Title

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

Journal

American Journal of Respiratory and Critical Care Medicine, 190(10)

ISSN

1073-449X

Authors

Wei, Fang

Lin, Chien-Chung

Joon, Aron

et al.

Publication Date

2014-11-15

DOI

10.1164/rccm.201406-1003oc

Peer reviewed

ORIGINAL ARTICLE

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

Fang Wei[1]*, Chien-Chung Lin[2,3]*, Aron Joon[4], Ziding Feng[4], Gabriel Troche[5], Maruja E. Lira[5], David Chia[6], Mao Mao[5], Chung-Liang Ho[7], Wu-Chou Su[2,3], and David T. W. Wong[1]

[1]School of Dentistry and [6]Department of Pathology, University of California, Los Angeles, California; [2]Institute of Clinical Medicine, [3]Department of Internal Medicine, and [7]Department of Pathology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan; [4]Section of Early Cancer Detection and Biomarkers, The University of Texas, MD Anderson Cancer Center, Houston, Texas; and [5]Oncology Research Unit, Pfizer, Inc., San Diego, California

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

(Received in original form June 2, 2014; accepted in final form October 10, 2014)

*These authors contributed equally to this study.

Supported by Faculty Seed Grants from the UCLA School of Dentistry grant 441901-69749 (F.W.), the Taiwan Department of Health Clinical Trial and Research Center of Excellence grant DOH102-TD-C-111-003 (C.-C.L. and W.-C.S), and the Taiwan Department of Health Cancer Research Center of Excellence grant MOHW103-TD-B-111-06 (C.-C.L. and W.-C.S).

Author Contributions: F.W., C.-C.L., D.T.W.W., W.-C.S., and Z.F. wrote and edited the manuscript. F.W., C.-C.L., D.T.W.W., and W.-C.S. conceptualized the overall strategy and developed the clinical translation and implementation. C.-C.L. and W.-C.S. designed the clinical protocol, enrolled patients to the protocol, and managed the patients with lung cancer. F.W. and D.T.W.W. designed the EFIRM platform. G.T., M.E.L., and M.M. designed and performed the *in vitro* and *in vivo* testing. C.-L.H. designed and performed the *EGFR* mutation test in tissue. D.C. designed the clinical study and oversaw the statistical analysis of the clinical study. A.J. and Z.F. designed and performed the statistical analyses. W.-C.S. and D.T.W.W. were the study's principal investigators.

Correspondence and requests for reprints should be addressed to David T. W. Wong, D.M.D., D.M.Sc., UCLA School of Dentistry, UCLA Dental Research Institute, 73-042 Center for Health Sciences, 10833 Le Conte Avenue, University of California, Los Angeles, CA 90095-1668. E-mail: dtww@ucla.edu; or Wu-Chou Su, M.D., Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, No. 35, Xiaodong Road, North District, Tainan 704, Taiwan. E-mail: sunnysu@mail.ncku.edu.tw

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 190, Iss 10, pp 1117–1126, Nov 15, 2014

Originally Published in Press as DOI: 10.1164/rccm.201406-1003OC on October 15, 2014
Internet address: www.atsjournals.org

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) (13), 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 × *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 × *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 GGC 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

