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■ ORIGINAL ARTICLE ■

Comparison of Direct Sequencing, PNA Clamping–Real Time Polymerase Chain Reaction, and Pyrosequencing Methods for the Detection of *EGFR* Mutations in Non–small Cell Lung Carcinoma and the Correlation with Clinical Responses to EGFR Tyrosine Kinase Inhibitor Treatment

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Background: The aims of this study were to evaluate the abilities of direct sequencing (DS), peptide nucleic acid (PNA) clamping, and pyrosequencing methods to detect epidermal growth factor receptor (*EGFR*) mutations in formalin-fixed paraffin-embedded (FFPE) non-small cell lung carcinoma (NSCLC) samples and to correlate *EGFR* mutational status as determined by each method with the clinical response to EGFR tyrosine kinase inhibitors (TKIs). **Methods:** Sixty-one NSCLC patients treated with EGFR TKIs were identified to investigate somatic mutations in the *EGFR* gene (exons 18-21). **Results:** Mutations in the *EGFR* gene were detected in 38 of the 61 patients (62%) by DS, 35 (57%) by PNA clamping and 37 (61%) by pyrosequencing. A total of 44 mutations (72%) were found by at least one of the three methods, and the concordances among the results were relatively high (82-85%; kappa coefficient, 0.713 to 0.736). There were 15 discordant cases (25%) among the three different methods. **Conclusions:** All three *EGFR* mutation tests had good concordance rates (over 82%) for FFPE samples. These results suggest that if the DNA quality and enrichment of tumor cells are assured, then DS, PNA clamping, and pyrosequencing are appropriate methods for the detection of *EGFR* mutations.

Key Words: Lung neoplasms; Receptor, epidermal growth factor; Mutation; Sequencing analysis, DNA; Peptide nucleic acids; Pyrosequencing

Two pivotal studies published in 2004 showed an association between activating mutations in the epidermal growth factor receptor (*EGFR*) gene and a dramatic clinical response to EGFR tyrosine kinase inhibitors (TKIs) in patients with non-small cell lung carcinoma (NSCLC).[1,2] Therefore, molecular testing for *EGFR* mutations is of increasing clinical importance in routine practice.

A variety of methods are available for the detection of *EGFR* mutations, and different methods are being used in different countries. Although several guidelines for molecular testing have been proposed by several working groups, there is thus far no consensus regarding the best method to detect *EGFR* mutations when using clinical samples.[3-6] Direct sequencing (DS) and pyrosequencing were the only approved diagnostic meth-

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ods in Korea up until 2011, and the peptide nucleic acid (PNA) clamping method was only recently approved by the Korean Food and Drug Administration (FDA). The PNA clamping method is known to be more sensitive than DS, and allows for the detection of mutations in samples containing as few as approximately 1% mutant alleles.[7-9] Pyrosequencing is a non-electrophoretic real-time sequencing technology using luminometric detection.[10] This technique is well suited for the detection of somatic mutations, which may be present in a small fraction of tumor cells within a background of normal tissue.[11,12] A study to determine the concordance of these three methods in a single institution and to correlate the results of these mutational analyses with clinical responses has not been conducted. Because *EGFR* mutations, rather than demographic features, are independently associated with a favorable prognosis for NSCLC patients treated with EGFR TKIs, the optimization of *EGFR* mutation tests is very important for selecting appropriate therapeutic strategies for NSCLC patients.[6,13]

The aims of this study were 1) to evaluate the efficacy of the DS, PNA clamping, and pyrosequencing methods for detecting *EGFR* mutations and 2) to assess clinical responses to EGFR TKIs in groups defined by these different detection methods.

