ment strategies. We believe that substantial enhancement of detection is stochastically dependent on the PCR. We also believe that PCR can be enhanced by increasing the ratio of primer to DNA. The limitations with whole-peripheral-blood DNA may be attributable to the absolute amount of DNA that can be incorporated into the PCR and the ability of the primer to find and anneal to the specific sequence. Magnetic cell sorting before PCR enriches the microchimeric DNA within the PCR and provides the additional benefit that positively and negatively selected cell populations can be analyzed separately. We sorted the peripheral blood cells for CD4+ and CD8+ T cells for a previous study (3). Sorting for other cells, e.g., CD3, before PCR may be just as effective; this postulation, however, needs to be tested.

Some of the differences observed between magnetic cell separation and whole peripheral blood may be attributable to the different methods of DNA extraction. The positively selected cells were insufficient in number to be extracted by the salting-out method used for whole peripheral blood, which requires large volumes, and even if scaled down, the yield of DNA would have been insufficient. Furthermore, extracting DNA from the positively selected cells with use of the QIAamp Blood Kit would have overloaded the column with DNA, in which case the DNA would be contaminated with proteins. Because we used two different DNA extraction methods, we analyzed DNA from a total of 500 000 cells. In addition, it is unlikely that microchimeric DNA and/or cells would have been selectively lost in either of the extraction methods.

The frequency of microchimeric cells found in many of the peripheral blood samples examined was comparable to that found in early pregnancy, where magnetic cell sorting is used extensively to isolate microchimeric fetal cells for prenatal diagnosis. The high numbers of microchimeric cells may be attributable to immune activation because some of these women have autoimmune diseases; however, it is striking that microchimeric cells are not detected as easily in the whole peripheral blood from the same women. These results suggest that PCR amplification of whole-peripheral-blood DNA for the detection of microchimeric cells is not suitable possibly because of the high background of autologous cells. Isolation of cells by magnetic cell separation before PCR increases the detection of male microchimeric cells by a factor of >250.

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References

Real-Time Reverse Transcription-PCR Detects KS1/4 mRNA in Mediastinal Lymph Nodes from Patients with Non-Small Cell Lung Cancer, Michael Mitas,1 David J. Cole,1 Loretta Hoover,2 Mostafa M. Fraig,2 Kaidi Mikhitarian,1 Mark I. Block,3 Brenda J. Hoffman3, Robert H. Haines3, William E. Gillanders,1 and Michael B. Wallace3 (Departments of 1Surgery and 2Pathology and Laboratory Medicine, and 3Digestive Disease Center, Medical University of South Carolina, Charleston, SC 29425; address correspondence to this author at: Hollings Cancer Center, Medical University of South Carolina, 68 Jonathan Lucas St., Room 313, PO Box 250956, Charleston, SC 29425; fax 843-792-3940)

Non-small cell lung cancer (NSCLC) is the most common cancer-related cause of death for both men and women in the US. Standard therapies for patients with NSCLC
include surgery, chemotherapy, and radiation therapy, and the stage of disease dictates choice of therapy. The current staging system for lung cancer uses the American Joint Committee on Cancer TNM system, and its goal is to classify patients into groups based on the extent of disease. This system relies heavily on the pathologic evaluation of the primary tumor (T), regional nodes (N), and distant metastases (M). Patients in whom mediastinal lymph node (MLNs) are involved (N2 or N3) are classified with stage III disease. This report.

The recent identification of genes overexpressed in lung cancer (2–4) combined with advances in real-time reverse transcription-PCR (RT-PCR) provide the opportunity to establish sensitive and specific ways to analyze MLNs. In addition, molecular biology approaches using real-time RT-PCR are well suited to the analysis of lymph node tissue procured through minimally invasive procedures such as endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). This technique enables reliable biopsy of MLNs without the need for general anesthesia or surgery (5). Given the advantages of EUS-FNA, we investigated the possibility that metastatic disease could be reliably detected in MLNs of NSCLC patients by real-time RT-PCR.

To define the ability of real-time RT-PCR to detect metastatic NSCLC in MLNs, we procured by EUS-FNA nine MLNs containing metastatic NSCLC (five adenocarcinomas, one large cell carcinoma, one squamous cell carcinoma, and two uncharacterized carcinomas). For negative controls, we collected 30 cervical lymph nodes obtained by surgical resection. Protocols for tissue procurement and patient consent governing all aspects of this study were reviewed and approved by the Medical University of South Carolina Institutional Review Board.

