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RNA is Favorable for Analyzing *EGFR* Mutations in Malignant Pleural Effusion of Lung Cancer

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ABSTRACT:

Malignant pleural effusion (MPE) is a useful specimen allowing for the evaluation of *EGFR* status in non-small-cell lung cancer (NSCLC). However, direct sequencing of genomic DNA from MPE samples was found not sensitive for *EGFR*-mutation detection.

To test whether *EGFR* analysis from RNA is less prone to interference from nontumor cells which have no or lower *EGFR* expression, we compared three methods (sequencing from cell-derived RNA versus sequencing and mass-spectrometric analysis from genomic DNA) parallelly for *EGFR*-mutation detection from MPE samples in 150 lung adenocarcinoma patients receiving first-line TKIs.

Among these MPE samples, *EGFR* mutations were much more frequently identified by sequencing using RNA than by sequencing and mass-spectrometric analysis from genomic DNA (for all mutations, 67.3% versus 44.7% and 46.7%; for L858R or exon 19 deletions, 61.3% versus 41.3% and 46.7%). The better mutation-detection yield of sequencing from RNA was coupled with the superior prediction of clinical efficacy to first-line TKIs. In patients with acquired resistance, *EGFR* sequencing from RNA provided satisfactory detection of T790M (54.2%).

These results demonstrated that *EGFR* sequencing using RNA as template greatly improves sensitivity for *EGFR*-mutation detection from samples of MPE, highlighting

RNA as the favorable source for analyzing *EGFR* mutations from heterogeneous MPE specimens in NSCLC.

Keywords: non-small-cell lung cancer; epidermal growth factor receptor (EGFR); tyrosine kinase inhibitors; malignant pleural effusions

INTRODUCTION

Lung cancer, predominantly non-small-cell lung cancer (NSCLC), is the leading cause of cancer-related death worldwide [1]. Most patients have advanced disease at the time of diagnosis, and, if left untreated, have a median survival of 4-5 months [2]. Molecular therapeutics targeting epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of advanced NSCLC [3, 4]. Recently, a strong association of somatic mutations in the tyrosine kinase domain of *EGFR* with clinical efficacy to two small-molecule EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, has been clearly demonstrated [5-8]. These mutations exist in exons 18 to 21 of the *EGFR*. As reported in the literature, the two major *EGFR* mutations, in-frame deletions in exon 19 and a single amino acid substitution at position 858 (L858R) in exon 21, were the best-documented mutations associated with response to EGFR TKIs [6-9]. By contrast, the acquisition of a second site *EGFR* mutation, T790M in exon 20, is associated with acquired TKI resistance [10-11].

Since somatic *EGFR* mutations are the major determinant of tumor response to TKIs, molecular assays in clinical samples may become an integral part of care for advanced NSCLC patients [12]. However, even in prospectively conducted clinical trials, less than 50% of patients had specimens available for mutation analysis [13, 14]. Malignant pleural effusion (MPE) is a common complication of NSCLC. As pleural

effusion sampling is usually easy, relatively non-invasive and repeatable, tumor-derived DNA in the MPE samples could be a useful source of information on the status of *EGFR* in NSCLC patients [15-18].

It is known that direct sequencing is not exquisitely sensitive in heterogeneous samples [19]. Thus, using sequencing of cell-derived genomic DNA from MPE samples, previous studies frequently reported an *EGFR*-mutation rate lower than expected [15-18]. Interesting, using RNA as the template for *EGFR* sequencing, we recently reported a much higher mutation rate in MPE samples of lung adenocarcinoma [20]. Although this variability probably reflected patient selection and geographic differences, assay methodology might have substantial attribution. Because contaminated nontumor cells within MPEs may have no or lower *EGFR* expression, using RNA instead of genomic DNA as the source for *EGFR* sequencing could minimize the influence of nontumor cells [21, 22]. However, clinical data with in parallel comparison will be needed to address this hypothesis and practical issue.

