Original Article

A prospective phase 2 study of expeditious *EGFR* genotyping and immediate therapeutic initiation through extracellular vesicles (EV)-based bronchoalveolar lavage fluid (BALF) liquid biopsy in advanced NSCLC patients

In Ae Kim[1], Jae Young Hur[1,2], Hee Joung Kim[1,3], Wan Seop Kim[1,2], Kye Young Lee[1,3]

[1]Precision Medicine Lung Cancer Center, Konkuk University Medical Center, Seoul, Republic of Korea; [2]Department of Pathology, Konkuk University School of Medicine, Seoul, Republic of Korea; [3]Department of Pulmonary Medicine, Konkuk University School of Medicine, Seoul, Republic of Korea

Contributions: (I) Conception and design: IA Kim, HJ Kim, KY Lee; (II) Administrative support: IA Kim, HJ Kim, WS Kim; (III) Provision of study material or patients: All authors; (IV) Collection and assembly of data: IA Kim, JY Hur, HJ Kim, KY Lee; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Kye Young Lee, MD, PhD. Precision Medicine Lung Cancer Center and Department of Pulmonary Medicine, Konkuk University Medical Center and Konkuk University School of Medicine, 120-1 Hwayang-dong, Gwangjin-Gu, Seoul 05030, Republic of Korea. Email: kyleemd@kuh.ac.kr.

Background: In our previous study, epidermal growth factor receptor (*EGFR*) genotyping using extracellular vesicles (EV)-derived DNA isolated from bronchoalveolar lavage fluid (BALF) was proven to be highly concordant with conventional tissue-based genotyping and its turn-around-time (TAT) was only 1–2 days. On this background, we prospectively validated the performance of EV-based BALF liquid biopsy for *EGFR* genotyping in the real practice of advanced non-small cell lung cancer (NSCLC) patients.

Methods: After screening 120 newly diagnosed stage III–IV NSCLC patients, 51 cases were detected as *EGFR*-mutated by EV-based BALF *EGFR* genotyping and 40 patients were enrolled for gefitinib treatment. BALF EV were isolated by ultracentrifuge method and *EGFR* genotyping was performed with PCR-based PNA-clamping assisted fluorescence melting curve analysis. The objective response rate, progression-free survival (PFS), TAT, time to treatment initiation (TTI), and concordance rate were analyzed with clinical parameters.

Results: There was only one false positive case among the 120 screened patients and the overall concordance rate between tissue biopsy and EV-based BALF liquid biopsy was 99.2% including the subtype of *EGFR* mutations. TAT for EV-based BALF *EGFR* genotyping was 1.9±1.1 days, while tissue-based TAT was 12.1±7.2 days (P<0.001). *EGFR* genotyping was determined even before obtaining histopathologic report in most cases. TTI in BALF *EGFR* genotyping was faster than tissue genotyping (7.8±6.5 *vs.* 13.8±12.9 days). Therapeutic outcomes of response rate and PFS were almost similar to tissue-based results.

Conclusions: We demonstrated, for the first time, that EV-based BALF liquid biopsy should be an excellent platform for expeditious *EGFR* genotyping and rapid therapeutic intervention even before obtaining the result of histopathology in advanced NSCLC patients.

Keywords: Extracellular vesicles (EV); liquid biopsy; epidermal growth factor receptor mutation (*EGFR* mutation); gefitinib; non-small cell lung cancer (NSCLC)

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Introduction

Epidermal growth factor receptor (*EGFR*) mutation testing is an essential step for the therapeutic decision in newly diagnosed advanced non-small cell lung cancer (NSCLC) patients (1,2) and it is usually performed using tumor tissue DNA after histologic confirmation. Obtaining tumor tissue for *EGFR* mutation testing is basically invasive and sometimes challenging in the cases of small-sized tumor, risky location for percutaneous targeting, and radiologic characteristics, such as cavity, consolidation, or ground glass type lesions. Surgical biopsy is sometimes adopted for adequate tissue biopsy. Turn-around-time (TAT) is another important unmet need for both the patients and the physicians on the decision of adequate treatment, because it is usually 2–3 weeks. Recently, plasma liquid biopsy using circulating tumor DNA has been introduced, but it has an intrinsic limitation of low sensitivity to be used in real clinical routine practice (3,4). This low sensitivity issue could be overcome with the use of molecular barcoding and deep sequencing; however, they are still too pricy for routine practice. On this background, we have developed a novel platform for high-speed *EGFR* genotyping using extracellular vesicles (EV)-derived DNA from bronchoalveolar lavage fluid (BALF) and obtained the results almost equally concordant with tissue-based genotyping. EV are ideal carriers of cancer biomarkers, as cancer cells secret an abundance of EV and the contents of tumor cell originated EV reflect the molecular and genetic composition (double-stranded DNA and various subtypes of RNA, proteins and lipids) of parental cells (5,6). EV are potential sources of tumor genetic material for *EGFR* mutation tests. Furthermore, the lipid bilayer of EV enables EV in the body fluid to exist for stable cargoes from enzymatic degradation (7-10).

