ORIGINAL ARTICLE

ctDNA Determination of *EGFR* Mutation Status in European and Japanese Patients with Advanced NSCLC: The ASSESS Study

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ABSTRACT

Introduction: To offer patients with *EGFR* mutation–positive advanced NSCLC appropriate EGFR tyrosine kinase inhibitor treatment, mutation testing of tumor samples is required. However, tissue/cytologic samples are not always available or evaluable. The large, noninterventional diagnostic ASSESS study (NCT01785888) evaluated the utility of circulating free tumor-derived DNA (ctDNA) from plasma for *EGFR* mutation testing.

Methods: ASSESS was conducted in 56 centers (in Europe and Japan). Eligible patients (with newly diagnosed locally advanced/metastatic treatment-naive advanced NSCLC) provided diagnostic tissue/cytologic and plasma samples. DNA extracted from tissue/cytologic samples was subjected

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to *EGFR* mutation testing using local practices; designated laboratories performed DNA extraction/mutation testing of blood samples. The primary end point was level of concordance of *EGFR* mutation status between matched tissue/cytologic and plasma samples.

Results: Of 1311 patients enrolled, 1288 were eligible. Concordance of mutation status in 1162 matched samples was 89% (sensitivity 46%, specificity 97%, positive predictive value 78%, and negative predictive value 90%). A group of 25 patients with apparent false-positive plasma results was overrepresented for cytologic samples, use of less sensitive tissue testing methodologies, and smoking habits associated with high *EGFR* mutation frequency, indicative of false-negative tumor results. In cases in which plasma and tumor samples were tested with identical highly sensitive methods, positive predictive value/sensitivity were generally improved.

Conclusions: These real-world data suggest that ctDNA is a feasible sample for *EGFR* mutation analysis. It is important to conduct mutation testing of both tumor and plasma samples in specialized laboratories, using robust/sensitive methods to ensure that patients receive appropriate treatments that target the molecular features of their disease.

Keywords: NSCLC; *EGFR* mutation; ctDNA; Plasma; Real-world

Introduction

NSCLC accounts for approximately 83% of all lung cancers, and most patients present with advanced disease at diagnosis.[1] Adenocarcinoma (ADC), which is among the most common of the NSCLC histological subtypes, has been shown to be associated with activating mutations in the *EGFR* gene in 13% of European and 47% of Japanese patients (corresponding rates for non-ADC 5% and 7%, respectively).[2] Response to EGFR tyrosine kinase inhibitors (TKIs) is acknowledged to be greater in patients with tumors harboring activating mutations in the tyrosine kinase domain of the *EGFR* gene versus wild-type *EGFR*.[3] Furthermore, EGFR TKIs have demonstrated efficacy superior to that of doublet chemotherapy in patients with *EGFR* mutation–positive advanced NSCLC.[4–8] Clinical guidelines[9,10] and several working groups[11,12] thus advocate mutation testing of tumor sample DNA from patients with nonsquamous advanced NSCLC to confirm suitability for EGFR TKI treatment. However, tissue/cytologic samples are not always available or evaluable for diagnosis and mutation testing, leaving some patients unable to have the *EGFR* mutation status of their tumors determined.[13]

Several studies have demonstrated that it is feasible to assess *EGFR* mutation status by using circulating free tumor-derived DNA (ctDNA), which can be isolated from the plasma or serum of patients with NSCLC.[4,14] Indeed, nucleic acid released from tumor cells into the circulation represents an alternative source of tumor-derived DNA, an approach referred to as liquid biopsy.[13] Indicative of the clinical relevance of ctDNA in mutation analysis, the presence of *EGFR* mutations in ctDNA has been found to predict response to EGFR TKIs[7,13,15]; similar objective response rates and progression-free survival have also been observed between patients with *EGFR* mutation–positive NSCLC detected by testing of tissue/cytologic samples and patients with *EGFR* mutations detected in their ctDNA.[4] Indeed, an amendment to the summary of product characteristics for the EGFR TKI gefitinib stating that ctDNA may be used for mutation analysis of NSCLC when tumor samples are unavailable[16] was adopted by the Committee for Medicinal Products for Human Use in September 2014.

