Evolution and Clinical Impact of EGFR Mutations in Circulating Free DNA in the BELIEF Trial

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ABSTRACT

Introduction: Longitudinal evaluation of mutations in blood samples was a prespecified secondary objective in the BELIEF trial of erlotinib and bevacizumab in advanced EGFR-positive NSCLC. Here, we report the testing results and explore the correlation of EGFR status in blood with clinical outcomes.

Methods: Blood samples were prospectively collected from patients at baseline, at response evaluation, and at progression and sent to a central laboratory. Circulating free DNA was purified and EGFR mutations were analyzed with a validated real-time quantitative polymerase chain reaction assay.

Results: EGFR exon 19/21 mutations were detected in 55 of 91 baseline blood samples (60.4%) and correlated with a significantly worse progression-free survival: 11.4 months (95% confidence interval [CI]: 9.0–14.8 mo) for the patients who were positive versus 22.9 months (95% CI: 9.5–33.9 mo) for those who were negative (log-rank $p = 0.0020$). Among the 74 samples at response, exon 19/21 mutations were detected only in three samples (4.1%). In contrast, 29 of 58 patients (50.0%) were exon 19/21 positive at progression and showed a significantly worse median overall survival of 21.7 months (95% CI: 17.0–30.9 mo) compared with 37.4 months (95% CI: 22.6–53.1 mo) for those who were negative (log-rank $p = 0.011$). Blood samples at the three time points were available for 48 patients. Of those, among 14 exon 19/21 EGFR-negative at presentation, 13 (93%) were persistently negative for the sensitizing mutations after progression and the p.T790M could only be detected in the blood of two patients.

Conclusions: Longitudinal testing of EGFR mutations in blood can offer valuable clinical information. In patients of the BELIEF study, detection of EGFR mutations in circulating free DNA at presentation was associated with shorter progression-free survival, whereas positivity at progression correlated with shorter overall survival. Finally, patients negative in blood at presentation were almost invariably negative at relapse.

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Keywords: NSCLC; EGFR mutations in blood; cfDNA; Survival

Introduction

Tumor-derived circulating free DNA (cfDNA), also known as circulating tumor DNA (ctDNA), is routinely used to determine EGFR mutations at presentation, especially in patients whose tumor tissue is insufficient for genetic analysis.15 In a prospective study, we reported 72% objective responses and a median progression-free survival (PFS) of 11 months6 in patients with advanced NSCLC selected for tyrosine kinase inhibitor treatment solely on the basis of EGFR-testing performed in blood. Similar results were obtained in a phase II trial of 183 patients on gefitinib, with 72.1% response rate and a median PFS of 9.5 months.7

In addition to its diagnostic value, the prognostic role of EGFR mutations in blood has been evaluated in several clinical trials, sometimes with contradictory results. The first study in which mutations were assessed in ctDNA was the phase III EURTAC trial of erlotinib versus chemotherapy in patients with EGFR mutation. Activating EGFR mutations in baseline blood samples were centrally assessed with a peptide nucleic acid (PNA) probe-based 5′ nuclease real-time quantitative polymerase chain reaction (PNA-Q-PCR) assay.8 Positivity in baseline blood was found to be significantly associated with shorter overall survival (OS) in patients carrying the p.L858R mutation ($p = 0.03$) but not in those with exon 19 deletions.9 In the case of the LUX-Lung 3 and LUX-Lung 6 trials of afatinib versus chemotherapy in patients with EGFR mutation, blood mutations were centrally tested using a validated allele-specific quantitative real-time PCR kit (Therascreen).10 Patients with EGFR mutations in cfDNA showed a significantly shorter PFS and OS than those whose mutations could be detected exclusively in tissue ($p = 0.0037$ and 0.0003 for PFS and OS respectively in LUX-Lung 3 and both $p < 0.0001$ in LUX-Lung 6). This association was found not only in p.L858R patients but also in those with exon 19 deletions. Regarding dynamic alterations of EGFR status in blood during treatment, several studies report a longer PFS to tyrosine kinase inhibitors (TKIs) in patients whose EGFR mutations are undetectable in blood in samples obtained during treatment10,11 or in those showing clearance of EGFR mutations.7 In addition, an exploratory analysis of blood samples from the CTONG0901 clinical trial found a longer PFS in those patients whose p.L858R mutation in blood increased to its highest level at progression to erlotinib or gefitinib.12