In a previous study, an EV-based BALF liquid biopsy for *EGFR* genotyping showed a sensitivity of 97.8%, specificity of 96.9%, and concordance rate of 97.7% when compared to tissue genotyping in 224 advanced stage III–IV NSCLC patients (11). In addition, its TAT of 1–2 days was significantly shorter than the conventional tissue-based *EGFR* testing. Thus, therapeutic decision process for EGFR-TKIs or even other therapeutic modalities can be accelerated with the knowledge of the *EGFR* genotype, especially in the patients with symptomatic disease. In addition, EV-based BALF liquid biopsy can be highly effective when tissue biopsy is risky or impractical to access. In this study, we had prospectively validated the diagnostic and therapeutic performance of EV-based BALF *EGFR* genotyping in the aspects of both the speed and accuracy in advanced NSCLC patients. Gefitinib in previous research has shown to be beneficial as a first line treatment in patients with *EGFR* mutations (1). However, there is no prospective clinical trial reporting the efficacy of first line gefitinib treatment based on EV-based BALF liquid biopsy. This study aimed to prove the diagnostic performance of EV-based BALF liquid biopsy and show the possibility of EGFR-TKI treatment without tissue biopsy in real clinical practice. This article is presented in accordance with the TREND reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-892/rc).

Highlight box

Key findings

- EV-based BALF liquid biopsy performed on the patients with suspicious advanced lung cancer reveals identical concordance with tissue *EGFR* genotyping, while it is less invasive and faster than tissue biopsy.

What is known and what is new?

- *EGFR* mutation testing to newly diagnosed advanced NSCLC patients is an essential step for the therapeutic decision.
- However, obtaining tumor tissue for *EGFR* mutation testing is often challenging.
- This work is the first prospective study reporting the efficacy of first line gefitinib treatment based on EV-based BALF liquid biopsy.

What is the implication, and what should change now?

- EV-based BALF liquid biopsy provides great advantages in the cases of difficult tissue biopsy due to small tumor size, risky location, or the nature of radiologic findings such as ground glass type or cavitary tumor.
- The decision for EGFR-TKI treatment can be made through EV-based BALF liquid biopsy even without tissue biopsy.

Methods

Study design and patient population

This study was single arm, phase II, single center and prospective clinical trial to investigate the clinical performance of EV-based BALF liquid biopsy for EGFR genotyping. From January 2018 to August 2020, 120 treatment-naive and newly diagnosed advanced stage III–IV NSCLC patients were screened for EV-based BALF liquid biopsy for *EGFR* genotyping after obtaining BALF from tumor site through bronchoscopic examination

in the referred patients with suspected lung cancer. We preferentially screened patients of the cohort in the previous study (11) using EV-based BALF liquid biopsy with favorable factors for *EGFR* mutation: female, never smoker or minimally exposed smoker. Heavy smokers and the patients with central-type lung cancer were not included because likelihood of harboring *EGFR* mutations is significantly low (12).

This study was planned to provide gefitinib to the selected patients before histologic confirmation to reduce the risk of misdiagnosis. Routine diagnostic work-up for histologic confirmation and staging was simultaneously done in all patients. After confirming *EGFR* mutation positivity by EV-based BALF liquid biopsy, oral gefitinib 250 mg/day (Iretinib®, Chong Kun Dang Pharm.) treatment was immediately initiated by investigator after acquisition of patients' consent for clinical trial even before the acquisition of pathologic report. Inclusion criteria to select patients for gefitinib treatment were: age ≥19 years; histopathologic confirmed and treatment-naive NSCLC patients with stage IIIB or IV advanced NSCLC; active *EGFR* mutation (E21L858R, E19DEL, E21L861Q, G719X, S768I) or combination with rare *EGFR* mutation in BALF; ECOG performance status of 0–2; treatment-naive; measurable target lesion according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1; life expectancy of ≥12 weeks; normal organ function [absolute nucleophile counts >1,500, platelet ≥100,000/mm^3, hemoglobin ≥9.0 g/dL, AST/ALT/ALP ≤3 times of the upper limit of normal (ULN), total bilirubin ≤2.0 mg/dL, serum creatinine ≤ ULN]. Patients were excluded, if they had been treated with cytochrome P450 inhibitor within 1 week; had other active malignancy; had pre-existing interstitial lung disease/pulmonary fibrosis; were pregnant or lactating women; had symptomatic or uncontrolled brain metastasis; had NYHA ≥3 congestive heart failure, uncontrolled hypertension, unstable angina, myocardial infarction within 6 months. Disease stages were based on the 8th TNM classification criteria (13). Clinical and demographic data of the enrolled patients were reviewed. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the institutional review board of Konkuk University Medical Center (No. KUH1010868). Written informed consent was obtained from all patients. This prospective clinical trial was also approved by Ministry of Food and Drug Safety, South Korea (KFDA Registration No. 201700442, Approval No. 31413).