As ctDNA analysis is technically challenging, it is important that the accuracy, suitability, and feasibility of use of ctDNA for mutation analysis in clinical practice be established. In the open-label IFUM study of white patients with *EGFR* mutation–positive NSCLC, between 652 evaluable matched tissue/cytologic and plasma (ctDNA) samples, mutation status concordance was 94%, demonstrating a high accuracy of ctDNA-based mutation testing with respect to tissue-based testing.[4] However, there is a need to confirm such findings outside of the controlled clinical trial environment to improve local diagnostic practice, enable wider access to ctDNA mutation testing, and provide more patients with the opportunity to receive therapies personalized to the mutation status of their tumors. The large, multicenter, noninterventional, diagnostic ASSESS study (NCT0178588) investigated the utility of ctDNA for *EGFR* mutation testing in a real-world diagnostic setting.

Materials and Methods

Study Design and Participants

Eligible patients were those at least 18 years of age (≥20 years in Japan) with histologically/cytologically confirmed, systemic treatment-naive, locally advanced NSCLC (stage IIIA/B according to the American Joint Committee on Cancer staging system) unsuitable for curative treatment or chemoradiotherapy or with metastatic disease (stage IV) and those with recurrent disease after previous curative treatment (resection and/or adjuvant chemotherapy). Provision of a diagnostic tissue/cytologic sample and a fresh blood (plasma) sample at enrollment was mandatory. The blood sample was taken before initiation of any treatment.

The primary end point of ASSESS was the level of concordance between *EGFR* mutation status obtained by tissue/cytologic testing and that obtained by blood (plasma)-based testing. Secondary end points included *EGFR* mutation testing practices and mutation frequency and correlations between mutation status and demographic data/disease status and treatment decisions after mutation testing (not reported).

All patients provided written, informed consent. Study approval was obtained from the independent ethics committees at each institution. The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization/Good Clinical Practice, applicable regulatory requirements for noninterventional studies, and AstraZeneca's policy on bioethics and human biological samples.

Procedures

EGFR mutation testing and results data for tumor samples obtained before enrollment in ASSESS were used where available (see Fig. 1*A*). For tests conducted in ASSESS, diagnostic tissue/cytologic samples were subjected to *EGFR* mutation testing as per local practices after histopathological review (WHO classification[17]) to ensure that samples were adequate for use. Each patient provided a 10-mL blood sample, which was processed to plasma, frozen, and transported to designated laboratories for mutation testing. In Europe, 43 academic, hospital, and commercial laboratories performed the tissue/cytologic testing and five central/regional expert laboratories were used for blood-based testing. In Japan, two commercial laboratories were used for both tissue/cytologic and blood-based testing.

Outcomes

Testing methodologies, sample types and availability, testing turnaround time, and testing success rate/mutation detection rate were captured to assess *EGFR* mutation testing practices. Concordance of *EGFR* mutation status between matched tissue/cytologic and plasma samples was assessed by using the following: concordance rate; sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) (Supplementary Table 1); and exact two-sided 95% confidence interval (CI).

Statistical Analyses

Concordance of *EGFR* mutation status between matched tissue/cytologic and plasma samples was calculated for the evaluable population (all eligible patients with known tumor [tissue/cytologic] and plasma sample mutation status). The sample size of ASSESS was calculated to allow accurate determination of sensitivity between tumor- and blood-based testing. For 100

