CLINICAL INVESTIGATION

Changing the paradigm: circulating tumor DNA as a 'liquid biopsy' for clinical biomarker assessments

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Clinical development of targeted anticancer therapies often involves interrogation of key driver alterations through diagnostic assessment of tumor DNA. However, predictive biomarker assessment of tumor tissue has substantial limitations. Recent clinical studies of circulating tumor DNA suggest such DNA may be a paradigm-changing medium for the diagnosis and management of cancer patients that can provide an up-to-date 'liquid biopsy' for use in clinical diagnostic assessment. The field is evolving rapidly and numerous studies have shown that highly sensitive technologies allow for detection of genomic alterations in circulating tumor DNA. Here, we consider how these advances have the potential to shape pharmacodynamic and predictive biomarker assessments in clinical trials by providing comprehensive, real-time molecular assessment in a minimally invasive manner.

Keywords: biomarkers • ctDNA • diagnostics • liquid biopsy • mutation detection • targeted therapy

In the rapidly evolving genomics era, patients are increasingly treated with targeted anticancer therapies that are tailored to inhibit critical cancer driving nodes, rather than standard chemotherapy chosen primarily based on the anatomical location of the primary tumor. Such therapies often target mutant kinases that can be aberrantly activated by different mechanisms, including gene amplification (e.g., *HER2*), somatic mutations in key kinase domains (e.g., *EGFR*) and translocations (e.g., *BCR-ABL*) [1]. Through the molecular profiling of human cancers using various sequencing methodologies, researchers have identified additional activated kinases, such as *BRAF*, *KRAS, PIK3CA, MET* and *ALK*. The relevance of targeting these cancer-driving kinases has been assessed in biomarker-defined patient populations, and in these examples has been shown to provide highly effective targeted therapy. Recent evidence supporting the rationale for targeting the underlying genetic abnormality include the approval of the ALK inhibitors (crizotinib and ceritinib) and EGFR inhibitors (erlotinib and gefitinib) in lung cancer and the BRAF inhibitor (vemurafenib) in melanoma [2–7]. Though patients may show rapid and durable responses to such agents, resistance almost always arises and, in most cases, occurs though diverse mechanisms [8]. An example of this phenomenon has been the elucidation of resistance mechanisms to EGFR inhibitors in non-small-cell lung cancer by Sequist *et al.*, who performed longitudinal serial biopsies in patients receiving erlotinib and profiled tumor samples to reveal distinct resistance mechanisms that include intragenic mutations in EGFR, c-Met amplification, activation mutations in *PIK3CA* and histological transformation [9]. Clearly, effectively treating a patient who has developed resistance to an EGFR inhibitor requires knowledge of the underlying genetic changes in the resistant tumor in order for the treating oncologist to administer an effective subsequent therapy. However, in many cases, it is not feasible or practical to collect serial biopsies in general oncology practice when the patient presents with resistant disease. More typically, predictive bio-

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marker evaluation has relied upon molecularly profiling archival tumor tissue that may have been surgically resected years prior to the patient beginning therapy. In addition, a single biopsy tumor resection represents only a static snapshot of the patient's disease and may not accurately reflect the overall portrait of a patient's cancer due to clonal heterogeneity and evolution of the original tumor during proliferation and metastasis. To more accurately characterize a patient's tumor, enrollment into clinical trials may require patients undergo a fresh biopsy to assess baseline biomarker status prior to enrollment onto clinical studies of potentially attractive experimental medicines. This approach, however, will exclude patients that fail to consent to provide a fresh biopsy or whose tumor is not amenable to a surgical procedure (e.g., it is deemed unsafe) to obtain a fresh biopsy. As such, clinical development teams and academic researchers are continuously assessing novel approaches to utilize surrogates that may accurately represent the real-time status of the patient's tumor. Over the past few years methodologies to detect and quantitate circulating tumor DNA (ctDNA) have gained momentum as a mechanism to aid the diagnosis and clinical management of cancer patients for a few key reasons. First, ctDNA provides a way to obtain a fresh liquid biopsy from the patient without the need for an invasive procedure. Second, the technologies to profile ctDNA have advanced in analytical sensitivity and specificity, enabling the detection of rare mutations. Finally, the analysis of ctDNA from the blood is not limited to a single tumor site or biopsy specimen, but rather the shed ctDNA originates from multiple tumor sites and thus may represent the true tumor heterogeneity better than a single biopsy specimen [10–12]. In addition, the analysis of ctDNA holds promise for early detection of cancer, the assessment of residual disease after surgical resection and, as discussed, potentially for real-time longitudinal monitoring to help identify acquired resistance mechanisms that may lead to tailoring an appropriate follow-on therapy prior the determination of radiographical or clinical progression [13–17]. This review will focus on recent advances of this rapidly developing technology, with a particular emphasis on the utility of ctDNA for both predictive and pharmacodynamic biomarker assessment in clinical trials. Research into the utility of ctDNA to detect somatic mutations has been carried out in numerous tissue types with a number of papers available. Here, we highlight recent clinical results from four main tissue types (lung cancer, colorectal cancer, breast cancer and melanoma) that illustrate the general principles and show where ctDNA analyses have shown promise in providing noninvasive biomarker information that may eventually translate to clinical utility.

