Noninvasive Saliva-based EGFR Gene Mutation Detection in Patients with Lung Cancer

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Abstract

Rationale: Constitutive activation of the epidermal growth factor receptor (EGFR) is prevalent in epithelial cancers, particularly in non-small cell lung carcinoma (NSCLC). Mutations identified in EGFR predict the sensitivity to EGFR-targeted therapy. Detection of these mutations is mainly based on tissue biopsy, which is invasive, expensive, and time consuming.

Objectives: Noninvasive, real-time, inexpensive detection and monitoring of EGFR mutations in patients with NSCLC is highly desirable.

Methods: We developed a novel core technology, electric field–induced release and measurement (EFIRM), which relies on a multiplexible electrochemical sensor that can detect EGFR mutations directly in bodily fluids.

Measurements and Main Results: We established EFIRM for the detection of the EGFR mutations in vitro and correlated the results with tumor size from xenografted mice. In clinical application, we demonstrated that EFIRM could detect EGFR mutations in the saliva and plasma of 22 patients with NSCLC. Finally, a blinded test was performed on saliva samples from 40 patients with NSCLC. The receiver operating characteristic analysis indicated that EFIRM detected the exon 19 deletion with an area under the curve of 0.94 and the L858R mutation with an area under the curve of 0.96.

Conclusions: Our data indicate that EFIRM is effective, accurate, rapid, user-friendly, and cost effective for the detection of EGFR mutations in the saliva of patients with NSCLC. We termed this saliva-based EGFR mutation detection (SABER).

Keywords: lung cancer; EGFR mutation; saliva diagnostics; electrochemical sensor

Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for 29% of all cancer deaths in men and 26% of all cancer deaths in women (1, 2). Recent understanding of the pathogenesis and molecular oncology of lung cancers has contributed to the discovery of the importance of acquired genetic alterations in epidermal growth factor receptor (EGFR), which encodes a pharmacologically targetable tyrosine kinase (3, 4). In 2009, the first randomized clinical trial (the Iressa

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At a Glance Commentary

Scientific Knowledge on the Subject: In non-small cell lung cancer (NSCLC), epidermal growth factor receptor (EGFR) mutations have emerged as important biomarkers in predicting the response to the EGFR tyrosine kinase inhibitors. The identification of these mutations is based on invasively obtained biopsy samples, which is often not acceptable in a clinical setting. The analysis of circulating tumor DNA or circulating tumor cells in the blood is an alternative approach but is often complicated, technique dependent, and time consuming. A noninvasive, readily available, diagnostic procedure with minimal preparation that provides immediate information on EGFR mutation status is desirable.

What This Study Adds to the Field: In patients with NSCLC, Electric Field–Induced Release and Measurement (EFIRM) can detect EGFR mutations directly in saliva. This enables clinicians to adjust their therapeutic strategies in a timely fashion, consequently improving the clinical outcome of EGFR-targeted therapy.

Pan-Asia Study) demonstrated that, for patients with advanced non-small cell lung carcinoma (NSCLC) carrying an activating EGFR mutation, initial treatment with an EGFR–tyrosine kinase inhibitor (TKI) was superior to standard platinum-based chemotherapy (5). EGFR mutations analysis is performed on tumor cells in biopsy or cytology obtained from bronchoscopy, computed tomography–guided biopsy, surgical resection, or drainage from malignant pleural effusions. However, sampling tumor tissue other than surgical resection has significant inherent limitations; tumor tissue is a single snapshot in time and is subject to selection bias resulting from tumor heterogeneity, and it can be difficult to obtain enough DNA for EGFR mutation test if there is a lack of tumor cells (6). Due to the invasive procedure and the progressive development of drug-resistant mutations, the initial detection and continuous monitoring of EGFR mutations are still unmet clinical needs. Because blood harbors the same genetic lesions as the primary tumor, blood-borne biomarkers such as circulating tumor cells (CTCs) and circulating tumor DNA are promising for detecting somatic mutations derived from malignant tumors (7). Limitations exist on the uncertainty of collection methods and diversity of phenotypes of CTCs in blood (8). And detecting circulating tumor DNA in plasma requires molecular methods such as polymerase chain reaction–based technology (9, 10), high-performance liquid chromatography (11), and mutant-enriched liquid chips (12), which are complicated, technique dependent, and time consuming. The ideal method should be noninvasive, be readily available, need minimal or no sample preparation, and provide immediate information on EGFR mutation status, which is important for the long-term management of patients with NSCLC to enable clinicians to adjust therapeutic strategies, improving the outcome of targeted therapy.

