ORIGINAL ARTICLE

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A Highly Sensitive and Quantitative Test Platform for Detection of NSCLC *EGFR* Mutations in Urine and Plasma

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Received 20 April 2016; accepted 24 May 2016
Available online - 24 July 2016

ABSTRACT

Introduction: In approximately 60% of patients with NSCLC who are receiving EGFR tyrosine kinase inhibitors, resistance develops through the acquisition of *EGFR* T790M mutation. We aimed to demonstrate that a highly sensitive and quantitative next-generation sequencing analysis of *EGFR* mutations from urine and plasma specimens is feasible.

Methods: Short footprint mutation enrichment next-generation sequencing assays were used to interrogate *EGFR* activating mutations and the T790M resistance mutation in urine or plasma specimens from patients enrolled in TIGER-X (NCT01526928), a phase 1/2 clinical study of rociletinib in previously treated patients with *EGFR* mutant–positive advanced NSCLC.

Results: Of 63 patients, 60 had evaluable tissue specimens. When the tissue result was used as a reference, the sensitivity of *EGFR* mutation detection in urine was 72% (34 of 47 specimens) for T790M, 75% (12 of 16) for L858R, and 67% (28 of 42) for exon 19 deletions. With specimens that

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Drs. Reckamp, Melnikova, and Karlovich equally contributed to this work.

Disclosure: Dr. Reckamp reports personal fees from Trovagene and institutional clinical trial support from Clovis during the conduct of the study. Drs. Melnikova, Kosco, Chroucher, Mr. Samuelsz, Drs. Vibat, Geiss, and Erlander are employees of Trovagene. Dr. Karlovich, Ms. Mann, Mr. Rolfe, and Dr. Raponi are employees of Clovis Oncology and hold stock in Clovis Oncology. Dr. Sequist reports institutional trial support from Novartis during the conduct of the study; non-compensated consulting for Boehringer Ingelheim, Clovis Oncology, Novartis, Merrimack Pharmaceuticals, and Taiho; and compensated consulting for AstraZeneca and Ariad. Dr. Camidge reports personal fees from Clovis outside the submitted work. Dr. Wakelee reports personal fees from Peregrine and ACEA; grants from Novartis, Bristol-Myers Squibb, XCovery, Celgene, Roche/Genentech, MedImmune, Gilead, AstraZeneca, and Lilly; and grants and personal fees from Pfizer outside the submitted work. Dr. Perol reports personal fees from Clovis Oncology, Astra-Zeneca, Roche, and Boehringer-Ingelheim outside the submitted work. Dr. Oxnard reports personal fees from AstraZeneca, Clovis, Sysmex, Boehringer-Ingelheim, and Inivata outside the submitted work; in addition, Dr. Oxnard has a patent development and application of NSCLC plasma genotyping using digital polymerase chain reaction pending. Dr. Gadgeel reports personal fees from Roche/Genentech and AstraZeneca outside the submitted work. The remaining author declares no conflict of interest.

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ISSN: 1556-0864

http://dx.doi.org/10.1016/j.jtho.2016.05.035

met a recommended volume of 90 to 100 mL, the sensitivity was 93% (13 of 14 specimens) for T790M, 80% (four of five) for L858R, and 83% (10 of 12) for exon 19 deletions. A comparable sensitivity of *EGFR* mutation detection was observed in plasma: 93% (38 of 41 specimens) for T790M, 100% (17 of 17) for L858R, and 87% (34 of 39) for exon 19 deletions. Together, urine and plasma testing identified 12 additional T790M-positive cases that were either undetectable or inadequate by tissue test. In nine patients monitored while receiving treatment with rociletinib, a rapid decrease in urine T790M levels was observed by day 21.

Conclusions: DNA derived from NSCLC tumors can be detected with high sensitivity in urine and plasma, enabling diagnostic detection and monitoring of therapeutic response from these noninvasive “liquid biopsy” samples.

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Keywords: Urine; Circulating tumor DNA; NSCLC; *EGFR* mutations; T790M

Introduction

A major challenge for assessing *EGFR* mutation status in advanced NSCLC is the availability of suitable biopsy tissue for molecular testing. Clinical studies suggest that 10% to 20% of all NSCLC biopsies are inadequate for molecular analysis because of a lack of either sufficient tumor cells or amplifiable DNA.[1,2] Biopsies also pose an economic burden and health risk to patients, with biopsy-associated patient morbidity (e.g., pneumothorax) observed in 12% to 21% of image-guided transthoracic needle tissue biopsies.[3] Moreover, despite guidelines recommending *EGFR* testing at diagnosis for guiding first-line treatment decisions,[4,5] up to 25% of patients with lung cancer receive treatment before *EGFR* mutation assessment.[6] Physicians cite tumor histologic features (i.e., squamous), insufficient tumor samples, poor health status of the patient, long turnaround times for tests, and patient’s desire to initiate therapy as reasons for failure to undergo timely molecular testing.[6]

In NSCLC patients receiving first-line tyrosine kinase inhibitors (TKIs) targeting *EGFR* mutation–positive tumors (i.e., erlotinib, gefitinib, and afatinib), resistance to therapy develops through the emergence of a second mutation in *EGFR,* T790M, in approximately 60% of cases.[7] Performance of a repeat biopsy of these patients is still an emerging standard of care, and up to 25% of patients may be medically ineligible owing to comorbidities or the lack of an accessible lesion.[8] False-negative results could occur with tissue biopsies, likely because of the underlying intratumoral and intertumoral heterogeneity often associated with resistance mechanisms such as T790M.[9,10]

