Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

# Comparison of epidermal growth factor receptor mutation analysis results between surgically resected primary lung cancer and metastatic lymph nodes obtained by endobronchial ultrasound-guided transbronchial needle aspiration

Hironobu Okada[1]*, Takashi Anayama[1]*, Motohiko Kume[1], Kentaro Hirohashi[1], Ryouhei Miyazaki[1], Manabu Matsumoto[2] & Kazumasa Orihashi[1]

1 Department of Surgery II, Kochi Medical School, Kochi Universtiy, Nankoku, Kochi, Japan
2 Laboratory of Diagnostic Pathology, Kochi Medical School Hospital, Nankoku, Kochi, Japan

**Keywords**
EBUS-TBNA; EGFR mutation; lung cancer.

**Correspondence**
Takashi Anayama, Department of surgery II, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku-city, Kochi 783-8505, Japan.
Tel: +81 88 880 2375
Fax: +81 88 880 2376
Email: anayamat@kochi-u.ac.jp

Received: 8 January 2012;
accepted 1 February 2012.

doi: 10.1111/j.1759-7714.2012.00122.x

*These two authors contributed equally to this work.

## Abstract

**Background:** Lung cancers with mutations in the epidermal growth factor receptor (EGFR) gene respond well to treatment with EGFR inhibitors. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is considered a useful modality to obtain samples from the mediastinal and hilar lymph nodes. However, the EGFR gene status of EBUS-TBNA samples may not always match that of primary tumors.
**Methods:** In 14 node-positive patients diagnosed by EBUS-TBNA, EGFR mutation analysis results were compared between EBUS-TBNA samples and surgically removed primary tumors. EGFR mutation was screened with peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp followed by direct sequence analysis. For one controversial case, gene mutation analyses were performed for the multiple micro-fractions of a metastatic lymph node, which exhibited the heterogeneous immunohistochemical features.
**Results:** EBUS-TBNA diagnosed one case of exon 21 point mutations, one case of exon 19 deletion, and 12 cases of wild-type EGFR. Results were consistent with those of surgically removed primary tumors in 13 of 14 cases. One case of wild-type EGFR diagnosed by EBUS-TBNA exhibited exon 21 point mutation in the surgically removed primary tumor. The metastatic lymph node targeted by EBUS-TBNA mostly consisted of cancer cells with wild-type EGFR; however, a minor component positive for thyroid transcription factor-1 (TTF-1) and surfactant-associated protein A (PE-10) exhibited EGFR mutation.
**Conclusion:** The combination of EBUS-TBNA and PNA-LNA clamp is useful for EGFR mutation analysis. However, EGFR mutation status in EBUS-TBNA samples may not be consistent with that of the primary tumor when the tumor contains few EGFR mutations.

## Introduction

Platinum-based combination chemotherapy has been commonly used as the first line of treatment to improve overall survival for advanced non-small cell lung cancer (NSCLC).[1] However, the efficacy of this approach is only about 30%.[2] Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are being increasingly used. EGFR-TKIs are effective in the treatment of lung cancer, especially in female and non-smoking patients of East Asian origin with adenocarcinoma histology.[3,4] The presence of a mutation in the epidermal growth factor receptor (EGFR) gene is a

strong predictor of a better outcome with gefitinib or erlotinib.[5–9]

Although EGFR mutations can be examined in resected specimens of primary cancer in surgically treated patients, an alternative method is required if the cancer is not resectable. Several studies have reported non-surgical approaches to obtaining cancer cells for EGFR mutation analysis. Several methods have been used, such as computed tomography-guided percutaneous fine needle aspiration biopsy (CT-guided FNA),[10] transbronchial lung biopsy (TBLB), transbronchial needle aspiration (TBNA),[11] and bronchoscopic microsampling (BMS).[12]

On the other hand, mediastinal or hilar metastatic lymph nodes are also promising sites for the collection of tumor cells. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is minimally invasive and provides high sensitivity and a high negative predictive value in the diagnosis of mediastinal and hilar lymph node metastasis.[13–15] In advanced lung cancer with mediastinal and/or hilar lymph node metastasis, multiple EBUS-TBNA passages can be performed in a target lymph node to obtain an adequate amount of samples for both cytological diagnosis and DNA mutation analysis without causing severe complications, such as pneumothorax or endobronchial hemorrhage, which occasionally occur during CT-guided FNA and TBLB.

