Quantification of epidermal growth factor receptor (EGFR) mutation may be a predictor of EGFR-tyrosine kinase inhibitor treatment response

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Keywords
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Abstract
Background: Epidermal growth factor receptor (EGFR) gene mutation is a reliable predictive factor for response to EGFR-tyrosine kinase inhibitors (TKIs). The quantified EGFR value may also predict response and survival within an EGFR mutated group.

Methods: We conducted a retrospective study of 836 lung cancer patients. The patient sample was divided into two groups based on the mean delta cycle threshold (ΔCt) value. EGFR mutation tests using peptide nucleic acid (PNA)-mediated clamping polymerase chain reaction (PCR) were performed. The efficiency of PCR clamping was determined by measuring the Ct value and EGFR quantification was determined by the corrected ΔCt value.

Results: EGFR mutation positivity was 30.1% and there were 235 single activating mutations. In this mutation group, the higher corrected ΔCt value (≥ mean value) group showed better objective response (70.9% vs. 54.9%, P = 0.022) and clinical benefit rates (86.4% vs. 68.3%, P = 0.003) than the lower group. In addition, corrected ΔCt values were significantly and inversely correlated with disease response (r = -0.184, P = 0.017). In multivariate analysis, both female gender (P = 0.014) and higher corrected ΔCt value (P = 0.012) were independent predictive factors for better clinical benefit rate. The higher corrected ΔCt value group had a tendency for longer progression-free survival than the lower group (P = 0.050).

Conclusion: The corrected ΔCt value, which refers to EGFR quantification by PNA-mediated PCR clamping, can predict better clinical response to EGFR-TKI therapy. However, further study is warranted to determine its value as a biomarker to reflect survival.

Introduction
Lung cancer is the major cause of cancer-related mortality worldwide.¹⁻³ The discovery of epidermal growth factor receptor (EGFR) gene mutation has changed diagnosis and treatment methods for advanced non-small cell lung cancer (NSCLC).⁴⁻⁶ Many randomized clinical trials have shown that EGFR-tyrosine kinase inhibitors (TKIs) are a better treatment option than standard platinum double chemotherapy in patients with active EGFR mutations.⁷⁻¹¹ EGFR mutations, such as deletions in exon 19 (19del) and point mutations in exon 21 (L858R) are reliable predictive factors of a response to EGFR-TKIs.¹²,¹¹ In contrast, chemotherapy is a better choice than EGFR-TKIs in wild-type NSCLC.⁹,¹⁰,¹⁴,¹⁵ Therefore, EGFR gene mutation status is very important when deciding which first-line treatment modality to apply in patients with advanced NSCLC.
Several methods for detecting EGFR gene mutations have been developed. Highly sensitive methods, such as the scorpion amplified refractory mutation system (ARMS) and peptide nucleic acid (PNA) clamping are being used with less sensitive direct sequencing techniques. All of these methods report that EGFR mutation status provides binary results, such as positive or negative. A study of EGFR mutation positive NSCLC patients reported a response rate of 68% and progression-free survival (PFS) of 12 months. However, not all EGFR mutation positive NSCLC patients are equally responsive to EGFR-TKIs. Until now, no EGFR mutation test has been available to report biological continuous variables. We hypothesized that the more EGFR mutated genes present, the better response to EGFR-TKIs. Therefore, this study examined whether the quantification of EGFR gene mutation status can be a predictive marker of clinical response and survival using EGFR-TKI treatment, especially in NSCLC patients with sensitive EGFR mutations.

Methods

Patients and materials

This study is a retrospective observational study and included all patients who underwent EGFR PNA clamping at the Chonnam National University Hwasun Hospital between January 2013 and June 2015. Among the total 836 patients tested for EGFR mutations, 265 patients received EGFR-TKIs and 252 were positive for EGFR mutations (Fig 1). The PNA clamping test to detect EGFR genes was performed using DNA acquired from formalin-fixed paraffin-embedded tumor tissue or cytology specimens. The hospital institutional review board approved the study (IRB approval number: CNUHH-2016-012) and written informed consent was waived because of the retrospective design.

