Assessment of Intratumoral Heterogeneity of Oncogenic Driver Mutations in Surgically-resected Lung Adenocarcinoma: Implications of Percutaneous Biopsy-based Molecular Assay for Target-directed Therapy

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Abstract. *Aim: The present study investigated whether there is intratumoral heterogeneity of oncogenic driver mutations within surgically-resected tumors and between surgical specimens and percutaneous biopsy samples. Patients and Methods: Thirty-four patients who underwent surgery for lung adenocarcinoma were studied. We obtained four to five snap-frozen samples from each surgical specimen. Mutational analyses of epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral homolog (KRAS), v-raf murine sarcoma viral oncogene homolog B (BRAF), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit-alpha (PIK3CA) genes were performed and then compared in multiple surgical specimens and between surgical and percutaneous biopsy samples. Results: EGFR and KRAS mutations were detected in 19 and 2 patients, respectively. Multiple surgical samples from different areas of the tumor had the same mutation genotype in all cases except for one. The 14 biopsy specimens had the same mutational profiles as the corresponding surgical specimens. Conclusion: Heterogeneous distributions of oncogenic driver mutations were not found in surgically-resected lung adenocarcinoma. Small tumor specimens obtained with percutaneous biopsy were suitable for EGFR analyses, thus providing critical information for personalized therapy.*

Advances in our understanding of oncogenic driver mutations and signaling pathways have led to the development of targeted-therapies for cancer treatment (1). In non-small cell lung cancer (NSCLC), epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib have been extensively studied in many clinical trials (2-5). Efforts to identify which patients are most likely to benefit from EGFR-TKIs revealed that those harboring an activating EGFR mutation (exon 19 deletion and L858R in exon 21) achieve a higher response rate and prolonged progression-free survival (PFS) (6-9). Therefore, the presence of an EGFR-activating mutation is considered a predictive marker for treatment response and PFS.

However, it should be noted that not all patients with activating EGFR mutations respond to EGFR-TKIs and, conversely, some patients with wild-type EGFR do respond to EGFR-TKIs (10-12). These unpredictable responses to EGFR-TKIs regardless of the presence of activating EGFR mutations can be explained by intratumoral genetic heterogeneity (13). If genetically-distinct cells are present within the same tumor, the results of mutational analysis may be inconsistent among biopsies obtained from different areas of the tumor and thus the predictiveness of biomarkers might be compromised depending on the distribution of the mutation. In addition, even patients who responded to EGFR-TKIs on initial treatment may develop resistance during treatment (14, 15). This phenomenon can be explained by the out-growth of pre-existing unresponsive cells harboring additional mutations in EGFR-dependent signaling molecules, such as components of the Kirsten rat sarcoma viral homolog

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(KRAS)-v-raf murine sarcoma viral oncogene homolog B (BRAF) and the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA)-phosphatase and tensin homolog (PTEN)-v-akt murine thymoma viral oncogene homolog 1 (AKT) pathways (16-18). Because cancer diagnosis is made based on biopsies from a small region of a tumor that may not necessarily represent the mutational status of the entire tumor, intratumoral genetic heterogeneity poses a challenge for personalized medicine and ultimately affects the efficacy of targeted therapies.

Therefore, understanding tumor heterogeneity in NSCLC with regard to *EGFR, KRAS, BRAF,* and *PIK3CA* genotypes is crucial in order to establish the appropriate method for obtaining lung cancer tissues for molecular assays and setting treatment guidelines based on the molecular findings. In the present study, we obtained at least four different snap-frozen samples from surgically-resected lung tumors and investigated the incidence and heterogeneity of EGFR, KRAS, BRAF, and PIK3CA mutations using highly sensitive assay techniques. In addition, we compared the results of these mutational analyses with those of low-volume tissue samples that were procured separately for histological examination during a diagnostic biopsy procedure in the context of routine clinical workflow.

Materials and Methods

Patient population. From August 2010 to January 2012, 46 consecutive patients who underwent pulmonary resection for operable NSCLC at our Institution were prospectively enrolled in this study. The study was reviewed and approved by the Institutional Review Board of Samsung Medical Center. All patients gave written informed consent for study participation and for genomic and molecular analyses (SMC 2008-03-006). Among these, 25 patients also underwent preoperative percutaneous transthoracic core needle biopsy.