MATERIALS AND METHODS

Patients and specimens

Formalin-fixed paraffin-embedded (FFPE) tissues from 103 patients with NSCLC (26 biopsy and 77 resection samples) were obtained from the Seoul National University Bundang Hospital (SNUBH), Korea, between May 2003 and July 2010. All patients received EGFR TKIs gefitinib and erlotinib. We first analyzed 103 patients using DS and PNA clamping. However, 42 patients could not be tested by pyrosequencing either because the amount of available tissue was too small or because paraffin blocks were unavailable; consequently, 61 patients were included in this study. The patients included consisted of 26 men and 35 women. The mean age was 61.3 years (standard deviation, 10.6 years; range, 26 to 84 years), and the mean tumor size was 4.2 cm (standard deviation, 2.5 cm; range, 1.7 to 14.0 cm). All patients had undergone biopsy or surgical treatment (biopsy, n = 1; wedge resection, n = 1; lobectomy, n = 56; and pneumonectomy, n = 3).

The hematoxylin and eosin-stained slides were independently reviewed by two pathologists (H.J.L. and J.H.C.) to confirm the original diagnosis of each patient based on the World Health Organization criteria.[14]

The pathologic stage (p-stage) was determined at the time of the initial diagnosis using the 7th edition of the tumor-node-metastasis (TNM) classification.[15] The stage Ia-IIIa patients had received EGFR TKIs when they relapsed. Patients were categorized as follows: never smokers (< 100 lifetime cigarettes), former smokers (quit ≥ 1 year ago), or current smokers (quit < 1 year ago). Additional data, including response, progression of the disease, survival status, and cause of death, were obtained from patients' medical records and/or through interviews with the families of the patients. The median follow-up period for all patients was 30.0 months, with a range of 2 to 111 months. All patient samples were tested with informed consent.

DNA extraction

Genomic DNA was extracted from FFPE tissue as described previously.[16,17] A QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used for genomic DNA isolation according to the manufacturer's instructions.

Mutational analyses of *EGFR* genes: DS

EGFR mutations in exons 18 to 21 were identified by nested polymerase chain reaction (PCR) and DS as described previously.[16,17] All sequence variants were confirmed by sequencing the products of independent PCR amplifications in both directions. These sequences and chromatographs were manually compared with the *EGFR* reference sequence by two pathologists (H.J.L. and J.H.C.).

Mutational analyses of *EGFR* genes: PNA clamping

EGFR mutations were identified using the PNA Clamp EGFR mutation detection kit (Panagene, Daejeon, Korea) according to the manufacturer's instructions and as described previously.[7-9] A CFX 384 real-time PCR instrument was used (Bio-Rad, Hercules, CA, USA). The threshold cycle (Ct) was automatically calculated from PCR amplification plots in which fluorescence was plotted against the number of cycles. Delta-Ct values were calculated as the Ct value from PCR with the PNA control minus the Ct value from PCR of the samples. A higher delta-Ct value indicates that the mutant was efficiently amplified. A cut-off value of 2 was used to differentiate the presence and absence of mutant DNA in the clinical samples.

Mutational analyses of *EGFR* genes: pyrosequencing

EGFR mutations in exons 18 to 21 were identified by pyrosequencing as described previously.[12] An aliquot of 40 μL of PCR product was bound to streptavidin Sepharose HP (GE Health-

care, Uppsala, Sweden), purified, washed, denatured in 0.2 mol/L NaOH solution, and washed again. Then, 0.3 μmol/L of pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was performed on a PyroMark ID system (Qiagen) following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA) Progression-free survival was assessed from the date of biopsy or surgical treatment to the earliest sign of disease progression, as determined using the Response Evaluation Criteria in Solid Tumors (RECIST), or to death from any cause.[18] Overall survival was defined as the time from the date of biopsy or surgical treatment to the last follow-up visit or cancer-related death. Statistical significance was defined as $p < 0.05$.

RESULTS

Comparison of the DS, PNA clamping, and pyrosequencing methods

EGFR mutations were detected in 38 of the 61 patients (62%) by DS, in 35 (57%) using the PNA clamping method, and in 37 (61%) by pyrosequencing. There was good concordance (over 82%) in the assessment of *EGFR* mutations between DS and PNA clamping (concordant cases [n = 50, 82%] and discordant cases [n = 11, 18%]; kappa coefficient, 0.736), between DS and pyrosequencing (concordant cases [n = 51, 84%] and discordant cases [n = 10, 16%]; kappa coefficient, 0.716), and between PNA clamping and pyrosequencing (concordant cases [n = 52, 85%] and discordant cases [n = 9, 15%]; kappa coefficient, 0.713). Overall, there were concordant cases (n = 46, 75%) and discordant cases (n = 15, 25%) between the three different methods. In concordant cases, *EGFR* mutations were detected in 29 of the 61 patients (48%). A total of 44 mutations (72%) were found by at least one of the three methods.

