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Early Prediction of Response to Tyrosine Kinase Inhibitors by Quantification of *EGFR* Mutations in Plasma of NSCLC Patients

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Introduction: The potential to accurately quantify epidermal growth factor receptor (*EGFR*) mutations in plasma from non–small-cell lung cancer patients would enable more rapid and more frequent analyses to assess disease status; however, the utility of such analyses for clinical purposes has only recently started to explore.

Methods: Plasma samples were obtained from 69 patients with *EGFR*-mutated tumors and 21 negative control cases. *EGFR* mutations in plasma were analyzed by a standardized allele-specific polymerase chain reaction (PCR) test and ultra-deep next-generation sequencing (NGS). A semiquantitative index (SQI) was derived from dilutions of known *EGFR* mutation copy numbers. Clinical responses were evaluated by Response Evaluation Criteria in Solid Tumors 1.1 criteria and expressed as percent tumor shrinkage.

Results: The sensitivity and specificity of the PCR test and NGS assay in plasma versus tissue were 72% versus 100% and 74% versus 100%, respectively. Quantitative indices by the PCR test and NGS were significantly correlated ($p < 0.001$). *EGFR* testing at baseline and serially at 4 to 60 days during tyrosine kinase inhibitor therapy revealed a progressive decrease in SQI, starting from day 4, in 95% of cases. The rate of SQI decrease correlated with percent tumor shrinkage at 2 months ($p < 0.0001$); at 14 days, it was more than 50% in 70% of patients (rapid responders). In two patients with slow response, an early increase in the circulating levels of the T790M mutation was observed. No early T790M mutations were seen in plasma samples of rapid responders.

Conclusions: Quantification of *EGFR* mutations from plasma with a standardized PCR test is feasible. To our knowledge, this is the first study showing a strong correlation between the *EGFR* SQI in the first days of treatment and clinical response with relevant implications for patient management.

Key Words: EGFR mutations, NSCLC, Plasma, Next-generation sequencing, Predictive medicine.

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Assessment of epidermal growth factor receptor (*EGFR*) mutations in non–small-cell lung cancer (NSCLC) patients is mandatory for proper treatment choice and is usually performed on tissue or cytological samples.[1–4] However, the lack of neoplastic tissue may be a critical issue preventing molecular characterization. For example, in the phase 3 IPASS study, only 42% of the patients had biopsied tissue suitable for molecular testing, and in the phase 3 INTEREST study, only 31% had adequate tissue.[5,6] Several reports indicate that *EGFR* mutations can be detected in plasma or serum of NSCLC patients.[7–12] Although mutation analysis in tissue has been required as a reflection of disease state, the development and validation of analytical tools using plasma as the primary material has been pursued only recently.

Neoplastic tissue remains the definitive standard for molecular analysis, and available in vitro diagnostic assays for clinical application have been validated using tumor tissue. However, the

potential for analyzing plasma to assess disease mutation status is attractive for several reasons, including noninvasive sampling compared with biopsy, enabling increased sampling frequency, testing in critically ill patients, and testing at diagnosis when tumor samples are unavailable. Blood collection is less invasive than tissue sampling, can be used where tissue specimens are limited, can be repeated frequently over time, or may be the only option for critically ill patients. Finally, plasma testing may reflect overall disease status compared with a conventional, small sample from a single tumor site. During treatment, plasma analysis could reveal the emergence of *EGFR* treatment–resistance mutations, possibly well in advance of clinical progression.

As DNA analytical methods have become more sensitive, attempts to develop methods to assess tumor mutation status by evaluating patient plasma samples are now being pursued. This study evaluated the ability to quantify the presence of *EGFR*-mutated alleles in plasma of NSCLC patients for early prediction of response to tyrosine kinase inhibitor (TKI) therapy. We compared two different methods for quantifying *EGFR* mutations in NSCLC patient plasma samples, specifically, the cobas® *EGFR* Mutation Test ("PCR test") and ultra-deep next-generation sequencing (NGS). We then quantitatively monitored *EGFR* mutations in plasma samples from an independent prospective series of patients, at baseline and serially, during the first days of treatment with TKIs. *EGFR* mutation data were compared with clinical response.