Several studies have performed EGFR mutation analysis using samples obtained by EBUS-TBNA. EGFR mutation analysis in EBUS-TBNA samples has been reported in combination with highly sensitive detection methods,[16] such as the loop-hybrid mobility shift assay, which detects 1% of EGFR mutation-positive tumor cell populations.[17] In addition, micro-dissection techniques can be used to isolate tumor cells from samples containing other non-neoplastic lymph node cell contents.[18] However, the micro-dissection technique is complicated for use in daily clinical practice, and a sufficient amount of tissue cannot necessarily be extracted by EBUS-TBNA. We retrospectively carried out EGFR mutation analysis in EBUS-TBNA samples using the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method,[19,20] which has been reported to be a high sensitivity detection method when compared with conventional direct sequencing. We then compared these results with EGFR mutation status in surgically removed primary tumors, in order to validate the usefulness of EGFR mutation analysis carried out using a combination of tissue sampling by EBUS-TBNA and PNA-LNA clamping.

## Methods

### Patient eligibility

From April 2006 to October 2009, 14 consecutive surgical patients with lung cancer preoperatively diagnosed by EBUS-TBNA as hilar (N1) or single ipsilateral mediastinal lymph node metastatic disease (single station N2), were enrolled in the study (Table 1). The target lymph nodes for EBUS-TBNA were hilar or mediastinal lymph nodes identified as enlarged (>10 mm) by computed tomography (CT), or those showing abnormal uptake of 2-fluoro-2-deoxy-D-glucose (FDG) on positron emission tomography (PET) (Fig. 1a,b). Lymph node stations were identified according to the International Association for the Study of Lung Cancer lung cancer staging guidelines.[21] The series included nine male and five female

**Table 1** Patient Characteristics and comparison of epidermal growth factor receptor (EGFR) mutation status in endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) samples and surgically removed primary tumors

| | | | | EBUS-TBNA | | | |
|---|---|---|---|---|---|---|---|
| Case# | Age/gender | c-Stage | Histology | LN Station | Diameter (mm) | EGFR Status of lymph node | Primary tumor |
| 1 | 68M | T4N1M0 | Sq | #11R | 18 × 8 | WT | WT |
| 2 | 64F | T2N2M0 | Ad | #11R | 11 × 9 | WT | WT |
| 3 | 75M | T2N2M0 | Large | #7 | 22 × 19 | WT | WT |
| 4 | 60M | T2N2M0 | Large | #7 | 14 × 8 | WT | Ex21: L858R |
| 5 | 84F | T2N2M0 | Ad | #4R | 16 × 7 | Ex21: L858R | Ex21: L858R |
| 6 | 58M | T3N1M0 | Ad | #10L | 28 × 15 | WT | WT |
| 7 | 49M | T2N2M0 | Ad | #4R | 22 × 16 | WT | WT |
| 8 | 59M | T4N2M0 | Ad | #4L | 11 × 6 | WT | WT |
| 9 | 72M | T2N2M0 | Ad | #7 | 18 × 12 | WT | WT |
| 10 | 78M | T1N2M0 | Ad. | #7 | 20 × 11 | Ex19: L747-E749 del | Ex19: L747-E749 del |
| 11 | 68M | T2N2M0 | LCNEC | #7 | 30 × 13 | WT | WT |
| 12 | 72F | T2N2M1 | Ad. | #7 | 15 × 4 | WT | WT |
| 13 | 56F | T2N2M0 | Ad. | #7 | 17 × 11 | WT | WT |
| 14 | 38F | T1N2M0 | Ad. | #4R | 12 × 5 | Ex21: L858R | Ex21: L858R |

Ad, adenocarcinoma; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; EGFR, epidermal growth factor receptor; Large, large cell carcinoma; LCNEC, large cell neuroendocrine cell carcinoma; n/a, not applicable; Sq, squamous cell carcinoma; WT, Wild type.

