Assessment of SHOX2 methylation in EBUS-TBNA specimen improves accuracy in lung cancer staging

K. Darwiche1, P. Zarogoulidis2*, K. Baehner1, S. Welter3, R. Tetzner4, J. Wohlschlaeger5, D. Theegarten5, T. Nakajima6 & L. Freitag1

1Department of Interventional Pneumology, Ruhrlandklinik, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; 2Pulmonary Department—Oncology Unit, ’G. Papanikolaou’ General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece; 3Department of Thoracic Surgery, Ruhrlandklinik, University Hospital Essen, University of Duisburg-Essen, Essen; 4Molecular Oncology, Epigenomics AG, Berlin; 5Institute of Pathology and Neuropathology, University Hospital of Essen, University of Duisburg-Essen, Essen, Germany; 6Department of General Thoracic Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan

Received 22 May 2013; revised 12 July 2013; accepted 16 July 2013

Background: Endobronchial ultrasound with transbronchial needle aspiration (EBUS-TBNA) is a well-established method to assess mediastinal lymph nodes for lung cancer. However, a proportion of patients require further investigation, due to the low negative predictive value (NPV). The objective of this study was to determine whether the assessment of short stature homeobox 2 (SHOX2) DNA methylation level in lymph node tissue obtained by EBUS-TBNA improves the accuracy of mediastinal staging.

Patients and methods: EBUS-TBNA was carried out for suspicious lymph nodes of 154 patients. Negative or ambiguous histological results were confirmed by surgical means and clinical follow-up over 6 months. EBUS-TBNA was assessed on 80 positive and 85 negative classified lymph nodes and compared with the result of the SHOX2 DNA methylation real-time PCR analysis. Relative methylation measured by delta–delta cycle threshold (ΔΔCt) was used to classify the samples. Clinical performance of the EBUS-TBNA procedure with and without the additional SHOX2 assessment was calculated against the final classification according to the gold standard.

Results: Based on data from 105 patients, an average 80-fold increase in the SHOX2 methylation level was measured for positive compared with negative lymph nodes. SHOX2 results with a ΔΔCt value of <6.5 indicate positive lymph nodes. Applying this molecular analysis to EBUS-TBNA cases, not diagnosed by pathologic assessment, the sensitivity of staging was improved by 17%–99%. The NPV increased from 80% to 99%.

Conclusions: The combination of EBUS-TBNA and SHOX2 methylation level strongly improves the assessment of the nodal status by identifying additional malignant lesions and confirming benign nodes and therefore avoiding invasive follow-up procedures.

Key words: lung cancer, SHOX2, DNA methylation

Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of tumor-related death worldwide [1]. Accurate mediastinal lymph node staging is essential in clinical workup, because it has a prognostic value and determines the treatment. Patients with ipsilateral mediastinal lymph node metastasis are treated with neoadjuvant chemo- or radiochemotherapy before tumor resection to improve the clinical outcome [2-4].

Endobronchial ultrasound with transbronchial needle aspiration (EBUS-TBNA) is a well-established method to assess mediastinal lymph nodes for lung cancer staging [5-7]. EBUS-TBNA has been shown to have a high sensitivity and diagnostic yield for mediastinal lymph node staging in lung cancer. However, due to the low negative predictive value (NPV), a high proportion of patients require further investigations or procedures after a nondiagnostic EBUS-TBNA [8]. Invasive procedures such as cervical mediastinoscopy are used to rule out lymph node metastasis after a nondiagnostic EBUS-TBNA [9].

In lung cancer cells, epigenetic changes are common, occurring frequently as hypermethylation of specific regions of the genome [10]. Hypermethylation usually occurs at tumor suppressor gene loci in or in the proximity of the promoters of genes and may lead to cancer development [11, 12]. DNA methylation of the human homeobox gene SHOX2 (short stature homeobox 2) has been previously assessed in lung cancer patients. In lung cancer tissue, gene amplification correlated with hypermethylation of the SHOX2 gene locus [13]. Recently, in a study with >500 patients suspected to have lung cancer, hypermethylation of SHOX2 in bronchial aspirates
could be found in 68% of patients, and methylated \textit{SHOX2} appears to be a clinically useful tumor marker where histological and cytological findings after bronchoscopy are ambiguous \cite{14}.

