Epidermal Growth Factor Receptor Mutation and Anaplastic Lymphoma Kinase Gene Fusion: Detection in Malignant Pleural Effusion by RNA or PNA Analysis

Yi-Lin Chen1,2,3,8, Chung-Ta Lee1,3,4, Cheng-Chan Lu1,3,4,5,8, Shu-Ching Yang1,3,8, Wan-Li Chen1,3,8, Yang-Cheng Lee6, Chung-Hsien Yang7, Shu-Ling Peng1, Wu-Chou Su1,3,4,5, Nan-Haw Chow1,3,4,5, Chung-Liang Ho1,2,3,4,5,*

1 Molecular Diagnostics Laboratory, Department of Pathology, National Cheng Kung University Hospital, Tainan, Taiwan, 2 Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University Hospital, Tainan, Taiwan, 3 College of Medicine, Molecular Medicine Core Laboratory, Research Center of Clinical Medicine, National Cheng Kung University Hospital, Tainan, Taiwan, 4 The Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University Hospital, Tainan, Taiwan, 5 The Institute of Molecular Medical, College of Medicine, National Cheng Kung University Hospital, Tainan, Taiwan, 6 Division of Hematology/Oncology, Department of Internal Medicine, Tainan Municipal Hospital, Tainan, Taiwan, 7 Yuan’s General Hospital, Kaohsiung, Taiwan, 8 The Association of Medical Technologists, Tainan, Taiwan

* clh9@mail.ncku.edu.tw

Abstract

Analyzing EGFR mutations and detecting ALK gene fusion are indispensable when planning to treat pulmonary adenocarcinoma. Malignant pleural effusion (MPE) is a devastating complication of lung cancer and sometimes the only source for mutation analysis. The percentage of tumor cells in the pleural effusion may be low; therefore, mutant enrichment is required for a successful analysis. The EGFR mutation status in MPE was determined using three methods: (1) PCR sequencing of genomic DNA (direct sequencing), (2) mutant-enriched PCR sequencing of genomic DNA using peptide nucleic acid (PNA-sequencing), and (3) PCR sequencing of cDNA after reverse transcription for cellular RNA (RNA-sequencing). RT-PCR was also used to test cases for ALK gene fusion.

PNA-sequencing and RNA-sequencing had similar analytical sensitivities (< 1%), which indicates similar enrichment capabilities. The clinical sensitivity in 133 cases when detecting the common EGFR exon 19 and exon 21 mutations was 56.4% (75/133) for direct sequencing, 63.2% (84/133) for PNA-sequencing, and 65.4% (87/133) for RNA-sequencing. RT-PCR and sequencing showed 5 cases (3.8%) with ALK gene fusion. All had wild-type EGFR. For EGFR analysis of MPE, RNA-sequencing is at least as sensitive as PNA-sequencing but not limited to specific mutations. Detecting ALK fusion can be incorporated in the same RNA workflow. Therefore, RNA is a better source for comprehensive molecular diagnoses in MPE.
Introduction

Of the 1.5 million new cases of lung cancer every year, about 85% are non-small-cell lung cancer (NSCLC), the leading cause of cancer death worldwide.[1] Approximately 70–80% of patients with NSCLC present with advanced disease and a poor prognosis.[1] There are many driver mutations in NSCLC, among which the epidermal growth factor receptor (EGFR) mutation and ALK fusion are the 2 most common therapeutic targets.[2]

The EGFR gene is mutated in a considerable proportion of primary lung adenocarcinomas, especially in patients who respond to gefitinib and erlotinib, which are small-molecule EGFR tyrosine kinase inhibitors (TKIs).[3] Patients with sensitive EGFR mutations were recommended for first-line TKI treatment. Both the response rate (RR) and progression-free survival (PFS) of patients with EGFR mutations who were treated with TKIs were better than those of patients treated with chemotherapy.[4,5] In contrast, patients with wild-type EGFR did not benefit from EGFR TKI treatment. Therefore, determining the EGFR status is paramount for EGFR TKI treatment. The EGFR mutation frequency is about 22–64% in Asia[6] and 9–21% in western countries.[2,7] It is more frequent in non-smokers and East Asians.[6] However, smokers can have a positive rate as high as 30%. Therefore, no clinical parameter can be used to pre-select patients for EGFR TKI treatment.[6] It is recommended that all patients with NSCLC have their EGFR gene status determined before TKI therapy. In addition, detecting somatic mutations of exons 19 and 21 in EGFR may provide prognostic information for patients with advanced NSCLC.[8,9] Therefore, the EGFR mutation is both a prognostic factor and a predictive factor for the response of TKI targeting therapy.

A fusion protein between the N-terminal portion of the echinoderm microtubule-associated protein-like 4 (EML4) protein and the intracellular signaling portion of ALK[10] tyrosine kinase receptor, which has recently[11] been identified in a small subset of patients with NSCLC, is now recognized as an important oncogenic driver in NSCLC. Multiple EML4-ALK variants in NSCLC have been identified, all containing the same C-terminal kinase domain with a gain of function.[12] The incidence of the EML4-ALK fusion gene in NSCLC is around 3–5% with no significant differences between Asian and western countries.[13,14] Currently, three different techniques are available for detecting ALK rearrangement: fluorescence in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemistry (IHC), each with advantages and limitations.[15] RT-PCR is a highly sensitive technique that can define both the fusion partner and the variant of ALK gene fusion.