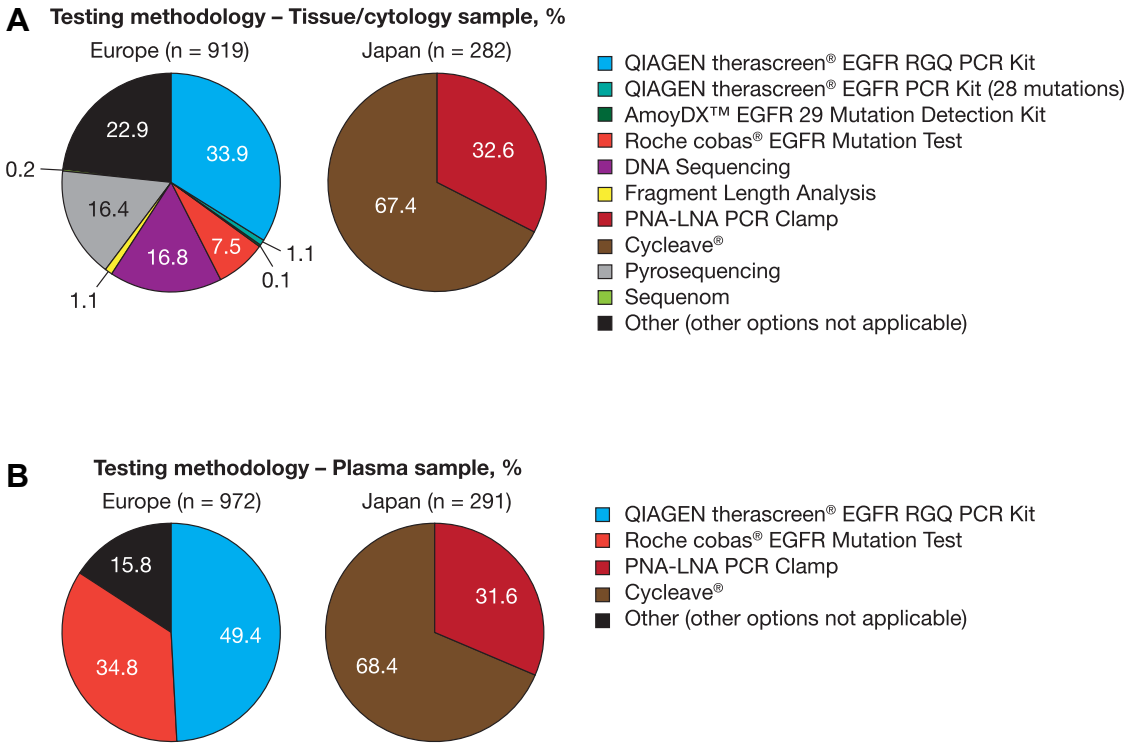

Figure 1. Mutation testing methods used for tissue/cytologic (*A*) and plasma (*B*) samples in Europe and Japan (enrolled population). Other refers to Fragment Length Analysis/PNA-LNA PCR Clamp. PCR, polymerase chain reaction; PNA-LNA, peptide nucleic acid-locked nucleic acid; RGQ, Rotor-Gene Q.

Table 1. Patient Demographics (Enrolled Population)

Characteristic	Europe (n = 997)	Japan (n = 291)	Overall (n = 1288)
Mean age ± SD	65.4 ± 9.74	70.2 ± 9.0	66.5 ± 9.8
Men, n/N (%)	675 of 997 (68)	192 of 291 (66)	867 of 1288 (67)
Race, n/N (%)			
White	976 of 997 (98)	0 of 291 (0)	976 of 1288 (76)
Asian	5 of 997 (1)	291 of 291 (100)	296 of 1288 (23)
WHO performance status, n/N (%)			
0	388 of 997 (39)	114 of 291 (39)	502 of 1288 (39)
1	453 of 997 (45)	117 of 291 (40)	570 of 1288 (44)
2	136 of 997 (14)	37 of 291 (13)	173 of 1311 (13)
3	17 of 997 (2)	18 of 291 (6)	35 of 1288 (3)
4	3 of 997 (0)	5 of 291 (2)	8 of 1288 (1)
Smoking status			
Never-smoker, n/N (%)	174 of 996 (17)	78 of 291 (27)	252 of 1287 (20)
Pack-years, median	40.0	45.0	40.0

Note: Pack-years = (number of cigarettes smoked per day × number of years smoked)/20.

patients with *EGFR* mutation-positive NSCLC, if the sensitivity was 50%, the 95% CI would be expected to be 40% to 60%. Assuming a 10% prevalence of *EGFR* mutations in Europe, it was estimated that 1000 patients should be tested to obtain 100 with *EGFR* mutation–positive NSCLC. As an independent replication, sensitivity was obtained in patients from Japan; assuming a 30% prevalence of *EGFR* mutations in Japan, it was estimated that 300 patients should be tested to obtain 100 with *EGFR* mutation–positive NSCLC.