PATIENTS AND METHODS

Patients

Two hundred and three peripheral blood samples were collected from 85 subjects including: (1) 42 NSCLC patients harboring *EGFR* mutations in primary tumor tissue, enrolled in the TRIGGER trial (see below), where plasma samples were collected at baseline, before first-line treatment with erlotinib (Tarceva; Genentech), and immediately after progression; (2) 27 previously untreated stage IIIB-IV NSCLC patients, carrying *EGFR* mutations in tumor specimens, for whom plasma was collected at baseline and serially at 4 to 60 days during erlotinib therapy; and (3) 21 control cases, comprising 10 breast carcinoma patients negative for *EGFR* mutations and 11 healthy donors.

TRIGGER is a phase II, open-label study of TaRceva® (erlotinib) treatment In chemonaive patients with locally advanced or metastatic non-small-cell lunG cancer who present activatinG mutations in the tyrosine kinase domain of the Epidermal growth factor Receptor; clinical Trials.gov identifier: NCT01378962. The main objective of the study was to evaluate the efficacy of erlotinib (150 mg) in terms of 12-month progression-free survival. An exploratory objective of the TRIGGER study was to evaluate the correlation between *EGFR* testing in tumor biopsies and plasma samples at baseline and tumor progression. Study details will be described in a future publication.

Written informed consent was obtained from all patients under study. Approval from independent regional Ethics Committees was obtained for all patients. The study was conducted in accordance with the precepts of the Helsinki Declaration.

Blood Sample Collection and DNA Extraction

At each collection, two blood samples containing 5 ml were collected in BD VACUTAINER PPT K2EDTA tubes (BD Diagnostics, Buccinasco (Milan), Italy) and subjected locally to plasma separation, within 30 minutes, using a shared protocol. After centrifugation at 1000 rpm for 15 minutes, the plasma was aliquoted and stored at −80°C until DNA extraction by the Center of Predictive Molecular Medicine (University-Foundation, Chieti, Italy). DNA was extracted from 4 ml of plasma using the cobas® cfDNA Sample Preparation kit (under development by Roche Molecular Systems, Pleasanton, CA), according to the manufacturer's instructions. DNA was recovered in 200 µl of elution buffer. The DNA amount was not measured to avoid loss of material. Half of the volume was used for PCR assay and the other half for ultra-deep NGS analysis.

Assessment of *EGFR* Mutations from Tissue Samples and Plasma DNA

EGFR mutation analysis in tissue samples at the time of diagnosis was conducted in the collaborating clinical centers by Sanger sequencing or other conventional techniques and confirmed by Sanger sequencing. The plasma test used in this study is an allele-specific polymerase chain reaction (PCR) assay designed to detect 41 *EGFR* mutations: G719A/C/S in exon 18; 29 deletions in exon 19; S768I, T790M, five insertions in exon 20; and L858R in exon 21. Analysis was confirmed by negative and positive controls contained in the kit. The PCR reactions were run on the cobas® z 480 analyzer with *EGFR* Blood Analysis Package Software (in development, Roche Molecular Systems, Pleasanton, CA). A semiquantitative index (SQI) was created to reflect a trend for the proportion of mutated versus wild-type copies of the *EGFR* gene. The SQI was derived from a dilution series containing known copy numbers of mutated *EGFR* and a fixed amount of wild-type *EGFR*, with the wild-type DNA serving as an internal control during real-time PCR. The SQI is reported as an automated result from the cobas® z 480 software when an EGFR mutation is detected in ctDNA. A positive correlation of the SQI to copy number and reproducibility of the data was demonstrated by linearity studies with spike-in control mutations (data not shown).

Ultra-deep NGS was performed as follows. DNA fusion primers containing genome-specific sequences, along with one of seven distinct 10-base pair multiplex identifier sequences, and sequencing adapters were used to amplify specific regions of exons 18, 19, 20, and 21 of the *EGFR* gene (NM_005228.3), as previously described, and were used to distinguish samples run on the same plate.[13] Different strategies to maximize sensitivity and avoid cross-contamination were adopted as previously described.[14] Processed and quality-filtered reads were analyzed with the GS Amplicon Variant Analyzer software version 2.7 (454 Life Sciences). NGS analysis was repeated in cases with mutations in less than 1% of the DNA molecules. The percentage of the mutant allele in the whole number of sequenced molecules was used as a quantitative index.

