A Brief Retrospective Report on the Feasibility of Epidermal Growth Factor Receptor and *KRAS* Mutation Analysis in Transesophageal Ultrasound- and Endobronchial Ultrasound-Guided Fine Needle Cytological Aspirates

Olga C. J. Schuurbiers, MD, Monika G. Looijen-Salamon, MD, PhD,†*
*Marjolijn J. L. Ligtenberg, PhD,†‡ and Henricus F. M. van der Heijden, MD, PhD**

Introduction: Molecular testing for epidermal growth factor receptor (*EGFR*) and *KRAS* mutations is of increasing clinical importance in daily practice. In this study, we aimed to investigate the yield and applicability of molecular testing for *KRAS* and *EGFR* mutations in cytologic specimens obtained by EUS or endobronchial ultrasound (EBUS)-guided fine needle aspiration (FNA).

Methods: We selected all patients with an EUS- or EBUS-guided FNA positive for lung adenocarcinoma from the database of our tertiary care center for endosonography. Direct smears were Giemsa and Papanicolaou stained. The remaining material was processed in cell blocks. Both cell blocks and smears were considered suitable for molecular analysis when >40% of the aspirated cells were tumor cells. All eligible samples were investigated for *KRAS* and *EGFR* mutations by polymerase chain reaction followed by direct sequencing.

Results: Four hundred sixty-two patients underwent EUS or EBUS-FNA using 22-gauge needles.

In 35 patients, FNA showed lung adenocarcinoma. In eight patients, molecular analysis could not be performed because of insufficient material after routine and immunocytochemistry ($n = 3$), a low percentage (<40%) of tumor cells ($n = 3$), or an insufficient DNA quality ($n = 2$). The average percentage of tumor cells was 73% ± 23%.

Molecular analysis could reliably be performed in 27 patients (77%). Mutation analysis showed *KRAS* and *EGFR* mutations in tumor samples from 10 (37%) and two (7%) patients, respectively. In one patient, two *EGFR* mutations (p.Thr790Met and p.Leu858Arg) were detected.

Conclusions: Molecular analysis for *KRAS* and *EGFR* mutations can be performed routinely in cytologic specimens from EUS- and EBUS-guided FNA.

Departments of *Pulmonary Diseases, †Pathology, and ‡Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.
Disclosure: The authors declare no conflicts of interest.
Address for correspondence: Henricus F.M. van der Heijden, MD, PhD, Department of Pulmonary Medicine, Radboud University Nijmegen Medical Centre, 454, PO Box 9101, Nijmegen 6500 HB, The Netherlands. E-mail: H.vanderHeijden@long.umcn.nl

ISSN: 1556-0864/10/0510-1664

Key Words: EBUS, EUS, EGFR, KRAS, Cytology, Mutation analysis, Adenocarcinoma, Lung, NSCLC.

(*J Thorac Oncol.* 2010;5: 1664–1667)

Determination of the mutation status of the epidermal growth factor receptor (*EGFR*) gene is of importance to adequately select patients with both early and advanced non-small cell lung cancer (NSCLC) for targeted treatment with tyrosine kinase inhibitors (TKIs) or monoclonal antibodies and to predict the prognosis and response to *EGFR*-targeted treatment and systemic chemotherapy.[1–3] Also, the *KRAS* mutational status is of importance because *KRAS* is an important proliferation step downstream of the EGFR and mutations in *KRAS* relate to resistance to EGFR-targeted therapy and adjuvant chemotherapy.[2,4,5] *KRAS* mutations may also be of relevance to other downstream pathways such as the PI3K/AKT pathway.[2,6]

Recent landmark studies investigating the effects of different TKIs in NSCLC used histologic specimens for molecular analysis of the mutation status. However, in clinical practice, the diagnosis of lung cancer is often based on cytologic specimens because tissue samples are increasingly obtained by ultrasound-guided techniques and on transthoracic fine needle aspirations (FNAs). Recent developments have shown that transesophageal ultrasound-guided (EUS) FNA (EUS-FNA) and endobronchial ultrasound (EBUS)-guided FNA are minimally invasive diagnostic and staging procedures, which have shown to be highly sensitive and accurate.[7–9] They allow safe cytologic sampling of mediastinal lymph nodes and centrally located intrapulmonary tumors or metastases in the upper abdomen including the left adrenal gland. Therefore, EUS-FNA and EBUS have been incorporated in the guidelines of the European Society of Thoracic Surgeons and American College of Chest Physicians,[10,11] and a combination of these techniques has been shown to have a better sensitivity and negative predictive value than cervical mediastinoscopy (the now debated gold standard).[9,12]