Circulating tumor DNA

Cell-free ctDNA can be identified in the blood of the majority of metastatic cancer patients to varying degrees, with notable exceptions being tumors localized to the brain or tumor types with mucinous features such as renal cell carcinoma, prostate and thyroid cancers [16]. ctDNA may be distinguished from overall cell-free DNA (cfDNA), which may be derived from normal or cancerous cells and shed into circulation, by the presence of markers specific for neoplastic cells such as oncogenic mutations, chromosomal alterations or methylation. In early studies, ctDNA was detected at higher levels in the blood of cancer patients when compared with healthy individuals, highlighting potential utility in the diagnosis of cancer [18]. The exact origin of ctDNA is debated, but the source is likely to be from tumor cells that have undergone apoptosis or necrosis, but where the debris has not been cleared by phagocytes [12,19–20]. A second hypothesis is that ctDNA could be actively released from cell into circulation [21–23]. A third hypothesis is that ctDNA may be derived from circulating tumor cells (CTCs), a concept that is less likely due to the low number of CTCs identified in a large number of cancer patients and the fact that ctDNA can often be detected in patients who do not harbor detectable CTCs [16]. In addition, the average length of ctDNA fragments in circulation is approximately 180 base pairs, a length that is consistent with the normal physiological apoptotic process, further supporting the former hypothesis [24]. A variety of methods have been used to assess somatic cancer alterations in ctDNA, and are shown in Table 1. In general, the most sensitive methods utilize polymerase-chain reaction (PCR)-based technology, which allow for detection of specific mutations at frequencies as low as 0.01% and can be very useful for monitoring longitudinal changes in mutation status over time and treatment. In contrast, next generation sequencing approaches have been shown to have lower sensitivity (typically 1–5%), but offer more potential for discovery of novel mutations and resistance mechanisms, since such approaches can interrogate a large number of genes simultaneously.

Depending on the indication (Table 2), the median reported levels of ctDNA has been reported to range from 7 ng/ml (range 2–50 ng/ml) in the plasma of melanoma cancer patients to 18 ng/ml (range 5–230 ng/ml) in the plasma of colorectal cancer patients [25]. Several studies have shown that ctDNA levels increase with tumor stage in the blood of breast, colorectal and pancreatic cancer patients [16]. Linked to this phenomenon, high levels of ctDNA have been associated with a poor prognosis in cancers such as breast, lung and melanoma [25–27]. The highly var-

ied detectable levels of ctDNA indicate that not all tumor types, and early stage cancer patients, will be applicable for analysis with the current technologies available, and that studying the relevance of ctDNA in metastatic cancer patients may be the best opportunity to demonstrate initial clinical utility. However, several recent reports have been able to detect cancer-driving alleles in early stage cancers, particularly in breast and colorectal cancers, and therefore with more sensitive technologies, ctDNA could also have great utility in detection of residual disease [16,17].

Predictive biomarkers & ctDNA

Clinically validated predictive biomarkers (i.e., biomarkers that predict therapeutic benefit to anti-cancer agents) in oncology are relatively limited to date. The majority of predictive biomarkers are associated with identification of patient subsets that may derive therapeutic benefit (e.g., HER2 expression for treatment with traztuzumab, pertuzumab and trastuzumab emtansine), rather than excluding patients from treatment, with the exception being *KRAS* mutations for the management of colorectal cancer patients treated with cetuximab or panitumumab [46,47]. In oncology, the assessment of predictive biomarkers is often carried out on formalin fixed paraffin embedded tissue, which is often resected months to years previously from the patient's primary tumor. Tumor tissue has its limitations in that it may not provide an up-to-date and fully representative biospecimen (Figure 1), since samples are typically obtained from just one lesion. As discussed in the upcoming sections, ctDNA holds promise to mitigate many of the limitations associated with archival formalin-fixed, paraffin-embedded (FFPE) tissue, but will only be successful if robust methods are prospectively implemented alongside tissue in carefully designed clinical studies.