The variables measured in the study were investigated for association by regression analysis and independent samples two-sided *t* test. A *p* value less than 0.05 was considered

as significant. Statistical analyses were performed using SPSS version 22 (SPSS, Chicago, IL).

RESULTS

Plasma Samples

A total of 203 plasma samples from 69 patients and 21 control cases were examined. The TRIGGER blood samples at baseline and progression were analyzed by both PCR and ultra-deep NGS, two different techniques which allow to quantify *EGFR* mutations in plasma. In an independent prospective series, monitoring of the *EGFR* SQI was performed by the PCR test at baseline and serially during the first days of TKI treatment.

EGFR Mutation Detection by PCR

EGFR mutation analysis in tissue samples of the 42 NSCLC patients enrolled in the prospective TRIGGER trial showed an exon 19 deletion in 31 cases (74%) and an L858R point mutation in exon 21 in 11 cases (26%) (Supplementary Table S1, Supplemental Digital Content 1, http://links.lww.com/JTO/A863). Plasma samples were collected from these 42 patients before treatment with erlotinib. Plasma samples were also obtained after progression in 15 patients during the 12-month follow-up. With baseline plasma, 30 cases (71%) were mutated with the *EGFR* PCR test and corresponded exactly with mutations present in matching tissue samples. At progression, 11 of 15 plasma samples (73%) showed the same sensitizing mutation detected in tumor tissues. In five of these 11 cases (45%), the T790M mutation was concomitantly detected. No mutations were present in the 21 control cases. Overall, the sensitivity and specificity of the PCR test were 72% and 100%, respectively.

Mutation Detection by Ultra-Deep NGS

Ultra-deep NGS was conducted with a mean coverage of 20,230 ± 1410 sequences per sample. In the series of 42 patient plasma samples at baseline, 31 cases (74%) were mutated, with mutations corresponding to those in the matching tumor sample. Eleven of the 15 plasma samples (73%) at progression showed the same sensitizing mutation detected at baseline. No mutations were detected in the 21 control cases. The ultra-deep NGS assay showed a sensitivity of 74% and a specificity of 100%. The T790M mutation was detected in the five plasma samples that were also positive for this mutation by the PCR test (Supplementary Table S1, Supplemental Digital Content 1 [http://links.lww.com/JTO/A863] and Table 1).

EGFR Mutation Detection: PCR versus Ultra-Deep NGS

Table 1 reports the quantification of *EGFR* mutations in plasma samples from the TRIGGER study, by the PCR test and ultra-deep NGS, in *EGFR* exons 19 and 21 at baseline, and for the T790M mutation in exon 20 at progression. The results indicate a strong exponential relationship between the PCR test and ultra-deep NGS indexes ($R^2 > 95\%$; $p < 0.0001$; Fig. 1). The PCR test is based on a mutant-specific PCR amplification, resulting in an enrichment of mutant molecules, whereas the ultra-deep NGS assay was performed with conventional PCR.

TABLE 1. Comparison of Quantification Data by PCR versus Ultra-Deep NGS

Case No.	Type of Mutation	NGS	PCR
21	Exon 19 Del	0.02	5.99
23	Exon 19 Del	0.05	8.29
6	Exon 19 Del	0.06	8.49
10	Exon 19 Del	0.06	8.62
4	Exon 19 Del	0.07	8.91
22	Exon 19 Del	0.24	9.50
35	Exon 19 Del	0.27	9.55
37	Exon 19 Del	0.29	10.04
42	Exon 19 Del	0.33	10.20
15	Exon 19 Del	0.80	12.07
31	Exon 19 Del	0.81	12.44
19	Exon 19 Del	0.88	12.45
25	Exon 19 Del	0.98	13.59
2	Exon 19 Del	1.51	13.74
8	Exon 19 Del	1.57	13.91
32	Exon 19 Del	0.81	13.97
30	Exon 19 Del	1.68	14.86
13	Exon 19 Del	3.05	15.82
5	Exon 19 Del	3.84	16.18
28	Exon 19 Del	6.42	16.88
16	Exon 19 Del	45.29	21.58
20	Exon 19 Del	55.83	20.62
26	Exon 19 Del	72.06	19.88
17	L858R	0.21	7.4
18	L858R	0.22	8.04
36	L858R	1.37	10.74
14	L858R	1.76	11.48
7	L858R	31.38	14.39
38	L858R	41.17	15.29
31	T790M	0.16	11.34
28	T790M	0.91	13.11
26	T790M	2.46	14.57
20	T790M	3.55	14.70
7	T790M	24.5	17.08

