Title: Direct Comparison of Three PCR Methods in Detecting EGFR Mutations in Patients with Advanced Non-Small-Cell Lung Cancer

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Running Title: Comparison of three PCR methods in the clinic

Key Words: non-small-cell lung cancer; EGFR mutations; mutant-enriched PCR; PNA-LNA PCR clamp; PCR invader

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Micro abstract

We compared three PCR methods (mutant-enriched PCR, PNA-LNA PCR and PCR clamp) to detect EGFR mutations in 50 patients with advanced NSCLC. Seventeen were harboring EGFR mutations, five of whom showed discrepancies between the results of different PCR methods. All five responded to gefitinib, which we consider to suggest that the discrepancies were false negatives.

Clinical Practice Points

• Several methods have been used to detect EGFR mutations in non-small-cell lung cancer (NSCLC): however, it is not clear which is the most suitable for use in the clinic.

• We compared three PCR methods (mutant-enriched PCR, PNA-LNA PCR and PCR clamp) in 50 patients with advanced NSCLC. Seventeen of the patients were harboring EGFR mutations, five of whom showed discrepancies between the results of different PCR methods. All five patients responded to gefitinib.

• We considered that all of the discrepancies might be false negatives because the patients responded to gefitinib. To clarify the reasons for the false negatives of each PCR method, and establish the clinical sensitivity and specificity of each method, a large prospective clinical trial is warranted.
Abstract

Background: Epidermal growth factor receptor (EGFR) mutations are predictive of response to EGFR tyrosine kinase inhibitors (EGFR-TKIs) in non-small-cell lung cancer (NSCLC). Several methods have been used to detect EGFR mutations: however, it is not clear which is the most suitable for use in the clinic. In this study, we directly compare the clinical sensitivity and specificity of three PCR methods. Patients and Methods: We compared the three PCR methods (mutant-enriched PCR, PNA-LNA PCR and PCR clamp) in patients with advanced NSCLC. A patient who showed sensitive mutations by at least one PCR method was treated with gefitinib. A patient who showed no sensitive mutations was treated with chemotherapy with cytotoxic agents. Results: Fifty patients with advanced NSCLC previously untreated with EGFR-TKIs were enrolled in this trial. Seventeen of the patients were harboring EGFR mutations, five of whom showed discrepancies between the results of different PCR methods. All five patients responded to gefitinib. All patients harboring EGFR mutations received gefitinib treatment and 21 of 33 EGFR-mutation-negative patients received chemotherapy with cytotoxic agents. Median progression-free survival of the gefitinib group and the chemotherapy group were 8.2 months and 5.9 months, respectively. Conclusion: We considered that all of the discrepancies might be false negatives because the patients responded to gefitinib. To clarify the reason for the false negatives of each PCR method, and establish the clinical sensitivity and specificity of each PCR method, a large prospective clinical trial is warranted.
Introduction

Gefitinib is an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that competes with adenosine triphosphate (ATP) for the ATP-binding site of EGFR tyrosine kinase.\textsuperscript{1,2} Approximately 20-30\% of patients with non-small-cell lung cancer (NSCLC) harbor EGFR gene mutations.\textsuperscript{3-5} Point mutations of exon18 and exon21, and deletions of exon19, are known to be sensitive for gefitinib.\textsuperscript{6} About 85-90\% of these mutations are deletions of exon19 and point mutations of exon21.\textsuperscript{7-9} These EGFR mutations are predictive of response to EGFR-TKIs in patients with NSCLC.\textsuperscript{10} Recently, two randomized phase 3 trials revealed that first-line gefitinib treatment in patients with advanced NSCLC harboring EGFR mutations of exon19 or exon21 showed longer progression-free survival (PFS) with less toxicity compared with platinum doublet chemotherapy.\textsuperscript{11,12} Considering these results, EGFR gene status is most important in selecting anticancer drugs for patients with advanced NSCLC.