Together with the prognostic value of EGFR mutations in blood, the presence and role of the p.T790M in treatment-naive tumors also constitute an area of active research. Despite some reports claiming that it might be artifactual,13,14 the presence of the p.T790M as a minor clone in a certain number of EGFR-mutant (EGFR-mut) tumors at presentation is now widely accepted.15-20 However, the frequencies reported vary from 1% to 70% of patients, probably as a consequence of the different sensitivities of the techniques employed to detect the mutation.15,20 Using a PNA-Q-PCR assay combined with microdissection of several tumor areas, we found the p.T790M in 30% to 35% of patients with EGFR mutation at presentation and reported that it associates with a shorter PFS to EGFR TKIs.21 To test this hypothesis clinically, we subsequently performed a proof-of-concept clinical trial, the BELIEF trial, in which
patients with EGFR-mut NSCLC stratified by the presence of the pretreatment p.T790M were administered erlotinib and bevacizumab, a combination that had demonstrated efficacy in p.L858R–p.T790M preclinical models. At a median follow-up of 21.4 months, the presence of the pretreatment p.T790M associated with a better outcome, with a median PFS of 16.0 months in the p.T790M-positive group versus 10.3 months in the p.T790M-negative patients.22

Here, we report the results of the EGFR-testing in the blood samples prospectively collected from the patients enrolled in the BELIEF trial and explore their correlation with clinical outcomes.

Materials and Methods

Cell Lines

PC9 was provided from Hofmann-La Hofmann Roche Ltd. under the authorization of Dr. Mayumi Ono, and BxPC-3 and H1975 human tumor cell lines were acquired from the American Type Culture Collection (Rockville, Maryland). Cells were cultured under standard conditions and authenticated by analyzing more than 20 polymorphisms by next-generation sequencing (NGS) and routinely tested for mycoplasma contamination. The PC9 and H1975 cell lines harbor exon 19 and p.T790M–p.L858R mutations in the EGFR gene, respectively, and the BxPC-3 cells are wild-type (wt) for EGFR. Genomic DNAs from the cell lines were purified using DNA Easy extraction kit (Qiagen, Hilden, Germany) and used as controls.

CfDNA Isolation From Serum and Plasma Samples

Paired serum or plasma samples (10 mL) were collected in vacutainer tubes (BD, Plymouth, United Kingdom) in each hospital participating in the BELIEF trial. A first centrifugation step (2300 rpm, 10 min) was performed in site; supernatants were transferred to a new tube, stored at −80°C, and sent to the central laboratory. Serum and plasma samples (1.2 mL) were submitted to a second centrifugation step followed by cfDNA purification with the QIAeasy DSP Virus and Pathogen Midi Kit, using a QIAasympyphony robot (Qiagen, Hilden, Germany). Extraction was performed from two aliquots of serum or two aliquots of plasma per patient. The final elution volume was 30 µL per aliquot.

PNA-Q-PCR Assay for EGFR Mutation Testing

EGFR mutations in cfDNA samples were determined using a fully validated and International Organization for Standardization 15189-accredited Q-PCR (Taqman) assay in the presence of a PNA clamp (Eurogentec, Seraing, Belgium) designed to inhibit the amplification of the wt alleles.6 The assay can detect and quantify the most common EGFR mutations, including p.T790M (Supplementary Table 1), with limits of detection of 2.5 pg mutant genotypes per µL and 0.005% mutant allelic fraction. In addition, the PNA-Q-PCR assay has shown a sensitivity of 76% and a specificity of 100% versus paired tumor tissue, comparable with other techniques such as COBAS, Therascreen, ddPCR, or NGS.6 Finally, we have recently performed a comparison study of the PNA-Q-PCR with the NGS GeneReader Platform (Qiagen, Hilden, Germany). Experimental details of the NGS on liquid biopsy samples have been described elsewhere.23,24 The study included 106 blood samples from patients with advanced NSCLC and found a concordance rate of 93.4% (99 of 106) between the two techniques for the testing of sensitizing EGFR mutations (Supplementary Table 2).