  

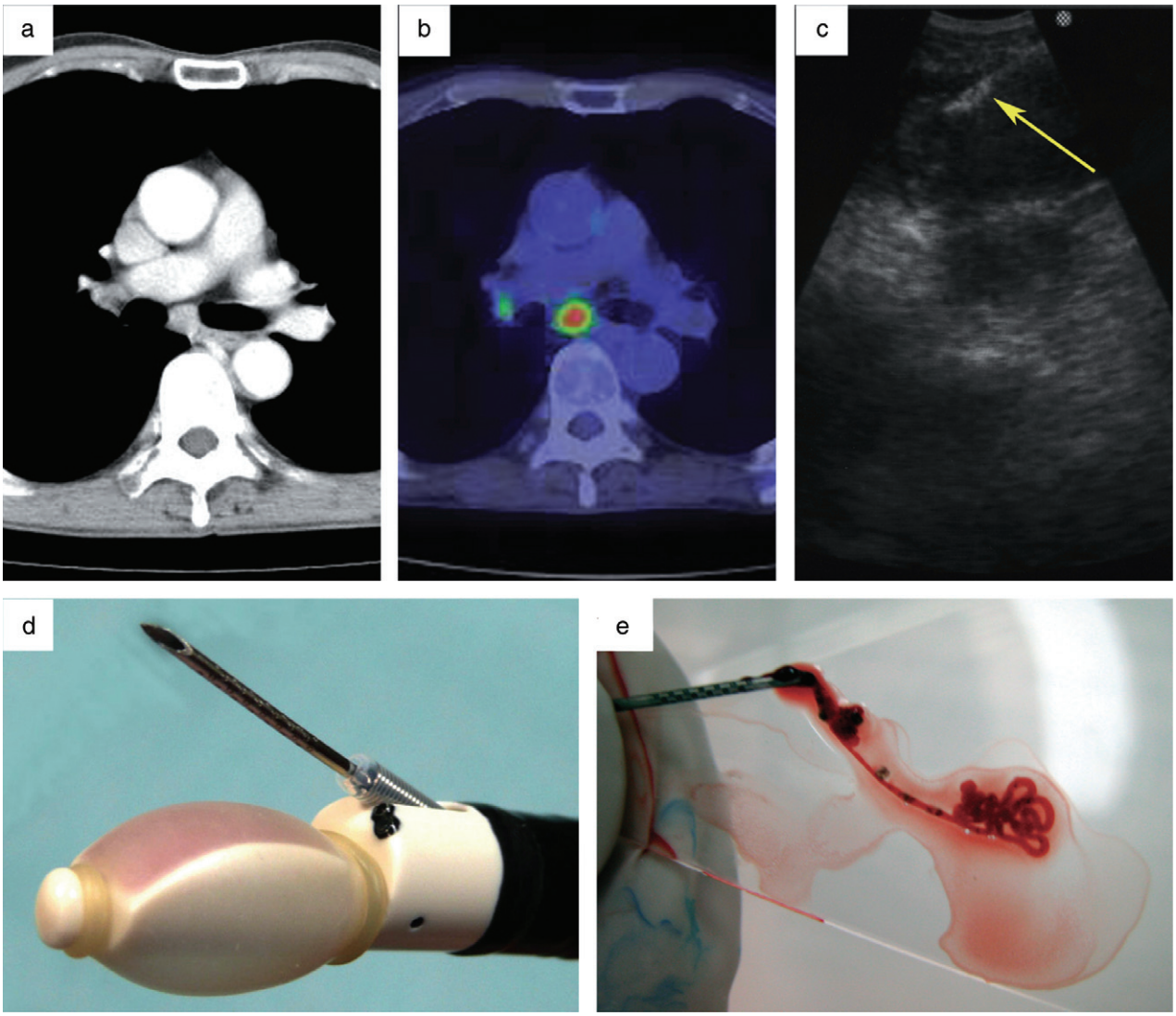


**Figure 1** Diagnostic radiological imaging and endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) in a representative case. (**a**) Computed tomogram showing subtracheal lymph node 10 mm in diameter. (**b**) Positron emission tomogram demonstrating increased uptake of 2-fluoro-2-deoxy-D-glucose (FDG) in the corresponding lymph node. (**c**) Endobronchial ultrasonogram (EBUS) showing a needle within the lymph node (yellow arrow). (**d**) The tip of convex probe EBUS. The balloon is filled with saline to optimize a contact of transducer to the bronchus. (**e**) Harvested specimen. The black portion contains carbon dust included in the lymph node parenchyma. The first drops of the liquid component were submitted for quick cytological diagnosis. After confirming the presence of malignant cells, an additional biopsy is obtained for epidermal growth factor receptor (EGFR) mutation analysis.

patients, with the patient age ranging from 38 to 84 years (mean age, 64.2 years). Surgical resection for single station N2 disease was based on previous reports suggesting that single station N2 portends five year survival rates comparable with those of N1 subsets.[22–25] Induction chemotherapy was not performed. The series included three patients with N1 disease and 11 patients with single station N2 disease. Primary tumors were surgically removed and evaluated for EGFR mutation status in order to examine the genetic homology between the primary tumor and the metastatic lymph node.