Lung biopsy and resection were performed within a 1-month time interval (median=14 days; range=6-24 days). All biopsies were performed by a single thoracic intervention-trained radiologist (H.Y.L.) who had performed more than 1,000 imaging-guided lung biopsy procedures during six years of experience. Lung biopsy was performed using an 18-gauge single-shaft gun biopsy needle with a 22-mm active needle (Gunbiopsy; M. I. Tech, Seoul, Korea) through C-arm cone-beam computed tomography (CT) guidance (Allura Xper FD20; Philips Healthcare, Best, the Netherlands) as previously described (19). For each target tumor, biopsy sampling was performed only once; therefore one core tissue specimen was obtained with each biopsy sampling. Approximately half of the specimen were freshly-frozen and sent to the genetics laboratory of our Institution for genetic analysis. The other half of the specimen was immersed in a 10% formalin solution for pathological confirmation of the presence of tumor cells (20).

In terms of the handling of specimens during surgery, samples were obtained immediately after removal of lung tumor specimens from patients. The tumor was cut into transverse sections at a region that appeared to have the maximum diameter. Four samples were harvested from the periphery of the tumor at the 12-, 3-, 6-, and 9-o'clock positions. If possible, one additional sample was harvested from the center of the tumor. Sample size should be at least 1×1×1 mm^3 to guarantee the success of molecular analyses. Samples were microdissected to minimize stromal contamination and snap-frozen in liquid nitrogen. The remaining part of the sample was delivered to the Pathology Department for further pathological diagnosis. Sample harvesting was performed according to a strict pre-decided protocol to reduce variability between sampling methods.

Tissue/nucleic acid preparation and mutation testing. The histopathological sections were examined by a pathologist who is an expert in lung cancer and the diagnosis of adenocarcinoma was confirmed in all cases. The tumor samples selected for molecular analysis contained at least 70% neoplastic tissue. DNA was extracted from fresh-frozen samples with the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For each sample, mutational analyses of *EGFR* (exon 18-21), *KRAS* (exon 2), *PIK3CA* (exon 9, 20), and *BRAF* (exon 14) were performed by directional sequencing of polymerase chain reaction (PCR) fragments amplified with genomic DNA from fresh tissue. PCR was performed in a 20 μL volume containing 100 ng of template DNA, 10× PCR buffer; 0.25 mM dNTPs, 10 pmol primers, and 1.25 U Taq DNA polymerase (iNtRON, Korea). PCR products were electrophoresed on 2% agarose gels and purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Bidirectional sequencing was performed using the BigDye Terminator v.1.1 kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl genetic analyzer (Applied Biosystems). Sequencer version 4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA) was used in combination with manual chromatogram review for sequence analysis. Confirmatory re-sequencing from replicate PCR amplification reactions was performed for any sequences that were ambiguous or deviated from wild-type, such that all abnormal sequences were confirmed in at least quadruplicate amplification reactions. The results were marked as mutation-positive if a mutation was detected in both the forward and reverse DNA strand.

Results

Patients' characteristics. Specimens of 25 patients who successfully underwent preoperative biopsy were inadequate for mutational analysis due to insufficient material (n=1) or preoperative induction treatment (n=10). One patient among 21 surgical sample-only patients was excluded because the integrity of tumor specimens was damaged by the surgical procedure (n=1). Therefore, 14 patients with both biopsy samples and surgical samples and 20 patients with multiple surgical sampling-only were finally included in our study. The total study population was 18 men and 16 women, with a mean age of 64 (range=42 to 77) years. All patients had adenocarcinoma. Seventeen patients were former- or current-smokers and the remaining 17 patients were never-smokers. Overall, there were 15 never-smoker female patients with adenocarcinoma. The extent of pulmonary resection was lobectomy in 30 patients (88.2%), wedge resection in three (8.8%), and bilobectomy in one (3%). Most patients had pathological stage IA (n=8) or IB (n=14) disease. The average mass size was 3.4 cm (range=1.8-8.9 cm). Details of patient characteristics are summarized in Table I.

Mutations in primary adenocarcinoma. We determined the mutational status of four major driver oncogenes in 34 pulmonary adenocarcinomas. Nineteen patients (55.9%) had *EGFR* mutation including four exon 21 L858R substitutions and 15 with exon 19 deletions. Two patients (5.9%) had a *KRAS* G12C mutation. There were no *PIK3CA* or *BRAF* mutations. EGFR and KRAS mutations were mutually exclusive (Table II).

Heterogeneity of EGFR and KRAS mutations in primary adenocarcinoma. In all cases except one, multiple surgical samples had the same mutation genotype in all five areas of tumor tested. However, one case (case 20) had a heterogeneous pattern. This patient was a 60-year-old female never-smoker with a 2×1.8 cm mass in her right upper lobe. Lobectomy was performed and the mass was diagnosed as adenocarcinoma with an acinar pattern. Two foci were wild-type for EGFR and three foci exhibited EGFR exon 19 deletion L747_T751 (Figure 1). To confirm heterogeneity in the tumor, we re-tested for *EGFR* mutation using archival formalin-fixed paraffin-embedded tissue. Three blocks were tested and had the same EGFR deletion mutation in all three areas.

