Analytic and Clinical Validation of an Ultrasensitive, Quantitative Polymerase Chain Reaction Assay for EGFR Mutation Analysis With Circulating Tumor DNA

Xiaowei Wang, PhD; Yunhua Gao, MS; Bei Wang, MS; Zhenrong Zhang, MD; Chaoyang Liang, MS; Hongxiang Feng, MS; Yongqing Guo, MS; Jiping Da, MS; Minli Mo, PhD; Mengyun Zhang, MS; Feng Ding, PhD; Zhao Chen, PhD; Hui Li, PhD; Deruo Liu, MD, PhD

Objectives.—To develop a timely and cost-effective assay that can accurately detect EGFR mutations in circulating tumor DNA and to evaluate the analytic and clinical performance of the assay.

Design.—Analytic assessment was conducted with a set of reference materials carrying classic EGFR mutations. A recently developed Poisson distribution–based approach was employed to understand the assay sensitivity. Clinical evaluation was performed with 224 pairs of plasma and matched tissues from patients with stage I to IV disease. EGFR mutation rates of 390 consecutive plasma samples processed in the central service laboratory were compared with previously reported prevalence in an Asian population.

Results.—Our results suggested that limit of detection for the EGFR quantitative polymerase chain reaction assay was 10 mutation copies, and the lowest detectable copy numbers could be extended to a single-digit level. The clinical sensitivity was 53.3% for all stages combined and 81.4% for late stages, with a high specificity of 100%. Clinical observations showed an overall positive finding rate of 32.5% and 41.4% for stage IV disease, which is consistent with previously reported EGFR mutation prevalence in an Asian population.

Conclusions.—Our results supported the clinical utility of the ultrasensitive, quantitative polymerase chain reaction assay for EGFR mutation analysis with circulating tumor DNA.


Non–small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide, and most patients are diagnosed at advanced stages and have poor prognoses.1 The status is being changed by the clinical application of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors.2-3 Somatic mutations in the EGFR gene in patients with NSCLC are associated with a significant increase in the response rate to EGFR–tyrosine kinase inhibitors, such as erlotinib, gefitinib, and afatinib, and improvement in progression-free and overall survival.5-6 The presence of EGFR-activating mutation needs to be tested before prescription of EGFR-tyrosine kinase inhibitors, and exon 19 deletions and exon 21 L858R point mutations account for approximately 90% of all EGFR mutations detected.5,7 Most patients, however, will develop resistance to therapy because of acquired-resistance mutations, among which, EGFR T790M is the most common one.8,9 Therefore, monitoring the emergence of resistance mutations is also needed to facilitate clinical decisions.

Currently, tumor tissue, generally from the primary tumor, is used to determine EGFR mutation status. However, tumor samples are not always available for patients with advanced NSCLC; the test accuracy may be confounded by tumor heterogeneity, and repeat biopsies to
monitor treatment response are difficult in clinical settings.\textsuperscript{5,10} Circulating tumor DNA (ctDNA) represents a promising solution to address these issues and holds great potential for molecular testing, such as EGFR mutation analysis.\textsuperscript{1,12}

A variety of techniques have been developed to identify somatic mutations in ctDNA, including real-time polymerase chain reaction (PCR), next-generation sequencing (NGS), BEAMing (beads, emulsion, amplification, and magnetics), and digital PCR.\textsuperscript{12–22} The advanced techniques, such as CAPP-Seq (cancer personalized profiling by deep sequencing), have achieved significant improvement in detecting multiple classes of somatic alterations with ctDNA.\textsuperscript{23} However, the high cost of most techniques limits accessibility to patients; the turnaround time—some are in weeks—is incompatible with the urgent need to make treatment decisions, and many techniques require dedicated molecular biology and bioinformatics support, which hinder the wide adoption for routine use in clinical practice.\textsuperscript{24}

To meet clinical needs, our team developed an ultrasensitive quantitative PCR (Q-PCR) assay to detect EGFR mutations in plasma samples: the EGFR Ultra assay. Systematic analytic and clinical validation was conducted to evaluate the assay’s performance and to report the results in the current study.