## EBUS-TBNA tissue sampling

EBUS-TBNA was performed under mild sedation with 2–4 mg of midazolam administered intravenously, and 2 mL of 2% lidocaine was introduced from a bronchoscopic channel into the trachea and bronchus several times. In those cases where additional samples for gene mutation analysis were obtained, 1 mg of Midazolam was administered intermittently, as necessary. A 7.5 MHz convex probe endobronchial ultrasound system (CP-EBUS; BF-UC260F-OL8; Olympus

Medical, Tokyo, Japan) was used for examination (Fig. 1d). Ultrasound images were obtained by CP-EBUS simultaneously, using a dedicated ultrasound scanner (EU-C2000; Olympus, Tokyo, Japan), which also applied conventional white light images. A disposable aspiration biopsy needle (NA-201SX-4022; Olympus, Tokyo, Japan) was used to puncture the lymph node under ultrasound guidance (Fig. 1c). After cleaning out the internal lumen of the needle using the internal sheath, negative pressure was applied using a syringe. The needle was moved back and forth inside the tumor. Finally, the needle was retrieved and the internal sheath was used to push out the histologic core (Fig. 1e). The first-drop EBUS-TBNA specimen was pushed out with the inner wire onto a glass slide for quick cytological diagnosis by on-site cytologists. EBUS-TBNA was repeated one to four times until an adequate tissue sample, including lymphocytes, was obtained. After the cancer cells were identified cytologically, the residual tissue sample was stored for DNA mutation analysis.

### DNA extraction and EGFR mutation analysis

DNA was extracted from all fresh samples obtained by EBUS-TBNA and surgically resected primary tumors using the QIA amp DNA Blood Kit (Qiagen, Venlo, The Netherlands). In case number four shown in Table 1, the surgically removed metastatic lymph node, which had been targeted for EBUS-TBNA, was examined in detail. DNAs were extracted from two fractions of different immunohistochemical features with the micro-extraction method by DEXPAT (TaKaRa, Tokyo, Japan), according to the manufacturer's instruction. Lysis buffer (50 μL) was applied to the desired point and then repeated pipetting was performed until the tissue had dissolved into a lysate. The lysate was heated for 10 minutes at 100°C and centrifuged for 10 minutes at 12 000 rpm and 4°C, and the dissolved DNA fraction was extracted according to the manufacturer's instructions.

### PNA-LNA PCR clamp assay

Extracted DNA was examined for EGFR mutations by means of the PNA-LNA PCR clamp method. Genomic DNA fragments containing mutation hot spots of the EGFR gene were amplified using a PCR assay in the presence of a peptide nucleic acid clamp primer synthesized from a peptide nucleic acid with a wild-type sequence. This method resulted in preferential amplification of the mutant sequence, which was then detected by a fluorescent primer that incorporates locked nucleic acids to increase specificity.[19,20]

### PCR-based direct sequencing for exons 19 and 21

Samples were screened by the PNA-LNA PCR clamp method, and the detailed gene mutation status of each sample was confirmed by the direct sequence method. EGFR mutations in the extracted DNA were examined using PCR-based direct sequencing for exons 19 and 21. Sequencing was carried out using the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA, USA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

### Immunohistochemistry in surgically resected tissue samples

Sections of 4-μm thickness were obtained from paraffin-embedded tumor blocks. The paraffin was removed, and endogenous peroxidase activity was then blocked. The heat-induced antigen-retrieval technique was used before immunostaining. The primary antibodies used in this study were mouse monoclonal antibodies (MAbs) against thyroid transcription factor-1 (TTF-1; Thermo Fisher Scientific Anatomical Pathology, Runcorn, UK; 1:100 dilution), as a marker of primary lung cancer, surfactant-associated protein A (PE-10; Dako Inc., Kyoto, Japan; 1:100 dilution), as a marker of pneumocyte origin,[26] and anti-cytokeratin 7 (anti-CK7; Dako Inc., Kyoto, Japan), as a marker indicating epithelial differentiation.[27] Sections were incubated with these antibodies for 24 hours at 4°C. Immunostaining was performed using the avidin-biotin-peroxidase complex technique with the Labeled Streptavidin-Biotin (LSAB) kit and peroxidase (Dako Inc., Kyoto, Japan), followed by light hematoxylin counterstaining.