Reliability of core biopsy in the assessment of driver mutations in adenocarcinoma. Regarding CT features of adenocarcinomas of 14 patients who underwent percutaneous transthoracic biopsy, seven tumors were solid, one had non-solid features, and six were part-solid. Three out of seven solid tumors showed heterogeneous enhancement. The mean CT attenuation value of all tumors was 11.7 Hounsfield units (HU), ranging from 15 to 44 HU. The mutational profiles of driver genes were the same in these 14 biopsy cases as in the corresponding surgical samples. Six cases of part-solid tumors had the identical EGFR mutation in four surgical specimens and a biopsy tissue. One solid tumor with heterogeneous enhancement had KRAS mutation in both surgical specimens and biopsy tissue. The other two solid tumors with heterogeneous enhancement were wild-type in all mutation profiling.

Table I. *Summary of clinical and pathological characteristics*.

	No.	%
Mean age, years (range)	64 (42-76)	
Gender		
Male	18	53
Female	16	47
Smoking status		
Never smoker	17	46
Current smoker	5	17
Ex-smoker	12	37
Pathological stage		
IA	8	23
IB	15	43
IIA	1	3
IIB	2	6
IIIA	5	14
IIIB	1	3
IV	3	9

Discussion

EGFR-activating mutation is considered a strong predictive biomarker for the treatment of EGFR-TKIs (6-9). However, this is not always reliable because not all patients who carry the activating mutation respond to treatment and some patients do respond despite the absence of mutations (10-12). Many investigators have tried to explain this by the phenomenon of intra-tumoral genetic heterogeneity (21-23). Therefore, we analyzed the mutational status of different regions of the same tumor in patients undergoing surgical resection for NSCLC to investigate the incidence of intra-tumoral genetic heterogeneity of driver mutations. Among 34 patients, nine (55.9%) had *EGFR* mutations and two (5.9%) had *KRAS* mutations. *PIK3CA* or *BRAF* mutations were not detected in any of the patients. Considering the clinical characteristics of our study population, the incidence of these mutations is in line with previous reports (24). One case exhibited a heterogeneous distribution of EGFR mutations among different areas obtained from the same primary tumor; two foci were wild-type and three foci had *EGFR* exon 19 deletion. Repeat testing using archival formalin-fixed paraffin-embedded tissue revealed that two tested regions had the same *EGFR* exon 19 deletion.

Several researchers have reported the rarity of intra-tumoral genetic heterogeneity in lung adenocarcinoma. Yatabe *et al*. evaluated the distribution of *EGFR* mutations within tumors of patients with lung adenocarcinoma and showed that heterogeneous distribution of *EGFR* mutations was extremely rare (25). They suggested that the intra-tumoral heterogeneity reported in previous studies might have been pseudo-heterogeneity resulting from a mutant allele-specific imbalance or heterogeneously-distributed *EGFR* amplification. However, despite such reports denying the heterogeneous distribution of *EGFR* or *KRAS* mutations, there is a growing body of experimental and clinical evidence to support the presence of intratumoral heterogeneity. Gerlinger and colleagues analyzed multiple regions of two primary clear cell renal carcinomas and associated metastatic sites and demonstrated that 63% to 69% of all non-synonymous somatic mutations were identified across multiple biopsies (26). Bai *et al*. evaluated intratumoral heterogeneity of *EGFR* mutations in 85 patients with stage IIIA to IV NSCLC who underwent palliative surgical resection and reported that approximately 30% of patients exhibited intratumoral *EGFR* mutational heterogeneity (21).

Table II. *Clinicopathological features and mutational profiles in individual patient.*

