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Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: A pilot study

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Abstract

Background: This article describes a pilot study evaluating a novel liquid biopsy system for non-small cell lung cancer (NSCLC) patients. The electric field-induced release and measurement (EFIRM) method utilizes an electrochemical biosensor for detecting oncogenic mutations in biofluids.

Methods: Saliva and plasma of 17 patients were collected from three cancer centers prior to and after surgical resection. The EFIRM method was then applied to the collected samples to assay for exon 19 deletion and p.L858 mutations. EFIRM results were compared with cobas results of exon 19 deletion and p.L858 mutation detection in cancer tissues.

Results: The EFIRM method was found to detect exon 19 deletion with an area under the curve (AUC) of 1.0 in both saliva and plasma samples in lung cancer patients. For L858R mutation detection, the AUC of saliva was 1.0, while the AUC of plasma was 0.98. Strong correlations were also found between presurgery and post-surgery samples for both saliva (0.86 for exon 19 and 0.98 for L858R) and plasma (0.73 for exon 19 and 0.94 for L858R).

Conclusion: Our study demonstrates the feasibility of utilizing EFIRM to rapidly, non-invasively, and conveniently detect epidermal growth factor receptor mutations in the saliva of patients with NSCLC, with results corresponding perfectly with the results of cobas tissue genotyping.

Introduction

Lung cancer is the most common cancer and leading cause of cancer death in China and worldwide.1–8 Non-small cell lung cancer (NSCLC) constitutes 80% of all lung cancer cases, and is typically diagnosed at an advanced stage when survival rates are low.9–15 The discovery of a relationship between epidermal growth factor receptor (EGFR)-activating mutations and EGFR-tyrosine kinase inhibitors (TKI) has taken the treatment of patients with EGFR mutations into an era of precision medicine.16–20 The 2009
Iressa Pan-Asia Study (IPASS) demonstrated that EGFR-TKIs are superior to traditional chemotherapeutic agents for patients with EGFR mutant lung cancer, and the current clinical EGFR-TKIs have become a standard first-line treatment for patients with non-squamous NSCLC carrying EGFR mutations. Genotyping for EGFR mutations in advanced non-squamous NSCLC patients is highly recommended in current clinical practice.

Epidermal growth factor receptor testing for mutations is traditionally performed on tissues acquired by surgery or biopsy. However, the majority of these diagnoses are on late stage patients in poor physical condition. In these instances, the performance of biopsy or surgery is often impractical because of poor patient health and the risk of additional clinical complications. Moreover, a high proportion of NSCLC patients will develop EGFR-TKI treatment resistance, and performing additional surgeries or biopsies to monitor for an additional change in EGFR mutation status would further increase the risk to patient health. As a result of these limitations, explorations have been made into the feasibility of detecting actionable EGFR oncogenic mutations in biofluids, such as serum or plasma.

Recent emerging work from the University of California, Los Angeles (UCLA) has demonstrated the feasibility of performing saliva-based EGFR mutation detection (SABER) based on a core technology called electric field-induced release and measurement (EFIRM). This method is an electrochemical method based on immobilized nucleic acid probes for capturing mutated sequences and applying electric fields to facilitate the hybridization process. Because of the speed and simplicity of the method, EFIRM has the potential to be a suitable tool for oncogenic mutation monitoring in a clinical context. Recently, a randomized, blind study using SABER for NSCLC patients demonstrated high concordance with tumor tissue based genotyping. Here, we report the first clinical validation pilot study of EFIRM in Mainland China.

Methods

Patients and clinical specimens

Inpatients with suspicious lesions for lung cancer, treated at three large-scale major hospitals in China, West China Hospital, Henan Cancer Hospital, and Jiangsu Cancer Hospital, were recruited as subjects for our study. Each study participant had 5 mL saliva and 10 mL blood collected twice on the day before surgery, the day after surgery, and approximately seven days after surgery. Blood plasma samples were collected by drawing blood into ethylenediaminetetraacetic acid tubes, centrifuging at 2500g for 10 minutes at −4°C, and then collecting the upper plasma layer. Samples were stored at −80°C until analysis. As per previous studies in salivary biomarkers, saliva samples were kept on ice during collection from patients and then centrifuged at 2600g for 15 minutes at 4°C. The supernatant was removed from the pellet, treated with RNase inhibitor (Superase-In, Ambion Inc., Austin, TX, USA), and stored at −80°C for future analyses. All subjects provided informed consent.

