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Mutation Detection of Epidermal Growth Factor Receptor and *KRAS* Genes Using the Smart Amplification Process Version 2 from Formalin-Fixed, Paraffin-Embedded Lung Cancer Tissue

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Recent evidence indicates that the presence of epidermal growth factor receptor (*EGFR*) or *KRAS* mutations in non-small cell lung cancer (NSCLC) can predict the response of the tumor to gefinitib. However, it is difficult to detect these mutations using formalin-fixed, paraffin-embedded (FFPE) tissues because the fixation process and aging can damage the DNA. In this study, we describe our work in adapting the Smart Amplification Process version 2 (SmartAmp2) to detect *EGFR* or *KRAS* mutations in DNA extracted from FFPE tissues. We were able to detect these mutations in 37 (97%) of 38 FFPE lung cancer tissue samples within 60 minutes with the SmartAmp2 assay and to confirm the correlation between *EGFR* mutations in FFPE tissues and gefitinib responsiveness. All mutations had previously been confirmed in the 38 samples using DNA extracted from frozen tissues. Electrophoresis results indicated that PCR analysis was not reliable for DNA extracted from FFPE tissue when primers with a long amplicon (>300 bp) were used. This study confirms that the SmartAmp2 assay is suitable for use with DNA extracted from FFPE as well as frozen tissues. *(J Mol Diagn 2010, 12:257–264; DOI: 10.2353/jmoldx.2010.090105)*

Lung cancer, which is responsible for 1.18 million deaths annually worldwide, is the most common cause of cancer mortality in men and the second most common cause in women.[1] Treatment involves a combination of surgery, chemotherapy, and radiation therapy determined based on histological results obtained in biopsy of cancer cells from the individual patient. Despite the greater availability of treatment and substantial research efforts, the prognosis for lung cancer remains poor, and the development of more effective treatments is one of the most important topics in oncology today.

Recent studies have indicated that mutations in the epidermal growth factor receptor (*EGFR*) gene and *KRAS* gene help physicians decide the course of chemotherapy in patients with non-small cell lung cancer (NSCLC). *EGFR* mutations[2–5] and *KRAS* mutations[6–10] occur in 8 to 10% and 33% of NSCLC patients, respectively, and in 27 to 56% and 5 to 15% of East Asian NSCLC patients, respectively. They are negatively correlated in NSCLC such that patients who have a mutation in the tyrosine kinase domain of the *EGFR* respond to tyrosine kinase inhibitors such as gefitinib and erlotinib, whereas patients with mutations of the *KRAS* gene do not respond to this treatment.[3,9,11] Consequently, NSCLC patients with *EGFR* mutations have a favorable prognosis,[12,13] whereas the prognosis for those with *KRAS* mutations is poor.[14–17] Therefore, to provide the optimal therapy, physicians must be able to determine whether patients have *EGFR* or *KRAS* gene mutations.

Many methods used to detect *EGFR* or *KRAS* mutations in clinical samples include restriction fragment length polymorphism,[18] single-strand conformation polymorphism,[19] PCR sequencing,[20] high-resolution melting analysis,[21,22] and Scorpions Amplified Refractory Mutation System.[23] All of these methods require careful DNA extraction and purification, involve many steps, and must be performed by skilled technicians. Some of these methods are more sensitive than simple sequencing but are unsuitable for routine clinical use because of their complexity and long turnaround times.

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Recently, Mitani et al[24] developed a rapid, simple, and sensitive mutation detection assay called the Smart Amplification Process version 2 (SmartAmp2). This assay has shown the ability to detect mutations in samples containing as little as 1% mutant allele.[25,26] This assay can be used in the clinical setting, and it allows for the detection of *EGFR* and *KRAS* gene mutations within 60 minutes (including sample preparation) and enables high-throughput screening.

There are a number of archival formalin-fixed, paraffin-embedded (FFPE) tissue banks worldwide. FFPE tissue is relatively cheap, is easy to ship and handle, provides superior morphological quality, and is compatible with nearly all relevant immunohistochemical antibodies. Consequently, most surgical specimens are stored in FFPE tissue for later analysis of gene mutations if necessary. However, it is time-consuming to extract DNA from FFPE tissue and often difficult to detect mutations because the fixation process and aging can damage DNA.[20,27] The present study demonstrates a technique for adapting the SmartAmp2 method to detect mutations from FFPE tissue. The procedure can detect mutations with high accuracy, and unlike any other method, it gives a reliable diagnostic result based exclusively on amplification. The SmartAmp2 assay provides reliable information from old specimens and marks a major advance in cancer diagnostics.

