Direct sequencing and amplification refractory mutation system for epidermal growth factor receptor mutations in patients with non-small cell lung cancer

HUILI CHU1,2, CHEN ZHONG1,2, GUOLIANG XUE1, XIUJU LIANG1, JUN WANG1, YINGXIN LIU1, SHIWEI ZHAO1, QIAN ZHOU2 and JINGWANG BI1

1Department of Oncology, General Hospital, Jinan Command of the People’s Liberation Army, Jinan, Shandong 250031; 2Department of Radiology, Jinan Central Hospital, Jinan, Shandong 250014, P.R. China

Abstract. Treatment with epidermal growth factor receptor (EGFR) tyrosine inhibitors (EGFR-TKIs) provides encouraging outcomes for advanced non-small cell lung cancer (NSCLC) patients with EGFR mutations. Pleural effusion is a common complication of NSCLC. We compared direct DNA sequencing and ADx Amplification Refractory Mutation System (ADx-ARMS) to detect EGFR mutations in malignant pleural effusion samples. We obtained 24 samples from pleural effusion fluid of NSCLC patients. Three common types of EGFR mutations were examined by direct sequencing and ADx-ARMS analysis. The sensitivity of the methods was compared and the relationship between EGFR mutations and response rates of the patients determined. In 14/24 patients, we detected EGFR mutations (58.3%) by ADx-ARMS, and in 10 samples (41.7%) by direct sequencing. In 6 samples, EGFR mutations were on exon 19, and in 8 samples, mutations were on exon 21 by ADx-ARMS. By contrast, we found EGFR mutations in 4 samples on exon 19, and in 6 samples on exon 21 by direct sequencing. Neither method showed concordance for the methods. In 18/24 patients, gefitinib treatment was administered, including 10 patients with mutations who showed improved response compared to 8 of the wild-type patients (P<0.05). In conclusion, EGFR mutation analysis by ADx-ARMS was the most sensitive compared to direct sequencing, and provided more reliable EGFR mutation assessments. ADx-ARMS could be introduced into the clinical practice to identify NSCLC patients likely to benefit from TKI treatment, especially those with malignant pleural effusion.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, and especially in China. The majority of these deaths are due to non-small cell lung cancer (NSCLC), which is the most common histologic type of lung cancer (1). It is current practice to treat advanced NSCLC with platinum based chemotherapy, although treatment outcomes are particularly poor (2,3). Therefore, target therapy for patients with advanced NSCLC are currently being evaluated.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (TK) that is frequently overexpressed and plays a central role in the development of NSCLC (4,5). Abnormal activation of EGFR can promote tumor cell proliferation, differentiation and migration. EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, which target EGFR, have demonstrated promising outcomes in the treatment of NSCLC patients (6-8). The efficacy of EGFR-TKIs is associated with Asian race, shows gender specificity to women, non-smokers and adenocarcinoma histology (9). Furthermore, an association between mutations in the EGFR TK domain and sensitivity to EGFR-TKIs has been previously reported (6,10).

EGFR mutations are located in EGFR exons 18 to 21 (9) and most mutations are observed as in-frame deletions in exon 19 and a point mutation L858R in exon 21 (11). Thus, testing for EGFR mutations may be prognostically important to identify potential responders who would benefit from treatment with EGFR-TKIs. This is particularly true for Chinese NSCLC patients with high EGFR mutation rates (12). The samples used for EGFR mutations are usually from resected tumor tissues, which could be stably and easily detected. It is difficult to obtain sufficient tumor tissues with advanced NSCLC, thus alternative specimens need to be established for testing EGFR mutations.

Malignant pleural effusion is a common complication of lung cancer. It is present in ~15% of lung cancer patients and in
60~10-50% of patients at the time of diagnosis (13). In about half
61of NSCLC patients with a pleural effusion, most effusions are
determined to be malignant consistent with the progress of the
disease. As sampling of pleural effusion fluid is usually a stan-
62dard and uncomplicated procedure, which is also non-invasive
and repeatable, we hypothesized that genetic alterations in the
63pleural effusion fluid of NSCLC patients could provide useful
guidelines with regard to the response to EGFR-TKIs therapy.

