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REVIEW ARTICLE

Circulating tumour DNA in *EGFR*-mutant non-small-cell lung cancer

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ABSTRACT

The advent of targeted therapy in non-small-cell lung cancer (NSCLC) has made the routine molecular diagnosis of *EGFR* mutations crucial for optimal patient management. Obtaining tumour tissue for biomarker testing, especially in the setting of re-biopsy, can present many challenges. A potential alternative source of tumour DNA is circulating cell-free tumour-derived DNA (ctDNA). Although ctDNA is present in low quantities in plasma, the convenience of sample acquisition and the increasing reliability of detection methods make this approach a promising one. The various performance characteristics of both digital and nondigital platforms are still variable, and a standardized approach is needed that will make those platforms reliable clinical tools for the detection of *EGFR* sensitizing mutations and resistance mutations, including the T790M resistance mutation. Information derived from ctDNA can be used to assess tumour burden, to identify genomic-based resistance mechanisms, and to track dynamic changes during therapy.

Key Words Lung adenocarcinoma, *EGFR*, circulating DNA, ctDNA, liquid biopsy

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INTRODUCTION

Clinical practice has changed since the discovery of mutations in the kinase domain of the *EGFR* gene in non-small-cell lung cancer (NSCLC). Patients with a tumour that harbours such mutations, especially exon 21 L858R and exon 19 deletions (which account for approximately 90% of all sensitizing mutations[1]), experience prolonged progression-free survival when treated with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs)[2–4]. However, most of those patients will ultimately progress and succumb to their cancer. The action of second-generation TKIs (afatinib and dacomitinib), which irreversibly inhibit members of the ErbB family receptor tyrosine kinases, has been less impressive, partly tempered by greater side effects; however, those agents will remain an important therapeutic option. Importantly, acquired resistance in approximately 60% of patients treated with the first-generation EGFR TKIs erlotinib and gefitinib is conferred by the point mutation T790M[5,6]. That mutation restores the kinase domain's binding affinity for adenosine triphosphate, rendering the TKIs ineffective. The high frequency of acquired resistance attributable to the T790M mutation has prompted the development of third-generation TKIs that can overcome that specific resistance mechanism. Furthermore, the presence of T790M in a tumour before treatment with a first-generation TKI is a marker for worse prognosis[7–9]. Routine detection of T790M at diagnosis and continual monitoring throughout TKI treatment and progression is even more important now that the third-generation EGFR TKI osimertinib, which specifically inhibits tumours harbouring the T790M mutation, has become clinically available.

In a hallmark example of precision oncology, the initial diagnostic biopsy material from pulmonary adenocarcinomas is now being routinely tested for *EGFR* sensitizing mutations (and *ALK* rearrangements), usually on formalin-fixed paraffin-embedded tissue sections. Given the increasing number of approved EGFR TKIs with differing specificities and resistance mechanism profiles, many institutions are now incorporating pretreatment molecular testing for the T790M point mutation. In many cases, the biopsy material limits that testing, and because most patients with NSCLC are diagnosed at an advanced stage, surgical acquisition of more tumour tissue for molecular testing is not a viable alternative. Moreover, monitoring resistance and sensitizing *EGFR* mutations during progression is determined by accessibility to tumour that can be biopsied. Intratumoural heterogeneity also complicates the matter, in that only a subset of somatic mutations (that is, truncal mutations) are shared by all tumour cells, and subclonal populations might not be thoroughly detected and characterized by the limited sampling.

Alternatively, circulating cell-free tumour-derived DNA (ctDNA) has been used to detect and monitor tumour progression in various cancers, including detecting sensitizing *EGFR* mutations in NSCLC. In oncology, including in

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NSCLC, ctDNA is rapidly gaining clinical utility; many studies have shown promise in monitoring treatment response in patients with sensitizing *EGFR* mutations undergoing EGFR TKI therapy, and in detecting the presence of the T790M resistance mutation in treatment-naïve patients and in those with progressive disease while taking the first-generation EGFR TKIs erlotinib and gefitinib.