In this paper, we explored the clinical usefulness of using saliva to detect EGFR mutations in patients with NSCLC by developing a core technology, electric field–induced release and measurement (EFIRM; Figure 1), for the detection of biomarkers in bodily fluids. Multiple targeting molecules have been studied in saliva (14–19), and EFIRM has been applied in a pancreatic cancer rodent model for biomarker development (16). Here we describe the optimization of EFIRM for detecting EGFR mutations. The resultant assay, saliva-based EGFR mutation detection (SABER), met the clinical requirements for EGFR mutation detection in a blinded study of patients with NSCLC and could be combined with tissue DNA testing or complement the biopsy, especially in cases where tumor is insufficient for DNA extraction.

Methods

Plasma and Saliva Collection from Patients with NSCLC

Because the exon19 deletion and the exon21 L858R point mutation represent 90% of EGFR sensitizing mutations (20, 21), only patients confirmed to be wild type or harboring these mutations before treatment were allowed to enroll in the study. We collected blood into ethylenediaminetetraacetic acid tubes and centrifuged it at 2,500 x g for 10 minutes at 4°C to collect plasma for the EFIRM study. The upper phase corresponding to the plasma was immediately stored at −80°C until analysis. Unstimulated whole saliva was collected and processed according to previously established protocols (22). Briefly, saliva samples were kept on ice during collection and were then centrifuged at 2,600 x g for 15 minutes at 4°C. The supernatant was removed from the pellet, treated with RNase inhibitor (Superase-In; Ambion, Inc., Austin, TX), and stored at −80°C before use.

SABER-based Detection of EGFR Mutations in Bodily Fluids

Figure 1 illustrates the core technology of EFIRM. The sensor is a conducting polymer-based electrochemical chip with an array of 16 bare gold electrode chips (GeneFluidics, Los Angeles, CA). Paired probes (capture and detector; Sigma, St. Louis, MO) specific for the two TKI-sensitive mutations were designed for EFIRM as follows: a capture probe for the exon 19 deletion, 5′-TGT TGC TTC CTT GAT AGC GAC G-3′; a detector probe for the exon 19 deletion, 5′-GGA ATT TTA ACT TTC TCA CCT FITC-3′; a capture probe for the L858R point mutation: 5′-CAG TTT GGG CCG CCC AAA ATC-3′; and a detector probe for the L858R mutation: 5′-TTG ACA TGC TGC GGT GTT TTC A-FITC-3′. As noted, the detector probes were labeled with fluorescein isothiocyanate. The capture probes (100 nM) were first copolymerized with pyrrole onto the bare gold electrodes by applying a cyclic square wave electric field at +350 mV for 1 second and +950 mV for 1 second. In total, polymerization proceeded for 5 cycles of 10 seconds each.

The samples, including cell-culture medium or 40-μl samples of blood or saliva, were mixed with the detector probes and transferred onto the electrodes after polymerization. Hybridization was performed at −200 mV for 1 second and +500 mV for 1 second for 5 cycles of 10 seconds each. Next we added 150 U/ml of anti-fluorescein antibody conjugated to horseradish peroxidase (1:1,000 dilution; Roche, Indianapolis, IN) in casein-phosphate-buffered saline (Invitrogen, Carlsbad, CA). Finally, we
loaded the 3,3',5,5'-tetramethylbenzidine substrate for horseradish peroxidase and measured the amperometric signal. The total detection time was less than 10 minutes, and the procedure required just 20 to 40 µl of biological sample.

**Statistical Analysis**

To evaluate the performance of EFIRM in detecting EGFR mutations, the receiver operating characteristic curve for each probe was plotted, and the area under the curve (AUC) and its 95% confidence interval (CI) were calculated. All analyses were performed using SAS 9.3 TS Level 1M1 (23). We used the G*power program to estimate the sample size needed for validation in a blinded group using one-way analysis of variance with a power of 0.95 at \( \alpha = 0.05 \).