In the present study, we used two approaches to detect
64major EGFR mutations in malignant pleural effusions from 24
patients presenting with advanced NSCLC and compared the
65acquired results. The relationship between EGFR mutations
66with the efficacy of gefitinib was also evaluated.

Patients and methods
67
Patients. Cytologically or pathologically confirmed pleural
68effusions were obtained from 24 Chinese patients presenting
69with advanced NSCLC. Jinan General Hospital of PLA
70approved this study, and written informed consent was
71obtained from all patients. Eligibility criteria included
72patients with stage IIIB-IV, ECOG performance status (PS)
73of 0-3, and a life expectancy of at least 3 months. The records
74of all patients consisted of age, gender, smoking habit, histo-
75logical type of NSCLC and treatment. The response of the
76patients to treatment with gefitinib was evaluated in accor-
77dance with the ‘Response Evaluation Criteria in Solid Tumors
78(RECIST)’ guidelines (14). No research results were entered
79into the records of any of the patients whatsoever or released
80to the patient or the physician of the patient. Each specimen
81was only labeled by a serial number without any identification.

Collection of pleural effusion fluid and DNA extraction. Pleural
82effusion fluid was collected from patients in heparinized tubes
83between 20th February and 22nd June 2012. No particular
84collection method was used. A 30 ml volume sample of the fluid
85was centrifuged at 250 x g for 10 min at room temperature, and
86the cell pellets were stored at -80˚C until used. Genomic DNA
87in the cell pellets was extracted by DNeasy tissue kits (Qiagen,
88Germany), and according to the manufacturer’s protocol. The
89concentration and purity of extracted DNA were assessed by
90spectrophotometry (Nanodrop, ADx, China).

Polymerase chain reaction amplification and direct sequencing.
91Exons 19, 20 and 21 of the EGFR gene were amplified by poly-
92merase chain reaction (PCR). The primers specific for EGFR
93were designed using Primer Designer Software (primer premier
945.0). The sequences of primers for EGFR exon 19 to 21 are
95described in Table I. Each 50 µl reaction specimen contained
962 µl of template DNA, 0.25 µl of Ampli
97Taq Gold DNA poly-
98merase (Roche, USA), 5 µl of 10X PCR buffer, and 10 µM of
99forward and reverse primer. The same PCR program was used
100for all amplicons: 95˚C for 3 min; 32 cycles of 95˚C for 30 sec,
10155˚C for 30 sec; 72˚C for 30 sec; 72˚C for 10 min. After PCR
102assay had completed, the resultant amplicons were further puri-
103fied by QIAquick PCR purification kit (Qiagen), and subjected
104to sequencing analysis in both sense and antisense directions.

ADx-ARMS for the detection of EGFR mutations. We used
105an EGFR Gene 4 Mutations Diagnostic kit (ADx, Xiamen,
China), which combines the two technologies of ARMS and
Bi-loop Probe, to detect mutations in real-time PCR reactions.
All reactions proceeded in 25 µl volumes according to the
manufacturer’s protocol. Real-time PCR was performed using
the Mx3000P™ real-time PCR system (Agilent, Germany)
under the following conditions: initial denaturation at 95˚C for
5 min, 15 cycles of 95˚C for 25 sec, 64˚C for 20 sec, 72˚C for
20 sec, and 31 cycles of 95˚C for 25 sec, 60˚C for 35 sec (with
fluorescence collection, set to FAM and HEX), and finally
72˚C for 20 sec. Data were analyzed using Stratagene Mxpro
software. The threshold cycle (Ct) was defined as the cycle at
the highest peak of the curve, which represents the point of
maximum curvature of the growth curve. Positive results were
defined as Ct<26. Analysis of each sample was carried out in
duplicate, and the whole test process required only 90 min.
The EGFR mutation kit is intended for detection of the major
somatic mutations in EGFR.

Statistical analysis. SPSS statistical software (version 13.0)
was used for statistical analysis. The Chi-square test was used
to compare the sensitivity between direct sequencing and
ADx-ARMS. Two-sided P-values of <0.05 were considered
statistically significant.