Among the discordant cases (Table 1), one patient (case no. 1) had at least one of the same mutations. In six patients (cases nos. 2-7), *EGFR* mutations were detected by PNA clamping or pyrosequencing but not by DS. For the four discordant cases (case no. 8-11) in which *EGFR* mutations were detected only by DS, the *EGFR* mutations were undesignated, and these patients had progressive disease (PD) when treated with TKIs. Table 2 presents detailed profiles of *EGFR* mutations identified by DS, not designed by PNA clamp *EGFR* mutation detection kit or pyrosequencing. In four cases (case nos. 12-15), an exon 21 mutation (L858R) was identified by DS, while the other two methods identified these as wild type in three cases (by PNA clamping) and in one case (by pyrosequencing).

Among the concordant cases (Table 3), the TKI response (complete response [CR], partial response [PR], and stable disease [SD]) was significantly higher for the cases with exon 19 deletions or exon 21 point mutations than for those with wild-type *EGFR* (28% and 13% vs 4%). Of particular interest, an

Table 1. Summary of discordant cases (n = 15, 25%) of *EGFR* mutation profiles using direct sequencing, PNA clamping, and pyrosequencing according to clinicopathologic characteristics, including EGFR TKI responses

Case No.	Direct sequencing	PNA clamping	Pyrosequencing	TKI Response	Sex	Smoking	Histology
1	G719X, L861Q	G719X, L861Q	G719X	**SD**	F	N	ADC
2	Wild	Wild	Exon 19 del	**PR**	M	C	**ASC**
3	Wild	Wild	Exon 19 del	PD	F	N	ADC
4	Wild	G719X	Wild	**SD**	M	C	ADC
5	Wild	Exon 19 del	Exon 19 del	**PR**	M	C	ADC
6	Wild	S768I	Wild	PD	F	N	ADC
7	Wild	L858R	L858R	**PR**	M	N	ADC
8	Exon 19 del	Wild	Wild	PD	F	N	ADC
9	Exon 19 del	Wild	Wild	PD	M	FS	**ASC**
10	Exon 20 duplication	Wild	Wild	PD	F	N	ADC
11	R776H	Wild	Wild	PD	M	N	**LCC**
12	L858R	L858R	Wild	PD	M	N	ADC
13	L858R	Wild	L858R	**PR**	F	N	ADC
14	L858R	Wild	L858R	**SD**	F	N	ADC
15	L858R	Wild	L858R	PD	F	N	ADC

EGFR, epidermal growth factor receptor; PNA, peptide nucleic acid; TKI, tyrosine kianse inhibitor; SD, stable disease; F, female; N, never smoker; ADC, adenocarcinoma; PR, partial response; M, male; C, current smoker; ASC, adenosquamous cell carcinoma; PD, progressive disease; FS, former smoker; LCC, large cell carcinoma.

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Table 2. *EGFR* mutation profiles as identified by direct sequencing, not designed by PNA clamp *EGFR* mutation detection kit or pyrosequencing

Case No.	Exon	Alteration	Direct sequencing		TKI response
			Nucleotide alteration	Amino acid alteration	
8	19	Deletion	2239-2263del	L747-755Adel	PD
9	19	Deletion	2253-2276del	S752_I759del	PD
10	20	Duplication	dup 2311-2319 AACCCCCAC	D770_N771insNPH	PD
11	20	Point mutation	2327G>A	R776H	PD

EGFR, epidermal growth factor receptor; PNA, peptide nucleic acid; TKI, tyrosine kinase inhibitor; Del, deletion; PD, progressive disease; Dup, duplication.