Pharmacodynamic biomarkers & ctDNA

The relatively noninvasive nature of ctDNA analysis and the fact that multiple longitudinal samples can be obtained over time and treatment for a single patient suggest that ctDNA could have applicability to pharmacodynamic analysis of drug effects in treated patients or as a surrogate of tumor response. Indeed, several groups have now reported monitoring specific alterations in key oncogenes over treatment and, further, demonstrating that a decrease in the frequency of the mutant allele is associated with initial response to treatment, followed by a rise in allele frequency associated with disease progression [13,34,48]. A limitation of the current approach is that unless a high prevalence oncogene is tracked (e.g., *PIK3CA* in breast cancer or *BRAF* in melonoma), the approach needs to be individualized thought the identification of tumor mutations (possibly by performing deep sequencing on baseline ctDNA or tumor tissue) and the subsequent development of customized patient-specific assay that can be performed on longitudinal samples to examine changes in allele frequency in response to treatment. An encouraging alternative to developing a customized patient-specific ctDNA assay was described by Fackler *et al.*, who developed a panel of ten commonly methylated DNA markers (cMeth DNA) and showed that this assay could be used to detect breast cancer, monitor tumor burden and faithfully predict response to chemotherapy in a pilot experiment with 29 patients [49]. As discussed in other sections of this review, ctDNA also has shown promise in the related question of early detection of acquired resistance mutations in gynecological as well as colorectal cancers.

Table 1. Showing the various methods used to detect somatic mutation from circulating tumor DNA.

Technology	Platform	Sensitivity (%)
Sanger sequencing	Many	10
Next-generation sequencing	Illumina, Life Technologies	2
TAm-Seq	Illumina	2
Quantitative-PCR	Cobas	2
ARMS-PCR	Many	0.1
Scorpion-PCR	Many	0.1
PNA-PCR	Many	0.1
Digital-PCR	Bio-Rad, Life Technologies	0.01
Droplet-PCR	BEAMing, Bio-Rad, Raindance	0.01
CAPP-Seq	Illumina	0.01

ARMS: Amplification refractory mutation testing; PCR: Polymerase-chain reaction; PNA: Peptide nucleic acid.

ctDNA & lung cancer

EGFR is a member of the receptor tyrosine kinase family that is mutationally activated in approximately 10% of lung adenocarcinomas, most commonly through deletions in exon 19 or point mutation in exon 21 that result in aberrant activation of the pathway and downstream activation of the PI3K-AKT and MAPK survival pathways [9,50]. Small molecules that target this pathway include EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, which have been approved for the treatment of *EGFR* mutant non-small-cell lung cancer patients in the front line setting [4–6,51]. Numerous reports have demonstrated that *EGFR* mutations can be detected

Table 2. Highlighting relevant circulating tumor DNA studies in lung, breast, colorectal and melanoma cancers.

Tissue	Platform	n	Result	Ref.
Lung	PNA	109	53, 61, 84, 100 and 75%	[4]
	PCR-based	31	concordance respectively for *EGFR*†	[28]
	PCR-based	25		[29]
	DxS	4		[30]
	PCR-based	4		[31]
Breast	ARMS	45	95% concordance for *PIK3CA*†	[32]
	BEAMing	51, 41	71% (asynchronous) and 100% (synchronous) concordance for *PIK3CA*†	[33]
	ddPCR	29	100% concordance for *PIK3CA*†	[17]
	Sequencing	30, 2	PD modulation with treatment	[15,34]
	d-PCR	58	68% concordance for HER2 amp	[35]
Colorectal	Scorpion-PCR	71	31% (plasma) and 25% (serum) for *KRas*†	[36]
	PNA-PCR	15	100% concordance for *KRas*†	[37]
	PCR-based	95	100% and 92% concordance for *BRaf*† and *KRas*†, respectively	[38]
			85% concordance for *KRas*†	
	ARMS	108	78% concordance for *KRas*†	[39]
	DxS	108	*KRas* emergence as a resistance mechanism to anti-EGFR therapies	[40]
	BEAMing	1, 2	MET amplification as a resistance mechanism to anti-EGFR therapies	[41,42]
	PCR-based	3	PD modulation with treatment	[43]
	BEAMing	18		[13]
Melanoma	ARMS	94	56% concordance for *BRaf*†	[44]
	PCR-based	91	84% and 91% concordance for *BRaf* V600E† and V600K†, respectively	[45]