### Ethics committee approval

The Ethics Committee Board of the Kochi Medical School Hospital approved this study. Written informed consent for EBUS-TBNA and EGFR biomarker assessment was obtained from all patients. The study conformed to the provision of the Declaration of Helsinki.

## Results

The mean diameter of EBUS-TBNA-targeted hilar and mediastinal lymph nodes, was 18.1 ± 10.3 mm (Table 1). EBUS-TBNA was carried out without any complications in all 14 patients. The target lymph node was station 7 (subcarinal lymph node) in seven patients, station 11R (lobar lymph node) in two patients, station 4R (right lower paratracheal lymph node) in three patients, station 4 L (left lower paratracheal lymph node) in one patient, and station 10 L (hilar lymph node) in one patient. EGFR mutation analysis of all 14 EBUS-TBNA samples showed wild-type EGFR in 11 patients, exon 21:L858R mutation in one patient, and exon 19:L747-E749 deletion in one patient. Surgically removed primary tumors were also examined. Tumor histology revealed the presence of adenocarcinoma, large cell carcinoma, and squamous cell carcinoma in 10 patients (71%), three patients

  

(21%), and one patient (7%), respectively. EGFR status obtained from EBUS-TBNA specimens was identical to that of primary tumors in 13 of the 14 surgically treated patients (92.9%). One case of large cell carcinoma exhibited contradictory results. In this case, EGFR mutation was not present in the EBUS-TBNA specimen obtained from the metastatic lymph node, however, the surgically removed primary tumor and the surgically removed metastatic subcarinal lymph node exhibited exon 21:L858R EGFR mutations.

In this particular case, further immunohistochemistry and EGFR mutation analysis was performed for both the primary tumor and the surgically removed metastatic subcarinal lymph node that had been targeted for EBUS-TBNA. In hematoxylin and eosin (HE) staining, the primary tumor exhibited a nest-like or sheet-like growth pattern without glandular or squamoid differentiations. Immunohistochemically, the primary tumor was focally positive for TTF-1 and PE10. The tumor was diagnosed as a large cell carcinoma, lymphoepithelioma-like carcinoma. Histological and genetic findings of the metastatic lymph node were shown in Figure 2. HE staining revealed that the component in area A contained nests without any squamous or glandular differentiation (Fig. 2a1), while other tumor components in area B were aligned amorphously (Fig. 2b1). Immunohistochemistry showed that the cytoplasm of a small number of tumor cells was positive for PE-10 (Fig. 2a2). In the same area, the nuclei of tumor cells were focally positive for TTF-1 (Fig. 2a3). Many other parts of the same metastatic tumor were negative for both PE-10 (Fig. 2b2) and TTF-1 (Fig. 2b3). The positivities of TTF-1 and PE10 were lower in the metastatic lymph node than those in the primary tumor. Expression of CK7 was diffusely scattered throughout the tumor (Fig. 2a4,b4). Pinpoint DNA extraction by DEXPAT was performed for both area A and area B, followed by direct sequence analysis. DNA extracted from area A exhibited L858R mutation in exon 21 (Fig. 2a5), which was consistent with the gene signature of the primary tumor. In contrast, area B exhibited wild-type EGFR (Fig. 2b5).

