Non-Small Cell Lung Cancer can be Detected and its Subtypes Differentiated by a Blood Test of Methylation in Cell-Free DNA from Plasma

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Abstract

Introduction: Non-small cell lung cancer (NSCLC)-related mortality is a major health hazard in the world. Current detection modalities rely on imaging, which cannot reliably differentiate between the two main subtypes of NSCLC—adenocarcinoma (ADC) and squamous cell carcinoma (SCC). In this report we investigated the potential of DNA methylation analysis for detection of ADC and SCC using cell-free plasma DNA (cfDNA) as a substrate, which could be acquired non-invasively.

Methods: Methylation patterns of cfDNA were determined in three groups (30 samples each) using a previously developed microarray-based methylation assay (MethDet 56). Two different statistical techniques—a fixed cutoff approach with naïve Bayes classification and a continuous variable approach with linear discrimination analysis—were used with 25 rounds of 5-fold cross-validation to identify informative genes and assess the sensitivity and specificity of differentiating between ADC and healthy controls, SCC and healthy controls, and ADC vs. SCC samples.

Results: The continuous variable approach provided better discrimination for all comparisons with an accuracy between 80 and 90%. Four genes (GSTP, HIC1, SOCS1, and THBS) were informative for comparison of ADC vs. healthy controls, seven genes (BRCA1, CCND2, CDKN1A, GSTP, MYF3, RPL15, and TRANCE) for SCC vs. healthy controls, and nine genes (CCND2, CDKN1A, MGMT, MCJ, P73, RASSF1A, SLC19A1, SOCS1, and SYK) for ADC vs. SCC.

Conclusions: This proof-of-principle data showed that differential methylation of promoters in cfDNA can be used to develop a biomarker assay that can detect NSCLC and differentiate between its subtypes.

ABBREVIATIONS

NSCLC: Non-Small Cell Lung Cancer; ADC: Adenocarcinoma; SCC: Squamous Cell Carcinoma; cfDNA: Cell-Free DNA; Methdet-56: Methylation Detection 56.

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality and accounts for over one million deaths every year [1]. Non-small cell lung carcinoma (NSCLC) is the most frequent histological
Central type and is detected in over 80% of lung cancer cases [2]. NSCLC has two major subtypes—squamous cell carcinoma (SCC) and adenocarcinoma (ADC), which comprise approximately 80% of all NSCLC cases [3]. Prognosis for NSCLC is generally poor, with an overall five-year survival ranging from 67% for patients with stage I A disease to 1% for patients with stage IV disease [4]. The prevalence of SCC and ADC and the poor prognosis for patients with advanced stages of these subtypes of NSCLC signify the need for their timely detection, before clinical symptoms indicate the presence of advanced disease.

Screening for asymptomatic lung cancer based on chest X-rays and sputum cytology fail to show mortality reduction [5]. A much more sensitive technique, computed tomography (CT), detected a significantly larger number of early stage cancers [6], but also did not show a reduction in mortality [7] (preliminary results of the National Lung Screening Trial indicated that spiral CT reduced lung cancer-related mortality by 20% and overall mortality by 7% compared to chest X-ray [8]). As a result, current Clinical Practice Guidelines do not recommend screening for lung cancer by chest radiography, sputum cytology, or CT scanning [9]. New technologies are obviously required.

In this communication we report a blood-based detection technique, which utilized differential methylation of cell-free plasma DNA (cfDNA) that can be used to identify NSCLC and to differentiate between ADC and SCC. Our results reinforce previous findings indicating aberrant methylation of several genes in the blood of patients with NSCLC [10-16] and also expand on these results to include detection of the different subtypes of NSCLC, providing a proof-of-principle for development of an accurate and minimally invasive diagnostic procedure.

MATERIALS AND METHODS

This project was approved by the Institutional Review Board of Rush University Medical Center.