**MATERIALS AND METHODS**

The Ultrasensitive Q-PCR Assay for EGFR Mutation Detection

The EGFR Ultra assay was designed to detect 45 EGFR mutations in exons 18, 19, 20, and 21 with a Human EGFR Gene Mutations Detection Kit (real-time fluorescent PCR) (Beijing ACCB Biotech, Beijing, China) on an Agilent Mx3000P real-time PCR machine (Agilent, Palo Alto, California) with the following settings: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Interpretation of the result was based on absolute cycle threshold (Ct) values when samples passed quality standards of positive control, negative control, and internal controls. The baseline was set by selecting “for baseline (adaptive baseline).” The threshold value was set so that the threshold line was above the amplification curve of the negative control, and the negative control curve was displayed as “no Ct.” Samples with Ct value of 37 or less were classified as “mutation detected”; Ct values of more than 39 or of no amplification were “mutation not detected”; Ct values between 37 and 39 were further confirmed by repeating the experiment, and new Ct values between 37 and 39 were considered “mutation detected.” The assay results depended on mutant frequency, adequate specimen integrity, absence of PCR inhibitor, and sufficient DNA.

**Reference Materials**

A set of EGFR reference materials (RMs) was developed by National Institute of Metrology (Beijing, China). The set was composed of one plasmid with wild-type EGFR exons 18, 19, 20, and 21, and 11 plasmids carrying 21 EGFR variants (Table 1). The sequences of whole plasmids were confirmed by Sanger sequencing using ABI 3730 (Thermo Fisher Scientific, Waltham, Massachusetts). Reference materials were systematically quantified by high-resolution, inductively coupled plasma mass spectrometry (ELEMENT XR, Thermo Fisher Scientific) and aliquoted and stored at a stock concentration of 1 μg/mL. The purity determination and stability analysis were conducted by ultracentrifugation and chromatography. The molecular weight, analyzed by mass spectrometry, was used to calculate the copy number of molecules in the stock.

**Analytic Assessment**

Reference materials were spiked into a background genomic DNA (gDNA) extracted from human embryo kidney (HEK) 293 cells (ATCC, Manassas, Virginia) at different concentrations for analytic assessment. In each Q-PCR run, positive and negative controls were run in sextuplicate. For limit of detection (LOD) determination, 20 and 10 copies of RMs were added into each well, to a final concentration of 10 ng/μL and 1 ng/μL, respectively. For each RM at each concentration, 20 wells were tested per Q-PCR run, and 3 independent Q-PCR runs were performed with 3 different kit lots. All 11 RMs carrying mutant variants were used in LOD determination. For specificity, EGFR wild-type samples at 5 ng/μL and 1 ng/μL were analyzed 30 times in 3 independent Q-PCR runs with 3 different kit lots. For the series dilution experiments, RM1 at 1000, 100, 10, and 1 copies were diluted into 10 ng/μL of background gDNA, and experiments were performed 8 times per run with 3 independent runs to test E19del. To determine the lowest detectable copy number, RM2 was diluted into 2 sets of 60 wells to obtain an average copy number of 3 and 5 per well at a gDNA concentration of 10 ng/μL. The dilution panels were tested for E19del, L858R, and G719X. The experiment was repeated 3 times. For analytic assessment, samples with Ct value of 39 or less were considered positive.

**Patient Information**

For clinical validation, patients were enrolled in this study from January 2014 to June 2015 at the Department of Thoracic Surgery, China-Japan Friendship Hospital (Beijing, China). Pathologic diagnosis was established as lung adenocarcinoma or adenosquamous carcinoma, stages I to IV, with both plasma and tumor tissue samples collected. Patients presenting one or more metastatic sites to bone, liver, lung, brain, and pleura were classified as having stage IV disease. This study was conducted under an institutional review board–approved protocol and was monitored for assurance of human subjects’ protection. Written, informed consent was obtained from each patient before specimen collection.

For clinical observation, 390 consecutive samples were sent to the state-certified central service laboratory of Beijing ACCB Biotech from 15 different institutions in China for EGFR-mutation testing service from April 2015 to December 2015. Written, informed consent was obtained from each patient before specimen collection.

**Plasma Isolation and Cell-Free DNA Extraction**

To isolate high-quality, cell-free DNA, 10 ml of whole blood was obtained in Streck cell-free DNA blood collection tubes (Streck, La Vista, Nebraska) to prevent the release of genomic DNA. Blood samples were centrifuged at 820g for 10 minutes to precipitate cellular debris and gDNA at the bottom of tubes. Supernatant (2 ml) was used for cell-free DNA isolation with an OMEGA circulating DNA kit (Omega Bio-Tek, Norcross, Georgia) without an RNA carrier, per manufacturer instruction, and the DNA was eluted in a final volume of 30 μL. The quantity and quality of the isolated DNA were analyzed using Qubit 2.0 fluorometer (Thermo Fisher Scientific). gDNA was isolated from no less than 5 consecutive 5-μm slides of formalin-fixed, paraffin-embedded specimens with a QiAamp DNA tissue kit (Qiagen, Hilden, Germany), per manufacturer’s instruction. DNA concentration was measured with a Qubit 2.0 fluorometer. HEK293 cells were cultivated in Dulbecco’s Modified Eagle’s Medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Cells were cultured at 37°C in a 5% CO₂-humidified incubator. gDNA was isolated from HEK293 cells with Qiagen DNeasy blood and tissue kit (Qiagen), and quantified with a Qubit 2.0 fluorometer.