Malignant pleural effusion (MPE) is a common and devastating complication of NSCLC, especially adenocarcinoma. In some patients, MPE is an initial manifestation and thus an easy path for collecting malignant cells for molecular investigation.[16–19] Sampling MPE is generally repeatable and safe. Therefore, sampling the MPE of NSCLC is a practical approach for investigating EGFR mutations, especially when patients present with advanced and unresectable NSCLC.[20–23] There are several testing methods available for mutational analysis in pleural fluid, including PCR direct sequencing, mutant-enriched PCR, pyrosequencing, and real-time PCR. The sensitivity of PCR direct sequencing is about 10–20%.

Enrichment methods are required because the tumor cells in the effusion may coexist with copious background inflammatory cells and mesothelial cells. To ensure sufficient cancer cells for analysis, the enrichment is usually done using macro-dissection, manual micro-dissection, or laser-capture micro-dissection of the smear or cell blocks.[24]

Supplementary techniques that target specific mutations have been developed, however, such as the peptide nucleic acid (PNA) approach to improve mutation detection (PNA-sequencing).[25–29] The rationale for using these technologies is that approximately 90% of
EGFR mutations are either exon 19 deletion (exon 19 del) or L858R in exon 21.[9] On this base, PNA-sequencing on common mutations was developed to meet clinical needs.[9]

A recent cohort study [16] reported using RNA as a favorable source for analyzing EGFR mutations in MPE. It was believed that mutant EGFR mRNA from tumor cells was enriched in the RNA samples from pleural effusions because the non-tumor cells produced little wild-type EGFR mRNA.[16]

The aim of this study was to assess the sensitivity of EGFR mutation analysis using direct sequencing, PNA-sequencing, and RNA-sequencing of MPE in lung adenocarcinoma.

Materials and Methods

Patients

Effusion with abnormal cytology (malignancy, suspicious, atypical) was centrifuged and the cell pellet was stored for molecular testing. From January 2011 to June 2015, there were 160 molecular tests for pleural effusion ordered at our hospital. Their cytology diagnoses included: 157 “malignancy”, 2 “suspicious for malignancy”, and 1 “atypical cells”. Eighty-five of the 157 malignant cases had subtyping information in the diagnoses: 71 adenocarcinoma and 14 NSCLC not otherwise specified (NSCLC-NOS). Forty-one cases had biopsy tissue proof, all of which were adenocarcinoma. One hundred forty-two cases had enough cells for both DNA and RNA tests, 16 for RNA tests only, and 2 for DNA tests only. The 142 cases were used for methodological comparison. Seventy-three of the 142 malignant cases had subtyping information in the diagnoses, including 62 adenocarcinoma and 11 NSCLC-NOS. The RNA test covers exons 18–21 of EGFR; the PNA tests for exon 19 del and L858R in exon 21 have good performance and cover approximately 90% of all EGFR mutations. Other mutations are much less cost-effective if PNA were used. Because our PNA test was not designed to detect mutations in exons 18 and 20, we excluded 9 such cases. All 9 patients were diagnosed by RNA-sequencing, including five exon 18 mutations (1 G709A, 3 G719A, and 1 G719S) and 4 exon 20 mutations (2 p.Ala767_Val769 dup, 1 p.Ser768_Asp770 dup, and 1 p.Val769_Asp770 ins CG). The remaining 133 cases contained either wild-type EGFR or the common mutations (exon 19 del or L858R).

This study was approved by National Cheng Kung University Hospital’s Institutional Review Board (IRB #: B-BR-101-137). All patients signed a written informed consent form for molecular analysis.

Demographic and clinical information of these patients was recorded: age, sex, smoking history, tumor staging, performance status (Eastern Cooperative Oncology Group performance status (ECOG PS),[30] treatment regimens, and maximal response. Patients who had smoked fewer than 100 cigarettes in their lifetime were categorized as never smokers, and those who had smoked more than 100 cigarettes were categorized as former smokers. The response to gefitinib was evaluated in accordance with Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[31–33]

Specimen collection and preparation

Pleural effusion was collected in sterile tubes. Samples (15 mL) were centrifuged at 250 × g for 10 min, and the cell pellet was stored in a reagent (RNAlater; Qiagen, Hilden, Germany) at −80°C until use. If the cellularity was high enough, samples were also submitted for DNA extraction. Both RNA and DNA for molecular analysis were extracted in approximately 89% (142/160) of the MPEs.
Extracting genomic DNA and RNA from cell pellets
One kit (QIAmp DNA Mini Kit; Qiagen) was used to extract genomic DNA from cell pellets, and another (RNeasy; Qiagen) to extract RNA from stored cell pellets.

PCR sequencing of cDNA from cell-derived RNA (RNA-sequencing)
The reverse transcriptase (RT)-PCR was done using reverse transcriptase (Superscript II; Invitrogen, Carlsbad, CA) as previously described.[34] Exons 18–21 of the EGFR were amplified using specific primer sets (Table 1). The amplicons (557 bp) were purified and sequenced using a kit (BigDye Terminator Sequencing Kit; Applied Biosystems, Foster City, CA). The sequencing products underwent electrophoresis using ABI 3500 genetic analyzer (ABI Prism 3500; Applied Biosystems). The sequences were compared with the EGFR sequences in the GeneBank from the National Center for Biotechnology Information (NCBI) (accession number NM_005228.3). Both forward and reverse sequences were analyzed, and the original chromatograms were manually examined by two medical technologists.

