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ORIGINAL ARTICLE

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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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 (C_t) value. The ΔC_t-1 value (standard C_t value minus sample C_t 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 ΔC_t-1 value derived from the receiver operating characteristic curve was 5.32. A survival benefit was observed in the group with an ΔC_t-1 value > 5.32 or with a common *EGFR* mutation type compared to the group with an ΔC_t-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 ΔC_t-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,[3,8,9] 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.[6,11] 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).[13] Therefore, we used the ΔC_t-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 (C_t) value is based on the fluorescence values measured during the annealing step and the ΔC_t-1 value is automatically calculated by subtracting sample C_t from standard C_t:[15]

$$\Delta C_t - 1 = [\text{Standard } C_t] - [\text{Sample } C_t]$$

Treatment response

Patients underwent radiologic evaluation at baseline and every three months after treatment to assess treatment response.[16] 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

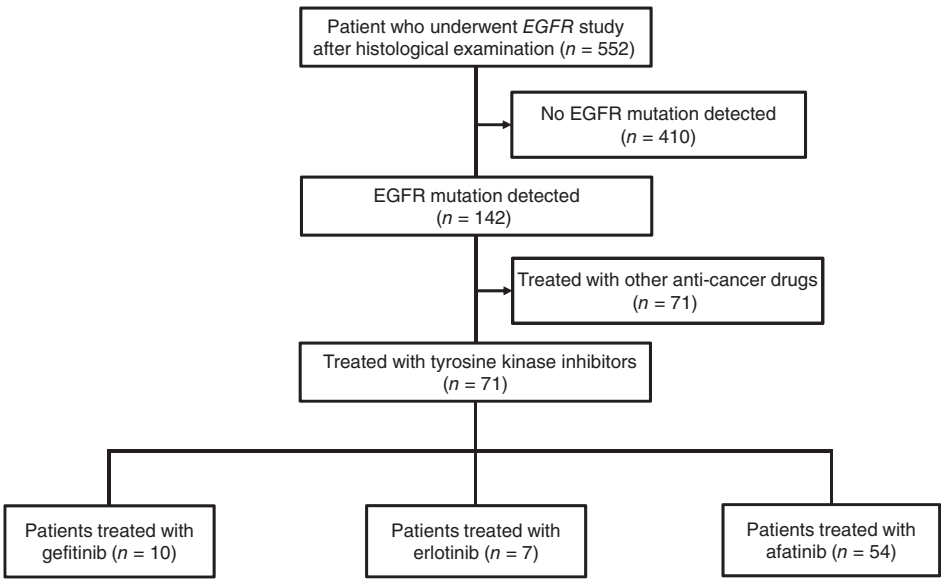

Figure 1 Flow diagram of study patients.

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.

Table 1 Baseline characteristics of NSCLC patients harboring *EGFR* mutation

Variables	Total (n = 71)	$\Delta C_t-1 \geq 5.32$† (n = 38)	$\Delta C_t-1 < 5.32$† (n = 33)	P
Age (years)				0.546
< 65	36 (50.7)	18 (50.0)	18 (50.0)	
≥ 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
IIIA/B	9 (12.7)	5 (55.6)	4 (44.4)	
IV	62 (87.3)	33 (53.2)	29 (46.8)	
Common *EGFR* mutation§	64 (90.1)	36 (56.2)	28 (43.8)	0.163
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 ΔC_t-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.

Progression-free survival analysis

The mean progression-free survival (PFS) of all patients was 14.7 months (95% confidence interval [CI] 12.2–17.2). In the group with an ΔC_t-1 value < 5.32, mean PFS was 14.0 months (95% CI 9.7–18.3), while in the group with an ΔC_t-1 value > 5.32, mean PFS was 14.9 months (95% CI 12.1–17.8). This difference was not statistically significant (P = 0.806) (Fig 2a).

When analyzed by *EGFR* subtype, the mean PFS was 14.9 months (95% CI 12.2–17.5) for patients with common *EGFR* mutations, including exon 19 deletions and L858R. Among these patients, those with an ΔC_t-1 value < 5.32 had mean PFS of 15.0 months (95% CI 10.4–19.6) while those with an ΔC_t-1 value > 5.32 had mean PFS of 14.6 months (95% CI 11.5–17.6), again without statistical significance (P = 0.872) (Fig 2b). In patients with uncommon *EGFR* mutations, the overall PFS was 13.5 (95% CI, 5.2–21.8), but those with an ΔC_t-1 value < 5.32 had a mean PFS of 6.6 months (95% CI 6.4–6.8), while those with an ΔC_t-1 value > 5.32 had a mean PFS of 20.4 months (95% CI 20.4–20.4); 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 ΔC_t-1 value < 5.32, mean OS was 16.5 months (95% CI 12.3–20.7), while in the group with an ΔC_t-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 ΔC_t-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 ΔC_t-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 ΔC_t-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 ΔC_t-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 ΔC_t-1 value was statistically significant in the common