PRINCIPLES OF CIRCULATING DNA

Discovered by Mandel and Metais, the presence of circulating cell-free DNA has been known since the late 1940s[10]. Every living cell actively secretes small fragments of DNA into the circulation, and the concentration of those secretions increases in certain conditions such as trauma, inflammation, apoptosis, or necrosis[11]. Circulating DNA consists of small double-stranded fragments that are approximately 150 bp in length[12], matching the length of DNA in a nucleosome. The fragments are rapidly cleared—a 99% clearance rate within 2 hours having been observed in multiple studies[13,14]. Plasma concentrations of circulating DNA vary widely, and a significant difference in quantity is seen between individuals with malignant disease and those who have nonmalignant disease or who are healthy[15].

The biologic role of circulating DNA is still far from completely understood. Studies have shown that circulating DNA in healthy individuals plays an important antimicrobial role as a principal component of neutrophil extracellular traps[16]. It is thought that release of those traps by neutrophils serves as an innate form of immune response that is capable of degrading virulence factors and killing bacteria. The circulating DNA component of the neutrophil extracellular traps also plays a crucial role in activating the coagulation system and is thought to be regulated by DNAse in the bloodstream.

The Human Genome Project provided the impetus for the technological progress in molecular analyses in the 1990s. The surge of newer molecular techniques allowed for the clinical application of circulating cell-free DNA. Indeed, the cell-free DNA present in the circulation, together with its accessibility by the minimally invasive technique of venipuncture, has led to its use in various clinical scenarios, including prenatal diagnosis of fetal trisomies[17]; prediction of outcomes in traumatic and burn injuries, myocardial infarctions, and stroke[11]; and monitoring of allograft rejection in organ transplantation[18].

Experimental evidence that tumour cells also release their DNA into the circulation has been available since 1989[19], and cancer-specific point mutations (*NRAS* point mutations in myelodysplastic syndrome and acute myeloid leukemia, and *KRAS* mutations in pancreatic cancer) were first detected in 1994[20,21]. Detection of other cancer-specific molecular alterations in the circulation, including microsatellite instability[22], gene amplifications[23,24], and the hypermethylation status of promoter regions in tumour suppressor genes[25,26], were discovered soon after. It was also noted that the total circulating DNA concentration was higher overall in patients with lung cancer (among other cancers) than in healthy subjects[27,28].

Recently, Underhill *et al.*[28] demonstrated that, in patients with lung cancer (and other tumours), the fragment lengths of ctDNA are shorter than those of normal circulating DNA (for lung cancer: 277 bp vs. 283 bp; $p = 0.002$). Additionally, by selecting the fraction of circulating DNA with base pairs shorter than the peak fragment length of that individual's library (approximately 20–50 bp shorter), they were able to increase the allele frequency of T790M mutation signals, suggesting an improvement in mutant call sensitivity by fractional selection of shorter fragment lengths. The realization that ctDNA has the potential to be used as a reliable biomarker for the presence and overall burden of a tumour has prompted many investigators to discover better, more sensitive methods of detection[29].

The challenge in the detection of ctDNA is that it is markedly dilute compared with the background circulating germline DNA (0.01%–10%)[30], requiring highly sensitive methods to increase its weak signal. Standard approaches, including Sanger sequencing and pyrosequencing, often fail to detect ctDNA fragments except in individuals with a very high tumour burden or level of ctDNA. Current approaches for detecting ctDNA can be broadly categorized in two ways: targeting specific molecular alterations and targeting all possible molecular alterations in DNA (including targeted and whole-genome or whole-exome sequencing).

MUTATION DETECTION METHODS

A variety of polymerase chain reaction (PCR)–based methods have been used to assess ctDNA for specific molecular alterations. These techniques generally require less DNA input material, have a good signal-to-noise ratio, and are efficient for use in cancers with few, but important, molecular alterations, including NSCLC with *EGFR* sensitizing mutations and the T790M resistance mutation. However, with the exception of digital PCR, these methods are more reliable for qualitative than for precise quantitative assessments. The amplification refractory mutation system uses sequence-specific PCR primers that allow amplification only if the target allele is present; it is broadly used in detecting *EGFR* sensitizing mutations[31,32]. Peptide nucleic acid clamping PCR relies on preferential amplification of mismatched sequences to enrich non-target sequences of a mixed template[33].

