Clinical Cancer Research

Exosome-Based Detection of *EGFR* T790M in Plasma from Non–Small Cell Lung Cancer Patients

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Abstract

Purpose: About 60% of non–small cell lung cancer (NSCLC) patients develop resistance to targeted epidermal growth factor receptor (EGFR) inhibitor therapy through the *EGFR* T790M mutation. Patients with this mutation respond well to third-generation tyrosine kinase inhibitors, but obtaining a tissue biopsy to confirm the mutation poses risks and is often not feasible. Liquid biopsies using circulating free tumor DNA (cfDNA) have emerged as a noninvasive option to detect the mutation; however, sensitivity is low as many patients have too few detectable copies in circulation. Here, we have developed and validated a novel test that overcomes the limited abundance of the mutation by simultaneously capturing and interrogating exosomal RNA/DNA and cfDNA (exoNA) in a single step followed by a sensitive allele-specific qPCR.

Experimental Design: ExoNA was extracted from the plasma of NSCLC patients with biopsy-confirmed T790M-positive ($N = 102$) and T790M-negative ($N = 108$) samples. The T790M mutation status was determined using an analytically validated allele-specific qPCR assay in a Clinical Laboratory Improvement Amendment laboratory.

Results: Detection of the T790M mutation on exoNA achieved 92% sensitivity and 89% specificity using tumor biopsy results as gold standard. We also obtained high sensitivity (88%) in patients with intrathoracic disease (M0/M1a), for whom detection by liquid biopsy has been particularly challenging.

Conclusions: The combination of exoRNA/DNA and cfDNA for T790M detection has higher sensitivity and specificity compared with historical cohorts using cfDNA alone. This could further help avoid unnecessary tumor biopsies for T790M mutation testing. *Clin Cancer Res; 24(12); 2944–50. ©2018 AACR.*

Introduction

Non–small cell lung cancer (NSCLC) comprises ~85% of all diagnosed lung malignancies (1). More than half of patients with NSCLC already have advanced disease at diagnosis, leading to a 5-year survival rate of only ~15%. The field has been successful in developing therapies targeting specific molecular pathways (2). Extensive research on the molecular landscape of NSCLC has shown that the presence of mutations in the kinase domain of the epidermal growth factor receptor (EGFR) correlate directly with sensitivity to first-generation *EGFR* tyrosine kinase inhibitors (TKI) such as erlotinib and gefitinib (3–6). Unfortunately, around 60% of these patients develop resistance to anti-EGFR treatment through a missense point mutation resulting in an amino-acid change from threonine to methionine in exon 20 of *EGFR* (*EGFR* T790M; refs. 7–12). This mutation is thought to not only appear during treatment, but in rare cases (<5%) can also be found in primary tumors not previously treated with TKIs (5, 7, 13–16). Detecting the T790M mutation is therefore of critical importance in guiding the treatment of NSCLC patients. Currently, this is mostly performed based on a direct biopsy of the tumor tissue.

Obtaining tissue biopsies for molecular analysis can be challenging. There is a significant risk of bleeding, infection, and other complications in up to 20% of patients. For as many as 49% of patients, physicians are unable to obtain a biopsy for molecular analysis either due to comorbidities or insufficient tumor tissue (17, 18). Thus, there is a critical need for a noninvasive liquid biopsy approach to assess tumor mutations as well as enable easy repeat testing throughout treatment (19–23).

Recently, the Food and Drug Administration (FDA) approved the first liquid biopsy companion diagnostic with the cobas EGFR Mutation Test version 2, where T790M mutation status in plasma was specifically indicated as an aid for use of osimertinib (TAGRISSO). The cobas test analyzes the mutations present in the cfDNA fraction, but its performance for *EGFR* T790M detection is limited by 58% sensitivity and 80% specificity (24).

