Introduction

Lung cancer is the most frequently diagnosed cancer and the most common cause of cancer-related mortality worldwide.[1,2] Treatment with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have significantly improved the prognosis of patients with advanced lung adenocarcinoma, particularly those with EGFR gene mutations.[3,4]

Currently, tumor tissues obtained by surgery or biopsy (including thoracoscopic pleural metastatic tissue biopsy) are commonly used for EGFR mutation test,[5–7] but unfortunately, it is difficult to obtain adequate amount of tumor tissues from patients with advanced lung adenocarcinoma. Some studies have analyzed the EGFR mutation rate in pleural effusions and the relationship between the mutation rate and patient response to gefitinib,[8–11] whereas a previous study investigated the EGFR mutation rate in malignant pleural effusions (MPEs) of lung adenocarcinoma and compared it with the mutation rate in surgically resected specimens of lung adenocarcinoma from patients without MPEs.[12] However, to the best of our knowledge, no study has been done to compare EGFR mutation statuses between MPEs and their matching metastatic pleural tumor tissues (MPTTs). Furthermore, the sensitivity and specificity of EGFR gene mutation detection in MPEs remain unknown comparing with that in MPTTs. The purpose of this study is to analyze the EGFR gene mutation rates in MPEs and matching MPTTs obtained by thoracoscopic pleural metastatic tissue biopsy from patients with advanced lung adenocarcinoma and determine if MPEs are good substitutes for MPTTs in EGFR gene mutation test. Meanwhile, the sensitivity and specificity of the mutation tests from MPE supernatants and their matching cell

Abstract

**Background:** Though the possibility of using malignant pleural effusions (MPEs) as alternatives for metastatic pleural tumor tissues (MPTTs) in epidermal growth factor receptor (EGFR) mutation test has been examined, due to the lack of studies comparing the results in matching MPEs and MPTTs, the clinical value of MPEs for advanced adenocarcinoma patients with pleural effusions is not confirmed.

**Methods:** EGFR mutation statuses in matching MPTTs, MPE supernatants and cell blocks, of 41 patients with advanced lung adenocarcinoma as diagnosed by thoracoscopy were analyzed using amplification refractory mutation system (ARMS).

**Results:** EGFR mutations were detected in 46.3% (19/41) of MPTTs, 43.9% (18/41) of MPE supernatants and 56.3% (18/32) of MPE cell blocks by ARMS analysis. Generally, the same EGFR statuses were identified in both MPTTs and matching MPE cell blocks of 81.3% patients (26/32), whereas MPTTs and matching MPE supernatants of 87.8% (36/41) patients shared the same EGFR status. Compared with EGFR mutation detection in MPTTs, the sensitivity of EGFR mutation detection in MPE-cell blocks was 87.5% (14/16), specificity was 75.0% (12/16), while the sensitivity of EGFR mutation detection in MPE-supernatants was 84.2% (16/19), specificity was 90.9% (20/22).

**Conclusions:** The high concordance of EGFR mutation statuses between MPEs and MPTTs in lung adenocarcinoma patients with pleural metastasis as determined by ARMS analysis suggests that MPEs, particularly MPE supernatants, may be substitutes for MPTTs in EGFR mutation test.
blocks were compared to determine which are of more clinical value.