Detection and monitoring of cancer-specific genomic alterations in blood, specifically through the assessment of circulating tumor DNA (ctDNA), is a minimally invasive alternative to a tissue biopsy that has shown promise in overcoming some of the challenges associated with sampling from tissue.[11] However, ctDNA presents its own challenges for clinical diagnostics. It is highly fragmented, may be very rare (<0.01%) as a proportion of all circulating free nucleic acids in blood, and may be especially difficult to detect in certain cancers such as those localized in the central nervous system.[12,13] Highly sensitive assays have been developed and continue to be improved upon to address these challenges.[13–24]

ctDNA in the systemic circulation is eventually excreted into urine, where it is thought to undergo further degradation.[25–28] Urine analysis is a truly noninvasive alternative to tissue biopsy that integrates DNA from multiple sites, thus potentially addressing the challenges posed by tumor heterogeneity. To date, only a limited number of published studies have examined the feasibility of ctDNA detection from urine.[25–28] Although to our knowledge, none of these studies describe ctDNA detection from the urine of patients with NSCLC, patient-matched tissue and plasma and urine studies in colorectal cancer (*KRAS*) and histiocytic disorders (*BRAF*) indicate good concordance of DNA mutation status across all three biopsy specimens.[29–31]

We report herein, for the first time, the development and characterization of an *EGFR* mutation detection platform that identifies L858R, exon 19 deletions, and T790M in ctDNA from both plasma and urine samples from patients with NSCLC. We evaluated the clinical performance of this platform in matched pretreatment urine and plasma samples and examined the feasibility of longitudinal monitoring of *EGFR* mutations from the urine of patients with NSCLC in the TIGER-X study, which is a phase 1/2 study of the third-generation EGFR-TKI rociletinib (CO-1686).

Patients and Methods

Patients

A blinded, retrospective study was conducted on matched urine and plasma specimens collected from 63 patients with stage IIIB to IV disease who were enrolled in the TIGER-X trial (NCT01526928). Patients in TIGER-X were required to have histologically or cytologically confirmed NSCLC and documented evidence of one or more *EGFR* mutations. All patients signed an ethics committee/institutional review board–approved consent

before any procedures. Further details regarding TIGER-X study design have been previously published.[14,32]

Sample Collection and Processing

Tissue biopsies were collected within 60 days of initiation of treatment with rociletinib. For all formalin-fixed, paraffin-embedded tissue specimens, tumor content was assessed by board-certified pathologists using hematoxylin and eosin–stained slides. Tumor specimens were considered evaluable if any tumor cells were identified. For seven cases, DNA was extracted from one 5-μm section and central laboratory tissue testing was performed with the cobas EGFR Mutation Test (Roche Molecular Systems, Pleasanton, CA). For 55 cases, DNA was extracted from two 5-μm sections and central laboratory tissue testing was performed with the therascreen EGFR RGQ Polymerase Chain Reaction (PCR) Kit (Qiagen, Valencia, CA). A local EGFR test result was used for one case in which tissue was not submitted to the central laboratory.

Blood and urine samples were obtained serially, before administration of the first dose, and with every 21-day cycle of rociletinib treatment. Blood samples were collected in K2 ethylenediaminetetraacetic acid BD Vacutainer tubes (BD Biosciences, San Jose, CA), processed into plasma within 30 minutes (1800 *g* for 10 minutes at 18°C–23°C), and stored at or below –70°C. For plasma DNA analysis, 1.5 to 4 mL of plasma was extracted using the QIAamp DNA Circulating Nucleic Acid Kit (Qiagen, Manchester, U.K.) according to the manufacturer's instructions. Urine samples between 10 and 100 mL were collected in the clinic into 120-mL cups, supplemented with preservative, and stored at or below –70°C. For urinary DNA extraction, urine was concentrated to 4 mL using Vivacell 100 concentrators (Sartorius Corp., Bohemia, NY) and incubated with 700 μL of Q Sepharose Fast Flow quaternary ammonium resin (GE Healthcare, Pittsburgh, PA). Tubes were spun to collect Sepharose and bound DNA. The pellet was resuspended in a buffer containing guanidine hydrochloride and isopropanol, and the eluted DNA was collected as a flow-through using polypropylene chromatography columns (BioRad Laboratories, Irvine, CA). The DNA was further purified using QiaQuick columns (Qiagen, Hilden, Germany). DNA in plasma and urine was quantitated using a droplet digital PCR (ddPCR) assay that amplifies a single-copy RNaseP reference gene (QX200 ddPCR system, Bio-Rad, CA) as described previously.[30]

Urine and Plasma EGFR *Mutation Analysis*

Quantitative analysis of the T790M resistance mutation and *EGFR* activating mutations (L858R and 69 deletion variants in exon 19) was performed using a mutation enrichment PCR coupled with next-generation sequencing (NGS) detection (Trovagene, San Diego, CA). Selective amplification of mutant fragments was accomplished by short amplicon (42–44 base pairs [bp]) kinetically driven PCR that amplifies the mutant fragments while suppressing the amplification of the wild-type (WT) sequence using a blocker oligonucleotide. PCR primers contained a 3′ gene-specific sequence and a 5′ common sequence that was used in the subsequent sample barcoding step. The PCR enrichment cycling conditions utilized the initial 98°C denaturation step followed by the assay-specific 5 to 15 cycles of pre-amplification PCR and 17 to 32 cycles of mutation enrichment PCR. Custom DNA sequencing libraries were constructed and indexed using the Access Array System for Illumina Sequencing Systems (Fluidigm Corp., San Francisco, CA). The indexed libraries were pooled, diluted to equimolar amounts with buffer and the PhiX Control library, and sequenced to 200,000× coverage on an Illumina MiSeq platform using 150-V3 sequencing kits (Illumina, San Diego, CA). Primary image analysis, secondary base calling, and data quality assessment were performed on the MiSeq instrument using RTAv1.18.54 and MiSeq Reporter v2.6.2.3 software (Illumina). The analysis output files (FASTQ) from the runs were processed using custom sequencing reads counting and variant calling algorithms to tally the sums of total target gene reads, WT, or mutant *EGFR* reads, which passed predetermined sequence quality criteria (q score $\geq$ 20). A custom quantification algorithm was developed to accurately determine the absolute number of mutant DNA molecules in the source ctDNA sample. To that end, each single multiplexed NGS run contained a set of standard curve samples in addition to clinical samples and controls. For each run the standard sample set was assayed in parallel with patient samples starting with PCR enrichment of mutant *EGFR* DNA followed by NGS. The number of mutant copies detected was determined by interpolation from a standard curve derived from the standard sample set. To account for time of residency in urine across serially collected samples, the results were standardized by normalizing the number of copies detected in the sample to a constant number of WT DNA genome equivalents (GEqs) observed in an average urine sample (i.e., 330 ng WT DNA = 100,000 GEqs).

