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).¹² 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.³⁶ Direct sequencing (DS) and pyrosequencing were the only approved diagnostic meth-
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. Pyrosequencing is a non-electrophoretic real-time sequencing technology using luminometric detection. 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. 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 mutations, rather than demographic features, are independently associated with a favorable prognosis for NSCLC patients treated with EGFR TKIs, the optimization of mutation tests is very important for selecting appropriate therapeutic strategies for NSCLC patients.

The aims of this study were 1) to evaluate the efficacy of the DS, PNA clamping, and pyrosequencing methods for detecting 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.

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. 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. 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. 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. 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. 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. 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. In 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

| 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        | L858R         | PR           | F   | N       | ADC       |
| 13       | L858R            | Wild         | L858R         | SD           | F   | N       | ADC       |
| 14       | L858R            | L858R        | L858R         | PD           | F   | N       | ADC       |

*EGFR*, epidermal growth factor receptor; PNA, peptide nucleic acid; TKI, tyrosine kinase 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.

http://www.koreanjpathol.org http://dx.doi.org/10.4132/KoreanJPathol.2013.47.1.52
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 |
|----------|------|------------|------------------|--------------|
| 8        | 19   | Deletion   | 2239-2263del     | L747-755del  | PD           |
| 9        | 19   | Deletion   | 2252-2270del     | S752_I759del | PD           |
| 10       | 20   | Duplication| dup 2311-2319 AACCOCAC | D770_N771insPH | 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       |
|               | PR (n=3)     | F   | N       | ADC       |
|               |              | M   | C       | ADC       |
| SD (n=9)      |              | F   | N       | ADC       |
|               |              | M   | C       | ADC       |
| PD (n=5)      |              | F   | N       | ADC       |
|               |              | M   | C       | ADC       |
| Exon21        | PR (n=5)     | F   | N       | ADC       |
|               |              | M   | N       | ADC       |
|               |              | M   | C       | ADC       |
| SD (n=5)      |              | F   | N       | ADC       |
|               |              | M   | C       | ADC       |
| PD (n=5)      |              | F   | N       | ADC       |
|               |              | M   | C       | ADC       |

Wild          | PR (n=17, 28%)| M   | FS      | ADC       |
|               | SD (n=1, 3%)  | F   | N       | ADC       |
|               | PD (n=15)     | F   | N       | SCC       |
|               |              | F   | C       | ADC       |
|               |              | M   | N       | SCC       |
|               |              | M   | C       | ADC       |

Wild          | PR (n=17, 28%)| M   | FS      | ADC       |
|               | SD (n=1, 3%)  | F   | N       | ADC       |
|               | PD (n=15)     | F   | N       | SCC       |
|               |              | F   | C       | ADC       |
|               |              | M   | N       | SCC       |
|               |              | M   | C       | ADC       |

EGFR, epidermal growth factor receptor; PNA, peptide nucleic acid; TKI, tyrosine kinase 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...
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 wild-type *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
complementary sequence in DNA; the binding capacity of PNA is stronger than that of DNA. 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%. 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 (SNU BH). During formalin fixation, the formaldehyde within tissues gradually changes to formic acid, which hydrolyzes DNA. DNA quality is affected by the fixation time and the type of fixative used. Greer et al. and Liu et al. 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. Goto et al. 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.

In this study, 15 cases (25%) with discrepant results for the
In six patients (cases nos. 2-7) (Table 1), EGFR mutations were detected only by PNA clamping or pyrosequencing. These cases might have had less than 25% mutant DNA in the tested sample, therefore making these novel mutations.

The types of EGFR mutations identified in this study were in accordance with those found in previous studies. 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. 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-
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.

Acknowledgments

This work was supported by a Grant-in-Aid from the Korea Healthcare technology R&D project, Ministry of Health and Welfare, Republic of Korea (A111405 to J.H.C.) and partly supported by a grant of National Research Foundation of Korea (2011-0025344 to J.E.K.).

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