Table 3. Summary of concordant cases (n=46, 75%) of *EGFR* mutation profiles using direct sequencing, PNA clamping, and pyrosequencing according to clinicopathologic characteristics, including EGFR TKI responses

EGFR mutation	TKI response	Sex	Smoking	Histology
Exon19	**CR (n=2, 3%)**	F	N	ADC
(n=18, 29%)	**PR (n=3)**	F	N	ADC
		F	N	**SCC**
		M	C	ADC
	SD (n=9)	F (n=5)	N	ADC
		M (n=2)	FS	ADC
		M (n=2)	C	ADC
	PD (n=5)	F (n=3)	N	ADC
		M (n=2)	C	ADC
Exon21	**PR (n=5)**	F (n=3)	N	ADC
(n=11, 18%)		M	N	ADC
		M	C	ADC
	SD	M	C	ADC
	PD (n=5)	F (n=3)	N	ADC
		F (n=2)	C	ADC
Wild	**PR**	M	FS	ADC
(n=17, 28%)	**SD (n=1, 3%)**	F	N	ADC
	PD (n=15)	F (n=4)	N	ADC
		F	N	**SCC**
		F	C	ADC
		F	C	**LCNEC**
		M	N	**SCC**
		M (n=2)	FS	ADC
		M	C	ADC
		M (n=2)	C	**SCC**
		M (n=2)	C	**SarCa**

EGFR, epidermal growth factor receptor; PNA, peptide nucleic acid; TKI, tyrosine kianse inhibitor; CR, complete response; F, female; N, never smoker; ADC, adenocarcinoma; PR, partial response; SCC, squamous cell carcinoma; M, male; C, current smoker; SD, stable disease; FS, former smoker; PD, progressive disease; LCNEC, large cell neuroendocrine carcinoma; SarCa, sarcomatoid carcinoma.

exon 19 deletion was identified in the one patient with squamous cell carcinoma (SCC) histology; this patient exhibited a PR. Seven patients with wild-type *EGFR* exhibited PD, four of whom had SCC and three of whom had other histological types.

Patient characteristics and *EGFR* mutation status

EGFR mutations detected by the three methods were found more frequently in female patients (71% by DS, 60% by PNA clamping, and 69% by pyrosequencing), never smokers (72% by DS, 61% by PNA clamping, and 67% by pyrosequencing), and adenocarcinoma (ADC) histology (70%, p=0.014, by DS; 68%, p<0.001, by PNA clamping; 70%, p=0.002, by pyrosequencing) (Table 4). Of particular interest, a significant number of male patients (50% by DS, 54% by PNA clamping, and 50% by pyrosequencing) and ever smokers (former and current smokers; 49% by DS, 46% by PNA clamping, and 46% by pyrosequencing) were found to have *EGFR* mutations by these three methods. Of the 61 patients, 1 had a CR, 13 had a PR, 14 had SD, and 33 had PD.

Clinical outcomes among subgroups of patients treated with EGFR TKIs

As shown in Fig. 1, overall survival (p=0.046 for grouping by DS vs p=0.032 for PNA clamping vs p=0.002 for pyrosequencing) and progression-free survival (p=0.002 for grouping by DS vs p=0.021 for pyrosequencing) were significantly longer in patients with *EGFR* mutations detected by the three methods than in patients with wild-type *EGFR*.

The objective response rate (OR) in the overall population was 23% (Table 5). The OR was significantly higher in the patients with *EGFR* mutations detected by pyrosequencing than in patients with wild-type *EGFR* (35% vs 4%, p=0.005). However, the number of samples was too low to make any definitive conclusions. The disease control rate (DCR) in the overall population was 46%. The DCR was higher in patients with ADC histology than in those with non-ADC histology (52% vs 19%, p=0.051), although this difference did not reach statistical significance. The DCR was significantly higher in the patients with *EGFR* mutations than in those with wild-type *EGFR* (p=0.019 for grouping by DS; p=0.001 for PNA clamping; p<0.001 for pyrosequencing).