Briefly, amplification was performed in 12.5 µL volumes using 3 µL (for exon 21 analysis) or 1 µL (for exon 19 and p.T790M analysis) of cfDNA; 6.25 µL of Genotyping Master Mix (Applied Biosystems); 0.96 pmol of each primer; 1.2 pmol of probes; and 6.25 pmol (for exon 21 and p.T790M) or 62.4 pmol (for exon 19) of PNA. Samples were submitted to 50 cycles of 15 seconds at 92°C and 1.5 minutes at 60°C, in a QuantStudio 6 real-time PCR System (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA). The primers and probes used are available on request. Analyses were performed using two samples of serum or two samples of plasma cfDNA per patient. All samples were also assayed in the absence of PNA to confirm the presence of DNA and were considered suitable for analysis if there was amplification of the wt allele. Genomic DNAs from the PC9 or the H1975 cell lines at 5 ng/µL and at a one per 1000 dilution in DNA from the BxPC-3 cell line were used as positive controls for exon 19 deletions or p.T790M and exon 21 mutations, respectively. Extraction and non-template controls were added in each run. A sample was considered positive if the same mutant allele amplified at least in two of four aliquots of serum or plasma. If only one aliquot showed amplification, the four aliquots were retested and the sample was considered positive if the mutant allele amplified in at least one of them. Finally, the allelic fractions of the mutation in the positive samples were determined, as previously described.6

Statistical Analysis

Updated PFS and OS were analyzed in this study. PFS was defined as time from study enrollment until disease progression or death from any cause, whereas OS was defined as until death from any cause. Median PFS and OS, and also 24-month rates were estimated using the Kaplan-Meier method. Corresponding 95% confidence intervals (CI) using log-log transformation were also calculated. Differences in efficacy between mutation groups of patients were tested on the basis of log-rank statistic.

Blood (serum or plasma) samples were collected from patients at three time points: at baseline, at
response evaluation (6 weeks after start of treatment), and at progression (tumor rebiopsy at progression was not mandated by the trial protocol). Detection of mutation either in serum or plasma corresponds to positive mutation in blood. In addition, results on exons 19 (deletion 19) and 21 (L858R) were combined and analyzed together (EGFR exon 19/21); that is, positivity in either of them was treated as a cfDNA EGFR-positive result.

In an exploratory analysis, differences in patient and tumor baseline characteristics between cfDNA EGFR-positive and -negative patients at specific time points were assessed using either Fisher’s exact or Mann-Whitney U test for the categorical and continuous characteristics, respectively.

Time-varying Cox proportional hazards models were used to assess the longitudinal effect of cfDNA EGFR mutations over time on PFS and OS, adjusting for the following clinicopathological characteristics: Exon 19/21 blood, sex, smoking history, histology, performance status, pretreatment T790M mutation status in tissue, BRCA1, AEG1, radiotherapy, chemo-radiotherapy, age, brain metastasis, tumor volume (defined as the sum of diameters of target lesions at baseline measurement), and number of metastatic sites (defined as the number of nontarget lesions at baseline measurement). The backward elimination procedure with removal criterion \( p \) greater than 10% was used for inclusion of the significant covariates. Covariate categories with few patients were combined when appropriate. In the primary time-varying models, it was assumed that the cfDNA EGFR values at progression become true after a specific number of months from enrollment and hold true until the end of follow-up period. For patients without documented progression, the cfDNA measurement at response, if available, was used until the end of follow-up period. Sensitivity analyses for the time intervals in which the measurement at progression holds had been performed.

In addition, patients who were persistently cfDNA EGFR-positive, that is, positive at baseline and at progression, were compared with the remaining patients being negative on at least one time point.

