Presence of *EGFR* mutation in pathologically non-malignant specimens from computed tomography-guided lung needle biopsies

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Abstract. Activating mutations of the epidermal growth factor receptor (*EGFR*) gene are characteristic of non-small cell lung cancer (NSCLC). *EGFR* mutations were previously detected in histologically normal lung tissue around NSCLC tumors. Computed tomography-guided lung needle biopsy (CTNB) is an accurate and useful technique for the diagnosis of lung tumors. However, pathologically non-malignant cases occasionally become apparent following lung tumor resection. In this study, we determined the *EGFR* mutational status of lung tumors diagnosed as non-malignant in CTNB specimens, but diagnosed as NSCLC following surgical resection. Between 2000 and 2008, 1,109 CTNBs were performed at Okayama University Hospital. Among them, 15 cases were initially diagnosed as non-malignant by CTNB, but diagnosed as NSCLC following surgical resection as a result of a high likelihood of malignancy by clinical findings. Twelve paired DNAs of CTNB and corresponding resected specimens were available to examine the *EGFR* mutational status using a mutant-enriched PCR assay. *EGFR* mutations were detected in one out of 12 CTNB specimens and three of the corresponding resected tumors. This case harbored the same *EGFR* mutation in the CTNB specimen and resected tumor, but not in the distant corresponding non-malignant lung tissue. Our results indicated that the detection of *EGFR* mutations may therefore aid the diagnosis of NSCLC in pathologically non-malignant CTNB specimens.

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Introduction

Activating mutations of the epidermal growth factor receptor (*EGFR*) gene are specific to non-small cell lung cancer (NSCLC), particularly adenocarcinoma (1), indicating its diagnostic value for lung tumor. The status of *EGFR* mutation is also therapeutically significant for the selection of patients for treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) (2). Approximately 90% of *EGFR* mutations are composed of either in-frame deletions in exon 19 or a specific missense mutation in exon 21 (L858R) (1-3). Direct DNA sequencing is the most accurate detection assay for *EGFR* mutations, although we and others have developed a number of highly sensitive assays capable of detecting extremely low numbers of *EGFR* mutant alleles among numerous wild-type alleles (4-7). Of note, *EGFR* mutations were previously detected in histologically normal respiratory epithelium surrounding histologically malignant regions (8).

Computed tomography (CT) imaging enables small lung tumors to be identified (9). CT-guided lung needle biopsy (CTNB) is useful for the pathological diagnosis of such tumors (10,11). However, correct diagnosis of tumors is occassionally impossible since there are no malignant cells in the CTNB specimens, particularly when materials are insufficient. CTNB specimens may also be used for molecular analysis, including those for genetic mutations (4,5). We previously demonstrated that *EGFR* mutations are capable of being detected in CTNB specimens even when the proportion of cancer cells is low, suggesting that the detection of *EGFR* mutations in the cells aids the diagnosis of NSCLC (4,7).

In this study, the *EGFR* mutational status of pathologically non-malignant CTNB specimens were examined using a modified mutant-enriched polymerase-chain reaction(PCR) assay and compared with the *EGFR* mutational status of surgically resected tumors that had a pathological diagnosis of NSCLC.

Materials and methods

Materials. Between April 2000 and August 2008, CTNB for lung tumors was performed in 1,109 patients at Okayama

Table I. Clinical characteristics of 12 computed tomography-guided lung needle biopsy specimens diagnosed as non-malignant.

Case	Age	Gender	Smoking status	[a]p-Stage	Histology of resected specimens	Size (mm)	*EGFR* mutation Tumor	CTNB
1	61	F	Never	IA	AD	11	ex19 del.	ex19 del.
2	77	M	Ever	IA	AD	13	ex19 del.	WT
3	75	M	Ever	IA	AD	22	ex19 del.	WT
4	76	F	Never	IA	AD	12	WT	WT
5	70	M	Ever	IA	AD	14	WT	WT
6	76	M	Ever	IA	SQ	15	WT	WT
7	57	M	Ever	IB	AD	40	WT	WT
8	75	M	Ever	IA	AD	30	WT	WT
9	72	F	Never	IA	AD	12	WT	WT
10	77	M	Ever	IA	AD	22	WT	WT
11	58	M	Ever	IA	AD	13	WT	WT
12	73	M	Ever	IA	AD	10	WT	WT

F, female; M, male; Never, never-smoker; Ever, ever-smoker; AD, adenocarcinoma; SQ, squamous cell carcinoma; Size, tumor diameter in surgically resected specimens; Tumor, specimen of surgically resected tumor; CTNB, computed tomography-guided lung needle biopsy; ex19 del., exon 19 deletion; WT, wild-type. [a]p-Stage, pathological stage was determined according to International Union Against Cancer TNM classification of malignant tumors (6th edition).