The cell-free DNA (cfDNA) in biofluids is released through cell death mechanisms such as necrosis and apoptosis (25). In

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Translational Relevance

Tumor tissue biopsies carry risks and are not always possible, making liquid biopsies an attractive way to acquire molecular information from cancer patients. The *EGFR* T790M mutation is a critical biomarker in non–small cell lung cancer and has introduced new challenges in how these patients are managed. With the approval of osimertinib for patients failing first-generation EGFR inhibitor therapy, the use of a liquid biopsy to detect *EGFR* T790M would reduce the number of unnecessary repeat biopsies. Detection of *EGFR* T790M using cfDNA has proved to be challenging due to low abundance in blood. Here, we present a novel *EGFR* T790M assay based on a platform validated in a Clinical Laboratory Improvement Amendment laboratory that simultaneously monitors the mutation on exosomal RNA/DNA and cfDNA from plasma that achieves 92% sensitivity and 89% specificity.

contrast, exosomes and other extracellular vesicles are actively released from living cells (26) and contain both RNA and DNA (Fig. 1). Mutations such as T790M can be found in both cfDNA and exosomal RNA/DNA, so combining these sources of nucleic acids increases sensitivity (27–29). This improves detection in patients with limited copies of circulating T790M in the cfDNA fraction, such as those with early-stage disease or intrathoracic (M0/M1a) lung cancer patients (~25%–30% of all newly diagnosed cases; ref. 19). In addition to the increased sensitivity of mutation detection, it has been shown that mutations on exoNA correlate better to overall survival compared with cfDNA mutation analysis (27). Here, we present a highly sensitive, highly specific and easily performed liquid biopsy test for T790M, using a single step isolation of exosomal RNA/DNA and cfDNA followed by allele-specific qPCR.

Materials and Methods

Assay design

The test consists of four steps: coisolation of exosomal RNA/DNA and cfDNA in a single step using a cGMP manufactured isolation kit (ExoLution Plus, Exosome Diagnostics, Inc.); reverse transcription (RT), preamplification with an allele-specific *EGFR* exon 20 wild-type blocker, and a triplex TaqMan-based quantitative PCR (qPCR) step that uses an amplification-refractory mutation system (ARMS). The test also analyzes *EGFR* exon 7 and a nonhuman control sequence (QBeta RNA) that is spiked into the sample before the RT step, which serve as controls of sample integrity, inhibition, and qPCR (Fig. 2; Exosome Diagnostics, Inc.).

Analytical and clinical validation

Analytical and clinical validation of the test was performed in a Clinical Laboratory Improvement Amendments (CLIA) certified laboratory, following CLSI EP7-A2 and published guidelines (30, 31). The analytical validation was designed to address precision (repeatability and reproducibility), sensitivity (limit of detection, LoD), specificity, and the effect of interfering substances. The LoD for the analytical part of the test was calculated as previously described (32). A more detailed description can be found in the Supplementary Methods and Supplementary Figures section.

Clinical samples

The study included a total of 210 subjects, of which 102 were from T790M-positive NSCLC patients (101 confirmed positive by tissue biopsy using institutionally approved methods and one by