BALF sample collection, EV-based EGFR genotyping

Patients with suspected lung cancer based on chest tomography (CT) underwent bronchoscopy at initial lung cancer work-up for biopsy. BALF was retrieved in a trap by gentle aspiration through operating channel after instilling approximately 70–100 mL of sterile isotonic saline by wedging the bronchoscope at the segment or sub-segment where the tumor lesion was located. At least, more than 10–15 mL of BALF were collected from each patient. After sending for the routine cytological examination, 1 mL of residual BALF sample was used for the isolation of EV. Cells and debris were removed using centrifugation at 1,000 g for 10 min at 4 ℃. Cells and debris free BALF were spun in ultracentrifuge tube at 200,000 g for 1 h at 4 ℃ using a Beckman rotor (Beckman Coulter, Brea, CA, USA). The supernatant was carefully removed, and the pellet was suspended in 200 µL of phosphate-buffered saline. The EV were lysed by mixing cell lysis buffer and detergent, and the EV-derived DNA was purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The concentration and purity of DNA samples were measured using the NanoDrop (Thermo Scientific, Waltham, MA, USA). The length of the purified DNA was analyzed using a 4200 Tapestion and Genomic DNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA). For detecting *EGFR* mutations, PANAMutyper™ R *EGFR* kit (Panagene, Daejeon, Korea) with the peptide nucleic acid (PNA)-mediated PCR clamping method (14) and CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) were used. PCR and the melting curve step were performed according to the manufacturer's protocol (Panagene, Korea). All reactions had a total volume of 25 µL containing 70 ng of template DNA. Fluorescence was measured on all four channels (FAM, ROX, Cy5, and HEX). With the use of a mutant-type DNA specific PNA detection probe that had a fluorescent dye and quencher, *EGFR* mutations could be genotyped by melting peak analysis (15,16). To prevent false positive or negative results, the same test was performed at least twice times on each sample.

EGFR genotyping of tissue DNA

The tumor samples were prepared as formaline-fixed, paraffin-embedded (FFPE) tissues and tumor DNAs were purified using the TANBead OptiPure FFPE DNA Tube

(Taiwan Advanced Nanotech, Taoyuan, Taiwan) according to the manufacturer's protocol. Then, *EGFR* genotyping was done through PANAMutyper™ R *EGFR* kit (Panagene, Daejeon, Korea) according to the manufacturer's protocol. To prevent the bias, two pathologists read tissue and BALF samples separately in a blinded manner.

Treatment protocol and follow-up assessment

At screening, demographic data and medical history were recorded. All the assessments were made at screening/baseline period (week −2 to week 0). For the patients who were proven to have sensitive *EGFR* mutation by EV-based BALF liquid biopsy even before histopathologic result, 250 mg gefitinib (Iretinib®, Chong Kun Dang Pharm.) treatment was immediately initiated after receiving the consent for trial. All the patients received oral gefitinib at a dose of 250 mg/day until tumor progression or death or occurrence of intolerable adverse event (AE) or adverse drug reaction. Dose reductions or temporarily interruption of gefitinib treatment were permitted, if patients encountered any grade ≥3 drug-related AEs [assessed according to National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0]. Tumor assessment by computed tomography scan was performed every 8 weeks by masked independent radiologist. The primary endpoint was to assess the objective response rate (ORR) at best response according to RECIST criteria version 1.1. ORR was defined as the proportion of patients with complete response (CR) or partial response (PR) to gefitinib. The secondary endpoints were the assessment of progression-free survival (PFS), the concordance rate of *EGFR* mutation between BALF and tissue, disease control rate (DCR), TAT, and time-to-treatment initiation (TTI). TTI was determined with the time from the date of bronchoscopy or tissue biopsy to the date of starting treatment. All patients with *EGFR* mutations were treated with Gefitinib based on their BALF genotyping, therefore, the TTI for BALF was determined. TTI for conventional-tissue biopsy was determined with the time from the date of tissue biopsy to the starting date of cytotoxic chemotherapy after the confirmation of no *EGFR* mutation in tissue. PFS was calculated from the start of treatment to PD or death from any cause, or it was censored on the date of the last follow-up. ORR was defined as the proportion of patents with CR and PR in response to the treatment. DCR was defined as the proportion of patients with CR, PR, or stable disease (SD) in response to the treatment. TAT was determined by the time from the day of bronchoscopy for obtaining BALF to the day of reporting of *EGFR* mutation result. Drug safety evaluation was performed according to the NCI-CTCAE version 3.0.

Statistical analysis

Sample size calculation

The sample size was determined by the exact single-stage phase II design. The response rate of gefitinib was 70% (55–84%) in previous study (2). On the hypothesis that the *EGFR* mutation detection rate using BALF liquid biopsy is comparable (non-inferiority) to that using tissue biopsy (d=0.1, non-inferiority difference 0.07, α=5%, 1−β=0.9), the expected actual number was 32 patients. Assuming that the concordance rate of the tissue and BALF test was 90%, mismatched results occur in 3 patients. Therefore, 35 patients are required, and 40 patients were recruited considering a drop-out rate of 10%.