Materials and Methods

Clinical Samples

Tumor samples surgically resected from NSCLC patients at the Gunma University Hospital (Gunma, Japan) between 2003 and 2007 were used. Institutional approval and informed consent from all patients was obtained in writing. All tumor tissue was diagnosed for lung cancer by H&E stain. After surgical removal, a portion of each sample was immediately frozen and stored at −80°C until DNA extraction; the remainder was preserved in paraffin blocks after formalin fixation.

Sample Selection

A total of 43 samples that were available in both frozen and FFPE form were selected for the study. The SmartAmp2 assay was used to determine that both *EGFR* and *KRAS* gene mutations were present in the frozen tissue. Previous examination with a microscope confirmed that each FFPE tissue sample contained a sufficient number of tumor cells for analysis.

DNA Extraction

To suppress the tumor heterogeneity and to obtain a sufficient number of tumor cells, thin sections sliced from the tumor at the surface with maximum diameter were selected and cut into small pieces. DNA was extracted from a 3- to 5-mm cube collected from the small pieces using a DNA Mini Kit (Qiagen, Hilden, Germany), and the solution was serially diluted to a concentration of 20 ng/μl. For each tumor, the FFPE block with the maximum number of tumor-rich areas was selected and sliced into three 5-μm-thick sections. The tumor area of the section was macrodissected, and DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen). The extracted solution was not diluted. We added RNase during the DNA extraction, although this was an optional step according to the protocol included with the kit. We obtained concentrations of at least 40 ng/μl in all of the extracted solutions. Although the concentration was lower for the first extraction of some specimens, we extracted again using more sections until the concentration reached 40 ng/μl. After extraction, all DNA templates were stored at −20°C until use.

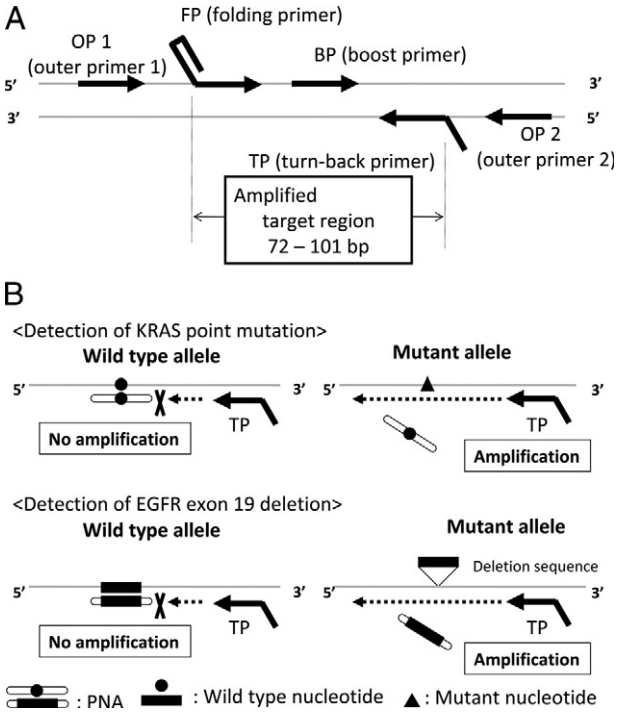

Figure 1. A: Amplification by the SmartAmp2 assay requires five unique primers: turn-back primer (TP), folding primer (FP), boost primer (BP), and two outer primers (OP1 and OP2). The genomic sequence between and including the TP and FP primers is the target region to be amplified in the SmartAmp2 assay. **B:** SmartAmp2 assay PNA clamp. The PNA clamp competitive probe is designed for the wild-type allele sequence. The greater stability of the PNA probe in hybridization inhibits SmartAmp2 amplification and suppresses wild-type allele amplification. Amplification of the mutant allele is not inhibited by PNA regardless of the point mutation (*KRAS*) or deletion (*EGFR* exon 19).