The statistical analysis was implemented using SAS version 9.4.

Results

Updated Efficacy Results of the BELIEF Trial

The description of the cohort of 109 patients enrolled in the BELIEF trial from June 2012 to October 2014 has been already published. As of September 17, 2018, one patient (0.9%) was still on combination treatment (52.7 mo follow-up), whereas four (3.7%) continued with erlotinib single-agent (47.0, 48.4, 57.8, 66.6 mo follow-up). At a median follow-up of 52.5 months (interquartile range: 45.8–59.1 mo) for the full cohort of the 109 BELIEF patients, 98 (90%) PFS events had occurred with a 24-month PFS rate of 33% (95% CI: 24%–42%) and median PFS of 13.3 months (95% CI: 10.4–15.6 mo). The 37 tissue T790M-positive patients yielded a 24-month PFS rate of 45% (95% CI: 28%–60%) and median PFS of 18.4 months (95% CI: 12.6–33.5 mo), whereas the respective results for the 72 tissue T790M-negative patients were 27% (95% CI: 17%–38%) and 10.9 months (95% CI: 9.4–14.2 mo).

In addition, 71 deaths (65%) had been recorded. The 24-month OS rate was 58% (95% CI: 48%–67%) with median OS of 30.2 months (95% CI: 23.1–39.6 months) for all patients, 62% (95% CI: 44%–76%) and 30.9 months (95% CI: 17.7–50.7 mo), respectively, for the tissue T790M-positive, and 24-month OS rate of 57% (95% CI: 44%–67%) with median OS of 28.7 months (95% CI: 21.7–40.0 mo) for the tissue T790M-negative ones.

Among the 98 patients with PFS events, 87 had documented tumor progression. Of those, 19 patients (21.8%) did not receive second-line treatment, eight (9.2%) continued with treatment-beyond-progression on erlotinib or radiotherapy alone, whereas 60 (69.0%) received another second-line treatment (Supplementary Table 3). Of those 60, 17 patients had received osimertinib as second-line treatment. As an exploratory remark, the median OS for the 17 patients receiving second-line treatment with osimertinib was not reached (95% CI: 39.6–not evaluable) with 24-month OS rate of 94% (95% CI: 65%–99%), whereas for the 43 patients receiving other second-line treatment, the median OS was 27.7 months (95% CI: 21.4–32.9 mo), with 24-month OS rate of 56% (95% CI: 40%–69%) (log-rank \( p = 0.0016 \)). Of note, five of the 17 osimertinib-treated patients (29.4%) and nine of the 43 differently treated (20.9%) were T790M tissue-positive at baseline.

Baseline Detection of Exon 19/21 EGFR Mutations in Blood Associates With Shorter PFS

A total of 223 blood samples were available for genetic analysis: 91 at baseline, 74 at response evaluation, and 58 at progression. Of the 91 baseline samples, 55 of 91 (60.4%) were positive for EGFR exon 19/21 mutations (cfDNA EGFR+) (Supplementary Fig. 1, Supplementary Table 4). The detection rate of sensitizing mutations when only serum was available was 57%, as compared with plasma which was 64% (Supplementary Table 5). The presence of the mutation in pretreatment blood (serum or plasma) was significantly associated with shorter PFS. Median PFS was 11.4 months (95% CI: 9.0–14.8 mo) for the 55 cfDNA EGFR-
positive patients versus 22.9 months (95% CI: 9.5–33.9 mo) for the 36 cfDNA EGFR-negative patients (log-rank \( p = 0.0020 \), Fig. 1, and adjusted Cox \( p = 0.0036 \), Supplementary Table 6). In contrast, EGFR status in pretreatment blood did not significantly associate with median OS, which was 27.0 months (95% CI: 19.6–39.6 mo) for the cfDNA EGFR-positive patients versus 36.6 months (95% CI: 17.4–51.8 mo) for cfDNA EGFR-negative patients, (log-rank \( p = 0.45 \), Supplementary Fig. 2, adjusted Cox \( p = 0.61 \), Supplementary Table 7).