This study investigated whether the assessment of \textit{SHOX2} DNA methylation level in lymph node tissue obtained by EBUS-TBNA could be used to improve the NPV of EBUS-TBNA and to provide greater accuracy in endoscopic lung cancer staging.

\textbf{materials and methods}

\textbf{patients}

Between January and May 2012, consecutive patients referred for EBUS-TBNA with enlarged (>10 mm) mediastinal or hilar lymph nodes in computed tomography were enrolled in this prospective study. EBUS-TBNA was carried out from suspicious lymph nodes. Negative or ambiguous endoscopic histological results were confirmed by surgical procedures (mediastinoscopy and open thoracotomy) and clinical follow-up, including computed tomography or positron emission tomography–computed tomography (PET-CT) over 6 months. Lymph nodes from patients who underwent workup for suspected lung cancer and did not show any evidence of malignant lung disease in this time period were considered as negative. The study was approved by the Ethics Committee of the University of Duisburg-Essen (approval no. 11-4921-BO) and registered at ClinicalTrials.gov (NCT01653002).

\textbf{EBUS-TBNA}

After conventional flexible bronchoscopy, EBUS-TBNA examination was carried out as described previously with an EBUS-TBNA bronchoscope (model BF-UC 180F, Olympus, Japan), which was connected to an ultrasound scanner (EU-ME1, Olympus) \cite{7,15–17}. All procedures were carried out under general anesthesia. All mediastinal and hilar lymph node stations were assessed systematically and punctured three times with a 22-gauge needle (NA-2015X-4022, Olympus) for pathologic examination if their diameter exceeded 5 mm. Further three EBUS-TBNAs of the same lymph node were carried out, and the obtained tissue was stored in 1–2 ml of isotonic saline solution and kept at −15 to −25°C for up to 3 months.

\textbf{SHOX2 DNA methylation}

For the determination of \textit{SHOX2} DNA methylation levels in EBUS-TBNA samples, the CE marked in vitro diagnostic test Epi pro\textit{Lung BL Reflex Assay} (Epigenomics AG, Berlin, Germany) was used as previously described \cite{14}.

Briefly, the cellular fraction of TBNA aspirates is used for cell lysis by proteinase K treatment followed by bisulfite conversion of cellular DNA. After final purification, the bisulfite-converted DNA is subject to PCR amplification. This duplex PCR amplifies methylated \textit{SHOX2} and actin \textit{β} (\textit{ACTB}) DNA. The latter serves as a reference for the quantification of total input DNA and is assessed using a Taqman probe specific for the \textit{ACTB} gene locus. A calibrator sample with a defined methylation level was used. PCR amplification was carried out on an ABI 7500 Fast Dx Real-Time PCR instrument (Applied Biosystems, CA, USA), and SDS Software (Applied Biosystems) was used for analysis.

The relative amount of methylated \textit{SHOX2} was calculated for each sample according to the delta−delta cycle threshold (ΔΔCt) method \cite{14}. Cts were measured in triplicate. Samples were excluded from the study when more than two replicates of the total DNA quantification assay (ACTB) showed cycle threshold values > 31.5. The cutoff to distinguish between malignant and benign lymph node tissue derived from the published data, which reports \textit{SHOX2} methylation of <1% in normal tissue adjacent to lung cancer tissue \cite{13}. This leads to a ΔΔCt of 6.5 (2−ΔΔCt = 0.01). Clinical performance of initial classification by EBUS-TBNA and methylated \textit{SHOX2} were compared with the final classification confirmed by surgical means and clinical follow-up over 6 months.

\textbf{results}

One hundred and fifty-four patients [48 females, 64 years (range 30–85)] presenting with 259 enlarged mediastinal or hilar lymph nodes who met our inclusion criteria were enrolled into the study. The size and location of the lymph nodes are pointed out in supplementary Table S1, available at \textit{Annals of Oncology} online.

In 10 subjects, positive and negative lymph nodes from different ipsilateral lymph node stations have been found. These patients (15 lymph nodes) passed to neoadjuvant therapy and were removed from the analysis group. Eleven subjects (13 lymph nodes) withdrew consent to invasive procedures or follow-up and were excluded from the study. In 66 lymph nodes, the amount of DNA was not sufficient to allow \textit{SHOX2} assessment (28 subjects). The data presented refer to the remaining 165 lymph nodes from 116 patients (supplementary Figure S1, available at \textit{Annals of Oncology} online).