Case No.	Gender	Age (years)	Size (cm)	Sub-type	Location	TNM	Smoker	Mutation
1	M	57	2.8	A	LUL	pT1bN1M1b	Y	*EGFR L858R*
2	M	77	2.5	A,L	LUL	cT1bN0M0	Y	*EGFR L858R*
3	F	76	4.1	A,P,MP	LLL	pT2aN0M0	N	*EGFR E746_A750del*
4	M	53	3.0	A,P	LUL	pT1bN0M0	Y	*EGFR E746_A750del*
5	F	52	1.5	P	RUL	pT1aN2	N	*EGFR E746_A750del*
6	F	65	2.9	A,P	LLL	pT1bN0M0	N	*EGFR E746_A755del*
7	F	63	2.0	A,P,L	LLL	pT1aN0	N	*EGFR L747_A750>P*
8	F	69	1.3	A	LLL	pT1bNOM1a	N	*EGFR V769_D770insASV*
9	F	66	2.8	A,L	RUL	pT1bN0M0	N	*EGFR P772_H773insNP*
10	M	57	8.9	S,A	LUL	T3N0M1	Y	*KRAS Gly12Cys*
11	F	52	2.3	A	RUL	pT1bN2M0	N	
12	F	68	3.1	P,MP	LLL	pT2aN0	N	
13	M	64	3.5	S,A	LUL	pT2aN0	Y	
14	M	57	7.0	A	LUL	pT2aN0M0	Y	
15	M	76	2.4	A	RUL	pT3N2M0	N	*EGFR L858R*
16	F	76	2.5	A,P	RML	pT2N0M0	N	*EGFR L858R*
17	F	64	2.6	A,L	LLL	pT1bN0M0	Y	*EGFR E746_A750del*
18	M	56	3.0	A,L	LLL	pT1bN0M0	Y	*EGFR E746_A750del*
19	M	71	3.2	A,MP,S	LLL	T1bN2M1b	Y	*EGFR E746_S752>V*
20	F	60	2.0	A	RUL	pT1aN0M0	N	*EGFR L747_T751del**
21	F	59	2.8	A	RUL	pT2N2M0	N	*EGFR A763_Y764insFQEA*
22	M	69	4.7	A,L	RUL	pT2aN0M0	Y	*EGFR V769_D770insASV*
23	M	73	4.7	P,MP	RLL	pT2aN0	Y	*EGFR D770_N771insSVD*
24	F	75	3.2	A	LLL	pT2aN0M0	N	*EGFR H773_V774insH*
25	M	68	3.6	A,MP	RLL	pT2aN0M0	Y	*KRAS Gly12C*ys
26	M	47	4.3	A,S	RML	pT2aN0M0	N	
27	M	61	4.7	P,MP	LLL	pT2aN1	Y	
28	M	59	4.5	S,A	RLL	pT2aN0M0	Y	
29	M	73	3.5	Enteric	RUL	pT2aN0M0	Y	
30	M	76	2.4	A	RUL	pT1bN2	Y	
31	M	60	3.5	S	RUL	pT2N1M0	Y	
32	F	66	3.0	A	RLL	pT2N0	N	
33	F	42	1.8	S,P	LLL	pT1aN0M0	N	
34	F	62	5.0	P,A	LUL	pT2aN0M0	N	

1-14: Biopsy and four sites from resection; 15-34: five sites from resection; ADC: adenocarcinoma; A: acinar, L: lepidic, P: papillary, MP: micropapillary, S: solid, RUL: right upper lobe, RML: right middle lobe, RLL: right lower lobe, LUL: left upper lobe, LLL: left lower lobe, Y: current or ex-smoker, N: never-smoker; EGFR: epidermal growth factor receptor; KRAS: Kirsten rat sarcoma viral homolog.

The heterogeneous distribution of mutations raises several important issues in clinical practice. First of all, mutational analyses based only on a small fraction of a tumor may not be reliable if the biopsy sample does not represent the mutational status of the entire tumor. If a tumor consists entirely of homogeneous wild-type or mutant cells, sampling any portion of the tumor would result in identical mutational status. However, if wild-type and mutant cells are mixed within the same tumor, topological segregation of cells with or without activating *EGFR* mutation can lead to lack of consistency among core biopsies obtained from different areas within the same tumor. Another important issue is that intratumoral genetic heterogeneity may contribute to resistance to EGFR-TKIs. Patients who responded to EGFR-TKIs on initial treatment may develop resistance during treatment. A possible underlying mechanism is that targeted therapies might favor the clonal selection and outgrowth of preexisting resistant cells that harbor secondary mutations in genes encoding the target proteins (13, 26). The concept of intratumoral genetic heterogeneity implies that these resistant cells are already present before the onset of therapy, although they were not predominant at the time of diagnosis. For example, the positive selection of NSCLC cells with secondary T790M mutations in EGFR confers resistance to gefitinib (14, 15) and cells harboring met proto-oncogene (MET) amplification confer resistance to EGFR-TKI by activating the v-erb-b2 avian erythroblastic leukemia viral oncogene homolog-3 (ERBB3) pathway (27).