**Detection of Exon 19/21 EGFR Mutations in Blood at Progression Associates With Shorter OS**

Among the 74 blood samples collected at response evaluation after 6 weeks of treatment, exon 19/21 EGFR mutations could be detected only in three samples (4.1%). In contrast, 29 of 58 patients (50.0%) were positive at progression. Remarkably, the detection of EGFR mutations in blood at relapse significantly associated with a shorter OS (Fig. 2). Median OS was 37.4 months (95% CI: 22.6–53.1 mo) for the 29 cfDNA EGFR-
negative patients at progression, but only 21.7 months (95% CI: 17.0–30.9 mo) for the remaining 29 samples in which EGFR mutations could be detected (log-rank \( p = 0.011 \)). In a multivariate Cox analysis, the detection of exon 19/21 mutations in blood at progression, together with brain metastases, emerged as the only independent factor associated with OS (adjusted Cox \( p = 0.025 \); Supplementary Table 6).

The T790M resistance mutation was detected in the blood of 19 of 58 patients (33%) at progression (Supplementary Table 9); but in contrast to EGFR sensitizing mutations, it did not significantly associate with outcome. Patients positive for p.T790M showed median PFS and OS of 9.4 months (95% CI: 7.8–13.1 mo) and 27.0 months (95% CI: 17.0–34.8 mo), respectively; not significantly different from the 11.4 months (95% CI: 8.6–16.0 mo) and 30.9 months (95% CI: 18.4–45.1 mo) observed in the patients with p.T790M-negative blood at progression.

**Longitudinal Analysis Indicates Association of EGFR Mutations in Blood at Any Time Point With Worse Outcome**

Blood samples at all the three time points were available for 48 patients (Fig. 3, Supplementary Table 10). Fourteen patients were exon 19/21-negative in blood at presentation. All of them were also negative at response evaluation and sensitizing mutations appeared only in one blood sample (7%) after progression, whereas the T790M was detected in two patients at progression (14%). In contrast, among the 34 patients positive at presentation, three (9%) were persistently positive at response evaluation and relapse, whereas the exon 19/21 mutations reappeared at progression in 24 patients (71%). At progression, the T790M was found in 17 blood samples, all except one, corresponding to patients also positive at the sensitizing mutations (Fig. 3).

The 13 patients consistently negative for EGFR mutations in blood had PFS and OS of 13.6 months (95% CI: 4.9–26.9 mo) and 35.9 months (95% CI: 12.6–45.1 mo), which were not significantly different from the seven patients positive only at presentation and negative at response and progression (13.1 mo and “not reached” median PFS and OS, respectively) (Fig. 3). Regarding the 24 patients with reemergence of the exon 19/21 mutation, PFS was 10.6 months (95% CI: 8.4–14.7 mo) and OS 22.4 months (95% CI: 17.0–34.8 mo). Finally, the three patients positive at all time points showed particularly poor PFS (1.4, 4.1 and 4.3 mo) and OS (4.2, 16.9 and 22.8 mo).

Overall, the longitudinal effect of cfDNA EGFR 19/21 mutation on PFS and OS was further explored through the use of time-varying Cox models (Table 1), using information on cfDNA EGFR mutation at all time points and adjusting for variables of clinical interest. The analysis indicated a significant effect of the existence of cfDNA EGFR 19/21 mutation, with patients EGFR 19/
21-positive at any time point and having worse results, both on PFS (with hazard ratio \[\text{HR}_{\text{Positive versus Negative}} = 2.34, 95\% \text{ CI: 1.33–4.11, } p = 0.0031\]) and OS (with \[\text{HR}_{\text{Positive versus Negative}} = 2.12, 95\% \text{ CI: 1.14–3.91, } p = 0.017\]). Sensitivity tests confirmed these significant effects.