Results

Patients. Sixteen male and 8 female patients were enrolled for
the study. The median age was 58 years. Fourteen patients had
no history of cigarette smoking; the ten current smokers were
all male (Table).

Results of direct sequencing analysis. EGFR mutations were
observed in 10 samples by direct sequencing of DNA, 4 dele-
tions in exon 19, and 6 L858R mutations in exon 21 (Fig. 1).
We did not detect any mutations in exon 20 (data not shown).
ADx-ARMS analysis. ADx-ARMS analysis of EGFR mutations are shown in Fig. 2. The wild-type showed one increased curve, which was the positive control, and the mutant type showed two increased curves, which were the mutant and positive control curves, respectively. Using the EGFR Mutations Diagnostic kit, 6 deletion mutations in exon 19, and 8 L858R mutations in exon 21 of EGFR were detected. We confirmed that there was no mutation in exon 20 (not shown).

Comparison between direct sequencing and ADx-ARMS. We found gene mutations in EGFR in only 10 patients by the direct sequencing assay. Thus, direct gene sequencing was less sensitive than ADx-ARMS analysis. In 24 patients, EGFR mutations were detected in 14 samples (58.3%) by ADx-ARMS, while 10 mutations (41.7%) were detected by direct sequencing. However, no significant difference was seen between these approaches (χ²=1.333, P=0.248). Among the test results of 24 patients, there was an 83.3% concordance between direct sequencing and ADx-ARMS. Four EGFR mutation-negative samples found by direct sequencing were mutation-positive by ADx-ARMS.

Correlation between EGFR mutation and clinical response. For patients treated with gefitinib, EGFR mutations were detected in cells from malignant pleural effusions in ten of the 18 patients (Table III). Among those 10 EGFR mutant samples, 8 patients achieved partial response, and 2 presented with stable disease after 28 days of gefitinib therapy. In the 8 patients who partially responded, 6 of them showed decreased levels of pleural effusion, and reduced size of the tumor (Fig. 3). Six of the eight patients who had no demonstrable EGFR mutations progressed to develop the disease. While defining a patient with partial response as a responder, the frequency of EGFR mutations was significantly higher in gefitinib responders (8/9) than was found in non-responders (2/9, P=0.02).

Discussion
In this study, we demonstrated the feasibility of using DNA from malignant pleural effusion as an alternative to tumor samples for the detection of EGFR mutations from advanced NSCLC patients.

We used the pleural effusion samples to detect EGFR mutation status and compared two methods: i) gene sequencing and; ii) ADx-ARMS. We also showed that patients with mutant EGFR had a better response to treatment with EGFR-TKIs.

Table I. Primers used for EGFR mutation screening by direct sequencing.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>CCAGCAATATCAGCCTTAGGTG</td>
<td>GGGGAGGGAGTTATACCCACTA</td>
</tr>
<tr>
<td>20</td>
<td>GTCACTTCACAGCCCTGCGTA</td>
<td>GCTACTTCACAGCCCTGCGTA</td>
</tr>
<tr>
<td>21</td>
<td>CTGGAGGACCGTGCCTTG</td>
<td>GAGAGACTGAAACCTAACATTGCTA</td>
</tr>
</tbody>
</table>
In our study, the response rate was 80% (8 of 10 patients achieved partial response) in EGFR mutation patients, while EGFR wild-type patients had only a 12.5% response rate (1 of 8 patients achieved partial response). Patients with mutant EGFR had a response rate which was significantly higher than patients with wild-type EGFR (P<0.05). The data are in agreement to other previously reported studies (15-17).

Direct gene sequencing has been regarded as a gold-standard method for gene mutation analysis in the last decades. Direct sequencing usually requires sufficient tumor tissue as the testing sample with a sensitivity of ~30% (18,19). However, it is challenging to obtain sufficient tissue for gene sequencing in advanced NSCLC. In addition, gene sequencing is both time-consuming and technically demanding (17).

Many studies have shown that gene sequencing is unable to provide satisfactory data for the detection of pleural effusion fluid samples that contain mixtures of DNA from normal cells (20,21), thus it cannot be widely used in clinical practice. Therefore, alternative clinical samples with more sensitive methodological approaches are urgently needed for individualized therapy of EGFR-TKIs.