Sample collection and DNA isolation

Whole blood was obtained by venipuncture, collected in Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) containing EDTA, and stored at 4°C for a maximum of 2 h. Each tube was centrifuged twice (2600×g) for 10 minutes at 4°C to separate the plasma from cellular elements. The plasma was stored at -80°C. DNA was isolated and quantified as previously described [17,18] using DNAzol BD (Invitrogen, Carlsbad, CA, USA) and proteinase K.

Microarray-mediated methylation assay

This assay was performed as previously described [18]. Briefly, each DNA sample was split into two equal aliquots, and one of them was digested with Hin6I (Fermentas, Glen Burnie, MD, USA) while the other was mock-digested. Both samples were amplified via a multiplexed, nested polymerase chain reaction (PCR). 5-Aminovalyl dUTP (Biotium Inc., Hayward, CA, USA) was added for the second PCR; products of the Hin6I-digested DNA were labeled with Cy3, while products of the mock-digested DNA were labeled with Cy5. Both labeled products were mixed and hybridized to custom printed DNA microarrays (Microarrays Inc., Huntsville, AL, USA). The slides were then washed and scanned using a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA), and the data were analyzed using the GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA).

Data processing for statistical analysis

The microarray was composed of three identical subarrays with 64 (8×8) spots printed on each subarray. DNA probes for gene promoters occupied 56 spots, 5 spots had controls for nonspecific binding, and 3 empty spots were used to assess and subtract the background signal. If the hybridization signal for a spot was less than 2 times the average of the five control spots, the spot was removed from analysis (Filter 1).

The second filter removed any gene promoter that had less than two informative spots (NA) among the three subarrays. Finally, the methylation ratio of Cy5/Cy3 was calculated, and methylation status was determined for each gene (either methylated or unmethylated). For the continuous variable approach, the mean of the ratios was calculated for each gene of every patient or control subject. For each group, genes missing in more than 25% of the samples were removed from analysis (Filter 3).

Statistical analysis

Data were processed using a fixed cutoff approach of the methylation ratio as previously described [19] by dichotomizing results into methylated and unmethylated promoters, applying Fisher’s exact test to rank differentially methylated promoters, and selecting the most informative combination by naive Bayes algorithm with k-fold cross-validation.

Alternatively, a Student’s t-test was performed on the log-transformed methylation ratio for each gene, and those with a p value of <0.05 were selected for further analysis. Unsupervised principal component analysis and hierarchical clustering in addition to supervised stepwise linear discrimination analysis coupled with cross-validation were used to determine informative genes and the sensitivity and specificity of differentiation between groups.

RESULTS AND DISCUSSION

Patients

Caucasian patients were recruited in Italy [20] and the US. No site-related bias of the results was observed. Basic demographics, smoking status, and disease stage for each group were very similar (Table 1). Differences in age (Table 1A) and smoking history (Table 1B) were insignificant (p >0.4 for both, as determined by ANOVA), while gender distribution in the SCC group (Table 1C) reflected its prevalence in males [21]. This difference, however, was unlikely to affect the results because only minimal gender-specific variability in DNA methylation has been reported [22]. Plasma samples were collected before any treatment. The pathology was determined from final pathology reports.

DNA concentration

DNA in plasma was measured as described previously [17] using Quant-iT PicoGreen dsDNA stain (Molecular Probes, Eugene, OR, USA). As previously reported [23,24], the mean
Table 1: Clinicopathological and demographic characteristics of tested groups: age (A), smoking status (B), and stage of the disease for patients with ADC and SCC (C).