Direct sequencing and PNA-sequencing using cell-derived genomic DNA
Exons 18, 19, 20, and 21 of the EGFR gene were amplified using culture-independent nested PCR (Table 1) as previously described.[17,21,35] For PNA-sequencing, PNA is used to construct the PCR clamp reactions; the PNA clamp suppresses the amplification of wild-type DNA, thereby increasing the preferential amplification of the mutant sequences. PNA oligos were synthesized by Bio-Synthesis, Inc. (Lewisville, TX). The mutant-enriched PCR was done in a mixture of PCR primers (10 μM each), PNA oligo (100 μM each), genomic DNA (25 ng),

| Table 1. PCR primers and the PNA sequences. |
|---------------------------------------------|
| **PCR primer (DNA)**                        |
| Exon 19 forward                             |
| 5’-AAC GTC TTC TCT CTC CTG TCA T-3’         |
| Exon 19 reverse                             |
| 5’-AGC GGT GTC TAG AGC AGA GCA GCT GCC-3’   |
| Exon 21 forward                             |
| 5’-ATC TGT CCC TCA CAG CAG GGT C-3’         |
| Exon 21 reverse                             |
| 5’-GSC TGA CCT AAA GCC ACC T-3’             |
| **Sequencing primer (DNA)**                 |
| Exon 19 forward                             |
| 5’-AGC GGT GTC TAG AGC AGA GCA GCT GCC-3’   |
| Exon 21 forward                             |
| 5’-CCC TCA CAG CAG GGT C-3’                 |
| Exon 21 reverse                             |
| 5’-GAC CTA AAG CCA CCT C-3’                 |
| **PCR primer (RNA)**                        |
| Exons 18–21 forward                         |
| 5’-GGA GCC TCT TAC ACC CAG TG-3’            |
| Exons 18–21 reverse                         |
| 5’-TGC CTC CTT CTG CAT GGT AT-3’            |
| **Sequencing primer (RNA)**                 |
| Exons 18–21 forward                         |
| 5’-GCC TCT TAC ACC CAG TGG AG-3’            |
| Exons 18–21 reverse                         |
| 5’-TGC CTC CTT CTG CAT GGT AT-3’            |
| **PNA (Peptide Nucleic Acid)**              |
| Exon 19                                    |
| 5’-AGA TGT TGC TCT TCT TAA-3’               |
| Exon 21 (L858R)                             |
| 5’-CAG TTT GGC CA G CCC A-3’                |

*Subsequent nucleotide is a locked nucleic acid.

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and a DNA polymerase (Super Therm Gold Master Mix; Bionovas Biotechnology, Toronto, Ontario, Canada). The PCR amplicons were purified and then subjected to bidirectional sequencing.

PCR amplification

The PCR was done using a thermal cycler (G-Storm; GMI, Inc., Ramsey, MN). The cycling was as follows: 95°C for 6 min, 35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Nested PCR for EGFR mutation analysis was done using the same reaction conditions.

Constructing plasmid clones of EGFR exons 19 and 21 sequences and of mixed cell lines

The pGEMT easy vector plasmids, which contain EGFR exon 19 del (E746_A750 del) or L858R mutations, were derived from human primary NSCLC tissue using PCR cloning. The sequence of each plasmid was confirmed using Sanger sequencing. To evaluate the sensitivity of PNA-sequencing, plasmid DNAs containing EGFR mutants were serially diluted with genomic DNA from A549 cells with wild-type EGFR. To determine the detection limit of EGFR mutations at the cellular level, genomic DNA with E746_A750 del was extracted from H1650 cells and mixed with genomic DNA from A549 cells (wild-type EGFR) in ratios ranging from 1:1 to 1:100. The A549 and H1650 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Similarly, to determine the detection limit of EGFR mutations at the RNA level, total RNA from H1650 and A549 cells was mixed in ratios ranging from 1:1 to 1:100.

RT-PCR quality control

For quality control, two primer pairs were used to amplify the GAPDH (165 bp) and β2-microglobulin (256 bp) genes from 25 ng of each sample.[36] Primers for GAPDH were: GAA GGT GAA GGT CGG AGT C and TGG AAT TTG CCA TGG GTG GA; primers for β2-microglobulin were: TGG AGG CTA TCC AGC GTA CT and CGG CAG GCA TAC TCA TCT TT. The final concentrations of the primers were 0.8 μM in a 25-μL reaction. PCR conditions were as follows: 95°C for 30 s, followed by 35 cycles each of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final 10-min extension at 72°C. Gel electrophoresis was done using a 2% agarose gel.

Detecting the EML4-ALK fusion gene using multiplex PCR

The procedure for EML4-ALK multiplex PCR was adapted from Sanders et al.[14] and Soda et al.,[11] using Super Therm Gold Master Mix (Bionovas) in one tube. The primers used are shown in Table 2. The most common fusion types of KIF5B-ALK were detected using the primers in Table 2. Reaction conditions were: one cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and then one cycle of 72°C for 10 min. RT-PCR amplicons were purified and sequenced using a kit (Big Dye Terminator Sequencing Kit; Applied Biosystems). The different variants were determined and then compared with previously published reports.

Statistical analysis

Patient characteristics (sex, tumor histology, and smoking habit) were tabulated in relation to the mutation status. Fisher’s exact test was used to analyze associations between patient characteristics and the presence of EGFR mutations. Significance was set at p < 0.05 (two-sided). SPSS 17.0 for Windows (SPSS Inc., Chicago, IL) was used for all analyses.
Evaluating the response to EGFR-TKI

The tumor response to EGFR-TKI was evaluated using chest X-rays of the disease sites every 2–4 weeks, and computed tomography every 8–12 weeks after treatment. Treatment responses were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) using the unidimensional method.