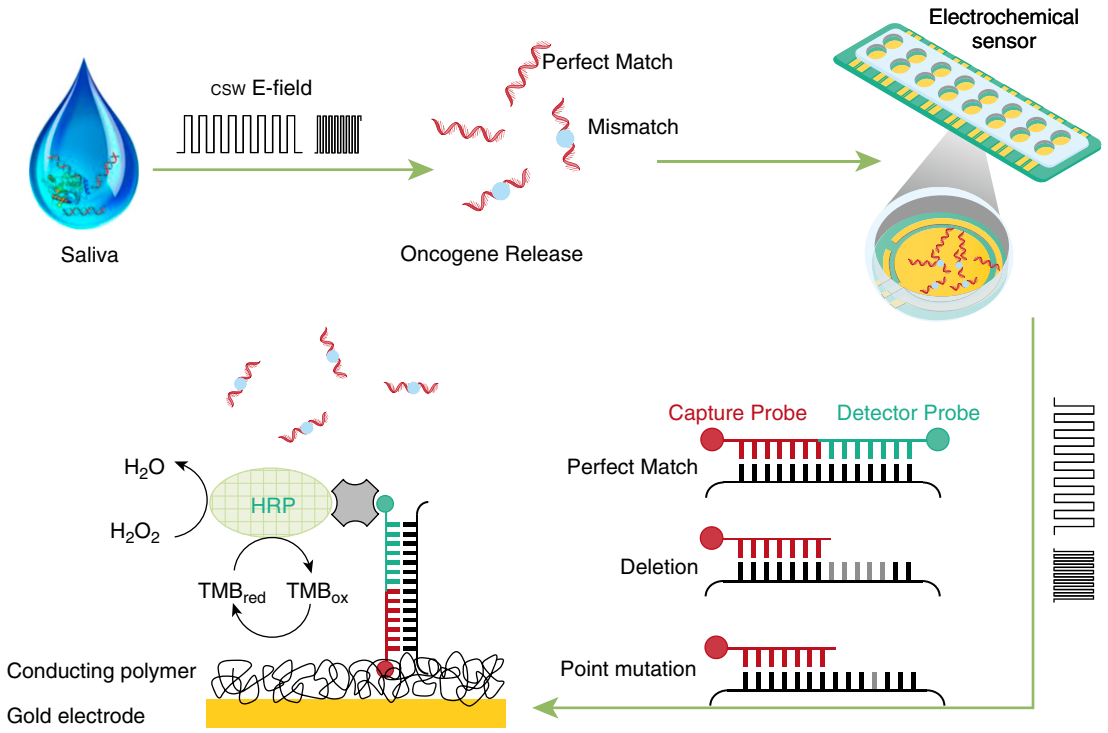

Figure 1. Electric field–induced release and measurement (EFIRM) technology for the detection of epidermal growth factor receptor (EGFR) mutations in bodily fluids of patients with lung cancer. The cyclic-square wave of the electrical field (csw E-field) was applied to release and detect the *EGFR* mutations. *EGFR* sequences were measured on the electrochemical sensor with a capture probe precoated in conducting polymer. The horseradish peroxidase (HRP)-labeled reporter probe generated amperometric signals when there was a reaction with the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate under a −200 mV electrical field.

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 in the presence of wild-type DNA. For the p.L858R point mutation, as little as 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 mm^3), (*3*) medium tumors (500–900 mm^3), and (*4*) large tumors (>900 mm^3). Mice xenografted with the HCT 116 cell line, which carried the *EGFR*