Determination of ctDNA EGFR *Mutation Detection Cutoffs in Urine and Plasma*

Clinical *EGFR* mutation detection cutoffs for urine and plasma were determined for each assay by assessing the level of nonspecific signal present, if any, from urine and plasma DNA samples obtained from 54 to 64 unique healthy volunteers and metastatic patients with

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non-NSCLC cancers (~50%:50%). Detection cutoffs were standardized to 100,000 WT GEqs yielding adjusted clinical detection cutoffs of 5.5, 5.5, and 12.6 copies per10^5 GEqs for exon 19 deletions, L858R, and T790M, respectively.

Statistical Analysis

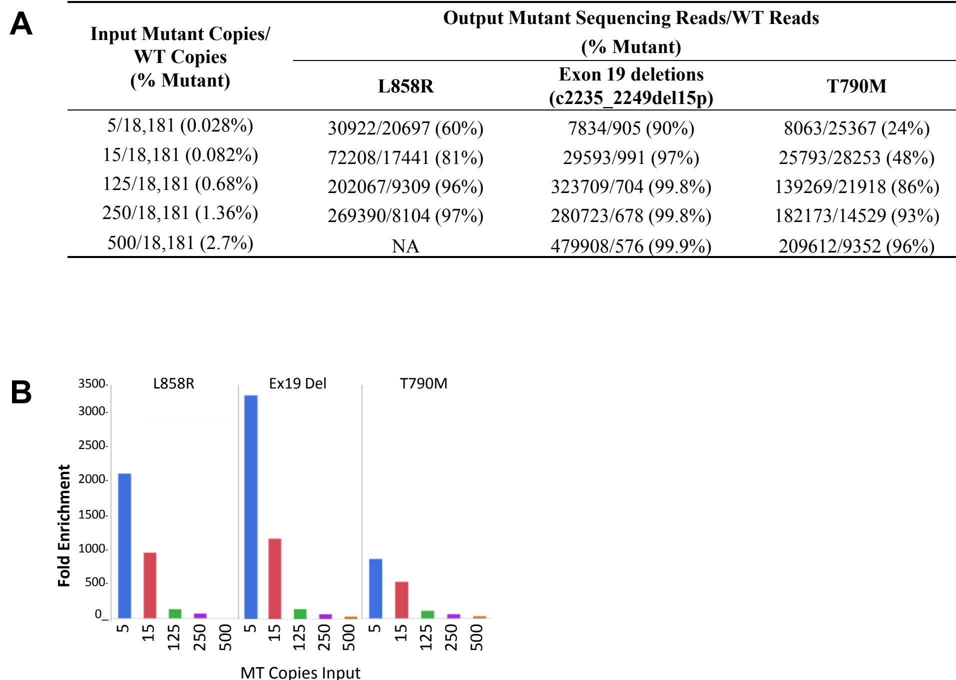
The correlation between input and output absolute *EGFR* mutant copies in the analytical spike-in experiments were examined using Spearman's correlation, which is robust against nonlinearity. Analysis of trends observed in urine ctDNA *EGFR* signal upon patient treatment with rociletinib was assessed using a two-sided Wilcoxon's paired two-sample test. p Values less than 0.05 were considered statistically significant. All statistical analyses were carried out using R v3.2.3 computer software (R Foundation for Statistical Computing, Vienna, Austria).

Results

Development of EGFR Mutation Enrichment NGS Assays for ctDNA

A three-pronged approach was taken to overcome the inherent technical challenges of obtaining sensitive detection and robust quantification of ctDNA mutations in plasma and urine. First, ultrashort footprint PCR assays were developed to increase the likelihood of amplifying highly degraded ctDNA. Three ultrashort footprint assays were developed to detect the most common *EGFR* mutations: (1) a 42-bp *EGFR* exon 19 deletion assay recognizing 69 annotated deletions, (2) a 46-bp *EGFR* exon 21 L858R assay, and (3) a 44-bp *EGFR* exon 20 T790M assay. Secondly, mutant ctDNA fragments were enriched by a PCR-based method to maximize sensitivity for detecting ctDNA mutations having rare prevalence (i.e., ~0.01%). Preferential PCR enrichment of mutant *EGFR* ctDNA was accomplished by using WT *EGFR* oligonucleotides that block the ability of PCR primers to anneal and amplify WT *EGFR* DNA, thereby increasing the likelihood of amplifying mutant *EGFR* templates (see Patients and Methods). Lastly, absolute ctDNA mutation copy numbers from a patient sample were quantitated by NGS methodology. This was achieved with the aid of a standard sample set spiked with known copies of mutant *EGFR* molecules.

Enrichment performance of *EGFR* mutant DNA was assessed by spiking five to 500 copies of mutant DNA into 18,181 GEqs of WT DNA (0.028% to 2.7%). Fold enrichment of *EGFR* mutant fragments increased as the proportion of mutant versus WT fragments decreased



A

Input Mutant Copies/ WT Copies (% Mutant)	Output Mutant Sequencing Reads/WT Reads (% Mutant)		
	L858R	Exon 19 deletions (c2235_2249del15p)	T790M
5/18,181 (0.028%)	30922/20697 (60%)	7834/905 (90%)	8063/25367 (24%)
15/18,181 (0.082%)	72208/17441 (81%)	29593/991 (97%)	25793/28253 (48%)
125/18,181 (0.68%)	202067/9309 (96%)	323709/704 (99.8%)	139269/21918 (86%)
250/18,181 (1.36%)	269390/8104 (97%)	280723/678 (99.8%)	182173/14529 (93%)
500/18,181 (2.7%)	NA	479908/576 (99.9%)	209612/9352 (96%)