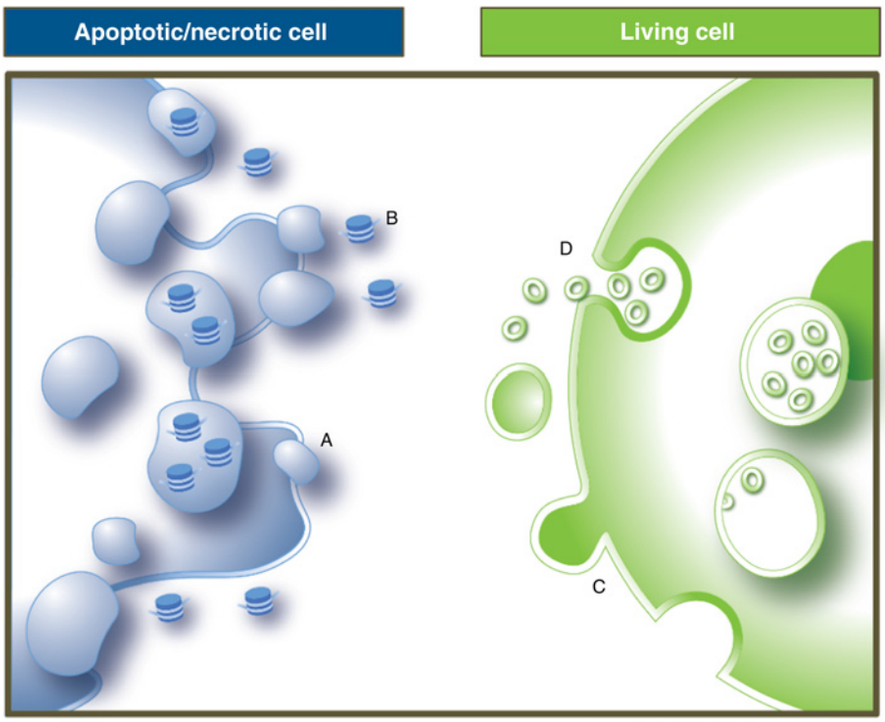

Figure 1.
Two distinct sources of cell-free nucleic acids in plasma. Apoptotic or necrotic cells may release cfDNA in apoptotic vesicles either (**A**) as free DNA or (**B**) associated with circulating nucleosomes. Exosomes are actively released by living cells either (**C**) directly from the plasma membrane or (**D**) through the multivesicular body pathway, carrying RNA, DNA, and other cargo (e.g., lipids and proteins) into the circulation.

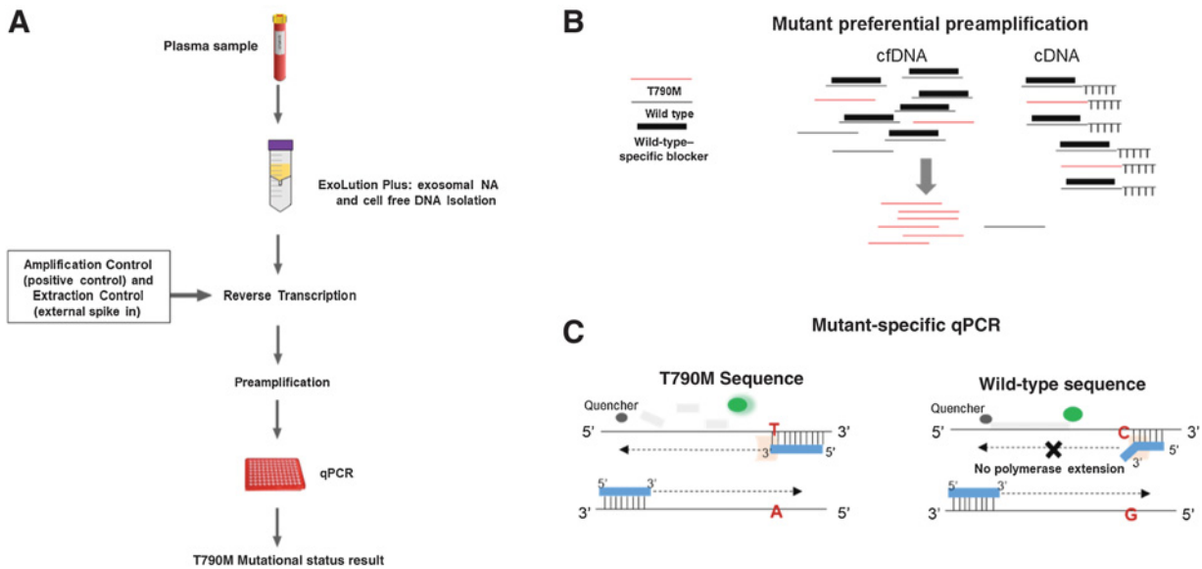

Figure 2.
Assay workflow overview. **A,** exoNA is coisolated from plasma and reverse transcribed. At the reverse transcription step, an amplification control (DNA) and an RNA spike-in control are added to ensure reverse transcription and subsequent amplifications occur (preamplification and triplex qPCR). **B,** Multiplex preamplification reaction includes a wild-type blocker for exon 20 of *EGFR*, which favors amplification of mutant molecules from cfDNA and cDNA. **C,** Allele-specific qPCR.