Saliva-based epidermal growth factor receptor (EGFR) mutation detection in bodily fluids

Epidermal growth factor receptor mutations in serum and saliva were detected by EFIRM in blinded samples by trained laboratory personnel. Each plasma and saliva sample was measured in duplicate. Paired probes (capture and detector; TsingKe, Beijing, China) specific to the two TKI-sensitive mutations were used: for the exon 19 deletion (19 del), a capture probe 5'-TGT TGC TTC CTTGAT AGC GAC G-3' and a detector probe 5'-GGA ATT TTA ACT TTC TCA CCT FITC-3'; for the L858R point mutation, a capture probe: 5'-CAG TTT TGT GCC CCC CCC AAA ATC-3'and detector probe: 5'-TTG ACA TGC TGC GGT GTT TTC A-FITC-3'. The detector probes were labeled with fluorescein isothiocyanate. The EFIRM detection method involves four primary steps. First, copolymerization of capture probes with pyrrole onto the bare gold electrodes by applying a cyclic square wave electric. Second, hybridization of the samples with detector and capture probes. Third, the combination of anti-fluorescein antibody conjugated to horseradish peroxidase (1:1000 dilution; Roche, Indianapolis, IN, USA). Fourth, chromogenesis by 3,3',5,5'-tetramethylbenzidine substrate for horseradish peroxidase and measurement of the amperometric signal. The total detection time of the protocol was less than 10 minutes, and the procedure required 20 to 40 μL of the biological sample.

EGFR mutation detection in tumor tissues

Epidermal growth factor receptor mutations in tumor tissues were detected by cobas assay, which is an allele-specific real-time polymerase chain reaction (PCR) system that qualitatively measures the amplification of DNA to detect EGFR gene mutation from DNA derived from freshly frozen tissues. DNA from these tissue specimens was extracted according to the standard procedure delineated in the cobas DNA Sample Preparation Kit (Roche Molecular Systems Inc., Pleasanton, CA, USA). This processed involved first incubating the sample with protease and buffer ATL at 56°C until dissolution. Buffer AL was then added for an additional 10 minutes at 70°C. Next, DNA samples were collected by the DNeasy Mini spin column (Qiagen, Valencia, CA, USA). After DNA samples were collected, the amount of DNA was analyzed by spectrophotometer and
adjusted to a concentration of 2 ng/μL. Finally, the prepared DNA was amplified and detected by cobas 4800 (Roche Molecular Systems Inc.) following the instructions for the cobas EGFR mutation test. The results were automatically reported in cobas 4800 software.

**Statistical analysis**

Receiver operating characteristic curves (ROC) and the area under the curve (AUC) with 95% confidence intervals (CI) were calculated to access the ability of EFIRM in detecting EGFR mutations. Correlation coefficients were calculated using Pearson parametric methods. All analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

**Results**

**The clinical characteristics of patients**

A total of 17 randomized patients with adenocarcinoma with EGFR mutation from three different cancer centers in China (10 from West China Hospital, 4 from Henan Cancer Hospital, and 3 from Jiangsu Cancer Hospital) were enrolled. The clinical characteristics of these patients, including gender, age, smoking status, cancer stage, and pathological type, are presented in Table 1.