University Hospital (Japan). Histological diagnosis was determined using hematoxylin and eosin (H&E)-stained sections of CTNB specimens and surgically resected tumors. Of these tumors, 24 tumors were diagnosed as non-malignant, but the clinical findings suggested a high likelihood of malignancy. Finally, 15 of the 24 tumors were diagnosed as primary lung cancer. Twelve of the tumors were available for further study. The clinical data of these 12 cases are shown in Table I. DNA was extracted from the paraffin-embedded specimens of the CTNB and the surgically resected tumors without microdissection using the QIAamp® DNA FFPE tissue kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions.

Methods. EGFR gene mutations in exons 19 and 21 were determined using mutant-enriched PCR, a two-step PCR with intermittent restriction digestion to selectively eliminate wild-type alleles and to enrich mutated alleles (4,7). Our previously developed mutant-enriched PCR assay was initially used on all 12 CTNB samples. However, amplification was not achieved in some CTNB samples, particularly when they were small specimens. Therefore, we developed a modified mutant-enriched PCR to detect exon 19 deletions in which the amplicon was shorter than that of the previous version (79 bp versus 138 bp). Primer sequences were as follows: 5'-AAGATAAAATTCCCGTCGCT-3' (forward primer) and 5'-CTCACATCGAGGATTTCCTTG-3' (reverse primer), and PCR conditions were the same as those of our previous study. The first round of amplification used 5-100 ng sample DNA, 150 µmol/l deoxynucleotide triphosphate, 2 pmol of each primer and 0.25 units HotStarTaq DNA polymerase (Qiagen, Inc.). Intermittent restriction digestion of 2 µl first-round PCR

products was carried out with 10 IU *Mse*I at 37°C for 6 h. An aliquot of first-round PCR product was used as a template for second-round amplification under the same conditions as the first-round PCR. The product of the second amplification was analyzed by ethidium bromide staining of 12% polyacrylamide gel electrophoresis and reproducibility was confirmed in two independent experiments.

Results

Clinical characteristics. The histological diagnoses of CTNB specimens and surgically resected specimens were re-evaluated to confirm that the CTNB samples of all 12 cases showed no malignancy, whereas all of the pathological diagnoses of corresponding resected specimens were NSCLCs. The characteristics of the cases are shown in Table I.

Mutations. We determined the *EGFR* mutational status of 12 surgically resected specimens, and identified three deletions in exon 19 (25%), but no *EGFR* exon 21 L858R mutations. The mutational status of CTNB samples was blindly evaluated using the modified mutant-enriched PCR assay, with the finding that one sample harbored an *EGFR* exon 19 deletion (8.3%). This result was confirmed in two independent experiments. The histological images of CTNB and the resected tumor of Case 1 are shown in Fig. 1. The pathological findings of the Case 1 tumor revealed a clear separation of malignant and non-malignant cell areas (Fig. 1A). Of note, the same type of *EGFR* exon 19 deletion (E746 to A750) was present in the CTNB specimen and the resected tumor of Case 1 (Fig. 2, sequencing data is not shown). In addition, the *EGFR* mutation was not present in the distant peripheral normal lung
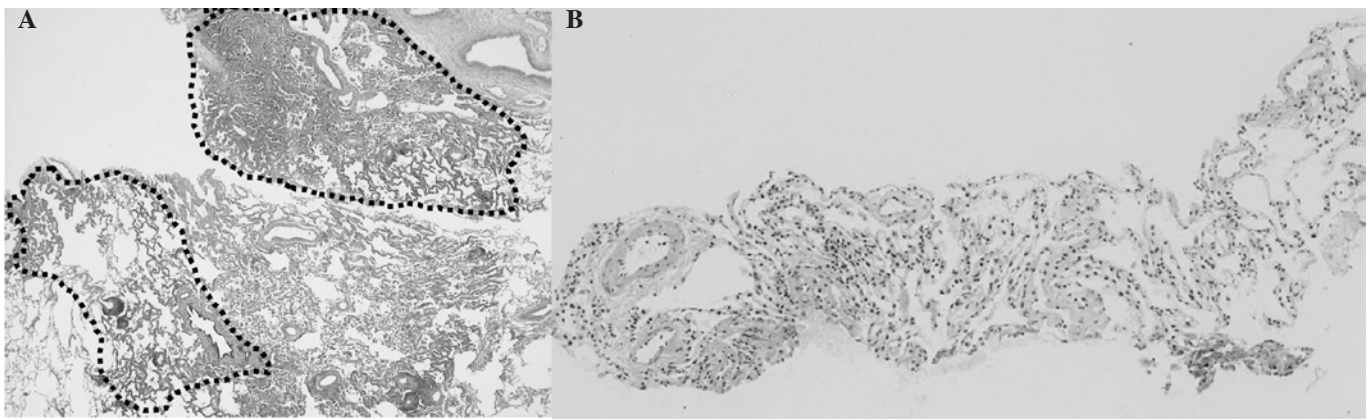
Figure 1. Microscopic findings of (A) the surgically resected and (B) computed tomography-guided lung needle biopsy (CTNB) specimens in Case 1. The portion circled by the black dotted line revealed malignant tumor cells in the surgically resected specimen. Malignant cells were not detected in the CTNB specimen. Magnification, x2 and x4, respectively.
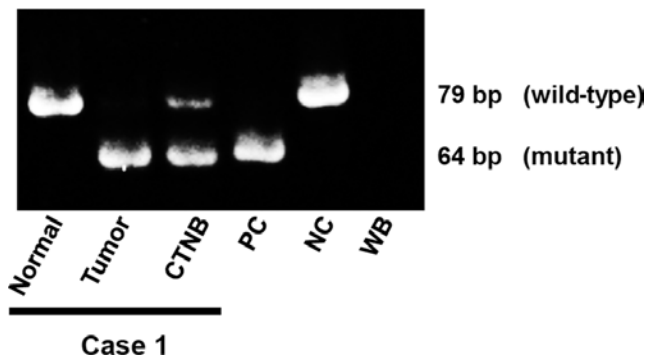