However, in contrast to cervical mediastinoscopy, EUS-FNA and EBUS will result in cytologic specimens, preferably processed on slides for rapid onsite evaluation and

vials for cell blocks. Because these samples are often the only available proof of lung cancer, mutation analysis performed on cytologic specimens is of increasing interest.[13] Furthermore, differences in mutations in the primary tumor site compared with metastatic disease sites have been shown and may be of importance to daily clinical practice.[14] Therefore, in case of recurrent disease, renewed tissue sampling needs to be considered increasing the necessity to use minimal invasive techniques for sampling of tumor material on which molecular analyses can be performed.

Recently, two studies reported on *EGFR* analysis in cell block-based cytologic specimens from EBUS.[15,16] In this study, we aimed to investigate the yield and applicability of molecular testing for both *KRAS* and *EGFR* mutations in all available cytologic specimens, both cell blocks and direct smears, obtained by EUS-FNA- or EBUS-guided FNA in routine daily practice.

METHODS

Between January 2006 and January 2010, 462 patients were evaluated in our tertiary care university hospital center for endosonography. EUS was available since December 2005 and EBUS from December 2008 onward. Of these 462 patients, 289 were referred for diagnosis or staging of proven or suspected lung cancer.

All patients were investigated in an outpatient procedure using midazolam (2.5–5 mg) for conscious sedation. EUS and EBUS were performed using Pentax EG 3870UTK or EB-1970UK echo endoscopes in combination with a Hitachi EUB 6500 or 7000HV ultrasound scanner. In all patients, FNA was performed using 22-gauge needles (MediGlobe); on average 2.9 ± 1.1(standard deviation; range: 1–5) aspirations per diagnostic site were performed. Direct smears were made for Giemsa staining and Papanicolaou staining. Giemsa-stained smears were processed and analyzed onsite for rapid onsite evaluation by a cytotechnician. The remaining material was processed in cell blocks, from which 4-μm slides were cut for hematoxylin and eosin staining and immunocytochemistry. To minimize the chance of false-negative results, on the basis of our experience in histologic NSCLC and colorectal cancer specimens,[17] both cell blocks and smears were considered suitable for molecular analysis when DNA could be isolated from regions with >40% tumor cells. For DNA isolation from the cell blocks, the relevant regions were manually microdissected from two to three 20-μm sections using flanking hematoxylin and eosin-stained slides as a reference and incubated overnight at 56°C in 200 μl lysis buffer (10 mM Tris pH 8.5, 1 mM ethylenediaminetetraacetic acid (EDTA); 0.05% tween20, 5% Chelex-100, and 2 μg/μl Proteinase K) followed by 10 minutes at 95°C. After centrifugation, the supernatant is used for subsequent analyses. For the DNA isolation from the smears, regions with more than 40% tumor cells are scraped from the glass slides and incubated overnight at 56°C in 400 μl lysis buffer (10 mM Tris pH7.5, 2 mM EDTA, 400 mM NaCl, 1% sodiumdodecylsulfate, and 2 μg/μl Proteinase K) followed by a salt precipitation with 140 μl 6 M NaCl and ethanol precipitation of the supernatant. The DNA pellet is dissolved in 20 μl Tris (10 mM Tris pH 7.5 and 1 mM EDTA). Mutation analysis of *EGFR* exons 18, 19, 20, and 21 and *KRAS* codons 12, 13, and 61 was performed using 1 μl DNA solution in polymerase chain reactions (PCRs) followed by Big-Dye terminator sequencing (BigDye Terminators (v 1.1); Applied Biosystems, USA) and analysis on an ABI 3730 DNA Analyzer (Applied Biosystems; primer sequences and PCR conditions are available on request).

As the molecular analyses performed in this study have been performed as part of routine diagnostic daily practice, there was no need to consulting our ethics committee. Full disclosure of results was given to the patients, and results were discussed in our multidisciplinary tumor board meetings to determine the optimal treatment strategies.

RESULTS

In 112 patients (82 men and 30 women), cytologic proof of lung cancer was obtained by EUS (n = 77), EBUS (n = 30), or combined EUS and EBUS (n = 5). The average age was 64.8 ± 9.7 years (range 36–90 years). Giemsa and Papanicolaou and immunocytochemical stainings showed squamous cell carcinoma in 38 cases (34%), adenocarcinoma in 35 cases (31%), large cell or undifferentiated NSCLC in 26 patients (23%), and small cell lung cancer in 13 cases (12%) (Table 1).