For EUS-FNA, a fine-needle apparatus commercially produced for use with EUS (EUS-N-1; Wilson Cook Co.) was advanced into a target lymph node under high-frequency (7.5 mHz) ultrasound guidance. An occluding stylet within the needle lumen was used to minimize contamination from pass-through structures and was removed once the needle was in position. As suction was applied with use of a syringe, the needle was moved back and forth within the lymph node for ~2 min. This procedure typically retrieved a specimen of pure lymph node cells of ~0.5–2 cm$^3$. Material from the EUS-FNA was placed on multiple slides. One set of slides was air dried, stained with Diff Quik stain (Mercedes Medical), and interpreted in the endoscopy suite for specimen adequacy and for the presence or absence of metastatic NSCLC.

Another set of slides was fixed in 950 mL/L alcohol and stained later with Papanicolaou stain. Criteria for metastatic carcinoma were the presence of one or more cohesive clusters of neoplastic cells with characteristic epithelial morphology and the presence of numerous lymphocytes in the background. Duplicate samples were placed on ice and taken immediately for RNA processing and real-time RT-PCR.

For potential molecular markers of NSCLC metastatic disease, we tested the epithelial carcinoma-associated genes KS1/4 (6), lunx (7), [also known as palate, lung and nasal epithelium carcinoma associated (Plunc) gene (8, 9)], CEA, CK19, and muc1. $\beta_2$-microglobulin was used as an internal reference control gene. The sequences for primers used in this study are listed in Table 1. Primers for KS1/4 and lunx were designed using Primer Express Software (PE Biosystems). RNA isolation and real-time RT-PCR conditions were as described previously (10), with the exception that 0.1 U of UngErase enzyme and 0.25 U of AmpliTaq Gold were used per 10-$\mu$L reaction. The amplification efficiencies (AE) of KS1/4, lunx, and CEA, were 100%, 100%, and 98%, respectively; they were obtained by using the formula: $AE = 10^{1/m} - 1$ (11), where $m$ is the slope of the line determined from linear regression analysis (Microsoft Excel software) of serial 10-fold dilutions of cDNA (data not shown) prepared from the lung cancer cell line A549.

### Table 1. Primer pairs/amplicons analyzed by real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of selected primer pair, 5′-3′</th>
<th>Amplicon length, nt</th>
<th>$T_{m}$ °C</th>
<th>Primer Ref.</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS1/4</td>
<td>CCGAGCTCAGAAAGATGTTG TAGA GTACACTGCGCA TGGACGA</td>
<td>88</td>
<td>Predicted: 77, Measured: 78.6</td>
<td>This report</td>
<td>9/9</td>
<td>10/10</td>
</tr>
<tr>
<td>lunx</td>
<td>CCCTGGAAGCCTGCAAATT GAACCAACTCATGCAGGAGCTTT</td>
<td>110</td>
<td>Predicted: 81, Measured: 81.6</td>
<td>This report</td>
<td>7/9</td>
<td>10/10</td>
</tr>
<tr>
<td>muc1</td>
<td>ACCATCTATGAGGAGATAC GCCACATACATTGCAAGAAAC</td>
<td>107</td>
<td>Predicted: 83, Measured: 83.1</td>
<td>(10)</td>
<td>8/9</td>
<td>9/10</td>
</tr>
<tr>
<td>CEA</td>
<td>TGTAGCTGTGGAAATGTTAGAAAGAAGC GGGCCCATCGCATCATGATTGG</td>
<td>131</td>
<td>Predicted: 82, Measured: 81.0</td>
<td>(10)</td>
<td>6/9</td>
<td>7/10</td>
</tr>
<tr>
<td>CK19</td>
<td>CATGAAAGGCGCTGGAGGAAAGA GATTCGCGCGCTACGATACAG</td>
<td>138</td>
<td>Predicted: 84, Measured: 86.3</td>
<td>(10)</td>
<td>8/9</td>
<td>8/10</td>
</tr>
</tbody>
</table>

*aForward primer is shown as upper sequence in the respective primer pair. Reverse primer is the lower sequence.
*bPredicted $T_{m}$ determined using Primer Express software. Measured $T_{m}$ determined from dissociation profile analysis (12).
*cSensitivity of respective gene for NSCLC detection in Cy5+ samples.
*dSpecificity of gene with respect to negative control EUS-FNA samples.
efficiencies of other genes used in this study were determined previously (10).