A growing number of studies are investigating the utility of ctDNA in cancer management, and the field is moving toward digital methods of detection. Digital methods of PCR technology incorporate a number of techniques to improve the specificity and sensitivity of mutation detection. Digital droplet PCR sample preparation includes separation of template molecules into individual reaction vessels, which can then be individually assessed for the presence of mutation. That approach converts the analog nature of PCR into a linear digital signal and permits absolute quantification of variant alleles[34,35]. A specific subtype of the latter approach, BEAMing (beads, emulsion, amplification, magnetics), places single DNA molecules onto magnetic beads, upon which thousands of copies are made, creating a one-to-one representation of the starting DNA molecule per bead[36]. The technology has been commercialized, and although being actively used[37], it has not gained traction because the protocol is laborious and requires specific bead-bound oligonucleotides for each mutation tested.

Oxnard *et al.*[38] used BEAMing digital PCR to retrospectively detect the presence of the T790M mutation in patients with acquired EGFR TKI resistance receiving osimertinib.

More affordable approaches to targeted sequencing based on oligonucleotide DNA capture has been used to sequence target gene panels and even the entire human exome[39]. That approach relies on sequencing library construction, followed by hybridization to DNA or RNA oligonucleotides complementary to selected regions. The hybrid molecules are sequestered and amplified with universal primer pairs complementary to the adaptors. Because ctDNA exists as smaller nucleic acid fragments, ligation-based chemistry can be used to directly prepare libraries[40], bypassing the standard next-generation sequencing methods for library preparation that require either shearing of larger fragments before adaptor ligation or transposon-based library construction, which simultaneously fragments and tags DNA in a single reaction. Pioneering work by Newman *et al.*[41] incorporated that technology to quantify ctDNA in patients with NSCLC. To select their regions-of-interest panel, those authors used data from the Catalogue of Somatic Mutations in Cancer and The Cancer Genome Atlas to include regions of the genome that contain the most recurrent mutations in NSCLC. Their CAPP-seq (cancer personalized profiling by deep sequencing) panel targets 521 exons and 13 introns from 139 recurrently mutated genes, covering approximately 125 kb, and identifies insertions, deletions, point mutations, and structural alterations, including *ALK* rearrangements. They demonstrated that, compared with radiographic imaging, ctDNA analysis better assesses early response to treatment and could be used to distinguish between residual disease and treatment-related changes. They have also now integrated molecular barcoding, which incorporates sequencing adapters that act as "barcodes" to allow for reconstruction of the parental DNA duplexes after amplification, as well as *in silico* filtering of common recurrent background errors that reflect oxidative damage arising *in vivo* or *ex vivo* (that is, G>T transversions and C>T or G>A transitions). The latter work has resulted in an error rate lower by a factor of 15 than that achieved with the original CAPP-seq approach[42].

Whole-exome sequencing does not require *a priori* knowledge of a tumour's molecular profile. As proof of principle, Murtaza *et al.*[43] used whole-exome sequencing to monitor treatment response in 6 patients with advanced breast, ovarian, and lung cancers. They demonstrated an overall strong concordance between mutations detected in the primary tumour tissue and in ctDNA. They observed that the variant allele frequency largely reflected the levels of ctDNA in each sample. However, the relatively high cost, large target size for coverage, and high DNA input requirement (>100 ng/mL) currently limits the practicality of this approach in assessing ctDNA.

CORRELATION OF TISSUE AND PLASMA SAMPLES

The performance characteristics of the many technology platforms used to detect *EGFR* sensitizing mutations in plasma vary considerably. Table I summarizes the various methods and the ranges of their diagnostic accuracy. Nondigital methods have high specificity, but suffer from lower sensitivities. Digital methods, including digital droplet PCR and BEAMing, have high sensitivities and specificities, but require more technical expertise and laborious protocols.