The median follow-up period for the analysis of progression-free survival was 16 months. The median progression-free survival was 19-20 months for the patients with *EGFR* mutations

Table 4. Patients' characteristics and *EGFR* mutation status using direct sequencing, PNA clamping, and pyrosequencing

	n (%)	Direct sequencing *EGFR* mutation			PNA clamping *EGFR* mutation			Pyrosequencing *EGFR* mutation		
		(+)	(-)	p-value	(+)	(-)	p-value	(+)	(-)	p-value
Total	61 (100)	38 (62)	23 (38)		35 (57)	26 (43)		37 (61)	24 (39)	
Sex										
Female	35 (57)	25 (71)	10 (29)	0.113	21 (60)	14 (40)	0.794	24 (69)	11 (31)	0.188
Male	26 (43)	13 (50)	13 (50)		14 (54)	12 (46)		13 (50)	13 (50)	
Smoking history[a]										
Never	36 (59)	26 (72)	10 (28)	0.066[b]	22 (61)	14 (39)	0.600[b]	24 (67)	12 (33)	0.294[b]
Former	6 (10)	3 (50)	3 (50)		2 (33)	4 (67)		2 (33)	4 (67)	
Current	19 (31)	9 (47)	10 (53)		11 (58)	8 (42)		11 (58)	8 (42)	
Histology										
ADC	50 (82)	35 (70)	15 (30)	0.014[c]	34 (68)	16 (32)	<0.001[c]	35 (70)	15 (30)	0.002[c]
SCC	5 (8)	1 (20)	4 (80)		1 (20)	4 (80)		1 (20)	4 (80)	
Others	6 (10)	2 (33)	4 (67)		0	6 (100)		1 (17)	5 (83)	
Tumor size (cm)										
≤3	26 (43)	21 (81)	5 (19)	0.016	19 (73)	7 (27)	0.040	21 (81)	5 (19)	0.008
>3	35 (57)	17 (49)	18 (51)		16 (46)	19 (54)		16 (46)	19 (54)	
Operation method										
Biopsy	1 (2)	1 (100)	0	1.000	1 (100)	0	1.000	1 (100)	0	1.000
Resection	60 (98)	37 (62)	23 (38)		34 (57)	26 (43)		36 (60)	24 (40)	
EGFR TKI response										
CR	1 (2)	1 (100)	0	0.537[d]	1 (100)	0	0.122[d]	1 (100)	0	0.005[d]
PR	13 (21)	9 (69)	4 (31)		10 (77)	3 (23)		12 (92)	1 (8)	
SD	14 (23)	12 (86)	2 (14)		12 (86)	2 (14)		12 (86)	2 (14)	
PD	33 (54)	16 (48)	17 (52)		12 (36)	21 (64)		12 (36)	21 (64)	

EGFR, epidermal growth factor receptor; PNA, peptide nucleic acid; ADC, adenocarcinoma; SCC, squamous cell carcinoma; TKI, tyrosine kianse inhibitor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
[a]Never smokers were defined as patients who had a lifetime smoking exposure of <100 cigarettes and former smokers were defined as patients who had stopped smoking at least 1 yr before diagnosis; [b]Comparison between never smokers and others; [c]Comparison between adenocarcinoma and nonadenocarcinoma; [d]Comparison between CR, PR and SD, PD.

detected by the three methods and 10-12 months for those with wild-type *EGFR* (p=0.008 for grouping by DS; p=0.020 for PNA clamping; p=0.018 for pyrosequencing). The median follow-up period for the analysis of overall survival was 30 months. The median overall survival was 34-35 months for the patients with *EGFR* mutations detected by the three methods and 24-25 months for those with wild-type *EGFR*. These results suggest that *EGFR* mutations, not clinical predictors such as sex, smoking history, or histology, are associated with better outcomes with EGFR TKI treatment in terms of progression-free survival.

DISCUSSION

In this study, three different methods for the detection of *EGFR* mutations–DS, PNA clamping, and pyrosequencing–were compared using samples from 61 NSCLC patients who were treated with EGFR TKIs. A total of 44 mutations (72%) were found by at least one of the three methods, and the concordances among the results were relatively high (82-85%; kappa coefficient, 0.713 to 0.736).