SmartAmp2 Assay

The SmartAmp2 assay has been described previously.[25,26] It is the first one-step mutation–detection technology to enable the precise amplification of only target sequences. Using a new DNA polymerase (*Aac* pol) and a unique five-set primer design, we performed rapid and sensitive assays under isothermal conditions (Figure 1A).[24] The assays were performed in parallel sets using the same template, with one assay detecting the wild-type sequence, and the other detecting the mutant

Table 1. PCR and Sequencing Primers for the *EGFR* and *KRAS* Genes

Exon	Primer name	Forward primer	Reverse primer	Amplicon length (bp)	Annealing temperature (°C)	Refs.
EGFR	19-1	5′-ACCATCTCACAATTGCCAGTTAAC-3′	5′-GAGGTTCAGAGCCATGGACC-3′	192	60	28
Exon19	19-2	5′-CCAGATCACTGGGCAGCATGTGGCACC-3′	5′-AGCAGGGTCTAGAGCAGAGCAGCTGCC-3′	265	60	27
	19-3	5′-CCTTAGGTGCGGCTCCACAGC-3′	5′-CATTTAGGATGTGGAGATGAGC-3′	349	62	20
EGFR	21-1	5′-TCACAGCAGGGTCTTCTCTGTTT-3′	5′-ATGCTGGCTGACCTAAAGCC-3′	212	61	28
Exon21	21-2	5′-TCAGAGCCTGGCATGAACATGACCCTG-3′	5′-GGTCCCTGGTGTCAGGAAAATGCTGG-3′	297	62	27
	21-3	5′-CAGCCATAAGTCCTCGACGTGG-3′	5′-CATCCTCCCCTGCATGTGTTAAAC-3′	374	60	20
KRAS	KRAS-1	5′-TCATTATTTTTATTATAAGGCCTGCTGAA-3′	5′-CAAAGACTGGTCCTGCACCAGTA-3′	189	59	21
	KRAS-2	5′-ACTGGTGGAGTATTTGATAG-3′	5′-ACTCATGAAAATGGTCAGAG-3′	288	59	29
	KRAS-3	5′-TGAAGTACAGTTCATTACGATACACG-3′	5′-GGAAAGTAAAGTTCCCATATTAATGGT-3′	499	58	30

Annealing temperature in PCR thermal cycle is indicated as above.
The amplicon length and annealing temperature used in the PCR thermal cycle are indicated.

sequence. Detection of the wild-type sequence served as a positive control, allowing us to distinguish between assay failure and a true negative result.

In the present study, we used two versions of the SmartAmp2 assay, conventional and peptide nucleic acid (PNA) clamp. In the conventional SmartAmp2 assay, the target sequence was detected by primer genotyping. When the primer sequence was completely complementary to the target sequence, an amplified product was produced. Therefore, we needed two different primer sets to detect wild-type alleles and mutant alleles using this method. For the PNA clamp SmartAmp2 assay, we used PNA and a single primer set which was designed to amplify the target sequence in both the wild-type and mutant allele. The PNA was exactly homologous to the wild-type allele. However, because PNA is not recognized by the polymerase as natural DNA, it cannot serve as a primer for polymerization or as a substrate for Taq polymerase exonuclease activity. In addition, the melting temperature of a perfectly matched PNA-DNA duplex is higher than that of DNA-DNA of the same length, although a single mismatch destabilizes the PNA-DNA hybrids, causing a melting temperature shift of 10 to 18°C. The greater stability of the hybridized PNA probe inhibits the SmartAmp2 amplification; thus, amplification of the wild-type allele is suppressed (Figure 1B). When the assay is performed with both the primer set and PNA, any mutant sequences in the template are amplified. All template sequences are amplified when using the same primer set without PNA, which served as a control. Conventional SmartAmp2 was used to detect some *EGFR* deletions and the L858R mutation (sample numbers 1–13 and 19–30) and PNA clamp SmartAmp2 was used to detect other *EGFR* deletions and *KRAS* mutations (sample numbers 14–18 and 31–38). In this article, "SmartAmp2" refers to both assay methods. The mutation detection kits were from K.K. DNAFORM (Yokohama, Japan). The SmartAmp2 assay was used to detect mutations in both frozen and FFPE tissues.

PCR and Electrophoresis

PCR was performed on DNA extracted from both frozen and FFPE tissues. We used several sets of primers to amplify the *EGFR* and *KRAS* gene mutations. Each primer was designed to produce various amplicon lengths, including that of the mutation site (Table 1). Although the PCR primer sequences for *EGFR* and *KRAS* amplification were used previously,[20,21,27–30] the other experimental conditions were unique to this study. The PCRs were performed in a total volume of 25 μl containing 10× PCR Gold Buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxyribonucleotide triphosphates, 500 nmol/L each primer, 1 U of TaqDNA Gold Polymerase (Applied Biosystems Japan, Tokyo, Japan), and 2 μl of extracted genomic DNA. The thermal cycling regimen was as follows: 5 minutes at 94°C, followed by 35 cycles at 94°C for 15 s, annealing for 30 s, 72°C for 1 minute, and 1 cycle of 72°C for 5 minutes. Annealing temperatures were based on the results of the PCR gradient in a pilot study (see Table 1).