The mean ΔΔCt measured in these lymph nodes was 3.0 for positive and 9.4 for negative lymph nodes (Figure 1). These results indicate an ~80-fold increased \textit{SHOX2} methylation level for positive lymph nodes (0.5% versus 39%). Analysis of patient subgroups showed no significant differences for different types of tumor related to the cases (supplementary Figure S2, available at \textit{Annals of Oncology} online).

Initial pathologic analysis confirmed tumor cells \((n = 27,\text{ adenocarcinoma}; n = 15,\text{ squamous cell carcinoma}; n = 20,\text{ small-cell lung cancer}; n = 17,\text{ other malignant neoplasm};\) supplementary Table S2, available at \textit{Annals of Oncology} online) in 79 of 165 lymph node specimen. Seventy-four of the 79 were correctly classified by \textit{SHOX2}, with a result of ΔΔCt of <6.5. In five malignant lymph nodes, ΔΔCt was found to be above the cutoff. In two of them, an atypical carcinoid has been diagnosed. Six negative lymph nodes finally staged as sarcoidosis were correctly classified as negative by \textit{SHOX2}.

In 80 lymph nodes, EBUS-TBNA was not diagnostic and further surgical biopsy \((n = 21,\text{ thoracotomy}; n = 18,\text{ mediastinoscopy}; n = 8,\text{ transthoracic biopsy}; n = 5,\text{ thoracoscopy}) or clinical follow-up \((n = 28,\text{ CT scan and/or PET–CT}) was required. Seventeen lymph nodes have been finally found to contain epithelial tumor cells \((n = 6,\text{ squamous cell carcinoma}; n = 4,\text{ adenocarcinoma}; n = 2,\text{ small-cell lung cancer}; n = 5,\text{ other malignant neoplasm})\). Sixteen of those 17 cases were correctly classified by \textit{SHOX2} with a ΔΔCt of <6.5. The remaining 63 lymph nodes without malignancy were predominantly reactive enlarged nodes (supplementary Table S3, available at \textit{Annals of Oncology} online).

For 62 of these 63 cases, \textit{SHOX2} resulted in a ΔΔCt higher than 6.5 confirming the benign status. The only false positive case showed a ΔΔCt of 2.6 and chronic lymphocytic leukemia was diagnosed.

In summary, \textit{SHOX2} classified 78 of the 80 uncertain cases correctly with a sensitivity of 94% and specificity of 98%. The performance was maintained even when including the EBUS-TBNA clarified cases. On the entire cohort, \textit{SHOX2} classified 90
Utilizing online). Utilizing 30 results with the pathologic results, the results for all lymph nodes analyzed within the study classiﬁed the most accurately. Light-grey box: conﬁrmed positive nodes. Sixty-eight of 69 negative lymph nodes showed a ΔΔCt above the cutoff (99%). Ninety of 96 positive lymph nodes showed a ΔΔCt below the cutoff (94%). (B) Receiver operator characteristic (ROC) for SHOX2 results on lymph nodes obtained by EBUS-TBNA. Data obtained for all 165 lymph nodes. (C) Data obtained for the subgroup of 80 lymph nodes with negative or ambiguous results after pathologic assessment. The area under the curve (AUC) was calculated to 0.97 for both groups.

EBUS-TBNA: endobronchial ultrasound transbronchial needle aspiration.

Table 1. Statistical results of the lymph node assessment by pathologic evaluation alone and additional application of the SHOX2 assay

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathology only</td>
<td>82</td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>SHOX2 only</td>
<td>94</td>
<td>99</td>
<td>92</td>
<td>99</td>
<td>96</td>
</tr>
<tr>
<td>Combined</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
</tbody>
</table>

NPV, negative predictive value; PPV, positive predictive value.

of 96 malignant nodes (94%) and 68 of the 69 negative lymph nodes (99%) correctly. The clinical performance of SHOX2 can be displayed as an receiver operating characteristic (ROC, Figure 1). For both the entire cohort and subgroup of nodes not clearly by pathologic evaluation, the area under curve is 0.97.

