**SHOX2 DNA Methylation Is a Biomarker for the Diagnosis of Lung Cancer in Plasma**

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**Introduction:** Recently, analysis of DNA methylation of the SHOX2 locus was shown to reliably identify lung cancer in bronchial aspirates of patients with disease. As a plasma-based assay would expand the possible applications of the SHOX2 biomarker, this study aimed to develop a modified SHOX2 assay for use in a blood-based test and to analyze the performance of this optimized SHOX2 methylation assay in plasma.

**Methods:** Quantitative real-time polymerase chain reaction was used to analyze DNA methylation of SHOX2 in plasma samples from 411 individuals. A training study (20 stage IV patients with lung cancer and 20 controls) was performed to show the feasibility of detecting the SHOX2 biomarker in blood and to determine a methylation cutoff for patient classification. The resulting cutoff was verified in a testing study composed of 371 plasma samples from patients with lung cancer and controls.

**Results:** DNA methylation of SHOX2 could be used as a biomarker to distinguish between malignant lung disease and controls at a sensitivity of 60% (95% confidence interval: 53–67%) and a specificity of 90% (95% confidence interval: 84–94%). Cancer in patients with stages II (72%), III (55%), and IV (83%) was detected at a higher sensitivity compared with stage I patients. Small cell lung cancer (80%) and squamous cell carcinoma (63%) were detected at the highest sensitivity when compared with adenocarcinomas.

**Conclusions:** SHOX2 DNA methylation is a biomarker for detecting the presence of malignant lung disease in blood plasma from patients with lung cancer.

**Key Words:** Lung cancer, SHOX2, DNA methylation, Plasma.

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DNA methylation plays a key role in fundamental biological processes, and aberrant DNA methylation of certain loci has been reported to play a major role in carcinogenesis. Such epigenetic modifications are highly informative and robust and are therefore a promising source for cancer biomarkers for improving diagnosis and treatment of cancer. Recently, real-time polymerase chain reaction (PCR) analysis of SHOX2 DNA methylation was described as a valuable biomarker for detecting lung cancer in bronchial aspirates obtained during bronchoscopy. In a case-control study with more than 500 patients, lung cancer could be detected with sensitivity of 68% and at high specificity of 95%. In addition, SHOX2 DNA methylation allowed for accurate detection of lung cancer even in patients having no visible tumor in bronchoscopy and a negative cytology. A test for SHOX2 methylation has recently become commercially available in Europe as an in vitro diagnostic test to aid pathologists in the diagnosis of lung cancer. The test that analyzes methylated SHOX2 DNA, derived from tumor cells present in bronchial aspirates, is highly specific and sensitive. However, the test is limited to patients undergoing bronchoscopy and hence is not suitable for screening asymptomatic patients or for combining molecular biomarkers with noninvasive diagnostic procedures (e.g., CT scanning). For such approaches, tests based on standard specimens, such as serum or plasma, are required. During cancer development and progression, tumor cells release DNA into the bloodstream. Such circulating DNA can be analyzed in serum or plasma for DNA biomarkers, including DNA methylation biomarkers.

In the present study, the real-time PCR-based assay for analysis of SHOX2 methylation was tested in blood plasma from 411 individuals to distinguish between malignant lung disease and controls (healthy individuals, benign lung disease, and patients with prostate cancer). A training study (20 patients with stage IV lung cancer and 20 controls) was first performed to show the feasibility of detecting the SHOX2 biomarker in blood and to determine a methylation cutoff for patient classification. The resulting cutoff was then verified in a testing study composed of 371 plasma samples.

**MATERIALS AND METHODS**

**Patients**

Plasma samples were collected at three independent sites with appropriate written consent under approval of the local ethics committees. Sixty-one specimens (testing study) were provided by the Charité University Hospital (Berlin, Germany), 289 (testing study) and 40 specimens (training study) were obtained from Oncomatrix, Inc., (San Marcos, CA), and 21 samples (testing study) were from Rainier Clinical Research Center, Inc. (Renton, WA). Samples from 343 patients (testing study) passed the sample quality control acceptance criterion as described in the section “Data and Statistical Analysis” and were suited for analyzing the SHOX2 DNA methylation. The characteristic of this population (testing study only) is described in more detail in Table 1. Gender and age data were not available for many patients (Table 1). Therefore, the analyzed cases and controls were neither sex- nor age-matched. However, a previous study based on the analysis of bronchial aspirates showed that age and sex had no impact on the SHOX2 DNA methylation level.