<table>
<thead>
<tr>
<th>A. Age(yrs)</th>
<th>Healthy</th>
<th>ADC</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>67.15(49-83)</td>
<td>66.93(50-80)</td>
<td>67.50(68-80)</td>
</tr>
<tr>
<td>Male</td>
<td>67.0(53-79)</td>
<td>67.81(53-78)</td>
<td>66.18(53-82)</td>
</tr>
<tr>
<td>Total</td>
<td>67.08</td>
<td>67.37</td>
<td>66.84</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Smoking (pk*yr)</th>
<th>Healthy</th>
<th>ADC</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>56.86(0-100)</td>
<td>47.05(15-120)</td>
<td>49.25(29-87)</td>
</tr>
<tr>
<td>Male</td>
<td>60.28(0-120)</td>
<td>60.36(10-104)</td>
<td>57.92(20-120)</td>
</tr>
<tr>
<td>Total</td>
<td>58.57</td>
<td>53.71</td>
<td>57.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Stage</th>
<th>Healthy</th>
<th>ADC</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Total</td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>3</td>
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<td>III</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>17</td>
<td>30</td>
</tr>
</tbody>
</table>

**Abbreviations:** ADC – adenocarcinoma; SCC – squamous cell carcinoma; yrs – years; pk*yr – pack-year

**Figure 1** DNA concentrations in the plasma of healthy controls and patients with ADC and SCC.

The concentration of cfDNA was much higher in patients with NSCLC compared to healthy controls (Figure 1), with statistically significant differences observed between healthy controls and patients with ADC and SCC by Wilcoxon rank sum test (p <0.01 for both comparisons) and only marginally significant for comparison between ADC and SCC patients (p = 0.0707). The variability of DNA concentration in ADC and SCC groups was significantly higher than in controls (compare the standard deviations in controls and in patients with NSCLC), and their coefficients of variation (CV) were 1.17, 1.15, and 0.54 for ADC, SCC, and control subjects, respectively. No stage-specific differences in concentration were observed.

**Selection of biomarkers using the fixed cutoff approach**

In this approach a common threshold of Cy5/Cy3 ratio (R = 4) [25] was used for all genes; all genes with a Cy5/Cy3 ratio below the threshold (R <4) were designated as methylated, and all genes with a Cy5/Cy3 ratio above the threshold (R >4) were designated as unmethylated. Fisher’s exact test was used to compare methylation in healthy controls and patients with ADC and SCC as previously described [18] using a modest threshold of p <0.1 to select potentially informative genes. For the comparison of healthy controls and ADC a total of six genes met this threshold, while 15 genes were identified for the comparison.
Table 2: Differentiation of groups by the fixed cutoff approach. A-C: Accuracy of differentiation expressed as the fraction of correctly and incorrectly identified samples in each comparison. D-F: Informative genes for each differentiation. Methylation of informative gene in corresponding groups is expressed as percent of samples where the gene has been scored as methylated.

<table>
<thead>
<tr>
<th></th>
<th>Identified % (range)</th>
<th></th>
<th>Identified % (range)</th>
<th></th>
<th>Identified % (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy</td>
<td>ADC</td>
<td></td>
<td>Healthy</td>
<td>SCC</td>
</tr>
<tr>
<td>Healthy</td>
<td>65.1% (48.0-82.2)</td>
<td>34.9%</td>
<td></td>
<td>Healthy</td>
<td>80% (65.7-94.3)</td>
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<tr>
<td>ADC</td>
<td>35.5% (47.4-81.6)</td>
<td></td>
<td>SCC</td>
<td>21.3%</td>
<td>78.7% (64.0-93.4)</td>
</tr>
</tbody>
</table>

D. E. F.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Genes</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction of Samples with Methylated Genes, %</td>
<td>Fraction of Samples with Methylated Genes, %</td>
<td>Fraction of Samples with Methylated Genes, %</td>
</tr>
<tr>
<td>Healthy</td>
<td>ADC</td>
<td></td>
</tr>
<tr>
<td>CALCA</td>
<td>80.0</td>
<td>46.7</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>83.3</td>
<td>46.7</td>
</tr>
<tr>
<td>S100A2</td>
<td>86.7</td>
<td>46.7</td>
</tr>
<tr>
<td>SOCS1</td>
<td>46.7</td>
<td>10.0</td>
</tr>
<tr>
<td>P73</td>
<td>13.3</td>
<td>66.7</td>
</tr>
<tr>
<td>RPL15</td>
<td>76.7</td>
<td>100.0</td>
</tr>
<tr>
<td>VHL</td>
<td>23.1</td>
<td>73.3</td>
</tr>
</tbody>
</table>