Guardant 360 analysis; Supplementary Table S1 and Supplementary Fig. S1A and S1B). All 102 T790M-positive patients had received prior treatment with at least one first-generation TKI. The negative samples were from either patients with a confirmed T790M-negative biopsy ($n = 21$) or tumor-free donors ($N = 87$). For the clinical samples, plasma was collected either during or after tissue biopsy, but before initiation of third-generation TKI. The study included both retrospective and prospectively collected patient samples. Retrospective samples were provided by Clovis Oncology, Inc., Addario Lung Cancer Medical Institute (ALCMI; Wilmington, NC), and Massachusetts General Hospital (Boston, MA), while the prospectively collected samples (including 13 patients with confirmed *EGFR* T790M-positive tumors) were from Memorial Cancer Institute (Hollywood, FL), Althia Health and Ochsner Medical Center (New Orleans, LA). All samples were collected under clinical study protocols approved for this purpose by the relevant Institutional Review Boards (IRB).

Sample preparation and qPCR analysis

All samples (1–2 mL of plasma from each patient) were analyzed by Exosome Diagnostics, Inc. using the ExoDx Lung (T790M) test. In summary, exosomal RNA/DNA and cfDNA was isolated from 1 to 2 mL plasma samples using the ExoLution Plus platform (Exosome Diagnostics, Inc.), based on a spin column that simultaneously captures exosomes and cfDNA, which are then eluted and reverse transcribed as described previously (27). The cDNA/DNA was subjected to a mutation specific preamplification in the presence of a blocker oligonucleotide that prevents wild-type *EGFR* from being efficiently amplified (Fig. 2). The amplification includes an inhibition control (QBeta RNA) as well as an endogenous sample integrity control (exon 7 of *EGFR*). The preamplified material is then subjected to an allele-specific qPCR for *EGFR* T790M as well as a standard qPCR for QBeta and exon 7 of *EGFR*.

Training and validation on clinical samples

The clinical cohort was split into stage-matched training and validation cohorts. The training cohort consisted of 105 subjects selected randomly in a cancer stratified manner to keep the ratio of M0/M1a, M1b, and MX disease stages constant between training and left-out validation set, from which 51 samples were *EGFR* T790M-positive and 54 T790M-negative. From the 51 positive patients, 14 were M1a, 32 were M1b, 2 of M0, and 3 were MX.

The independent validation cohort consisted of 105 samples, with 51 T790M-positive and 54 T790M-negative samples with approximately the same disease stage distribution as the training cohort. Allele-specific PCR amplification of a single-nucleotide variant will eventually lead to background signal due to the presence of the wild-type allele. The lowest cycle threshold (CT) value allowed for exon 7 (which serves as an estimate for wild-type *EGFR* was 14). To estimate the optimal CT cutoff for T790M detection in plasma to best match tissue status, we used the training cohort of 105 subjects. To evaluate the performance and generalization abilities of the model, we performed 100 bootstrap experiments, where the training cohort was split into a stratified 80% subtraining and 20% subtesting cohort. A qPCR CT cutoff was estimated on each of the subtraining cohorts by maximizing Youden's J statistic to be able to achieve the highest values for sensitivity and specificity (33). Eventually, the CT cutoff was reestimated by maximizing Youden's J statistic on the full training cohort using the results of the bootstrapping. We validated the clinical performance of the test using this optimized CT cutoff on the independent clinical validation cohort of 105 subjects.

Results

Analytical validation

The assay test performance was evaluated in a set of controls that consisted of DNA derived from admixtures of wild-type and a genetically engineered T790M *EGFR*-mutant cell line (Supplementary Table S2). The assay was validated on clean admixtures as well as in a plasma background (nucleic acid from plasma extraction) to assess performance in a complex background. The test was able to detect mutant allele frequencies down to 0.05% (the lowest validated admixture; Supplementary Table S2).

The analytical parameters, including LoD reported here, were assessed using T790M spike-ins in 2 mL of plasma background. The estimated LoD was 6 copies (at 50% detection rate), independent of operator (2 operators) or day (3 different days; Supplementary Table S3). As shown in the table, the test can detect single copies of the mutation (i.e., ∼1 copy 20% of the time), but the performance is limited by the Poisson distribution of mutant molecules. Because the final readout from this assay is a qualitative assessment of the T790M mutation similar to the cobas EGFR Mutation Test v2 (Roche), this copy-number information is only used to address analytical assay performance specifications.