**Mutation Analysis by NGS**

Libraries were prepared using the NextDaySeq EGFR minipanel on the Ion Torrent System (manufactured at Beijing ACCB Biotech), according to the manufacturer’s instructions. Briefly,
Abbreviations: AA, amino acid; CDS, coding sequence; RM, reference material.

disease stages. A test was performed to compare clinical sensitivity with different predictive value, and 95% CIs were calculated. Analysis of variance sensitivity, specificity, positive prediction value, and negative reliable, and cost-effective detection of PCR assay (Genomics Viewer (Broad Institute, Cambridge, Massachusetts).

onto Ion 318 chips to generate sequencing data with a minimum kit, according to manufacturer’s protocol. The libraries were loaded ing was performed with the Ion PGM Sequencing Supplies 200 v2 were enriched on the Ion OneTouch enrichment system. Sequenc-

OneTouch 2 instrument. Template-positive ion sphere particles protocol. Briefly, the library pool was clonally amplified in an Genome Machine) system, according to the manufacturer’s library pool was sequenced with Ion Torrent PGM (Personal fluorometer, diluted to a concentration of 3 ng/ double-stranded DNA (dsDNA) HS assay kit on a Qubit 2.0 barcodes. After purification, libraries were quantified with a Qubit

using pooled primer pairs, followed by ligation with adaptors and barcodes. After purification, libraries were quantified with a Qubit

density function for analyze lowest-detectable copy range. The Poisson probability was used for data analysis. Poisson distribution was employed to

In the past 2 years, we adopted an ultrasensitive EGFR Ultra Assay Analytic Sensitivity

and Specificity

Statistical Analysis

SPSS software (version 19.0, IBM Software, Armonk, New York) was used for data analysis. Poisson distribution was employed to analyze lowest-detectable copy range. The Poisson probability density function for $\lambda$, as 3 and 5 was used to calculate the expected numbers of wells that contain a certain number of copies for the 3-copy and 5-copy dilution panel. Clinical assessment was performed with a $2 \times 2$ diagnostic table, and parameters, such as sensitivity, specificity, positive prediction value, and negative predictive value, and 95% CIs were calculated. Analysis of variance tests were performed to compare clinical sensitivity with different disease stages. A $t$ test was used for pairwise comparison.

RESULTS

Assessment of the EGFR Ultra Assay Analytic Sensitivity and Specificity

In the past 2 years, we adopted an ultrasensitive EGFR Q-PCR assay (EGFR Ultra) in a clinical setting to allow timely, reliable, and cost-effective detection of EGFR mutations and facilitate therapeutic decision making for patients at risk for NSCLC. The EGFR Ultra assay was intended for qualitative detection of 45 somatic mutations in exons 18, 19, 20, and 21 of EGFR in DNA derived from NSCLC tumor tissue and ctDNA of plasma. The assay was based on an amplification-refractory mutation system, and included one negative control and 7 test reactions targeting 45 mutations. Before clinical implementation, a systematic assessment was conducted to evaluate the analytic sensitivity and specificity.

A series of RMs was previously developed by the National Institute of Metrology (Beijing, China) for analytic assessment of molecular diagnostic assays. The EGFR RM set was composed of one plasmid with wild-type exons 18, 19, 20, and 21 of the gene and 11 plasmids carrying 21 types of EGFR variants, including 7 point mutations, such as L858R and T790M, and 14 indels, such as E746-A750del and E747_A750>P (Table 1).

The minimum copy number of mutant DNA that produces correct results 95% of the time, also known as the LOD, was determined for all 21 mutations present in the RMs (Supplemental Table 1; see supplemental material file at www.archivesofpathology.org in the July 2017 table of contents). The detection rates of 7 representative mutations, one for each assay reaction, are summarized in Table 2. Our results showed 95% or greater detection rates across all mutations, demonstrating a LOD at 10 mutation copies per reaction. Test results obtained from the EGFR wild-type samples at 5 ng/µL and 1 ng/µL had no mutations detected, suggesting high analytic specificity.