A growing body of evidence about the diagnostic utility and performance of various methods for the noninvasive measurement of the T790M resistance mutation in ctDNA is accumulating (Table II). As in the case of *EGFR* sensitizing mutations, nondigital methods maintain relatively high specificity, but lower sensitivity. The concordance rates between the studies vary widely, possibly for several reasons. First, small-volume tumours might not shed enough DNA into the circulation. Second, intratumoural heterogeneity could explain a positive call on the plasma even though the primary tumour tests negative. Third, detection discordance between tumour tissue and plasma could reflect the differing sensitivities of the platforms used.

CIRCULATING DNA TO ASSESS TUMOUR BURDEN

There are many advantages to using ctDNA as a way to assess overall tumour burden. Several studies have investigated the use of total circulating DNA quantification as a surrogate for tumour burden in cancer patients, especially in metastatic disease—analogous to assessing HIV viral load[47,49]. Investigators have shown that, in lung cancer patients, increasing concentrations of total circulating DNA are correlated with tumour stage and overall survival[60–62]. Using quantitative real-time PCR, Szpechcinski *et al.*[63] compared total circulating DNA concentrations in patients with chronic respiratory inflammation (chronic obstructive pulmonary disease, sarcoidosis, or asthma) and in patients with NSCLC. They determined that increased total circulating DNA is 90% sensitive and 80.5% specific for plasma from lung cancer patients compared with non-cancer patients, even in the presence of confounding pulmonary pathology.

The recent TRACERx study[64] also provides valuable insight into predictors of ctDNA detection in early-stage NSCLC. The study authors discovered that plasma-based detection is increased in tumours with non-adenocarcinoma histology, necrosis, lymphovascular invasion, and a higher Ki-67 proliferation index. Tumour volume was also seen to correlate with plasma ctDNA variant allele frequencies, and a primary tumour volume of 10 cm^3 predicted a ctDNA plasma variant allele frequency of 0.1%.

Chen *et al.*[65] investigated the clinical value of urinary samples as source of ctDNA in patients with NSCLC. The study enrolled 150 patients with sensitizing *EGFR* mutations who were receiving a first-generation EGFR TKI (erlotinib or gefitinib) and compared DNA from primary tumour tissue with ctDNA from blood and urine. Serial urinary and plasma ctDNA measurements were also performed every month for 9 months. The authors reported a concordance rate of 88% between primary tumour tissue and urinary ctDNA for *EGFR* mutation, and a concordance rate of 98% between plasma and urinary ctDNA. As expected, ctDNA concentrations dropped in both urine and plasma after TKI initiation. By the final time point, 53% of patients in the study cohort had developed the T790M mutation, which had been absent at baseline; the median period of mutation emergence for the group overall was 6 months after TKI initiation. Additionally,

TABLE I Assay performance in detecting *EGFR*-sensitizing mutations in circulating tumour DNA

Method	Sensitivity[a] (%)	Specificity[a] (%)	Concordance[a] (%)	References
ARMS	50–75	85–100	72.7–94.3	Kimura *et al.,* 2006[32]; Kuang *et al.,* 2009[44]; Xu *et al.,* 2012[45]; Liu *et al.,* 2013[46]; Douillard *et al.,* 2014[47]
Cobas[b]	60.7	96.4	91.3	Weber *et al.,* 2014[48]
PNA-PCR	17.1	100	27.5	Kim *et al.,* 2013[49]
CAPP-Seq	85	96	—	Newman *et al.,* 2014[41]
ddPCR	66–92	87–100	70–93	Yung *et al.,* 2009[50]; Ishii *et al.,* 2015[51]; Lee *et al.,* 2016[52]; Seki *et al.,* 2016[53]; Takahama *et al.,* 2016[54]; Del Re *et al.,* 2017[55]; He *et al.,* 2017[56]
BEAMing	82–87	97	90–93	Thress *et al.,* 2015[57]
cSMART	72.7–100	94.0–98.3	86.9–98.4	Chai *et al.,* 2016[58]

[a] Sensitivities, specificities, and concordance rates are presented in reference to the mutation call from the corresponding tumour tissue, which was defined as the "gold standard."
[b] F. Hoffmann-La Roche, Basel, Switzerland.
ARMS = amplification refractory mutation system; PNA–PCR = peptide nucleic acid–mediated polymerase chain reaction; CAPP-Seq = cancer personalized profiling by deep sequencing; ddPCR = digital droplet polymerase chain reaction; BEAMing = beads, emulsion, amplification, and magnetics; cSMART = circulating single-molecule amplification and re-sequencing technology.