## Discussion

Among 14 surgically treated patients, one patient exhibited conflicting EGFR status between the EBUS-TBNA lymph node sample and the surgically removed primary tumor. Although the EBUS-TBNA lymph node sample exhibited wild-type EGFR, the surgically removed primary tumor exhibited L858R mutation in exon 21 of the EGFR gene. Further investigation of the surgically resected metastatic lymph node tissue sample clarified that the metastatic lymph node included a small number of cancer cells with epithelial differentiations and expression of PE-10 and TTF-1 proteins (area A; Fig. 2a1–a4). DNA extraction from area A revealed an EGFR mutation including two overlapping peaks of guanine and adenine (Fig. 2a5), which indicates that both the L858R and the wild-type gene coexisted in the same area. On the other hand, only wild-type EGFR was detected in area B, the part of the tumor negative for both PE-10 and TTF-1 (Fig. 2b1–b4). In this case, EBUS-TBNA possibly obtained cancer cells from the subset of the tumor represented as area B, which contained wild-type EGFR and did not contain a detectable number of EGFR mutations. This result suggests that the population of cancer cells with EGFR mutation might be different depending on the part of the tumor sampled, therefore, EGFR mutation analysis results obtained by EBUS-TBNA might differ according to the point of sampling. The difference in the phenotype between the primary tumor and the metastatic tumor may also lead to the inconsistent results of EGFR mutation analyses.

We confirmed that the result of EGFR mutation status in EBUS-TBNA samples was consistent with that of the primary tumor in 13 of 14 patients (92.5%). This finding supports previous reports indicating that EBUS-TBNA assessment of EGFR mutation is useful for patients with unresectable lung cancer in whom tissue specimens cannot be easily obtained. When the target lymph node contains only a small amount of metastatic lesion, the sample obtained by EBUS-TBNA will contain a small number of cancer cells consisting predominantly of lymphocytes. Methods that are more sensitive than direct sequencing for detecting EGFR mutations are required for EGFR mutation analysis in small specimens containing only a few EGFR mutation-positive cancer cells. The PNA-LNA clamp method is considered to be one such highly sensitive method, which is capable of detecting EGFR mutation in one cancer cell among 100 cells with wild-type DNA.

Since the assessment of EGFR mutation has been widely used for guidance in the clinical management of lung cancer, extreme care must be taken when working with small amounts of tissue samples. The occurrence of false negative EGFR mutation may be prevented with the use of larger amounts of template DNA; therefore, it is desirable to collect as many passage samples as possible when performing EBUS-TBNA.

## Conclusion

In conclusion, the combination of EBUS-TBNA and highly sensitive EGFR mutation detection methods, such as PNA-LNA clamp, was found to be useful. However, the genetic heterogeneity of the tumor should be taken into consideration, especially when using EBUS-TBNA needle biopsy specimens.

## Acknowledgements

We appreciate the assistance of Mr. Tamotsu Takahashi, C.T. (IAC), chief cytotechnologist, Laboratory of Diagnostic Pathology Kochi Medical School Hospital, who contributed the rapid on-site cytological diagnosis of EBUS-TBNA samples.

 