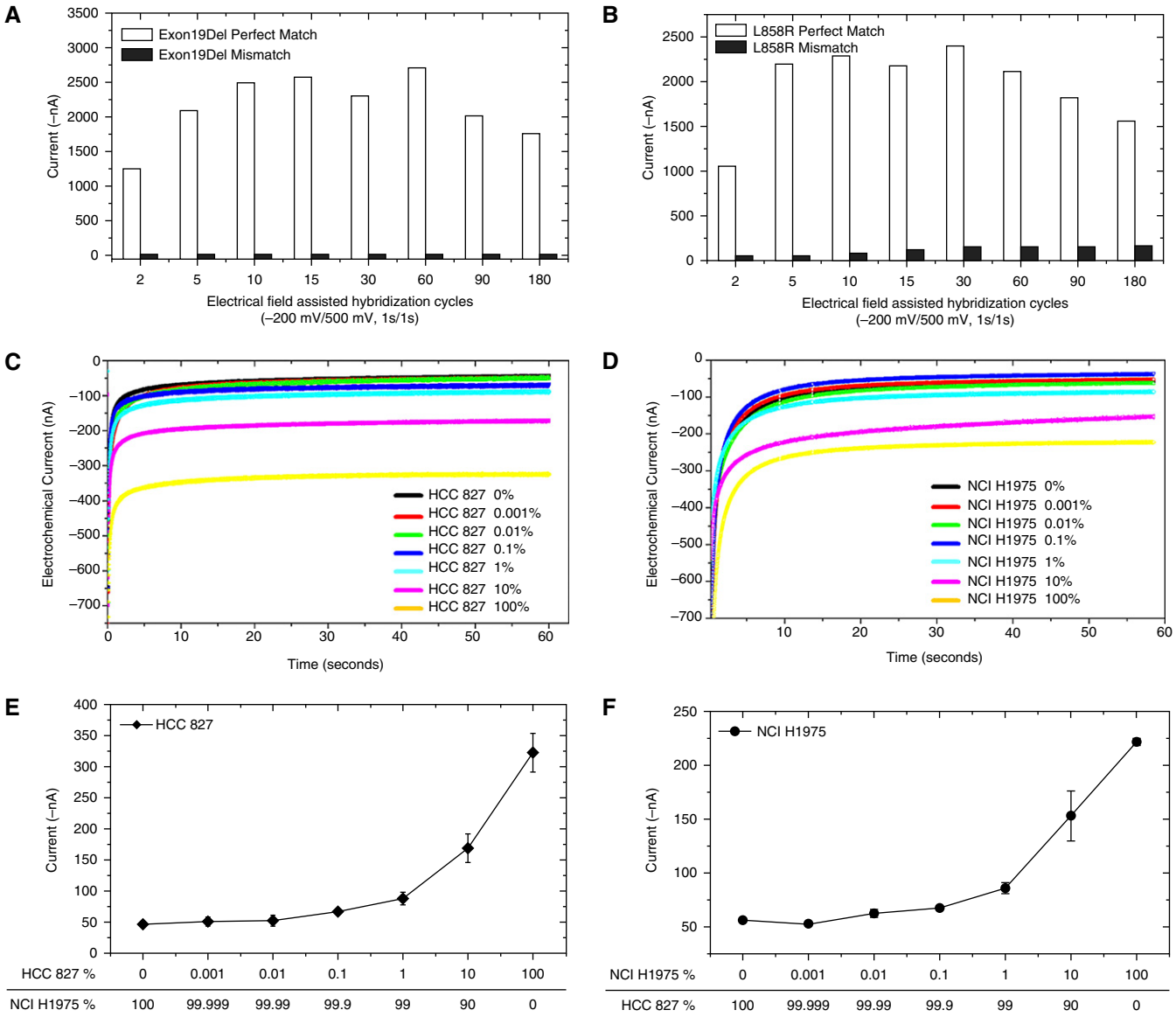

Figure 2. *In vitro* optimization of electric field–induced release and measurement (EFIRM) for specific human epidermal growth factor receptor (EGFR) mutation detection. Cycle numbers for the application of EFIRM for the detection of oligos carrying the (*A*) exon 19 deletion and the (*B*) L858R mutation. Targeting oligos were dissolved in Tris-HCl buffer at a final concentration of 1 nM. Wild-type sequences were used as mismatch sequences. The tyrosine kinase inhibitor–sensitive *EGFR* mutations, including the exon 19 deletion (from HCC827 cells) and L858R (from NCI-H1975 cells), were assayed by decreasing the ratio of targeted oncogene sequence to other sequences. Electrochemical current readouts are listed in amperometric signals for (*C*) the exon 19 deletion and (*D*) L858R. Reactions were performed in triplicate using 20 ng of input DNA. Means and SDs from triplicate experiments are provided for (*E*) the exon 19 deletion and (*F*) L858R.

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.