The effect of interfering substances was also assessed following CLSI EP7-A2 guidelines and showed that TKIs or any of the other interfering substances assessed at therapeutic levels did not affect the test performance (Supplementary Methods).

Clinical performance

CT values of the *EGFR* T790M qPCR assay from the training cohort (51 *EGFR* T790M-positive and 54 T790M-negative subjects) were used to train the test to discriminate between *EGFR* T790M-positive and -negative samples as determined by tissue biopsy. In the training cohort, the test had a sensitivity of 91%, a specificity of 95%, and the area under the receiver operator characteristics curve (AUC) was 0.94 (Fig. 3A, Table 1C; Supplementary Table S4).

The clinical performance on the independent validation cohort (51 *EGFR* T790M-positive and 54 T790M-negative subjects) was 92% sensitivity, 89% specificity, and an AUC of 0.96 (Fig. 3B and Table 1C). Of the 51 *EGFR* T790M-positive patients, 47 were classified as true positives (TP), 4 as false negatives (FN), and of the 54 *EGFR* T790M-negative subjects, 48 as true negatives (TN) and 6 as false positives (FP) compared with tissue biopsy (Table 1A). From the false positive samples, one was from a T790M-negative NSCLC patient and the remaining were from presumably healthy subjects.

The test correctly classified 88% (14/16) of patients with intrathoracic (M0/M1a) disease, ∼94% (30/32) with M1b disease, and 100% (3/3) of unknown M stage (MX; Table 1B).

Discussion

Liquid biopsies are a promising tool for patient stratification as well as longitudinal monitoring of cancer patients. However, a primary challenge for the liquid biopsy field is the low allelic frequencies of the mutation (often below 0.5%) and low copy numbers of the mutation target. Technical advancements in recent years have enabled detection of mutations at very low allelic frequencies (34), but cannot solve the problem of low/no copy numbers in the sample.

Given these challenges, it is crucial to examine the different sources of nucleic acids in the circulation to maximize the chance of detecting the target mutation at an earlier disease