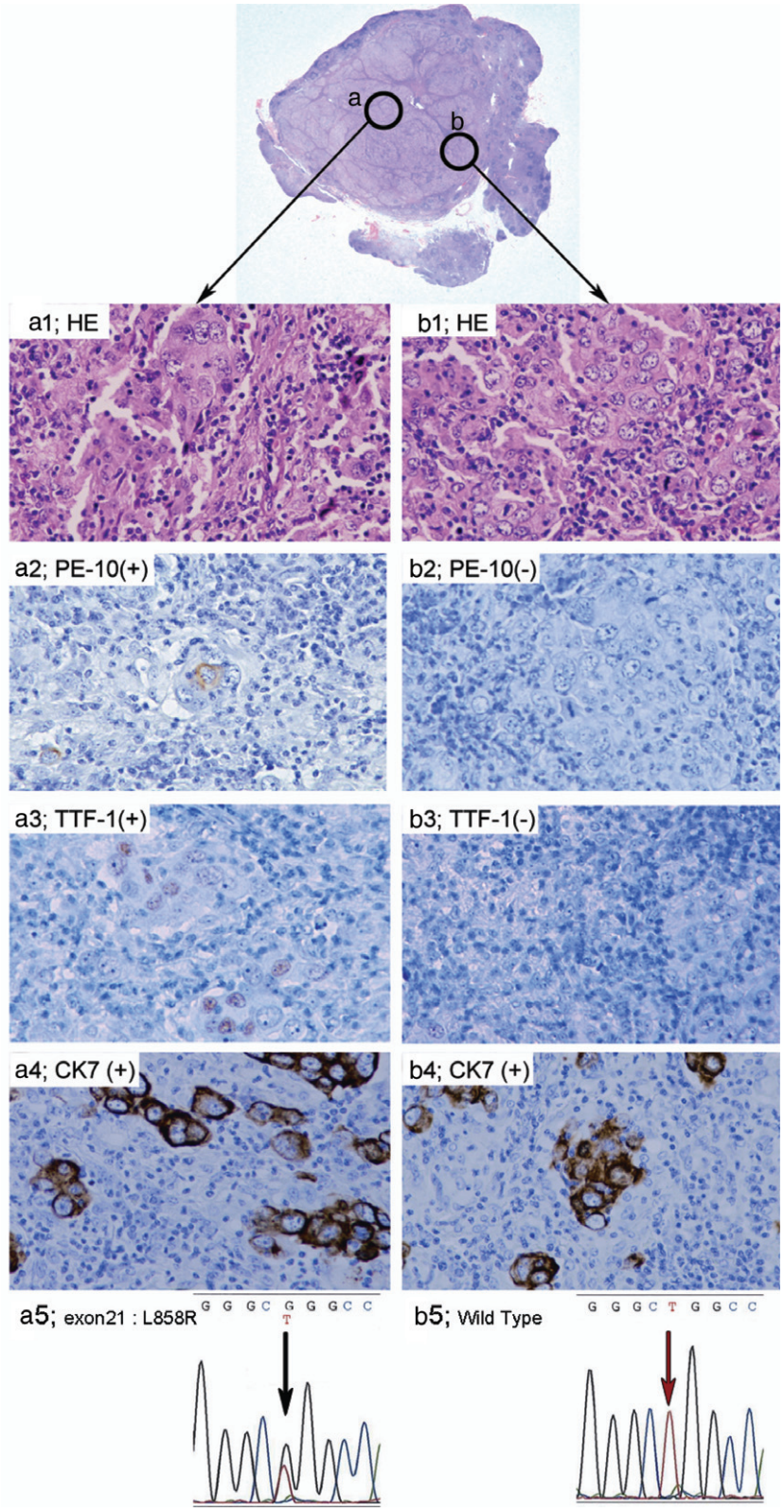


**Figure 2** Surgically removed subcarinal metastatic lymph node of case four. Two components with different histological findings exhibited different epidermal growth factor receptor (EGFR) status. Area a1 consists of nests without squamous or glandular differentiation, while tumor cells consisted of a few nests and were aligned amorphously in area b1. Area a was positive for PE-10 (a2) in parts of the cell cytoplasm but was negative in area b (b2). Approximately 30% of nuclei in the tumor cells were positive for TTF-1 (a3), whereas all cells were negative in b3. CK7 was positive in both a4 and b4. EGFR mutation analysis of area a revealed both mutant gene sequence CGG and the wild-type CTG coexisted in the 858th codon of exon 21 (a5; blue arrow), while only the wild-type CTG was detected in area b (b5; red arrow).

## Disclosure

No authors report any conflict of interest.

## References


1 NSCLC Meta-analyses Collaborative Group. Chemotherapy in addition to supportive care improves survival in advanced non-small-cell lung cancer: a systematic review and meta-analysis of individual patient data from 16 randomized controlled trials. *J Clin Oncol* 2008; **26**: 4617–25.

2 Wakelee H, Belani CP. Optimizing first-line treatment options for patients with advanced NSCLC. *Oncologist* 2005; **10** (**Suppl.**): 1–10.

3 Yang C-H, Shih J-Y, Chen K-C *et al*. Survival outcome and predictors of gefitinib antitumor activity in East Asian chemonaive patients with advanced nonsmall cell lung cancer. *Cancer* 2006; **107**: 1873–82.

4 Morita S, Okamoto I, Kobayashi K *et al*. Combined survival analysis of prospective clinical trials of gefitinib for non-small cell lung cancer with EGFR mutations. *Clin Cancer Res* 2009; **15**: 4493–8.

5 Paez JG, Jänne PA, Lee JC *et al*. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.

6 Pao W, Miller V, Zakowski M *et al*. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004; **101**: 13306–11.

7 Mitsudomi T, Kosaka T, Endoh H *et al*. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005; **23**: 2513–20.

8 Mok TS, Wu YL, Thongprasert S *et al*. Gefitinib or carboplatin–paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009; **361**: 947–57.