**Results**

**Optimization of EFIRM for EGFR Mutation Detection Using Lung Cancer Cell Lines**

We optimized EFIRM (Figure 1) to detect two EGFR mutations: p.L858R (point mutation c.2573T>G in exon 21) and p.E746-A750del (c.2236_2250del15 in exon 19, a 15–base pair deletion). Genomic DNA samples from the human lung cancer cell lines NCI-H1975 and HCC827, which harbored the respective mutations, were used for the EFIRM optimizations.

We first optimized the electrical field profile for the appropriate number of hybridization cycles (Figures 2A and 2B). The hybridization signal increased rapidly after two cycles of electrical waves. After five cycles, perfect match signals reached a plateau, whereas the mismatch sequences generated only background signal levels. We therefore defined the optimized hybridization cycle as five for all subsequent studies.

The specificity and sensitivity of EFIRM detection for the respective EGFR mutations were investigated by decreasing the ratio of mutant EGFR DNA to wild-type EGFR DNA (Figures 2C–2F). For the p.E746-A750del, as little as 0.1% mutant DNA was detected when a control sample was used. We used 10 µl of 2 ng/µl DNA for these experiments. These data demonstrated that EFIRM was able to detect EGFR mutations with high sensitivity and specificity.

**Detection of EGFR Mutations in the Plasma of a Mouse Model with Xenografted Lung Cancer**

Circulating DNA and RNA, including the oncogene KRAS, have been detected in patients with cancer (24) and tumor-bearing animals (25) and are correlated with tumor stage and size. We investigated whether EFIRM could detect the EGFR mutations in plasma and also correlate with tumor size in mice with human lung cancer xenografts. We used banked plasma from a mouse model xenografted with the human lung cancer cell line NCI-H1975 that harbored the EGFR point mutation p.L858R at increasing tumor burdens in four different stages: (1) no tumor (nonxenograft mice), (2) small tumors (100–500 mm³), (3) medium tumors (500–900 mm³), and (4) large tumors (>900 mm³). Mice xenografted with the HCT 116 cell line, which carried the EGFR
wild type, were used as a control (26). To determine if the optimized EFIRM could detect the EGFR mutations in the plasma of these xenografted mice, 40 μl of mouse plasma was assayed in triplicate with EFIRM at defined time intervals of Day 0, 11, 16, and 19 (Figure 3A). In all of the studies, the wild-type groups showed very low signal levels, as the probes were designed specifically for the L858R mutation. We observed a positive linear relationship between the electrochemical current and tumor size (Figure 3B, 10 μl of plasma \( R = 0.86 \); Figure 3C, 20 μl of plasma \( R = 0.98 \); and Figure 3D, 40 μl of plasma \( R = 0.95 \)). Increased plasma volume yielded improved discrimination between different tumor sizes (Figures 3B–3D). Naive mice were associated with the lowest amount of signal (Figures 3B–3D). Small tumors (100–500 mm\(^3\)) were associated with signal levels that significantly differed from the naive group, and larger tumors had higher current signals (Figures 3B–3D). After 19 days of growth, DNA from mice with large tumors generated electrochemical currents on the order of several hundred nA (Figures 3C and 3D). These results indicate that EFIRM can detect and monitor progressive tumor development by measuring the plasma of xenografted mice. Amperometric readouts are provided in Figures 3E–3G.
EFIRM Detection of EGFR Mutations in Plasma and Saliva from Patients with NSCLC

Tumor-specific EGFRvIII can be released into the blood to merge with the plasma membranes of cancer cells that lack EGFRvIII (27), and cancer-derived microvesicles can alter the contents of secreted microvesicles of salivary gland cells (28). We therefore hypothesized that an EGFR mutation could be detected in the plasma and saliva of patients with NSCLC.

We examined whether the two most common TKI-sensitive EGFR mutations (p.L858R and p.E746-A750del) could be detected by the optimized EFIRM technology in the plasma and saliva of patients with NSCLC. We collected tissue, blood, and saliva samples from patients with NSCLC. The EFIRM technology was used to detect the presence of EGFR mutations in these samples.