Pleural effusion fluid, which has DNA from tumor cell pellets or the free DNA from the tumor provide a good alternative (17,20,22). The advantage of collecting free DNA or cell pellets is that it is a relatively simple approach, it is non-invasive and a repeatable technique. Thus, it could dynamically guide clinical approaches. Due to different methods and the selectivity of lung cancer patients with pleural effusion fluid, the frequency of mutant EGFR is in the range of 12.5-73% (17,20,21,23-25).

In our study, the frequency of EGFR mutations (deletion mutations and L858R mutations) detected by sequencing and by ADx-ARMS was found to be 41.7 and 58.3%, respectively. ADx-ARMS appeared to be the more sensitive approach as compared with direct sequencing in this study. The mutations detected by ADx-ARMS consisted of an in-frame deletion in exon 19 (E746_A750 del: 2235_2249del15 and 2236_2250del15), an insertion mutation in exon 20 (T790M), and a point mutation in exon 21 (L858R). Other deletion patterns in exon 19 and other mutations in the tyrosine kinase domain of EGFR could not be detected by this assay.

Among the 24 patients, there was 83.3% concordance between direct sequencing and ADx-ARMS. Our findings of a correlation between EGFR mutations and tumor response to therapy with TKIs was consistent with previous studies (15,16). Due to the small number of our samples, the EGFR mutation rate showed no significant difference between these two methods ($\chi^2=1.333$, P=0.248). At this point, it is worthwhile mentioning two limitations of our study. One is that we did not compare EGFR mutations between effusion cells and primary tumors, the main reason being that some tumor samples were not available. In addition, our results need further study based on the relationship between EGFR mutations and progressive-free survival and overall survival.

In summary, the clinical responses of NSCLC to EGFR-targeted therapy are closely associated with EGFR sensitive mutations. Screening of EGFR mutations by the ADx-ARMS approach using malignant pleural effusion as the source specimen is more sensitive and faster as compared with traditional gene sequencing approaches. These observations support

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of patients (n=24)</th>
<th>Percentage of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤70</td>
<td>14</td>
<td>58.3</td>
</tr>
<tr>
<td>&gt;70</td>
<td>10</td>
<td>41.7</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>66.7</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>Smoking habit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never-smoker</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>Current or former smoker</td>
<td>16</td>
<td>66.7</td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>20</td>
<td>83.3</td>
</tr>
<tr>
<td>Non-adenocarcinoma</td>
<td>4</td>
<td>16.7</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy naïve</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Previous chemotherapy</td>
<td>18</td>
<td>75</td>
</tr>
</tbody>
</table>

| Table III. EGFR mutations and the response treated with gefitinib in 18 patients. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| No. of patients | Response        | Gender | Age (yrs.) | EGFR mutation |
| 1               | PR             | F     | 61          | Exon 21        |
| 2               | PR             | F     | 49          | Exon 21        |
| 5               | SD             | M     | 77          | Exon 19        |
| 16              | PR             | M     | 84          | Exon 19        |
| 7               | PR             | M     | 51          | Exon 21        |
| 8               | PR             | F     | 59          | Exon 19        |
| 11              | PR             | F     | 65          | Exon 21        |
| 2               | PR             | M     | 81          | Exon 19        |
| 9               | PR             | M     | 73          | Exon 21        |
| 3               | SD             | M     | 51          | Exon 21        |
| 14              | PD             | M     | 63          | WT             |
| 17              | PD             | M     | 49          | WT             |
| 19              | SD             | F     | 52          | WT             |
| 24              | PD             | F     | 73          | WT             |
| 20              | PD             | F     | 61          | WT             |
| 23              | PD             | M     | 73          | WT             |
| 12              | PR             | M     | 61          | WT             |
| 4               | PD             | M     | 55          | WT             |

M. male; F, female; WT, wild-type; PR, partial response; SD, stable disease; PD, progressive disease.
the utility of this technology in routine clinical practice, an approach that can benefit patients presenting with advanced NSCLC.

References