EGFR mutation testing practices (enrolled population) and *EGFR* mutation frequency (evaluable tumor/plasma populations) were summarized by using appropriate descriptive statistics.

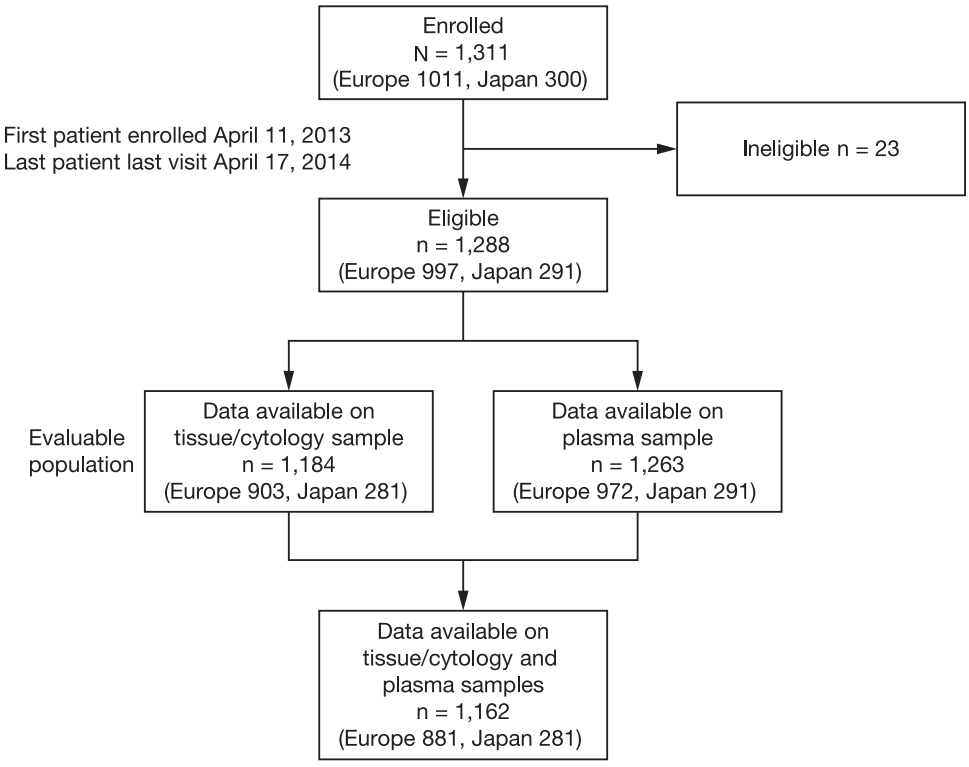

Figure 2. Patient flow diagram.

Results

Patients

From April 11, 2013, to April 17, 2014, a total of 1311 patients were enrolled (Table 1), 1288 of whom were eligible (Fig. 2). A total of 23 patients were enrolled in error (they did not fulfil the inclusion/exclusion criteria) and were not included in the final analysis (Supplementary Table 2).

Sampling Methodologies and EGFR *Mutation Testing Practices*

Tissue/cytologic samples were mostly collected during current diagnosis (Supplementary Fig. 1*A*) and most often sampled from the primary tumor (Supplementary Fig. 1*B*). The most frequent sampling sites were the lungs/lymph nodes (Supplementary Fig. 1*C*). Bronchoscopy was the most frequently used sample collection method (Supplementary Fig. 1*D*). A range of mutation testing methods for tissue/cytologic samples (>10) were observed in Europe versus only two methods in Japan (Fig. 1*A*). The mutation testing methods observed for plasma were limited and differed between Europe and Japan (Fig. 1*B*).