NGS, next-generation sequencing; PCR, polymerase chain reaction.

Quantification data from ultra-deep NGS show that 18 of the 34 mutations (53%) detected in plasma were present in less than 1% of the reads, indicating that sensitive mutation detection techniques must be used to screen plasma samples for *EGFR* mutations. The PCR test was also extremely sensitive, detecting mutations present in as low as 0.02% of the molecules (Table 1).

In 12 plasma samples (29%) from TRIGGER patients at baseline, the PCR assay failed to find *EGFR* mutations. Results from two cases analyzed by NGS were in agreement with tissue samples but were not detectable by PCR

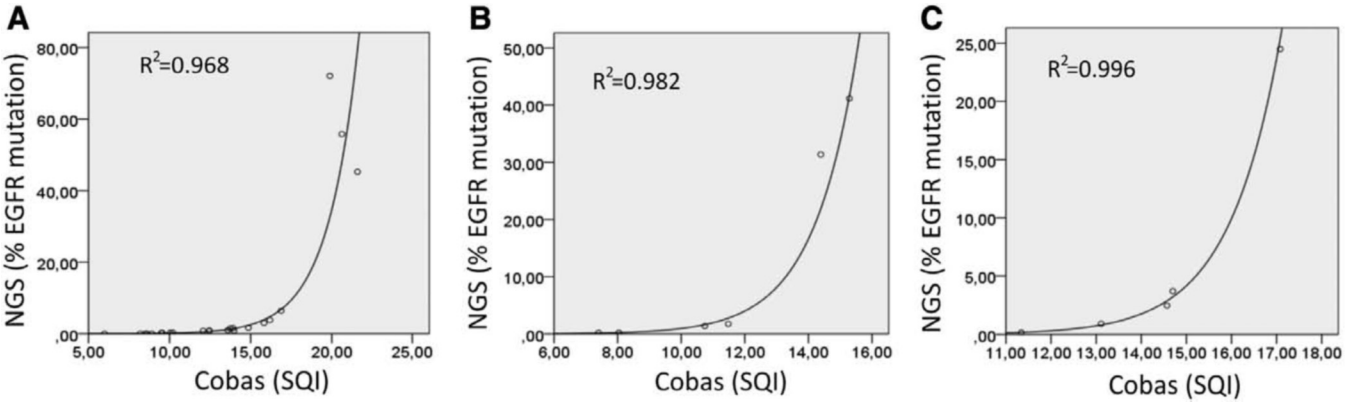

FIGURE 1. Detection of *EGFR* mutations with PCR versus ultra-deep NGS. Data were plotted for deletion in exon 19 (23 patients) (*A*), L858R in exon 21 (six patients) (*B*), or T790M in exon 20 (five patients) (*C*). NGS, next-generation sequencing; PCR, polymerase chain reaction.

testing (an extremely rare type of exon 19 deletion, p.E747_A749del and the L861Q mutation) (Supplementary Table S1, Supplemental Digital Content 1, http://links.lww.com/JTO/A863); in 11 of TRIGGER plasma samples (26%) at baseline, the ultra-deep NGS analysis could not detect an *EGFR* mutation. In one of these cases identified with PCR (patient number 23), an L858R mutation was observed in a very low proportion of the molecules (SQI, 4.99), in keeping with tissue data (Supplementary Table S1, Supplemental Digital Content 1, http://links.lww.com/JTO/A863). Overall, by using the two different approaches, 32 of 42 cases (76%) in the TRIGGER series were found to carry an *EGFR* mutation in plasma.