The sensitivity for mutation detection in heterogeneous samples may also be increased if technologies that could identify low-abundance mutations are introduced [19, 23]. Our laboratory recently established a sensitive matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) platform for DNA analysis of *EGFR* mutations [24, 25]. To

determine whether the use of RNA may improve the sensitivity of *EGFR* testing, we conducted this study to compare three analytical methods (sequencing from cell-derived RNA versus sequencing and MALDI-TOF MS analysis of genomic DNA) in parallel for the analysis of *EGFR* mutations from MPE samples of lung adenocarcinoma, along with the prediction of EGFR TKI efficacy in the first-line setting.

METHODS

Patients and specimens

Between June 2005 and October 2009, we consecutively collected 150 samples of MPE from 150 individual patients with advanced lung adenocarcinoma who received gefitinib or erlotinib as the first-line antitumor treatment. This study was approved by the Institutional Review Board of the National Taiwan University Hospital. All patients had signed an informed consent form for the use of samples in molecular analysis. The adenocarcinoma histology was confirmed by the pathology reports for biopsy of the primary tumors or cell blocks of MPEs with positive thyroid transcription factor-1 (TTF-1) stains. Among the 150 pleural effusion samples, 94 were obtained at initial diagnosis, while the other 56 were collected with progression of the disease. Patients

who had smoked fewer than 100 cigarettes in their lifetime were categorized as never smokers.

Extraction of genomic DNA and RNA from cell lysates of effusion samples

The pleural effusion fluid was collected and centrifuged at 250×*g* for 10 min at 4□, with the cell pellet frozen. The processing of samples (from sampling to freezing) was less than 2 hours. Genomic DNA was extracted from cell lysates using a QIAmp DNA Mini Kit (Qiagen, Valencia, CA). For RNA purification, the cell pellet was submerged in RNA*later* (Qiagen) for storage until isolation using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction.

Direct sequencing using cell-derived RNA

The reverse transcription-polymerase chain reaction (RT-PCR) was performed using a Qiagen One-Step RT-PCR Kit (Qiagen), with the condition as previously described [26]. Exons 18–21 of *EGFR* were amplified with a forward primer (5′-GGA-TCG-GCC-TCT-TCA-TGC-3') and reverse primer (5′-TAA-AAT-TGA-TTC-CAA-TGC-CAT-CC-3′). Amplicons were purified and sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing products underwent electrophoresis on an automatic ABI PRISM 3700 genetic analyzer (Applied Biosystems). Both the forward and reverse

sequences obtained were analyzed and chromatograms were examined manually by two reviewers. *EGFR* mutations detected in the initial round of sequencing were confirmed by the subsequent round of independent RT-PCR and sequencing reaction.

Direct sequencing using cell-derived genomic DNA

Exons 18, 19, 20 and 21 of the *EGFR* were amplified separately by nested PCR, using specific primers (listed in Table S1 in the Online Supplement) and conditions as described previously [6, 7, 27-29]. The PCR amplicons were purified and bidirectional sequencing was performed on the PCR products. Only specimens in which a mutation was confirmed in the subsequent PCR and sequencing reaction were recognized as mutation positive.

MALDI-TOF MS analysis of cell-derived genomic DNA

We performed *EGFR*-mutation detection of genomic DNA by MALDI-TOF MS according to user's manual of MassARRAY system (Sequenom, San Diego, CA). Briefly, after PCR of genomic DNA to amplify the loci of L858R, exon 19 deletions and T790M, single nucleotide extension with probes was performed, followed by the analysis using MALDI-TOF MS. *EGFR* mutants could be distinguished from wild-type ones due to the mass difference of an incorporated single nucleotide. For each sample, at least two duplications were performed. The sequences of PCR primers and corresponding probes for identifying T790M, L858R and exon 19 deletions are listed in

Table S2 and illustrated in Figure S1 in the Online Supplement. For exon 19 deletions, detection probes were designed for nine of the most common types of deletions.

Evaluation of EGFR TKI Efficacy

The antitumor response of the patients was evaluated by chest radiography every 2-4 weeks and by computed tomography of the disease sites every 8-12 weeks after the start of treatment. Treatment responses were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) using the unidimension method, and were reported as best response achieved [30]. Progression-free survival was calculated from the first day of TKI administration until the earliest sign of disease progression or death from any cause. Acquired resistance was defined as progression of the disease after previously documented response or durable (≥6 months) stable disease from continuous treatment with TKIs [31].