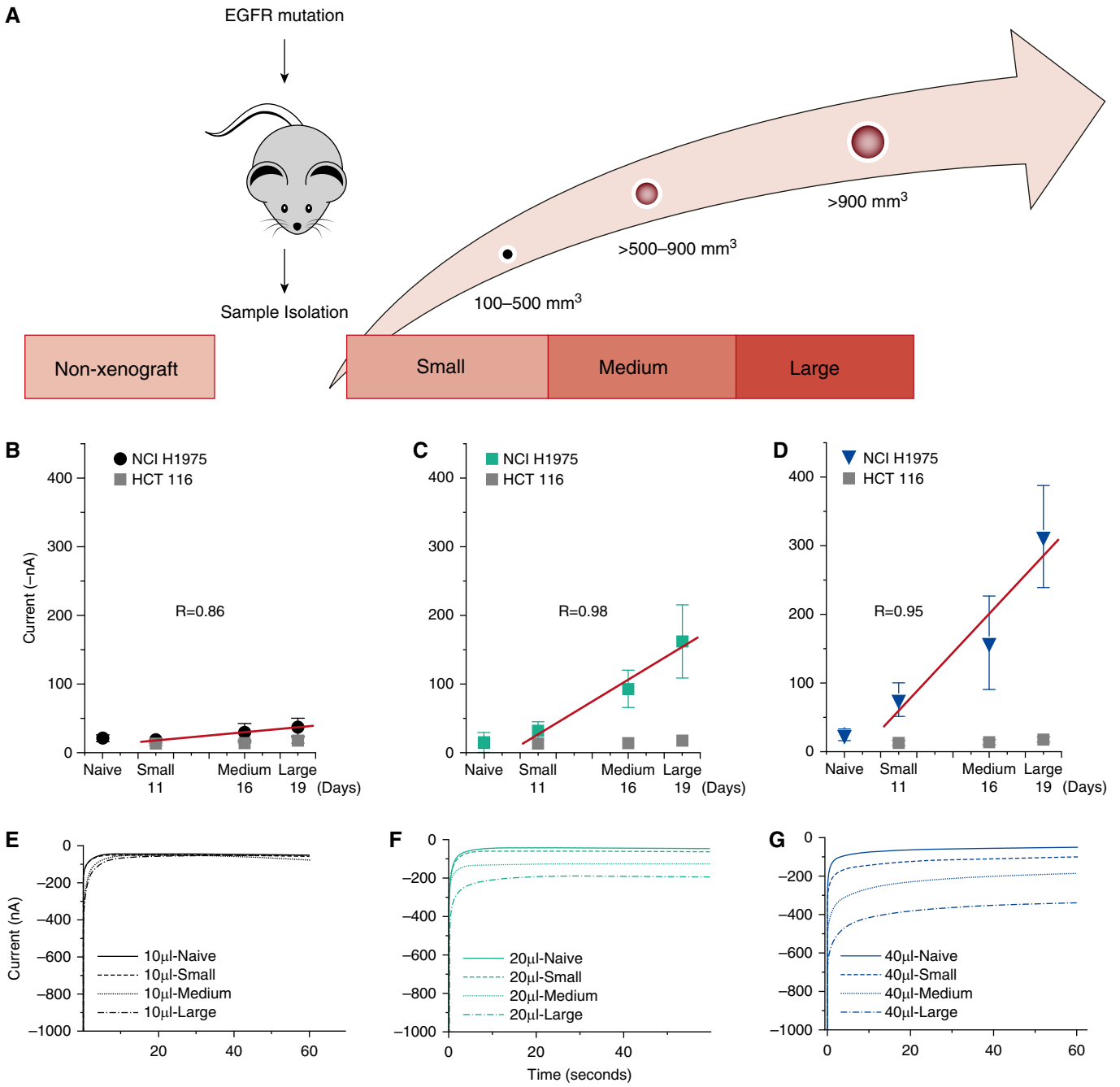

Figure 3. Detection of the epidermal growth factor receptor (EGFR) L858R point mutation in xenografted lung cancer mice via electric field–induced release and measurement (EFIRM). (*A*) Design of the tumor burden study using *EGFR* L858R xenografted mice. EFRIM of the four groups of mice (three mice 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.

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,

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 and saliva specimens were procured before the 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			
Total	22	40	1.00†
Male	12 (54.5)	22 (55.0)	
Female	10 (45.5)	18 (45.0)	
Smoker	7 (31.8)	11 (27.5)	0.95†
Stage			1.00‡
III	5 (22.7)	8 (20.0)	
IV	17 (77.3)	32 (80.0)	
EGFR mutant type			0.98†
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 (%).
*t test.
†Chi-square test.
‡Fisher test.