Plasma Monitoring of *EGFR* Mutations

To assess the potential clinical utility of monitoring *EGFR* mutations in plasma at initiation of treatment with TKIs, we assessed *EGFR* mutations in plasma samples of 27 previously untreated patients carrying *EGFR* mutations in their primary tumors at baseline and serially at 4, 8, 13, 18, 25, 35, and 60 days during TKI therapy. Seven of these patients (26%) were negative at baseline. SQI curves indicating the amount of mutant *EGFR* in plasma of 20 patients during the first days of treatment are reported in Figure 2. With serial testing, the *EGFR* SQI demonstrated a progressive decrease starting from day 4 of therapy in 95% of cases. After 4 days of treatment, we observed an average percent decrease of 13.5%. At 8 days, the average percent decrease was 41.6% and at 14 days 63.5%. In all but two patients, plasma samples became negative for mutant *EGFR* alleles in a time range of 8 to 60 days (mutation clearing time). The rate of SQI decrease was greater than 50% at 14 days in 14 patients (70%) (rapid responders) and lower than 50% at 14 days in six patients (30%) (slow responders) (Fig. 3) and was directly correlated with PTS at 2 months, evaluated by Response Evaluation Criteria in Solid Tumors 1.1 criteria (Table 2). The mean percentage of tumor shrinkage (PTS) was 59.1 ± 1.8 in the rapid responders and 18.3 ± 3.7 in the slow responders ($p < 0.0001$). An *EGFR* mutation in the plasma of two slow responders was not completely cleared by TKI treatment. In these two patients, the T790M mutation was unexpectedly detected very early after initiating therapy, at 35 days, and further increased in the following days (Fig. 3). These cases were defined as early resistant.

DISCUSSION

The aim of this study was to evaluate the feasibility of finding *EGFR* mutations in plasma and to test whether determining mutant *EGFR* levels during the first days of treatment with EGFR TKIs may reflect clinical response. SQI values for *EGFR* mutations in plasma were generated by the PCR test. Results were confirmed by ultra-deep NGS we have recently developed and applied to detect rare *EGFR* mutations in cytological samples and circulating tumor cells.[13,15]

Two quantification methods were tested on a series of 42 *EGFR*-positive NSCLC patients enrolled in the multicenter, prospective TRIGGER trial and 21 negative controls. Plasma samples were collected at baseline and immediately after progression. The sensitivity and specificity of *EGFR* detection in plasma versus tissue were, respectively, 72% and 100% for the PCR test and 74% and 100% for ultra-deep NGS. The concordance rate between the two assays was 95%. The detection sensitivity at baseline and progression were very similar (71% and 73% by the PCR test and 74% and 73% by ultra-deep NGS, respectively). Our results are in keeping with published data.[8,9,11,16]

Quantitative results reported in Table 2 and Figure 2 indicate a strong correlation between data obtained by PCR and ultra-deep NGS. This can be ascribed to the fact that the PCR assay uses mutation-specific primers that greatly enhance the detection of mutated alleles, whereas the ultra-deep NGS assay is based on deep sequence analysis of normal and mutant *EGFR* molecules amplified by intronic primers.

EGFR quantification data in plasma revealed that the percentage of mutated alleles in the series of patients analyzed varied from 0.02% to 55% as revealed by ultra-deep NGS. Remarkably, mutated *EGFR* represented less than 1% of the total *EGFR* DNA in plasma for about 50% of the patients. Clearly, highly sensitive techniques must be used to detect low levels of mutant DNA. We have previously shown, through a