**EGFR Mutations in Blood Correlate With Mutations in Baseline Biopsies**

At all time points, the type of exon 19/21 EGFR mutations identified in blood were systematically concordant with those found in baseline biopsies. The correspondence of pretreatment p.T790M mutation status and the results in blood at progression is presented in Table 2. Of 17 tissue T790M-positive patients, the 41\% were also positive in blood at progression; whereas of the 41 patients negative in tissue, 29\% were positive in blood at progression (Fisher’s exact \(p = 0.54\)). If a cutoff of a 0.05\% allelic fraction in tissue was selected, seven of 12 (58\%) patients p.T790M-positive in tumor at presentation showed the resistance mutation at progression, compared with 12 of 46 (26\%) of the p.T790M-negative patients in baseline biopsy (\(p = 0.045\)). Remarkably, among patients harboring the p.T790M mutation at baseline, the sensitizing mutation in blood samples at progression was systematically accompanied by the p.T790M (Supplementary Fig. 3).

### Discussion

To our knowledge, BELIEF is the first trial in which the predictive value of EGFR mutation status in blood at presentation, response evaluation, and progression, in a cohort of patients treated with an EGFR TKI, has been systematically evaluated. Genetic testing of serum or plasma samples at these three time points was incorporated as a prespecified secondary analysis. The highest frequency of positivity in blood was found in samples at presentation (60\%), followed by liquid biopsies at progression (50\%); whereas only three of 74 patients (4\%) were positive in blood at response evaluation. Although a 46\% detection rate of EGFR mutations in pretreatment cfDNA has been reported in the large ASSESS diagnostic study, and a detection rate of 29\% to 61\% in the LUX-Lung 3 and 6 trials in most liquid biopsy studies, the detection rate is around 70\% to 75\%. The relatively low frequency of detection of EGFR mutations in blood in our study can be explained by the fact that in many cases, only serum was collected at presentation, and sensitivity of mutation detection in this fluid has been described to be lower than in plasma. However, even when the most sensitive detection techniques are used, the percentage of patients with EGFR mutation positive in liquid biopsies at presentation only reached 80\% to 85\%. The negative samples correspond to tumors not shedding ctDNA to blood owing to a variety of factors, such as lack of

### Table 1. Time-Varying Multivariable Cox Proportional Hazards Models for PFS and OS Adjusted for Variables of Clinical Interest on the Full Cohort of 109 BELIEF Patients

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>Cox Results for PFS (98 Events)</th>
<th>Cox Results for OS (71 Events)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio 95% CI p Value</td>
<td>Hazard Ratio 95% CI p Value</td>
</tr>
<tr>
<td>EGFR Exon 19/21 in blood (time-varying)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive vs. Negative</td>
<td>2.340 (1.332, 4.112) 0.0031</td>
<td>2.115 (1.144, 3.910) 0.017</td>
</tr>
<tr>
<td>Not Available vs. Negative</td>
<td>0.009 (0.617, 1.649) 0.97</td>
<td>1.392 (0.771, 2.514) 0.27</td>
</tr>
<tr>
<td>Brain metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes vs. No</td>
<td>1.723 (1.043, 2.848) 0.034</td>
<td>2.487 (1.444, 4.284) 0.0010</td>
</tr>
</tbody>
</table>

The following variables were initially used in the model selection process: Exon 19/21 blood (time-varying), sex, smoking history, histology, performance status, T790M in tissue, BRCA1, AEG1, radiotherapy, chemo-radiotherapy, age, brain metastasis, tumor volume, and number of metastatic sites.

PFS, progression-free survival; OS, overall survival; CI, confidence interval.