Results

Sensitivity assays using cloned EGFR DNA fragments

To determine the sensitivity of PNA-sequencing, cloned DNA fragments (mutants) were serially diluted in genomic DNA from A549 cells (wild-type EGFR) with various calculated gene copy numbers. The analytical sensitivity of PNA-sequencing was estimated to be less than (better than) 1% for both E746_A750 del (Fig 1A) and L858R mutations (Fig 1B). Consistent with general experiences with frame-shift mutations, PCR direct sequencing showed a sensitivity of 10% for E746_A750 del (Fig 1A).

Sensitivity assays using cell lines with EGFR mutations

We used mixed cell lines to estimate the sensitivities at the cellular level. Genomic DNA from H1650 cells (E746_A750 del) and A549 cells (wild-type EGFR) was mixed in ratios ranging from 1:1 to 1:100. Consistent with the above assay using cloned DNA fragments, the analytical sensitivity of PNA-sequencing for E746_A750 del was estimated to be < 1% at the cellular level (Fig 1C). For RNA-sequencing, complementary DNA was obtained using RT-PCR sequencing. The sensitivity of RNA-sequencing was similar to that of the PNA-sequencing at the cellular level (< 1%) (Fig 1C).

Detecting EGFR mutations using direct-, PNA- and RNA-sequencing

The analytical sensitivities of PNA-sequencing and RNA-sequencing met our criterion for EGFR mutation analysis (< 1%). They were subsequently used for routine clinical testing. PCR direct sequencing was also used as a companion test to PNA-sequencing. If a mutation was detected using any one of the 3 methods, a positive report was issued. From January 2011 to June 2015, there were 160 molecular tests done for pleural effusion; 101 were positive for EGFR mutations (63%).

Table 2. Primers used for multiplex PCR gene fusion detection.

| Gene and primers | Location |
|------------------|----------|
| **EML4-ALK detection** |
| 5’-AAG ATC ATG TGG CCT CAG TG-3’ | Forward primer: EML4 exon 2 |
| 5’-CTG CAG ACA AGC ATA AAG ATG-3’ | Forward primer: EML4 exon 6 |
| 5’-GAC TCA GGT GGA GTC ATG C-3’ | Forward primer: EML4 exon 13 |
| 5’-CCA GGA CAC TGG TGG GAT TT-3’ | Forward primer: EML4 exon 17 |
| 5’-CAG ATA TGG AAG GTG CAC TG-3’ | Forward primer: EML4 exon 20 |
| 5’-TCT TGC CAG CAA AGC AGT ATG TGG-3’ | Reverse primer: ALK exon 20 |
| **KIF5B-ALK detection** |
| 5’-TCA AGC ACA TCA CAA GAG CAA GTG-3’ | Forward primer: KIF5B exon 2 |
| 5’-GAC GAT TGG AGG AAT CTG TCG ATG-3’ | Forward primer: KIF5B exon 17 |
| 5’-CAT CTG AAG GAG TGA AAG CTT TGG-3’ | Forward primer: KIF5B exon 24 |

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One hundred forty-two of the 160 cases had enough cells for both DNA and RNA tests. The mean age of the patients was 69.0 years (range: 31–100 years). EGFR mutation was detected in 96 cases (67.6%). EGFR mutations were not associated with sex (men: 30.9% versus women: 36.6%; \( p = 0.275 \)) and smoking history (37.3% of never smokers versus 7.0% of former or current smokers; \( p = 0.321 \)), but associated with age \( \geq 65 \) \( p = 0.008 \). (S1 Table).

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Fig 1. Sensitivity assays using cloned DNA fragments or cell lines. Cloned DNA fragments containing the (A) EGFR E746_A750 del or (B) EGFR L858R mutation were serially diluted in genomic DNA from A549 cells with wild-type EGFR. Direct sequencing of PCR products (direct sequencing; left column) and mutant-enriched PCR sequencing using peptide nucleic acid (PNA-sequencing; right column) were done to determine their analytical sensitivities. The arrows indicate the detected mutation sites. (C) Genomic DNA or total RNA from H1650 cells (E746_A750 del) and A549 cells (wild-type EGFR) were mixed in ratios ranging from 1:1 to 1:100. The DNA underwent direct sequencing and PNA-sequencing. The RNA was undergone PCR sequencing of cDNA after reverse transcription (RNA-sequencing). The arrows indicate the detected mutation sites. The sequencing chromatograms from the lowest detectable ratios are shown in the panel.

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Comparing the three detection methods

Because the PNA-sequencing method used in this study detects only exon 19 del and L858R mutations, we excluded 9 cases with other mutations for the comparison. In the remaining 133 cases, direct sequencing detected $\text{EGFR}$ mutations in 75 cases (56.4%), PNA-sequencing detected $\text{EGFR}$ mutations in 84 cases (63.2%), and RNA-sequencing detected $\text{EGFR}$ mutations in 87 cases (65.4%) of MPE. DNA direct sequencing detected 32 cases with exon 19 deletions and 43 with exon 21 L858R mutations. PNA-sequencing detected 36 cases with exon 19 deletions, 47 cases with L858R mutations, and one case with double mutations (E745_A750 del and L858R). RNA-sequencing detected 39 cases with exon 19 deletions and 48 cases with L858R mutations (Table 3).

Discrepancy using different detection methods

Parallel analysis showed that RNA-sequencing and PNA-sequencing were significantly ($p < 0.0001$) correlated (Table 4).