PNA clamping and quantification of EGFR gene mutation

DNA was extracted from five paraffin sections (10-μm) of the tumor tissue. Before DNA extraction, the tissue was deparaffinized in xylene and then washed in 70% ethanol. DNA was isolated with a Gene All Tissue DNA Purification Kit (General Biosystem Inc., Seoul, Korea) according to the manufacturer’s protocol. The DNA obtained was eluted in 50 μL of elution buffer, and the concentration and purity of the extracted DNA were assessed by spectrophotometry with a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The extracted DNA was stored at -20°C until use.

We used the PNA Clamp Mutation Detection Kit (Panagene Inc., Daejeon, Korea) to detect EGFR gene mutations by real-time polymerase chain reaction (PCR). All reactions were carried out in a volume of 20 μL with template DNA, a primer and PNA probe set, and fluorescent dye PCR master mix. Real-time PCR was performed using a CFX 96 Kit (Bio-Rad, Philadelphia, PA, USA). PCR cycling conditions were performed with a five minute hold at 94°C, followed by 40 cycles at 94°C for 30 seconds, 70°C for 20 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. Detection of each of the 29 EGFR gene mutations was possible through one-step PNA mediated real-time PCR clamping. PCR efficiency was determined by measuring the cycle threshold (Ct) values, which were automatically calculated from PCR amplification plots of fluorescence versus the number of cycles (Fig 2).

The delta (Δ)Ct-1 value (standard Ct value minus sample Ct value) was calculated to identify the presence of EGFR mutation. When the ΔCt-1 value was more than 2, the sample was considered as mutant. When the ΔCt-1 value was between 0 and 2, the ΔCt-2 value (sample Ct value minus non-PNA Ct value) was calculated. For EGFR quantification, we used the corrected ΔCt value, which standardized the variations in sample quality. The formula used is as follows: corrected ΔCt = standard Ct – sample Ct x 25/non PNA.

Response evaluation

The response to EGFR-TKIs was assessed by chest computed tomography (CT) every two months until radiologic evidence of disease progression or symptomatic deterioration. The radiologic response was evaluated
according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1: (i) complete response (CR), disappearance of all clinical and radiological evidence of the tumor; (ii) partial response (PR), decrease of 30% or more in the sum of the longest diameters of all target measurable lesions; (iii) progressive disease (PD), increase of more than 20% of the sum of the longest diameters of all target measurable lesions or the appearance of new lesions; and (iv) stable disease (SD), all other circumstances.21

The objective response rate (ORR) is defined as the percentage of patients who achieve CR or PR. The disease control rate (DCR) is defined as the percentage of patients who achieve CR, PR, or SD. The clinical benefit rate (CBR) is defined as CR or PR or at least six months of SD.22

Statistical analysis

Pearson’s chi-square or Fisher’s exact tests were used to assess the relationship between various clinical factors, corrected ΔCt, and response to EGFR-TKIs. PFS and overall survival (OS) rates were analyzed using the Kaplan–Meier method. We performed linear regression for multivariate analysis. All analyses were performed using IBM SPSS version 22.0 (IBM Co., Armonk, NY, USA). A P value less than 0.05 indicated statistical significance.

Results

Patient and specimen characteristics

The baseline characteristics of the 836 enrolled patients and specimens are summarized in Table 1. The mean age of the patients was 67.27 years. Most patients were male (63.3%), and adenocarcinoma (80.4%) was the major histologic type, followed by squamous cell carcinoma (11.6%). EGFR wild-type accounted for 69.9% and EGFR mutation 30.1% (15.7% exon19 deletion, 12.4% L858R or L861Q) of the sample.

Of the specimens analyzed, 679 (81.2%) were biopsy samples and 157 (18.8%) were cytology samples from cell-blocks. Most biopsy samples were obtained via bronchoscopy, endobronchial ultrasound-transbronchial needle aspiration, or transthoracic needle biopsy from lung. EGFR, epidermal growth factor receptor. Values are presented as number (%) unless otherwise specified.