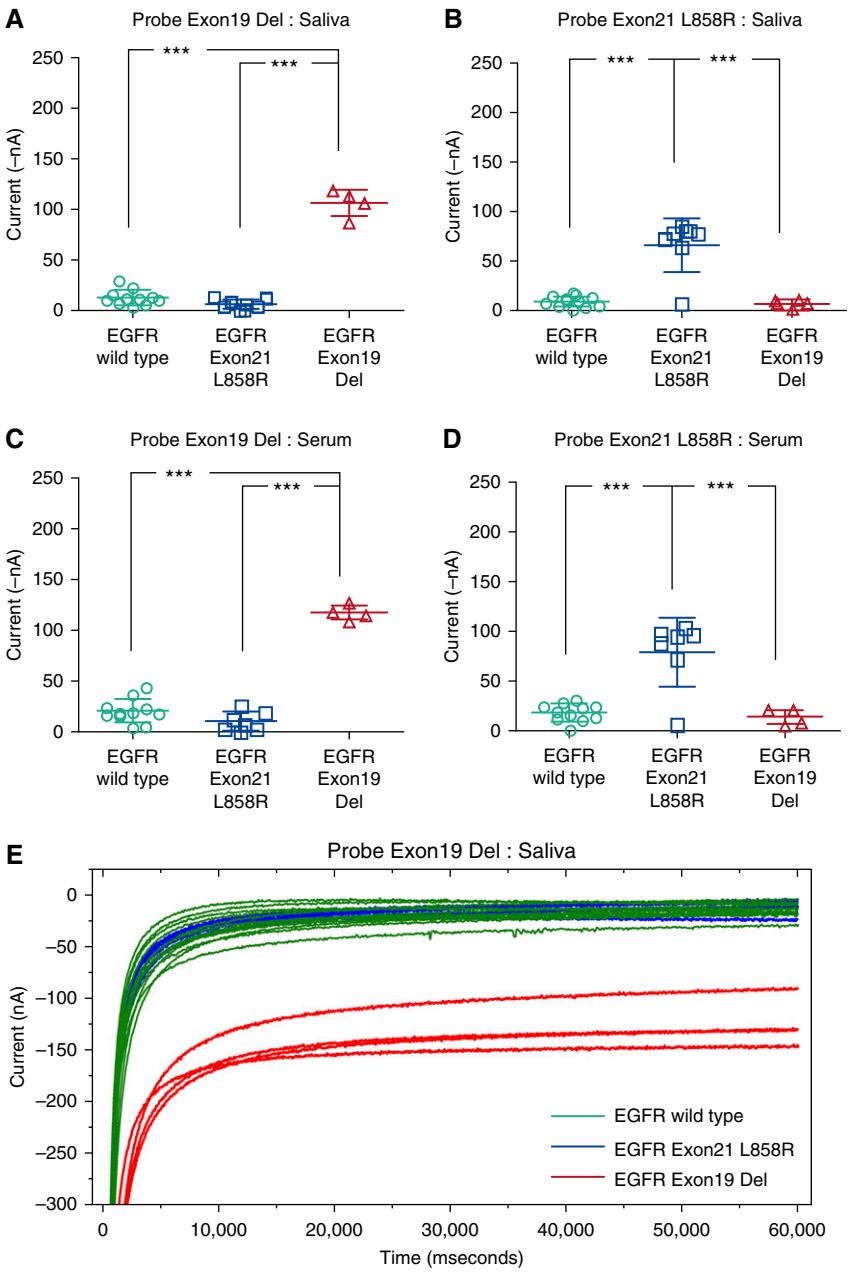

Figure 4. Detection of epidermal growth factor receptor (EGFR) mutations by electric field–induced release and measurement (EFIRM) in plasma and saliva from patients with non-small cell lung cancer. We compared EFIRM with biopsy results to directly determine the *EGFR* mutation type in the plasma and saliva of patients with lung cancer. Corresponding probes were applied to the electrochemical sensor to detect the specific mutation type. (*A*) The probe for exon 19 deletion in saliva; (*B*) the probe for L858R in saliva; (*C*) the probe for exon 19 deletion in plasma; and (*D*) the probe for L858R in plasma. (***$P < 0.0001$, one-way analysis of variance and Bonferroni *post hoc* test). (*E*) Amperometric current results with the probe for exon 19 deletion in saliva from patients with lung cancer.

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).