The median *EGFR* test turnaround time for tissue/cytologic samples was 11 days for Europe (95% CI: 14.0–17.3) and 8 days for Japan (95% CI: 8.2–14.1); mutation testing success rates were high in both Europe (98%) and Japan (<100%). Mutation tests were not performed on the tissue/cytologic samples of 110 patients and results were not obtained from the tested samples of 17 patients. The most frequent reason for not testing was insufficient material provided (60% [47 of 78 responses to the question] in Europe and 56% [five of nine] in Japan). There were no obvious differences in sampling methods between evaluable and nonevaluable tissue/cytologic samples.

EGFR *Mutation Frequency*

The overall *EGFR* mutation frequency was 16% for evaluable tissue/cytologic samples and 9% for evaluable plasma samples (Table 2). Consistent with previous reports,[2] in Europe, 14% of ADC tissue/cytologic samples (99 of 712) and 3% of non-ADC samples (six of 180) were *EGFR* mutation–positive. In Japan, 40% of ADC tissue/cytologic samples (78 of 195) and 8% (six of 77) of non-ADC samples were *EGFR* mutation–positive.

Concordance of EGFR *Mutation Status between Matched Tissue/Cytologic and Plasma Samples*

Overall concordance of mutation status was 89% (sensitivity 46%, specificity 97%, PPV 78%, and NPV 90%) (Table 3). An *EGFR* mutation–positive result in plasma and mutation-negative result in tissue/cytologic testing were observed in samples from 25 patients (false-positive rate 22%); the demographics and sampling/mutation testing methodologies associated with these patients are summarized in Supplementary Table 3. False-negative results were obtained for 102 patients (10%).

When tissue/cytologic and plasma samples were tested with identical, sensitive methods (Qiagen therascreen EGFR Rotor-Gene Q Polymerase Chain Reaction (PCR) Kit, Peptide Nucleic Acid–Locked Nucleic Acid PCR Clamp [Qiagen, Manchester, UK], Roche cobas EGFR Mutation Test [Roche Molecular Diagnostics, Pleasanton, CA], or Cycleave [Takara Bio Inc., Kusatsu, Japan]), the PPV and sensitivity were generally improved (see Table 3).

Furthermore, in a subset of 94 Japanese patients, sensitivity increased from an initial result of 17% (five of 29) to 52% (15 of 29) when samples that had originally had ctDNA extracted using the Qiagen QIAamp MinElute Virus Spin Kit for DNA (400 μL plasma) had extraction repeated using the Qiagen QIAamp Circulating Nucleic Acid Kit (3 mL plasma) specifically designed to isolate smaller, fragmented DNA, even though the same

Table 2. *EGFR* Mutation Frequency by Sample Type, Region, and Histologic Subtype (Population with Evaluable Tissue/Cytologic and/or Plasma Samples)

	EGFR mutation frequency	
	Tissue/Cytologic Samples	Plasma
Characteristic	n/n (%)	n/n (%)
Overall	191 of 1184 (16)	119 of 1263 (9)
Country		
Europe	105 of 903 (12)	82 of 972 (8)
Japan	86 of 281 (31)	37 of 291 (13)
Histological subtype		
ADC	177 of 907 (20)	109 of 952 (11)
Non-ADC	12 of 257 (5)	9 of 288 (3)
TNM stage		
IIIA	5 of 66 (8)	3 of 75 (4)
IIIB	3 of 105 (3)	2 of 119 (2)
IV	183 of 1006 (18)	114 of 1063 (11)
TNM stage IV		
M1a	57 of 235 (24)	17 of 252 (7)
M1b	82 of 490 (17)	67 of 528 (13)
EGFR mutation subtype		
Exon 19 deletions	97 of 191 (51)	68 of 119 (57)
Exon 19 deletions + T790M	0 of 191 (0)	0 of 119 (0)
L858R	73 of 191 (38)	38 of 119 (32)
L858R + T790M	0 of 191 (0)	2 of 119 (2)
T790M only	0 of 191 (0)	3 of 119 (3)
T790M + other[a]	1 of 191 (1)	1 of 119 (1)
Other[b]	20 of 191 (10)	7 of 119 (6)

[a]Any other mutation that occurred in combination with T790M that is not L858R or Exon 19 deletion.
[b]This category included double mutations not specified.
ADC, adenocarcinoma.