Statistical method

Thirty-eight patients with sensitive *EGFR* mutation were analyzed, and efficacy of EGFR-TKI was assessed one year after the initiation of gefitinib treatment of the last entered patient. The survival curves, median value of PFS and corresponding 95% confidence interval (CI) were calculated using the Kaplan-Meier method. *EGFR* status assessment of histological tumor samples was considered as a standard reference for the calculation of concordance. Categorical variables were summarized by calculating frequencies and percentages. The means and standard deviations were used to determine numerical variables. We used *t*-tests, Fisher's exact tests, and χ^2 statistics. All statistical analyses were carried out using SPSS Statistics version 25.0 (IBM Corp, Chicago, IL, USA) and R software version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria), and a P value <0.05 was regarded as the indicator of statistical significance.

Results

Patient characteristics

The mean age of the enrolled patients was 70.3±11.6 years. Females were 49.2% and non-smokers were 55.0%. Most of the patients were histologically classified as adenocarcinoma (80.8%). Stage IIIB were 7.5%, stage IVA were 55.8% and stage IVB were 36.7% (*Table 1*).

Table 1 Clinical characteristics of study participants, stratified by EV-based BALF liquid biopsy (n=120)

Variables	Total	BALF *EGFR* (+)	BALF *EGFR* (–)	Gefitinib
All patients	120	51	69	40
Age, years	70.3±11.6	70.6±9.6	70.0±13.0	72.1±8.06
<65	37 (30.8)	14 (27.5)	23 (33.3)	9 (22.5)
≥65	83 (69.2)	37 (72.5)	46 (66.7)	31 (77.5)
Sex				
Male	61 (50.8)	22 (43.1)	39 (56.5)	17 (42.5)
Female	59 (49.2)	29 (56.9)	30 (43.5)	23 (57.5)
Smoking history				
Never-smoker	66 (55.0)	34 (66.7)	32 (46.4)	29 (72.5)
Ex-smoker	31 (25.8)	14 (27.5)	17 (23.6)	11 (27.5)
Current smoker	23 (19.2)	3 (5.8)	20 (30.0)	0 (0)
Stage				
IIIB	9 (7.5)	3 (5.9)	6 (8.7)	1 (2.5)
IVA	67 (55.8)	30 (58.8)	37 (53.6)	25 (62.5)
IVB	44 (36.7)	18 (36.7)	26 (37.7)	14 (35.0)
Performance status				
0–1	94 (78.4)	39 (76.4)	55 (79.8)	30 (75.0)
2	26 (21.6)	12 (23.6)	14 (20.2)	10 (25.0)
Histology				
Adenocarcinoma	97 (80.8)	46 (90.2)	51 (73.9)	37 (92.5)
NSCLC, NOS	16 (13.4)	4 (7.8)	12 (8.7)	2 (5.0)
Squamous cell carcinoma	6 (5.0)	0 (0)	6 (27.4)	0 (0)
SCLC	1 (0.8)	1 (2.0)	0 (0)	1 (2.5)
EGFR type (tissue)				
19 del	29 (24.2)	29 (56.8)	0 (0)	24 (60.0)
21 L858R	20 (16.6)	21 (41.2)[†]	0 (0)	14 (35.0)
G719C/S768I	1 (1.0)	1 (2.0)	0 (0)	1 (2.5)
WT	70 (58.2)	0 (0)	69 (100.0)[†]	1 (2.5)

Data are shown as mean ± SD or n (%). [†], one case where 21L858R mutation was initially detected with BALF-based test was later verified to have a wild type in the tissue-based test. The number of 21L858R cases is one more and the number of WT case is one less in BALF than in tissue due to this false positive EGFR case. EV, extracellular vesicles; BALF, bronchoalveolar lavage fluid; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; NOS, not otherwise specified; SCLC, small cell lung cancer; WT, wild type; SD, standard deviation.

Among the 120 screened patients, 51 cases were detected to harbor *EGFR* mutations through EV-based BALF liquid biopsy. Eleven patients among them were excluded because 4 were transferred, 2 had symptomatic brain metastasis, 2 had other organ cancers, 3 did not consent the trial. Among 40 cases that were enrolled for immediate initiation of gefitinib treatment, two were dropped out early due to 1 transfer, 1 small cell lung cancer histology, and finally 38 patients were included to be analyzed. The data cutoff for this analysis was September, 2021 (*Figure 1*). The median