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

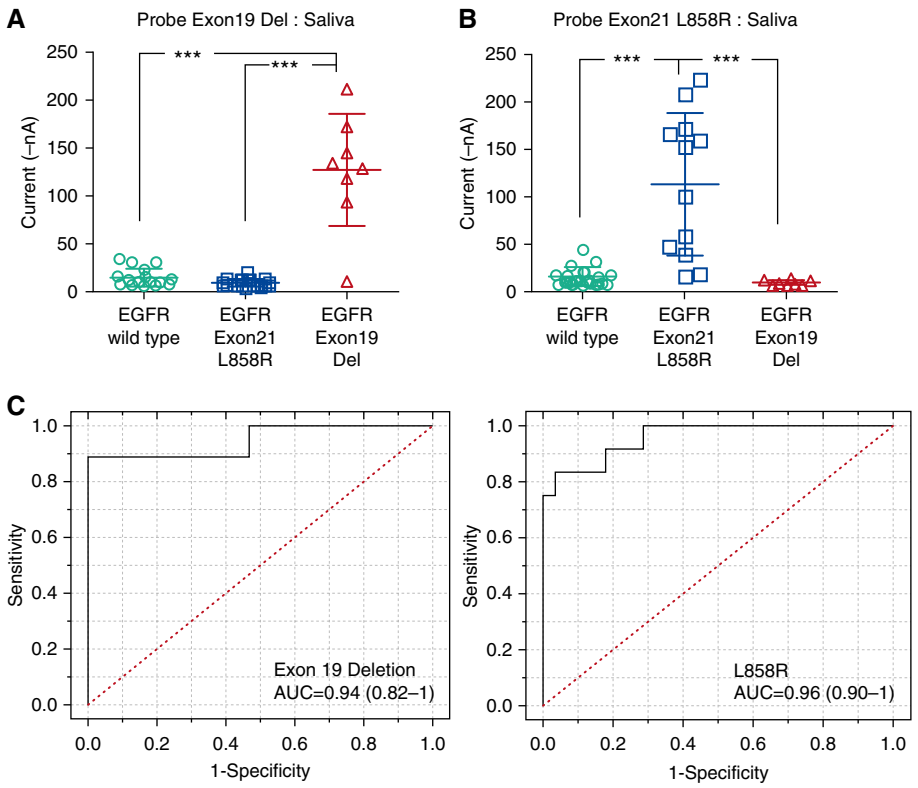

Figure 5. Blinded and randomized clinical electric field–induced release and measurement (EFIRM)-based detection of epidermal growth factor receptor (EGFR) mutations in saliva. We performed EFIRM in duplicate. The absolute values of the signal associated with (*A*) the exon 19 deletion and (*B*) the L858R point mutation according to patient subgroup are presented (***$P < 0.0001$, one-way analysis of variance and Bonferroni *post hoc* test). (*C*) The receiver operating characteristic curves for detecting (left to right) the exon 19 deletion (area under the curve [AUC] = 0.94, 95% confidence interval [CI], 0.82–1) and the L858R mutation, respectively (AUC = 0.96, 95% CI, 0.90–1).

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 computed tomography–guided or bronchoscopic biopsy. Recent evidence has suggested that regionally separated heterogeneous somatic mutational events can lead to sampling bias, which impairs the interpretation of genomics data derived from single-tumor biopsies (34, 35).

Clinical Applications of SABER

We named the saliva-based EFIRM detection of *EGFR* mutation "SABER." Applying SABER directly to detect *EGFR* mutations in the saliva of patients with NSCLC provides information on *EGFR* mutation status in a noninvasive, rapid, and cost-effective way. Saliva offers several benefits for diagnosing *EGFR* mutations compared with blood-based platforms such as CTCs and circulating free tumor DNA. Saliva collection is noninvasive and stress-free for patients and therefore is a more suitable biofluid than plasma for 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