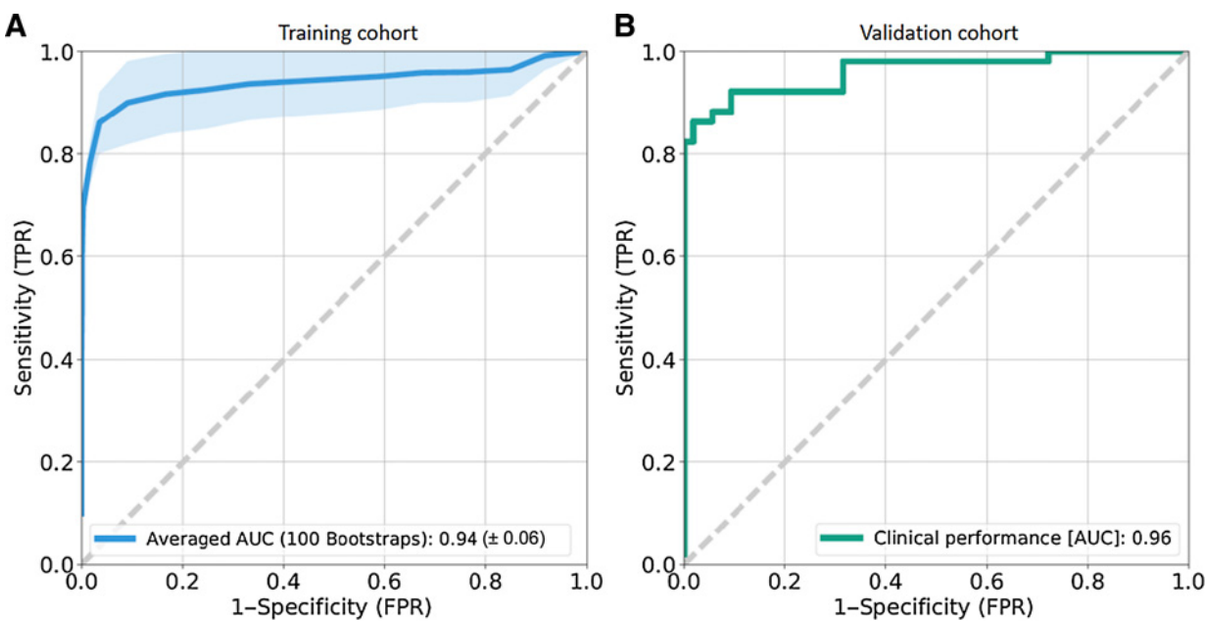

Figure 3.

Receiver operating characteristic (ROC) curve analysis. The x-axes show 1 − specificity or the false positive rate (FPR), and y-axes show the sensitivity or true positive rate (TPR). **A,** Training cohort with averaged performance across 100 bootstrap experiments. For each bootstrap, the training cohort was split into 80% training data and 20% testing data to estimate the optimal CT cutoff and the performance of the trained model. **B,** Performance of the test was confirmed in an independent validation cohort of 105 clinical samples.

Table 1. Validation cohort performance

		Plasma results using cfDNA and exosomal RNA		Total number
		+	−	
A				
Tissue result	+	47	4	51
	−	6	48	54

B	
M stage	**Sensitivity**
M0/M1a	14/16 (88%)
M1b	30/32 (94%)
MX	3/3 (100%)

C		
Parameter	**Training data (SD)**	**Clinical validation data**
AUC	0.94 (± 0.06)	0.96
Specificity	0.95 (± 0.06)	0.89
Sensitivity	0.91 (± 0.09)	0.92
Accuracy	0.93 (± 0.06)	0.91
Precision	0.95 (± 0.06)	0.89
Negative predictive value	0.92 (± 0.07)	0.92
Positive predictive value	0.95 (± 0.06)	0.89

NOTE: A, Confusion matrix that correlates T790M results in tissue with plasma exoNA. The sensitivity and specificity were 92% and 89%, respectively. B, Sensitivity of the plasma exoNA test with cfDNA and exosomal RNA based on M status. C, Averaged performance measurements across 100 bootstrap experiments on the training and results from the clinical validation data.

stage. While many liquid biopsies only interrogate the cfDNA fraction, it is well known that mutations are also present in the RNA (26).

T790M is an important disease progression biomarker, and mutation status is required for decision on the best patient care. The primary treatment for acquired resistance through T790M is osimertinib, which has gone through regulatory approval in the United States, Europe, and Japan. However, tissue biopsies have several challenges, including safety and cost. As many as 19% of patients receiving a lung biopsy suffer from adverse effects and the median cost of a biopsy procedure with complications is over $37,000 (18). Additionally, there is a significant fraction of patients where a tissue biopsy cannot be performed.

The assay presented here provides a novel liquid biopsy that utilizes all sources of mutations (both RNA and DNA) to ensure highly sensitive detection of T790M. To determine test performance, the study evaluated a total 210 clinical samples, of which 102 were T790M-positive to approximate the T790M prevalence in patients with acquired resistance. Even though the cohort had ∼37% intrathoracic or unknown disease stage (M0/M1a-MX) patients, the exoNA-based assay achieved an overall performance of 92% sensitivity and 89% specificity.

Detecting the T790M mutation is especially difficult in patients with confined thoracic disease (stages M0/M1a). Recent studies show that even highly sensitive methods that rely on cfDNA as input material struggle to reliably detect T790M. For example droplet digital PCR could only detect T790M in 18% (2/11) of M1a patients (35), 51% (121/243) detection by the cobas EGFR Mutation Test v2 (36), and 27% (4/15) when using BEAMing (19). In contrast, the exoNA-based test presented here shows a clinical sensitivity of 88% (14/16) and 94% (30/32) for disease stages M0/M1a and M1b respectively.