For each NSCLC-associated gene, ΔCt values for cervical control lymph nodes and cytology-positive (Cy⁺) MLNs were obtained from triplicate reactions. The ΔCt value is the difference between the threshold cycle (Ct) for a NSCLC cancer-associated gene and that for a β2-microglobulin internal reference control gene. Relative gene expression of the samples was derived from real-time RT-PCR data using the equation: (1 + AE)ΔCt (10), where AE is the amplification efficiency of the gene of interest, and ΔCt is the difference between the mean ΔCt value in cervical control lymph nodes and the ΔCt value in the respective test sample. ΔCt threshold values for marker positivity were set at 3 SD away from the mean of the cervical control data set (Fig. 1). We observed that of the markers examined, KSI/4 had the highest sensitivity for detection of NSCLC metastatic disease (nine of nine samples; 100%). The sensitivities for the other genes are listed in Table 1. There was no apparent difference in expression profiles according to tumor histology (e.g., squamous vs adenocarcinoma; data not shown). Lunx was overexpressed in seven of nine samples (Table 1 and Fig. 1); it was not overexpressed in one adenocarcinoma sample and in one uncharacterized NSCLC.

During the EUS-FNA procedure, a needle is passed through the esophagus into a MLN. Contamination from the esophagus and other pass-through structures is prevented or minimized by an occluding stylet within the needle lumen, which is removed after the needle is positioned within the lymph node. To verify that gene overexpression observed in the Cy⁺ samples was not attributable to an artifact associated with the EUS-FNA procedure itself, we determined gene expression for 10 negative control (subcarinal MLNs) EUS-FNA samples (Fig. 1). Control EUS-FNA samples were obtained from 10 consecutive patients who had no history of cancer and who were undergoing endoscopy for other indications (e.g., evaluation of benign pancreato-biliary disease). We observed that KSI/4 and lunx were not overexpressed in any of the 10 EUS-FNA negative-control samples [i.e., specificity, 10 of 10 (100%); Table 1], providing evidence that overexpression of these genes in the Cy⁺ samples was attributable to metastatic cancer. Regarding the other markers, we observed that CK19, CEA, and muc1 were overexpressed in three, two, and one EUS-FNA control nodes, respectively. These results suggest that overexpression of CK19, CEA, or muc1 can be associated with noncancer events. Hence, these genes may have limited utility as NSCLC molecular markers for samples obtained by EUS-FNA. Although our sample size was small, the results of our analyses of control and Cy⁺ EUS-FNA samples suggest that KSI/4 is the most informative molecular marker of NSCLC metastatic disease.

To explore the possibility that real-time RT-PCR might be capable of detecting occult micrometastases, we analyzed 40 cytology-negative (Cy⁻) MLNs from 27 NSCLC patients who had no evidence of metastatic disease. Overexpression of the high-specificity markers KSI/4 and lunx was detected in 2 of 40 and 0 of 40 Cy⁻ MLNs, respectively. Overexpression of the lower specificity markers muc1, CEA, and CK19 was detected in 2 of 40, 10 of 40, and 18 of 40 Cy⁻ MLNs, respectively. These results suggest that real-time RT-PCR has the potential to detect occult NSCLC micrometastatic disease.

In summary, we tested five molecular markers (lunx, KSI/4, CEA, CK19, and muc1) for use with real-time RT-PCR of EUS-FNA specimens and evaluated their sensitivity and specificity for the detection of NSCLC metastases. Overexpression of KSI/4 as well as at least one other marker gene was detected in all nine Cy⁺ specimens. The mean number of markers overexpressed was 3.8. Overexpression of lunx or KSI/4 was not detected in any of the control lymph nodes and thus had the highest specificity for NSCLC. These results demonstrate that real-time RT-PCR combined with EUS-FNA has potential value for staging patients with NSCLC. Furthermore, this is the first study to establish that KSI/4 is an informative molecular marker of metastatic NSCLC and that its apparent diagnostic accuracy is superior to other candidate markers.

This work was supported by US Department of Defense Grant DOD N63116000MDM0U01-SP0007 (to M.M.) and National Cancer Institute/NIH Grant R21 CA97875-01 (to M.B.W.)