Materials and Methods

Patients and samples
This study was carried out at Changhai Affiliated Hospital of the Second Military Medical University (Shanghai, China) and the procedures were approved by the Institutional Ethics Committee of Changhai Hospital. All patients had signed an informed consent form for the use of these samples in molecular analysis. Patients were eligible for inclusion in the study for further analysis if they met the following criteria: (1) the patients who were highly suspect for lung malignant disease; (2) patients already have pleural effusion at clinic; (3) thoracoscopy was required to make clear the causes of pleural effusion and collect the tumor sample biopsy; (4) metastatic lung cancer were diagnosed by biopsy; (5) Performance status (Eastern Cooperative Oncology Group performance status (ECOG PS)) ≤ 2. From April 2011 to June 2013, 23 males and 18 females with a median age of 55 years (range, 29 to 78 years), including 13 smokers and 28 non-smokers, were enrolled at the Respiratory Department of Medicine of Changhai Hospital. All patients were pathologically diagnosed as lung adenocarcinoma with pleural metastasis. None of them had received prior EGFR-TKIs therapy. Detailed patient information is listed in Table 1. Paired MPTT and MPE samples were collected for EGFR mutation analysis. MPTT samples were obtained through a semi-rigid thoracoscope (LTF-240, Olympus Optical Co Ltd, Tokyo, Japan). MPE samples (250–500 mL) were collected from each patient and centrifuged at 1,000 g for 10 min at room temperature within one hour of collection and all MPE samples were collected before the MPTT procedure to reduce the confounder factors. Ten milliliters of supernatant was stored at 37°C for further analyses. The cell pellets were fixed in 10% neutral-buffered formalin, and then embedded in paraffin to make the MPE cell blocks. Each formalin-fixed, paraffin-embedded (FFPE) sample (MPTT, MPE-cell block) was cut into 5 μm-thick sections that were incubated at 37°C for 3 hr and then stored at room temperature.

Table 1. Patients information.

| Characteristics (n = 41) | Patients number | Percentage (%) |
|-------------------------|----------------|----------------|
| Age                     |                |                |
| Average                 | 55±12          |                |
| Range                   | 29–78          |                |
| Sex                     |                |                |
| Male                    | 23             | 56.1           |
| Female                  | 18             | 43.9           |
| History of Smoking      |                |                |
| Smoking                 | 28             | 68.3           |
| Non-smoking             | 13             | 31.7           |
| Therapy                 |                |                |
| None                    | 39             | 95.1           |
| Chemotherapy            | 2              | 4.9            |
| Radiotherapy            | 0              | 0              |
| Targeted Therapy        | 0              | 0              |

All slides from MPTT and MPE-cell blocks were reviewed and diagnosed as lung adenocarcinoma by three different pathologists. All FFPE tissues were pathologically examined to confirm the presence of lung adenocarcinoma and to determine the percentage of tumor cells. Since the sensitivity of ARMS analysis was approximately 1%, only tissue samples with 1% tumor cells or more were selected for the EGFR mutation analysis. Accordingly, all FFPE MPTT samples and 32 of 41 MPE-Cell blocks were qualified for further EGFR mutation analysis.

Genomic DNA extraction and EGFR mutation analysis
Genomic DNA in FFPE samples (10–12 serial sections) and MPE supernatants were extracted by QIAamp DNA FFPE tissue kits (Qiagen, Hilden, Germany) and QIAamp circulating nucleic acid kits (Qiagen, Hilden, Germany) by following the manufacturer’s protocols. The concentration of DNA samples were measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, USA). The DNA was diluted to 2–3 ng/μl to be used in EGFR mutation test.

EGFR mutations were analyzed by using of a Human EGFR Gene Mutations Fluorescence PCR Diagnostic Kit (Amoy Diagnostics, Xiamen, China), which is based on the ARMS technology. The assay can identify the 29 most common types of EGFR mutations currently described in lung cancers. These EGFR mutations included 19 types of deletions in exon 19, 3 types of insertions in exon 20, T790M, L858R, L861Q, G719X and S768I point mutations. All experiments were done by following the manufacturer’s protocols. Briefly, 10 ng genomic DNA was added to 45 μL PCR master mix containing PCR buffer, DNA polymerase, PCR primers, fluorescent Taqman probe specific for each individual EGFR mutation. After 47 amplification cycles, the fluorescent signal was collected from FAM and HEX channels.