†Mutations analyzed in both tissue and plasma.
ARMS: Amplification refractory mutation testing; BEAMing: Beads, emulsion, amplification and magnetics; PCR: Polymerase-chain reaction; PD: Pharmacodynamics; PNA: Peptide nucleic acid.

in the plasma and serum of NSCLC patients. Several clinical studies have shown potential for diagnosing EGFR mutation status based on mutational analysis of ctDNA. In the pivotal EURTAC trial that was the basis for the approval of erlotinib as an alternative to platinum-based chemotherapy in newly diagnosed metastatic non-small cell lung cancer (NSCLC) patients with *EGFR* activating mutations, *EGFR* mutations were detected in the serum in 58 of 109 (53%) tissue positive patients [4]. Other studies, although with smaller sample sizes, have shown a more favorable detection rate of *EGFR* mutations in the blood of NSCLC patients. Brevet *et al.* demonstrated that *EGFR* mutations were identified in 61% (19 of 31) of tissue positive cases [28], Similarly, Akca *et al.* showed a detection rate of 84% (21 of 25) [29], Punnoose *et al.*, demonstrate a 100% concordance rate in four tissue positive cases and Kimura *et al.* identified *EGFR* mutations in three of four tissue positive patients (75%) [30]. Interestingly, in two of these studies, Kimura *et al.* detected an additional plasma *EGFR* mutation in a patient that was wild-type by tissue, and Brevet *et al.* identified an additional two patients that were plasma positive, but tissue negative [28,31]. These discrepancies may be due to the tumor heterogeneity that exists within cancer patients, but additional studies comparing ctDNA status with multiple metastatic sites would be required to validate this hypothesis. Finally, Taniguchi *et al.* demonstrated that mutations could be identified in 74% (32 of 44) patients [52]. Consistent with the studies that compared erlotinib and gefitinib to standard of care chemotherapeutics, Punnoose *et al.* showed that the four plasma *EGFR* mutant patients had a better prognosis to erlotinib

in combination with pertuzumab compared with 20 plasma wild-type patients in a randomized Phase II clinical trial [30]. Similarly, comparing outcome based on either tissue or plasma analysis, Brevet *et al.* demonstrated that plasma EGFR-positive patients had an almost identical overall survival benefit with erlotinib when compared with patients that were positive by tumor tissue analysis [28]. Lastly, Kimura *et al.* demonstrated that serum *EGFR* mutations were predictive of response to erlotinib compared with serum *EGFR* wild-type patients [31]. Moreover, the acquired resistance mechanisms to EGFR TKIs, a secondary T790M mutation that occurs within the ATP-binding pocket, was detected in 10 of 23 *EGFR* mutant patients who progressed following EGFR tyrosine kinase therapy, whereas in EGFR TKI naïve patients, the T790M resistance mutations was detected in only one of 21 EGFR-positive patients. This phenomenon is consistent with clonal evolution of the T790M resistance allele suggested by Engelman *et al.* [9]. Looking forward, a blood-based assay may prove critical for monitoring the emergence of the T790M resistance allele in patients, and potentially in the future for selecting subsequent therapies, especially given that next generation of EGFR inhibitors have been designed to inhibit the T790M mutant EGFR protein.

ctDNA & breast cancer

Breast cancer has emerged as a promising indication where ctDNA-based analyses may have utility both in early and late stage disease. A recent study by Bea-