The PCR products of all primers were confirmed by electrophoresis in 2% agarose gel containing ethidium bromide.

Sequencing Analysis

The PCR products generated from FFPE tissues were purified using the QIAquick PCR Purification Kit (Qiagen) and processed for DNA sequencing reaction using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems Japan) with a forward primer of EGFR 19-1, EGFR 21-1, and KRAS-1 (Table 1). Sequence data were generated using the ABI PRISM 3100 DNA Analyzer (Applied Biosystems Japan).

The presence of mutations in the frozen tissue was confirmed by direct sequencing and PNA-enriched sequencing in our previous study. The PNA-enriched sequencing method has been described previously.[31–34]

Results

EGFR and KRAS Mutation Detection Using the SmartAmp2 Assay and Direct Sequencing

We performed the SmartAmp2 assay on 43 DNA samples extracted from both frozen and FFPE tissues. In the frozen tissue, 38 mutations were detected: 18 samples had *EGFR* mutations in exon 19, 12 samples had *EGFR* mutations in exon 21, and 8 samples had *KRAS* mutations in exon 2. No mutations were detected in five samples (Table 2). The presence of these mutations was previously examined by PNA-enriched sequencing, and the results were completely concordant with SmartAmp2

Table 2. Results of SmartAmp2 Assay, Electrophoresis, and Direct Sequencing

Sample no.	DNA extracted from frozen sample as templates			DNA extracted from FFPE sample as templates				
		Visual band by any primers	Direct sequencing		Visual band			Direct sequencing
	SmartAmp2 mutation site and type			SmartAmp2	Short	Medium	Long	
1	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Mut	Mut	+	−	−	Mut
2	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Wt	Mut	+	−	−	Mut
3	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Mut	Mut	+	+	+	Mut
4	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Mut	Mut	+	−	−	Wt
5	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Mut	Mut	+	−	−	Mut
6	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Mut	Mut	+	+	−	Mut
7	*EGFR* Ex 19 E746-A750 del (c.2235-2249del15)	+	Mut	Mut	+	+	−	Wt
8	*EGFR* Ex 19 E746-A750 del (c.2236-2250del15)	+	Wt	Mut	+	−	−	Wt
9	*EGFR* Ex 19 E746-A750 del (c.2236-2250del15)	+	Mut	Mut	+	−	−	Mut
10	*EGFR* Ex 19 E746-A750 del (c.2236-2250del15)	+	Mut	Mut	+	−	−	Mut
11	*EGFR* Ex 19 E746-A750 del (c.2236-2250del15)	+	Wt	Mut	+	−	−	Wt
12	*EGFR* Ex 19 E746-A750 del (c.2236-2250del15)	+	Wt	Mut	+	−	−	Mut
13	*EGFR* Ex 19 E746-A750 del (c.2236-2250del15)	+	Mut	Mut	+	−	−	Mut
14	*EGFR* Ex 19 T747-T751 del	+	Wt	Mut	+	−	−	Mut
15	*EGFR* Ex 19 L747-E749 del, A750P	+	Mut	Mut	−	−	−	Wt
16	*EGFR* Ex 19 L747-E749 del, A750P	+	Mut	Mut	+	−	−	Wt
17	*EGFR* Ex 19 E746-E749, S752-P753 del	+	Wt	Mut	+	−	−	Mut
18	*EGFR* Ex 19 L747-K754 del, K754N	+	Mut	Mut	+	+	−	Mut
19	*EGFR* Ex 21L858R	+	Wt	Mut	+	−	−	Wt
20	*EGFR* Ex 21L858R	+	Wt	Mut	+	−	−	Wt
21	*EGFR* Ex 21L858R	+	Mut	Mut	+	−	−	Mut
22	*EGFR* Ex 21L858R	+	Wt	Mut	+	−	−	Mut
23	*EGFR* Ex 21L858R	+	Mut	Mut	+	−	−	Wt
24	*EGFR* Ex 21L858R	+	Mut	Mut	+	+	−	Wt
25	*EGFR* Ex 21L858R	+	Wt	Wt	+	+	+	Wt
26	*EGFR* Ex 21L858R	+	Wt	Mut	+	−	−	Mut
27	*EGFR* Ex 21L858R	+	Mut	Mut	+	−	−	Wt
28	*EGFR* Ex 21L858R	+	Mut	Mut	+	−	−	Wt
29	*EGFR* Ex 21L858R	+	Wt	Mut	+	−	−	Wt
30	*EGFR* Ex 21L858R	+	Wt	Mut	+	−	−	Wt
31	*KRAS* Ex2 G12V	+	Wt	Mut	+	−	−	Wt
32	*KRAS* Ex2 G12C	+	Wt	Mut	+	−	−	Wt
33	*KRAS* Ex2 G12A	+	Wt	Mut	+	−	−	Wt
34	*KRAS* Ex2 G12V	+	Mut	Mut	+	−	−	Wt
35	*KRAS* Ex2 G12V	+	Mut	Mut	+	+	−	Wt
36	*KRAS* Ex2 G12V	+	Mut	Mut	+	−	−	Mut
37	*KRAS* Ex2 G12F	+	Mut	Mut	+	−	−	Wt
38	*KRAS* Ex2 G12V	+	Mut	Mut	+	−	−	Mut
39	Wt	+	Wt	Wt	+	−	−	Wt
40	Wt	+	Wt	Wt	+	+	−	Wt
41	Wt	+	Wt	Wt	+	−	−	Wt
42	Wt	+	Wt	Wt	+	+	−	Wt
43	Wt	+	Wt	Wt	+	−	−	Wt