Comparisons of DS with PNA clamping and pyrosequencing with respect to TKI responses have recently been published, and the results of those studies were quite different from our results.[7,8,12,19] Kim *et al.*[7] reported that *EGFR* mutations were detected in 63 of 240 NSCLC patients (26%) by DS, whereas PNA clamping detected *EGFR* mutations in 83 patients (35%). The patients in that study were from eight centers of the Korean Molecular Lung Cancer Group (KMLCG).[7] The PNA clamping method was reported to detect 22 additional *EGFR* mutations-positive samples (10 in exon 19, 9 in exon 21, and 3 in both exons) and to identify more mutations than DS, although the clinical outcomes were not significantly different between the groups defined by each method. Dufort *et al.*[12] reported that pyrosequencing is a highly accurate method for detecting *EGFR* mutations in patients with NSCLC. They found that three *EGFR* mutations-positive samples were detected only by pyrosequencing and not by DS, reflecting the lower sensitivity of the classical sequencing method.

PNA is an artificially synthesized polymer that can bind to a

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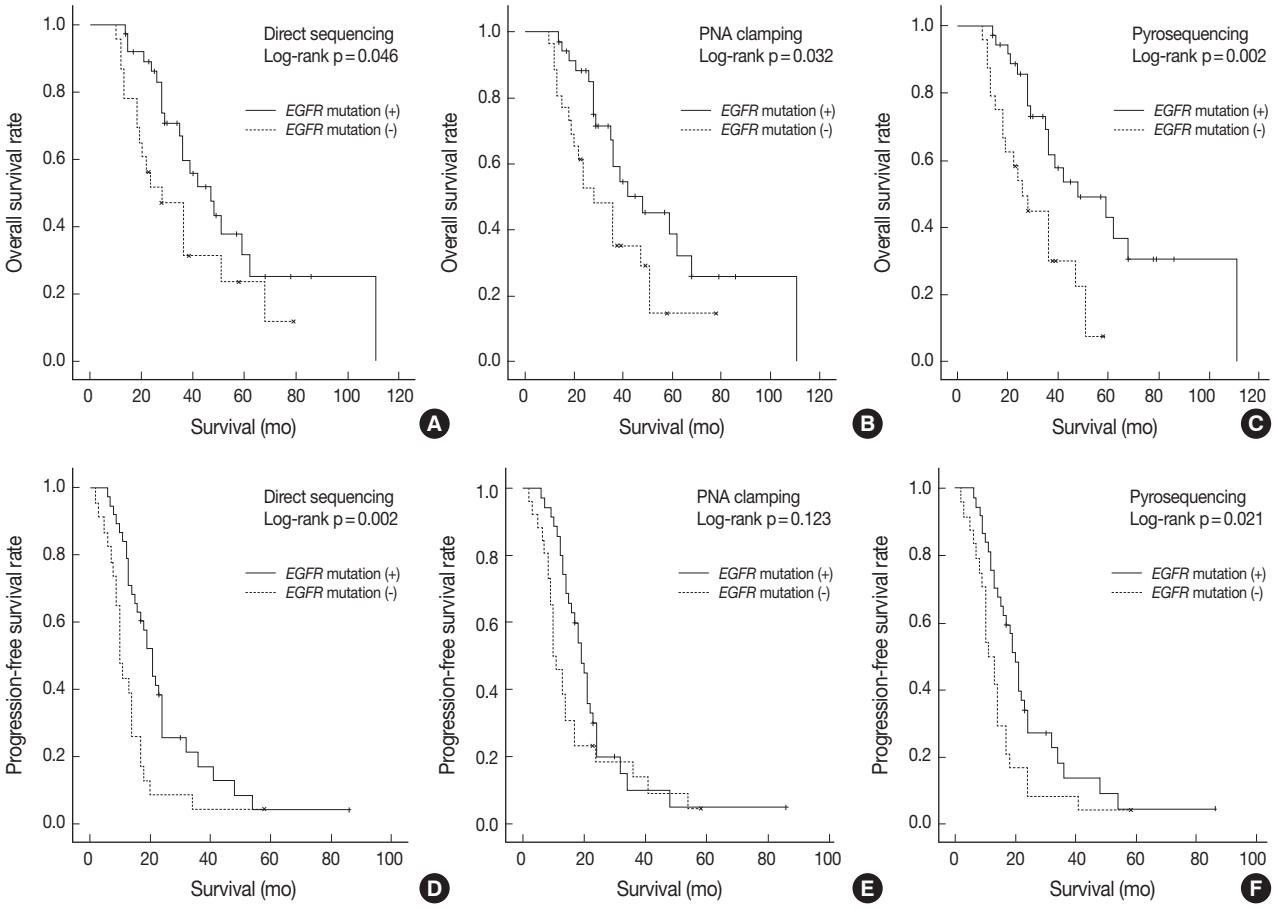