We further explored whether the sensitivity of our assay allowed detection at a single-digit copy-number level. RM2 at 1000, 100, 10, and 1 copies were diluted into 10 ng/µL background gDNA and tested for EGFR E19del. Whereas samples with 10 copies and higher were consistently Table 1. EGFR Reference Materials With Alternative Annotation

<table>
<thead>
<tr>
<th>RM</th>
<th>Exon 18 CDS</th>
<th>Exon 18 AA</th>
<th>Exon 19 CDS</th>
<th>Exon 19 AA</th>
<th>Exon 20 CDS</th>
<th>Exon 20 AA</th>
<th>Exon 21 CDS</th>
<th>Exon 21 AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Wild type</td>
</tr>
<tr>
<td>2</td>
<td>2155G&gt;A</td>
<td>G719S</td>
<td>2235–2249del</td>
<td>E746–A750del</td>
<td>2369C&gt;T</td>
<td>T790M</td>
<td>2573T&gt;G</td>
<td>L858R</td>
</tr>
<tr>
<td>3</td>
<td>2156G&gt;C</td>
<td>G719A</td>
<td>2236–2250del</td>
<td>E746–A750del</td>
<td>2303G&gt;T</td>
<td>S768I</td>
<td>2582T&gt;A</td>
<td>L861Q</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>2239–2256del</td>
<td>L747–S752del</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2237–2255&gt;T</td>
<td>E746–S752&gt; \ V</td>
<td>2308–2309insCCAGCGTGGG</td>
<td>D770–N771insG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2236–2253del</td>
<td>E746–T751del</td>
<td>2309–2313insGGT</td>
<td>H773–V774insH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2239–2248&gt;C</td>
<td>E747_A750&gt;P</td>
<td>2310–2311insGGT</td>
<td>D770–N771insSVD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 11 | 2239–2247del| L747–E749del| 2311–2312insGGTGG
| 12 | 2239–2258>CA | L747_P753>Q | 2312insGGTGGCAC |

Abbreviations: AA, amino acid; CDS, coding sequence; RM, reference material.

a The alternative annotation is provided in parentheses.

EGFR genomic regions of EGFR exons 18, 19, 20, and 21 were amplified using pooled primer pairs, followed by ligation with adaptors and barcodes. After purification, libraries were quantified with a Qubit double-stranded DNA (dsDNA) HS assay kit on a Qubit 2.0 fluorometer, diluted to a concentration of 3 ng/µL, and pooled. The library pool was sequenced with Ion Torrent PGM (Personal Genome Machine) system, according to the manufacturer’s protocol. Briefly, the library pool was clonally amplified in an emulsion PCR reaction using ion sphere particles on the Ion OneTouch 2 instrument. Template-positive ion sphere particles were enriched on the Ion OneTouch enrichment system. Sequencing was performed with the Ion PGM Sequencing Supplies 200 v2 kit, according to manufacturer’s protocol. The libraries were loaded onto Ion 318 chips to generate sequencing data with a minimum depth of 10 000 reads. Variants were identified with the Integrative Genomics Viewer (Broad Institute, Cambridge, Massachusetts).
positive, some samples with one expected copy showed weakly positive signals (Figure 1). To understand the potential of our assays, we applied a recently developed Poisson distribution–based approach for testing boundaries of real-time PCR assays. Briefly, at a very low concentration, for example, at less than 10 copies per well, it is unlikely to make a dilution panel with all wells containing exactly the same number of copies as the template, which poses a hurdle to classic analytic assessment of LOD and sensitivity. Instead, the distribution of molecules per well followed the Poisson distribution (Figure 2, a and b), and the positive rates were analyzed with Poisson distribution statistics. We prepared 2 sets of dilution panels of 60 wells with 5 and 3 expected copies of RM2 per well (Figure 2, a and b) and ran the Q-PCR assay. If the assay’s lowest detectable copy number was X, any samples containing copy numbers no less than X should theoretically be detected, which is illustrated in Figure 2, c. Of 60 samples, 49 wells (81.7%) were positive for the 5-copy panel, indicating a lowest detectable copy number for EGFR E19del between 3 and 4 copies (95% CI, 1–9) (Figure 2, c). For the 3-copy panel, 31 wells showed as positive, indicating the same range of lowest detectable copy number at 3 to 4 copies. The same ranges were obtained from 3 independent experiments (Supplemental Table 2). The 2 sets of dilution panels were also tested for G719S and L858R (Supplemental Table 2), and results indicated the lowest detectable copy numbers between 4 and 5 (95% CI, 1–9) for G719S and between 3 and 4 (95% CI, 1–9) for L858R. Overall, our results suggested the EGFR Ultra assay achieved high sensitivity and specificity, and the detection boundaries could possibly be further extended to the single-digit copy-number level.