Statistical analysis

Progression-free survivals after first-line TKIs were analyzed by the Kaplan-Meier method, and were compared between groups by the log-rank test. Analysis of factors associated with progression-free survival was performed using the Cox's proportional-hazards model. Two-sided *P* values less than 0.05 were considered statistically significant. All analyses were performed using SPSS software (version 12.0 for Windows; SPSS Inc., Chicago, IL).

RESULTS

Patient's characteristics

The clinical characteristics of these 150 patients are summarized in Table 1. Their mean age was 68.2±13.7 years (range 30-92 years). Six patients received erlotinib, while the remaining 144 patients received gefitinib as the first-line anti-tumor therapy.

Detection of *EGFR* mutations by sequencing using genomic DNA versus RNA

All of the specimens were successfully amplified and sequenced. To determine whether the use of genomic DNA or RNA may influence the sensitivity of analysis, we compared the sequencing results using either type of template (Table 2). Among the 150 MPE samples in total, *EGFR* mutations were identified in 67 (44.7%) by sequencing of genomic DNA and in 101 (67.3%) by sequencing from RNA. Of the 67 samples with mutations detected by genomic DNA sequencing, 38 (25.3%) had L858R, 24 (16.0%) had exon 19 deletions, and 5 (3.3%) had other mutations. The mutations detected by sequencing from RNA included L858R in 62 (41.3%) samples, exon 19 deletions in 30 (20.0%) samples, and other types of mutations in 9 (6.0%) samples. Mutations other than L858R or exon 19 deletions detected by using genomic DNA or RNA are listed in Table S3 in the Online Supplement.

All the mutations identified using genomic DNA were also detected using RNA, except in one sample in which the mutation (exon 19 deletion) was identified by genomic DNA sequencing only. By contrast, of the 83 samples that were identified as *EGFR*-mutation negative by genomic DNA sequencing, 35 were found to have mutations by sequencing from RNA. Of note, analysis of the sequencing chromatograms comparing both types of template illustrated the remarkable effect of enriching mutant *EGFR* from tumor cells by using RNA as template (Fig. 1). In data revealing heterozygous mutations (double peak), the presence of mutant allele was commonly easier to identify by using RNA, with a higher mutant/wild-type signal ratio which corresponded to percentage of the mutant allele.

Detection of L858R and exon 19 deletions by MALDI-TOF MS analysis of genomic DNA versus sequencing from RNA

As our MALDI-TOF MS platform was designed for identifying mutations of L858R and exon 19 deletions, we focused on comparison of the mutation-detection yields between mass-spectrometric analysis of genomic DNA and EGFR sequencing from RNA for these two major *EGFR* mutations. In total, there were 70 (46.7%) samples found to have L858R or exon 19 deletions by MALDI-TOF MS analysis (Table 2). Our results showed that sequencing from RNA was more sensitive than mass-spectrometric analysis of genomic DNA for the detection of these two mutations.

Thirty samples with negative L858R or exon 19 deletions by MALDI-TOF MS analysis were identified as positive by sequencing from RNA. By contrast, sequencing from RNA missed only 8 samples for the detection of these mutations, as compared with MALDI-TOF MS analysis.

Using immunocytochemical analysis (Methods in the Online Supplement), we evaluated the proportion of tumor cells to total nucleated cells within MPEs on 29 effusion cell blocks, along with their influence on the yields of *EGFR*-mutation detection by the three methods (Table S4 in the Online Supplement). *EGFR* sequencing from RNA and MALDI-TOF MS analysis of genomic DNA exhibited similar mutation-detection yields while tumor cells constituted more than 5% of total cells within MPEs. On the contrary, the sensitivity of genomic DNA sequencing obviously decreased if tumor cells accounted for less than 15% of total cells.