Table 1 Clinical features of all patients (n = 836)

| Variables                  | Number (%)       |
|----------------------------|------------------|
| Age (year)                 |                  |
| Mean ± standard deviation  | 67.27 ± 120.945  |
| Median (range)             | 69 (20–93)       |
| Gender                     |                  |
| Male                       | 530 (63.4)       |
| Female                     | 306 (36.6)       |
| Smoking status             |                  |
| Never smoker               | 378 (45.2)       |
| Former or current smoker   | 458 (54.8)       |
| Pathology                  |                  |
| Adenocarcinoma             | 672 (80.4)       |
| Squamous cell carcinoma    | 97 (11.6)        |
| Mixed or other types       | 67 (8.0)         |
| EGFR mutation type         |                  |
| Wild-type                  | 584 (69.9)       |
| Exon 19 deletion           | 131 (15.7)       |
| L858R or L861Q             | 104 (12.4)       |
| Other types†               | 17 (2.0)         |
| Specimen                   |                  |
| Tissue biopsy              | 679 (81.2)       |
| Surgery                    | 107 (12.8)       |
| Small biopsy‡              | 485 (58.0)       |
| Lymph node excision biopsy | 87 (10.4)        |
| Cytology                   | 157 (18.8)       |
| Bronchial washing          | 62 (7.4)         |
| Pleural fluid              | 94 (11.2)        |
| Sputum                     | 1 (0.1)          |

†Other types consisted of insertion three duplication (7 patients), G719X (7), S768I (2), and T790M (1). ‡Small biopsy specimens were produced by bronchoscopy, endobronchial ultrasound-transbronchial needle aspiration, or transthoracic needle biopsy from lung.
We assessed the treatment response to EGFR-TKIs using ORR, DCR, and CBR (Table 2). The ORR, DCR and CBR to EGFR-TKIs were significantly better in female than male patients ($P < 0.001$). Non-smokers had better ORRs ($P = 0.012$), DCRs ($P = 0.002$), and CBRs ($P = 0.005$) than smokers. Adenocarcinoma histology had better DCRs (76.1%) than squamous cell carcinoma (44.4%, $P = 0.029$).

The EGFR mutation positive group had better ORRs, DCRs, and CBRs than the wild-type group ($P < 0.001$). The group treated with EGFR-TKIs as first-line had better ORRs, DCRs, and CBRs than those treated with EGFR-TKIs as second and third or further line treatment ($P < 0.001$). There is disparity between the number of patients that were treated with EGFR-TKIs (265) and the number of patients with EGFR mutations (253). Not all patients with EGFR mutations could be treated with EGFR-TKIs because of poor performance status. In addition, patients without EGFR mutations treated with EGFR-TKIs as second-line treatment were included in this study.

We performed EGFR quantification by corrected $\Delta Ct$ value within the EGFR mutation positive group and the mean corrected $\Delta Ct$ value was 6 (Table S1). The group with higher corrected $\Delta Ct$ values ($\Delta Ct \geq 6$) showed a better ORR (70.9% vs. 54.9%, $P = 0.022$) and CBR (86.4% vs. 68.3%, $P = 0.003$) than the lower group. If categorized into a median value of corrected $\Delta Ct$ as 6.42, the higher corrected $\Delta Ct$ value ($\Delta Ct \geq 6.42$, $n = 124$) group showed a better CBR (86.8% vs. 71.3%, $P = 0.009$) than the lower group ($\Delta Ct < 6.42$, $n = 128$).

According to RECIST version 1.1, the best measurable responses ranged from a 100% reduction ($-100\%$) to a 250% increase in tumor size. Corrected $\Delta Ct$ values were significantly and inversely correlated with disease response in the 168 patients with measurable target lesions ($r = -0.184$, $P = 0.017$; Fig 3).

In addition, we performed multivariate analysis, including variables such as corrected $\Delta Ct$, gender, smoking history, pathology, EGFR mutation type, and EGFR-TKI treatment line in patients who had EGFR mutations ($n = 252$). Both female gender (odds ratio [OR] = 3.058, $P = 0.014$) and higher corrected $\Delta Ct$ value (OR = 2.734, $P = 0.012$) were independent predictive factors for a better CBR (Table 3).