The average percentage of tumor cells in the 35 specimens containing adenocarcinoma was 73% ± 23%. Molecular analysis could not be performed in eight patients because of insufficient material after routine cytology and immuno-

TABLE 1. Patients with EUS- or EBUS-Guided Cytology-Proven Lung Cancer

Patient Characteristics (n = 112)	
Age (yr)	64.8 ± 9.7 (range, 36–90)
Gender: female:male (n (%))	30:82 (27:73)
Histology (n (%))	
SCC	38 (34)
AC	35 (31)
LC/undifferentiated NSCLC	26 (23)
SCLC	13 (12)
Adenocarcinoma (n = 35), n (%)	
Samples available for analysis	27 (77)
Gender (female:male)	11:16 (41:59)
Percentage tumor cells	73% ± 21%
KRAS mutation positive (n (%))	10 (37)
c.35G>T (p.Gly12Val)	5
c.35G>A (p.Gly12Asp)	2
c.34G>T (p.Gly12Cys)	1
c.182A>T (p.Glu61Leu)	1
c.37G>T (p.Gly13Cys)	1
EGFR mutation positive (n (%))	2 (7)
c.2369C>T (p.Thr790Met) and c.2573T>G (p.Leu858Arg)[a]	1
c.2239_2248delinsC (p.Leu747_Ala750delinsPro)	1
KRAS and *EGFR* mutation negative (n (%))	15 (56)

[a] Double mutation in one patient.

AC, adenocarcinoma; LC, large cell undifferentiated NSCLC; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; SCC, squamous cell carcinoma; EGFR, epidermal growth factor receptor.

cytochemistry($n = 3$), a low percentage (<40%) of tumor cells ($n = 3$), or an insufficient DNA quality ($n = 2$).

Molecular analysis could reliably be performed in 27 patients (77%). The average percentage of tumor cells in the 27 samples was 79% ± 12%. Analysis was performed on material obtained from cell blocks ($n = 19$), Giemsa-stained ($n = 3$) or Papanicolaou-stained smears ($n = 5$).

Mutation analysis showed *KRAS* mutations in tumor samples from 10 patients (37%). Six mutations were detected in cell block preparations and four in material from Papanicolaou-stained smears. *EGFR* mutations were found in two patients (7.4%). One patient had an activating exon 19 (p.Leu747_Ala750delinsPro) mutation detected in material from a Papanicolaou-stained smear. The second patient, with recurrent, TKI-naive adenocarcinoma, had two *EGFR* mutations (p.Thr790Met and p.Leu858Arg) detected in a cell block specimen (Table 1 and Figure 1).

DISCUSSION

This study shows that molecular analysis of *EGFR* and *KRAS* mutations on cytologic material obtained by EUS or EBUS is feasible and can be performed in daily practice. Molecular analysis could be performed in 77% of the adenocarcinoma samples. Biomarker analysis on cytologic material is becoming increasingly available. A far as we know, this is the first study to report successful mutation analysis of both the *KRAS* and *EGFR* gene on both cytologic smears and cell blocks obtained by EUS or EBUS.

In 27 of 35 patients (77%) with cytologically proven lung adenocarcinomas, we were able to perform molecular analysis using PCR and subsequent sequence analysis. This is in agreement with the study by Garcia-Olive et al.,[15] where *EGFR* gene analysis of the EBUS-TBNA sample was feasible in 26 (72.2%) of the 36 patients with lymph node metastasis and similar methods were used. Nakajima et al.[16] were able to perform molecular analysis in histologic cores obtained by EBUS in 43 of 46 lung adenocarcinoma patients (94%). In this study, the assessment of tumor cells is not described, but the short axis of the sampled lymph nodes with *EGFR* mutations was approximately similar to that of our study (16.8 ± 7.0 mm).[16] The percentage of *EGFR* mutations found in the aforementioned Spanish and Japanese studies was 10%

