Review Article on Mechanisms of Resistance to EGFR-targeted Therapy

Applications of cell-free circulating tumor DNA detection in EGFR mutant lung cancer

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Abstract: Analyses of cell-free tumor DNA (ctDNA) have provided a non-invasive strategy for cancer diagnosis, the identification of molecular aberrations for treatment identification, and evaluation of tumor response. Sensitive and specific ctDNA sequencing strategies have allowed for implementation into clinical practice for the initial genotyping of patients and resistance monitoring. The specific need for EGFR mutation detection for the management of lung cancer patients has been an early imperative and has set the stage for non-invasive molecular profiling across other oncogenic drivers. Ongoing efforts are demonstrating the utility of ctDNA analyses in the initial genotyping of patients, the monitoring resistance clones, and the initial evaluation of response.

Keywords: Epidermal growth factor (EGFR); osimertinib; circulating tumor DNA (ctDNA); BEAMing; droplet digital PCR; next generation sequencing (NGS)

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EGFR as a model of non-invasive detection monitoring

The assessment of molecular alterations for patients with non-squamous non-small cell lung cancer (NSCLC) has been adopted into guidelines to determine suitability for targeted treatments (1,2). Tissue adequacy in detecting resistance mutations is an important reason to consider plasma testing. Up to 30% of NSCLC patients cannot provide sufficient tumor samples for molecular testing at diagnosis or progression (3). Circulating tumor DNA serves as an alternative method to non-invasively detect molecular aberrations in NSCLC when tissue is not available (4) (*Figure 1*).

Plasma detection of *EGFR* mutations serves as a model to demonstrate the clinical utility of a non-invasive testing study for EGFR-TKI selection in the era of precision medicine (5). The therascreen (Qiagen) EGFR RGQ PCR Kit and cobas® EGFR Mutation Test v2 are qPCR assays approved by the EMA and FDA, respectively, for the analysis of plasma ctDNA (6). The cobas® EGFR Mutation Test (v1) is a qualitative real-time polymerase chain reaction (RT-PCR) test approved in 2013 for exon 19 del and L858R substitution mutations in FFPE NSCLC tissue specimens (7). The FDA approved the cobas® EGFR Mutation Test v2 in 2015 for both FFPE and plasma specimens and includes the assessment of T790M mutations, exon 18 substitutions, exon 19 deletions, and exon 20 and 21 substitutions when tissue is not available (7,8). There have been several plasma based laboratory developed tests that utilize next generation sequencing (NGS) strategies, and these are currently pending regulatory approvals.

Initial genotyping with PCR technologies

The need to initially genotype patients with prompt turn-around times is fundamentally important in therapeutic decision-making. Basing front-line approaches on the results

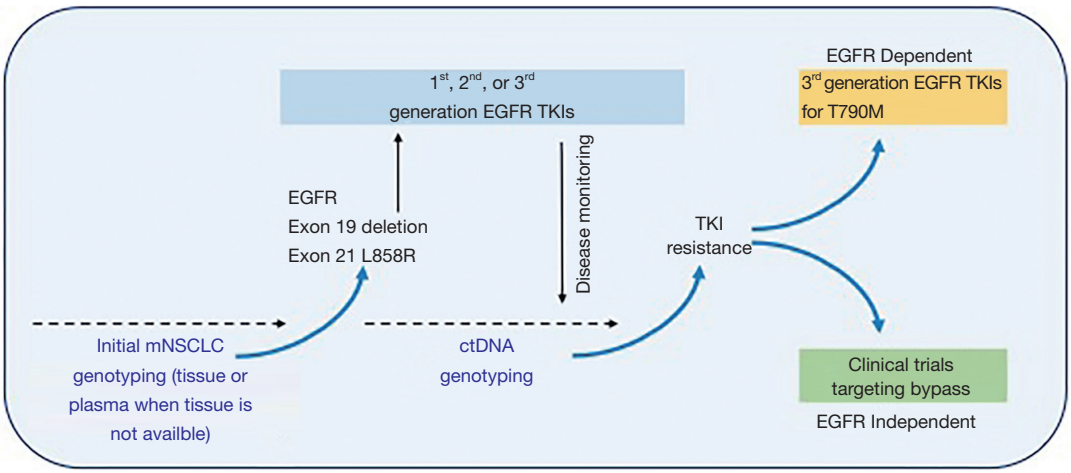

Figure 1 Applications of non-invasive mutation detection for the initial genotyping and resistance monitoring on EGFR tyrosine kinase therapy.

of a patient's molecular profile has maximized efficacy and mitigated potential toxicity with sequential treatments. The overall survival of patients on targeted therapies has superseded historical controls without targeted approaches.