**Sample and Calibrator Preparation**

DNA extraction from plasma samples, bisulfite conversion, and purification were performed using the Epi proColon Plasma DNA Preparation Kit (Epigenomics AG, Berlin, Germany). A calibrator sample with a defined methylation level was prepared by mixing bisulfite-converted DNA from sperm cells with bisulfite-converted methylated DNA (Millipore, Billerica, MA). DNA extraction from sperm cells and the bisulfite conversion of sperm DNA and methylated DNA were carried out as described previously. The DNA concentration of sperm and universal methylated DNA was determined by ultraviolet spectrophotometry using a NanoDrop ND-1000 spectral photometer (NanoDrop Technologies, Wilmington, DE).

**Real-Time PCR**

Real-time PCR assays were duplexed in one reaction: an assay for quantification of total input DNA and a methylation.

### TABLE 1. Characteristics of the Patient Population (Testing Set Only)

<table>
<thead>
<tr>
<th>Stage (UICC), n (%)</th>
<th>Total</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>37 (20)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>29 (15)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>53 (28)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>42 (22)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>27 (14)</td>
<td></td>
</tr>
</tbody>
</table>

Clinical data of the 343 analyzed patient samples (188 [55%] cases and 155 [45%] controls) Twenty-eight samples failed the sample quality control because their DNA yield was too low. They were excluded from analysis and are not included in this table.

* The prostate cancer patients were classified as controls in the determination of specificity of the SHOX2 biomarker for lung cancer.

SCLC, small cell lung cancer; UICC, Union for International Cancer Control.
lation-specific HeavyMethyl (HM) assay for quantification of methylated SHOX2. The assay for total input DNA quantification was composed of two methylation-unspecific oligonucleotides and a Taqman probe for amplifying a region within the ACTB gene locus. The quantitative methylation-specific SHOX2 assay (HM assay) is composed of two methylation-unspecific primers, two methylation-specific blockers (one blocking each primer), and a Taqman probe, which specifically amplified methylated DNA within the SHOX2 gene locus. PCR was performed in a total volume of 40 μl containing 20 μl Epi pro Lung BL Master Mix (Epigenomics AG), 15 μl DNA eluate from plasma extraction, and 5 μl water. Real-time PCR was performed using a 7500 Fast real-time PCR instrument (Applied Biosystems, CA) with the following temperature profile: 20 min/95°C, 55 cycles with 15 sec/95°C, and 30 sec/56°C.

Data and Statistical Analysis

For each sample, a relative methylation value was determined using the ΔΔCT method.20 In contrast to Schmidt et al.,13 the calculation was modified as follows: ΔΔCT Sample = ΔCT Sample - ΔCT Calibrator, where ΔCT Sample = CT Sample/Methylation Quantification Assay - CT Sample/Total Quantification Assay and ΔCT Calibrator = CT Calibrator/Methylation Quantification Assay - CT Calibrator/Total Quantification Assay. ΔΔCTs were measured in triplicate. Percentage methylation was calculated using the following formula: Methylation Sample = Methylation Calibrator × 2 -ΔΔCT.

Samples were excluded from the study when more than two replicates of the total DNA quantification assay showed cycle threshold values more than 35.

A methylation cutoff was assigned based on the results of the training study for dichotomization of the methylation value. The performance of the assay was reported by means of sensitivity and specificity. Sensitivity was defined as the ratio of correctly assigned positive lung cancer samples in all lung cancer samples. Specificity was defined as the ratio of correctly assigned negative samples in all normal/benign lung and other cancer (prostate cancer) samples. The prostate cancer samples were classified as controls in the determination of specificity of the SHOX2 biomarker for lung cancer.

The impact of tumor stage and histology on SHOX2 DNA methylation was tested by logistic regression for grouped data. The area under the curve of the receiver operating characteristic (ROC) was computed using the trapezoidal rule. ROC analyses and logistic regression were carried out using IBM SPSS Statistics (IBM, Armonk, NY).

RESULTS

The real-time PCR duplex assay originally developed for sensitive quantification of methylated SHOX2 DNA in a background of high amounts of unmethylated DNA in bronchial aspirates13 was modified to meet the conditions for plasma analysis. The modified assay was based on replacement of the Scorpion probe with Taqman technology and optimized buffer conditions. The analytical performance of the assay is shown in Figure 1. Different amounts (0–720 pg) of bisulfite-converted, artificially methylated DNA were spiked into a background of 50,000 pg unmethylated DNA from sperm to characterize the performance of the assay. The assay allowed for the reliable detection (12 of 12 replicates) of 15 pg (~5 haploid human genome equivalents) of methylated DNA in a background of 50,000 pg unmethylated DNA, respectively (1:3400). Lower amounts of methylated DNA, down to one haploid genome equivalent (~3 pg), were sporadically detected, which is to be expected when analyzing single copies of DNA.

