recurrence of the tumor depends on whether or not the microscopic residual tumor is located on the mucosa, on the extramucosa, on the bronchial wall, or in the lymphatics of the bronchial stump (so-called lymphangiosis carcinomatosa).

Because we did not perform microdissections of the bronchial margins, our methylation changes in this study are not anatomically specific and instead represent the global methylation status of the mucosa, bronchial wall, and lymphatics. Unfortunately, this study was not powered to detect any correlations of methylation changes with local recurrences. Our sample size of 20 patients is too small, and the number of events of local recurrence are too few. Based on a power of 80% and assuming the proportions of patients with and without recurrences will be similar to our current data set, we would need a total cohort of 339 patients to answer this question effectively. The large proportion of patients with adenocarcinoma subtypes in our study may also have been a limiting factor because adenocarcinoma is associated with a higher rate of distant metastases, especially metastases to the brain (30).

Ahrendt et al. (19) were the first to establish that bronchoalveolar fluid exhibited hypermethylation changes consistent with those observed in the primary lung tumor. Using a single gene, p16, they found positive methylation by methylation-specific polymerase chain reaction in 12 of 19 (63%) patients with tumors positively methylated for p16. Using multiple genes, we increased the sensitivity of methylation-specific polymerase chain reaction to 75% (15 of 20 patients), and our sensitivity for single gene p16 methylation in the bronchoalveolar fluid of 44% (4 of 9) compared favorably with that of Ahrendt et al. (19). Also, in our study, the concordance of any gene methylated in the bronchoalveolar fluid compared with the primary tumors was high (Table 2). These data suggest that overall the frequencies of methylation of the different genes found in bronchoalveolar fluid and primary tumors were comparable. These concordance data can also be interpreted as the bronchoalveolar fluid not reflecting the presence of tumor cells per se but as the bronchoalveolar fluid detecting a field effect present in the bronchial mucosa in close proximity to the tumor.

The fact that the bronchoalveolar fluid was obtained not only from the lobe but also from the segments containing the tumor mass supports this hypothesis. We did not sample bronchial mucosa further away from the tumor mass to see whether there were less frequent hypermethylation changes than what was observed in the mucosa near the tumor. Interestingly, in this study, when the bronchoalveolar fluid failed to show DNA hypermethylation changes, the tumors were all peripheral and small. This is consistent with the findings of a recent analysis of 107 smokers without cancer by Zochbauer-Muller et al. (31), who showed that bronchial brushings and bronchoalveolar fluid had more DNA methylation changes in the central airways compared with the peripheral airways.

In summary, our data demonstrate that the histologically negative bronchial margins of resected non-small cell lung cancer exhibit frequent aberrant DNA promoter hypermethylation changes for many genes. These methylation events are reflective of the epigenetic changes in the tumor itself but may not represent minimal residual disease. Instead, this hypermethylation may represent a large field defect ofpreneoplastic changes that occurs early in carcinogenesis. Because these hypermethylation changes at the bronchial margin probably occur early, before a critical threshold of events can cause tumor progression, it remains to be determined whether or not they will be predictive of clinical outcomes, such as local tumor recurrence, which is determined largely by tumor stage and progression. The results of this study serve as a reminder that caution and much validation must be used before we can translate biomarkers from the bench to the bedside.

REFERENCES

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