Fig. 1. Overall survival (A-C) and progression-free survival (D-F) with respect to the epidermal growth factor receptor (*EGFR*) mutation status of non-small cell lung carcinoma patients as determined by direct sequencing (A, D), peptide nucleic acid (PNA) clamping (B, E), and pyrosequencing (C, F).

complementary sequence in DNA; the binding capacity of PNA is stronger than that of DNA.[7-9,20] The PNA clamping method is known to be more sensitive, rapid and simple to perform, and can detect mutant alleles even when present at levels 100-fold lower than wild-type alleles, whereas the minimum percentage of mutant DNA for analysis by DS is more than 25%. The minimum percentage of mutant DNA needed for analysis by pyrosequencing is at least 20%.[12] However, the detection rates for *EGFR* mutations were not significantly different among the three methods used in this study. This lack of a significant difference might be due to 1) a higher proportion of tumor cells in the samples used in this study; 2) the macro- or microdissection of tumor cells prior to *EGFR* mutation tests by pathologists; and 3) the meticulous control of the turnaround time between the submission of the specimen to the pathology laboratory and formalin fixation in a single institution (SNUBH). During formalin fixation, the formaldehyde within tissues gradually changes to formic acid, which hydrolyzes DNA.[6] DNA quality is affected by the fixation time and the type of fixative used.[21] Greer *et al.*[22] and Liu *et al.*[23] suggested that tissues used for molecular tests should not be fixed for more than one day. To acquire a high proportion of tumor cells, a pathologist can, using a microscope, select an appropriate area from which DNA should be extracted. Thus, proper tissue handling (e.g., the timing of tissue sample acquisition, a shorter fixation time, and DNA quality control) by the pathologist is very important to improve the sensitivity of *EGFR* mutation tests.[5] Goto *et al.*[24] reported that it should be recognized that the detection rate of mutations by DS is largely influenced by the level of optimization in the processes implemented by the laboratory, and that the differences in reagents, DNA quality, software, and, crucially, primer design and amplicon size can affect the detection rate for DS. In particular, these researchers examined all the FFPE samples prepared by a single pathologist and generally found them to be of high quality and high tumor content.[24]

In this study, 15 cases (25%) with discrepant results for the

Table 5. Clinical outcomes among subgroups of patients who are treated with EGFR TKIs

	n (%)	Objective response[a]	p-value	Disease control rate[b]	p-value	Median TTP (mo)	p-value	MST (mo)	p-value
Total	61 (100)	14 (23)		28 (46)		16.0		30.0	
Sex									
Female	35 (57)	7 (20)	0.553	15 (43)	0.613	18.0	0.440	29.0	0.445
Male	26 (43)	7 (27)		13 (50)		13.5		35.0	
Smoking history[c]									
Never	36 (59)	9 (25)	0.762[d]	17 (47)	1.000[d]	17.0	0.300[d]	32.0	0.795[d]
Former	6 (10)	1 (17)		3 (50)		19.5		44.5	
Current	19 (31)	4 (21)		8 (42)		11.0		28.0	
Histology									
ADC	50 (82)	12 (24)	0.726[e]	26 (52)	0.051[e]	17.5	0.043[e]	30.0	0.100[e]
SCC	5 (8)	1 (20)		1 (20)		11.0		28.0	
Others	6 (10)	1 (17)		1 (17)		10.0		28.0	
Direct sequencing									
EGFR mutation (+)	38 (62)	10 (26)	0.537	22 (58)	0.019	20.0	0.008	34.5	0.428
EGFR mutation (-)	23 (38)	4 (17)		6 (26)		10.0		24.0	
PNA clamping									
EGFR mutation (+)	35 (57)	11 (31)	0.122	23 (66)	0.001	19.0	0.020	34.0	0.606
EGFR mutation (-)	26 (43)	3 (12)		5 (23)		10.5		24.0	
Pyrosequencing									
EGFR mutation (+)	37 (61)	13 (35)	0.005	25 (68)	<0.001	19.0	0.018	35.0	0.294
EGFR mutation (-)	24 (39)	1 (4)		3 (13)		12.0		25.0	