For clinical performance evaluation of the assay in plasma, DNA methylation was quantified by real-time PCR in two case-control studies comprising a total of 411 plasma samples (222 cases and 189 controls). A small case-control study including plasma samples from 20 patients with stage IV lung cancer (cases) and from 20 healthy individuals and patients with benign lung disease (controls) was conducted to show that SHOX2 DNA methylation is a useful lung cancer
biomarker in blood plasma. The results of this training study are shown in Figure 2. Higher SHOX2 DNA methylation can be found in plasma samples from cases in comparison with the controls (Figure 2). As described for bronchial aspirates samples,13 background DNA methylation of the SHOX2 gene was found in most samples, thus necessitating the implementation of a clinical cutoff to dichotomize the quantitative methylation value into a qualitative result (test negative or test positive). Based on the results, the cutoff was chosen to reduce the false-positive rate to 5% for controls, resulting in a cutoff of 0.05%. According to this clinical cutoff, samples with methylation values more than 0.05% were rated as test positive, whereas samples with methylation values less than 0.05% were rated as test negative. Using this cutoff, 15 of 20 patients with lung cancer were classified as SHOX2 methylation positive resulting in a sensitivity of 75%. Nineteen of 20 control samples were classified as SHOX2 methylation negative leading to a specificity of 95% (Figure 2).

The cutoff (0.05%) was further verified and the clinical performance evaluated in a testing study composed of 371 plasma samples from patients with suspected lung cancer. In total, 371 plasma samples (169 controls and 202 cases) were analyzed. Valid results were obtained from 343 (92%) samples (155 controls and 188 cases). One hundred twelve cases were SHOX2 positive, resulting in an overall sensitivity of 60%. Sixteen controls were SHOX2 methylation positive, resulting in a specificity of 90%.

Stage and histology-specific performance of the SHOX2 DNA methylation biomarker using plasma samples from patients with suspected lung cancer. In total, 371 plasma samples (169 controls and 202 cases) were analyzed. Valid results were obtained from 343 (92%) samples (155 controls and 188 cases). One hundred twelve cases were SHOX2 positive, resulting in an overall sensitivity of 60%. Sixteen controls were SHOX2 methylation positive, resulting in a specificity of 90%.

SCLC, small cell lung cancer.
detected with higher sensitivity when compared with stage I patients (27%). Overall, small cell lung cancer (SCLC; 80%) and squamous cell carcinoma (63%) were detected at the highest sensitivity when compared with adenocarcinomas (39%). The association between clinicopathological features (stage and histology) and SHOX2 DNA methylation status (positive and negative) was analyzed in more detail using univariate logistic regression. Histology ($p = 0.032$) and stage ($p < 0.0001$) were determined to have significant correlation with SHOX2 DNA methylation.

Both the stage-dependent and histology-dependent distribution of sensitivity in bronchial aspirates are strongly concordant with the findings of Schmidt et al.\textsuperscript{13}

**DISCUSSION**

DNA methylation has been shown to play an important role in carcinogenesis at an early stage.\textsuperscript{11} That makes DNA methylation alterations among the most promising candidates in biomarker research. Several previous studies specifically targeted DNA methylation biomarkers for their potential to improve clinical lung cancer management.\textsuperscript{19,21–25} Recently, DNA methylation of the SHOX2 gene was shown to be a useful tumor marker for aiding in the diagnostic workup for suspected lung cancer.\textsuperscript{13} Bronchial aspirates are used as the sample material for this assay, and the test is therefore restricted to patients with suspected lung cancer undergoing bronchoscopy. However, bronchoscopy is not considered for screening purposes on asymptomatic individuals. Successful detection of the DNA methylation biomarker SHOX2 in blood might enable its use for screening purposes.