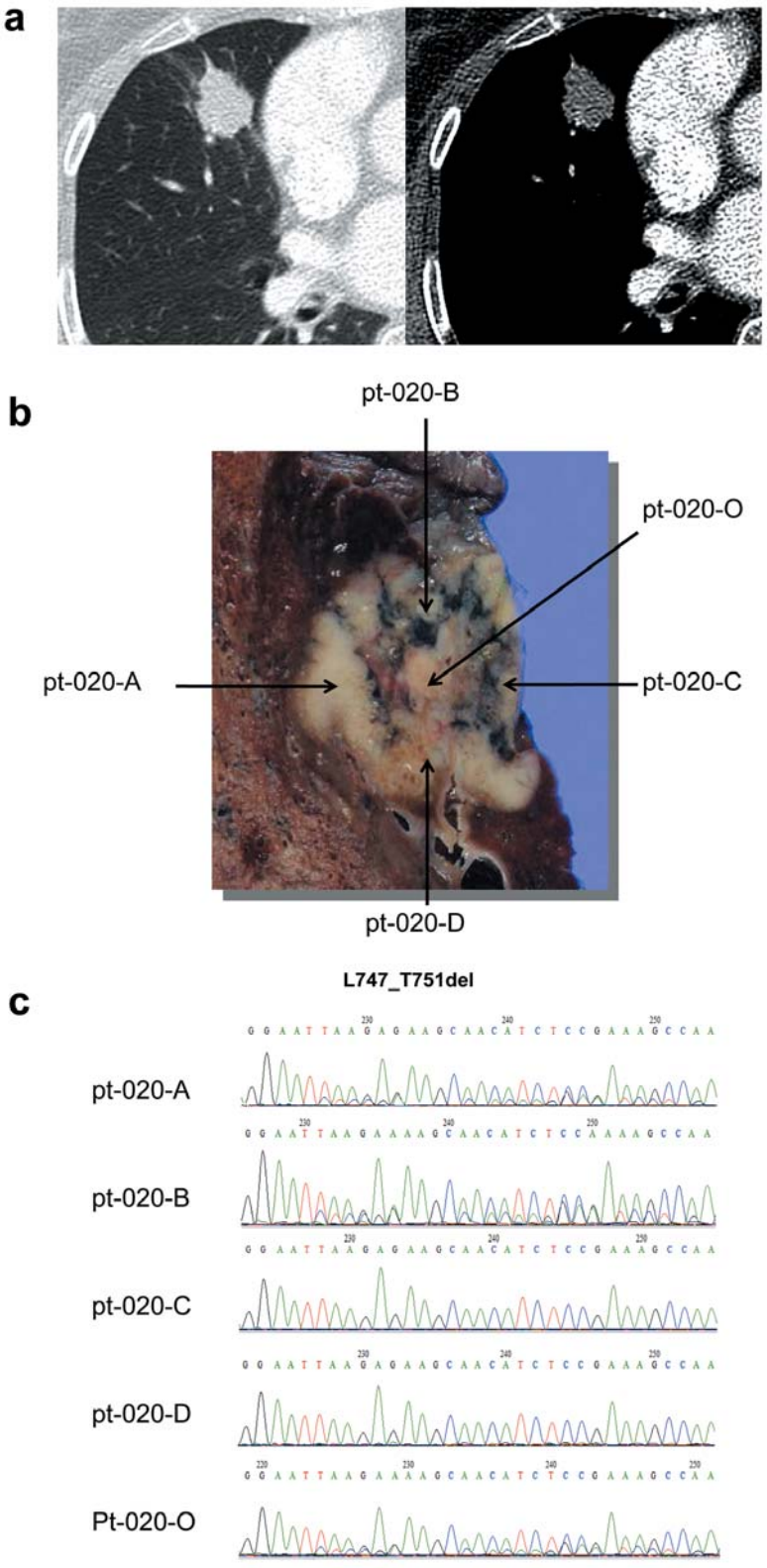

Figure 1. *Invasive adenocarcinoma in a 60-year-old woman. a: Transverse computed tomography scans showing a 2-cm round, lobulated, solid mass in the right upper lobe, which presents a homogenous attenuation with mean attenuation value of 45 HU and standard deviation of attenuation of 28 HU. b: The lobectomy specimen shows a solid mass with the five sites of sampling for mutation analysis indicated. c: Chromogram from five regions of lung adenocarcinoma. Sequencing showed wild-type sequence in two samples and epidermal growth factor receptor (EGFR) deletion mutation in three samples.*

On the basis that there is sufficient evidence supporting the existence of intratumoral heterogeneity, our findings can be interpreted in several ways. Firstly, since driver mutations such as *EGFR* or *KRAS* occur early in the carcinogenesis of lung adenocarcinoma, subsequent clonal expansion will distribute the mutation throughout the tumor. This means that somatic genetic alterations present at the early stages of tumor development are likely to occur ubiquitously at all sites of the tumor. However, ubiquitous distribution of driver mutations does not necessarily mean that the mutational profiles are genetically-homogeneous throughout the tumor. Intra-tumoral genetic heterogeneity cannot be denied based only on the fact that it is difficult to detect. In our study, we used conventional Sanger sequencing, which has a relatively low sensitivity and this might have affected our results.