Table 3. Concordance of *EGFR* Mutation Status between Matched Tissue/Cytologic and Plasma Samples (Population with Evaluable Tissue/Cytologic and Plasma Samples)

Characteristic	Concordance Rate		Sensitivity		Specificity		PPV		NPV	
	n/n (%)	95% CI	n/n (%)	95% CI	n/n (%)	95% CI	n/n (%)	95% CI	n/n (%)	95% CI
Overall (n = 1162)	1035 of 1162 (89)	87.1-90.8	87 of 189 (46)	38.8-53.4	948 of 973 (97)	96.2-98.3	87 of 112 (78)	68.8-85.0	948 of 1050 (90)	88.3-92.0
Japan (n = 281)	227 of 281 (81)	75.7-85.2	34 of 86 (40)	29.2-50.7	193 of 195 (99)	96.3-99.9	34 of 36 (94)	81.3-99.3	193 of 245 (79)	73.1-83.7
Europe (n = 881)	808 of 881 (92)	89.7-93.4	53 of 103 (51)	41.4-61.4	755 of 778 (97)	95.6-98.1	53 of 76 (70)	58.1-79.8	755 of 805 (94)	91.9-95.4
Qiagen therascreen EGFR RGQ PCR Kit (n = 138)	131 of 138 (95)	89.8-97.9	16 of 22 (73)	49.8-89.3	115 of 116 (99)	95.3-100	16 of 17 (94)	71.3-99.9	115 of 121 (95)	89.5-98.2
Roche cobas EGFR Mutation Test (n = 23)	22 of 23 (96)	78.1-99.9	3 of 4 (75)	19.4-99.4	19 of 19 (100)	82.4-100	3 of 3 (100)	29.2-100	19 of 20 (95)	75.1-100
Cycleave (n = 190)	161 of 190 (85)	78.8-89.5	29 of 57 (51)	37.3-64.4	132 of 133 (99)	95.9-100	29 of 30 (97)	82.8-99.9	132 of 160 (83)	75.5-88.0
PNA-LNA PCR Clamp[a] (n = 91)	76 of 91 (84)	74.3-90.5	15 of 29 (52)	32.5-70.6	61 of 62 (98)	91.3-100	15 of 16 (94)	69.8-99.8	61 of 75 (81)	70.7-89.4

[a]PNA-LNA PCR Clamp concordance data using optimized ctDNA extraction procedure.
PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; RGQ, Rotor-Gene Q; PCR, polymerase chain reaction; PNA-LNA, peptide nucleic acid-locked nucleic acid; ctDNA, circulating free tumor-derived DNA.

detection method (peptide nucleic acid–locked nucleic acid PCR Clamp) had been used. Including the newer Japanese subset data in the overall data increased the concordance rate (90% [1036 of 1158]), sensitivity (50% [97 of 194]), PPV (80% [97 of 122]), and NPV (91% [939 of 1036]).

Discussion

To our knowledge, ASSESS is the most comprehensive study of real-world *EGFR* mutation testing practices in Europe and Japan. ASSESS was designed to evaluate how data obtained using local practices for tissue/cytologic and plasma sample testing compare with data obtained from well-controlled clinical testing that is generally performed in a single expert laboratory.

The mutation status concordance observed between 1162 matched tissue/cytologic and plasma samples (89%) suggests that ctDNA is a feasible sample for real-world *EGFR* mutation analysis if robust/sensitive DNA extraction and mutation analysis methodologies are used. ctDNA comprises less than 1% of total circulating free DNA[18]; including data from the subset of patients who had DNA reextracted with an optimized method increased the overall concordance rate and sensitivity.