Comparing the SHOX2 results with the pathologic results, the biomarker reached a higher sensitivity. Whereas pathologic assessment reached a sensitivity of 82%, the SHOX2 biomarker alone detected 94% of the positive lymph nodes, while the speciﬁcity was 100% for pathologic assessment and 99% for SHOX2 (supplementary Tables S4 and S5, available at Annals of Oncology online). Utilizing SHOX2, the NPV is increased from 80% to 92%, compared with pathologic evaluation alone.

The highest demand for conﬁrmative information is required for the group of initially nondiagnostic nodes (80 of 165, see Figure 1). For the presented cohort, this proposed procedure results in overall accuracy of 99% (Table 1; supplementary Table S6, available at Annals of Oncology online).

**discussion**

EBUS-TBNA has proven to be an excellent method for diagnosing and staging of non-small-cell lung cancer with a sensitivity of 84%–94% [7, 18, 19]. Results with considerable lower sensitivity had been reported by other authors leading to question the reliability of lung cancer staging when EBUS-TBNA is carried out by less-experienced bronchoscopists or specimens are assessed by less-experienced pathologists [20, 21]. The use of biomarkers can contribute to the diagnostic performance since the biomarker assessment does not depend on individual experience of investigators.

The prevalence of lymphatic micrometastases not detected by conventional pathologic evaluation can lead to early recurrent disease and is associated with worse outcome [22–25]. Therefore, micrometastases are most likely the reason for unsatisfactory survival of stage I lung cancer patients. Morphologic methods have been used; however, molecular detection of genetic or epigenetic modiﬁcations such as CK19, KRAS and p53 has been shown to enhance the sensitivity of micrometastases detection in mediastinal lymph nodes [22, 24–29]. However, no marker reaches sensitivity and speciﬁcity high enough to be valuable in lung cancer diagnostic workflow.

The detection of micrometastases in lymph nodes before resection would give the opportunity of neoadjuvant therapy and may promote better outcome as indicated in previous studies [20, 30, 31]. Based on our results in this study, the assessment of SHOX2 DNA methylation identiﬁed 16 of 17 malignant lymph nodes not detected by conventional pathology, making this assay an applicable and relatively fast method to detect micrometastases in mediastinal and hilar lymph nodes, and improves sensitivity and the NPV of lung cancer staging with EBUS-TBNA.

Applying SHOX2 DNA measurement to pathologic evaluation will not only improve the diagnosis of lymph nodes affected by malignant diseases, but with its high speciﬁcity also supports benign results and therefore, aids in avoiding unnecessary invasive procedures. Due to possible sampling error, the risk of missing a malignant lesion can never be
excluded by any test. However, a detection error will be minimized and a benign disease such as sarcoidosis can be diagnosed with more certainty. In this study, 52 subjects with no signs of malignancy were referred to surgical biopsy. In 70% of these cases, SHOX2 measurement indicated a benign result and invasive diagnostic procedures might have been avoided. It is important to note that the only one falsely positive classified case showed a ΔΔCt of 2.6 and was diagnosed as chronic lymphocytic leukemia. Diagnosing this disease by pathologic assessment of EBUS-TBNA specimen is eventually challenging [32]. The evaluation of aberrant methylation may reveal malignancy in lesions affected by leukemia not detected by pathology [33].

The SHOX2 methylation levels observed in lymph nodes are comparable with the levels reported for primary tumors [13]. However, the SHOX2 biomarker sensitivity of 94% and specificity of 99% obtained in this study exceeds the results reported by others. Whereas sensitivity of 68%–78% and specificity of 95%–95% have been reported for bronchial aspirates, the detection of lung cancer based on the measurement of SHOX2 in plasma was 60% at specificity of 90% [14, 34]. The direct measurement of tissue potentially containing cancer cells has a higher sensitivity compared with the assessment of fluid specimens. On this basis, the selected ΔΔCt cutoff of 6.5 used in this study should be verified in an additional independent cohort.

The provided preanalytical protocol was adapted to enable the analysis of tissue material. The cutoff for the reference gene (ACTB) was also established at ΔΔCt of 31.5, which lead to a relatively high number of invalid results due to insufficient DNA amount but resulted in a better test performance. We expect to improve the validity rate for the SHOX2 analysis with implementation of the assay and increased laboratory experience.