Wt, wild type; Mut, mutant type.

PCR was performed using primers that produced each length of amplicon to amplify the sequence, including that of the suspected mutations (see Table 1). We defined three primers for each exon as "short," "medium," or "long" in ascending order of amplicon size. PCR was performed using all primers for samples 39–43.

+, PCR product of a primer produced a visual band in the electrophoresis gel; −, no visual band was detected.

(data not shown). In DNA extracted from the FFPE tissue, we used the SmartAmp2 assay to detect 37 of the 38 mutations (97%): 18 samples had *EGFR* mutations in exon 19, 11 had *EGFR* mutations in exon 21, and 8 had *KRAS* mutations in exon 2. No mutations were detected in six samples (Table 2). The L858R mutation could not be detected in one FFPE tissue (sample 25). A typical SmartAmp2 assay is shown in Figure 2. The results from all of the SmartAmp2 assays were obtained within 60 minutes. Several samples of wild-type lung cancer DNA were extracted from FFPE tissue and assayed in concentrations ranging from 20 to 200 ng/μl; no false-positive results were observed.

Direct sequencing was also performed on 43 DNA samples extracted from frozen and FFPE tissues, and the results were compared with those obtained with SmartAmp2 from frozen tissue. The mutation in *EGFR* exon 19 was detected by direct sequencing in 12 of 18 samples (67%) from frozen tissue. Similarly, the mutations in *EGFR* exon 21 and *KRAS* exon 2 were detected in 5 of 12 samples (42%) and in 5 of 8 samples (63%), respectively, from frozen tissue. Meanwhile, the mutation in *EGFR* exon 19 was detected by direct sequencing in 12 of 18 samples (67%) from FFPE tissue. Similarly, the mutations in *EGFR* exon 21 and *KRAS* exon 2 were detected in 3 of 12 samples (25%) and in 2 of 8 samples (25%), respectively,

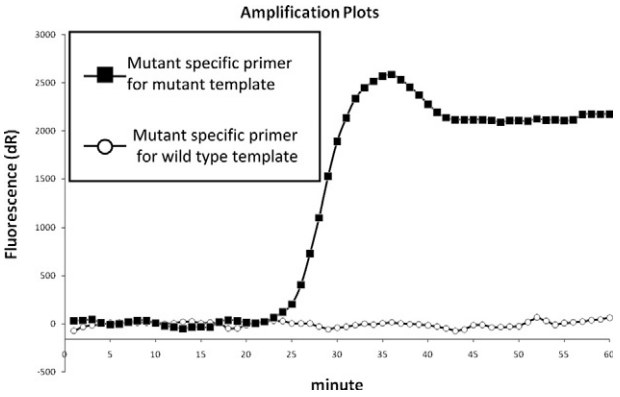

Figure 2. A typical SmartAmp2 assay result. The graphs represent amplification curves of the L858R mutation detection in wild-type and mutant templates. If the amplification started within 40 minutes, it was defined as a positive reaction.

from FFPE tissue. Taken together, the mutations for *EGFR* exon 19, *EGFR* exon 21, or *KRAS* exon 2 were detected in 22 of 38 samples (58%) from frozen tissue and in 17 of 38 samples (45%) from FFPE tissue (Table 2).