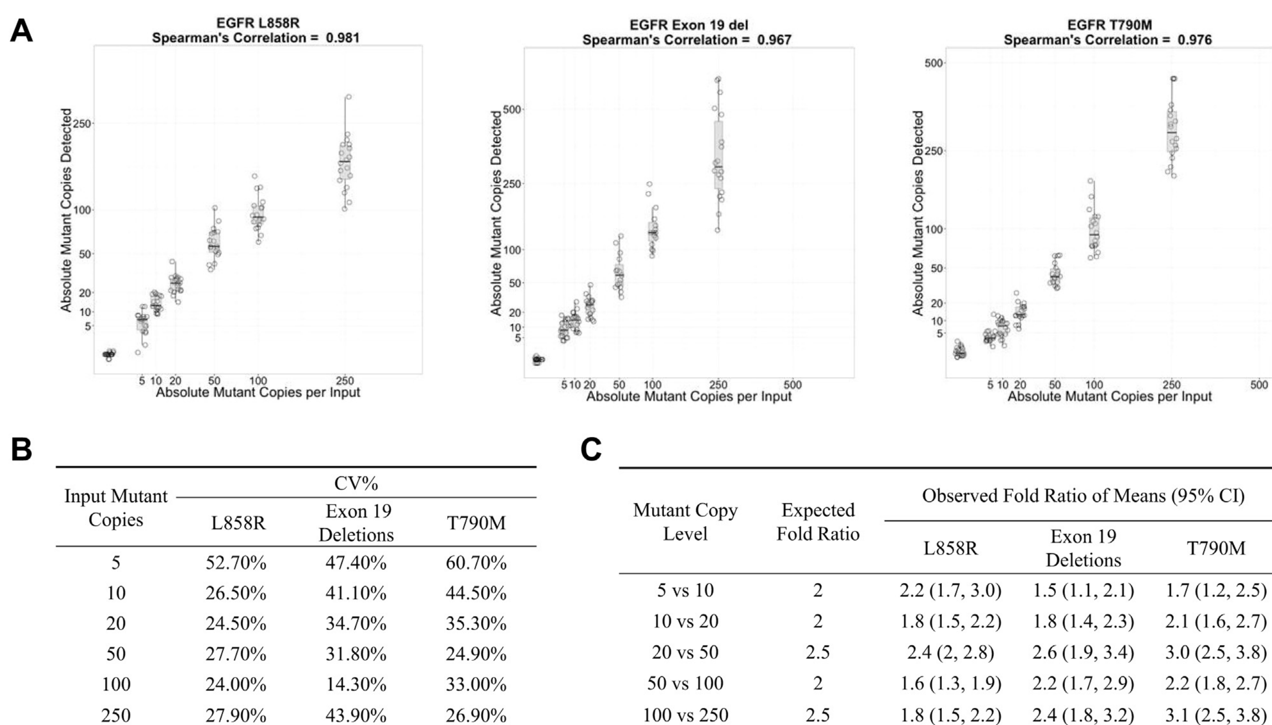
Figure 1. Mutation (MT) enrichment next-generation sequencing. (*A*) Comparison between input ratio of mutant-to-wild-type (WT) *EGFR* copies and output ratio of mutant-to-WT *EGFR* sequencing reads for five to 500 input mutant copies of indicated *EGFR* variants diluted in 60 ng (~18,180 genome equivalents) of WT DNA (mutation abundance of 0.028%-2.7%). Output sequencing reads are means of 18 replicates from six independent next-generation sequencing dilution series experiments performed on three different days by two operators on two MiSeq instruments. Percent mutant is calculated as a fraction of all copies or reads. (*B*) Data from (*A*) presented as mutation fold enrichment estimates. Fold enrichment is calculated as the percent of input mutant *EGFR* molecules divided by the percentage of output mutant *EGFR* sequencing reads.

from 2.7% to 0.028% (Fig. 1*A* and *B*). The resulting sequencing libraries comprised 24% to 99.9% mutant reads, thus enabling sensitive mutation detection by NGS (Fig. 1*A*). For the three assays, 857- to 3214-fold enrichment of *EGFR* mutation signal was obtained for an input of five copies of mutant *EGFR* DNA within 60 ng (18,181 GEqs) of WT DNA (Fig. 1*B*).

Analytical Performance of ctDNA EGFR *Mutation Assays*

The lower limit of detection (LLoD) for the *EGFR* mutation assays was determined by using a statistical model based on the Poisson distribution of rare mutant DNA molecules within a series of highly diluted *EGFR* mutant DNA samples (Supplementary Digital Content Methods). The observed frequency distribution of the number of copies detected within 80 replicates of samples with a mutant DNA spike-in level of one or two expected mutant DNA copies per replicate was within the 95% confidence intervals of expected frequency distribution within a Poisson model (Supplementary Table S1). These results indicated a LLoD of one copy in 18,181 WT GEqs (0.006%) for *EGFR* exon 19 deletion and L858R assays and a LLoD of two copies in 18,181 GEqs (0.01%) for the *EGFR* T790M assay.

The analytical accuracy and reproducibility of the *EGFR* mutation assays were determined by a dilution series of six replicates of 0, 5, 10, 50, 100, and 250 copies against a background of 18,181 WT DNA copies, spanning the linear range of the assays. The entire workflow was replicated six times with three dilution series replicates prepared by two different operators on three different days for a total of 18 measurements at each copy level; NGS analysis was performed on two different Illumina MiSeq instruments. The Spearman correlation between spiked-in absolute copy numbers (quantified by ddPCR) versus detected copy numbers (quantified by mutation enrichment NGS) ranged from 0.967 to 0.981 for the *EGFR* mutation assays (Fig. 2*A*). The mean coefficient of variation percentage was 34.5% across the



Input Mutant Copies	CV%		
	L858R	Exon 19 Deletions	T790M
5	52.70%	47.40%	60.70%
10	26.50%	41.10%	44.50%
20	24.50%	34.70%	35.30%
50	27.70%	31.80%	24.90%
100	24.00%	14.30%	33.00%
250	27.90%	43.90%	26.90%