### Survival

In survival analysis, the higher corrected $\Delta Ct$ value ($\Delta Ct \geq 6$) group had longer PFS than the lower group (303.4 vs. 223.9 days, $P = 0.050$), but this result was not statistically

| Variable | Best response |   |   |   |   |   |   |   |   |   |   |
|----------|---------------|---|---|---|---|---|---|---|---|---|---|
| Gender   |               | CR| PR| SD| ORR (%) | P  | DCR (%) | P  | CBR* (%) | P  |
| Male     |               | 3 | 51| 36| 38.3     | 0.000| 63.8     | 0.000| 49.6     | 0.000|
| Female   |               | 12| 68| 35| 59.7     | 0.000| 85.8     | 0.000| 75.4     |   |
| Smoking status |           | Non-smoker | 12| 78| 43| 55.6     | 0.012| 82.1     | 0.002| 69.8     | 0.005|
| Smoking status |           | Smoker     | 3 | 40| 28| 38.4     |       | 63.4     |       | 50.9     |   |
| Pathology |               | Adenocarcinoma | 13| 116| 68| 49.8     | 0.230| 76.1     | 0.029| 63.7     | 0.108|
| Pathology |               | Squamous cell carcinoma | 2 | 2 | 1 | 22.2     |       | 44.4     |       | 33.3     |   |
| Pathology |               | Other types | 2 | 1 | 1 | 42.8     |       | 57.1     |       | 42.8     |   |
| EGFR mutation |           | Wild-type | 2 | 8 | 27| 8.2      | 0.000| 18.5     | 0.000| 11.7     | 0.000|
| EGFR mutation |           | Exon 19 deletion | 9 | 67| 23| 56.7     |       | 48.3     |       | 25.6     |   |
| EGFR mutation |           | L858R or L861Q | 4 | 39| 18| 32.1     |       | 29.8     |       | 32.7     |   |
| EGFR mutation |           | Other types | 0 | 4 | 3 | 3        |       | 3.4      |       | 2.9      |   |
| EGFR-TKI  |               | Gefitinib  | 9 | 51| 36| 48.8     | 0.529| 78.0     | 0.077| 68.3     | 0.101|
| EGFR-TKI  |               | Erlotinib  | 4 | 58| 26| 48.8     |       | 69.3     |       | 57.5     |   |
| EGFR-TKI  |               | Afatinib   | 1 | 8 | 4 | 64.3     |       | 92.9     |       | 78.6     |   |

*Clinical benefit rate (CBR) is defined as complete response (CR) or partial response (PR) or at least six months of stable disease (SD). DCR, disease control rate; EGFR, epidermal growth factor receptor; ORR, objective response rate; TKI, tyrosine kinase inhibitor.
significant. There were no significant differences between higher and lower corrected ΔCt value groups according to EGFR mutation or specimen type (Fig 4). The OS difference between the higher and lower corrected ΔCt value groups was not significant (379.7 vs. 356.2 days, P = 0.655, Fig 5). In addition, we performed correlation analysis between ΔCt value and PFS. However, the Pearson correlation coefficient of 0.089 was considered to have no statistical significance.

**Discussion**

Despite the fact that sensitive EGFR mutations, such as 19del and L858R, were reliable predictive factors of a response to EGFR-TKIs, EGFR mutation status is not yet reported by continuous variable. In this study, we attempted EGFR gene quantification by PNA clamping and found that the corrected ΔCt value could predict a better clinical response to EGFR-TKI therapy. In addition, this value was inversely correlated with treatment response in EGFR mutated patients.

For more sensitive and rapid detection of EGFR gene mutations, new techniques, such as scorpion ARMS, Taqman PCR, and denaturing high-performance liquid chromatography, have been introduced. A PNA is an artificial polymer with the properties of both nucleic acids and proteins and can bind tightly to complementary sequences in DNA because of a lack of electrostatic repulsion. Therefore, when a PNA oligomer, designed to detect an EGFR mutation and to bind to the antisense strand of the wild-type EGFR gene, is used for real-time PCR, amplification is rapid and sensitive and displays similar sensitivity to scorpion ARMS. PNA clamping enables detection of EGFR gene mutations in samples containing as few as 1% mutant alleles. Because the efficiency of PNA-mediated PCR clamping was determined by measuring the Ct value, we tried to quantify EGFR mutation status by this value. In addition, small biopsy tissue or cytology specimens often contain a small subpopulation of mutant cells mixed with a greater level of normal tissue resulting in poor sample quality; therefore, we used corrected ΔCt value, which standardized the variations in sample quality using a non-PNA value.