The performance of the test described here can be compared with the FDA-approved cfDNA test for T790M (cobas EGFR Mutation Test v2, Roche), which showed a lower sensitivity of 58% and 80% specificity. The Roche test is currently used when tissue is not available. It has been suggested that liquid biopsies in the future could be used up front (23) and only when the liquid biopsy is negative, the patient would proceed to an invasive tissue biopsy. However, the low sensitivity of the cfDNA-based assay would lead to a lot of unnecessary invasive tissue biopsies for follow-up on a negative liquid biopsy result. By applying the exoNA-based assay presented here, the number of unnecessary follow-up biopsies could be reduced from 42% (with cfDNA) to 8% with the combined exosome platform.

Because exosomes are shed by living cells and cfDNA is released by necrotic/apoptotic cells, the combined detection of cfDNA and exosomal nucleic acids described here will analyze two different biological processes of the tumor and may facilitate earlier detection of the developing resistance mutation in a longitudinal setting. This combined exosome/cfDNA platform was recently shown to generate approximately 10-fold more copies of *EGFR*-activating mutations in a direct comparison with BEAMing analysis on cfDNA and a higher clinical sensitivity (29). Another study showed that longitudinal mutation analysis using this platform could correlate better to overall survival compared with methods using cfDNA alone (27).

Disclosure of Potential Conflicts of Interest

D.G. Grimm, V. Tadigotla and J.K. Skog have ownership interests (including patents) at Exosome Diagnostics. L.E. Raez reports receiving commercial research grants from Exosome Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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References