Figure 3. Detection of the epidermal growth factor receptor (EGFR) L858R point mutation in xenografted lung cancer mice via electric field–induced release and measurement (EFIRM). (A) Design of the tumor burden study using EGFR L858R xenografted mice. EFIRM of the four groups of mice (three mice per group) with (B) 10 µl of plasma (R = 0.86), (C) 20 µl of plasma (R = 0.98), and (D) 40 µl of plasma (R = 0.95). The reactions were performed in triplicate with both the means and SDs provided. Linear fits to the data appear in red and the correlation coefficient R is provided. Data from the control group with wild-type EGFR are illustrated in gray squares. The amperometric current readout is listed with (E) 10 µl of plasma, (F) 20 µl of plasma, and (G) 40 µl of plasma.
plasma, and saliva from each patient with NSCLC at National Cheng Kung University Hospital (NCKUH). After sample collection, we performed biopsy-based EGFR genotyping at NCKUH (see Methods). Plasma and saliva-based detection of the two EGFR mutations were assayed by EFIRM at the University of California, Los Angeles (UCLA).

Twenty-two patients with NSCLC (12 men and 10 women, mean age of 62.0 ± 12.7 yr, mostly nonsmokers) met the enrollment criteria and were enrolled in the study (Table 1). The details of the patients’ clinical characteristics, including disease burden and TNM stage, are described in the online supplemental. The EGFR mutation rate was comparable to other studies that detected EGFR mutations in Asian lung adenocarcinoma, ranging from 38% (29) to 55% (30). The plasma specimens were procured before first treatment. Figure 4 illustrates the EFIRM results from saliva and plasma showing the respective EGFR mutations compared with biopsy-based genotyping. The original amperometric current signals from the detection of the p.E746-A750del exon 19 deletion group in saliva samples are shown in Figure 4E, with data that were read out at 60 seconds. The amperometric currents of the exon 19 deletion group (p.E746-A750del) detected by EFIRM using an exon 19 probe were significantly higher than those in the wild-type and p.L858R mutant groups based on saliva (Figures 4A and 4E; 106.3 ± 13.2 in the p.E746-A750del group vs. 12.8 ± 7.5 in the wild-type group and 6.3 ± 4.7 in the p.L858R mutant group; P < 0.0001). The amperometric currents of the exon 21 mutant group (p.L858R) detected by EFIRM using the L858R probe were significantly higher than those in the wild-type and p.L858R mutant groups (Figure 4B; 66.5 ± 27.2 in the p.L858R mutant group vs. 9.5 ± 5.3 in the wild-type group and 7.7 ± 4.2 in the p.E746-A750del group; P < 0.0001).

Similar results were obtained from plasma using the probe designed for the exon 19 deletion group (Figure 4C; 117.2 ± 8.1 in the p.E746-A750del group vs. 20.7 ± 11.7 in the wild-type group and 10.4 ± 9.2 in the p.L858R mutant group; P < 0.0001) and for the p.L858R mutant group (Figure 4D; 79.0 ± 34.2 in the p.L858R mutant group vs. 18.1 ± 8.9 in the wild-type group and 13.5 ± 7.4 in the p.E746-A750del group; P < 0.0001). Although the sample size was limited, we suggest a cutoff at 2 SDs above the mean value from the control group to differentiate the mutant and control groups. These findings indicated that EFIRM could be used to detect specific EGFR mutations in the plasma and saliva of patients with NSCLC.

Blinded Study to Detect EGFR Mutations in Saliva of Patients with NSCLC Using EFIRM

To validate the clinical performance of EFIRM detection of EGFR mutations in the saliva of patients with NSCLC, we performed a blinded clinical study of an independent cohort of 40 randomized patients with NSCLC from NCKUH. Forty saliva samples were obtained from patients with advanced NSCLC collected at NCKUH, blinded, and sent to UCLA for EFIRM assays. Biopsy-based EGFR genotyping was performed at NCKUH. The blinded samples were further randomized by a biostatistician from a third institution at MD Anderson Cancer Center (MDACC) followed by EFIRM measurements of the two EGFR mutations at UCLA. The patient cohort consisted of 22 men and 18 women, with a mean age of 58.8 ± 10.4 years; 32 cases (80%) exhibited stage IV cancer, and most patients were nonsmokers (Table 1). The clinical characteristics, including tumor stage and EGFR mutations in the blinded group, were similar to those in the testing group.