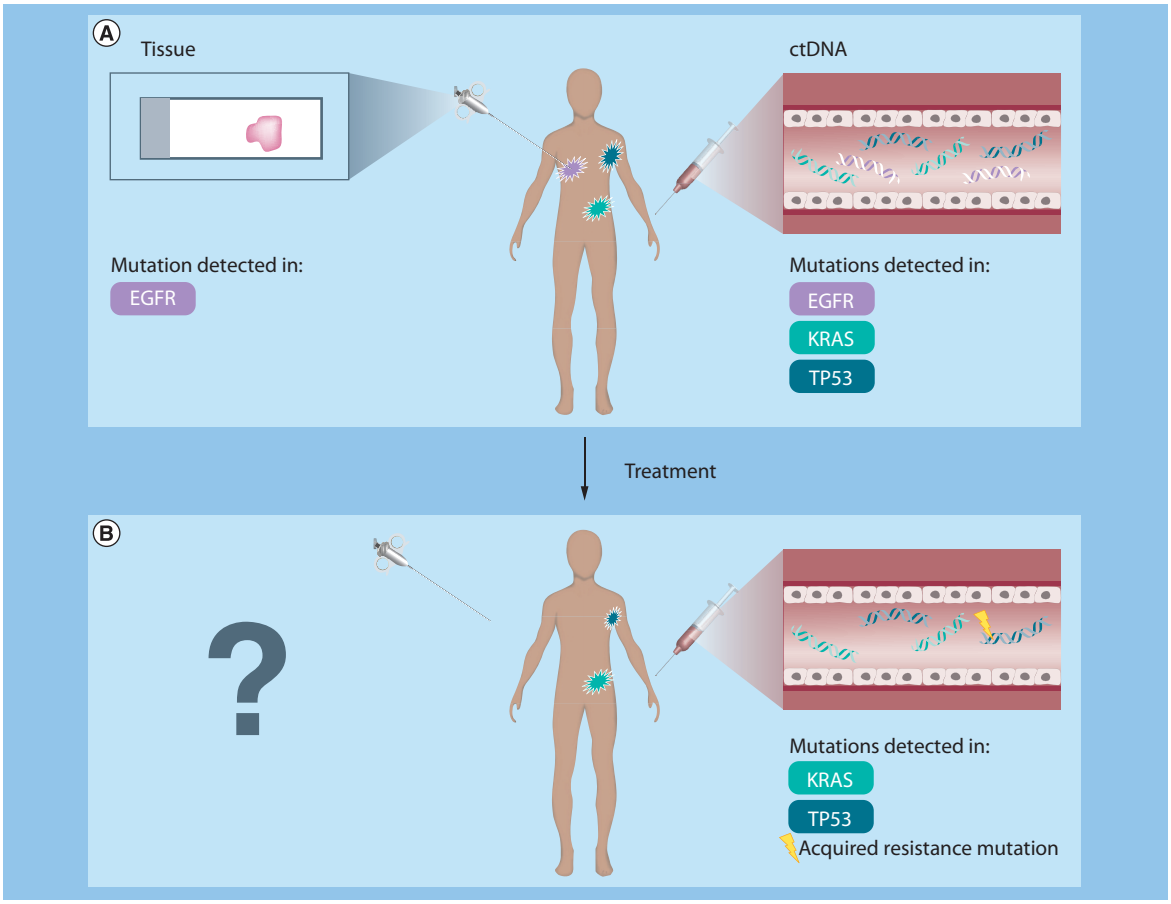

Figure 1. The paradigm for genotyping a patient by tissue (left hand side) or by blood (right hand side). When a patient presents with cancer, the treating physician will order a tumor biopsy, if feasible, and make a treatment decision based on the genotyping results. This approach only takes into consideration the biopsied lesion, and neglects the other metastatic sites, as indicated by the green and blue tumors. Following treatment of a targeted therapy, for example, an EGFR tyrosine kinase inhibitor backbone regimen, the EGFR mutant lesion will derive a great benefit from this therapy (purple tumor). In addition, a second lesion, as indicated by the blue tumor, may have a partial response as chemotherapy is often co-administered. Finally, the third lesion, as indicated by the green tumor may derive no clinical benefit, due to a dominant *KRAS* mutation. By using circulating tumor DNA, the treating oncologist may be able to **(A)** accurately capture the genetic landscape of all tumor lesions and **(B)** assess resistance mechanisms following progression on therapy, potentially identifying a relevant follow on therapy or a relevant clinical trial in which the patient can be enrolled.

ver *et al.* used droplet digital PCR (ddPCR) to identify *PIK3CA* mutations in plasma from patients prior to and after breast surgery [17]. They found that the ddPCR technique could identify *PIK3CA* mutations in plasma with 93% sensitivity. Most importantly, the group found that *PIK3CA* mutations could still be detected even post operatively in five of 10 patients. This latter finding suggests that *PIK3CA* ctDNA present in the plasma post surgery could potentially be reflective of residual disease and hence might be used to identify patients who might most benefit from adjuvant therapy to eradicate disseminated cancer cells. The possibility has been further supported by a study from Turner *et al.*, who performed a similar study and, further, demonstrated that the presence of residual *PIK3CA* ctDNA in the plasma was associated with disease recurrence in four of five patients [53]. Confirmation of this finding will require large prospective studies that will take many years to complete but could pave the way for ctDNA to be used for monitoring residual disease post surgery, and specifically to identify and select patients for adjuvant therapy based on the presence of tumor derived DNA as an indicator of patients harboring residual cancer.