Clinical response to $\text{EGFR}$-TKIs

Forty-seven of the 87 patients with $\text{EGFR}$ mutations (exon 19 deletions or L858R) received TKIs treatment in our hospital. The clinical decisions were all based on $\text{EGFR}$ mutations detected in MPE. The best clinical responses according to RECIST after TKIs treatment were: 15 partial response (32%), 22 stable disease (47%), and 10 progressive disease (21%). Fig 2 shows one patient as an example. The MPE was tested negative by DNA-sequencing (Fig 2A), but RNA and PNA-sequencing showed exon 19 E746_A750 del (Fig 2B and 2C). The tumor shrank from 2.1 to 1.7 cm (19%) after 18 months of TKI treatment (Fig 2D and 2E).

Table 3. Distribution of $\text{EGFR}$ Exon 19 and 21 mutations detected using the three methods.

|                      | Direct sequencing | PNA-sequencing | RNA-sequencing |
|----------------------|-------------------|----------------|----------------|
| **Total cases (n = 133)** |                   |                |                |
| Wild-type (%)        | 58 (43.6)         | 49 (36.8)      | 46 (34.6)      |
| Mutation type (%)    | 75 (56.4)         | 84 (63.2)      | 87 (65.4)      |
| **Detected as a single mutation** |   |   | |
| Exon 19              | 32                | 36             | 39             |
| Exon 21              | 43                | 47             | 48             |
| **Detected as double mutations** |   |   | |
| Del+L858R            | 3                 | 1a             |               |

*DNA: WT, PNA: Del+L858R, RNA: L858R

Table 4. Comparison of PNA-sequencing and RNA-sequencing in 133 MPE samples.

| Method         | RNA-sequencing |
|----------------|----------------|
| PNA-sequencing |                |
| WT             | MT             |
| WT             | 46             | 3*            |
| MT             | 0              | 84            |

WT: wild-type; MT: mutant type; Del 19, L858R; Significance set at $p < 0.0001$.

*Del 19, L858R; Significance set at $p < 0.0001$. 

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Fig 2. Example of a case of detected EGFR exon 19 mutation using PNA- and RNA-sequencing but not direct sequencing. (A-C) The mutation (EGFR exon 19 E746-A750 del) in the cellular component of MPE was detected using PNA- and RNA-sequencing but not direct sequencing. The arrow indicates a mutation. Wild-type sequence: GCTTCTCTTAATTCCTT; mutant sequence: TTGATAGCGACGGGAAT. Pretreatment (D) and post-treatment (E) CT images. The tumor size decreased from 2.1 cm to 1.7 cm (19%) after 18 months of TKI treatment.

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Multiplex RT-PCR for detecting ALK gene fusion

Multiplex RT-PCR, which was then used to test all 113 cases for EML4-ALK and KIF5B-ALK gene fusion, detected that five of 46 (10.8%) wild-type EGFR cases had EML4-ALK fusion. All of them were variant 1 (E13; A20), which was confirmed using Sanger sequencing (Fig 3). The 67 cases with exon 19 del or L858R mutations showed negative results.

Clinical response to ALK-TKIs

Two of the 5 patients with ALK fusions received Ceritinib treatment in our hospital. The clinical decisions were based on ALK Fluorescent in Situ Hybridization performed on tissue biopsy following the detection of ALK fusion in MPE. The best clinical responses according to RECIST after TKIs treatment were: 1 partial response and 1 progressive disease. Fig 3 shows the EML4-ALK fusion gene sequencing and the CT images of the patient with partial response. The tumor shrank from 2.4 to 1.5 cm (36%) after 6 months of TKI treatment (Fig 3B and 3C).

Discussion

The traditional method for EGFR mutation analysis is direct-sequencing, a technique in which non-malignant cells are likely to interfere with in a heterogeneous tissue sample.[24,39] Tsai et al.[16] reported that RNA-sequencing was significantly more sensitive for detecting EGFR mutations (67.3% vs. 44.7%). Consistent with other studies,[16] in 133 cases, we detected only 75 (56.4%) exon 19 del and L858R mutations using direct-sequencing compared with 87 (65.4%) using RNA-sequencing. Such a result is compatible with the notion that RNA-sequencing is more sensitive than direct-sequencing (using DNA) because of the natural enrichment of the mutant EGFR mRNA in the cells of pleural effusion.

We found that RNA-sequencing was slightly more sensitive than PNA-sequencing in clinical samples (65.4% vs. 63.2%, respectively). This is because the sensitivity of RNA-sequencing is determined by the total EGFR expression level, not by the number of background cells (mesothelial and inflammatory cells). In contrast, the sensitivity of DNA-based PNA-sequencing depends solely upon the number of background cells. The tumor cells have the mutant genome and they express the mutant EGFR mRNA. The background cells within MPEs have considerably lower EGFR expression in comparison with the overexpression of EGFR in NSCLC cells. Because PNA does not completely (100%) block the wild-type background signal, all of the genomic DNA of the background cells will contribute to wild-type signals in PNA-sequencing. However, if the background cells express a very low level of EGFR, the mutant EGFR expressed by the tumor cells will be highly enriched in RNA-sequencing, no matter how many background cells there are. Using H1650 as a background, PNA-sequencing and RNA-sequencing had similar sensitivities (Fig 1). It is possible that the background cells in MPE have even lower EGFR expression than do H1650 cells, which makes the RNA-sequencing more sensitive than PNA-sequencing in clinical samples.