**Clinical Validation of the EGFR Ultra Assay With Paired Plasma and Tissues**

The 224 blood samples with paired tissues were collected from patients with NSCLC from January 2014 to June 2015. Of the 224 patients, 216 (96.4%) had adenocarcinoma, and 8...
(3.6%) had adenosquamous carcinoma; 47, 49, 26, 34, and 68 patients had stage I, II, IIIA, IIIB, and IV disease, respectively (Table 3; Supplemental Table 3). In total, 49 plasma samples were positive for the EGFR mutation. Clinical sensitivity for the EGFR Ultra assay with plasma compared with tissue was 53.3% (95% CI, 42.6–63.6), with a specificity of 100.0% (95% CI, 96.5–100.0), a positive prediction value of 100.0% (95% CI, 90.9–100.0), and a negative prediction value of 75.4% (95% CI, 68.2–81.5) (Table 3; Supplemental Table 3). The clinical sensitivity was significantly different in the 5 disease stages (P < .001). The sensitivity was as low as 10.0% (95% CI, 1.7–33.1) for stage I samples and increased enormously to 85.7% (95% CI, 66.4–95.3) for stage IV samples (P < .001) (Table 3; Supplemental Table 3). The sensitivity for late stage disease (stages IIIB and IV) was 81.4% (95% CI, 66.1–91.8), which was significantly higher than that of early stage disease (stages I to IIIA) at 28.5% (95% CI, 17.0–43.5) (P < .001). The assay had a high specificity of 100% (95% CI, 96.5–100.0) and a positive prediction value of 100% (95% CI, 90.9–100), thanks to the absence of false-positive results in the plasma samples. The clinical evaluation was further performed for specific mutations, which suggested good sensitivity for major mutations, such as, 56.1% for E19del and 58.5% for L858R (Supplemental Table 4). The clinical validation suggested the EGFR Ultra assay was reliable for patients with late-stage disease when tissue samples are not available.

Clinical Observations From 390 Consecutive Samples From Patients With NSCLC

In the central service laboratory, we used the EGFR Ultra assay to analyze EGFR mutation status in blood samples. Of the first 390 consecutive samples processed, 8 samples (2.1%) failed the quality control, either at the DNA extraction or the PCR steps. Of 382 plasma samples, 124 samples (32.4%) carried EGFR mutations for an overall positive rate at 32.5% (Table 4). The positive rates were significantly different at different disease stages (P < .001), probably because of different sensitivity for each stage. Of the 124 samples with positive results, 16 samples (12.9%) carried double mutations, including 4 samples (25.0%) carrying a combination of responsive mutation and resistant mutation (Supplemental Table 5). Previous studies reported the prevalence of EGFR mutations in NSCLC in an East Asian population as 41%,7 with a double-mutation rate ranging from 3% to 5%.26,27 Considering the assay sensitivity at the different stages of disease, the positive rates were consistent with previously reported EGFR mutation prevalence. Most mutations were L858R and E19del, whereas rare mutations were also detected (Figure 3, a), which is consistent with previously reported EGFR mutation distributions.5,7

We performed deep sequencing with a mini-EGFR sequencing panel targeting exons 18, 19, 20, and 21 and sequenced samples with weak, positive signals (37 < Ct value ≤39) at more than 10 000 times the coverage depth for all 4 exons to confirm the existence of the detected mutations. Among 12 samples with weak, positive Q-PCR signals, 6 (50%) had enough residual DNA for a confirmatory NGS assay. The NGS assay confirmed all 6 samples harboring expected mutations at a very low abundance. Figure 3, b, shows a representative IGV screen shot of one sample with an EGFR E19del at a mutation abundance of 0.12% (Figure 3, b). The key challenge for NGS to detect low concentrations of mutated DNA is that the real signal is often obscured by the noise, which might lead to a significant number of false-positives; therefore, the EGFR NGS assay remained as a confirmatory test.