Secondly, sampling four or five different regions within the same tumor for mutational analyses may not be sufficient to reveal the presence of intra-tumoral genetic heterogeneity. If the driver mutations are truly ubiquitous throughout the tumor, we should have obtained a much larger number of samples from different sites in the tumor in order not to miss relatively small areas of genetically-distinct cells, because it cannot be guaranteed that four or five regions would represent the rest of the tumor. In contrast to our study, Bai *et al*. obtained 28 to 34 foci per tumor by microdissection of bulk tumor tissue, in their study, evaluating the intratumoral heterogeneity of *EGFR* mutations (21). Yatabe *et al*. divided lung adenocarcinoma specimens into 100 even parts and found identical *EGFR* mutations throughout individual tumors (25). Moreover, cross-sectioning the tumor and obtaining samples from four or five different foci appears to be inadequate given that mutations are ubiquitous throughout the three-dimensional structure of the tumor.

Thirdly, intra-tumoral heterogeneity may have been missed due to pooling of numerous non-neoplastic cells in individual samples, even though there were a substantial number of mutated cells present. Admixture of non-neoplastic cells, such as stromal or inflammatory cells, within the tumor can easily result in a false-negative result. This may be related to the fact that more patients with early-stage NSCLC were included in the study population than in previous reports. As image-guided lung biopsies are being increasingly performed (28), the current challenge of image-guided core needle biopsy is actually the risk of sampling error. However, our results show that accurate determination of oncogenic driver mutation status is possible with percutaneous core needle biopsy. The presence of a sub-solid component within a nodule or heterogeneous enhancement of a tumor on CT images was also not an obstacle to obtaining sufficient and reliable material for mutational analysis. Furthermore, in every case with available biopsy sample, all of the mutational profiles of driver genes were same in the biopsy as in multiple surgical samples. Therefore, if sufficient tumor tissue can be obtained, percutaneous transthoracic core needle biopsy performed by an experienced radiologist can be reliably used to retrieve tissue for molecular profiling. In our study, C-arm cone-beam CT-guided percutaneous transthoracic biopsy was performed for a tumor with central necrosis that could be identified and consequently avoided in the needle path based on CT images (19). On the other hand, because the resolution of CT scan images is not high enough to enable the identification of minor necrosis, there could be sampling error in lesions with minor necrosis. In such a case, positron emission tomography/CT would be helpful to depict the viable portion of the target lesion. In addition, we previously found that acquisition of specimens larger than 5 mm significantly increased the probability of a successful gene study compared with specimens of 5 mm or smaller because the sampling error decreased (19). On-site pathologists are not always stationed at the procedure field to confirm that diagnostic material has been sufficiently obtained. Thus, gross confirmation of image-guided viable tumor targeting and sample size evaluation of the target lesion is the most feasible approach in daily practice.

Nevertheless, we need to consider identifying patients at high risk of a false-negative result when the analysis is conducted using small samples (29, 30). As an alternative, assessment of tumor heterogeneity using imaging may be another potential approach to investigate tumor heterogeneity (31). Imaging methods assess how grainy or coarse a tumor appears on imaging. PET and CT have both been used to derive tumor textural information and the appearance on such scans has been shown to relate to patient outcome in esophageal and colorectal cancer, as well as NSCLC (32-34). It is important to investigate whether textural heterogeneity on imaging relates to underlying genomics. Given the challenges and expense of measuring tumor genomic signatures, imaging may be a more viable option. Targeted biopsy and sampling based on the textural heterogeneity may be helpful to identify hot spots that reflect the genomic signature of the target lesion. The strength of this radiogenomic approach is that it allows for the quantification of tumoral heterogeneity as a whole and *in vivo*.

In summary, we investigated the incidence and heterogeneous distributions of oncogenic driver mutations within surgically resected lung adenocarcinoma. In the majority of cases, no heterogeneity was found among multiple surgical specimens. This may be because driver mutations such as *EGFR* or *KRAS* are widespread throughout the tumor, but technical issues regarding sampling methods should also be considered. Comparison of the mutational profiles between multiple surgical specimens and percutaneous biopsy samples showed that accurate determination of oncogenic driver mutation status was possible on percutaneous core needle biopsy. More meticulous examination needs to be conducted to investigate intra-tumoral genetic heterogeneity for mutations associated with resistance to target-directed therapy.