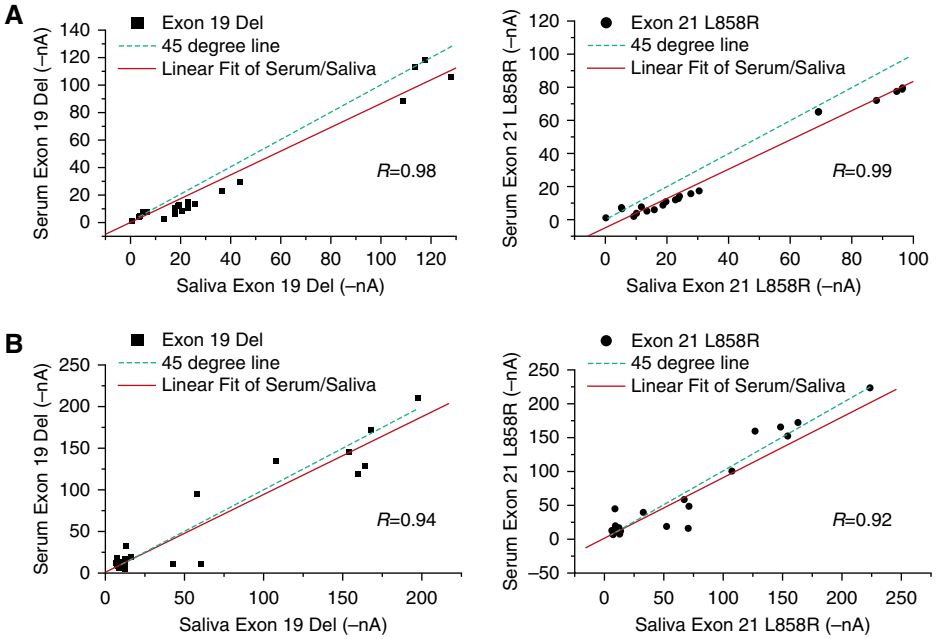

Figure 6. Correlation of epidermal growth factor receptor (EGFR) mutation statuses between plasma and saliva using electric field–induced release and measurement (EFIRM). The *scattergram* shows the correlation and linear regression between amperometric currents recorded using plasma and saliva. Each *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.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64:9–29.
2. Targeting resistance in lung cancer. *Cancer Discov* 2013;3:OF9.
3. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, *et al*. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
4. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, *et al*. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–1500.
5. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, *et al*. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–957.
6. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol* 2014;32:579–586.
7. Bidard FC, Weigelt B, Reis-Filho JS. Going with the flow: from circulating tumor cells to DNA. *Sci Transl Med* 2013;5:207ps214.
8. Wicha MS, Hayes DF. Circulating tumor cells: not all detected cells are bad and not all bad cells are detected. *J Clin Oncol* 2011;29:1508–1511.
9. Kimura H, Kasahara K, Kawaishi M, Kunitoh H, Tamura T, Holloway B, Nishio K. Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer. *Clin Cancer Res* 2006;12:3915–3921.
10. Kimura H, Suminoe M, Kasahara K, Sone T, Araya T, Tamori S, Koizumi F, Nishio K, Miyamoto K, Fujimura M, *et al*. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). *Br J Cancer* 2007;97:778–784.
11. Bai H, Mao L, Wang HS, Zhao J, Yang L, An TT, Wang X, Duan CJ, Wu NM, Guo ZQ, *et al*. Epidermal growth factor receptor mutations in plasma DNA samples predict tumor response in Chinese patients with stages IIIB to IV non-small-cell lung cancer. *J Clin Oncol* 2009;27:2653–2659.
12. Zhang H, Liu D, Li S, Zheng Y, Yang X, Li X, Zhang Q, Qin N, Lu J, Ren-Heidenreich L, *et al*. Comparison of EGFR signaling pathway somatic DNA mutations derived from peripheral blood and corresponding tumor tissue of patients with advanced non-small-cell lung cancer using liquidchip technology. *J Mol Diagn* 2013;15:819–826.
13. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008;10:1470–1476.
14. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, Wong DT. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin Cancer Res* 2009;15:5473–5477.
15. Zhang L, Farrell JJ, Zhou H, Elashoff D, Akin D, Park NH, Chia D, Wong DT. Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology* 2010;138:949–957.e1–7.