1. Bremnes RM, Busund LT, Kilvaer TL, Andersen S, Richardsen E, Paulsen EE, et al. The role of tumor-infiltrating lymphocytes in development, progression, and prognosis of non-small cell lung cancer. J Thorac Oncol 2016; 11:789–800.
2. Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, et al. EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 2005;23:857–65.
3. Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell 2012;150:1121–34.
4. Zhang Z, Stiegler AL, Boggon TJ, Kobayashi S, Halmos B. EGFR-mutated lung cancer: a paradigm of molecular oncology. Oncotarget 2010;1:497–514.
5. Suda K, Onozato R, Yatabe Y, Mitsudomi T. EGFR T790M mutation: a double role in lung cancer cell survival? J Thorac Oncol 2009;4:1–4.
6. Morita S, Okamoto I, Kobayashi K, Yamazaki K, Asahina H, Inoue A, et al. Combined survival analysis of prospective clinical trials of gefitinib for non-small cell lung cancer with EGFR mutations. Clin Cancer Res 2009; 15:4493–8.
7. Gazdar AF.Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene 2009;28:S24–31.
8. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
9. Balak MN, Gong Y, Riely GJ, Somwar R, Li AR, Zakowski MF, et al. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. Clin Cancer Res 2006;12:6494–501.
10. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res 2006;12:5764–9.
11. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res 2013;19:2240–7.
12. Mok TS, Wu YL, Ahn MJ, Garassino MC, Kim HR, Ramalingam SS, et al. Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. N Engl J Med 2017;376:629–40.
13. Mulloy R, Ferrand A, Kim Y, Sordella R, Bell DW, Haber DA, et al. Epidermal growth factor receptor mutants from human lung cancers exhibit enhanced catalytic activity and increased sensitivity to gefitinib. Cancer Res 2007;67: 2325–30.
14. Vikis H, Sato M, James M, Wang D, Wang Y, Wang M, et al. EGFR-T790M is a rare lung cancer susceptibility allele with enhanced kinase activity. Cancer Res 2007;67:4665–70.
15. Rosell R, Molina MA, Costa C, Simonetti S, Gimenez-Capitan A, Bertran-Alamillo J, et al. Pretreatment EGFR T790M mutation and BRCA1 mRNA expression in erlotinib-treated advanced non-small-cell lung cancer patients with EGFR mutations. Clin Cancer Res 2011;17: 1160–8.
16. Su KY, Chen HY, Li KC, Kuo ML, Yang JC, Chan WK, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. J Clin Oncol 2012;30:433–40.
17. Mao C, Yuan JQ, Yang ZY, Fu XH, Wu XY, Tang JL. Blood as a substitute for tumor tissue in detecting EGFR mutations for guiding EGFR TKIs treatment of nonsmall cell lung cancer: a systematic review and meta-analysis. Medicine (Baltimore) 2015;94:e775.
18. Lokhandwala T, Bittoni MA, Dann RA, D'Souza AO, Johnson M, Nagy RJ, et al. Costs of diagnostic assessment for lung cancer: a medicare claims analysis. Clin Lung Cancer 2017;18:e27–e34.
19. Karlovich C, Goldman JW, Sun JM, Mann E, Sequist LV, Konopa K, et al. Assessment of EGFR mutation status in matched plasma and tumor tissue of NSCLC patients from a phase I study of rociletinib (CO-1686). Clin Cancer Res 2016;22:2386–95.
20. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol 2013;10: 472–84.
21. Karachaliou N, Mayo-de las Casas C, Queralt C, de Aguirre I, Melloni B, Cardenal F, et al. Association of EGFR L858R mutation in circulating free DNA with survival in the EURTAC Trial. JAMA Oncol 2015;1:149–57.
22. Diaz LA Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014;32:579–86.
23. Oxnard GR, Thress KS, Alden RS, Lawrance R, Paweletz CP, Cantarini M, et al. Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer. J Clin Oncol 2016;34:3375–82.
24. FDA: summary of safety and effectiveness data (SSED). website: https://www.accessdata.fda.gov/cdrh_docs/pdf15/P150044B.pdf. In: FDA, editor.
25. Schwarzenbach H, Stoehlmacher J, Pantel K, Goekkurt E. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. Ann N Y Acad Sci 2008;1137:190–6.
26. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008; 10:1470–6.
27. Möhrmann L, Huang HJ, Hong DS, Tsimberidou AM, Fu S, Piha-Paul SA, et al. Liquid biopsies using plasma exosomal nucleic acids and plasma cell-free DNA and clinical outcomes of patients with advanced cancers. Clin Cancer Res 2018;24:181–8.
28. Enderle D, Spiel A, Coticchia CM, Berghoff E, Mueller R, Schlumpberger M, et al. Characterization of RNA from exosomes and other extracellular vesicles isolated by a novel spin column-based method. PLoS One 2015; 10:e0136133.
29. Krug AK, Enderle D, Karlovich C, Priewasser T, Bentink S, Spiel A, et al. Improved EGFR mutation detection using combined exosomal RNA and circulating tumor DNA in NSCLC patient plasma. Ann Oncol 2017. [Epub ahead of print]
30. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 2010;23:550–76.
31. Leighl NB, Rekhtman N, Biermann WA, Huang J, Mino-Kenudson M, Ramalingam SS, et al. Molecular testing for selection of patients with lung cancer for epidermal growth factor receptor and anaplastic lymphoma kinase tyrosine kinase inhibitors: American Society of Clinical Oncology endorsement of the college of american pathologists/international association for the study of lung cancer/association for molecular pathology guideline. J Clin Oncol 2014;32:3673–9.

32. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–22.
33. Youden WJ. Index for rating diagnostic tests. Cancer 1950;3:32–5.
34. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20:548–54.
35. Thress KS, Brant R, Carr TH, Dearden S, Jenkins S, Brown H, et al. EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform comparison of leading technologies to support the clinical development of AZD9291. Lung Cancer 2015;90:509–15.
36. Jenkins S, Yang JC, Ramalingam SS, Yu K, Patel S, Weston S, et al. Plasma ctDNA analysis for detection of the EGFR T790M mutation in patients with advanced non-small cell lung cancer. J Thorac Oncol 2017;12:1061–70.
37. McKillop D, Partridge EA, Kemp JV, Spence MP, Kendrew J, Barnett S, et al. Tumor penetration of gefitinib (Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor. Mol Cancer Ther 2005;4:641–9.
38. Tsubata Y, Hamada A, Sutani A, Isobe T. Erlotinib-induced acute interstitial lung disease associated with extreme elevation of the plasma concentration in an elderly non-small-cell lung cancer patient. J Cancer Res Ther 2012; 8:154–6.