9 Rosell R, Moran T, Queralt C *et al*. Screening for epidermal growth factor receptor mutations in lung cancer. *N Engl J Med* 2009; **361**: 958–67.

10 Otani H, Toyooka S, Soh J *et al*. Detection of EGFR gene mutations using the wash fluid of CT-guided biopsy needle in NSCLC patients. *J Thorac Oncol* 2008; **3**: 472–6.

11 Horiike A, Kimura H, Nishio K *et al*. Detection of epidermal growth factor receptor mutation in transbronchial needle aspirates of non-small cell lung cancer. *Chest* 2007; **131**: 1628–34.

12 Yasuda H, Soejima K, Nakayama S *et al*. Bronchoscopic microsampling is a useful complementary diagnostic tool for detecting lung cancer. *Lung Cancer* 2011; **72**: 32–8.

13 Yasufuku K, Chiyo M, Sekine Y *et al*. Real-time endobronchial ultrasound-guided transbronchial needle aspiration of mediastinal and hilar lymph nodes. *Chest* 2004; **126**: 122–8.

14 Rivera MP, Mehta AC. Initial diagnosis of lung cancer: ACCP evidence-based clinical practice guidelines 2nd edition. *Chest* 2007; **132** (Suppl.): 131S–48S.

15 Lim E, Shah PL. Role of endobronchial ultrasound-guided transbronchial needle aspiration for mediastinal lymph node staging of lung cancer. *Thorac Cancer* 2010; **1**: 2–3.

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 Oshita F, Matsukuma S, Yoshihara M *et al*. Novel heteroduplex method using small cytology specimens with a remarkably high success rate for analysing EGFR gene mutations with a significant correlation to gefitinib efficacy in non-small-cell lung cancer. *Br J Cancer* 2006; **95**: 1070–5.

18 Garcia-Olivé I, Monsó E, Andreo F *et al*. Endobronchial ultrasound-guided transbronchial needle aspiration for identifying EGFR mutations. *Eur Respir J* 2010; **35**: 391–5.

19 Nagai Y, Miyazawa H, Huqun *et al*. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. *Cancer Res* 2005; **65**: 7276–82.

20 Tanaka T, Nagai Y, Miyazawa H *et al*. Reliability of the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based test for epidermal growth factor receptor mutations integrated into the clinical practice for non-small cell lung cancers. *Cancer Sci* 2007; **98**: 246–52.

21 Rusch VW, Asamura H, Watanabe H, Giroux DJ, Rami-Porta R, Goldstraw P, Members of IASLC Staging Committee. The IASLC lung cancer staging project: a proposal for a new international lymph node map in the forthcoming seventh edition of the TNM classification for lung cancer. *J Thorac Oncol* 2009; **4**: 568–77.

22 Riquet M, Bagan P, Le Pimpec Barthes F *et al*. Completely resected non-small cell lung cancer: reconsidering prognostic value and significance of N2 metastases. *Ann Thorac Surg* 2007; **84**: 1818–24.

23 Keller SM, Vangel MG, Wagner H *et al*. Prolonged survival in patients with resected non-small cell lung cancer and single level N2 disease. *J Thorac Cardiovasc Surg* 2004; **128**: 130–7.

24 Inoue M, Sawabata N, Takeda S, Ohta M, Ohno Y, Maeda H. Results of surgical intervention for p-stage IIIA (N2) nonsmall cell lung cancer: acceptable prognosis predicted by complete resection in patients with single N2 disease with primary tumor in the upper lobe. *J Thorac Cardiovasc Surg* 2004; **127**: 1100–06.

25 Riquet M, Assouad J, Bagan P *et al*. Skip mediastinal lymph node metastasis and lung cancer: a particular N2 subgroup with a better prognosis. *Ann Thorac Surg* 2005; **79**: 225–33.

26 Nicholson AG, McCormick CJ, Shimosato Y, Butcher DN, Sheppard MN. The value of PE-10, a monoclonal antibody against pulmonary surfactant, in distinguishing primary and metastatic lung tumours. *Histopathology* 1995; **27**: 57–60.

27 Broers JLV, Ramaekers FCS, Rot MK *et al*. Cytokeratins in different types of human lung cancer as monitored by chain-specific monoclonal antibodies. *Cancer Res* 1988; **48**: 3221–29.


 