Conflicts of Interest

There were no conflicts of interest relevant to this article.

Acknowledgements

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References

1 Sun S, Schiller JH, Spinola M and Minna JD: New molecularly targeted therapies for lung cancer. J Clin Invest *117*: 2740-2750, 2007.

2 Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP and Baselga J: Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small cell lung cancer (The IDEAL 1 Trial) [corrected]. J Clin Oncol *21*: 2237-2246, 2003.

3 Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, Nishiwaki Y, Ohe Y, Yang JJ, Chewaskulyong B, Jiang H, Duffield EL, Watkins CL, Armour AA and Fukuoka M: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med *361*: 947-957, 2009.

4 Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, Fujita Y, Okinaga S, Hirano H, Yoshimori K, Harada T, Ogura T, Ando M, Miyazawa H, Tanaka T, Saijo Y, Hagiwara K, Morita S, Nukiwa T; North-East Japan Study Group: Gefitinib or chemotherapy for non-small cell lung cancer with mutated EGFR. N Engl J Med *362*: 2380-2388, 2010.

5 Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabárbara P, Seymour L; National Cancer Institute of Canada Clinical Trials Group: Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med *353*: 123-132, 2005.

6 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science *304*: 1497-1500, 2004.

7 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small cell lung cancer to gefitinib. N Engl J Med *350*: 2129-2139, 2004.

8 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M and Varmus H: 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 *101*: 13306-13311, 2004.

9 Mitsudomi T, Kosaka T, Endoh H, Horio Y, Hida T, Mori S, Hatooka S, Shinoda M, Takahashi T and Yatabe Y: 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 *23*: 2513-2520, 2005.

10 Uramoto H and Mitsudomi T: Which biomarker predicts benefit from EGFR-TKI treatment for patients with lung cancer? Br J Cancer *96*: 857-863, 2007.

11 Han JY, Park K, Kim SW, Lee DH, Kim HY, Kim HT, Ahn MJ, Yun T, Ahn JS, Suh C, Lee JS, Yoon SJ, Han JH, Lee JW, Jo SJ and Lee JS: First-SIGNAL: First-line single-agent iressa versus gemcitabine and cisplatin trial in never-smokers with adenocarcinoma of the lung. J Clin Oncol *30*: 1122-1128, 2012.

12 Yang CH, Yu CJ, Shih JY, Chang YC, Hu FC, Tsai MC, Chen KY, Lin ZZ, Huang CJ, Shun CT, Huang CL, Bean J, Cheng AL, Pao W and Yang PC: Specific EGFR mutations predict treatment outcome of stage IIIB/IV patients with chemotherapy-naive non-small cell lung cancer receiving first-line gefitinib monotherapy. J Clin Oncol *26*: 2745-2753, 2008.

13 Swanton C: Intratumor heterogeneity: Evolution through space and time. Cancer Res *72*: 4875-4882, 2012.

14 Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG and Halmos B: EGFR mutation and resistance of non-small cell lung cancer to gefitinib. N Engl J Med *352*: 786-792, 2005.

15 Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med *2*: e73, 2005.

16 Eberhard DA, Johnson BE, Amler LC, Goddard AD, Heldens SL, Herbst RS, Ince WL, Jänne PA, Januario T, Johnson DH, Klein P, Miller VA, Ostland MA, Ramies DA, Sebisanovic D, Stinson JA, Zhang YR, Seshagiri S and Hillan KJ: Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small cell lung cancer treated with chemotherapy alone and in combination with erlotinib. J Clin Oncol *23*: 5900-5909, 2005.

17 Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borrás AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J, Tracy S, Zhao X, Heymach JV, Johnson BE, Cantley LC and Jänne PA: Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. J Clin Invest *116*: 2695-2706, 2006.

18 Ludovini V, Bianconi F, Pistola L, Chiari R, Minotti V, Colella R, Giuffrida D, Tofanetti FR, Siggillino A, Flacco A, Baldelli E, Iacono D, Mameli MG, Cavaliere A and Crinò L: Phosphoinositide-3-kinase catalytic alpha and KRAS mutations are important predictors of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in patients with advanced non-small cell lung cancer. J Thorac Oncol *6*: 707-715, 2011.

19 Yoon HJ, Lee HY, Lee KS, Choi YL, Ahn MJ, Park K, Ahn JS, Sun JM, Kim J, Kim TS, Chung MJ and Yi CA: Repeat biopsy for mutational analysis of non-small cell lung cancers resistant to previous chemotherapy: Adequacy and complications. Radiology *265*: 939-948, 2012.