The amperometric currents of the exon 19 deletion group detected by EFIRM using an exon 19 probe were significantly higher than those in the wild-type and p.L858R mutant groups (Figure 5A; 126.6 ± 58.6 in the p.E746-A750del group vs. 14.5 ± 3.5 in the wild-type group and 9.6 ± 3.7 in the p.L858R mutant group; P < 0.0001). Similar results were obtained using a probe designed for the p.L858R mutant group (Figure 5B; 113.2 ± 75.1 in the p.L858R mutant group vs. 15.9 ± 9.6 in the wild-type group and 9.5 ± 3.2 in the p.E746-A750del group; P < 0.0001). The receiver operating characteristic analysis (Figure 5C) indicated that the AUCs were 0.94 (95% CI, 0.82–1) and 0.96 (95% CI, 0.90–1) (Figure 5C) for probes carrying the p.E746-A750del or the p.L858R mutation, respectively.

Correlation of EGFR Mutation Status between Plasma and Saliva Using EFIRM

We compared the findings from the plasma with those from the saliva to evaluate whether saliva could be as informative as plasma and serve as an additional bodily fluid for mutation testing. For the testing group, the amperometric currents of saliva were correlated with those from plasma using the two different probes (Figure 6A; R = 0.98, P < 0.0001 in the p.E746-A750del groups and R = 0.99, P < 0.0001 in the p.L858R groups). Similar results were observed in the blinded group (33 plasma

| Table 1. Patient Characteristics of the Testing and Blinded Validation Groups |
|----------------------------------|----------------------------------|------------------|
| Nonblinded Cohort | Blinded Validation Cohort | P Value |
| Age, yr | 62.1 ± 12.7 | 58.8 ± 10.4 | 0.28* |
| Sex | | | |
| Male | 22 | 40 | 1.00† |
| Female | 10 (45.5) | 18 (45.0) | |
| Smoker | 7 (31.8) | 11 (27.5) | 0.95† |
| Stage | | | |
| III | 5 (22.7) | 8 (20.0) | 1.00† |
| IV | 17 (77.3) | 32 (80.0) | |
| EGFR mutant type | | | |
| Wild | 11 (50.0) | 20 (50.0) | |
| L858R | 7 (31.8) | 12 (30.0) | |
| Exon 19del | 4 (18.2) | 8 (20.0) | |

Definition of abbreviation: EGFR = epidermal growth factor receptor.

Data are presented as mean ± SD or n (%).

*Chi-square test.
†t test.
‡Fisher test.
journey

Discussion

EFIRM for Detection of EGFR Mutations

Current oncogene mutation detection technologies are mainly polymerase chain reaction (PCR)-based, requiring sample pretreatment and several hours for processing. EFIRM exploits (1) the simple and effective release of biomarkers from bodily fluids, (2) enhanced sample mixing and accumulation, (3) enhanced hybridization of the oncogene (EGFR), and (4) suppression of nonspecific interference. When exposed to a nonuniform electrical field, DNA or RNA is rapidly released in situ. The specificity of each mutation is expected to yield a unique electric field profile in terms of voltage, cycle numbers, duration, and other parameters. The continuous flapping of the electrical field in EFIRM permits only perfectly matched sequence hybridization; mismatched sequences are removed. By individually optimizing the electric profile for each target sequence of interest, EFIRM achieves sensitivity and specificity that are comparable with those of quantitative PCR, while only requiring a few microliters of the clinical sample.

The mechanism underlying the existence of tumor-specific oncogenic mutations in saliva remains unclear. Our study showed that EGFR DNA was the major contributor to the EFIRM signal in saliva when readouts after treatments with DNase and RNase on the saliva were compared (data not shown). We also demonstrated that the amperometric currents of the EFIRM signals from plasma were highly correlated with those from saliva using the probe designed for p.E746-A750del and p.L858R. These results suggest that the amount of EGFR mutant DNA in saliva and plasma are correlated. This implies that the EGFR mutant DNA in saliva may come from plasma. However, the mechanisms by which lung cancer cells release the EGFR mutant DNA and disperse it via the blood to distant sites remain to be established.