Clinical samples such as surgical specimens, lung biopsy and cytology ordinarily contain a few malignant cells and many normal cells. Thus, an assay is required to detect EGFR mutations against a large background of wild-type genes. Some polymerase chain reaction (PCR) methods have been used to detect EGFR mutations in the clinic. Mutant-enriched PCR is a highly sensitive PCR assay in two stages. The first stage entails amplification of mutant and wild-type sequences, followed by a selective restriction enzyme digestion of the wild-type sequence. The second stage involves amplification of the undigested mutant sequence. Kahn et al. reported detection of mutant K-ras genes using mutant-enriched PCR\textsuperscript{13} and Asano et al. developed the method to detect EGFR gene mutations in lung cancer.\textsuperscript{14} The peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp method has high sensitivity
for detecting mutations through the use of PNA clamp primers that bind to the wild-type sequence and suppress their amplification. Nagai et al. have reported that the PNA-LNA PCR clamp method can detect EGFR mutations with high sensitivity. The PCR invader method detects mutations using cleavase enzyme that recognizes overlapping structures of the invader probes and mutation sequence. Previous authors have reported that the PCR invader method was useful for detecting polymorphisms and identifying genotypes. These three methods are known to be highly sensitive: their sensitivities are approximately the same.

These PCR methods are in common clinical use; however, they have not been directly and prospectively compared with respect to clinical sensitivity and specificity. In this study, we directly compared these three PCR methods in the detection of EGFR mutations prospectively in patients with advanced NSCLC, and evaluated the false-positive and -negative rates in the clinic.

Patients and methods

Eligibility criteria

Patients who satisfied the following criteria were enrolled: stage IIIb or IV, and recurrence after operation; age ≥ 20 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 2; adequate organ functions; and no medical problems to prevent compliance with the protocol. We obtained signed informed consent from patients with NSCLC who visited Nagasaki University Hospital, and analyzed their EGFR mutations using the mutant-enriched PCR, PNA-LNA PCR clamp and PCR invader methods. EGFR-sensitive-mutation-positive patients received gefitinib treatment and mutation-negative patients received chemotherapy with
Tumor Samples

Samples were obtained from paraffin-embedded sections of specimens, frozen lung cytology specimens that were obtained from bronchoscopy (washing and brushing), or malignant pleural effusions. Each sample was divided into three parts, and each PCR method was performed. In the samples from paraffin-embedded sections, the micro-dissection method was not used.

Mutant-enriched PCR method

The deletion region in exon19 was amplified by PCR with forward primer: 5′-ATCCCAGAGTGAGAAAGATAAAATTC-3′ and reverse primer: 5′-CCTGAGGTTCAGAGCCATGGA-3′. The PCR products were digested with MseI (New England BioLabs, Inc., Beverly, MA, USA). After the digest was amplified, the second PCR products were separated by 6% polyacrylamide gel electrophoresis (PAGE) and visualized by ethidium bromide staining. The point mutation region in exon21 was amplified by PCR using forward primer: 5′-CAGCCAGGAACGTACTGGTGA-3′ and reverse primer: 5′-TCCTGGTGTCAGGAAAATGCT-3′. The PCR products were digested with MscI (New England BioLabs, Inc.). After the digest was amplified, the second PCR products were digested with AsuI (Fermentas International, Inc., Ontario, Canada). The digests were then subjected to separation by 8% PAGE and visualized by ethidium bromide staining.14,19-21
**PNA-LNA PCR clamp method**

The PNA-LNA PCR clamp method preferentially amplifies mutation sequences and detects mutations. The method needs peptide nucleic acid (PNA) clamp primer and locked nucleic acid (LNA) probe. PNA clamp primers bind to the wild-type sequence and suppress their amplification; LNA probes are designed to specifically detect mutant sequences and enhance their amplification in the presence of wild-type sequences, because PNA clamp primers competitively inhibit mutant LNA probes to bind to the wild type.\textsuperscript{15,22,23} To detect mutations of exon18 (G719C, G719S and G719A), exon19 (E746-A750del (nt 2235-2249del), E746-A750 (nt 2236-2250), L747-A750del T751S, L747-S752del P753S, L747-E749del A750P, L747-S752del E746V and S752-I759del) exon20 (T790M) and exon21 (L858R and L861Q), 12 probes were used. The PNA-LNA PCR clamp method was performed by Mitsubishi Kagaku Bio-chemical Laboratories Inc. (Tokyo, Japan).