16. Lau C, Kim Y, Chia D, Spielmann N, Eibl G, Elashoff D, Wei F, Lin YL, Moro A, Grogan T, *et al*. Role of pancreatic cancer-derived exosomes in salivary biomarker development. *J Biol Chem* 2013; 288:26888–26897.
17. Lee YH, Kim JH, Zhou H, Kim BW, Wong DT. Salivary transcriptomic biomarkers for detection of ovarian cancer: for serous papillary adenocarcinoma. *J Mol Med (Berl)* 2012;90:427–434.
18. Xiao H, Zhang L, Zhou H, Lee JM, Garon EB, Wong DT. Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry. *Mol Cell Proteomics* 2012;11:M111.012112.
19. Zhang L, Xiao H, Karlan S, Zhou H, Gross J, Elashoff D, Akin D, Yan X, Chia D, Karlan B, *et al*. Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PLoS ONE* 2010;5:e15573.
20. Sequist LV, Bell DW, Lynch TJ, Haber DA. Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. *J Clin Oncol* 2007;25:587–595.
21. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 2007;7:169–181.
22. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, Eisele D, Abemayor E, Elashoff D, Park NH, *et al*. Salivary transcriptome diagnostics for oral cancer detection. *Clin Cancer Res* 2004;10: 8442–8450.
23. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988;44:837–845.
24. Spindler KL, Pallisgaard N, Vogelius I, Jakobsen A. Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. *Clin Cancer Res* 2012;18:1177–1185.
25. Thierry AR, Mouliere F, Gongora C, Ollier J, Robert B, Ychou M, Del Rio M, Molina F. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. *Nucleic Acids Res* 2010;38: 6159–6175.
26. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, *et al*. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* 2004;91:355–358.
27. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 2008;10: 619–624.
28. Lau CS, Wong DT. Breast cancer exosome-like microvesicles and salivary gland cells interplay alters salivary gland cell-derived exosome-like microvesicles in vitro. *PLoS ONE* 2012;7:e33037.
29. Huang YS, Yang JJ, Zhang XC, Yang XN, Huang YJ, Xu CR, Zhou Q, Wang Z, Su J, Wu YL. Impact of smoking status and pathologic type on epidermal growth factor receptor mutations in lung cancer. *Chin Med J (Engl)* 2011;124:2457–2460.
30. Ho HL, Chang FP, Ma HH, Liao LR, Chuang YT, Chang-Chien YC, Lin KY, Chou TY. Molecular diagnostic algorithm for epidermal growth factor receptor mutation detection in Asian lung adenocarcinomas: comprehensive analyses of 445 Taiwanese patients with immunohistochemistry, PCR-direct sequencing and Scorpion/ARMS methods. *Respirology* 2013;18:1261–1270.
31. Jänne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H, Lindeman N, Lee JC, Halmos B, Maher EA, *et al*. A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer Res* 2006;12:751–758.
32. Chin TM, Anuar D, Soo R, Salto-Tellez M, Li WQ, Ahmad B, Lee SC, Goh BC, Kawakami K, Segal A, *et al*. Detection of epidermal growth factor receptor variations by partially denaturing HPLC. *Clin Chem* 2007;53:62–70.
33. Endo K, Konishi A, Sasaki H, Takada M, Tanaka H, Okumura M, Kawahara M, Sugiura H, Kuwabara Y, Fukai I, *et al*. Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast TaqMan PCR assay. *Lung Cancer* 2005;50: 375–384.
34. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, *et al*. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* 2012;366:883–892.
35. Yap TA, Gerlinger M, Futreal PA, Pusztai L, Swanton C. Intratumor heterogeneity: seeing the wood for the trees. *Sci Transl Med* 2012;4: 127ps10.
36. Bonne NJ, Wong DT. Salivary biomarker development using genomic, proteomic and metabolomic approaches. *Genome Med* 2012;4:82.
37. Wu JY, Yu CJ, Yang CH, Wu SG, Chiu YH, Gow CH, Chang YC, Hsu YC, Wei PF, Shih JY, *et al*. First- or second-line therapy with gefitinib produces equal survival in non-small cell lung cancer. *Am J Respir Crit Care Med* 2008;178:847–853.
38. Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, *et al*. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;359:366–377.
39. Taniguchi K, Uchida J, Nishino K, Kumagai T, Okuyama T, Okami J, Higashiyama M, Kodama K, Imamura F, Kato K. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. *Clin Cancer Res* 2011;17:7808–7815.
40. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, Luke JJ, Butaney M, Kirschmeier P, Jackman DM, *et al*. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res* 2014;20:1698–1705.
41. Yung TK, Chan KC, Mok TS, Tong J, To KF, Lo YM. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. *Clin Cancer Res* 2009;15:2076–2084.