EGFR, epidermal growth factor receptor; TKI, tyrosine kianse inhibitor; TTP, time-to-progression; MST, median survival time; ADC, adenocarcinoma; SCC, squamous cell carcinoma.
[a]Objective response: complete response or partial response; [b]Disease control rate: complete response or partial response or stable disease; [c]Never smokers were defined as patients who had a lifetime smoking exposure of <100 cigarettes and former smokers were defined as patients who had stopped smoking at least 1 yr before diagnosis; [d]Comparison between never smokers and others; [e]Comparison between adenocarcinoma and nonadenocarcinoma.

three methods were identified. *EGFR* mutations were detected in nine cases by DS, six cases by PNA clamping, and eight cases by pyrosequencing. Because PNA clamping can detect 29 designed target mutations of clinical significance among the approximately 250 *EGFR* mutations[7] and because pyrosequencing has, in most cases, been used for the re-sequencing of a small number of selected hotspot codons,[25] these two methods cannot detect undesignated *EGFR* mutation sites. Four out of the 15 discrepant cases (cases nos. 8-11) (Table 2) were identified only by DS. The mutations in these cases were undesignated *EGFR* mutations and were thus not detectable by PNA clamping or pyrosequencing. The clinical significance of these rare mutations is still uncertain, and further analyses are needed. The weakness of PNA clamping and pyrosequencing is that these methods can only be used to detect mutations for which primers have been individually designed; in contrast, DS is able to uncover novel mutations.

In six patients (cases nos. 2-7) (Table 1), *EGFR* mutations were detected only by PNA clamping or pyrosequencing, but not by DS. These cases might have had less than 25% mutant DNA in the tested sample, therefore making these *EGFR* mutations undetectable by DS.

The types of *EGFR* mutations identified in this study were in accordance with those found in previous studies.[2,26] The most frequent mutations were an in-frame deletion in exon 19 (53-54%) and the L858R point mutation in exon 21 (35-39%); these mutations accounted for over 90% of the detected mutations. Patients who harbored activating *EGFR* mutations showed a positive clinical response to EGFR TKIs; the OR for these patients was 23% and the DCR was 46%. The OR and DCR were higher in *EGFR*-mutation-positive patients than in patients with wild-type *EGFR*, as reported in previous retrospective and prospective studies.[27] Only mutational analysis by pyrosequencing was statistically significantly correlated with OR. However, the number of samples was too low to make any definitive conclusions.

Mutational analysis by DS, PNA clamping, and pyrosequencing was successful and confirmed a strong and independent association between *EGFR* mutations and clinical outcome. As expected, *EGFR* mutations were more frequent in women who had never smoked and in those with ADC histology. In addition, a significant number of males (51%) and ever smokers (47%, current and former smokers) were found to have *EGFR* mutations by the three methods. Thus, it is necessary to test for *EGFR* mutations not only in female never smokers but also in males and ever smokers. Clinical predictors, such as sex, smok-

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ing history, and histology, added little predictive information to that provided by the mutational analysis. These data indicate that the mutational status of *EGFR* is the most important predictor of clinical outcome in EGFR TKIs-treated patients.[17]

In conclusion, all three *EGFR* mutation tests had good concordance rates (over 82%) for FFPE samples. These results suggest that if the DNA quality and enrichment of tumor cells are assured, then DS, PNA clamping, and pyrosequencing are appropriate methods for the detection of *EGFR* mutations. The presence of some cases with discordant results for the three different methods indicates that these methods must be further standardized and validated.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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