EGFR-mutation status and clinical response to first-line EGFR TKIs

We analyzed the relationship between *EGFR*-mutation status and TKI efficacy among the 94 patients with MPE samples obtained at initial diagnosis (Table 3). Of the 94 patients for analysis, partial response occurred in 79.7% (47 of 59) of patients with identified L858R or exon 19 deletions by sequencing from RNA, and in 79.5% (31 of 39) and 81.4% (35 of 43) of patients with these mutations detected by genomic DNA sequencing and MALDI-TOF MS analysis respectively. Of the patients without

mutations detected by genomic DNA sequencing and MALDI-TOF MS analysis, 42.3% (22 of 52) and 35.3% (18 of 51) of patients had clinical response. By contrast, partial response occurred in 5 (16.7%) of the 30 patients without identified mutations using sequencing from RNA. None of these five TKI responders had identified *EGFR* mutations by the highly sensitive Scorpion Amplified Refractory Mutation System method (EGFR RGQ PCR Kit; Qiagen) (Methods in the Online Supplement), suggesting the low risk of false negative results in these patients.

Patients with L858R or exon 19 deletions had significantly longer progression-free survival than those without these mutations, according to *EGFR*-mutation data either with genomic DNA sequencing (P=0.021 by log-rank test), sequencing from RNA (P<0.001), or MALDI-TOF MS analysis (P=0.018) (Fig. 2). Comparison of the hazard ratios for progression (0.64 [95% CI, 0.41-1.00] with genomic DNA sequencing, 0.25 [95% CI, 0.15-0.41] with sequencing from RNA, and 0.59 [95% CI, 0.38-0.92] with TOLDI-MOF MS analysis) revealed that *EGFR*-mutation status determined by sequencing from RNA provided the best prediction of progression-free survival (Fig. 2).

Detection of *EGFR* mutations associated with drug resistance

Of the total 150 samples, T790M was identified in 10 (6.7%) by genomic DNA sequencing, 19 (12.7%) by sequencing from RNA, and 24 (16.0%) by MALDI-TOF

MS analysis (Table 2). Among the 94 patients with samples collected at the time of initial diagnosis, de novel T790M was identified in 2 (2.1%) by genomic DNA sequencing, 3 (3.2%) by sequencing from RNA, and 8 (8.5%) by MALDI-TOF MS analysis. Of the 56 patients with samples obtained at the progression of disease after EGFR TKIs, we identified 24 patients to fulfill the criteria of acquired resistance to TKI treatment. Of these patients, T790M mutations were detected in 6 (25.0%) by genomic DNA sequencing, 13 (54.2%) by sequencing from RNA, and 14 (58.3%) by MALDI-TOF MS analysis.

Factors associated with progression-free survival to treatment with first-line EGFR TKIs

Using *EGFR*-mutation data determined by sequencing from RNA, we further analyzed factors that were associated with progression-free survival to first-line TKI treatment among the 94 patients with MPE samples obtained at initial diagnosis (Table 4). Patients with *EGFR* mutations of L858R or exon 19 deletions (hazard ratio, 0.28 [95% CI, 0.17-0.47]; $P<0.001$) and good performance status (hazard ratio, 0.58 [95% CI, 0.35-0.97]; $P=0.036$) were found to be independently associated with longer progression-free survival.

DISCUSSION

Molecular assays for *EGFR* mutations have shown promise in identifying advanced NSCLC patients who are likely to respond to EGFR TKIs. Because of the limited tissue availability for molecular analysis, the emerging issue concerning TKI treatment would be the development of reliable and practical *EGFR* testing for clinical samples that are commonly available [12, 13]. This study documented that, in contrast to analysis of genomic DNA, direct sequencing using cell-derived RNA from samples of MPE was very sensitive for *EGFR*-mutation detection without complex procedure or high cost in other high sensitive methods. This improved sensitivity was coupled with the superior prediction of treatment efficacy to first-line EGFR TKIs.