As described, with direct sequencing we could not detect mutations well, especially when FFPE tissue samples were used. However, with SmartAmp2 detection of mutations was almost 100% using both FFPE tissue and frozen tissue samples.

Agarose Gel Electrophoresis of PCR Products

Agarose gel electrophoresis of PCR products was performed on DNA extracted from the 43 frozen and FFPE tissues to elucidate the reason for SmartAmp2's superior performance. A visible band was apparent in all samples for all primers in the DNA extracted from the frozen tissue. In contrast, in the FFPE tissue, it was difficult to amplify DNA using primers with a long amplicon. The DNA showed visible bands in 42 samples in which the primers had a short amplicon; however, bands were visible in only 9 samples of primers with a medium amplicon and in only 2 samples with a long amplicon (Figure 3, Table 2). This indicates that DNA extracted from FFPE tissue was severely fragmented.

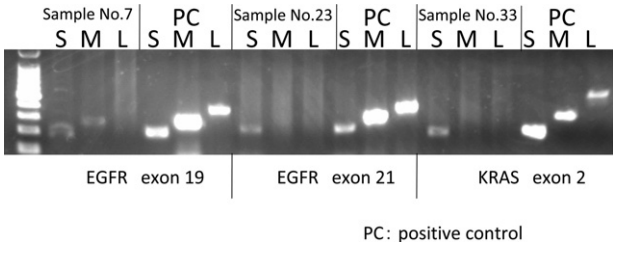

Figure 3. Electrophoresis results of samples 7, 23, and 33. The PCR products of DNA extracted from blood samples were used as a positive control. S, short; M, medium; L, long amplicon of each primer. For example, S lane of *EGFR* exon 19 represents the result of electrophoresis using the PCR product of the 192-bp amplicon of *EGFR* exon 19. The **left lane** is used as the 100-bp ladder marker.

Correlation between EGFR or KRAS Mutations and Gefitinib Responsiveness

To investigate whether the mutations of *EGFR* detected in FFPE tissue are associated with sensitivity to gefitinib, we followed the 43 patients and checked the history of gefitinib therapy. The response to gefitinib was assessed for the best response to therapy according to the use of Response Evaluation Criteria in Solid Tumors guidelines.[35] The clinical features and results of the mutational analyses in FFPE tissues are summarized in Table 3. By August 2009, 13 patients had been treated with gefitinib, and all had a recurrence of lung cancer. Of the 13 patients, 11 patients had *EGFR* mutations, 1 had a *KRAS* mutation, and 1 had wild-type alleles. Ten of the 13 patients had a partial response to gefitinib, all of whom had *EGFR* mutations. Therefore, the response rate to gefitinib in patients with *EGFR* mutations detectable in FFPE tissue was 91% (10 of 11). The patient with a *KRAS* mutation and the patient with wild-type alleles detectable in FFPE tissue were classified as having progressive disease and stable disease, respectively.

Discussion

In general, frozen tissue is preferable to FFPE tissue for genetic analysis because the DNA is not as degraded. However, it is difficult to keep frozen tissue in the general hospital because of space limitations or absence of equipments, and therefore, most clinical materials are preserved as FFPE tissues. There are several methods for detecting mutations in DNA from FFPE tissues, but they are time-consuming and complicated. Only a few studies have compared the detection of *EGFR* or *KRAS* mutations in DNA extracted from samples obtained from the same patient and prepared as both frozen and FFPE tissues, and the results may differ significantly.[36] This has led to the belief that it is difficult to obtain concordant results from both frozen and FFPE tissues.

The recent developments in SmartAmp2 have made rapid and sensitive detection of *EGFR* and *KRAS* mutations possible. The assay produces good results even with crude samples such as those taken directly from lysed blood and purification of DNA is not necessary.[24] In this study, we investigated the performance of the SmartAmp2 assay for detecting *EGFR* and *KRAS* mutations when we extracted DNA from FFPE tissues.