Abbreviations: ADC – adenocarcinoma; SCC – squamous cell carcinoma

Figure 2 Differentiation of groups by principle component analysis (A-C) and supervised hierarchical clustering (D-F). A, F–Analysis of healthy controls and patients with ADC; B, E–Analysis of healthy controls and patients with SCC; C, F–Analysis of patients with ADC and patients with SCC.
of healthy controls and SCC and 23 genes were identified for the comparison of ADC and SCC. Next, naive Bayes algorithm and 25 rounds of 5-fold cross-validation with independent selection of genes were used to identify components of the corresponding biomarkers and to test their performance. The sensitivity and the specificity of the biomarkers were averaged for all rounds of cross-validation (Table 2A-2C).

Promoters selected in over 75% of the cross-validation runs for each comparison are listed in (Table 2D-2F). From the 56 genes tested, the biomarker for detection of ADC included 4 genes (7.1%), the biomarker for SCC included 7 genes (12.5% of 56 tested), and the biomarker for differentiation of ADC and SCC included 13 genes (23.2% of 56 tested). Two of the six (33.3%) potentially informative genes (p < 0.1) were not selected for the ADC detection biomarker. Similarly, 8 out of 15 (53.3%) and 10 out of 23 (65.6%) genes were not selected for the SCC detection biomarker and ADC/SCC diagnosis biomarker, respectively. The frequency of methylation (%) for each gene (Table 2D-2F) was determined by dividing the number of patients that had methylated promoters by the total number of patients within the corresponding group.

Selection of biomarkers using the continuous variable approach

In this approach the ratio of Cy5/Cy3 signals (digested test vs. undigested control) was treated as a continuous variable for each gene, and the means of the log-transformed ratios were compared using the Student’s t-test. Ten genes were selected as potentially informative for comparison between healthy controls and patients with ADC, 21 genes were selected for comparison between healthy controls and patients with SCC, and 27 genes were selected for comparison between patients with ADC and patients with SCC (p < 0.05 for all comparisons).

Principle component analysis using these genes indicated that healthy controls, patients with ADC, and patients with SCC had substantially different methylation patterns (Figure 2A-2C), while supervised clustering analysis showed considerable separation of the different groups, albeit with notable crossover (Figure 2D-2F).

Linear discrimination analysis with cross-validation in a stepwise approach was then used to select the most informative genes for candidate biomarkers and evaluate their sensitivity and specificity (Table 3A-3C). Out of 10 potentially informative genes for detection of ADC, 4 (40%) were selected for the biomarker. Similarly, 7 of 21 (33.3%) and 9 of 27 (33.3%) were selected for detection of SCC and differentiation between ADC and SCC, respectively.

The continuous variable approach allowed for better detection of NSCLC and differentiation of ADC and SCC, with sensitivity ranging from 80.0% (95% CI: 65.7-94.3%) for SCC to 86.7% (95% CI: 74.5-98.9%) for ADC (Table 3A-3C). The specificity of detection varied from 73.3% (95% CI: 57.4-89.1%) for ADC to 86.7% (95% CI: 74.5-98.8%) for SCC. Significantly, differentiation of ADC and SCC achieved 86.7-90% accuracy, suggesting that differential detection of these subtypes of NSCLC by blood analysis is feasible.

The genes listed in (Table 3D-F) contributed to differentiation of healthy controls and patients with ADC and SCC. Significantly, SOCS1 contributed to differentiation regardless of the statistical approach used for biomarker development (compare (Table 2D,2F) with (Table 3D,3F). Similarly, CCND2, RPL15, P73, RASSF1A, and SYK were selected as components of one of the biomarkers by both the fixed cutoff and continuous variable approaches (Table 2 and Table 3).