**PCR invader method**

The invader method uses two oligonucleotide probes. An allele-specific single probe and an invader oligo probe hybridize to the single-stranded target DNA and form an overlapping structure. The 5'-flap of the single probe does not hybridize to the target DNA sequence. The 3'-end of the bound invader probe overlaps the primary probe by a single base of the mutation site. A cleavase enzyme recognizes this overlapping structure of the mutation site and cleaves the 5'-flap of the primary probe at the base of the overlap. If the probe does not hybridize completely at the site of target sequence, no overlapping structure is formed and cleavase does not cleave the 5'-flap of the primary probe so the target-specific product is not released. The
target-specific 5′-flap oligonucleotides hybridize to fluorescent resonance energy transfer (FRET), leading to the formation of an overlapping structure that is recognized by the cleavase enzyme. When the FRET is cleaved, a fluorophore is released from a quencher on the FRET and generates a fluorescence signal.\textsuperscript{16,17} Ten mutations of exon18 (G719A, G719C and G719S), exon19 {E746-A750del (nt 2235-2249del), E746-A750del (nt 2236-2250del) and L747-P753del ins S}, exon20 (S768I and T790M) and exon21 (L858R and L861Q) were determined. The PCR invader method was performed by BML (Tokyo, Japan).

*Treatment schedule*

EGFR-mutation-positive patients received gefitinib at a dose of 250 mg/day. EGFR-mutation-negative patients received chemotherapy. Chemotherapy regimens were left to the discretion of the attending physician. Treatments were continued until disease progression or intolerable toxicities became apparent or the patient refused further treatment.

*Evaluation*

We defined EGFR-mutation-negative patients as those in whom no EGFR-mutations were detected by any of the three PCR methods, and EGFR-mutation-positive patients as those in whom an EGFR mutation was detected by one or more PCR method. Mutation-positive patients were treated with gefitinib even when there were discrepancies in the PCR results. We defined false negative as an EGFR-mutation-negative case that responded to gefitinib, and false positive as an EGFR-mutation-positive case that did not respond to gefitinib.\textsuperscript{24}
The response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST).\textsuperscript{25} Adverse events were graded according to the Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v3.0) (http://www.jcog.jp/doctor/tool/CTCAEv3J_guideline_041027_2.pdf). PFS was defined as the time from the date of beginning treatment to the date of disease progression or death. Overall survival (OS) was assessed from the date of beginning treatment until death from any cause.

\textit{Statistical Analysis}

We analyzed differences in positive rate between tissue and cytology using Fisher’s exact test. In EGFR-mutation-positive patients receiving gefitinib and EGFR-mutation-negative patients receiving chemotherapy, we analyzed PFS. PFS and OS were calculated by the Kaplan-Meier method, with differences between the groups compared using the log-rank test. A P-value < 0.05 was considered statistically significant.

\textbf{Results}

From April 2008 to May 2010, 50 patients with advanced NSCLC previously untreated with EGFR-TKIs were enrolled in this study. Of these patients, 31 were men and 19 were women, with a median age of 68 years (range 24-89 years). Forty-seven of the patients had adenocarcinomas. Samples included 11 surgical specimens, 10 lung biopsies, 19 lung cytologies (washing and brushing), 5 pleural effusions and 5 lymph-node biopsies (Table 1). All samples were confirmed to contain malignant cells.

Sensitive EGFR mutations were detected in 17 samples. Using the
mutant-enriched method, 16 samples showed positive; the PNA-LNA PCR clamp method showed 14 positive samples; and the PCR invader method showed 15 positive samples (Table 2). There were five cases that showed discrepancies in their result. The mutant-enriched method showed one of these five cases to be negative: the other two methods showed two negatives, respectively. In case 4, the mutant-enriched method and the PNA-LNA PCR clamp method showed mutations in exon19 and 21, but the PCR invader method showed only the exon19 mutation (Table 3). In the 47 samples of adenocarcinoma, the positive rate was 46.2% (12 of 26) in tissue samples and 23.8% (5 of 21) in cytological samples, respectively. There was no statistically significant difference between them (Table 4).