Figure 2. Results of the mutant-enriched assay in the surgical and computed tomography-guided lung needle biopsy (CTNB) specimens in Case 1. Normal, normal section of surgically resected lung tissue distant from tumor; Tumor, specimen of surgically resected tumor; CTNB, CTNB specimen; PC, positve control; NC, negative control; WB, water blank. EGFR exon 19 deletions were present in both the CTNB specimen and surgically resected tumor in the same case using a modified mutant-enriched PCR assay. PCR, polymerase-chain reaction.

tissue of Case 1. No *EGFR* mutation was found in the CTNB samples of Cases 2 and 3.

Discussion

The detection of *EGFR* mutations is crucial for the diagnosis of NSCLC as well as for selecting patients for EGFR-TKI treatment (2,12,13). Sensitive assays have been developed to detect *EGFR* mutations in small clinical samples that are assumed to contain numerous non-malignant cells, including CTNB specimens, pleural effusion and circulating tumor cells (4-7,14). In the present study, *EGFR* mutations were detected in pathologically non-malignant CTNB specimens using our modified mutant-enriched PCR assay. This finding may be explained in two ways: i) the presence of a small portion of malignant cells only in DNA-extracted sections and not in the H&E-stained section, ii) the presence of *EGFR* mutations in the lung tissue of normal-appearing surrounding tumor tissue harboring *EGFR* mutations. The latter is supported by a previous study by Tang *et al* (8).

These authors determined *EGFR* mutational status in 21 lung adenocarcinoma cases and their adjacent and distant normal lung tissues to find that *EGFR* mutations were present in 43% of adenocarcinomas, 24% of adjacent normal tissues and none of the distant tissues (8). These authors also reported that the mutational patterns in the tumors and corresponding adjacent, non-malignant specimens were identical. Of note, more frequent mutations affecting normal epithelium were found in exon 19 (54%) compared with exon 21 (7%). The mutational type of the mutant case in this study in a non-malignant CTNB specimen was also an exon 19 deletion. Our results support the hypothesis that a localized type of field effect phenomenon may exist for *EGFR* mutations in the lung respiratory epithelium surrounding lung adeno-carcinomas (8).

Our results also have clinical implications. The administration of EGFR-TKIs is recommended for patients with *EGFR* mutation and is exclusively found in NSCLC (15). Thus, detection of an *EGFR* mutation from lung tumors may be considered to be NSCLC. As shown, sensitive assays may be capable of detecting *EGFR* mutations from pathologically non-malignant CTNB specimens, resulting in avoidance of additional invasive procedures for patients. Our study suggests that the *EGFR* mutation should be analyzed even in biopsied specimens with non-malignant diagnosis to determine whether patients should be treated with EGFR-TKI when they are clinically suspected as having NSCLC.

Acknowledgements

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