### Table 2. Association of T790M Mutation in Blood at Progression With Pretreatment T790M in Tissue (N = 58)

<table>
<thead>
<tr>
<th>T790M Mutation in Blood at Progression</th>
<th>Pretreatment T790M in Tissue</th>
<th>(\geq 0.05% \text{ AF} )</th>
<th>(&lt;0.05% \text{ AF} )</th>
<th>Median AF</th>
<th>Total No. of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>7 (41%)</td>
<td>12 (29%)</td>
<td>7 (58%)</td>
<td>12 (26%)</td>
<td>19</td>
</tr>
<tr>
<td>Negative</td>
<td>10 (59%)</td>
<td>29 (71%)</td>
<td>5 (42%)</td>
<td>34 (74%)</td>
<td>39</td>
</tr>
<tr>
<td>Fisher’s exact (p) value</td>
<td>(p = 0.54)</td>
<td>(p = 0.045)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All available patients</td>
<td>17 (100%)</td>
<td>41 (100%)</td>
<td>12 (100%)</td>
<td>46 (100%)</td>
<td>58</td>
</tr>
</tbody>
</table>

AF, Allelic fraction.
vascularization, low mitotic rate, or metastatic disease circumscribed to central nervous system.\textsuperscript{26-28}

The first relevant finding of our study refers to the serial monitoring of liquid biopsies. Among 14 patients with \textit{EGFR} mutation negative in blood at presentation, 13 (93\%) were persistently negative for sensitizing mutations after progression, and p.T790M was absent in the blood of 12 (86\%) (Fig. 3). This correlation indicates that most baseline “nonshedding” tumors\textsuperscript{26} maintain this status at relapse to targeted therapies. Consequently, mutation testing in liquid biopsies to monitor the course of the disease, including early detection of relapse, is unlikely to give relevant information in these cases. In addition, blood analyses to determine the presence of the p.T790M at progression to first- or second-generation \textit{EGFR} TKIs will usually yield negative results, making tissue rebiopsies necessary. In contrast, 79\% of patients positive in blood for the sensitizing mutations at presentation were also positive at progression, and 50\% showed a detectable p.T790M, demonstrating the usefulness of serial monitoring of liquid biopsies in shedding tumors.

Our study has also systematically evaluated the detection of \textit{EGFR} mutations in blood at presentation, response evaluation, and progression as a prognostic factor in patients treated with the combination of erlotinib and bevacizumab. In the case of baseline samples, we have found that patients who were positive for \textit{EGFR} mutation had a significantly shorter PFS (median 11.4 versus 22.9 mo, log-rank \( p = 0.0020 \)) to erlotinib and bevacizumab, which was the combination of drugs used in the trial. In contrast, although there was a trend toward a poorer OS (27.0 versus 36.6 mo), it did not reach statistical significance. These results are coincident with those reported for the LUX-Lung 3 and LUX-Lung 6 clinical trials of afatinib in patients with \textit{EGFR}-mut NSCLC. Outcome was significantly worse in patients of the LUX-Lung 3 study who were positive in serum, with PFS and OS of 8.3 months and 22.1 months, respectively, compared with 13.7 months and 33.6 months, respectively, in patients who were negative (\( p = 0.0037 \) and 0.0003 respectively). In the case of LUX-Lung 6, plasma was used instead of serum, and the results were 9.7 months and 20 months for patients who were positive versus 16.6 months and 35.6 months for those who were negative (both \( p < 0.0001 \)). Similar results were obtained in the ASPIRATION trial, in which patients with \textit{EGFR} mutation in tumor and plasma at presentation had PFS and OS to erlotinib treatment of 9.2 and 26.3 months, respectively, compared with 18.4 months and “not reached” in patients with \textit{EGFR} mutation detected in tumor but not in plasma.\textsuperscript{29}

A number of studies have also investigated the clinical value of \textit{EGFR} mutation analysis in blood at response evaluation. In the FASTC-2 trial of erlotinib intercalated with chemotherapy, 53 of 80 patients (66\%) were \textit{EGFR} mutation-negative in blood at cycle 3, and showed longer PFS and OS, with medians of 12.0 months and 31.9 months, respectively, versus 7.2 months and 18.2 months, respectively, in patients who were positive.\textsuperscript{11} Comparable results were obtained in the BENEFIT trial. Of 167 patients with blood samples available, 88\% showed clearance of \textit{EGFR} mutations in cfDNA at week 8 after initiation of gefitinib treatment, which correlated with a significantly longer PFS of 11.0 months versus 2.1 months in patients with persistence of the mutation in blood (\( p < 0.0001 \)).\textsuperscript{7} OS was not reported. In contrast, in the UMIN 13806 trial enrolling patients with \textit{EGFR} mutation for afatinib treatment, only four of 30 blood samples (13.3\%) at week 4 were positive, but median PFS was not significantly longer in patients with undetectable \textit{EGFR} mutations at baseline or response evaluation.\textsuperscript{30} In our cohort of 74 patients with cfDNA available at response evaluation, \textit{EGFR} mutations were only detected in three (4\%). Remarkably, these three patients had very poor outcomes, with PFS of 1.4, 4.1, and 4.3 months, in contrast with the median PFS of 10.9 and 11.7 months in patients with mutation clearance and double negative, respectively.