20 Cheung YC, Chang JW, Hsieh JJ, Lin G and Tsai YH: Adequacy and complications of computed tomography-guided core needle biopsy on non-small cell lung cancers for epidermal growth factor receptor mutations demonstration: 18-Gauge or 20-Gauge biopsy needle. Lung Cancer *67*: 166-169, 2010.

21 Bai H, Wang Z, Wang Y, Zhuo M, Zhou Q, Duan J, Yang L, Wu M, An T, Zhao J and Wang J: Detection and clinical significance of intratumoral EGFR mutational heterogeneity in Chinese patients with advanced non-small cell lung cancer. PLoS One *8*: e54170, 2013.

22 Chen ZY, Zhong WZ, Zhang XC, Su J, Yang XN, Chen ZH, Yang JJ, Zhou Q, Yan HH, An SJ, Chen HJ, Jiang BY, Mok TS and Wu YL: EGFR mutation heterogeneity and the mixed response to EGFR tyrosine kinase inhibitors of lung adenocarcinomas. Oncologist *17*: 978-985, 2012.

23 Grob TJ, Hoenig T, Clauditz TS, Atanackovic D, Koenig AM, Vashist YK, Klose H, Simon R, Pantel K, Izbicki JR, Bokemeyer C, Sauter G and Wilczak W: Frequent intratumoral heterogeneity of EGFR gene copy gain in non-small cell lung cancer. Lung Cancer *79*: 221-227, 2013.

24 Choi YL, Sun JM, Cho J, Rampal S, Han J, Parasuraman B, Guallar E, Lee G, Lee J and Shim YM: EGFR mutation testing in patients with advanced non-small cell lung cancer: A comprehensive evaluation of real-world practice in an East Asian tertiary hospital. PLoS One *8*: e56011, 2013.

25 Yatabe Y, Matsuo K and Mitsudomi T: Heterogeneous distribution of EGFR mutations is extremely rare in lung adenocarcinoma. J Clin Oncol *29*: 2972-2977, 2011.

26 Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA and Swanton C: Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med *366*: 883-892, 2012.

27 Almendro V, Marusyk A and Polyak K: Cellular heterogeneity and molecular evolution in cancer. Annu Rev Pathol *8*: 277-302, 2013.

28 Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC and Jänne PA: MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science *316*: 1039-1043, 2007.

29 Hiraki T, Mimura H, Gobara H, Iguchi T, Fujiwara H, Sakurai J, Matsui Y, Inoue D, Toyooka S, Sano Y and Kanazawa S: CT fluoroscopy-guided biopsy of 1,000 pulmonary lesions performed with 20-gauge coaxial cutting needles: Diagnostic yield and risk factors for diagnostic failure. Chest *136*: 1612-1617, 2009.

30 Fukui T, Ohe Y, Tsuta K, Furuta K, Sakamoto H, Takano T, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Asamura H, Tsuchida T, Kaneko M, Kusumoto M, Yamamoto S, Yoshida T and Tamura T: Prospective study of the accuracy of EGFR mutational analysis by high-resolution melting analysis in small samples obtained from patients with non-small cell lung cancer. Clin Cancer Res *14*: 4751-4757, 2008.

31 Lim EH, Zhang SL, Yu K, Nga ME, Ahmed DA, Agasthian T, Wong PS, Chua GC, Wong D, Tan L, Seto KY, Yap WS, Low SP, Khoo KL, Chang A, Ng A and Tan P: An alternative approach to determining therapeutic choices in advanced non-small cell lung carcinoma (NSCLC): Maximizing the diagnostic procedure and the use of low-volume lung biopsies. J Thorac Oncol *2*: 387-396, 2007.

32 Win T, Miles KA, Janes SM, Ganeshan B, Shastry M, Endozo R, Meagher M, Shortman RI, Wan S, Kayani I, Ell PJ and Groves AM: Tumor heterogeneity and permeability as measured on the CT component of PET/CT predict survival in patients with non-small cell lung cancer. Clin Cancer Res *19*: 3591-3599, 2013.

33 Hatt M, Visvikis D, Albarghach NM, Tixier F, Pradier O and Cheze-le Rest C: Prognostic value of ^{18}F-FDG PET image-based parameters in oesophageal cancer and impact of tumour delineation methodology. Eur J Nucl Med Mol Imaging *38*: 1191-1202, 2011.

34 Miles KA, Ganeshan B, Griffiths MR, Young RC and Chatwin CR: Colorectal cancer: texture analysis of portal phase hepatic CT images as a potential marker of survival. Radiology *250*: 444-452, 2009.

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