TABLE II Assay performance in the detection of T790M in circulating tumour DNA

Study	Matched samples (*n*)	Method	Concordance (%)	Sensitivity (%)	Specificity (%)
Douillard *et al.,* 2014[47]	652	ARMS	94.3	65.7	99.8
Ishii *et al.,* 2015[51]	18	ddPCR	83.3	81.8	85.7
Thress *et al.,* 2015[57]	72	BEAMing	90	81	58
Chai *et al.,* 2016[58]	61	cSMART	98.4	100	98.3
Seki *et al.,* 2016[53]	10	ddPCR	80	—	—
Takahama *et al.,* 2016[54]	41	ddPCR	65.9	64.5	70
Del Re *et al.,* 2017[55]	8	ddPCR	62.5	—	—
He *et al.,* 2017[56]	128	ddPCR	100	—	—
Wang *et al.,* 2017[59]	103	cSMART	90.29	50	91.92

ARMS = amplification refractory mutation system; cSMART = circulating single-molecule amplification and re-sequencing technology; ddPCR = digital droplet polymerase chain reaction; BEAMing = beads, emulsion, amplification, magnetics.

the quantity of urinary ctDNA was higher in patients who developed the T790M mutation, spiking upward a few months after detection of the resistant mutation. Urinary T790M was also prognostic, with the group testing positive for the mutation experiencing significantly worse overall survival.

Using ctDNA to More Comprehensively Characterize Resistance Mechanisms

The clinical impact of intratumoural geographic heterogeneity cannot be underscored further than by the characterization of resistance mechanisms. Tissue biopsies sample a particular geographic region of a tumour and will not always fully characterize the subclones present. Notably, earlier studies using ctDNA to characterize EGFR TKI resistance mechanisms were limited to methods specific for *EGFR* sensitizing mutations[37,57,66–70]. Newer studies using next-generation sequencing aim to more broadly capture and categorize drug-specific resistance mechanisms.

Chabon *et al.*[71] used CAPP-seq to analyze the ctDNA of 43 patients with T790M-mutant NSCLC who progressed on first- or second-generation EGFR TKIs and who entered a clinical trial for the third-generation TKI rociletinib. Analysis of ctDNA during progression but before rociletinib initiation showed additional molecular alterations constituting resistance mechanisms not previously detected in almost half the patients, including increased *MET* or *ERBB2* copy numbers, and additional single nucleotide variants in *EGFR*, *PIK3CA*, or *RB1*. Those patients experienced inferior responses and shorter progression-free survival when treated with rociletinib. Moreover, the authors described resistance mechanisms treatment-specific to third-generation TKIs, including a novel *EGFR* mutation (L798I) and an activating *KRAS* mutation as mechanisms of resistance to rociletinib. In contrast, *EGFR* C797S, a common resistance mutation found in patients treated with osimertinib[70], is not really found as a resistance mechanism in those treated with rociletinib. The authors also

noted that, as opposed to preclinical models[72,73] in which resistance mutations to third-generation TKIs have primarily involved additional mutations in the *EGFR* gene (for example, C797S), their patient cohort showed mostly bypass pathway activation, with *MET* copy number gain observed in 25% of patients as a common mechanism of acquired resistance[74]. In overcoming the limitations inherent in classic tissue biopsies, "liquid biopsies" are showing promise in more comprehensively characterizing EGFR TKI resistance mechanisms and will lead to more tailored combination or single-agent therapies.