References
10. Ubbink et al. (8) showed that it prevents in vitro increases for 2 h. Several studies have confirmed this effect (9), although others have not (10). In most studies, plasma Hcy from blood collected in fluoride tubes has been lower than in EDTA tubes (10–12).
Our aim was to evaluate the protective effect of fluoride against spurious increases of in vitro Hcy and to establish reference intervals for the AxSYM immunoassay (Abbott) for plasma Hcy from blood collected into fluoride tubes.
The Hcy assay was performed on the AxSYM analyzer according to the manufacturer’s instructions. Folic acid and vitamin B 12, measurements were performed on the Elecsys analyzer (Roche Diagnostics) according to the manufacturer’s instructions.
For the study of a possible fluoride protective effect, blood was drawn from 22 individuals and collected in EDTA and fluoride-oxalate tubes (Terumo). Fluoride-oxalate tubes contained 6.75 mg of lyophilized sodium fluoride (54 mmol/L of blood). Plasma was separated immediately (centrifugation started within 2 min) and after 1, 2, and 5 h at room temperature. The Hcy assay was performed on each aliquot. Two hundred and thirty women (mean age, 41 years; range, 24–59 years) and 126 men (mean age, 42 years; range, 23–63 years) who are workers in Erasme Hospital (Brussels) were included in the reference interval and vitamin correlation studies. The study was approved by the local ethics committee. For the establishment of plasma Hcy reference values, fasting venous blood was drawn in fluoride-oxalate tubes transported on crushed ice to the laboratory, where plasma was immediately separated from blood cells and analyzed within 2 h. For vitamin B12 and folic acid measurements, serum was analyzed within 4 h after collection. Statistical analyses were performed with Analyze-It for Microsoft

Reference Intervals for Plasma Homocysteine by the AxSYM Immunoassay after Collection in Fluoride Tubes, Frédéric Cotton, Jean-Claude Wautrecht, Véronique Léchevin, Pascale Macours, Philippe Thiry, Christine Gervy, and Jean-Marie Boeynaems (Hôpital Erasme, Université Libre de Bruxelles, 808 route de Lennik, B1070 Brussels, Belgium; * author for correspondence: fax 32-2-555-6655, e-mail fcotton@ulb.ac.be)

Homocysteine (Hcy) is an intermediate amino acid formed during the metabolism of methionine to cysteine. In the recent years, it has emerged as a risk factor for cardiovascular disease independent of known other factors (1–3). The first methods developed for total plasma Hcy measurements were HPLC, with or without derivatization, and gas chromatography. Later, immunoassays were developed, allowing automation and widespread use of the marker in the medical world. Plasma Hcy is influenced by various factors: genetics, diet, sex, age, ethnic group, drugs, and diseases. EDTA-, citrate-, or heparin-anticoagulated blood may be used, but plasma should be immediately separated from blood cells to avoid spurious increases (4). Addition of different substances has been suggested to reduce these spurious increases in Hcy concentrations. Acidic citrate has been shown to be effective in some studies (5) but not in others (6). A specific inhibitor of S-adenosylhomocysteine hydrolase (3-deaza-adenosine) was successfully used, but unfortunately, it is not compatible with immunoassays (7). Fluoride appears to be the most interesting additive. Ubbink et al. (8) showed that it prevents in vitro increases for 2 h. Several studies have confirmed this effect (9), although others have not (10). In most studies, plasma Hcy from blood collected in fluoride tubes has been lower than in EDTA tubes (10–12).

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<table>
<thead>
<tr>
<th>Time between collection and separation</th>
<th>EDTA</th>
<th>Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD), μmol/L</td>
<td>12.5 (6.3)</td>
<td>11.5 (5.7)</td>
</tr>
<tr>
<td>Change (SD) from time 0, %</td>
<td>8.5 (4.1)</td>
<td>2.5 (3.1)</td>
</tr>
<tr>
<td>Mean (SD) EDTA–fluoride difference, %</td>
<td>8.7 (3.1)</td>
<td>14.4 (3.7)</td>
</tr>
<tr>
<td>1 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD), μmol/L</td>
<td>13.6 (7.0)</td>
<td>11.8 (6.0)</td>
</tr>
<tr>
<td>Change (SD) from time 0, %</td>
<td>21.1 (5.4)</td>
<td>6.5 (4.7)</td>
</tr>
<tr>
<td>Mean (SD) EDTA–fluoride difference, %</td>
<td>14.4 (3.7)</td>
<td>21.5 (5.5)</td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD), μmol/L</td>
<td>15.0 (7.1)</td>
<td>12.2 (6.2)</td>
</tr>
<tr>
<td>Change (SD) from time 0, %</td>
<td>39.9 (9.9)</td>
<td>14.8 (6.5)</td>
</tr>
<tr>
<td>Mean (SD) EDTA–fluoride difference, %</td>
<td>21.5 (5.5)</td>
<td>28.3 (5.2)</td>
</tr>
<tr>
<td>5 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD), μmol/L</td>
<td>17.2 (7.4)</td>
<td>13.1 (6.3)</td>
</tr>
<tr>
<td>Change (SD) from time 0, %</td>
<td>39.9 (9.9)</td>
<td>14.8 (6.5)</td>
</tr>
<tr>
<td>Mean (SD) EDTA–fluoride difference, %</td>
<td>28.3 (5.2)</td>
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