Prognostic value of quantitative measurement of EGFR mutation using peptide nucleic acid clamping in advanced EGFR mutant non-small cell lung cancer patients

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Keywords
Epidermal growth factor receptor; lung cancer; molecular targeted therapy; peptide nucleic acid; prognosis.

Abstract
Background: The presence of EGFR mutation in patients with advanced non-small cell lung cancer (NSCLC) plays an important role in determining the appropriate treatment, response, and survival. Therefore, this study attempted to predict the prognosis of NSCLC patients using data from quantitative mutation measurements.

Methods: The data of patients with advanced NSCLC who underwent EGFR mutation testing using the peptide nucleic acid (PNA) mediated clamping method at the Pusan National University Hospital from October 2015 to December 2017 were retrospectively analyzed. The efficiency of PNA clamping was determined by measuring the threshold cycle (Ct) value. The ΔCt−1 value (standard Ct value minus sample Ct value) was calculated to quantify EGFR mutation.

Results: During the study period, 71 patients were treated with EGFR-tyrosine kinase inhibitors. The cutoff point for the ΔCt−1 value derived from the receiver operating characteristic curve was 5.32. A survival benefit was observed in the group with a ΔCt−1 value > 5.32 or with a common EGFR mutation type compared to the group with a ΔCt−1 value < 5.32.

Conclusion: EGFR mutation testing using PNA clamping may predict patient survival, especially in patients with common EGFR mutations, such as exon 19 deletion or L858R. A higher ΔCt−1 value correlates with better survival.

Introduction
Lung cancer is a significant cause of cancer-related death worldwide, and its incidence has been rapidly increasing.1 Non-small cell lung cancer (NSCLC) is the most common type, accounting for approximately 85% of all lung cancers.2,3 Adenocarcinoma is the most common histologic type of NSCLC, and can be subdivided using molecular diagnostic methods into patients harboring EGFR mutation or ALK translocation.4 Targeted therapeutics directed at these oncogenic alterations have delivered remarkable therapeutic results.5–7

The proportion of patients with EGFR mutations is higher than patients with ALK translocations,8,9 which enables more widely applicable targeted therapy. In addition, EGFR inhibition shows a well validated survival benefit and treatment response.6,7,10 However, previous studies have revealed different therapeutic responses with the same mutation type.5,11 Although the possible causes of the observed differential therapeutic responses have been studied, no plausible explanation has yet been determined.

There are many methods to detect EGFR mutation in patients, including direct sequencing, real-time PCR, and peptide nucleic acid (PNA) clamping. Direct sequencing has traditionally been used and remains the standard method to detect EGFR mutation in lung cancer.12 In contrast, PNA clamping is the latest molecular diagnostic technology, and has become favored in recent years because of
its simple processing steps, rapid output, and high sensitivity compared to the conventional method. However, some studies have shown that patients with the same mutation domain diagnosed by the same diagnostic method (PNA clamping) have different treatment responses to EGFR-tyrosine kinase inhibitors (TKIs). Therefore, we used the ΔCt−1 value from PNA clamping to better predict therapeutic response and prognosis in patients with EGFR mutations.

Methods

Study population

A total of 142 patients diagnosed with NSCLC and a confirmed EGFR mutation via PNA clamping treated at the Pusan National University Hospital (a university-affiliated, tertiary referral hospital in Busan, South Korea) between October 2015 and December 2017 were included in this retrospective study. Seventy-one patients were treated with EGFR-TKIs (Fig 1). As this was a retrospective study, the institutional review board of Pusan National University Hospital approved this work without requiring informed patient consent (approval no. H-1901-026-075).

Peptide nucleic acid clamping method

We used the PNA clamping method to determine the EGFR mutation status of each patient. This method uses PNA specific to the wild-type sequence to inhibit amplification of the wild-type EGFR gene. The resulting amplification signal occurs when mutant DNA is detected using intercalating dye. PNA clamping analysis was performed using a PNA clamp EGFR Mutation Detection Kit (Panagene, Deajeon, South Korea) following the manufacturer’s directions.

For a single amplification reaction, 7 μL of DNA template was mixed with 3 μL of PNA mixture and 10 μL reaction master mix. The reaction was amplified using a CFX96 real-time PCR instrument (Bio-Rad, San Francisco, CA, USA) with 5 minutes of initial denaturation at 94°C, followed by 40 cycles of amplification. Detection of the amplification signal was measured during the annealing step.14

The threshold cycle (Ct) value is based on the fluorescence values measured during the annealing step and the ΔCt−1 value is automatically calculated by subtracting sample Ct from standard Ct:15

\[ ΔCt−1 = \text{Standard Ct} - \text{Sample Ct} \]

Treatment response

Patients underwent radiologic evaluation at baseline and every three months after treatment to assess treatment response. Brain imaging and bone scans were performed when the patient had clinically suspicious disease progression findings.