The low PPV (78%) raised a concern that some of the plasma *EGFR* mutation results might be false positives, and 25 patients for whom testing yielded apparent false-positive plasma results were identified (Supplementary Table 3). This group was overrepresented for cytologic samples, which may not be representative of the disease, and/or the use of less sensitive DNA sequencing methodologies (56% of these 25 tumor samples were tested by DNA sequencing/pyrosequencing versus 25% of samples from the overall population [305 of 1201]). In addition, 80% of these patients were never-, former, or light smokers, a subgroup in which a higher *EGFR* mutation frequency is expected. Furthermore, in Europe, the *EGFR* mutation frequency by DNA sequencing/pyrosequencing of tissue/cytologic samples was 10% (37 of 370) versus 15% (68 of 465) for all other methods combined, suggesting that DNA sequencing/pyrosequencing may have missed some of the *EGFR* mutations present. This finding is in agreement with results of external quality assessment studies that have revealed a high rate of genotyping errors in Europe, where less sensitive techniques are still frequently used.[19,20] Overall, these data suggest that the low PPV was due to false-negative results in tumor rather than to false-positive results in plasma. False-negative results can occur because of tumor heterogeneity, inexperience, or use of methods with low sensitivity.

In contrast, it is clear that a negative result in plasma may be incorrect (false-negative), as ctDNA mutation analysis may not pick up *EGFR* mutations in all patients

who carry them in their tumors. The sensitivity of plasma tests, which is indicative of how likely they are to accurately detect *EGFR* mutations, varied (36%–100%) between countries. ASSESS revealed substantial differences within/between Europe and Japan in the mutation testing methodologies used, reflecting differences in available and/or accessible equipment and knowledge, which may contribute to this variation. However, overall sensitivity (46%) was markedly lower than the pooled sensitivities reported in two recent meta-analyses of concordance of *EGFR* mutation status between matched tissue/cytologic and plasma samples (62%–67%).[21,22]

Caution is advised when comparing data from clinical trials, in which testing is generally well controlled and performed in a single central laboratory, with the real-world ASSESS data. Of note, the concordance rate of 94% (sensitivity 66%, specificity 100%, PPV 99%, and NPV 94%) reported for IFUM was higher than in ASSESS.[4] Likewise, the sensitivity and PPV were higher in FASTACT-2[23] (75% and 94%, respectively) and sensitivity in the EURTAC study[24] was 78%. IFUM utilized the Qiagen therascreen EGFR Rotor-Gene Q PCR Kit for mutation analysis of matched tissue/cytologic and plasma samples. Interestingly, the ASSESS concordance data for matched tissue/cytologic and plasma samples tested with the therascreen kit were highly consistent with that of IFUM (concordance 95% [131 of 138], sensitivity 73% [16 of 22], specificity 99% [115 of 116], PPV 94% [16 of 17], and NPV 95% [115 of 121]). This confirms the findings of the IFUM study,[4] which indicated that when an *EGFR* mutation is detected in ctDNA, the result can be trusted and a prescriber can conclude that the patient is likely to benefit from TKI therapy.

In real-world practice, test sensitivity may be further complicated by the different stage/differentiation of the disease,[25] which likely influence release of ctDNA into blood. In ASSESS, a higher frequency of *EGFR* mutations was observed in the plasma of patients with M1b disease (13%) versus M1a disease (7%) despite the *EGFR* mutation frequency in tumor being similar in these two groups, which is consistent with the hypothesis of greater release of ctDNA in patients with distant metastases. It must be noted that analysis of ctDNA may allow identification of mutations in heterogeneous tumors that could carry a specific variant in only a minor tumor clone (i.e., beyond the "biopsy zone"). However, although plasma-based testing requires standardization as a technique under relatively early investigation, the ASSESS results suggest that further improvements in mutation analysis of tissue/cytologic samples are still required to reduce the occurrence of false-negative results.

In IFUM, 19% of patients had nonevaluable tissue/cytologic samples for reasons including low tumor content, insufficient sample quality/quantity, poor/inappropriate fixation, and no DNA.[4] In this respect, accurate and accessible ctDNA mutation testing to address the unmet need in patients without an available/evaluable tumor sample will be important to enable more patients to receive therapies personalized to the mutation status of their tumor.

Conclusions

These ASSESS real-world data suggest that ctDNA is a feasible sample for *EGFR* mutation analysis when tumor samples are unavailable. It is important to conduct mutation testing of both tumor and plasma samples in specialized laboratories, using robust and sensitive mutation testing methods to ensure accuracy of results.

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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 http://dx.doi.org/10.1016/j.jtho.2016.05.036.

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