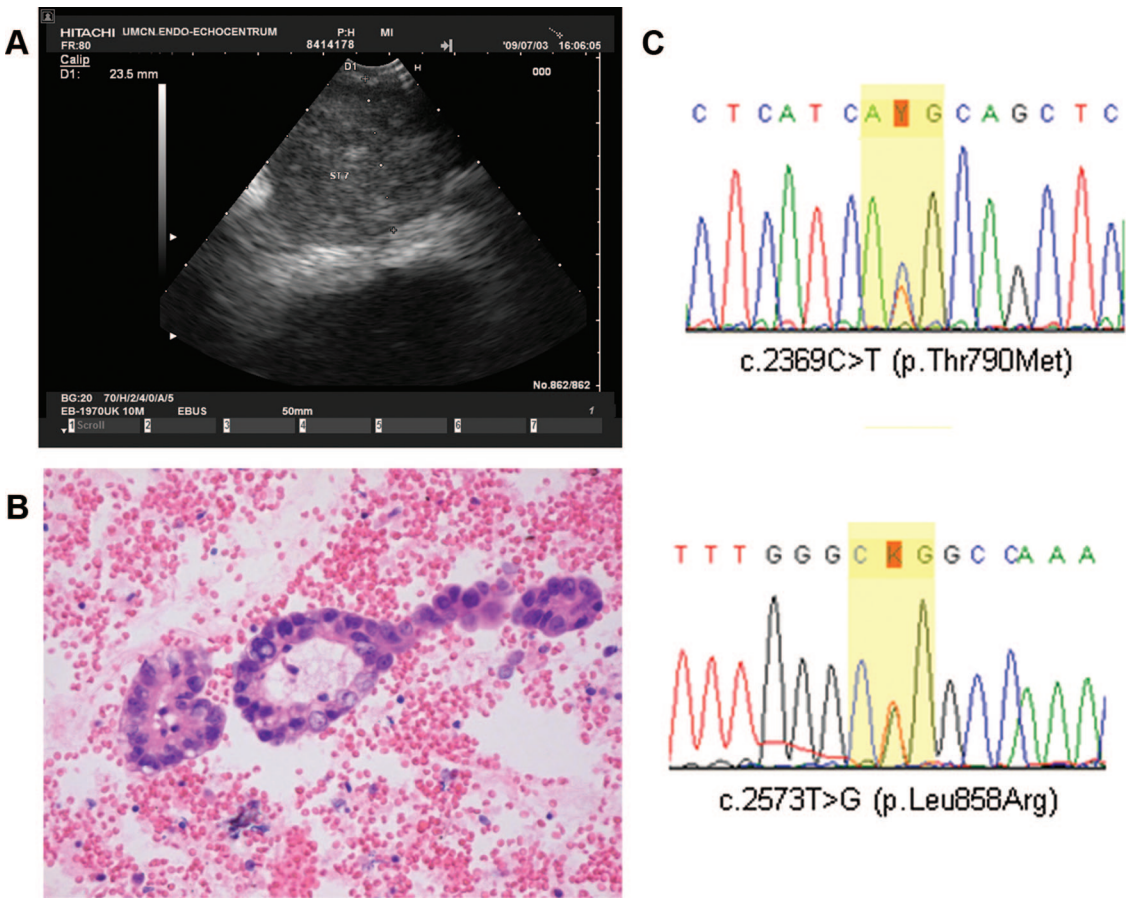

FIGURE 1. Representative sample of a 77-year-old patient referred for analysis of suspected recurrent lung cancer 4 years after the left upper lobe resection for a pT1aN0M0 adenocarcinoma. Endobronchial ultrasound (EBUS)-guided lymph node sampling revealed recurrent disease with a double epidermal growth factor receptor (*EGFR*) mutation: panel A, endosonographic EBUS image of the enlarged subcarinal lymph node at position number 7 (diameter, 23.5 mm); panel B, hematoxylin and eosin staining of lymph node aspirate (×200) revealing adenocarcinoma; and panel C, sequence chromatograms of *EGFR* mutations c.2369C>T (p.Thr790Met) (top) and c.2573T>G (p.Leu858Arg) (bottom).

and 26%, respectively. Especially the percentage from the latter study is much higher than the percentage of *EGFR* mutations (7.4%) found in our cohort. This might reflect a high percentage of cigarette smoking patients in our predominantly male, white group of patients. Unfortunately, exact data on smoking status are not available because the majority of our patients were referred for the diagnostic endosonographic procedure only and treated in the referring hospitals. However, in our own clinic for thoracic oncology, approximately 87% of patients are current or former smokers.

The percentage of *KRAS* mutations (37%) in our cohort is in line with other studies reporting *KRAS* mutations ranging from 15 to 22% and 18 to 43% in nonsmoking and smoking subjects, respectively.[4,14] Earlier studies used paraffin-embedded material from cell blocks,[15,16] whereas in this study, tumor cells could also be retrieved from direct cytologic smears. This may offer additional opportunities for mutation analysis, especially when histologic biopsies and cell blocks do not contain enough tumor cells.