A post hoc analysis of the comparisons made with the fixed cutoff approach revealed several interesting features of selected promoters, when the majority of them were informative for two biomarkers; however, the informative value of their methylation was very different for ADC and SCC. Overall, average methylation for the ADC pattern was only half that of the control (37% vs. 74%), while in the SCC pattern, methylation was two-fold higher than in the controls (81% vs. 37%) (Table 2D-2F). This observation was validated by comparison of the ADC and SCC patterns (77% average methylation for genes of the SCC pattern vs. 32% average methylation for genes of the ADC pattern), suggesting that informative genes were less methylated in ADC and more methylated in SCC compared to controls. The difference in methylation between ADC and SCC, normalized to controls, was 0.5 for ADC vs. 2.2 for SCC, suggesting significant differences in the biology of these tumors.

A post hoc analysis of the results for the continuous variable approach revealed characteristics of the promoters within the methylation patterns that were only partially similar to those observed with the fixed cutoff approach. The majority of promoters within each pattern were informative for only one biomarker and methylation tendencies were not as pronounced.

NSCLC is the leading cause of lung cancer-related deaths around the world. Its detection currently relies on imaging studies (e.g., spiral CT or chest radiography), which demonstrate increased sensitivity towards larger lesions. Significantly, detection of a solitary pulmonary nodule by imaging does not necessarily indicate the presence of cancer, as only about 30% of benign nodules are correctly identified as malignantly [26]. Blood-based biomarkers may facilitate earlier detection of lung cancer and reduce the cost by obviating the need for biopsy.

Besides detection of NSCLC as a group, it may be advantageous to identify the different histological subtypes of NSCLC. It is well-established that ADC and SCC of the lung are biologically different with distinctive patterns of gene expression [27,28] and unique prognostic markers [29,30]. These differences are reflected in the variable sensitivity of ADC and SCC to chemotherapy (e.g., pemetrexed [31]), suggesting that separate analysis of ADC and SCC specimens can reveal important biological information and is more appropriate than combined analysis of these diseases. Here we report the results of methylation profiling in patients with ADC and SCC using cfDNA. A previously developed microarray was used for these proof-of-principle studies to explore the feasibility of the approach. Accordingly, the results reported here should not be considered as a clinical-grade biomarker but rather as the basis for further studies investigating a much larger set of promoters. Considering that there are only a few communications reporting methylation analysis of plasma DNA for a handful of genes—e.g. CDKN2A [32], RASSF1A [13], SFN
[33], and APC [34]—our proof-of-principle study supports a wide-scale analysis of cfDNA methylation for diagnosis of lung cancer [35,36]. Ours appears to be the first study to report on the feasibility of potential biomarker development for noninvasive diagnosis of lung cancer using assessment of DNA methylation of multiple promoters in each sample.

Clinicopathological and demographic characteristics of the examined groups were very similar (Table 1), except for the larger number of males in the SCC group. Considering that no gender-related differences in methylation of cfDNA have been detected [22], this imbalance is unlikely to have influenced the results.

An increased concentration of cfDNA in patients with NSCLC (Figure 1) has been observed previously [24, 37], but including this measurement in the diagnostic workup does not improve the accuracy of lung cancer detection based on spiral CT [38], probably due to the high variability of the DNA concentration in plasma. Our results (Figure 1) indicated that the mean DNA concentration was the highest in patients with ADC (21.42 ng/ml) and somewhat lower in patients with SCC (14.16 ng/ml) compared to controls (7.80 ng/ml).

Using the fixed cutoff approach [17], differentiation between ADC and healthy controls could be achieved with a modest accuracy of 64-65% (Table 2A) using only four informative genes (CALCA, RASSF1A, S100A2, and SOCS1; (Table 2D). Differentiation between patients with SCC and healthy controls could be achieved with a much better accuracy (78-80%; Table 2B) using seven informative promoters (Table 2E), while 13 promoters (Table 2F) selected as differentially methylated in ADC and SCC samples provided considerable accuracy (80-81%; Table 2C ) for differential identification of these subtypes of NSCLC.