The IPASS (Iressa Pan-Asia Study), a phase 3 trial of first-line gefitinib versus chemotherapy for advanced pulmonary adenocarcinoma of never or light smokers conducted in East Asia, reported an *EGFR*-mutation rate of 59.7% in patients with clinical samples available for molecular analysis [14]. It is worth noting that the patients included in IPASS had favorable predictors for *EGFR* mutations and that a highly sensitive technique, Scorpion Amplification Refractory Mutation System, was used for *EGFR* testing. In the present study, using *EGFR* sequencing from RNA, we detected a comparable rate (67.3%) of *EGFR* mutation form MPE samples. Given the high mutation-detection yield, our results suggested that *EGFR* sequencing using RNA from MPE samples was highly sensitive for *EGFR*-mutation analysis in advanced lung

adenocarcinoma. The parallel comparison revealed that the improved sensitivity was attributed to the use of RNA instead of genomic DNA as template. Thus, in contrast to direct sequencing of genomic DNA which usually presents false-negative results, *EGFR* sequencing using RNA from MPE samples would be a very valuable assay for selecting advanced NSCLC patients to receive EGFR-directed therapy.

PCR-based sequencing is well established, widely available, and often quoted as the "gold standard" for DNA analysis [19, 23]. The most important limitation of sequencing is the lower sensitivity for detection of somatic mutations in clinical samples when tumor DNA constitutes a small fraction of the total DNA [19]. Strategies have been developed to make mutation assays less prone to interference from nontumor cells, such as macro- or micro-dissection to enrich tumor cells before analysis [23, 32]. Based on this study, we found that the use of RNA as template is another effective approach to improve the sensitivity of *EGFR* testing in heterogeneous MPE samples. Compared to the frequent overexpression of *EGFR* in NSCLC cells, not only inflammatory cells within MPE have no *EGFR* expression, but mesothelial cells also have considerably lower *EGFR* expression (see Fig. S2 in the Online Supplement) [21, 33-35]. When RNA is used as template, the differential expression of *EGFR* enriches mutant *EGFR* of tumor cells while minimizes the dilution of wild-type *EGFR* content from mesothelial cells (see Fig. S3 in the Online Supplement). This application of RNA

as template offers a new dimension for *EGFR* analysis especially for cytological samples, because microdissection is difficult to perform on cytological specimens [36]. Cytological samples, however, are more frequently used to diagnose NSCLC nowadays, and may constitute the only available specimens in advanced NSCLC patients who are inoperable.

Nucleotide mass spectrometry has been documented to be a sensitive assay for the analysis of oncogene mutations and genetic polymorphisms [19, 24, 37]. However, for samples of MPE, we found that MALDI-TOF MS analysis of genomic DNA was less sensitive than sequencing from RNA for *EGFR*-mutation detection. This possibly reflected the fact that MALDI-TOF MS analysis, though more sensitive than DNA sequencing, might be still unable to circumvent the highly heterogeneous character of MPEs, in which tumor DNA might constitute an extremely small fraction of total genetic content. This observation reinforced the strength of using RNA to enrich tumor *EGFR* for reliable mutation testing in highly heterogeneous samples. Furthermore, compared with other mutation-specific assays (such as MALDI-TOF MS and others) which focus on characterized mutations, direct *EGFR* sequencing has the advantage of detecting rare or novel mutations as well as the known ones. Therefore, *EGFR* sequencing should not be disregarded in clinical use, and efforts such as using RNA

instead of genomic DNA as a template should be taken to improve its performance for heterogeneous specimens.

Most studies to date have reported that 60-80% of *EGFR*-mutant NSCLCs respond to gefitinib or erlotinib [14, 38-41]. The present study showed a consistent response rate in patients with L858R or exon 19 deletions detected from MPEs. Using *EGFR* sequencing from RNA, we found that 16.7% of patients without identifiable *EGFR* mutations did show a partial response to TKIs. This result was equivalent to most of the literature, reporting 10-20% of response rate in mutation-negative cases and indicating that *EGFR* mutations are not the sole determinant of TKI response [7-9, 26, 42, 43]. By contrast, the response rates in mutation-negative group were much higher (42.3% and 35.3% respectively) with sequencing or MOLDI-TOF MS analysis of genomic DNA as the mutation-detection methods. Moreover, our results showed that *EGFR*-mutation status assessed with sequencing from RNA provided the better discrimination of progression-free survival to first-line EGFR TKIs, as revealed by the hazard ratios for progression. These findings demonstrated that *EGFR* sequencing using RNA from MPE samples provides superior basis for the prediction of first-line TKI efficacy.