In our feasibility study, we concentrated on the molecular analyses of adenocarcinoma; however, the technique will also be applicable in other histologic groups. Because the predictive value of *EGFR* amplification is still under debate.[18,19] Fluorescent in situ hybridization analysis was not routinely performed in our samples; however, in the only cytologic sample, in which fluorescent in situ hybridization analysis was performed, an *EGFR* amplification was detected.

CONCLUSION

This study shows that molecular analysis for *KRAS* and *EGFR* mutations can be performed routinely on cytologic specimens from EUS- and EBUS-guided FNA. Molecular analysis could be performed in 77% of the lung adenocarcinomas found in these cytologic samples. With this article, we hope to increase awareness of these techniques and show that also in daily clinical practice, *EGFR* and *KRAS* analysis can be performed in a high percentage of patients.

ACKNOWLEDGMENTS

The authors thank Sandra Hendriks-Cornelissen and Monique Goossens for technical assistance.

REFERENCES

1. Rosell R, Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009;361:958–967.
2. Shepherd FA, Tsao MS. Epidermal growth factor receptor biomarkers in non-small-cell lung cancer: a riddle, wrapped in a mystery, inside an enigma. *J Clin Oncol* 2010;28:903–905.
3. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–957.
4. Riely GJ, Marks J, Pao W. KRAS mutations in non-small cell lung cancer. *Proc Am Thorac Soc* 2009;6:201–205.
5. Takeda M, Okamoto I, Fujita Y, et al. De novo resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in EGFR mutation-positive patients with non-small cell lung cancer. *J Thorac Oncol* 2010;5:399–400.
6. Schuurbiers OCJ, Kaanders JH, van der Heijden HF, et al. The PI3-K/AKT-pathway and radiation resistance mechanisms in non-small cell lung cancer. *J Thorac Oncol* 2009;4:761–767.
7. Micames CG, McCrory DC, Pavey DA, et al. Endoscopic ultrasound-guided fine-needle aspiration for non-small cell lung cancer staging: a systematic review and metaanalysis. *Chest* 2007;131:539–548.
8. Varela-Lema L, Fernandez-Villar A, Ruano-Ravina A. Effectiveness and safety of endobronchial ultrasound-transbronchial needle aspiration: a systematic review. *Eur Respir J* 2009;33:1156–1164.
9. Wallace MB, Pascual JM, Raimondo M, et al. Minimally invasive endoscopic staging of suspected lung cancer. *JAMA* 2008;299:540–546.
10. De LP, Lardinois D, Van Schil PE, et al. ESTS guidelines for preoperative lymph node staging for non-small cell lung cancer. *Eur J Cardiothorac Surg* 2007;32:1–8.
11. Silvestri GA, Gould MK, Margolis ML, et al. Noninvasive staging of non-small cell lung cancer: ACCP evidenced-based clinical practice guidelines (2nd edition). *Chest* 2007;132:178S–201S.
12. Szlubowski A, Zieliński M, Soja J, et al. A combined approach of endobronchial and endoscopic ultrasound-guided needle aspiration in the radiologically normal mediastinum in non-small-cell lung cancer staging—a prospective trial. Eur J Cardiothorac Surg 2010;37:1175–1179.
13. Travis WD, Rekhtman N, Riley GJ, et al. Pathologic diagnosis of advanced lung cancer based on small biopsies and cytology: a paradigm shift. *J Thorac Oncol* 2010;5:411–414.
14. Schmid K, Oehl N, Wrba F, et al. EGFR/KRAS/BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. *Clin Cancer Res* 2009;15:4554–4560.
15. Garcia-Olive I, Monso E, Andreo F, et al. Endobronchial ultrasound-guided transbronchial needle aspiration for identifying EGFR mutations. *Eur Respir J* 2010;35:391–395.
16. Nakajima T, Yasufuku K, Suzuki M, et al. Assessment of epidermal growth factor receptor mutation by endobronchial ultrasound-guided transbronchial needle aspiration. *Chest* 2007;132:597–602.
17. Tol J, Dijkstra JR, Vink-Borger ME, et al. High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. *J Cell Mol Med* In press.
18. Yamamoto H, Toyooka S, Mitsudomi T. Impact of EGFR mutation analysis in non-small cell lung cancer. *Lung Cancer* 2009;63:315–321.
19. Dahabreh IJ, Linardou H, Siannis F, et al. Somatic EGFR mutation and gene copy gain as predictive biomarkers for response to tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* 2010;16:291–303.