We used the QIAamp DNA FFPE Tissue kit for DNA extraction and the SmartAmp2 assay for mutation detection. These are both easy, simple techniques that require no special skills and can be performed quickly. We initially extracted DNA without RNase, but mutation detection was not as good as expected, and the results were improved after we added the RNase treatment step. We needed more FFPE tissue sections to obtain a sufficient DNA concentration in the extracted solution with RNase than without it. Without RNase treatment, the RNA remaining in the extraction solution likely led to an overestimation of the DNA concentration, and consequently, the

Table 3. Characteristics of 13 Patients Treated with Gefitinib Therapy

Sample no.	Age	Sex	SmartAmp2 mutation site and type	Direct sequencing	Response to gefitinib
2	65	F	*EGFR* Ex 19 E746-A750 del	Mut	PR
3	69	F	*EGFR* Ex 19 E746-A750 del	Mut	SD
9	71	F	*EGFR* Ex 19 E746-A750 del	Mut	PR
10	50	F	*EGFR* Ex 19 E746-A750 del	Mut	PR
12	40	M	*EGFR* Ex 19 E746-A750 del	Mut	PR
13	66	F	*EGFR* Ex 19 E746-A750 del	Mut	PR
15	55	M	*EGFR* Ex 19 L747-E749 del, A750P	Wt	PR
16	63	F	*EGFR* Ex 19 L747-E749 del, A750P	Wt	RP
17	62	F	*EGFR* Ex 19 E746-E749, S752-P753 del	Mut	PR
21	74	F	*EGFR* Ex 21L858R	Mut	PR
27	66	M	*EGFR* Ex 21L858R	Wt	PR
37	71	F	*KRAS* Ex2 G12F	Wt	PD
42	74	F	Wt	Wt	SD

Wt, wild type; Mut, mutant type; F, female; M, male; PR, partial response; SD, stable disease; PD, progressive disease.
Age, gender, and the results of mutational analysis in FFPE tissue are indicated. Among the treated patients, 10 were PR and all had *EGFR* mutations detected in FFPE tissue.

actual amount of DNA used for the assay of some specimens might have been insufficient to allow the detection of mutations. The RNase treatment step was essential in our protocol.

With SmartAmp2, only 1 of 43 FFPE tissue samples gave a different result compared with the frozen tissue samples. This is a significant improvement over previous comparative analyses.[36] Additionally, the results of SmartAmp2 assay can be obtained within 60 minutes, considerably faster than other procedures. Unfortunately, we could not detect a mutation in sample number 25, despite using several FFPE blocks from different areas of the same tumor. We could find no reasonable explanation for our inability to detect the mutation in only FFPE blocks from sample number 25.

Direct sequencing was less effective at detecting mutations in DNA extracted from FFPE tissue than from frozen tissue, because mutations were detected in only 17 of the 38 FFPE tissue samples (45%) shown by SmartAmp2 to have mutations compared with 22 of 38 frozen tissue samples (58%).

To clarify the advantage of SmartAmp2 using FFPE tissue, we performed electrophoresis on the PCR products and demonstrated the difficulty of amplifying DNA in FFPE samples using PCR, especially with longer amplicon primers. This finding agrees with previous reports indicating that DNA from FFPE tissue degenerates into fragments, making longer amplicons (>300 bp) difficult to amplify.[27,36,37] The SmartAmp2 assay was able to detect amplify template and detect mutations in almost all FFPE tissues. This may be because SmartAmp2 primers are designed for amplification of a short target region (Figure 1A: *EGFR* exon 19, 101 bp; *EGFR* exon 21, 77 bp; *KRAS* exon 2, 72 bp), and thus, DNA fragmentation had little influence in the SmartAmp2 assay.[24–26] Certainly, with a short amplicon primer, most FFPE tissues (42 of 43; 98%) could be amplified by PCR.

As noted previously, most mutations in FFPE tissue could not be detected by direct sequencing, which requires the mutation be present in at least 20% of the sample.[38] This implies that most samples had a lower mutant to wild-type ratio, which allowed detection with the SmartAmp2 assay, but not with direct sequencing. On histological examination, these samples, as represented by sample number 31 (Figure 4), tended to have considerable inflammatory cell infiltrate or fibrotic changes, which might have provided a greater source of nonmutated DNA in these samples. Thus, although a sufficient DNA concentration was extracted from the tumor, it might have contained a higher percentage of nonmutated DNA and, consequently, a lower percentage of the mutant allele. In contrast to direct sequencing methods, the SmartAmp2 assay, owing to its high sensitivity, can detect a mutation present as <1% of sample DNA, as may be the case for severe tumor conditions like those in sample number 31.[25,26]