Seventeen patients harboring EGFR mutations were assigned to receive gefitinib and 33 patients without EGFR mutations were assigned to receive chemotherapy with cytotoxic agents. However, five patients were excluded from the chemotherapy group because of poor PS and seven were excluded because they received other treatments. Of the 21 patients who received chemotherapy, 15 received a platinum-containing regimen and 6 a single-agent regimen (Fig. 1).

Of the 17 patients who received gefitinib, 15 patients (88.2%) exhibited partial response and 2 exhibited stable disease. All five patients who showed discrepancies between the three PCR methods exhibited partial response. PFS was significantly longer in the gefitinib group than in the chemotherapy group (median 8.2 months vs. 5.9 months; HR 0.457; 95% CI 0.211-0.990; P=0.0472) (Fig. 2).

In the gefitinib group, the most common adverse events were grade 1 or 2 skin rash and grade 1 paronychia. One instance of grade 3 alanine aminotransferase/aspartate aminotransferase elevation was observed. Interstitial lung disease was reported in one patient. In the chemotherapy group, toxicities > grade 3
were neutropenia (33.3%), leucopenia (14.3%), thrombocytopenia (4.7%), oral mucositis (4.7%), corneal ulcer (4.7%), and hyponatremia (4.7%). While these toxicities seem severe compared with those associated with gefitinib treatment, they are comparable to previous reports of cytotoxic agents. There were no treatment-related deaths in the gefitinib group.

4. Discussion

In this study, we directly compared the clinical sensitivity of three PCR methods in patients with advanced NSCLC. Seventeen patients were harboring sensitive EGFR gene mutations, and five of them showed discrepancies from the three methods. In this study, all samples were histopathologically confirmed to contain malignant cells and as all five patients responded to gefitinib treatment, we considered that these discrepancies were all caused by false negatives.

Recently, two randomized phase 3 trials revealed that the efficacy of first-line gefitinib was superior to that of standard chemotherapy in patients with advanced NSCLC harboring sensitive EGFR mutations.\textsuperscript{11,12} Consequently, gefitinib has become adopted as first-line treatment for patients with advanced NSCLC harboring sensitive EGFR mutations in Japan.\textsuperscript{26} Compared with platinum-based chemotherapy, the toxicities of gefitinib are mild and acceptable.\textsuperscript{11,12} If false-positive patients are treated with gefitinib, the patients seldom suffer severe toxicities although the tumor does not respond. On the contrary, if false-negative patients are treated with platinum-based chemotherapy, there are severe toxicities and less clinical benefits. In addition, once the patients are diagnosed as not harboring sensitive EGFR mutations, physicians might not treat them with EGFR-TKIs even after the relapse of first-line chemotherapy, though second-line gefitinib was previously reported to show acceptable toxicity and
efficacy for patients with NSCLC harboring EGFR-sensitive mutations.\textsuperscript{27,28} Thus, a false negative seems to be a more severe problem for patients with NSCLC harboring EGFR-sensitive mutations.

At the beginning of this trial, we expected that detection of EGFR gene mutations in the clinic might give false positives but not as false negatives, because these PCR methods were reported to have high sensitivity. The mutant-enriched PCR method can detect EGFR mutations in the presence of 500-fold background levels of wild-type \textit{EGFR} in normal cells.\textsuperscript{14} The PNA-LNA PCR clamp method is reported to be able to detect a mutant \textit{EGFR} sequence in specimens containing 100 to 1000 excess copies of wild-type \textit{EGFR} sequence.\textsuperscript{15,29} Naoki et al. have reported that the PCR-invader method can detect a known EGFR mutation in lung cancer cells at 100–1000-fold dilution.\textsuperscript{30} Thus, we supposed that false negatives seldom arise in these PCR methods: however, we detected five false negatives in the 17 patients harboring EGFR mutations. The causes of the false negatives were unclear in this trial. Additionally, it was difficult to conduct the statistical analysis between PCR methods because discrepancies were detected in only five patients and the trial had a small sample size. However, the rate of false negative was high compared with previous reports.\textsuperscript{30} A prospective clinical mega-trial is needed to investigate what causes the false negatives in each PCR method.