We found that RNA-sequencing might reveal discrepancies between gene mutations in genomic DNA and in mRNA. PNA-sequencing found double mutations (E745_A750 del and L858R) in one case in this study, which RNA-sequencing showed as only singly mutated at L858R. Whether two common sensitive mutations (E745_A750 del and L858R) can reside in the same EGFR molecule is an interesting question.

The sensitivities of EGFR mutation detection were 56.4% for direct-sequencing, 63.2% for PNA-sequencing, and 65.4% for RNA-sequencing. The detection rates in our study were higher than those in Hung et al.,[20] who reported a 41% (12/29) detection rate using
Fig 3. Example of a case of detected EML4-ALK fusion using multiplex PCR. The RT-PCR product was sequenced and confirmed to be variant 1 (E13; A20) of EML4-ALK fusion. Pretreatment (B) and post-treatment (C) CT images. The tumor size decreased from 2.4 cm to 1.5 cm (36%) after 6 months of TKI treatment.

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direct-sequencing, but comparable to those in Tsai et al.,[16] who reported a 67.3% detection rate using RNA-sequencing. Altogether, EGFR mutation in the MPE of lung adenocarcinoma is generally between 60% and 70% in Taiwan, regardless of the patient’s sex, smoking status, and age. Additional prospective studies are needed to confirm our observation.

Among the 133 patients, 22 had available formalin fixed paraffin embedded (FFPE) tissue with amplifiable DNA by Qiagen Therascreen® EGFR RGQ PCR Kit. Nineteen of the 22 results were consistent with those of MPE (7 wild-type and 12 mutants). The remaining 3 (2 wild-type and 1 exon 19 del single mutation) were inconsistent. The corresponding results from MPE were: p. Glu746_Pro753 del Val, Ser (c.2237_2257>TCT), p. L747_T751 del ins Asn (c.2239_2253>AAT), and exon 19 del + T790M double mutations. The first 2 were less common exon 19 deletions which were not included in the 29 mutations to be detected by the Therascreen® kit. This demonstrated the ability of RNA-sequencing and/or PNA-sequencing for the detection of uncommon mutations.

Among the 46 EGFR wild-type cases, 9 had available FFPE tissue for immunohistochemistry. All results were consistent between MPE and FFPE tests (3 positive, 6 negative).

When clinically detecting EGFR mutations, several things need to be considered. For PNA-sequencing, the only modification is to add PNA to the PCR mixture. No additional cost for instruments is required. The technology is simple, rapid, and cost-effective for detecting specific mutant DNA sequences. For RNA-sequencing, careful handling to avoid ribonuclease (RNase) activity is critical for successful testing. Commercially available RNA stabilizing reagents together with refrigeration can minimize RNA degradation. Actually, fusion gene testing based on RT-PCR is a routine procedure for leukemia in many molecular laboratories and should not prohibit using RNA to test the cellular component of pleural effusions. However, the workflow of sample collection and transport should be streamlined to avoid RNA degradation. RNA is an ideal material for detecting fusion genes, not only for ALK, but also for ROS1 and RET found in lung adenocarcinoma.[40] The subtypes of gene fusions can be identified using RT-PCR and Sanger sequencing. It is conceivable that different subtypes respond differently to different TKIs. Such additional benefits may justify the workflow changes for RNA testing. The most sensitive method for EGFR and ALK testing might be true only when the testing specimen is pleural effusion. Generally, RNA-based techniques are not practical for general diagnosis, especially in reference laboratories that receive samples from many centers.

EGFR mutations and ALK gene fusion were thought to be mutually exclusive events in lung adenocarcinoma. Our finding that ALK gene-fusion-positive cases are all negative for EGFR mutations might support this notion. However, evidence that the co-occurrence of the two driver mutations is not uncommon, and that such cases might benefit from dual treatments with both EGFR and ALK TKIs, has recently been reported.[41] When treated with a single TKI, ALK TKI might be a better choice than EGFR TKI.[41] It is highly likely that in the near future, comprehensive molecular diagnoses for lung adenocarcinoma will include both EGFR and ALK testing. EGFR mutation analysis alone may not be adequate, because a patient with a sensitive EGFR mutation might have a concomitant ALK fusion, which might require ALK TKI for optimal treatment.

In conclusion, we have provided evidence that supports MPE as a practical specimen for molecular analysis in patients with NSCLC. RNA-sequencing is more sensitive than PNA-sequencing for detecting EGFR mutations for targeted therapy. PNA-sequencing can be used as an alternative when RNA quality is not optimal. We suggest RNA-sequencing for EGFR mutations and RT-PCR for ALK gene fusion for comprehensive molecular testing of NSCLC MPE.
Supporting Information

S1 Table. Patient characteristics and frequency of EGFR mutations.
(PDF)

Author Contributions
Conceived and designed the experiments: YLC CLH. Performed the experiments: CCL SCY WLC. Analyzed the data: CTL. Contributed reagents/materials/analysis tools: YCL CHY SLP WCS. Wrote the paper: NHC YLC CLH.