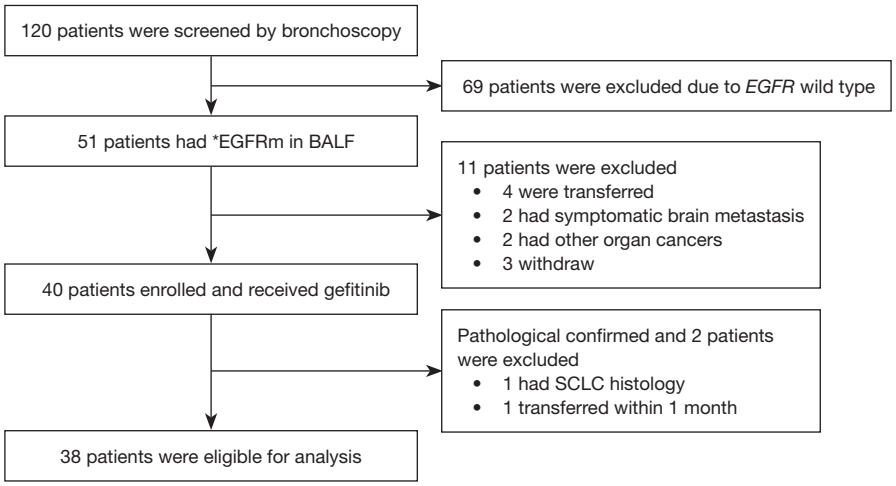

Figure 1 The study flow diagram of rapid diagnosis and EGFR-TKI initiation by EV-based BALF liquid biopsy in advanced NSCLC patients. EV, extracellular vesicle; BALF, bronchoalveolar lavage fluid; EGFR, epidermal growth factor receptor; SCLC, small cell lung cancer; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer.

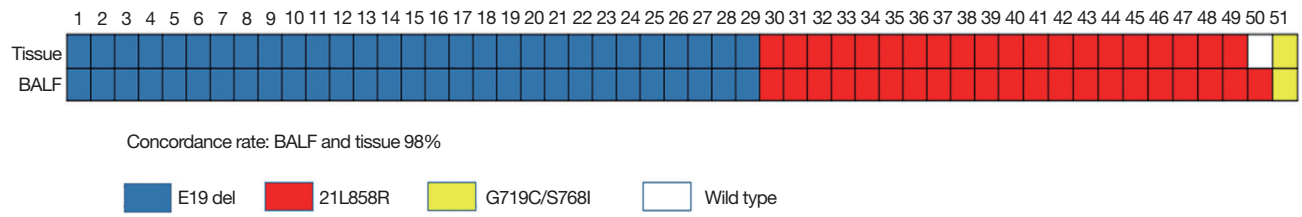

Figure 2 Concordance rate of EV-based BALF liquid biopsy for *EGFR* genotyping to tissue genotyping and plasma liquid biopsy (n=51). EV, extracellular vesicle; BALF, bronchoalveolar lavage fluid; EGFR, epidermal growth factor receptor.

follow-up period at the time of analysis was 18.7 months (range, 1–38 months). Two were lost to follow-up due to withdrawal and 36 patients were completed the follow-up.

Performance of EV-based BALF liquid biopsy for EGFR genotyping

Figure 2 displays the result of the concordance between tissue biopsy and EV-based BALF liquid biopsy for *EGFR* genotyping in 51 *EGFR*-mutated patients. There was only one mismatched case of E21L858R mutation which was proven to be false positive. The concordance rate in *EGFR* mutation positive group detected by EV-based BALF liquid biopsy was 98.0% (50/51). There was no false negative case in the wild-type *EGFR* patients by EV-based BALF liquid biopsy. Overall concordance rate in 120 screened patients was 99.2% (119/120). Tissue samples were unobtainable in two patients out of total 120 patients due to the complication at the time of diagnosis. Both of two patients were confirmed to have *EGFR* mutation later, one in pleural effusion and another in BALF cytology performed at progression time. The *EGFR* mutation results of these two patients obtained from BALF were consistent with the *EGFR* mutation results confirmed through other samples other than lung tissue.

The proportions of *EGFR* mutation subtypes were the same as reported in the previous research (4,12,17); the proportion of each sensitive mutation was 56.9% (29 cases) for E19del, 41.2% (21 cases) for E21L858R, and 2.0% (one case) for G719C/S769I compound mutation depending on BALF liquid biopsy. These data suggest that EV-based BALF liquid biopsy has almost the equivalent performance with conventional tissue biopsy for *EGFR* mutation testing except one case of mismatch.

Table 2 TAT and TTI of EV-based BALF liquid biopsy in comparison with tissue genotyping

Time	BALF genotyping	Tissue genotyping	P value
TAT (n=120) (day)	1.9±1.1	12.1±7.2	<0.001
TTI (day)			0.01
EGFR-TKI (n=40)	7.8±6.5		
Chemotherapy (n=49)	13.8±12.9		

Data are shown as mean ± SD. TTI, time with from the date of bronchoscopy or tissue biopsy to the date of treatment initiation (gefitinib or chemotherapy); EV, extracellular vesicles; BALF, bronchoalveolar lavage fluid; TAT, turn-around time; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; SD, standard deviation.