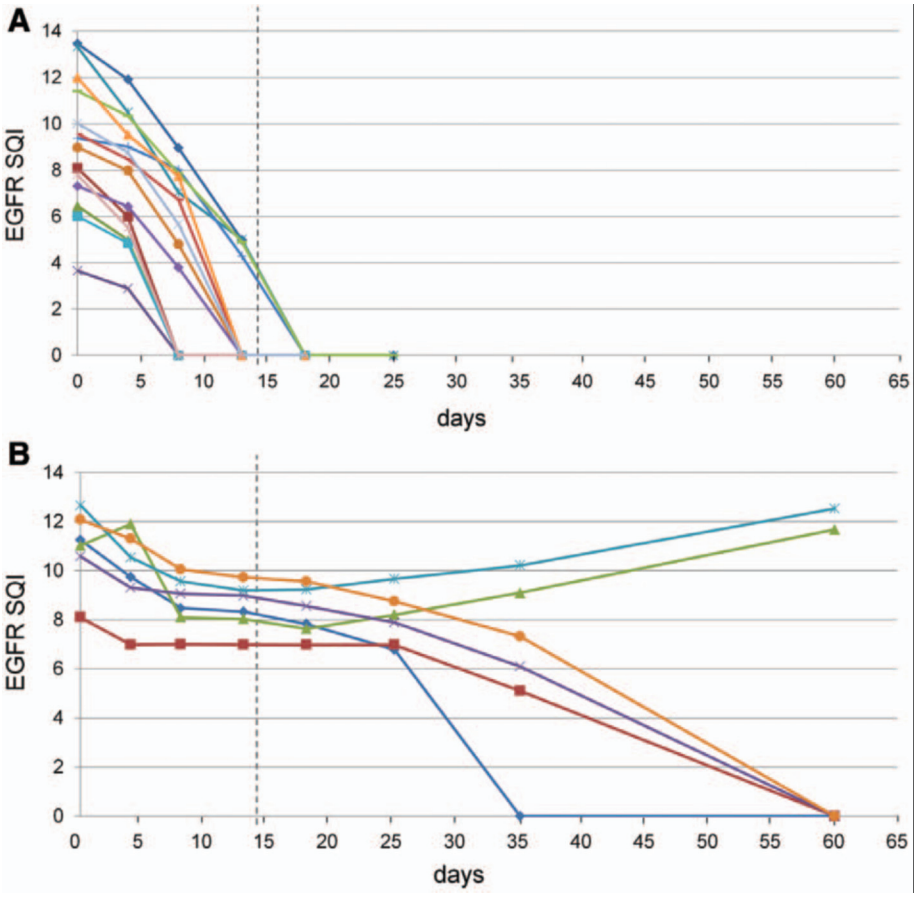

FIGURE 2. Quantification of mutated *EGFR* DNA from plasma of 20 patients by the PCR test after initiation of *EGFR* TKI therapy. *A,* Rapid responders. These 14 patients showed an *EGFR* SQI of 50% or greater at 14 days after initiating TKI treatment. *B,* Slow responders. These six patients showed an *EGFR* SQI below 50% at 14 days after initiating TKI treatment. The top two lines (turquoise and light green) are from two patients who developed T790M mutation. PCR, polymerase chain reaction; SQI, semiquantitative index; TKI, tyrosine kinase inhibitor.

series of dilution experiments, that ultra-deep NGS is one of the most sensitive methods for detecting *EGFR* mutations.[13,15]

Two different quantification approaches showed that the PCR test designed for detecting *EGFR* mutations is an extremely sensitive assay. PCR-based analyses such as this test are preferable to ultra-deep NGS in clinical practice because they are faster, easier, cheaper, and less prone to cross-contamination. However, because PCR-based assays use primers with known mutations to amplify mutated *EGFR* sequences, this approach will miss uncommon genetic alterations.

In an independent prospective series of NSCLC patients carrying *EGFR* mutations in tumor tissue and subjected to first-line treatment with EGFR TKIs, we show for the first time that accurate quantification of *EGFR* mutations, relative to NGS, in plasma may be useful for early prediction of clinical response. In 95% of the cases, we observed a progressive decrease of the *EGFR* SQI starting from the fourth day of treatment, with an average decrease of 63.5% at 14 days after initiation of treatment. Patients with a rapid versus slow decrease in mutant *EGFR* levels at 14 days were identified by using a cutoff of 50%. In rapid responders, a rapid decrease in mutated plasma *EGFR* DNA was associated with high levels of tumor shrinkage at 2 months. In slow responders, an attenuated decrease in mutated plasma *EGFR* DNA was associated with a lower PTS at 2 months. Two slow responders did not reach mutation clearance as measured by the PCR test, and an early increase in the circulating levels of the T790M mutation was observed. No T790M mutations were seen in serial plasma samples of the rapid responders. We therefore speculate that slow responders are more prone to develop early resistance. However, further clinical validation is required to assess the long-term impact of TKI treatment on rapid versus slow responders relative to progression-free and overall survival.