**Conclusion**

The presented results show for the first time that the assessment of SHOX2 methylation level in lymph node tissue obtained by EBUS-TBNA can improve the accuracy of lung cancer staging compared with conventional pathology alone, and results are received within 24 h. Utilizing the SHOX2 methylation level, sensitivity and NPV is increased, and unnecessary invasive procedures might be avoided.

**Acknowledgements**

The authors wish to thank Heiko Hang and Rüdiger Karpf-Wessel (both Ruhrlandklinik Essen, Germany) for their assistance in data collection.

**Disclosure**

RT owns shares of Epigenomics AG, whose products are discussed in this article, a conflict of interest form has been submitted for the editor in chief. KD, PZ, KB, SW, JW, DT, TN and LF reported that there are no potential conflicts of interest with any companies/organizations, whose products or services may be discussed in this article.

**References**

**Time to first cigarette and lung cancer risk in Japan**

H. Ito¹, S. Gallus², S. Hosono¹, I. Oze¹, K. Fukumoto¹,³, Y. Yatabe⁴, T. Hida⁵, T. Mitsudomi⁶, E. Negri², K. Yokoi³, K. Tajima⁷, C. La Vecchia²,⁸, H. Tanaka¹,⁹ & K. Matsuo⁹,¹⁰*

¹Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan; ²Department of Epidemiology, Istituto di Ricerche Farmacologiche ‘Mario Negri’, Milan, Italy; ³Department of Thoracic Surgery, Nagoya University Graduate School of Medicine, Nagoya; ⁴Department of Pathology and Molecular Diagnostics; ⁵Department of Thoracic Oncology, Aichi Cancer Center Central Hospital, Nagoya; ⁶Department of Surgery, Division of Thoracic Surgery, Kinki University Faculty of Medicine, Osaka-Sayama; ⁷Aichi Cancer Center Research Institute, Nagoya, Japan; ⁸Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy; ⁹Department of Epidemiology, Nagoya University Graduate School of Medicine, Nagoya; ¹⁰Department of Preventive Medicine, Kyushu University Faculty of Medical Science, Fukuoka, Japan

Received 12 March 2013; revised 22 July 2013; accepted 22 July 2013

**Background:** Cigarette smoking is the major cause of lung cancer (LC). Although the time to first cigarette (TTFC) of the day is a distinct indicator of nicotine dependence, little information is available on its possible relation to LC.

**Patients and methods:** This case–control study includes a total of 1572 incident LC cases and 1572 non-cancer controls visiting for the first time the Aichi Cancer Center Hospital between 2001 and 2005. We estimated the odds ratio (OR) and 95% confidence interval (CI) for TTFC using a logistic regression model after adjustment for several potential confounders.

**Results:** TTFC was inversely associated with the risk of LC. This association was consistent across histological subtypes of LC. For all LCs considered among ever smokers and after accurate allowance for smoking quantity and duration, besides other relevant covariates, compared with TTFC >60 min, the adjusted ORs were 1.08 (95% CI, 0.73–1.61) for TTFC of 31–60 min, 1.40 (0.98–2.01) for 6–30 min and 1.86 (1.28–2.71) for within 5 min ($P_{\text{trans}} < 0.001$). Statistically marginally significant heterogeneity by histological subtype was observed ($P_{\text{heterogeneity}} = 0.002$).

**Conclusions:** Nicotine dependence, as indicated by the TTFC, is associated with increased risk of LC and is therefore an independent marker of exposure to tobacco smoking.

**Key words:** nicotine dependence, smoking, addiction, lung cancer

**introduction**

The association between cigarette smoking and lung cancer (LC) risk was firmly established in the 1950s [1], and the direct associations of risk with younger age at smoking initiation, greater number of cigarettes per day (CPD), longer duration of cigarette smoking and the inverse one with years since quitting smoking, have been well established [2–7].

The time to first cigarette (TTFC) after waking is a specific indicator of nicotine dependence [8–13] and is also associated with other aspects of smoking behavior, including difficulty in smoking cessation, smoking relapse, and tolerance. It is one of