In this study, patients with EGFR mutations in the higher corrected ΔCt value (≥ mean value) group showed significantly better ORRs and CBRs than the lower group. Furthermore, corrected ΔCt values were inversely correlated with radiological response. This result suggests that EGFR mutation status may be reported with a biological continuous variable. However, there was no significant correlation between corrected ΔCt value and PFS. The corrected ΔCt value was an independent predictive factor in multivariate analysis; however, this value was not a significant factor in survival analysis. Thus, in the EGFR mutation positive group, the corrected ΔCt value could be an early predictive marker of clinical response to EGFR-TKIs, at least in the first six months, because of the better ORR and CBR results. It may reflect the rapid shrinkage and prolonged (≥6 months) stabilization of the EGFR sensitive tumor by EGFR-TKI treatment. However, the benefit of

### Table 3 Univariate and multivariate linear regression analysis for objective clinical benefit

| Variables                          | Univariate | Multivariate |
|-----------------------------------|------------|--------------|
|                                    | P          | OR | 95% Cl | P |
| Female (n = 105)                   | 0.025      | 3.058 | 1.252–7.468 | 0.014 |
| Non-smoker (n = 123)               | 0.606      | 0.548 | 0.216–1.394 | 0.207 |
| Adenocarcinoma (n = 182)          | 0.837      | 1.558 | 0.137–17.706 | 0.721 |
| Exon 19 deletion (n = 74)          | 0.638      | 0.665 | 0.297–1.486 | 0.320 |
| TKI for first line (n = 165)       | 1.000      | 0.000 | ND     | 1.000 |
| Corrected ΔCt ≥ 6.0 (n = 109)     | 0.003      | 2.734 | 1.248–5.988 | 0.012 |

Cl, confidence interval; Ct, cycle threshold; ND, not determined; OR, odds ratio; TKI, tyrosine kinase inhibitor.
Figure 4 This figure shows difference between corrected ΔCt < 6 (dotted line) and corrected ΔCt ≥ 6 values (solid line) in progression-free survival (PFS). PFS of (a) all patients; (b) in the exon 19 deletion mutation group; (c) the L858R or L861Q mutation group; (d) the surgical biopsy group; (e) the lymph node or small biopsy group; and (f) the cytology specimen group. ΔCt, delta cycle threshold.
Figure 5 The difference between corrected delta cycle threshold (∆Ct) < 6 (dotted line) and corrected ∆Ct ≥ 6 values (solid line) in overall survival (OS). OS of (a) all patients; (b) the exon19 deletion mutation group; (c) the L858R or L861Q mutation group; (d) the surgical biopsy group; (e) the lymph node or small biopsy group; and (f) the cytology specimen group. ∆Ct, delta cycle threshold.
the corrected ΔCt value as a long-term predictive and prognostic marker is not clear because we did not find statistically significant correlations in survival analysis. Most clinicians usually continue EGFR-TKIs beyond radiological progression; therefore, it is more difficult to find long-term response markers.

There are some limitations to the current study. First, this is a non-randomized retrospective study and enrolled a small number of patients in a single institution. Second, we could not perform tumor cell enrichment with tissue microdissection, despite the large proportion (58.0%) of small biopsy samples that were included. Microdissection of tiny tissues acquired from bronchoscopy or needle biopsy is not easily performed in daily clinical practice and our study may reflect the clinical reality. We also used a corrected ΔCt value to minimize the effect of normal cell contamination. Third, our study included a heterogeneous population, and all EGFR-TKIs were used as first-line treatment; therefore, the efficacy of the drug may be different according to the applied treatment line.

In conclusion, the corrected ΔCt value, which refers to EGFR quantification by PNA-mediated PCR clamping, can predict a better clinical response to EGFR-TKI therapy. However, further study is warranted to determine the value of corrected ΔCt as a biomarker to reflect PFS or OS.

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Disclosure

No authors report any conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1 Objective response rate (ORR), disease control rate (DCR), and clinical benefit rate (CBR) by corrected delta cycle threshold (ΔCt) value in epidermal growth factor receptor (EGFR) mutated patients (n = 252).