The analysis of ctDNA has also yielded promising early results in the metastatic breast cancer setting. *PIK3CA* mutations are particularly common in hormone receptor positive breast cancer and several groups have used the presence of these mutant alleles to help establish the concordance between the mutation status of the tumor tissue and ctDNA mutation status [54,55]. Board *et al.* found that the concordance between the *PIK3CA* mutation status in matched tumor and ctDNA was 95% of 45 tumor samples [32]. A separate study assessed the *PIK3CA* mutation status in plasma and in two different biopsy specimens, one archival sample and one that was collected at the same time as the plasma sample. The *PIK3CA* mutation status was 100% concordant (29 of 29) between the plasma and the tumor biopsy that was collected contemporaneously, while the concordance was 73% when the researchers compared the *PIK3CA* mutation status of the ctDNA with archival tissue samples (37 of 51) [33]. Thus, ctDNA may provide an accurate assessment of the current state of a patient's disease. The presence of *PIK3CA* mutations has also formed the basis of a strategy to identify patients with relatively high amounts of ctDNA utilizing sensitive methods such as ddPCR or BEAMing (Beads, Emulsion, Amplification and Magnetics) whose samples were subsequently analyzed by next generation sequencing as reported by Dawson *et al.* This group performed targeted and whole genome sequencing of ctDNA from 30 patients with metastatic breast cancer to develop personalized digital ctDNA assays that were unique for each patient. They then assessed these personalized ctDNA panels on serial plasma samples and compared changes in mutant allele frequency to radiographic imaging and the levels of the cancer antigen 15–3 (CA 15–3) [34]. They demonstrated that ctDNA levels were an early indicator of response and showed a greater correlation with tumor burden than either imaging or CA 15–3, suggesting that ctDNA is a specific and highly sensitive personalized biomarker of breast cancer. Another report from the same overall group used a similar approach to noninvasively study potential acquired resistance mechanisms in longitudinal samples from six patients, two of whom had advanced breast cancer [15]. The authors were able to identify mechanisms consistent with mediating resistance, including increases in *PIK3CA* frequency in a patient receiving epirubicin and paclitaxel, and increases in a *GAS6* mutation in a patient receiving HER2 targeted therapy. Advances in sequencing technology have recently been used to perform whole genome sequencing to broadly analyze the cancer genomes of 10 breast cancer patients and compared the results to cell-free DNA from normal subjects, suggesting a sensitive and specific, albeit still cost-prohibitive method for noninvasively detecting cancer that is not dependent on the availability of a fresh tumor biopsy [14]. A goal for the field should be to replicate these promising anecdotal findings in large, well-controlled clinical studies in order to clinically validate relevant resistance mechanisms and shed light on the prevalence of the various mechanisms. Longer term, one can imagine a scenario where such analyses could be used to dynamically monitor the emergence of acquired resistance and switch to an appropriate therapy based on the nature of the observed mechanism. A potential example that could pave the way for this approach would be *ESR1* mutations that arise in hormone receptor metastatic breast cancer patients treated with endocrine therapies [56–58].

ctDNA & colorectal cancer

Colorectal cancer has been suggested to evolve via a 'multiple hit' mutation model that occurs over several decades. One hypothesis is that mutations in the adenomatous polyposis coli (*APC*) gene are an initiation event that results in the generation of a small, locally confined adenoma of the colon. Subsequent activation mutations in the Ras pathway followed by loss-of-function mutations in tumor suppressor *TP53* pathway results in the generation of metastatic carcinomas [59].

Historically, doublet chemotherapy regimens (5-fluorouracil in combination with oxaliplatin or irinotecan) have been the standard treatment for patients with colorectal cancer. EGFR is expressed in a high percent-