Mutant Copy Level	Expected Fold Ratio	Observed Fold Ratio of Means (95% CI)		
		L858R	Exon 19 Deletions	T790M
5 vs 10	2	2.2 (1.7, 3.0)	1.5 (1.1, 2.1)	1.7 (1.2, 2.5)
10 vs 20	2	1.8 (1.5, 2.2)	1.8 (1.4, 2.3)	2.1 (1.6, 2.7)
20 vs 50	2.5	2.4 (2, 2.8)	2.6 (1.9, 3.4)	3.0 (2.5, 3.8)
50 vs 100	2	1.6 (1.3, 1.9)	2.2 (1.7, 2.9)	2.2 (1.8, 2.7)
100 vs 250	2.5	1.8 (1.5, 2.2)	2.4 (1.8, 3.2)	3.1 (2.5, 3.8)

Figure 2. Quantification of *EGFR* mutant and wild-type DNA blends by mutation enrichment next-generation sequencing. (*A*) Analysis of dilution series of indicated mutant *EGFR* variants spiked into 60 ng (~18,180 GEq) of WT DNA. An analysis algorithm was applied to transform the mutant *EGFR* sequencing reads into the absolute mutant copies detected. The box-and-whisker plots show the median (*center line*), 25th, and 75th percentiles (*box*) with the connecting "whiskers" extending from the first quartile minus 1.5 of the interquartile range (the third quartile minus the first quartile) and the third quartile plus 1.5 of the interquartile range. (*B*) Interrun reproducibility of the *EGFR* exon 19 deletions, L858R, and T790M mutation enrichment next-generation sequencing assays for the dilution series shown in (*A*). Coefficient of variation percent (CV%) is presented. (*C*) Quantification of twofold or 2.5-fold differences between subsequent mutant copy input levels. The expected fold ratio was calculated as the ratio of the two input mutant copy levels. The observed fold ratio was calculated as the ratio of two means for two measured mutant copy levels with a 95% confidence interval (CI).

reportable range of 5 to 250 copies for all three *EGFR* mutation assays (yielding an adjusted quantifiable range of 27.5 to 1375 copies per 100,000 GEqs), with the highest coefficient of variation percentage of 47.4% to 60.7% observed at the lowest input of five copies likely owing to Poisson limitations (Fig. 2*B*). Assay fold-discrimination performance was examined by comparing the expected copy ratio between two consecutive dilutions to the observed copy ratio (Fig. 2*C*). Known twofold and 2.5-fold differences within the dilution series were maintained and detected for all three *EGFR* mutation assays (Fig. 2*C*).

Clinical Performance of ctDNA EGFR *Mutation Assays in Urine*

Matched baseline tumor tissue biopsies and urine samples were obtained from the first 63 consecutive patients in TIGER-X who agreed to optional pretreatment urine sample collection. TIGER-X was a phase 1/2 trial of rociletinib in patients with stage IIIB to IV NSCLC who had been treated with at least one prior EGFR inhibitor and had an *EGFR* activating mutation in their medical record. The clinical characteristics of the 63 patients are shown in Table 1. Tumor tissue was processed by a central laboratory for *EGFR* mutation testing. Of the 63 tumor tissue biopsy samples, 60 were adequate for analysis, with 47 positive for T790M, 16 positive for L858R, and 42 positive for exon 19 deletion mutations. Thirteen of 60 evaluable cases were negative for T790M, and two of 60 cases were negative for either L858R or exon 19 deletion mutation by central laboratory testing. Urine volumes ranged from 10 to 100 mL, with 19 of 63 samples meeting the prespecified criteria for the recommended urine volume of 90 to 100 mL. Tumor tissue testing was used as a reference standard. For all samples (volumes 10–100 mL), the sensitivity of the urine assays for *EGFR* mutation detection was 72% (34 of 47 samples) for T790M, 75% (12 of 16) for L858R, and 67% (28 of 42) for exon 19 deletion mutations (Table 2). For samples with the recommended urine volume of 90 to 100 mL, the sensitivity of *EGFR* mutation detection was 93% (13 of 14) for T790M, 80% (four of five) for L858R, and 83% (10 of 12) for exon 19 deletions (see Table 2). For urine samples with lower volumes (10–89 mL), the sensitivity for *EGFR* mutation detection was 64% (21 of 33) for T790M, 73% (eight of 11) for L858R, and 60% (18 of 30) for exon 19 deletions (see Table 2).

The specificity of the *EGFR* urine assays was determined using urine samples obtained from healthy donors and patients with non-NSCLC metastatic cancers (see "Patients and Methods") and was 96% for T790M, 100% for L858R, and 94% for the exon 19 deletion mutations (see Table 2).

Table 1. Patient Demographic and Baseline Characteristics

Characteristics	Patients in TIGER-X with Pretreatment Urine Samples (n = 63)
Age, y	
Median	64
Range	40-85
Sex, n (%)	
Female	45 (71.4)
Male	18 (28.6)
Race, n (%)	
Asian	17 (27.0)
Black/African American	1 (1.6)
White	44 (69.8)
Other	0
Missing	1 (1.6)
Histologic diagnosis, n (%)	
Adenocarcinoma	63 (100)
ECOG at study entry, n (%0)	
0	18 (28.6)
1	45 (71.4)
No. previous therapies at baseline, n (%)	
0	0
1	21 (33.3)
2	11 (17.5)
3	7 (11.1)
4	9 (14.3)
5+	15 (23.8)
No. previous EGFR TKIs at baseline, n (%)	
1	34 (54.0)
2	17 (27.0)
3	12 (19.0)
Smoking history, n (%)	
Current smoker	1 (1.6)
Former smoker	22 (34.9)
Never-smoker	40 (63.5)

TIGER-X, Study to Evaluate Safety, Pharmacokinetics, and Efficacy of Rociletinib (CO-1686) in Previously Treated Mutant Epidermal Growth Factor Receptor (EGFR) in Non-Small Cell Lung Cancer (NSCLC) Patients; ECOG, Eastern Cooperative Oncology Group; TKI, tyrosine kinase inhibitor.