Among Japanese NSCLC patients on first-line gefitinib, 48% patients had EGFR exon 19 deletions and L858R mutations, and the identification of these mutations predicted response to gefitinib (9). In another study of forty-two patients receiving gefitinib, EGFR mutation detected by ctDNA was predictive of improved objective response rate (P=0.001) and median progression free survival (P=0.044) to gefitinib (10).

In a recent prospective trial called the Non-invasive *vs.* Invasive Lung Evaluation study (NILE), a hybridization captures targeted sequencing NGS assay detected FDA-approved mutations (EGFR, ALK, ROS1, BRAF) in previously untreated patients with high concordance of driver alleles compared to tissue genotyping tests (>98.2%). The utilization of cfDNA combined with tissue increased detection by 32% in patients with negative, not accessible, or insufficient tissue and median turn around time of 9 days (11). Another PCR based analysis showed a 91% overall concordance of EGFR mutations detected in plasma and tissue in 196 NSCLC patients studied (12). A high correlation was seen in a separate study with the EGFR gene as detected in plasma versus those detected in the matched tumor sample (87.7% concordance) (13). There have been earlier studies that have lacked this high level of concordance with potential underlying variables including differences in assay platforms, spatial and temporal factors in tumor burden, tumor heterogeneity, interval treatment, potential germline DNA contamination, and variants which may derive from clonal hematopoiesis (14).

The FASTACT-2 trial demonstrated the predictive value of serial ctDNA EGFR testing by exploring prospectively whether baseline tissue biopsy correlates with Cobas® EGFR ctDNA testing. Analysis of circulating tumor DNA was performed in patients with stage IIIB/IV NSCLC randomized to receive chemotherapy followed by maintenance with either erlotinib or placebo (15). A planned retrospective analysis of advanced NSCLC treated first-line with chemotherapy and erlotinib showed an 88% concordance between blood and tissue testing with a sensitivity and specificity of 75% and 96%, respectively. Median PFS for ctDNA was 13.1 months for EGFR mutant ctDNA-positive patients treated with erlotinib, and 6 months for patients treated with placebo. For EGFR wild-type ctDNA patients, erlotinib or placebo yielded similar survival (~6 months). Patients with EGFR mutant ctDNA detected at baseline who experienced a complete elimination at the end of an induction period of treatment had improved progression-free survival (12 *vs.* 7.2 months) and overall survival (31.9 *vs.* 18.2 months) (15,16).

Some additional studies have evaluated whether the upfront ctDNA load may correlate with clinical outcome endpoints. The EURTAC176 clinical trial tested ctDNA as a proxy for tissue EGFR testing with an RT-PCR (TaqMan) assay to assess for EGFR mutations. Patients with *EGFR* mutations detected in pre-treatment ctDNA predicted shorter OS in univariate analysis. Tissue confirmed L858R

mutation when detected in plasma correlated with a median OS of 13.7 months compared to 27.7 months for those with the mutation undetectable in cfDNA (HR 2.22) (17).

Tracking tumor burden with NGS based approaches

A number of studies have demonstrated that ctDNA load can correlate with changes in tumor burden as detected with ultrasensitive NGS approaches. Newman *et al.* evaluated the effectiveness of CAPP-Seq for minimal residual disease (MRD) detection and monitoring using plasma samples from healthy controls and a NSCLC cohort (18). In patients with Stage I-IV NSCLC, ctDNA was detected in all patients, in half of the patients with stage I and with a 96% mutant allele fraction specificity and a detection threshold of ~0.02%. Chabon *et al.* serially tracked EGFR tumor load and resistance mutations on therapy with rises that were concordant with CT scan tumor increases (19). Chaudhuri *et al.* evaluated CAPP-Seq in over 250 samples from forty patients being treated with curative intent diagnosed with stage I-III NSCLC and in fifty-four healthy controls. Plasma circulating DNA was detected post-treatment in the vast majority (94%) of patients having recurrence. ctDNA detection after treatment preceded progression seen on imaging modalities in about a third of patients by approximately 5 months. About half of the patients had actionable mutations in plasma, providing an opportunity for personalized adjuvant treatment (20). A novel technology called TEC-SEQ (Targeted Error Correction Sequencing) has identified genomic aberrations in lung cancer in stage I and II patients at a rate of approximately 45–50% (21). This strategy facilitates early MRD monitoring and has been utilized for tracking response to therapy (22). Ongoing studies are using fragment length to characterize tumor specific DNA from wild type DNA (23).

Resistance monitoring

Circulating tumor DNA has been used as a companion diagnostic to monitor for the EGFR gatekeeper T790M mutation after treatment with first- and second-generation inhibitors (24,25). Oxnard *et al.* showed similar efficacy outcomes with osimertinib with plasma digital PCR by BEAMing or tissue T790M testing (26). The positive predictive value (PPV) of ctDNA was 100% for L858R and EGFR 19 deletions, and 79% for T790M mutations. The study had a 30% false negative rate for plasma genotyping. Patients with a negative plasma T790M result have necessitated a primary tumor biopsy to confirm the absence of T790M.