**Table 1 The clinical characteristics of patients in the study**

| Patient No. | Gender | Age | Smoking status | Histologic type | Stage† | Cobas | Saliva | Plasma |
|-------------|--------|-----|----------------|-----------------|--------|-------|--------|--------|
| 1           | M      | 76  | N              | NSCLC           | No     | No    | No     | No     |
| 2           | M      | 53  | Y              | Adeno           | pT2aN1M0, IIA | Ex19 del | No     | No     | No     |
| 3           | M      | 53  | Y              | Squa            | pT2aN1M1a, IV | Ex19 del | No     | No     | No     |
| 4           | M      | 53  | Y              | Adeno           | pT2aN2M0, IIIA | Ex19 del | No     | No     | No     |
| 5           | M      | 65  | Y              | Squa            | pT3N0M0, IIB | No     | No     | No     | No     |
| 6           | F      | 72  | N              | NSCLC           | pT3N0M0, IIB | G719X   | No     | No     | No     |
| 7           | M      | 71  | Y              | Adeno           | pT2aN0M0, IIIB | L858R | L858R | L858R | L858R |
| 8           | M      | 46  | Y              | Adeno           | pT3N3M0, IIIIB | No     | No     | No     | No     |
| 9           | M      | 57  | Y              | Squa            | pT4N0M0, IIIA | No     | No     | No     | No     |
| 10          | F      | 61  | N              | Adeno           | pT2aN2M0, IIIA | Ex19 del | NE     | Ex19 del | NE     |
| 11          | M      | 47  | Y              | Adeno           | pT2aN2M0, IIIA | No     | No     | No     | No     |
| 12          | M      | 62  | Y              | Squa            | pT2aN0M0, IB | No     | No     | No     | No     |
| 13          | F      | 51  | N              | Adeno           | pT2aN0M0, IIB | L858R | L858R | L858R | L858R |
| 14          | M      | 71  | N              | Squa            | pT1aN1M0, IIA | No     | No     | No     | No     |
| 15          | M      | 50  | N              | Adeno           | pT2aN2M0, IIIA | No     | No     | No     | No     |
| 16          | M      | 60  | Y              | Squa            | pT2aN0M0, IB | No     | No     | No     | No     |
| 17          | M      | 51  | N              | Adeno           | pT2aN2M0, IIIA | L858R | L858R | L858R | L858R |

†Stage was based on National Comprehensive Cancer Network guidelines 2014, V3. Adeno, adenocarcinoma; EGFR, epidermal growth factor receptor; EFIRM, electric field-induced release and measurement; F, female; M, male; NE, no samples; NSCLC, non-small cell lung cancer; Squa, squamous lung cancer.
48.9 ± 8.0 nA in the L858R mutant and 51.1 ± 9.5 nA in the wild-type; \( P = 0.001 \) and four L858R mutations (Fig 4a, 111.4 ± 27.8 nA in the L858R mutant group vs. 46.2 ± 0.7 nA in the exon 19 del group and 51.9 ± 11.8 nA in the wild-type; \( P < 0.001 \) before surgery. Similar results were obtained from plasma collected after surgery (Fig 3b, 92.6 ± 18.8 nA in the exon 19 del group vs. 52.9 ± 6.1 nA in the L858R mutant and 51.8 ± 5.4 nA in the wild-type; \( P < 0.001 \); Fig 4b, 108.9 ± 22.2 in the L858R mutant group vs. 45.9 ± 1.7 nA in the exon 19 del and 52.4 ± 13.6 nA in the wild-type; \( P < 0.001 \)). The AUCs are shown in Fig 4c, respectively.

Cobas detection of EGFR mutations in tumor tissues

As a result of the approval of cobas for clinical use by the United States Food and Drug Administration and the China Food and Drug Administration, cobas assay is used as the gold standard method of EGFR mutation detection. We detected 17 tissue samples and found six samples harboring EGFR mutation (35.3%; 3 exon 19 del and 3 L858R mutations). We compared the results from EFIRM assay using saliva and plasma with the cobas assay using tumor tissues to evaluate the sensitivity and specificity of EFIRM assay in detecting EGFR mutation. The saliva and plasma samples of three patients that were identified to harbor EGFR exon 19 del with EFIRM were confirmed by cobas assay. Furthermore, these three patients with EGFR L858R mutations also perfectly matched the mutation results for saliva, plasma, and tissue samples. The other 11 patients were EGFR wild-type in both saliva and tissue samples, which meant that there was a single false positive L858R mutation in plasma sample detection by EFIRM. The specificity and sensitivity was 90.9% and 100%, respectively, by plasma. In addition, all of the patients with EGFR mutation were histologically diagnosed with adenocarcinoma (3 stage III, 2 stage II, and 1 stage I), which indicated that EFIRM assay could maintain a high sensitivity in different cancer stages.
The correlation of EGFR mutation detected before and after surgery

We compared the results from the saliva and plasma collected before and after surgery to evaluate whether surgery affected EGFR mutation status. The strength of the amperometric currents in the saliva samples collected before surgery correlated with those after surgery using the L858R and exon 19 probes (Table 2, $R = 0.86$ in exon 19 del; $R = 0.98$ in L858R). Similar results were obtained in plasma samples (Table 2, $R = 0.73$ in exon 19 del; $R = 0.94$ in L858R). Additionally, regardless of whether samples were collected before or after surgery, the amperometric currents between saliva and plasma had a strong correlation (Table 2, before surgery: $R = 0.87$ in exon 19 del and $R = 0.67$ in L858R; after surgery: $R = 0.79$ in exon 19 del and $R = 0.70$ in L858R).