Clinical Performance of ctDNA EGFR *Mutation Assays in Plasma*

Plasma was available for 60 of the 63 patients. When tumor tissue testing results were used as a reference standard, the detection sensitivity of the assays in plasma was 93% (38 of 41 samples [three of 44 available plasma samples failed NGS]) for T790M, 100% (17 of 17 samples) for L858R, and 87% (34 of 39 samples) for exon 19 deletions (see Table 2). The specificity of the *EGFR* plasma tests was determined using plasma samples obtained from healthy donors and patients with non-NSCLC metastatic cancers and was 94% for T790M,

Table 2. Performance of Mutation Enrichment NGS Assays for Detection of *EGFR* Mutations in Urine and Plasma

Characteristics	Specimen Type	Value, n %
T790M		
Sensitivity	All urine volumes, 10-100 mL	72% (34 of 47)
	Urine volumes, 90-100 mL[a]	93% (13 of 14)
	Urine volumes, 10-89 mL	64% (21 of 33)
	Plasma	93% (38 of 41)
Specificity	Urine	96% (54 of 56)
	Plasma	94% (60 of 64)
L858R		
Sensitivity	All urine volumes, 10-100 mL	75% (12 of 16)
	Urine volumes, 90-100 mL[a]	80% (4 of 5)
	Urine volumes, 10-89 mL	73% (8 of 11)
	Plasma	100% (17 of 17)
Specificity	Urine	100% (50 of 50)
	Plasma	100% (48 of 48)
Exon 19 deletions		
Sensitivity	All urine volumes, 10-100 mL	67% (28 of 42)
	Urine, volumes 90-100 mL[a]	83% (10 of 12)
	Urine, volumes 10-89 mL	60% (18 of 30)
	Plasma	87% (34 of 39)
Specificity	Urine	94% (47 of 50)
	Plasma	96% (47 of 49)

[a]Recommended urine volume: ≥90 mL.
NGS, next-generation sequencing.

100% for L858R, and 96% for exon 19 deletion mutations (see Table 2).

Urine and Plasma Identify Additional EGFR *T790M-Positive Cases Undetectable by Tissue Biopsy*

Contingency tables for the *EGFR* mutation detection in urine, plasma, and tissue are presented in Figure 3. For all urine sample volumes, there were 11 cases that were urine T790M–positive but tumor tissue T790M–negative or tissue sample–inadequate (Fig. 3*A*). Of these 11 cases, 10 were also T790M positive in plasma (one sample was T790M negative in plasma). Similarly, of the 11 discordant cases that were plasma T790M–positive but tissue T790M–negative or tissue sample–inadequate, 10 were also positive by urine T790M testing (one sample was T790M negative in urine). Together, urine and plasma T790M testing identified a higher proportion of positive cases (89% [56 of 63) than did tissue testing alone (75% [47 of 63]).

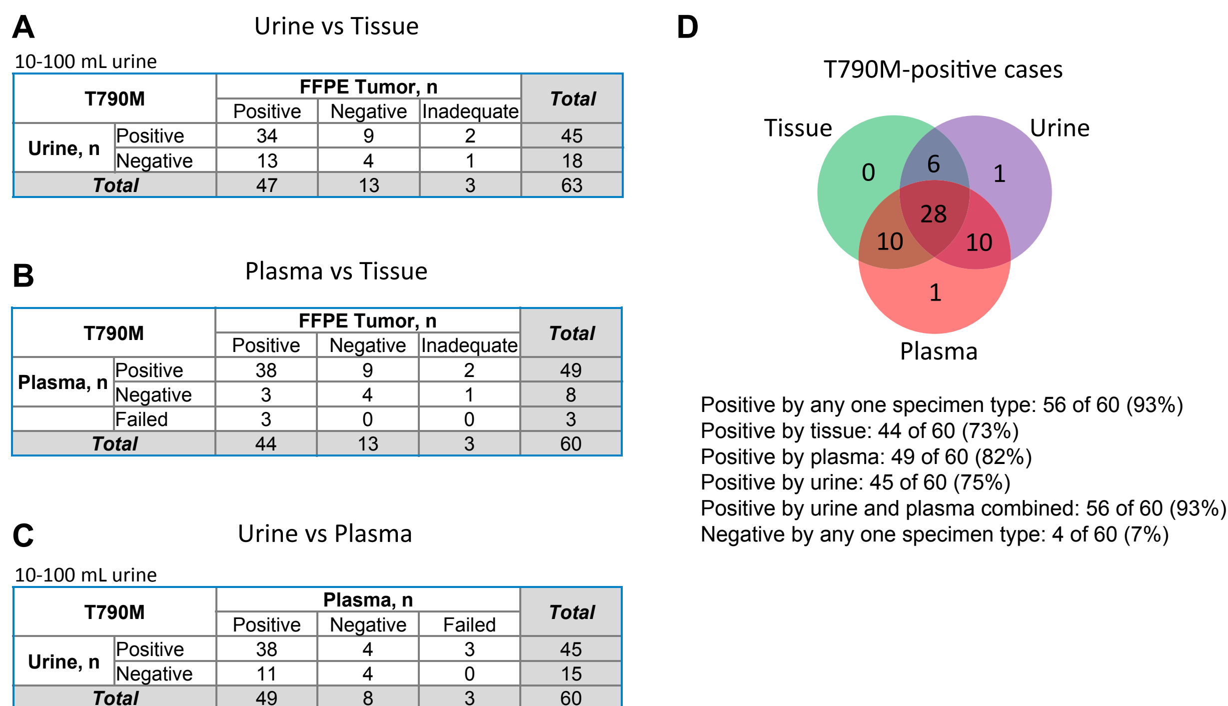
When 60 cases with all three patient-matched specimen types were considered, urine analysis identified seven T790M cases not detected or failed in plasma and 11 cases were not detected by tissue or found to be tissue sample–inadequate (Fig. 3*D*). Analysis of urine and blood combined detected T790M in 93% of patients (56 of 60). Tissue analysis alone detected T790M in 73% of patients (44 of 60). Four of 60 cases were negative according to testing of tissue, plasma, and urine samples.