Current Clinical Practices for Detecting EGFR Mutations in Patients with NSCLC

The most common method for detecting EGFR mutations is direct sequencing of

samples). We first demonstrated that EFIRM could be used to detect specific EGFR mutations in the plasma of these 33 patients (Figure E1). As in the testing cohort, we found that the amperometric currents of plasma detected by EFIRM correlated with those from saliva (Figure 6B; R = 0.94, P < 0.0001 in the p.E746-A750del groups and R = 0.92, P < 0.0001 in the p.L858R groups).
amplified DNA products. This strategy often takes up to a few days to yield results and is clinically limited by low sensitivity and false negatives or noninformative results, particularly for cytology specimens. Several new techniques, including the use of TaqMan PCR and denaturing high-performance liquid chromatography have been introduced (31–33), but none have been adopted as a standard clinical method for detecting EGFR mutations.

In addition, practical clinical obstacles often exist pertaining to the acquisition and availability of appropriate tissue samples and intratumoral genetic heterogeneity. In patients with advanced NSCLC, tumor tissue is not always available for EGFR mutation testing, either because only small amounts of tissue are collected or because the collected tissues have very low, or no, detectable amounts of tumor, especially from cancer screening (36). For the person collecting the sample, saliva poses minimal risk for contracting infectious diseases such as hepatitis C virus (HCV) and HIV. The method of processing saliva samples for EFIRM analysis is less complicated than those for extracting DNA or isolating circulating tumor cells from blood. Finally, the molecular methods and equipment for detecting circulating tumor DNA in plasma are complicated, technique-dependent, and not readily available. The advantages of EFIRM will enable clinicians to adjust their therapeutic strategies in a timely fashion, consequently improving the clinical outcome of EGFR-targeted therapy. Because EFIRM is based on an electrochemical platform, it can be transformed into high-throughput oncogenic mutation analysis lab assays for rapidly identifying oncogenic mutations. We have already developed a portable device that uses saliva samples for cancer detection based on SABER.

**Potential Limitations of the Study**

Our study focused on using EFIRM to detect EGFR L858R and exon 19 deletion but did not include other uncommon EGFR-TKI sensitive mutations that can be detected by reverse-transcription PCR kits such as the EGFR RGQ PCR kit (Qiagen, Valencia, CA), as described previously (37). This limitation restricted our comparison of EFIRM to current tissue biopsy, CTCs, or DNA for detecting EGFR mutations in predicting response to EGFR-TKI. A prospective study with probes specifically designed for these uncommon mutations is warranted to determine whether EFIRM is valuable in predicting treatment response to EGFR-TKI. Second, although we had as many cases in our study as other studies using CTCs (38, 39) or plasma DNA (9, 40, 41), this proof of principle study was not powered to decide the threshold. There were overlapping current values of the EGFR wild type and mutation type, especially at exon 21 L858R, which may have led to false-negative results. The possible reasons for the false negatives might include inadequate sensitivity of EFIRM or differences between circulating bodily fluids and localized tissue biopsy. A large prospective study will be necessary to determine the adequate and optimal...
Figure 6. Correlation of epidermal growth factor receptor (EGFR) mutation statuses between plasma and saliva using electric field–induced release and measurement (EFIRM). The scattergram shows the correlation and linear regression between amperometric currents recorded using plasma and saliva. Each dot represents data for one patient in (A) the testing group and (B) the blinded group.

estimate of a threshold to decide the balance between false-positive and false-negative rates. Nevertheless, we think the high AUCs of 0.94 and 0.96 in Figure 5C are very promising, as few diagnostic tests can reach that level of agreement with tissue biopsy results. Therefore, SABER can be combined with tissue DNA testing to enhance clinical confidence, or can be used to complement the biopsy of primary lesions as a source of EGFR mutation detection.

Author disclosures are available with the text of this article at www.atsjournals.org.