5. Conclusions

We prospectively compared the clinical sensitivity of three PCR methods to analyze EGFR mutations, and detected discrepancies in the results in five patients. We suggest that all of these discrepancies might be false negatives. To clarify the reason for the false negatives in each PCR methods, and establish the clinical
sensitivity and specificity of each PCR method, a prospective clinical trial is needed.

**Conflict of Interest Statement**

None declared.
References


Figure legends

Figure 1. Study Profile.

Figure 2. Progression-free Survival of Patients in the Present Study. Kaplan-Meier curves for progression-free survival. Tick marks indicate patients for whom data were censored at the data cutoff point.
Table 1
Patient Characteristics (n=50)

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Age, years</th>
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<tbody>
<tr>
<td></td>
<td>Median (Range) 68 (24-89)</td>
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<table>
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<th>Sex</th>
<th>No. of Patients</th>
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<td>Male</td>
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<td>Female</td>
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<td>AdSq</td>
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<tr>
<td>Lr</td>
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</tr>
<tr>
<td>PD</td>
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<th>Samples</th>
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<td>SS</td>
<td>11</td>
</tr>
<tr>
<td>LB</td>
<td>10</td>
</tr>
<tr>
<td>Cy</td>
<td>19</td>
</tr>
<tr>
<td>PE</td>
<td>5</td>
</tr>
<tr>
<td>LN</td>
<td>5</td>
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</table>

Ad, adenocarcinoma; Sq, squamous-cell carcinoma; AdSq, adenosquamous-cell carcinoma; Lr, large-cell carcinoma; PD, poorly differentiated carcinoma; SS, surgical specimen; LB, lung biopsy; Cy, lung cytology (washing and brushing); PE, pleural effusion; LN, lymph-node biopsy.
Table 2.
Results of the Three Methods for Detecting EGFR Mutations

<table>
<thead>
<tr>
<th>Method</th>
<th>Exon 19</th>
<th>Exon 21</th>
<th>Exon 19 + 21</th>
<th>Total</th>
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<td>Enriched</td>
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<td>9</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Clamp</td>
<td>4</td>
<td>9</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Invader</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Any of three methods</td>
<td>6</td>
<td>10</td>
<td>1</td>
<td>17</td>
</tr>
</tbody>
</table>

Enriched, mutant-enriched PCR; Clamp, PNA-LNA PCR clamp; Invader, PCR invader.
Table 3.
Characteristics of Five Patients Showing Discrepancy of Mutations

<table>
<thead>
<tr>
<th>Case</th>
<th>Sample</th>
<th>Enriched</th>
<th>Clamp</th>
<th>Invader</th>
<th>Type of mutation</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>SS</td>
<td>19</td>
<td>-</td>
<td>19</td>
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<tr>
<td>2</td>
<td>Cy</td>
<td>19</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3</td>
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<td>19</td>
<td>19</td>
<td>-</td>
<td>E746-A750del</td>
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<tr>
<td>4</td>
<td>SS</td>
<td>19, 21</td>
<td>19, 21</td>
<td>19</td>
<td>E746-A750del, L858R</td>
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<tr>
<td>5</td>
<td>SS</td>
<td>-</td>
<td>Unextracted</td>
<td>21</td>
<td>L858R</td>
</tr>
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SS, surgical specimen; Cy, lung cytology; LB, lung biopsy; Enriched, mutant-enriched PCR; Clamp, PNA-LNA PCR clamp; Invader, PCR invader.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total</th>
<th>Mutation positive</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Tissue</td>
<td>26</td>
<td>12 (46.2%)</td>
<td>0.376</td>
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<tr>
<td>Cytology</td>
<td>21</td>
<td>5 (23.8)</td>
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</tbody>
</table>
Figure 1.

NSCLC patients
n=50

Mutant-enriched PCR method
PNA-LNA PCR clamp method
PCR invader method

EGFR mutation (+)
n=17

EGFR mutation (-)
n=33

Poor PS n=5
Radiation n=2
Chemoradiation n=3
EGFR-TKI therapy n=2

Gefitinib-treated patients
n=17

Chemotherapy-treated patients
n=21
Figure 2.

- Mutation-positive patients received gefitinib
- Mutation-negative patients received chemotherapy

Progression-free Survival (%) vs. Month