Discussion

Liquid biopsy to detect actionable mutations in NSCLC in clinical practice is primarily based on digital droplet PCR (ddPCR) and/or next generation sequencing technologies with performance at ~70–80% concordance with tissue-based genotyping. Technologies that will permit 100% concordance detection (sensitivity) will provide the ultimate complementation to the tumor-specific fingerprint (specificity) for unambiguous detection of the tumor in a non-invasive setting. To our knowledge, this is the first pilot study aimed at evaluating a novel saliva-based EGFR mutation detection system for lung cancer in Mainland China. Our findings clearly show that saliva and plasma assays conducted using the EFIRM method have high concordance with biopsy tissue-based testing for lung cancer EGFR mutation status. EFIRM liquid biopsy is accurate, non-
invasive, and rapid. It is can be used in continuous moni-
toring EGFR mutation status during EGFR-TKI treatment.
We demonstrated that the amperometric currents of the
EFIRM signals from saliva were highly correlated with
those from plasma using the probe designed for p.E746-
A750del and p.L858R, regardless of whether the sample
was collected presurgery or post-surgery (0.70s range).
These results suggest that the amount of EGFR mutant
DNA in saliva and plasma is correlated. This may have a
mechanistic implication, suggesting a link between the
peripheral circulatory system and the salivary glands. Pre-
vious studies of this link have also found similarly strong
correlations.22 However, the precise mechanism by which
lung cancer cells release the EGFR mutant DNA and dis-
perse it via the blood to distal sites remains to be
established.

Another interesting finding in our study was the strong
correlations found between presurgery and post-surgery
saliva assays (0.86 and 0.98) and plasma (0.73 and 0.94).
This data implies that surgery may not influence EGFR
mutant DNA status in saliva or plasma. Saliva collected
after biopsy may result in tumor cells or cell-free DNA that
make their way into the saliva after tissue disruption,
demonstrating that the detection of EGFR mutation in
saliva was not a result of biopsy materials leaking into
saliva.

Although this cohort of tests was only conducted on a
small scale, there were still overlapping current values of
EGFR wild type and mutation type at exon 21 L858R in
plasma, which may have led to false-positive results. Previ-
ous studies have demonstrated a similar phenomenon at
L858R with an unclear threshold between wild type and
mutation type.22 A large prospective study will be con-
ducted to determine the adequate and optimal estimate of
a threshold to decide the balance between false positive
and false negative rates.

Electric field-induced release and measurement can
detect EGFR mutations in liquid biopsy from NSCLC

Figure 3 Blinded and randomized detection of epidermal growth factor receptor (EGFR) mutations by electric field-induced release and measure-
ment assay in plasma collected before surgery from patients with non-small cell lung cancer: (a) the probe for EGFR exon 19 deletion; (b) the probe
for L858R; and (c) receiver operating characteristic curves for detecting EGFR exon 19 deletion (area under the curve (AUC)= 1) and L858R mutations
(AUC = 0.98), respectively.
patients, but at present this work has mainly focused on EGFR L858R and exon 19 del mutations. Because the second T790M EGFR mutation accounts for half of the TKI-resistant cases, monitoring the T790M mutation would be useful to estimate EGFR-TKI resistance. Therefore, development of a new probe designed for EFIRM based on T790M mutation is necessary, and this approach, as with other liquid biopsy-based EGFR mutation assays (such as circulating tumor cells or cell-free circulating tumor DNA), might be a valid noninvasive alternative to biopsy, as well as a better tailoring of individualized precision therapy by monitoring the response to treatment in NSCLC patients.

In conclusion, our results provide evidence that EFIRM detection of EGFR mutation in the saliva of patients with lung cancer is accurate, non-invasive, and rapid.

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Disclosure

David Wong is a co-founder of RNAmeTRIX Inc., a molecular diagnostic company. He holds equity in RNAmeTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property invented by David Wong and patented by the University of California has been licensed to RNAmeTRIX.

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