EGFR sequencing from RNA, though identifying more activating *EGFR* mutations, appears to be less sensitive for detecting T790M from MPE samples than

MALDI-TOF MS analysis. One possible explanation is that, in addition to the mixing of nontumor cells in MPEs, tumor cells per se may also be heterogeneous, with T790M-harboring cells appearing in a small proportion of the total tumors cells especially before treatment with EGFR TKIs [44]. While the use of RNA as template has the advantage of less dilution of *EGFR* content from nontumor cells, the low de novel T790M alleles from the minority of T790M-containing tumor cells may not be readily detectable using conventional sequencing technique. On the contrast, we have shown that *EGFR* sequencing from RNA provided satisfactory detection of T790M (54.2%) in patients with acquired TKI resistance. This was probably because T790M had merged as a dominant allele through selective pressure of TKI treatment in these patients [44].

There was one limitation of this study that needs to be noted. The timing of sampling for MPEs varied, with 94 patients at the initial diagnosis and the other 56 patients at the disease progression after failure of EGFR TKIs. However, as the aim of this study was to compare different approaches in parallel for the detection of *EGFR* mutations from MPE samples, this limitation should not hamper our objective. Moreover, although our study suggested the promising of using RNA for *EGFR* analysis, the inherently labile nature of RNA, as well as the ubiquitous presence of

RNase, warrants the requisite of careful sample processing in RNA-based molecular testing [45].

In conclusion, we demonstrated that RNA is a more favorable source for *EGFR* testing than genomic DNA in the highly heterogeneous specimens of MPE related to lung cancer. Our study revealed that *EGFR* sequencing using RNA as template is very sensitive for mutation detection from MPE samples, which provides basis for satisfactory prediction of treatment efficacy to first-line EGFR TKIs in advanced NSCLC.

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Table 1. Clinical characteristics of patients

Total – no.	150
Sex – no. (%)	
Male	53 (35.3)
Female	97 (64.7)
Age yrs – no. (%)	
≤65	52 (34.7)
>65	98 (65.3)
Smoking status – no. (%)	
Never smoker	113 (75.3)
Smoker	37 (24.7)
ECOG performance status score – no. (%)	
0-1	112 (74.7)
≥2	38 (25.3)
Clinical stage* – no. (%)	
IIIb	17 (11.3)

IV	133 (88.7)

* Based on the sixth edition of the TNM classification of lung cancer.

ECOG, Eastern Cooperative Oncology Group.

Table 2. *EGFR*-mutation status of patients detected from malignant pleural effusion samples using genomic DNA sequencing, sequencing from RNA, or MALDI-TOF MS analysis of genomic DNA

	gDNA/sequencing	RNA/sequencing	gDNA/MALDI-TOF MS
Total subjects – no.	150	150	150
Subjects with activating mutations – no. (%)			
L858R, exon 19 deletions or other mutations	67 (44.7)	101 (67.3)	70 (46.7)
L858R or exon 19 deletions	62 (41.3)	92 (61.3)	70 (46.7)
Other mutations	5 (3.3)	9 (6.0)	–
Not detected*	83 (55.3)	49 (32.7)	80 (53.3)
Subjects with resistance mutations – no. (%)			
T790M	10 (6.7)	19 (12.7)	24 (16.0)

* Including those with silent mutations detected by sequencing from genomic DNA or RNA.

gDNA, genomic DNA; MALDI-TOF MS, matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry.

Table 3. Status of *EGFR* mutations assessed with different methods and clinical response to first-line EGFR TKIs in patients with malignant pleural effusion samples obtained at initial diagnosis

		gDNA/sequencing			RNA/sequencing			gDNA/MALDI-TOF MS	
	Subjects	L858R or Del19	Others	Not detected	L858R or Del19	Others	Not detected	L858R or Del19	Not detected
	no. (%)	No. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)
PR	53 (56.4)	31 (79.5)	0 (0.0)	22 (42.3)	47 (79.7)	1 (20.0)	5 (16.7)	35 (81.4)	18 (35.3)
SD	7 (7.4)	2 (5.1)	1 (33.3)	4 (7.7)	3 (5.1)	1 (20.0)	3 (10.0)	1 (2.3)	6 (11.8)
PD	34 (36.2)	6 (15.4)	2 (66.7)	26 (50.0)	9 (15.3)	3 (60.0)	22 (73.3)	7 (16.3)	27 (52.9)
Total	94	39	3	52	59	5	30	43	51

gDNA, genomic DNA; MALDI-TOF MS, matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry; Del19, exon 19 deletions; PR, partial response; SD, stable disease; PD, progressive disease.