Our finding that mutant allele frequency drop soon after therapy is intriguing. As circulating tumor DNA could come from breakdown of tumor cells, we would have expected that the EGFR SQI would initially increase and then decrease as the ctDNA is cleared. Our experimental data clearly indicate a different situation, at least starting from the fourth day of treatment. A possible explanation is that the amount of circulating DNA in blood depends mainly on the amount of tumor cells released in the bloodstream that could decrease under treatment. Necrotic or apoptotic cells could only be marginally involved in the process, and they could be locally removed by the immune system. However, we cannot exclude the possibility of an increment of mutant EGFR in plasma immediately after therapy.

CONCLUSION

In conclusion, our data suggest that quantification of mutant *EGFR* in plasma DNA in the first days of treatment may

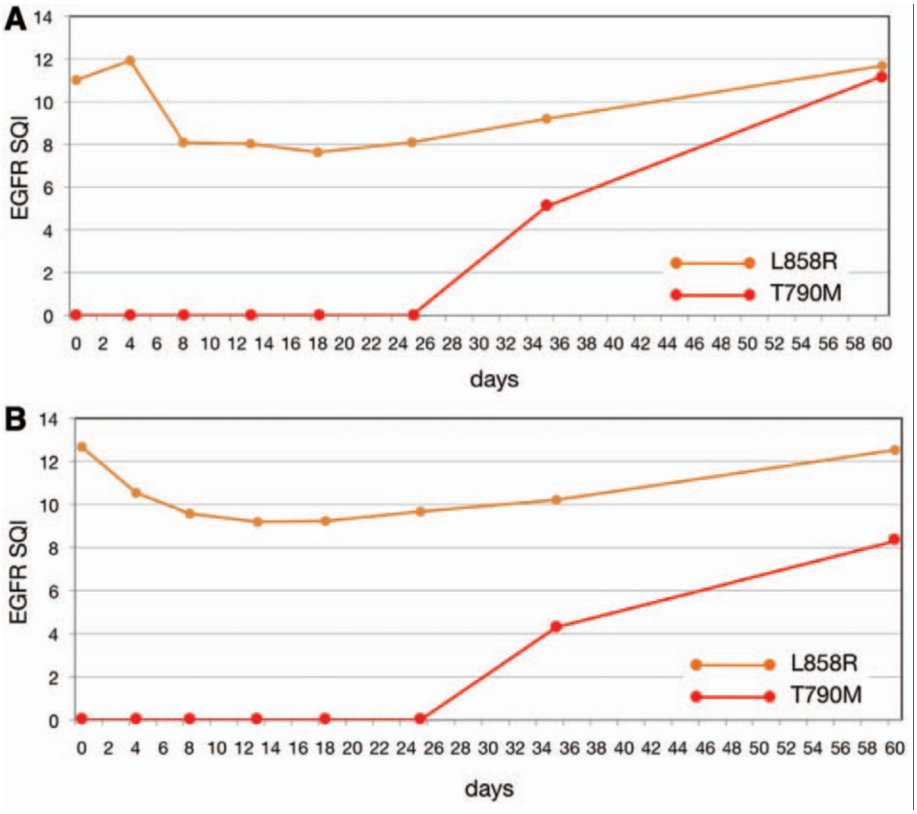

FIGURE 3. Quantification of mutated *EGFR* DNA from plasma of two slow responders with T790M mutation by the PCR test. The figures show the failure to clear the initial *EGFR* mutated DNA (L858R) and the emergence of T790M DNA during week 3 after initiating *EGFR* TKI treatment. PCR, polymerase chain reaction; SQI, semi-quantitative index; TKI, tyrosine kinase inhibitor.