It is worth noting that detection in FFPE tissues may have benefited from the *Aac* DNA polymerase used in the SmartAmp2 assay. It has very strong strand displacement activity, and because DNA in FFPE tissues is cross-linked to surrounding proteins, strand displacement activity may be necessary for amplifying DNA extracted from this tissue.[37]

In this study, a comparison between the use of the SmartAmp2 assay and direct sequencing for the detection of mutations in DNA extracted from FFPE tissue preparations demonstrated the superiority of SmartAmp2. However, extremely sensitive PCR-based detection methods

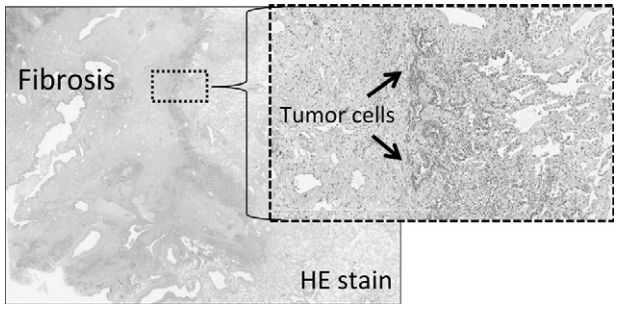

Figure 4. Microscopic examination of sample number 31. A typical case of a tumor with severe fibrotic changes. Most of the tumor had undergone fibrotic changes, and true tumor cells were found in only a small portion of the tumor, as indicated by the **arrows**. In such cases, mutations were difficult to detect by direct sequencing.

continue to be developed. For example, pyrosequencing, an advanced direct sequencing technology, is more sensitive than conventional direct sequencing and has been reported to be applicable for FFPE tissue analysis.[39–41] Although we might have obtained similar results with pyrosequencing, SmartAmp2 has advantages compared with sequencing-based methods, including pyrosequencing. First, because SmartAmp2 does not require a separate amplification process, because amplification itself is the signal for detection, it can be performed in fewer steps and within a shorter time than other methods. Second, the results of the SmartAmp2 assay are clear and very easy to read. In sequencing-based methods, the data must be carefully examined for the presence of signals, which may be difficult to discern for samples with a low percentage of mutation. Thus, these methods may not provide definitive results for guiding the development of therapeutic strategies. In contrast, with SmartAmp2, only the amplification of the sequence needs to be observable. The unambiguous results with SmartAmp2 can improve the therapeutic approach in patients. These two advantages are valuable, especially in clinical practice.

As shown above, we can detect very low levels of *EGFR* or *KRAS* mutations using SmartAmp2; however, the association between the effects of tyrosine kinase inhibitors such as gefitinib and the presence of extremely low levels of the mutant alleles has not been determined. In a recent study, patients with an *EGFR* mutation that was detected with SmartAmp2, but not by direct sequencing, were reported to respond to gefitinib.[25] Our results showing a high response rate to gefitinib in patients with *EGFR* mutations (Table 3) confirm earlier similar findings.[3,11] Moreover, tumors such as sample numbers 15, 16, and 27 with *EGFR* mutations detectable by SmartAmp2 assay, but not by direct sequencing, responded to gefitinib. Therefore, direct sequencing by itself may not provide enough information to determine therapeutic strategies. A more sensitive *EGFR* mutation detection method such as SmartAmp2 is essential to accurately predict a response to gefitinib. Only one tumor with a *KRAS* mutation, which was detected by SmartAmp2 but not by direct sequencing, was treated with gefitinib, and it did not respond to gefitinib. This suggests that low levels of *KRAS* mutations may correlate with resistance to gefitinib, further study with many samples will be needed to establish this.

In conclusion, we have compared the results of SmartAmp2 and direct sequencing using both FFPE and frozen tissue samples taken from the same tumor and have demonstrated that with SmartAmp2 we could detect mutations effectively in DNA extracted from the FFPE tissue. To our knowledge, this is the first report of applying SmartAmp2 to the analysis of FFPE tissue. The protocol for accurately identifying *EGFR* and *KRAS* mutations in DNA from FFPE tissue is quick, easy, and reliable. This new method will allow physicians to identify NSCLC patients who are the most likely to respond to tyrosine kinase inhibitors and ultimately provide better diagnostic options for these patients.

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