Statistical analyses
χ² test was used for categorical variables. The concordance rate of EGFR mutations and Cohen’s kappa coefficients were calculated between MPTTs and MPEs. Cohen’s kappa coefficient was calculated as: kappa = (Po–Pe)/(1–Pe), where Po is the observed concordance rate and Pe is the expected probability of chance agreement. In general, kappa values of 0.4–0.6 indicate moderate agreement and values more than 0.6 indicate a significant agreement between observations[13]. The Youden’s index, the difference between the true positive rate and the false positive rate was also calculated to reflect the reliability of EGFR mutation test, while indexes more than 0.7 indicate significant reliability. A P-value less than 0.05 was considered statistically significant. The statistical analyses were carried out using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA).

Results
EGFR mutations in MPTTs
All of the 41 MPTT specimens successfully passed pathological quality control (containing 5%–60% tumor cells). EGFR mutations were found in 46.3% (19/41) of MPTT samples. The most frequent mutations observed were the deletion mutation in exon 19 (12/19, 63.2%) and the point mutation (L858R) in exon 21 (7/19, 36.8%). As listed in Table 2, the rate of EGFR mutation was significantly higher in non-smokers (37.1%, 16/28) than in smokers (23.1%, 3/13) (P = 0.042). Although there was no statistical significance, the mutation rate was higher in women (61.1% (11/18) than in men (34.8%, 8/23) (P = 0.093). EGFR mutation status was not correlated with patients’ age (P = 0.754).
EGFR mutations in MPEs

As summarized in Supplementary Data (Table S1), the ARMS analysis showed that EGFR mutations were present in 56.3% (18/32) of MPE cell blocks. Among these mutations, 10 were exon 19 deletion, 7 were L858R, and 1 was S768I. The frequency of EGFR mutation in the 41 MPE supernatants was 43.9% (18/41). Among these samples, 10 were exon 19 deletions, 7 were L858R, and 1 was S768I. If EGFR mutation was present in either MPE supernatants or MPE cell blocks, the MPE samples was assessed as positive for EGFR mutation. Accordingly, the frequency of EGFR mutations in MPE samples (supernatants and cell blocks) was 53.7% (22/41). Among these mutations, 13 were deletions in exon 19, 8 were L858R, and 1 was point mutation in exon 20 (S768I). MPE cell blocks were generated from MPE samples, and subjected to EGFR mutation analysis accordingly.

The concordance of EGFR mutations in MPPT and MPE samples

The EGFR mutations in MPPT and MPE samples from the same patients were summarized in Table 3. Generally, the same EGFR statuses were identified in both MPPT samples and MPE cell blocks of 81.3% patients (26/32). Different EGFR mutations were identified in one patient's MPPT sample and MPE cell block, ARMS analysis showed that the MPPT sample harbored an exon 19 deletion, while the MPE cell block had S768I point mutation. Compared to the frequency of EGFR mutations in MPPT samples, the concordance with that in MPE cell blocks was 65.0% (13/20). ARMS analysis also revealed that MPPT samples and MPE supernatants of 87.8% (36/41) patients harbor the same EGFR status. The concordance between the frequency of EGFR mutations in MPPT samples and that in MPE supernatants was 76.2% (16/21). The concordance between EGFR status identified by ARMS analysis in MPPT samples and MPE samples was 85.4% (35/41). Compared to frequency of EGFR mutations in MPPT samples, the concordance of that in MPE samples was 73.9% (17/23). The concordance of the EGFR analysis results of MPE cell blocks and that of MPE supernatants was as high as 84.4% (27/32), indicating the high similarity of EGFR mutation status between these two materials.

Compared with EGFR mutation detection in MPPT, the sensitivity of EGFR mutation detection in MPE cell blocks was 87.5% (14/16), specificity was 75.0% (12/16), the false positive rate was 25.0% (4/16) and false negative rate was 12.5% (2/16) (Table 3); whereas the sensitivity of EGFR mutation detection in MPE supernatants was 84.2% (16/19), specificity was 90.9% (20/22), the false positive rate was 9.1% (2/22) and false negative rate was 15.8% (3/19) (Table 4). Statistical analysis showed that the kappa values between EGFR mutations in MPPT samples with that in MPE cell blocks, MPE supernatants and MPE samples were 0.625, 0.749 and 0.765, respectively, and they were statistically significant (P<0.001) (Table 5).