Association of EGFR *T790M Levels in Urine with Patient Response to Rociletinib*

Recent studies have suggested that the extent to which plasma *EGFR* mutation levels drop after introduction of EGFR TKI therapy may predict depth of response.[14,16] To assess this relationship in urine, longitudinal urine samples were obtained from 15 patients treated with therapeutic doses of rociletinib (500, 625, 750, or 1000 mg twice daily [hydrobromide formulation]). Of these 15 patients, five had progressive disease as their best overall confirmed response and 10 patients had either stable disease or a partial response (PR) as their overall confirmed response. Among these, nine patients were identified with quantifiable levels of baseline T790M in urine (>27.5 copies per 100,000 GEqs), with seven experiencing PR or stable disease as best overall confirmed response and two with progressive disease as best overall confirmed response. For all nine patients, there was a significant decrease in T790M levels in urine after cycle 1 relative to baseline irrespective of best overall confirmed response (range −51% to −100%; $p = 0.0091$, two-sided Wilcoxon test; $p < 0.0001$, two-sided *t*-test) (Fig. 4*A* and *B*). However, in the two patients with progressive disease, there was an observed attenuated decrease (−51% and −70%) compared with in patients with PR and stable disease as best overall confirmed response (range −83% to −100%).

Discussion

Herein we have described a highly sensitive method for detection of actionable *EGFR* mutations in the urine and plasma of patients with advanced NSCLC. In our study cohort of relapsed patients, the sensitivity of *EGFR* mutation detection in urine with tumor as a reference was 72% (34 of 47) for T790M, 67% (28 of 42) for exon 19 deletions, and 75% (12 of 16) for L858R mutations (all urine volumes). A higher sensitivity was achieved with urine samples at the recommended volume of 90 to 100 mL as compared with urine volumes less than 90 mL: 93% (13 of 14) for T790M, 71% (five of seven) for L858R, and 83% (10 of 12) for exon 19 deletions (90–100 mL) versus 64% (21 of 33) for T790M, 72% (eight of 11) for L858R, and 60% (18 of 30) for the exon 19 deletion mutations (volumes of 10–89 mL). The specificity of the urine *EGFR* assays in healthy volunteers or patients without NSCLC was 96% for T790M, 100% for L858R, and 94% for exon 19 deletion mutations. To our knowledge, this study represents the first successful demonstration of



A Urine vs Tissue

10-100 mL urine

T790M		FFPE Tumor, n			*Total*
		Positive	Negative	Inadequate	
Urine, n	Positive	34	9	2	45
	Negative	13	4	1	18
Total		47	13	3	63

B Plasma vs Tissue

T790M		FFPE Tumor, n			*Total*
		Positive	Negative	Inadequate	
Plasma, n	Positive	38	9	2	49
	Negative	3	4	1	8
	Failed	3	0	0	3
Total		44	13	3	60

C Urine vs Plasma

10-100 mL urine

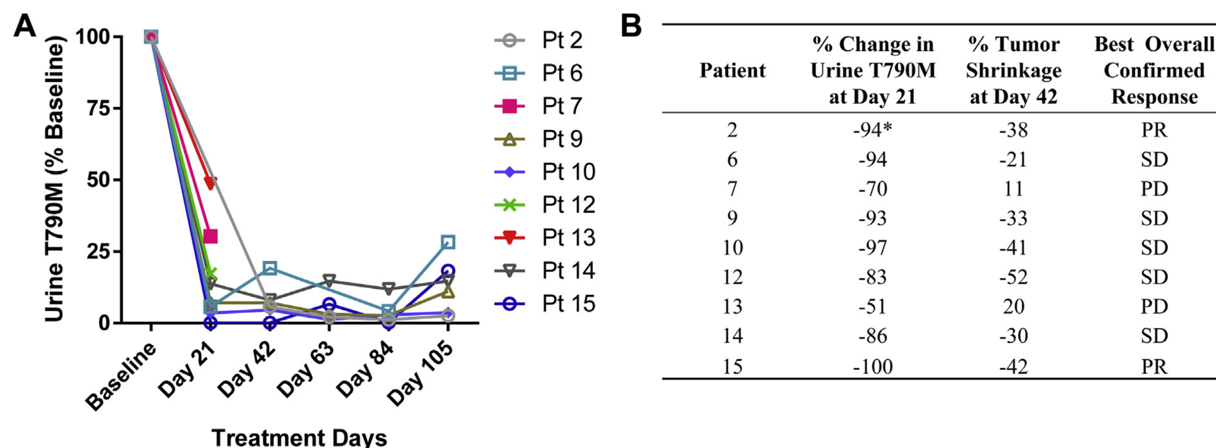
T790M		Plasma, n			*Total*
		Positive	Negative	Failed	
Urine, n	Positive	38	4	3	45
	Negative	11	4	0	15
Total		49	8	3	60

Positive by any one specimen type: 56 of 60 (93%)
Positive by tissue: 44 of 60 (73%)
Positive by plasma: 49 of 60 (82%)
Positive by urine: 45 of 60 (75%)
Positive by urine and plasma combined: 56 of 60 (93%)
Negative by any one specimen type: 4 of 60 (7%)

Figure 3. Contingency tables for the analysis of *EGFR* T790M mutation in matched tumor, urine, and plasma specimens from patients enrolled in the TIGER-X clinical trial. (*A*) Urine versus tumor analysis of T790M in 63 matched tumor and urine specimens. (*B*) Plasma versus tumor analysis of T790M in 60 matched tumor and plasma specimens. (*C*) Urine versus plasma analysis of T790M in 60 matched urine and plasma specimens. (*D*) Venn diagram showing T790M-positive status of 60 cases with available matched tumor, plasma, and urine specimens. Four cases not identified as T790M-positive by tumor, plasma, or urine are not depicted in the diagram. Abbreviation: FFPE, formalin-fixed, paraffin-embedded.