Statistical analysis

Descriptive statistics were used to summarize categorical and continuous variables, which were compared using the chi-square test for correlation analysis. The cutoff point, 5.32, was calculated using the receiver operation characteristic curve. Overall survival (OS) rates determined using

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Figure 1 Flow diagram of study patients.
the Kaplan–Meier method were assessed from the initiation of treatment until death from any cause. All analyses were conducted using SPSS version 22.0.

Results

Patients

A total of 142 patients were diagnosed with EGFR mutations during the study period, of which 71 were treated with EGFR-TKIs. Biopsy tissue was used to perform EGFR mutation testing using a PNA clamp. The treatment group consisted of 35 (49.3%) individuals aged > 65 years, 31 (43.7%) of which were male. The numbers of patients with stage III and IV NSCLC were 9 (12.7%) and 62 (87.3%), respectively. Sixty-four patients (90.1%) had common EGFR mutations (exon 19 deletion or L858R) and 27 patients (38.0%) had central nervous system metastasis. The EGFR-TKIs administered to the patients were: gefitinib in 10 patients (14.1%), erlotinib in 7 (9.9%), and afatinib in 54 (76.1%) (Table 1). EGFR-TKIs were used as first-line treatment in all patients.

Progression-free survival analysis

The mean progression-free survival (PFS) of all patients was 14.7 months (95% CI 14.0–15.4), while those with an ΔCt-1 value > 5.32 had a mean PFS of 24.1 months (95% CI 23.5–24.7), which was a statistically significant difference (P = 0.001) (Fig 2a). In patients with uncommon EGFR mutations, the overall PFS was 13.5 (95% CI 12.8–14.2), but those with an ΔCt-1 value > 5.32 had a mean PFS of 20.4 months (95% CI 20.4–20.7); however, this difference was not statistically significant (P = 0.09).

Overall survival analysis

The mean OS was 21.0 months (95% CI 18.5–23.5) in all patients. In the group with an ΔCt-1 value < 5.32, mean OS was 16.5 months (95% CI 12.3–20.7), while in the group with an ΔCt-1 value > 5.32, mean OS was 24.5 months (95% CI 22.4–26.6), which was a statistically significant difference (P = 0.001) (Fig 3a).

When stratified by EGFR subtype, mean OS was 21.6 months (95% CI 19.2–24.1) in patients with common EGFR mutations, including exon 19 deletions and L858R. Among patients with common EGFR mutations, an ΔCt-1 value < 5.32 corresponded to mean OS of 18.0 months (95% CI 13.5–22.5), while OS increased to 24.1 months in patients with an ΔCt-1 value > 5.32 (95% CI 21.8–26.3), which was also statistically significant (P = 0.014) (Fig 3b). In the uncommon EGFR mutation group, OS could not be evaluated because of the limited number of subjects.

Discussion

This study showed that when the ΔCt-1 value obtained by PNA clamping was greater than the cutoff value of 5.32, a survival benefit was observed among all patients, including those with common mutations. In the 71 patients with EGFR mutations who were treated with EGFR-TKIs, the patients with an ΔCt-1 value > 5.32 had a mean survival time of 24.5 months, which was a statistically significant survival benefit compared to those who did not. When the EGFR mutations were stratified by common or uncommon mutations, the difference in survival time according to the ΔCt-1 value was statistically significant in the common

Table 1 Baseline characteristics of NSCLC patients harboring EGFR mutation

| Variables          | Total (n = 71) | ΔCt-1 ≥ 5.32† (n = 38) | ΔCt-1 < 5.32† (n = 33) | P   |
|--------------------|---------------|------------------------|------------------------|-----|
| Age (years)        |               |                        |                        |     |
| < 65               | 36 (50.7)     | 18 (50.0)              | 18 (50.0)              | 0.546|
| ≥ 65               | 35 (49.3)     | 20 (57.1)              | 15 (42.9)              |     |
| Gender, female     |               |                        |                        |     |
|                    | 40 (56.3)     | 20 (50.0)              | 20 (50.0)              | 0.499|
| Never smoker       |               |                        |                        |     |
|                    | 47 (66.2)     | 23 (48.9)              | 24 (51.1)              | 0.278|
| Tumor stage‡       |               |                        |                        | 0.896|
| III/IV             | 9 (12.7)      | 5 (55.6)               | 4 (44.4)               |     |
|                   | 62 (87.3)     | 33 (53.2)              | 29 (46.8)              |     |
| Common EGFR mutation§ |           |                        |                        |     |
| CNS metastasis     | 27 (38.0)     | 11 (40.7)              | 16 (59.3)              | 0.091|
| EGFR-TKIs          |               |                        |                        | 0.200|
| Gefitinib†         | 10 (14.1)     | 3 (30.0)               | 7 (70.0)               |     |
| Erlotinib          | 7 (9.9)       | 5 (71.4)               | 2 (28.6)               |     |
| Afatinib           | 54 (76.1)     | 30 (55.6)              | 24 (44.4)              |     |