Table 3 Investigator-assessed objective response rate to gefitinib treatment by EV-based BALF liquid biopsy

Response	Value (N=38[†]), n (%)
CR	1 (2.6)
PR	28 (73.7)
SD	6 (15.8)
PD	3 (7.9)
ORR (CR + PR)	29 (76.3)
DCR (CR + PR + SD)	35 (92.1)

[†], 40 case enrolled but 2 cases dropped out early, 1 SCLC, 1 transfer. EV, extracellular vesicles; BALF, bronchoalveolar lavage fluid; CR, complete remission; DCR, disease control rate; ORR, objective response rate; PD, progressive disease; PR, partial remission; SD, stable disease; SCLC, small cell lung cancer.

TAT and TTI of EV-based BALF liquid biopsy

Turn-around time was defined by the time from the biopsy or bronchoscopy to the time of knowing the *EGFR* mutation result. Conventional TAT of tissue-based *EGFR* mutation testing took about 12.1±7.2 days in our center. In comparison, the BALF *EGFR* mutation testing only took about 1.9±1.1 days (P<0.001). Therefore, the time for initiating gefitinib treatment after the day of bronchoscopy was just 7.8±6.5 days and it was 7–10 days earlier than time of conventional tissue genotyping (13.8±12.9 days) (*Table 2*). It significantly saved the waiting time till initiating treatment and served to reduce the anxiety and suffering of the patients. These means that EV-based BALF liquid biopsy could be a novel platform for *EGFR* testing enough to replace the conventional tissue-based genotyping in all aspects of speed, accuracy, and less invasiveness. Looking into the surprising sensitivity and specificity, it might be highly feasible and valuable to investigate the therapeutic decision based on EV-based BALF liquid biopsy without tissue biopsy in *EGFR*-mutated NSCLC patients.

Therapeutic efficacy of gefitinib initiation based on EV-based BALF liquid biopsy

Therapeutic efficacy was evaluated in 38 patients who received gefitinib for more than 4 weeks. The median follow-up period at the time of analysis was 18.7 months (range, 1–38 months). The ORR as a primary endpoint was described in *Table 3*. During the observation period, one patient exhibited CR, 28 met the criteria for PR, 6 exhibited SD, and 3 exhibited PD. Thus, ORR (CR + PR) were 76.3%. The DCR as a secondary endpoint was 92.1% and the median PFS was 14.6 months (95% CI: 8.8–21.9) (*Figure 3*). A proportion of 76.3% ORR was better than 69.8–73.7% ORR of previous researches. Median PFS 14.6 months was longer than other previous studies (9.7–10.8 months) (2,18-21). The estimated one-sided confidence interval for gefitinib response rate, using BALF liquid biopsy was 0.635–1.0. The non-inferiority of the EGFR-TKI response rate of BALF liquid biopsy was proven, as the lower margin 0.635 of the calculated confidence interval was better than the pre-assumption lower margin 0.63 (0.7–0.07) before the study. In the case of BALF, the ORR 76.3% was not less than 70% (P=0.045).

The therapeutic outcomes of response and PFS were not inferior to the previous results. They are similar or better than the tissue-based results (2,17-20). Our efficacy endpoints, ORR 76.3% and PFS 14.6 months were numerically better than previous Gefitinib efficacy based by tissue biopsy. The early initiation of gefitinib treatment by BALF *EGFR* mutation before disease progression can improve the clinical outcomes such as PFS and tumor response.

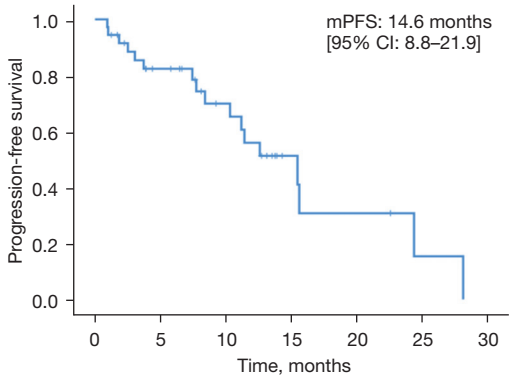

Figure 3 Kaplan-Meier curve of PFS in patients treated by gefitinib based on BALF liquid biopsy (n=38). CI, confidence interval; mPFS, median progression-free survival; PFS, progression-free survival; BALF, bronchoalveolar lavage fluid.

Safety analysis

All patients who had received at least one dose of Gefitinib were included in the safety analysis. Of the 40 patients who underwent gefitinib treatment, 34 (85%) reported the occurrence of AEs. Severe AEs were reported in 12 (30%) patients among the study population. Five (12.5%) patients discontinued the treatment due to drug-related AEs. The most common AEs were the skin eruption (57.5%), diarrhea (27.5%), and increasing liver enzyme (15%) (Table S1).