In this study, DNA methylation of SHOX2 was found to be a sensitive (60%; 95% CI: 53–67%) and specific (90%; 95% CI: 84–94%) biomarker for identifying patients with lung cancer based on the analysis of blood plasma. Sensitivity and specificity are slightly lower when using plasma when compared with bronchial aspirates.\textsuperscript{13} Compared with bronchial aspirates obtained directly at the region of interest, plasma samples are more challenging, as the total amount of lung-derived DNA and the fraction of tumor DNA are expected to be significantly lower. In addition, blood plasma contains a complex mixture of DNA originating potentially from any part of the body, i.e., the analytical performance requirements for analyzing these body fluids are higher and the markers need to be specific for lung tumor DNA to ensure a high specificity of the test. Recently, the methylation biomarker SEPT9 was described\textsuperscript{15} as a highly sensitive and specific DNA methylation biomarker for colorectal cancer based on the analysis of plasma. For the present study, the same protocols and components for DNA extraction, DNA bisulfite conversion, and purification were used. In addition, the SHOX2 assay for real-time PCR was optimized for detection of plasma-derived DNA in comparison with the previous study on bronchial aspirates.\textsuperscript{13} The new assay uses the same oligonucleotides for priming and blocking but changes the detection technology from Scorpion to TaqMan probes in an optimized buffer system to achieve maximum sensitivity and specificity. However, the method used for the preparation of bisulfite DNA from plasma was originally developed for usage in conjunction with the biomarker SEPT9. The presence of methylated SEPT9 is associated with the detection of invasive colorectal adenocarcinoma.\textsuperscript{15} In contrast, this study showed that SHOX2 DNA methylation is not a qualitative but a quantitative biomarker, which might require an adapted protocol for DNA preparation. Therefore, an optimized method, i.e., gentle bisulfite chemistry\textsuperscript{26} and optimized purification protocols to avoid PCR inhibition, might improve sensitivity and specificity of the SHOX2 biomarker. SHOX2 is located on chromosome 3q, a region that has been recognized as one of the most prevalent and significant for chromosomal rearrangements, i.e., genomic gain, in lung cancer.\textsuperscript{27–29} In a recent publication, amplification of the SHOX2 gene locus was frequently observed in lung cancer samples, and amplification correlated with hypermethylation of the SHOX2 gene.\textsuperscript{26} The high performance of SHOX2 DNA methylation as a biomarker, even in challenging samples such as plasma, might be due to combined effects of locus amplification and DNA methylation of SHOX2 in tumor cells. As a result, an increase in methylated SHOX2 DNA copies in tumor cells compared with normal cells also increases the SHOX2 DNA methylation level in plasma, thereby facilitating sensitive detection.

The methylation level of samples from patients with stage I disease was found to be significantly lower than from patients with more advanced malignant disease. This was also observed when bronchial aspirates were used as the sample type.\textsuperscript{13} The most likely explanation is the smaller size of the tumor resulting in a decreased amount of tumor-specific DNA being released into the bloodstream. Because of the lower
sensitivity of the SHOX2 biomarker for stage I tumors, additional biomarkers may be required for early detection of the disease. However, SHOX2 DNA methylation alone allows for the detection of stage II tumors with high sensitivity (72%) and it would represent an improvement of the current standard of care. For bronchial aspirate samples, the investigation of the impact of histological lung cancer subtypes on the methylation levels of bronchial aspersions showed that patients with SCLC and squamous cell carcinoma have higher levels of methylation than patients with adenocarcinoma. This differential detection was confirmed by the present study, suggesting that a second biomarker specific for adenocarcinoma might further increase the sensitivity of a blood-based test.

The assay used in this study for determining the DNA methylation of SHOX2 in blood is a quantitative assay providing information on the relative abundance of the tumor marker in the blood. This information might be further exploited, for instance, for monitoring SHOX2 methylation-positive patients after surgery to detect a recurrence of the disease.

CT as a screening tool for early lung cancer detection is currently being evaluated in large ongoing trials. Recent results from the NLST provided clear evidence that lung cancer screening decreases mortality in the screened population, and therefore, CT is likely to play an important role in the future management of lung cancer. CT has already been shown to allow for the detection of early lesions, in particular, peripheral adenocarcinomas. A biomarker in combination with CT might further improve lung cancer screening results and should be evaluated in more detail. CT scans perform well for detecting small peripheral lesions, especially adenocarcinoma. However, CT scans fail to detect preinvasive lesions and early lung cancer in the central airways, specifically SCLC and early stages of squamous cell carcinoma, which comprise 17 to 29% of all lung cancers.30 McWilliams et al.31 found 28 lung cancer cases in a screened group of 561 high-risk patients (5%). Seven (25%) of the detected malignant cases were found by bronchoscopy but not by image evaluation. The addition of biomarkers in conjunction with a CT might help to identify these patients using diagnostic tools less invasive than bronchoscopy.

Recent advancements in CT technology allowed for the detection of smaller lung nodules leading to a high sensitivity of this method. In the NELSON trial for instance, round 1 screening showed 94.6% (95% CI: 86.5–98%) sensitivity resulting in negative predictive value of 99.9% (95% CI: 99.9–100%). Therefore, the performance requirements for a biomarker, which could further contribute clinically valuable information to a group of people with negative CT scans, are considerably high. On the other hand, the group with clearly positive CT results (i.e., nodules >500 mm\(^3\)) or doubling time <400 days, which represented 2.6% and 1.8% of the participants in the first and second round of the NELSON trial, respectively, is relatively small. However, for this group, further reliable diagnostic procedures, i.e., bronchoscopy, are highly recommended due to the high prevalence of lung cancer in this group. The DNA methyl-