†The cutoff value of ΔCt-1. †According to 8th edition Tumor Node Metastasis Staging system. §Common EGFR mutations: exon 19 deletion or L858R. CNS, central nervous system; TKI, tyrosine kinase inhibitor.
Our findings suggest that the ΔCt-1 value can be used as an indicator to predict survival benefit and therapeutic selection for patients with advanced NSCLC. Many studies of treatment responses and survival according to EGFR mutation subtypes and individual factors have been conducted. However, few studies have assessed the methods for predicting prognosis based on diagnostic testing of EGFR mutation status.

Alegre et al. reported that patients with a baseline total EGFR mutation copy level above the median value showed reduced OS and PFS compared to those who did not; however, measurement of plasma cell-free DNA using droplet digital PCR is not widely used in clinical practice to detect EGFR mutation. Imamura et al. used PCR amplification and deep sequencing to detect exons 19, 20, and 21 of the EGFR gene and to obtain a plasma mutation score, which

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**Figure 2** Kaplan–Meier curves of progression-free survival (PFS) stratified by ΔCt-1 value in (a) total patients and (b) patients with common EGFR mutations (exon 19 deletion or L858R mutation in exon 21). (—) Ct-1 ≥ 5.32 and (—) Ct-1 < 5.32.

**Figure 3** Kaplan–Meier curves of overall survival (OS) stratified by ΔCt-1 value in (a) total patients and (b) patients with common EGFR mutations (exon 19 deletion or L858R mutation in exon 21). (—) Ct-1 ≥ 5.32 and (—) Ct-1 < 5.32.
can be used to evaluate therapeutic response and monitor oncogenic status. However, the correlation between mutation test results and survival outcome in NSCLC patients was not evaluated and the study sample was relatively small. Karachaliou et al. tested for circulating free DNA using the PNA–mediated 5′ nucleic real-time PCR assay in advanced NSCLC patients with oncogenic EGFR mutations (exon 19 deletion or L858R mutation in exon 21) and reported that detectable a L858R mutation in tumor tissue and circulating free DNA correlated with shorter OS. Although their study was conducted with a relatively large study group and presented OS, PFS, and response rate, Karachaliou et al. only included European patients and did not assay survival using quantitative differences in EGFR testing.

Park et al. found that patients with a higher corrected $\Delta C_{\text{t}}$ value than the average had a better objective response, a tendency for longer PFS, and a better clinical outcome. Similar to our findings, this study also showed that if the $\Delta C_{\text{t}}$ value was above the cutoff point, there was a treatment benefit compared to the other patients. The study by Park et al. was similar to ours in that it was retrospective in nature, performed at a single institution, and used delta values. However, it used EGFR-TKIs not only as a first-line treatment, but also as second and third-line treatment. In addition, there were differences between the studies in terms of the delta value cutoff point, number of patients, and parameters of histological diagnosis; moreover, they did not measure OS differences because of discrepancies in test values. According to the National Comprehensive Cancer Network guidelines, EGFR-TKIs are generally recommended as first-line therapy for patients with EGFR mutations. However, the study by Park et al. deviated from the guidelines regarding the use of EGFR-TKI in clinical practice, and 20% of their patients had histological features other than adenocarcinoma.

There are several limitations to this study. First, it was designed as a retrospective study, which may have resulted in selection bias. Second, it was a single institution study with a small number of subjects. Third, there were three types of EGFR-TKI drugs used for the treatment of advanced NSCLC. The selection of drugs could introduce bias in treatment outcomes. These shortcomings will need to be addressed in future multicenter, prospective studies using a larger number of subjects. Fourth, because of the retrospective nature of this study, it was difficult to relate the $\Delta C_{\text{t}}$–1 values to the results of the quantitative analysis of EGFR mutations. Therefore, additional prospective studies including larger samples are required.

In conclusion, this study showed that the $\Delta C_{\text{t}}$–1 value derived from EGFR mutation testing using PNA clamping may predict patient survival, with a higher $\Delta C_{\text{t}}$–1 value suggesting improved survival. This holds for patients with common EGFR mutations, such as exon 19 deletion or L858R.

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**Disclosure**

No authors report any conflict of interest.

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