Discussion

In previous research, we demonstrated the accuracy and speed of BALF *EGFR* testing for advanced-stage lung cancer patients (11). To prove the performance of this novel platform in real clinical practice, we prospectively validated the diagnostic performance of EV-based BALF liquid biopsy and the efficacy of EGFR-TKI treatment based on the results of this BALF liquid biopsy. In this study, *EGFR*-mutated NSCLC patients were detected by EV-based BALF liquid biopsy usually within around two days after bronchoscopy, and gefitinib treatment was promptly initiated before tissue confirmation, while tissue-based *EGFR* genotyping was confirmed in 10–14 days after bronchoscopic examination. The gefitinib treatments were continued by the time of progression, withdrawal of trial, or death. To the best of our knowledge, this work is the first prospective study reporting the efficacy of first line gefitinib by treating the patients with advanced *EGFR*-mutated NSCLC detected by EV-based BALF liquid biopsy even before conventional tissue-based genotyping. This study provides clinical evidence for the utility of EV-based *EGFR* mutation status check to ascertain eligibility for EGFR-TKI treatment with no *EGFR* result from tissue biopsy needed. The earlier initiation of treatment with the help of EV-based *EGFR* mutation detection improves the treatment efficacy such as PFS and tumor response.

Among the 120 screened patients, 51 patients were detected as harboring *EGFR* mutation by EV-based BALF liquid biopsy. A proportion of 42.5% of *EGFR* mutation positivity was relatively higher than expected and it is because we preferentially screened the patients with favorable factors for *EGFR* mutations, such as female, never smoker or minimally exposed smoker and peripheral-type tumor (12). Patient with central type tumor and current heavy smokers were not included. As a result, the greater part of *EGFR* mutant patients detected was never smoker (66.7%) or ex-smoker (25.8%). The concordance rate of *EGFR* genotype between tissue biopsy and EV-based BALF liquid biopsy was 98% and there was only one case of mismatch which was a false positive case. In one false positive case, we retested the *EGFR* genotyping in remained BALF and tissue and confirmed that the *EGFR* mutation was negative. The PANAmutyper *EGFR* PCR method had 0.1–1% error rate. After the case, we performed the *EGFR* mutation genotyping testing twice to reduce false positive cases. There was no false negative case in the wild-type *EGFR* patients by EV-based BALF liquid biopsy. Overall concordance rate in 120 screened patients was 99.2%. This finding suggests that EV-based BALF liquid biopsy has almost identical performance with conventional tissue biopsy for *EGFR* genotyping. Especially in the cases of difficult tissue biopsy due to small tumor size, location, and the nature of radiologic findings such as ground glass type, consolidation-like tumor and cavitary tumor, EV-based BALF liquid biopsy provides great advantages to avoid invasive or risky biopsy or even surgical biopsy.

In routine practice, *EGFR* mutation testing is ordered after confirming pathologic report after biopsy. It takes usually 2–3 weeks, and it is an indispensable test item before therapeutic decision. There is a great unmet need to shorten its TAT, especially in the symptomatic patients. Patient's anxiety and doctor's waiting for the result to make a therapeutic decision are considerable factors. Even though plasma liquid biopsy using cell-free DNA (cfDNA) has been adopted, its role is only supplementary due to its low sensitivity (22). In our study, TAT for EV-based BALF liquid biopsy was only 1.9±1.1 days, while 12.1±7.2 days in

tissue genotyping. In other words, the time to get *EGFR* genotyping result can be reduced to only 1–2 days after bronchoscopy and that is around 10 days shorter than tissue genotyping. Also, this expeditious *EGFR* genotyping with high accuracy obtained by EV-based BALF liquid biopsy has been proven to provide a great advantage to initiate early therapeutic intervention in both EGFR-TKI treatment in mutant cases and chemotherapy in wild-type cases after histologic diagnosis. Gefitinib treatment was initiated in only 7.8±6.5 days after bronchoscopic examination.

The response of early gefitinib treatment based on EV-based BALF *EGFR* genotyping was not different from the usual data of tissue-based treatment. The proportion of achieving objective responses was 76.3% (32/38) and the disease control rate was 92.1% (35/38). The response rates of BALF *EGFR* genotyping were better than previous trials of tissue *EGFR* genotyping (ORR, 69.8–73.7%; DCR, 89–95%) (17,19,21). The median PFS was 14.6 months (95% CI: 8.8–21.9), which was longer than the values of previous research (20,23) (median PFS, 6–15 months). It might be because most patients in our study were non-smokers or minimally exposed smokers who had good prognosis and good response for EGFR-TKIs in general (24,25). The AEs from gefitinib (Iretinib®) in this study were not different from the common AEs of other EGFR-TKIs (26). The most common AEs were the skin eruption, diarrhea, and increasing liver enzyme (Table S1).