age of colorectal cancer specimens, however, activating somatic mutations in the *EGFR* gene in CRC samples is rarely observed [60,61]. Activation of wild-type EGFR in colorectal cancer cells occurs by binding of one of several ligands (epidermal growth factor, transforming growth factor α, amphiregulin, epiregulin etc.) and increased ligand expression has been shown to occur in colorectal cancer cells and is thought to play a role in the pathogenesis of the disease [62]. Antibodies that inhibit ligand binding to EGFR, such as cetuximab and panitumumab, prevent the activation of EGFR have been approved for use in colorectal cancers that are *KRAS* wild-type [46,47]). In addition, *BRAF* mutations have also been implicated as a *de novo* resistance mechanism to EGFR-directed therapies and, as such, detecting these alleles in blood has warranted interest [63]. Using Scorpion RT-PCR technology, Morgan *et al.* demonstrate that they could detect *KRAS* mutations in both plasma and serum at high specificity (97 and 100%, respectively), but at disappointing sensitivities (31 and 25%, respectively) from 71 patients [36]. In a similar study, Kuo *et al.* showed they could detect *KRAS* mutations in plasma at 100% (15 of 15) concordance with tissue using a peptide nucleic acid (PNA)-based PCR approach [37]. Interestingly, the group detected *KRAS* mutations in an additional 11 plasma samples from the 37 of the tissue that were categorized as wild-type. Thierry *et al.* demonstrated that they could detect 100% (5 of 5) *BRAF* mutations and 92% (36 of 37) *KRAS* mutations when compared with tissue in 95 paired plasma/tissue sets using Scorpion RT-PCR technologies [38]. Spindler *et al.* showed that they could detect KRAS mutations in 32 of 41 (78%) samples and, further, showed that high ctDNA *KRAS* burden was a poor prognostic feature [40]. In a second study, the group showed that they could detect 35 and 41 *KRAS* mutations in the plasma and tissue, respectively, of 108 paired baseline plasma and primary tissue sample sets [39]. Twelve patients that were tissue positive, plasma negative at baseline for *KRAS* or *BRAF*, mutations were subsequently detected in the plasma of four patients that progressed following anti-EGFR therapy. Moreover, and an additional four patients that were tissue and plasma negative prior to EGFR therapy, became mutation positive in the plasma at progression, suggesting the acquisition of *KRAS* or *BRAF* mutations may also be an acquired resistance mechanism as well as a *de novo* resistance mechanism. In support of this phenomenon, two independent landmark studies demonstrated that mutant *KRAS* ctDNA allele frequency increased over time in six of 10 and nine of 24 patients CRC patients treated with anti-EGFR therapies [41,42]. Moreover, the detection of *KRAS* mutations in plasma preceded both a radiographic progression event and an increase in the level of the circulating cancer marker carcinoembryonic antigen. Importantly, postprogression biopsies demonstrated the emergence of the *KRAS* mutations in resistant metastatic tissue. More recently, amplification of the *MET* proto-oncogene has been shown to be a resistance mechanism to anti-EGFR therapies in colorectal cancers that are *KRAS* wild-type [43]. After determining the genomic re-arrangement by exome sequencing of the liver metastasis and designing personalized breakpoint primers, Bardelli *et al.* were able to detect amplification of *MET* in the plasma of three metastatic colorectal cancer patients upon acquisition of resistance to anti-EGFR therapies. Finally, Diehl *et al.*, showed that by identifying specific mutations from the tumor, and subsequently using the sensitive BEAMing technology for selected mutations, the allele frequency could be followed over time in patients treated with chemotherapy [13]. Moreover, the presence of detectable ctDNA correlated with a poor prognosis and outperformed carcinoembryonic antigen monitoring, suggesting utility in potentially monitoring postsurgical minimal residual disease. These exciting data suggest that monitoring ctDNA for the emergence of resistant tumor clones may be a useful tool for managing treatment options.

ctDNA & melanoma

Melanoma is an indication that has served as an excellent test bed to help demonstrate the technical and clinical utility of ctDNA to enable diagnostic assessments for targeted therapies directed at the *BRAF* oncogene [64]. This is facilitated by the fact that approximately 80% of advanced melanoma patients have detectable tumor-derived ctDNA [16], though lower levels have been reported in early stages of disease. Most ctDNA studies of melanoma samples have utilized a qRT-PCR mutation detection assay format, and the importance of analytical validation of the assay was demonstrated by Aung *et al.*, who demonstrated that cut-offs for calling mutant alleles with the amplification refractory mutation testing (ARMS) PCR system need to be optimized for plasma and not just extrapolated from tissue based assays [65]. Theoretically, this may be due to the greater cross-linking of FFPE DNA from tissue necessitates more stringent cut-offs to eliminate false positives due to errant enzymatic priming on cross-linked DNA, a phenomenon not as relevant to blood-derived DNA. ctDNA mutation detection has been shown to have potential clinical utility in Phase II clinical trials of agents targeting the BRAF/MEK axis in BRAF mutant melanomas. Specifically, Board *et al.* compared *BRAF* mutation detection in matched tissue and plasma samples from a Phase II study of the MEK