Table 4. Multivariate analysis of factors associated with progression-free survival to first-line EGFR TKIs in patients with advanced lung adenocarcinoma

Variables	Hazard ratio	95% CI	*P* value
L858R or exon 19 deletions (with/without mutations)	0.28	0.17 – 0.47	⩽0.001
Sex (female/male)	0.77	0.42 – 1.41	0.394
Smoking history (never/current or former smokers)	0.98	0.50 – 1.92	0.942
ECOG performance status score (0-1/≥2)	0.58	0.35 – 0.97	0.036
Clinical stage* (IIIb/IV)	1.04	0.54 – 2.01	0.901

* Based on the sixth edition of the TNM classification of lung cancer

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group.

FIGURE LEGENDS

Figure 1. Representative sequencing chromatograms using genomic DNA or RNA as template for *EGFR* sequencing. Tracings are in the forward direction. The wide-type and mutant nucleotide sequences are shown in capital letters. Panel A shows the chromatograms in a patient with L858R in exon 21. The double peaks (vertical arrows) represent the heterozygous missense mutation (2573 T>G) in *EGFR* gene. Note that using RNA for *EGFR* sequencing, the signal of the mutant allele (G) is more intense than that of the wide-type (T). Panel B and C show chromatograms in two samples with in-frame deletions in exon 19 (both are deletion 2235-2249). Horizontal arrows are shown to demonstrate the breakpoint of the deletion. In panel B, with sequencing using RNA, the wave figure represents almost the signal of mutant allele. The signal of wild-type allele is decreased and difficult to be recognized. Chromatograms in panel C show that the mutant allele not detectable by using genomic DNA could be apparently recognized by using RNA. gDNA, genomic DNA; W, wild-type; M, mutant.

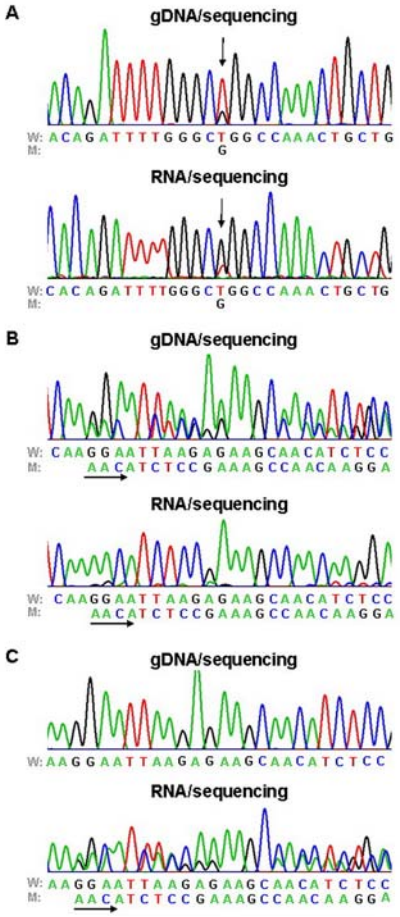

Figure 2. Kaplan-Meier curves for progression-free survival after the start of first-line EGFR TKI treatment among patients with various statuses of *EGFR* mutation assessed from malignant pleural effusion samples using genomic DNA sequencing (panel A), sequencing from RNA (panel B), and MALDI-TOF MS analysis of genomic DNA (panel C). Hazard ratios were calculated with the use of Cox regression analysis. gDNA, genomic DNA;

MALDI-TOF MS, matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry; PFS, progression-free survival; CI, confidence interval; HR, hazard ratio; Del19, exon 19 deletions.