In this study, we demonstrated for the first time that EV-based BALF liquid biopsy shows almost identical performance with conventional tissue biopsy for *EGFR* genotyping. We propose that the decision for EGFR-TKI treatment could be made through EV-based BALF liquid biopsy, even without tissue biopsy. If tissue evaluation is necessary in later time for such as detecting T790M mutation or next-generation sequencing (NGS) analysis, it can be performed with adequate time. At present, we are investigating the role of EV-based BALF liquid biopsy for detecting T790M mutation in the patients with acquired EGFR-TKI resistance. And also, we are developing optimal liquid NGS panel using BALF EV DNA (27). It is worthwhile to investigate prospective clinical trial for the 1st-line treatment of the 3rd generation EGFR-TKIs such as osimertinib or lazertinib through EV-based BALF liquid biopsy without tissue biopsy. Considering the risk and invasiveness of tissue lung biopsy, the paradigm shift from tissue-based diagnosis to liquid biopsy will be made in lung cancer soon. In this aspect, plasma liquid biopsy is extensively investigated at present, but the expectation is not optimistic when considering the complexity of blood samples. Furthermore, EV-based BALF liquid biopsy is much more promising, as this study reveals the concordance with tissue biopsy for *EGFR* genotyping.

Molecular testing of sensitizing *EGFR* mutations, *BRAF* V600E, as well as *ALK*, *ROS1*, and *NTRK* fusions, is now standard-of-care for patients with advanced NSCLC. Routine testing of *RET* fusions and *MET* exon 14 skipping mutations is also considered standard-of-care based on the recent guidelines. Thus, a comprehensive biomarker testing is recommended for all patients diagnosed with non-squamous NSCLC. Currently, our technique using BALF is limited for *EGFR* mutation not including other targetable mutations, and further development for detecting other mutations will be required. A safe, sensitive and accurate detection of *EGFR* mutation in BALF is, nevertheless, beneficial for specific sub-population with high mutation frequency, such as Asian non-smoker whose frequency is 40–50% (28,29). We used PANAMutyper for EV-based BALF liquid biopsy approved by Korean Ministry of food and Drug Safety, but other methods such as droplet digital PCR and other FDA-approved companion diagnostics kits such as therascreen® *EGFR* RGQ PCR Kit and cobas® *EGFR* Mutation Test can be used for EV-based BALF liquid biopsy.

Bronchoalveolar lavage (BAL) through the bronchoscope is a conventional and safe diagnostic technique for patients with a variety of pulmonary diseases or lung cancer (30). BAL makes it possible to obtain the cellular and non-cellular contents from the disease-located site such as distal airways and peripheral alveoli (31). Recently smoking-related central type lung cancers with visible endobronchial lesions decreases, while peripheral-type lung cancer, especially adenocarcinoma are steadily increasing (32). The matter of lung biopsy becomes a major task to overcome in the era of precision medicine because tissue is the issue. BALF cytology based diagnostic yield is quite low and that is the reason why BAL is not routinely performed in diagnostic work up for lung cancer. We would like to recommend taking supernatants rather than cellular components from BALF in which abundant amount of EVs released by tumor cells or tumor microenvironment. In our study, we demonstrated the usefulness of EVs isolated from BALF of lung cancer patients in special case of *EGFR* mutation testing (11). We propose that the application of EV-based BALF liquid biopsy will be extended to whole field of genetic, genomic or molecular diagnosis.

Conclusions

We have demonstrated for the time that EV-based BALF liquid biopsy is a novel platform for *EGFR* mutation testing that can lead to early therapeutic intervention with EGFR-TKI in advanced NSCLC patients. Compared to conventional tissue genotyping, EV-based BALF liquid biopsy in the patients with suspicious advanced lung cancer revealed almost identical concordance with TAT of 1–2 days and relatively less invasiveness. Its performance showed that it has the potential to replace tissue genotyping, overcoming the low sensitivity of plasma liquid biopsy using cfDNA. Large-scaled and multicenter clinical trials should be promptly initiated to obtain approvals for real-world application.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the institutional review board of Konkuk University Medical Center (No. KUH1010868) and written informed consent was obtained from all patients. This prospective clinical trial was also approved by Ministry of Food and Drug Safety, Republic of Korea (KFDA Registration No. 201700442, No. 31413).

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Supplementary

Table S1 Adverse events in patients treated by gefitinib based on BALF liquid biopsy (n=40)

Adverse events (N=40)	All grades, n (%)	Grades ≥3, n (%)
Skin eruption/itching	23 (57.5)	4 (10.0)
Diarrhea	11 (27.5)	3 (7.5)
Liver enzyme abnormality	6 (15.0)	2 (5.0)
Nausea/vomit	5 (12.5)	2 (5.0)
Anorexia	5 (12.5)	0 (0)
Insomnia	2 (5.0)	0 (0)
Dizziness/weakness	3 (7.5)	1 (2.5)
Headache	2 (5.0)	1 (2.5)
Pneumonitis/pneumonia	2 (5.0)	1 (2.5)
Creatine increased	1 (2.5)	1 (2.5)

BALF, bronchoalveolar lavage fluid.

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