Most of the promoters selected as informative for identification of each group have been reported as abnormally methylated in lung cancer. CALCA, S100A2, and P73 are hypermethylated in lung cancer cells [39-41], and SOCS1 is hypermethylated in tumor tissues [42]. Abnormal methylation of CCND2 [43-45], CDH1 [44,46], and hMLH1 [47] has been described in lung tumors, whereas methylation of CDH1 correlates with better survival [48]. Inactivation of RASSF1A in lung cancer tissues has been reported by many investigators [49-53], where its methylation is an early event [54] frequently correlating with more aggressive forms of cancer [55,56]. Abnormal methylation of RASSF1A can be also detected in sputum [57], plasma [13], and bronchoalveolar lavage [46] of patients with lung cancer. Abnormal methylation of EP300, ICAM1, MCT-1, RPL15, and VHL in lung cancer has not been previously reported.

With the exception of SOCS1, informative promoters were more frequently methylated in healthy controls than in patients with ADC (Table 2D) and less frequently in patients with SCC (Table 2E). The same tendency is seen in methylation of informative promoters used for differentiation of ADC and SCC—all of them are more frequently methylated in SCC than in ADC (Table 2F), suggesting significantly different mechanisms of development for these subtypes of NSCLC.

Important differences in methylation between the analyzed groups were detected using the continuous variable approach with unsupervised principal component analysis and hierarchical clustering (Figure 2). For all comparisons, the identified patterns of methylation separated the examined groups (Figure 2A-2C). Significantly, hierarchical clustering revealed much more
distinctive patterns for the comparisons of ADC vs. SCC and SCC vs. healthy controls than for comparison of ADC vs. healthy controls (Figure 2D-2F).

Four genes (Table 3D) selected as informative by the supervised linear discrimination analysis with cross-validation differentiate ADC from healthy controls with 86% sensitivity and 73% specificity (Table 3A), significantly improving on the fixed cutoff-based results. Similarly, differentiation of SCC vs. healthy controls (Table 3B) and ADC vs. SCC (Table 3C) was improved, although to a lesser degree, by using seven informative promoters for the former (Table 3E) and nine for the latter (Table 3F). Among the informative promoters are those known to be abnormally methylated in lung cancer tissues (HIC1 [58], BRCA1 [59], CDKN1A [60], and MGMT [42], [44], [54], [61], [62]) as well as promoters that have not been previously reported (GSTP, THBS, MYF3, TRANCE, MCJ, and SLC19A1).

Informative promoters selected for differentiation of ADC and SCC by the fixed cutoff approach were essentially a combination of informative promoters for differentiation of healthy controls from ADC and SCC patients, with the exception of CALCA and hMLH1 (Tables 2D-2F) and addition of EDNRB [63], SYK [64], [65], EP300, and MCT-1. Similarly, some informative promoters for differentiation of ADC and SCC were also informative for differentiation of healthy controls from ADC and SCC by the continuous variable approach (Table 3D-3F), although this intersection was less pronounced. Six promoters selected by both approaches (CCND2, SOCS1, P73, RASSF1A, RPL15, and SYK) may represent the nucleus of a putative diagnostic set.

CONCLUSION

In this proof-of-principle study, we showed that patients from three analyzed groups–ADC, SCC, and healthy controls–had distinct patterns of methylation in their cfDNA. This observation provides the basis for development of accurate blood-based markers that will facilitate differential diagnosis of these diseases. While further work with a larger set of promoters is needed to improve biomarker accuracy, our study presents strong evidence that such work will be successful.

ACKNOWLEDGEMENTS

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Conflict of interest

Victor Levenson and AnatolyMelnikov are co-founders of US Biomarkers, Inc and own part of the company, which has several patents submitted in the field of DNA methylation analysis in ultra-small samples. Victor Levenson and AnatolyMelnikov are co-inventors of several patents in the field of DNA methylation.

REFERENCES