EGFR mutation detection in the urine of patients with metastatic NSCLC.

The transrenal clearance of systemically derived DNA was first demonstrated in 2000 by Botezatu et al. who detected male-specific sequences in the urine of women transfused with male blood or pregnant with male fetuses.[25] In addition, this pioneering work demonstrated that circulating nucleic acid of tumor origin could be identified in the urine of patients with cancer, specifically those with colorectal or pancreatic cancer.[25]



Patient	% Change in Urine T790M at Day 21	% Tumor Shrinkage at Day 42	Best Overall Confirmed Response
2	-94*	-38	PR
6	-94	-21	SD
7	-70	11	PD
9	-93	-33	SD
10	-97	-41	SD
12	-83	-52	SD
13	-51	20	PD
14	-86	-30	SD
15	-100	-42	PR

Figure 4. Longitudinal dynamics of *EGFR* T790M signal in urine of patients treated with rociletinib. (*A*) A rapid decrease in urinary T790M signal from baseline was observed after one cycle of treatment (21 days) in all patients treated with rociletinib. (*B*) Clinical outcomes of patients in the longitudinal analysis. Best overall confirmed response was assessed by investigator. For patient 2, percent change in urine T790M from baseline was estimated at day 42. Abbreviations: PR, partial response; SD, stable disease.

Further studies suggested that the size of the systemically derived DNA fragments in urine can range from approximately 35 to 250 bp.[27,33–35] Subsequently, an anion exchange–based urinary DNA isolation technique coupled with ultrashort amplicon PCR detection was developed to maximize the detection of systemically derived DNA.[27,33,34] The method described in the present study builds on this work, incorporating an anion exchange–based method to preferentially isolate low-molecular-weight DNA from urine, short amplicon PCR with WT DNA suppression for mutation enrichment, and ultradeep sequencing to further enhance the identification of rare mutations. Using this approach, we show single-copy detection with spiked sample material.

Our methodology may have also contributed to the high clinical sensitivity that we observed for detecting *EGFR* mutations in plasma (range 87%–100%), which compares favorably with the published performance of real-time and ddPCR platforms. For example, T790M detection sensitivity in patients who experienced a relapse while receiving first-line EGFR TKIs was shown to range between 64% and 73% for the cobas test (a test platform based on real-time PCR), 73% to 81% for BEAMing (Beads, Emulsion, Amplification, and Magnetics, a technology based on digital PCR), and 77% for the ddPCR platform.[13,18,24] In the same studies, the sensitivity for detection of activating *EGFR* mutations was 73% to 84% for the cobas test, 82% to 84% for the BEAMing assay, and 74% to 82% for the ddPCR platform.[14,18,24] In five recent trials in patients with previously untreated NSCLC (NCT01203917, FASTACT-2, TRIGGER, EUROTAC, and NCT02279004), the sensitivity for detection of *EGFR* exon 19 deletions and L858R mutations in plasma was 78% for a real-time peptide nucleic acid clamp test, 69% to 86% for a ddPCR test, 62% to 68% for the therascreen *EGFR* RGQ PCR test, and 62% to 100% for the cobas test.[2,15,16,22,24] The specificities of our plasma assays in healthy volunteers and patients without NSCLC were 94% to 100%, which are similar to that found in urine. Limitations of this study include a small sample size for urine with volumes of 90 to 100 mL and a less than 100% assay specificity. Analysis of a larger cohort of patients is under way. An improved error correction bioinformatics algorithm is being developed to further increase assay specificity.

Assay sensitivity in the present study was calculated using tumor as the reference sample type. This method has limitations, particularly when applied to resistance mutations such as T790M that will have a significant false-negative rate in analysis of biopsy samples owing to tumor heterogeneity and low tumor cellularity.[9,10,14,24] In our study, the combination of urine and plasma testing identified 12 T790M-positive cases that were undetectable by central laboratory testing of tumor tissue. Although 10 of the 12 cases were positive by both ctDNA specimen types, one case each was unique to plasma and urine. Urine may therefore provide complementary information about a patient's mutational status that is not captured by plasma or tissue tests. These results indicate for the first time that either urine or plasma T790M testing may be considered as an alternative to tissue biopsy testing. Urine testing can be the preferable option because it represents a truly noninvasive alternative that can be collected in a patient's own home, and the results of both diagnostic and monitoring testing can be made available without scheduling visits to the clinic.

Given the ease of sample collection, urine holds promise for the serial monitoring of patients. Studies in plasma have already shown that early changes in ctDNA may predict response to targeted therapies and that emergence of resistance mutations can be identified before radiographic progression.[12,14,15,19,23] Reported here, T790M levels in urine rapidly decreased to a fraction of their pretreatment levels in patients treated with rociletinib, regardless of RECIST response status. These data are consistent with previous findings in plasma and suggest that rociletinib reduces proliferation and consequently turnover of T790M-positive clones even in patients with primary resistance.[9,14] It is an intriguing observation that there was an attenuated decrease in T790M levels for the two patients with progressive disease as best overall response. Further urine analysis of a substantially larger cohort of patients from the TIGER-X study is ongoing and should further inform the utility of longitudinal monitoring in this patient population.

In conclusion, our data demonstrate that urine testing using the mutation enrichment NGS method successfully identifies *EGFR* mutations in patients with metastatic NSCLC and has high concordance with tumor and plasma, suggesting that *EGFR* mutation detection from urine or plasma should be considered as a viable approach for assessing *EGFR* mutation status.

Acknowledgments

The authors thank patients for their participation in the study. We thank the study staff, who encouraged the optional collection of urine specimens; Onkar Dha and David Gustafson for coordinating biospecimen collection; Latifa Hassain for assistance with urine analyses; and Peter Morello, Sandeep Pingle, and Victoria Raymond for assistance with manuscript preparation.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at http://dx.doi.org/10.1016/j.jtho.2016.05.035.

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