DISCUSSION

The molecular analysis of ctDNA in patient plasma has become increasingly important in the management of patients with cancer, especially for patients with NSCLC, whose treatment options demand a clear understanding of the mutational profile of the key biomarkers such as EGFR. In the past decades, accumulated efforts have been made to develop sophisticated technologies permitting the isolation and identification of cancer-related mutations in plasma.12–22 Recently, 2 meta-analyses summarized the current progress by integrating clinical validation data with more than 2000 pairs of samples in both Asian and white populations,25,29 and the results emphasized high diagnostic accuracy, particularly high specificity, with ctDNA molecular testing. In the first meta-analysis, in which 19 of 20 studies
(95.0%) included were conducted within an Asian population, Luo et al\textsuperscript{28} reported pooled sensitivity and specificity as 67.4% (95% CI, 51.7–80.0) and 93.5% (95% CI, 88.8–96.3), respectively. Last year, 2 large, multicenter diagnostic studies, ASSESS (A Single-Arm, Investigator-Initiated Study of the Efficacy, Safety, and Tolerability of Intravitreal Aflibercept Injection in Subjects) and IGNITE (Ignite Genomics in Practice), investigated the utility of ctDNA for EGFR mutation analysis.\textsuperscript{2,7} In the ASSESS trial, in which 1162 pairs of plasma and matched tissues were examined, the sensitivity and specificity were 46% (95% CI, 38.8–53.4) and 97.4% (95% CI, 96.2–98.3).\textsuperscript{30} In the IGNITE trial, in which 1687 pairs of plasma and matched tissues from Asian Pacific patients were studied, the sensitivity and specificity were 49.6% (95% CI, 45.8–53.4) and 97.2% (95% CI, 96.0–98.1).\textsuperscript{7} In our study, the overall sensitivity and specificity were 53.3% (95% CI, 42.6–63.6) and 100.0% (95% CI, 96.5–100.0), which is consistent with previous data in the Asian population, as summarized above. Considering most previous studies were conducted with patients with late-stage disease, the performance of our assay with patients with late-stage disease, which was 81.4% (95% CI, 66.1–91.8) sensitivity and 100% (95% CI, 96.5–100.0) specificity, was, in fact, better than that in the 2 meta-analyses and 2 clinical trials, supporting the utility of the assay in clinical practice when tissue is not available.

Despite progress, several issues need to be solved before the wide adoption of the current assay and other ctDNA-based assays in daily clinical practice. First, we need to better understand the biology of ctDNA and how it influences the diagnostic sensitivity. Particularly, how much ctDNA is present in the circulation at each stage of disease and how tumor characteristics affect ctDNA availability. The information will help to define the intended use of the assay and the application of ctDNA-based assay and help clinicians understand the limitations and caveats of test results. Second, the preanalytic steps are important; we observed a variety of plasma sample-preparation protocols in various institutions, which introduces greater variability in assay performance. Therefore, we decided to adopt Streck cell-free DNA blood collection tubes, which contain a stabilizer preventing the release of gDNA, which dilutes ctDNA, making it more difficult to detect mutations. Meanwhile, we used a 2-step centrifugation to further remove cell debris and gDNA contamination. However, the preanalytic phase needs further optimization and standardization to achieve assay robustness, such as swift sample processing after blood draw and ctDNA quantification and quality control. In addition, although our assay could possibly detect mutations even with copy numbers less than 10, we defined the assay’s LOD as 10 copies, following the classic definition of LOD as the minimum copy numbers of mutated DNA copies that produce correct results 95% of the time. Thus, confirmation by other techniques is recommended for weak, positive samples. At the same time, to achieve a better clinical sensitivity, especially for patients with early stage disease, technology and innovations are critical for the development of timely and cost-effective assays that could achieve LOD of around 5 copies and less. Moreover, properly designed, prospective clinical studies that address key clinical issues will definitely help to take mutation analysis with ctDNA into clinical routine practice.

In summary, we have established a timely and cost-effective assay, the EGFR Ultra assay, to detect 45 EGFR mutations in plasma. The analytic assessment demonstrated
an LOD of 10 copy numbers, with a possible lowest detectable range at the single-digit level, which enables a blood-based mutation analysis with a moderately high clinical sensitivity, great specificity, and a mutation positive rate in patients with late-stage disease, consistent with previously reported EGFR mutation prevalence in Asian populations. All results supported the clinical utility of the ultrasensitive Q-PCR assay in EGFR mutation detection with ctDNA.

Figures 3.

**Figure 3.** Clinical observations from 390 consecutive samples. a, Distribution of EGFR E19del of a confirmatory next-generation sequencing assay with a weak, positive sample detected by the quantitative polymerase chain reaction assay.

References