Discussion

The number of lung cancer patients has been increasing and patients with advanced lung adenocarcinoma account for the majority of lung cancer-related death.[1,14] EGFR-TKIs are highly effective (71.2%) in the treatment of patients with advanced...
lung adenocarcinoma with activating mutations in the tyrosine kinase domain of EGFR, but have little or no effect on patients with no activating EGFR mutations.[3] Thus, choosing the right therapeutic approaches is critical to effectively treat patients with advanced lung adenocarcinoma, and decisions are made mainly based on the results of EGFR mutation test.[4,15] To those lung adenocarcinoma patients with pleural effusions, detection of EGFR mutation statuses in MPTTs obtained by thoracoscopic pleural biopsy provides useful information for choosing the right therapeutic approach. However, not all lung adenocarcinoma patients with pleural effusions are suitable for thoracoscopic pleural biopsy. Furthermore, since the status of EGFR mutation in patients with pleural effusions are suitable for thoracoscopic pleural biopsy, multiple biopsies may be required and this process of therapy, multiple biopsies may be required and this increases the complexity and cost of the test. To solve this problem, we compared the concordance of the EGFR mutation test results between MPE supernatants and MPTTs, and MPE cell blocks and MPTTs, respectively, and found that in the 41 pairs of MPE supernatants and cell blocks, the supernatants had higher concordance with MPTTs in terms of EGFR mutations. Furthermore, the Youden Index of the tests using the supernatants (0.749) was significantly higher than that of the tests using MPE cell blocks (0.625), suggesting that the results from supernatants could better reflect the real status of EGFR mutation in MPTTs. Herein, we concluded that compared with MPE cell blocks, MPE supernatants are better substitutes for MPTTs in EGFR mutation test and have the following advantages: (1) Their EGFR mutation status is highly concordant with that of MPTTs (87.8% in our studies) with the specificity of 90.9%, false negative rate of 9.1% and Youden index as high as 0.749; (2) By eliminating the need of embedding, the loss of specimen is minimized and the test procedure is simplified; (3) Unlike malignant cancer tissues, DNA in malignant pleural effusions is primarily derived from tumor cells, as long as it is accessible, it is qualified for EGFR test; for malignant cancer tissues, only tissues with a minimum of 1% malignant cancer cells are qualified for EGFR test, since the sensitivity of ARMS analysis was approximately 1%; (4) It is easier to purify DNA from MPE supernatants than from MPTTs.

### Conclusions

In this study, we have compared the EGFR mutation statuses in MPEs and their matching MPTTs from patients who have been diagnosed to have advanced lung adenocarcinoma by thoracoscopic pleural biopsy and demonstrated high concordance rate of EGFR mutations between MPEs and MPTTs as determined by the ARMS analysis. We also found that compared with MPE cell blocks, MPE supernatants showed higher concordance with MPTTs in EGFR mutations status. This result suggests that MPEs, particularly MPE supernatants, may be used as substitutes for MPTTs in EGFR mutation analysis. This information will benefit the treatment of advanced NSCLC patients in determining whether EGFR-TKIs are right to them.

### Supporting Information

**Table S1 Summary of EGFR mutations in MPTT and MPE of same patients.**

(DOCX)
Acknowledgments

We thank Changting Liu and Yanping Xu for their assistance in sample collection and preparation, Li Zheng for her pathological analysis, and Sunny Sun for her contribution in experimental data generation.

Author Contributions

Conceived and designed the experiments: CB. Performed the experiments: YH QL. Analyzed the data: GZ. Contributed reagents/materials/analysis tools: NW XN YX. Wrote the paper: DL YL ZH.

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