To complete our longitudinal analysis, we investigated the clinical value of \textit{EGFR} mutation detection at progression. Although several studies have reported frequent appearance of the \textit{EGFR} mutations in the blood of patients progressing to TKIs,\textsuperscript{11,30-33} the prognostic value of \textit{EGFR} status at this time point has not been fully evaluated. To the best of our knowledge, there is only one report in the literature in which blood was collected from 108 patients from the CTONG 0901 clinical trial carrying the p.L858R mutation and treated with erlotinib or gefitinib. In this cohort, median PFS in patients with the allelic fraction of the mutation increasing to its highest level when the disease progressed was 11.1 months, compared with 7.5 months in patients with a stable allelic fraction of the p.L858R at progression.\textsuperscript{12} OS was not evaluated. In our study, the detection of \textit{EGFR} sensitizing mutations in blood at progression emerged as the only independent factor associated with OS together with brain metastases. Patients with positive liquid biopsies at relapse had a median OS of 21.7 months compared with 37.4 months for negative patients. In contrast, the p.T790M, which appeared in the blood of 19 of 58 patients (33\%) at progression, did not significantly associate with outcome.

Finally, we analyzed the possible association of the p.T790M detected in blood samples at progression and the presence of the resistance mutation in pretreatment tumor biopsies. The p.T790M cannot be detected by standard techniques in most \textit{EGFR}-mut tumor samples at
presentation. However, the BELIEF clinical trial included testing of the pretreatment p.T790M using a sensitive PNA-Q-PCR technique designed to detect low abundance mutations. If a cutoff of a 0.05% allelic fraction was employed, seven of 12 patients (58%) positive for the p.T790M in tumor tissue baseline had a detectable resistance mutation in blood at progression, compared with only 12 of 46 patients (26%) p.T790M-negative baseline, and the association was marginally significant ($p = 0.045$). In addition, among patients p.T790M-positive at presentation, the reappearance of the sensitizing mutations in blood at progression was always accompanied by the emergence of the p.T790M. These findings support previous reports indicating that, at least in a percentage of patients with EGFR mutation, treatment with EGFR TKIs leads to a clonal amplification of the extremely low number of cells initially harboring the p.T790M. However, we also found a number of patients whose p.T790M emerged in blood at progression despite the absence of the mutation in baseline tumors biopsies, even as a minor clone. Therefore, at least in this group of patients, the p.T790M could emerge de novo caused by mechanisms such as the recently described expression of the activation-induced cytidine deaminase after EGFR TKI treatment, which deaminates the 5-methylcytosine to thymine at position c.2369 and generates the p.T790M mutation.

In summary, we have performed a comprehensive longitudinal analysis of the EGFR mutation status in liquid biopsies from patients enrolled in the BELIEF trial of erlotinib plus bevacizumab. Our results not only confirm previous reports indicating an association of positivity in baseline blood with shorter PFS to TKI treatment but also indicate that patients with EGFR sensitizing mutations undetectable in blood at progression have a significantly longer OS (adjusted Cox $p = 0.025$). Finally, our findings support the idea of distinct evolutionary trajectories of EGFR-mut tumors, so that the p.T790M at progression can derive from the selection of preexisting EGFR T790M-positive clones or emerge de novo in initially negative cells.

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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 https://doi.org/10.1016/j.jtho.2019.11.023.

References