TABLE 2. Correlation between Mutated Plasma *EGFR* Response and Percentage Tumor Shrinkage

Patient No.	Mutated *EGFR* Response at 14 Days	Tumor Shrinkage (%)
1	Rapid	69
2	Rapid	58
3	Rapid	67
4	Rapid	61
5	Rapid	49
6	Rapid	63
7	Rapid	66
8	Rapid	59
9	Rapid	52
10	Rapid	65
11	Rapid	55
12	Rapid	60
13	Rapid	56
14	Rapid	48
15	Slow	10
16	Slow	35
17	Slow	20
18	Slow	12
19	Slow	18
20	Slow	15

represent an early predictive parameter of clinical response. This new method of detecting *EGFR* mutation status could have important clinical applications, in that it could (1) complement or replace more expensive and invasive methods to assess response in TKI-treated patients; (2) allow early detection of the T790M mutations for possible changes to therapy; (3) represent a new way to compare the effectiveness of different drugs on *EGFR*-mutated tumors; and (4) be an additional tool to evaluate the best treatment regimen for patients.

With the strong correlation between *EGFR* SQI in plasma and clinical outcome, this study opens the way to prospectively design clinical trials to confirm these data and evaluate the diagnostic value of this test.

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REFERENCES

1. Keedy VL, Temin S, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. *J Clin Oncol* 2011;29:2121–2127.
2. Kerr KM, Bubendorf L, Edelman M, et al. Second ESMO consensus conference on lung cancer: pathology and molecular biomarkers for non-small-cell lung cancer. *Ann Oncol* 2014;25:1681–1690.
3. Reck M, Popat S, Reinmuth N, De Ruysscher D, Kerr KM, Peters S; ESMO Guidelines Working Group. Metastatic non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2014;25(Suppl 3):iii27–iii39.
4. Marchetti A, Felicioni L, Buttitta F. Assessing EGFR mutations. *N Engl J Med* 2006;354:526–528; author reply 526–528.

5. Kim ES, Hirsh V, Mok T, et al. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer (INTEREST): a randomised phase III trial. *Lancet* 2008;372:1809–1818.
6. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–957.
7. Luo J, Shen L, Zheng D. Diagnostic value of circulating free DNA for the detection of EGFR mutation status in NSCLC: a systematic review and meta-analysis. *Sci Rep* 2014;4:6269.
8. Douillard JY, Ostoros G, Cobo M, et al. Gefitinib treatment in EGFR mutated Caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. *J Thorac Oncol* 2014;9:1345–1353.
9. Li X, Ren R, Ren S, et al. Peripheral blood for epidermal growth factor receptor mutation detection in non-small cell lung cancer patients. *Transl Oncol* 2014;7:341–348.
10. Vallée A, Marcq M, Bizieux A, et al. Plasma is a better source of tumor-derived circulating cell-free DNA than serum for the detection of EGFR alterations in lung tumor patients. *Lung Cancer* 2013;82:373–374.
11. Weber B, Meldgaard P, Hager H, et al. Detection of EGFR mutations in plasma and biopsies from non-small cell lung cancer patients by allele-specific PCR assays. *BMC Cancer* 2014;14:294.
12. Zhao X, Han RB, Zhao J, et al. Comparison of epidermal growth factor receptor mutation statuses in tissue and plasma in stage I-IV non-small cell lung cancer patients. *Respiration* 2013;85:119–125.
13. Marchetti A, Del Grammastro M, Felicioni L, et al. Assessment of EGFR mutations in circulating tumor cell preparations from NSCLC patients by next generation sequencing: toward a real-time liquid biopsy for treatment. *PLoS One* 2014;9:e103883.
14. Marchetti A, Del Grammastro M, Filice G, et al. Complex mutations & subpopulations of deletions at exon 19 of EGFR in NSCLC revealed by next generation sequencing: potential clinical implications. *PLoS One* 2012;7:e42164.
15. Buttitta F, Felicioni L, Del Grammastro M, et al. Effective assessment of *egfr* mutation status in bronchoalveolar lavage and pleural fluids by next-generation sequencing. *Clin Cancer Res* 2013;19:691–698.
16. Thress K, Brant R, Carr H, et al. EGFR mutation detection in ctDNA from NSCLC patient plasma: a cross-platform comparison of technologies to support the clinical development of AZD9291. *J Clin Oncol* 2014;32:Abstr 8092.