Using ctDNA to Monitor Response to Therapy

He *et al.*[56] used digital droplet PCR to prospectively detect *EGFR* mutations in a cohort of 200 patients with NSCLC being treated with afatinib after developing resistance to a first-generation TKI. All patients underwent baseline blood sampling before any TKI treatment, and patients with *de novo* T790M were excluded. Eventually, 168 patients developed resistance to either erlotinib or gefitinib, and 128 patients were monitored to detect ctDNA variations. The authors reported 93.5% concordance between tissue and ctDNA for the *EGFR* sensitizing mutations L858R and ex19del, and 100% concordance for the T790M resistance mutation. Of the tested patients, 47% were positive for T790M ctDNA, and the average mutant ctDNA concentration in those patients was 660 ± 311 copies per millilitre. Interestingly, a correlative increase in ctDNA concentration was evident in patients who developed T790M, consistent with the reduced effectiveness of the first-generation TKIs. Serial monitoring of plasma samples after the start of afatinib treatment was able to capture the dynamic changes during treatment, with 46% of the patients receiving afatinib expeirencing a drop in ctDNA concentration, with a correspondingly favourable overall survival.

Clearly, the clinical utility of ctDNA as a means of genotyping and monitoring treatment response with *EGFR*-mutant NSCLC is rapidly becoming reality. To address the objective evaluation of ctDNA for monitoring the dynamic changes in NSCLC during TKI therapy, Kato *et al.*[75] proposed using a "MART" (mutation allele ratio in therapy) score as an index of therapeutic response. A diagnostic score called the plasma mutation score was defined as the number of reads with deletions (exon 19 deletions) or substitutions (exon 20: T790M; exon 21: L858R, L861Q) in 100,000 reads. The MART score is the ratio of the plasma mutation score for the activating mutation after therapy (taken at 2 or 4 weeks after initiation) compared with before therapy initiation. In a 52-patient cohort, all 3 patients who developed progressive disease had a MART score that exceeded 0.1.

Kato *et al.* also proposed a numeric index that defines the onset of disease progression. By defining the point at which ctDNA started to exceed the limit-of-quantification threshold and comparing it with the time point of objective disease progression by the Response Evaluation Criteria in Solid Tumors, they observed three types of responders (based on an arbitrary cut-off point of 100 days). In approximately 40% of patients, the interval between rising ctDNA and objective disease progression was within approximately 100 days and most likely indicated a parallel change in disease. However, in a small subset of patients (approximately 15%), ctDNA elevations preceded radiographic growth by more than 100 days and was characterized by more varied ctDNA dynamics. In 1 patient, ctDNA levels of the activating mutation rose and maintained at a certain level until disease progression, after which an accompanying increase in T790M ctDNA occurred. What is unclear is whether the early ctDNA elevations in these patients represent true disease progression or confounding secondary pathology. Lastly, for approximately 45% of the patients, ctDNA did not elevate with disease progression. Additional studies and further confirmation will be necessary to develop a consensus objective method for using ctDNA to monitor response to treatment.

SUMMARY

Precision oncology in NSCLC relies on the ability to detect "actionable" mutations in a precise and timely manner. That reliance is now truer than ever, given the availability of multiple generations of EGFR TKIs with action-specific targets and drug-specific resistance mechanisms. Analysis of ctDNA provides many benefits for real-time monitoring of tumour response to EGFR TKIs and for the detection of acquired resistance such as T790M. Obtaining plasma samples is easy, low-cost, and minimally invasive, with low morbidity. By sampling the full clonal spectrum, ctDNA also addresses the limitations of solid biopsies in capturing tumour heterogeneity. However, because ctDNA constitutes only a very minor percentage of total circulating DNA, ultrasensitive methods are necessary for ctDNA pipelines. Many studies now underway are looking at using ctDNA to assess the burden of *EGFR*-mutant NSCLC and its response to EGFR TKIs, and to demonstrate the prognostic value of acquired resistance mutations. Although the field is moving toward digital methods of ctDNA detection, the various assays have different sensitivity, specificity, and concordance profiles. The clinical utility of ctDNA will require more standardization and technical training in the newer digital platforms to ensure reliability as an adjunct tool in the management of *EGFR*-mutant NSCLC.

CONFLICT OF INTEREST DISCLOSURES

We have read and understood *Current Oncology*'s policy on disclosing conflicts of interest, and we declare the following interests: MST received grants and personal fees from AstraZeneca during the conduct of this study, and grants and personal fees from Merck, personal fees from Bristol–Myers Squibb, personal fees from Ventana/Roche, and grants and personal fees from Pfizer Canada outside the submitted work. MC has no conflicts to declare.

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