References
1. Group. NM-AC (2008) Chemotherapy in addition to supportive care improves survival in advanced non-small-cell lung cancer: a systematic review and meta-analysis of individual patient data from 16 randomized controlled trials. J Clin Oncol 26: 4617–4625. doi:10.1200/JCO.2008.17.7162 PMID: 18678835
2. Kris MG, Johnson BE, Berry LD, Kwiatkowski DJ, Iafrate AJ, Wistuba II, et al. (2014) Using Multiplexed Assays of Oncogenic Drivers in Lung Cancers to Select Targeted Drugs. Jama-Journal of the American Medical Association 311: 1998–2006.
3. Azzoli CG, Baker S Jr., Temin S, Pao W, Aliff T, Brahmer J, et al. (2009) American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. J Clin Oncol 27: 6251–6266. doi:10.1200/JCO.2009.23.5622 PMID: 19917871
4. X C., Z Q., W YL. (2012) Can EGFR-TKIs be used in first line treatment for advanced non-small cell lung cancer based on selection according to clinical factors?—A literature-based meta-analysis. J Hematol Oncol 5: 1–9.
5. Gridelli C, De Marinis F, Di Maio M, Cortinovis D, Cappuzzo F, Mok T (2011) Gefitinib as first-line treatment for patients with advanced non-small-cell lung cancer with activating epidermal growth factor receptor mutation: Review of the evidence. Lung Cancer 71: 249–257. doi:10.1016/j.lungcan.2010.12.008 PMID: 21216486
6. Shi Y, Au JS, Thongprasert S, Srinivasan S, Tsai CM, Khoa MT, et al. (2014) A prospective, molecular epidemiology study of EGFR mutations in Asian patients with advanced non-small-cell lung cancer of adenocarcinoma histology (PIONEER). J Thorac Oncol 9: 154–162. doi:10.1097/JTO.0000000000000033 PMID: 24419411
7. Krawczyk P, Ramlau R, Chorostowska-Wynimko J, Powrozek T, Lewandowska MA, Limon J, et al. (2015) The efficacy of EGFR gene mutation testing in various samples from non-small cell lung cancer patients: a multicenter retrospective study. Journal of Cancer Research and Clinical Oncology 141: 61–68. doi:10.1007/s00432-014-1789-x PMID: 25086987
8. Liu HP, Isaac Wu HD, Chang JW, Wu YC, Yang HY, Chen YT, et al. (2010) Prognostic implications of epidermal growth factor receptor and KRAS gene mutations and epidermal growth factor receptor gene copy numbers in patients with surgically resectable non-small cell lung cancer in Taiwan. J Thorac Oncol 5: 1175–1184. doi:10.1097/JTO.0b013e3181e2446 PMID: 20591511
9. Kobayashi K, Hagiwara K (2013) Epidermal growth factor receptor (EGFR) mutation and personalized therapy in advanced nonsmall cell lung cancer (NSCLC). Target Oncol 8: 27–33. doi:10.1007/s11523-013-0258-9 PMID: 23361373
10. Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. (2010) EML4-ALK Mutations in Lung Cancer That Confer Resistance to ALK Inhibitors. New England Journal of Medicine 363: 1734–1739. doi:10.1056/NEJMoa1007478 PMID: 20979473
11. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448: 561–566. PMID: 17625570
12. Choi YL, Takeuchi K, Soda M, Inamura K, Kogashi Y, Hatano S, et al. (2008) Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 68: 4971–4976. doi:10.1158/0008-5472.CAN-07-6158 PMID: 18593892
13. Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mehail T, et al. (2014) First-line crizotinib versus chemotherapy in ALK-positive lung cancer. N Engl J Med 371: 2167–2177. doi:10.1056/NEJMoa1404440 PMID: 25470694
14. Sanders HR, Li HR, Bruery JM, Scheerle JA, Meloni-Ehrig AM, Kelly JC, et al. (2011) Exon scanning by reverse transcriptase-polymerase chain reaction for detection of known and novel EML4-ALK fusion
variants in non-small cell lung cancer. Cancer Genet 204: 45–52. doi: 10.1016/j.cancergenet.2010.08.024 PMID: 21356191
15. Li Y, Pan Y, Wang R, Sun Y, Hu H, Shen X, et al. (2013) ALK-rearranged lung cancer in Chinese: a comprehensive assessment of clinicopathology, IHC, FISH and RT-PCR. PLoS One 8: e69016. doi: 10.1371/journal.pone.0069016 PMID: 23922677
16. Tsai TH, Su KY, Wu SG, Chang YL, Luo SC, Jan IS, et al. (2012) RNA is favorable for analysing EGFR mutations in malignant pleural effusion of lung cancer. European Respiratory Journal 39: 677–684. doi: 10.1183/09031936.0004511 PMID: 21719485
17. Kimura H, Fujiwara Y, Sone T, Kunitoh H, Tamura T, Kasahara K, et al. (2006) EGFR mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib. Br J Cancer 95: 1390–1395. PMID: 17060940
18. Guo H, Wan Y, Tian G, Liu Q, Kang Y, Li Y, et al. (2012) EGFR mutations predict a favorable outcome for malignant pleural effusion of lung adenocarcinoma with Tarceva therapy. Chang Gung Med J 29: 373–379. PMID: 17051834
19. Kimura H, Fujiwara Y, Sone T, Kunitoh H, Tamura T, Kasahara K, et al. (2006) High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients. Cancer Sci 97: 642–648. PMID: 16827805
20. Wu SG, Goh CH, Yu CJ, Chang YL, Yang CH, Hsu YC, et al. (2008) Frequent epidermal growth factor receptor gene mutations in malignant pleural effusion of lung adenocarcinoma. European Respiratory Journal 32: 924–930. doi: 10.1183/09031936.00167407 PMID: 18508816
21. Kubo A, Koh Y, Kawaguchi T, Isa S, Okamoto I, Fukuoka J, et al. (2011) Malignant pleural effusion from lung adenocarcinoma treated by gefitinib. Intern Med 50: 745–748. PMID: 21467709
22. Shamblin CJ, Tanner NT, Sanchez RS, Woolworth JA, Silvestri GA (2013) EGFR mutations in malignant pleural effusions from lung cancer. Current Respiratory Care Reports 2: 79–87.
23. Miyazawa H, Tanaka T, Nagai Y, Matsuoka M, Sutani A, Udagawa K, et al. (2008) Peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based detection test for gefitinib-refractory T790M epidermal growth factor receptor mutation. Cancer Sci 99: 595–600. doi: 10.1111/j.1349-7006.2007.00706.x PMID: 18271876
24. Tanaka T, Nagai Y, Miyazawa H, Koyama N, Matsuoka M, Sutani A, Udagawa K, et al. (2007) Reliability of the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based test for epidermal growth factor receptor mutations integrated into the clinical practice for non-small cell lung cancers. Cancer Sci 98: 246–252. PMID: 17233841
25. Araki T, Shimizu K, Nakamura T, Baba M, Kawai Y, Nakamura K, et al. (2011) Clinical screening assay for EGFR exon 19 mutations using PNA-clamp smart amplification process version 2 in lung adenocarcinoma. Oncology Reports 26: 1213–1219. doi: 10.3892/or.2011.1391 PMID: 21769434
26. Lee HJ, Xu X, Kim H, Jin Y, Sun P, Kim JE, et al. (2013) Comparison of Direct Sequencing, PNA Clamping-Real Time Polymerase Chain Reaction, and Pyrosequencing Methods for the Detection of EGFR Mutations in Non-small Cell Lung Carcinoma and the Correlation with Clinical Responses to EGFR Tyrosine Kinase Inhibitor Treatment. Korean J Pathol 47: 52–60. doi: 10.4132/KoreanJPathol.2013.47.1.52 PMID: 23453646
27. Skronski M, Chorostowska-Wynimko J, Szczepulska E, Szpechcinski A, Rudzinski P, Orłowski T, et al. (2013) Reliable detection of rare mutations in EGFR gene codon L858 by PNA-LNA PCR clamp in non-small cell lung cancer. Adv Exp Med Biol 756: 321–331. doi: 10.1007/978-94-007-4549-0_39 PMID: 22836650
28. Teckle P, Peacock S, McTaggart-Cowan H, van der Hoek K, Chia S, Melosky B, et al. (2011) The ability of cancer-specific and generic preference-based instruments to discriminate among clinical and self-reported measures of cancer severities. Health Qual Life Outcomes 9: 106. doi: 10.1186/1477-7525-9-106 PMID: 22123196
29. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45: 228–247. doi: 10.1016/j.ejca.2008.10.026 PMID: 19097774
30. Jackman D, Pao W, Riely GJ, Engelman JA, Kris MG, Janne PA, et al. (2010) Clinical definition of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. J Clin Oncol 28: 357–360. doi: 10.1200/JCO.2009.24.7049 PMID: 19949011
33. Nishino M, Jackman DM, Hatabu H, Yeap BY, Cioffredi LA, Yap JT, et al. (2010) New Response Evaluation Criteria in Solid Tumors (RECIST) guidelines for advanced non-small cell lung cancer: comparison with original RECIST and impact on assessment of tumor response to targeted therapy. AJR Am J Roentgenol 195: W221–228. doi:10.2214/AJR.09.3928 PMID: 20729419