In a separate retrospective study of EGFR mutant patients, BEAMing digital PCR was able to detect 70% T790M mutants. About a third (31%) of the patients with T790M-negative tumors had T790M positive ctDNA. In this study, response rates and PFS correlated with plasma positive T790M or tissue positive T790M (27). In another trial, an association between ctDNA load and OS was seen, whereas no correlation was noted with serial PET/CT tumor volume or avidity. Increased ctDNA independently associated with a shorter overall survival (28).

Tumor heterogeneity and acquired resistance

Co-occurring alterations or tumoral heterogeneity may explain drug resistance to EGFR inhibitors. Plasma ctDNA sampling can provide a comprehensive analysis across metastatic sites when detectable (6,29). Furthermore, a tumoral molecular profile may evolve dynamically over time as a result of selection pressures on therapy (30). Understanding the evolution of selective pressures contributing to resistance mutations may help guide sequential targeted therapy (31-34). Third generation EGFR tyrosine kinase inhibitors irreversibly inhibit *EGFR* T790M resistance and have received front-line approval in the metastatic setting for patients with EGFR mutation (35). Patients treated with osimertinib whom relapse may acquire new genetic alterations including the EGFR C797S mutation (36). A variety of additional alterations have been observed and may include KRAS mutations, BRAF V600 mutations, HER2 amplification, and MET amplification among others (35-37).

In patients with NSCLC treated with third generation EGFR TKIs, acquired EGFR C797S mutation has been identified on serial cfDNA specimens (38). Analysis of plasma collected from 15 patients treated with osimertinib detected three molecular subtypes emerging at resistance defined by EGFR C797S or a bypass mechanism (39). In a subset of patients who had progressed on rociletinib, different EGFR activating mutations (i.e., L798I) and bypass pathways with MET amplification have been detected in ctDNA and track with tumor resistance (19).

Early markers of response

A number of studies have shown that the dynamic

monitoring of ctDNA load can provide insights into the detection of treatment response (19,40). Shorter PFS and OS can be associated with early ctDNA detection independent of confounding factors including age, stage, and histological subtype (41,42). We have employed an ultrasensitive liquid biopsy approach (TEC-SEQ) to serially evaluate patients with advanced NSCLC who have received tyrosine kinase inhibitors including erlotinib, afatinib, osimertinib, and mavelertinib (PF-06747775). Analyses of 28 patients revealed molecular responders had a near complete elimination of ctDNA (>98%) on therapy. Molecular non-responders with limited or no reduction of ctDNA levels experienced a statistically shorter PFS (1.6 *vs.* 13.7 mos, P<0.0001) detected approximately 4 weeks sooner than detection on CT imaging (22).

Circulating tumor DNA has been detected in urine, and drug induced apoptosis has been modeled within days of TKI treatment in patients with detectable *EGFR* mutations. In a proof of concept study, we identified an initial spike within the first week of therapy followed by a significant decrease in the number of copies detected from baseline within a week. This work demonstrates that frequent ctDNA sampling may enable early evaluation of patient response or progression (43).

In a separate study, changes in EGFR mutation have been shown to correlate with early clinical response prediction to EGFR TKIs. EGFR-mutated tumors with ctDNA testing at baseline and serially during erlotinib therapy showed a decrease in 95% of cases. The rate of the decrease in ctDNA fraction correlated with radiological response (P<0.0001). The patients with rapid decrease in ctDNA fraction (>50–70%) had longer progression free survival (44).

Recent data from the FLAURA first line osimertinib study of 489 patients showed that the clearance of ctDNA at weeks 3 or 6 was associated with a longer PFS than those patients in whom there was no clearance of ctDNA. Plasma ctDNA analysis of treatment naïve patients with stage III/IV NSCLC were evaluated by ddPCR. EGFR mutation analysis was done at baseline, week 3, and week 6 after EGFR-TKI therapy. Early clearance of plasma EGFR mutation after EGFR-TKI therapy was associated with improved PFS. Patients with detectable baseline plasma ctDNA had shorter PFS than those without detectable EGFR mutation (45). The integration of ctDNA analyses in clinical trials to understand their correlation with clinical outcomes is an ongoing path forward.

Conclusions

There has been a pressing need for a highly sensitive and reliable non-invasive liquid biopsy strategy for screening and resistance monitoring. EGFR testing and tracking through serial ctDNA plasma analyses is being utilized for initial genotyping and resistance monitoring. Plasma assays are being increasingly utilized when tissue is not available. These tests may have high sensitivities and specificities, and several studies are ongoing demonstrating concordance with tissue testing. There has been integration of ctDNA analyses in clinical trials as endpoints to track with response. The development of additional assays will further improve our knowledge of drug resistance, clonal evolution, and combinatorial therapeutic strategies forward.

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