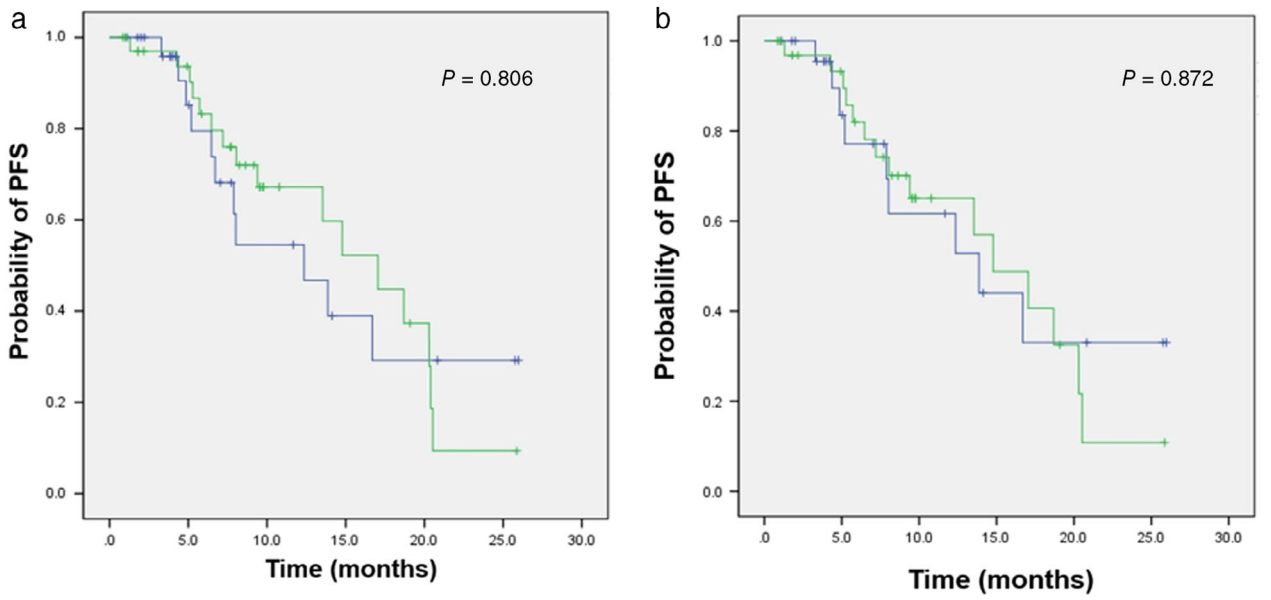

Figure 2 Kaplan–Meier curves of progression-free survival (PFS) stratified by ΔC_t-1 value in (**a**) total patients and (**b**) patients with common *EGFR* mutations (exon 19 deletion or L858R mutation in exon 21). (——) $C_t-1 \geq 5.32$ and (——) $C_t-1 < 5.32$.

EGFR group. Our findings suggest that the ΔC_t-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.[17–20] 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.[21] 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

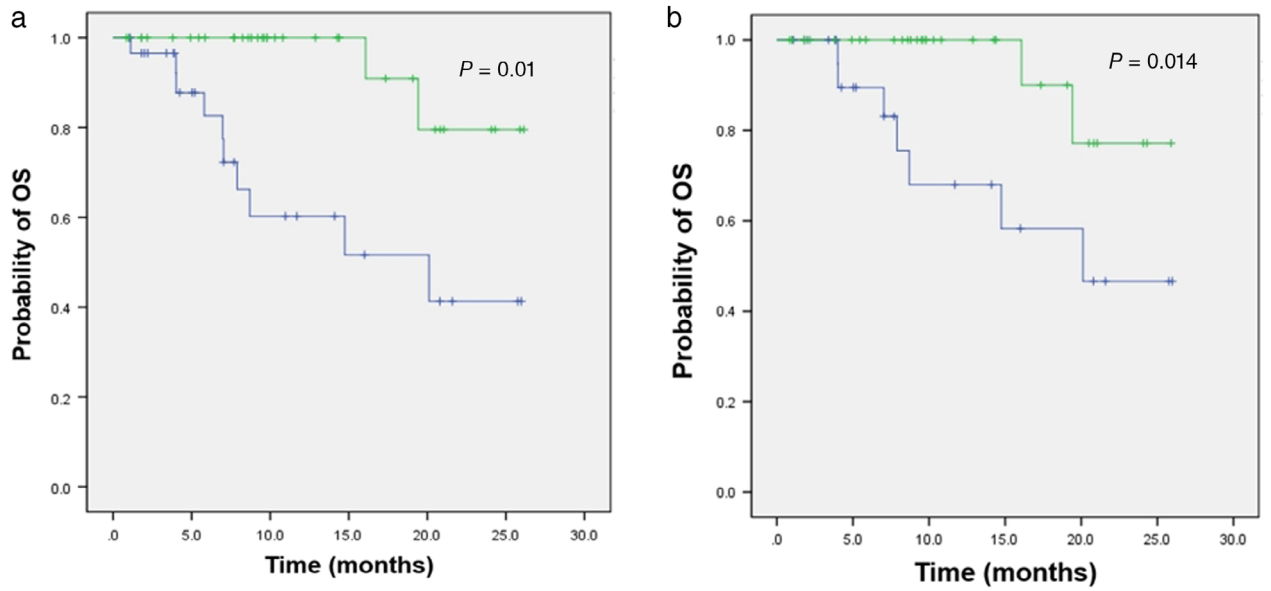

Figure 3 Kaplan–Meier curves of overall survival (OS) stratified by ΔC_t-1 value in (**a**) total patients and (**b**) patients with common *EGFR* mutations (exon 19 deletion or L858R mutation in exon 21). (——) $C_t-1 \geq 5.32$ and (——) $C_t-1 < 5.32$.

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.[22] Karachaliou *et al.* tested for circulating free DNA using the PNA–mediated 5′ nuclease 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.[23]

Park *et al.* found that patients with a higher corrected ΔC_t-1 value than the average had a better objective response, a tendency for longer PFS, and a better clinical outcome.[13] Similar to our findings, this study also showed that if the ΔC_t-1 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 $\Delta Ct-1$ 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 ΔC_t-1 value derived from *EGFR* mutation testing using PNA clamping may predict patient survival, with a higher ΔC_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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