34. Mitsudomi T, Kosaka T, Endoh H, Horio Y, Hida T, Mori S, et al. (2005) Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. J Clin Oncol 23: 2513–2520. PMID: 15738541

35. Nagai Y, Miyazawa H, Huqun, Tanaka T, Udagawa K, Kato M, et al. (2005) Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res 65: 7276–7282. PMID: 16105816

36. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. (2008) Somatic mutations affect key pathways in lung adenocarcinoma. Nature 455: 1069–1075. doi:10.1038/nature07423 PMID: 18948947

37. Lv C, Ma YY, Feng Q, Fang F, Bai H, Zhao BT, et al. (2013) A pilot study: sequential gemcitabine/cisplatin and icotinib as induction therapy for stage IIIB to IIIA non-small-cell lung adenocarcinoma. World Journal of Surgical Oncology 11.

38. William WN Jr., Pataer A, Kalhor N, Correa AM, Rice DC, Wistuba II, et al. (2013) Computed tomography RECIST assessment of histopathologic response and prediction of survival in patients with resectable non-small-cell lung cancer after neoadjuvant chemotherapy. J Thorac Oncol 8: 222–228. doi:10.1097/JTO.0b013e3182774108 PMID: 23287849

39. Yatabe Y, Matsuo K, Mitsudomi T (2011) Heterogeneous Distribution of EGFR Mutations Is Extremely Rare in Lung Adenocarcinoma. Journal of Clinical Oncology 29: 2972–2977. doi: 10.1200/JCO.2010.33.3906 PMID: 21730270

40. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. (2012) RET, ROS1 and ALK fusions in lung cancer. Nat Med 18: 378–381. doi:10.1038/nm.2658 PMID: 22327623

41. Won JK, Keam B, Koh J, Cho HJ, Jeon YK, Kim TM, et al. (2015) Concomitant ALK translocation and EGFR mutation in lung cancer: a comparison of direct sequencing and sensitive assays and the impact on responsiveness to tyrosine kinase inhibitor. Ann Oncol 26: 348–354. doi:10.1093/annonc/mdu530 PMID: 25403583