inhibitor AZD-6244 using an ARMS-based PCR assay [44]. They were able to detect plasma mutation in 56% of patient samples who were all *BRAF*-mutation positive based on the analysis of the tumor tissue. These researches further demonstrated that *BRAF* detection in ctDNA was not prognostic, since the presence or absence of *BRAF* mutations in ctDNA did not affect progression-free survival in patients with confirmed *BRAF* tumor mutations. Ascierto *et al.* studied *BRAF* mutations in ctDNA in a Phase II study of the BRAF inhibitor dabrafenib in patients with metastatic melanoma [45]. Analysis of baseline plasma and tumor tissue samples from 91 patients using a PCR-based assay showed an overall agreement of 84% between tumor and plasma DNA for *BRAF(V600E)* mutations, and slightly higher agreement (91%) for *BRAF(V600K)* mutations. Intriguingly, the amount of ctDNA correlated with overall tumor burden, and that patients with a high allele frequency of *BRAF* mutant ctDNA had a lower objective response rate and shorter progression-free survival. These studies are retrospective in nature and certainly require future prospective confirmation to show true clinical utility, but suggest that *BRAF* mutation detection in ctDNA could eventually play a role in the diagnosis and clinical management of patients with *BRAF* mutant melanoma.

Future perspective

In the not too distant future, it is hoped that personalized healthcare will become the standard for the treatment of cancer patients through the thoughtful and careful integration of multiple different diagnostic evaluations to select patients for appropriate therapies. This vision will only be realized through careful clinical evaluation of new technologies that can overcome some of the challenges of traditional tissue-based diagnostics. To date, the field of oncology has seen numerous examples that demonstrate the utility of selecting biomarker-defined patient populations for treatment with targeted therapies (e.g., agents that target ER, HER2, BRAF, EGFR, ALK). Targeted application of these agents has brought substantial clinical impact to many patients, and in some cases has changed the natural history of a particular cancer type. In this review, we have highlighted several examples of clinical studies that are paving the way for utilizing ctDNA to detect mutations in

Executive summary

- Circulating tumor DNA (ctDNA), which is released from tumor cells into the blood stream, can be found in the majority of cancer bearing patients at various levels. Mutations identified in ctDNA in general accurately reflect those identified in the tumor.
- Predictive biomarkers can accurately identify patients that are most likely to derive benefit from anticancer therapies. ctDNA can be used to determine the presence of somatic mutations and identify patients for therapies that target driving oncogenes. Pharmacodynamic biomarkers change over the treatment period and can be utilized to determine whether a patient is responding to therapy. As a blood draw is a minimally invasive sample collection, ctDNA provides a unique opportunity to gain insights into tumor dynamics.
- Over the past few years, more sensitive technologies have accelerated the research into ctDNA analysis and many published studies have demonstrated the utility in retrospective analysis.
- In breast cancer, mutations in the *PIK3CA* gene are common in the hormone receptor positive subtype. Several reports show a high concordance between tumor tissue and plasma, with a higher concordance observed in synchronous collections (i.e., tumor and plasma collected at the same time).
- Patients with EGFR driven lung cancers derive clinical benefit from treatment with tyrosine kinase inhibitors that target EGFR. Mutations in *EGFR* can be detected in the plasma of lung cancer patients, but also have been shown to be predictive of response to EGFR tyrosine kinase inhibitors in retrospective analysis.
- Colorectal cancer patients that express EGFR and wild type for KRAS are commonly treated with anti-EGFR monoclonal antibodies. Increases in mutant *KRAS* allele frequency and increased copy number of the RTK *MET* have been observed in plasma of patients that progress on anti-EGFR therapies. These observations provide evidence that *KRAS* mutations are an acquired resistance mechanism as well as a *de novo* mechanism to anti-EGFR therapies and suggest rationale for concomitant treatment with MAP-kinase inhibitors.
- *BRAF* mutant melanomas are treated with agents that inhibit the BRAF-MAP-kinase axis. A good concordance has been seen between plasma and tissue in *BRAF* mutant melanomas when assessed with the more sensitive PCR-based technologies.
- As tissue is not available for all cancer bearing patients due to inaccessible lesion site etc., ctDNA holds promise to provide an up-to-date snapshot of the tumor to guide an oncologists treatment options. However, all current published ctDNA studies have been retrospective in nature and will require prospective validation to be utilized as a treatment diagnostic. In the future, with ever increasing technology sensitivity and the hunt for the elusive predictive gene, ctDNA holds promise not only to identify these patients but also to monitor their response and enable early switching of therapy based on comprehensive analysis of ctDNA-based molecular changes.

EGFR or *BRAF* and select patients for appropriate therapy in patients with lung cancer or melanoma, respectively. In addition, predictive biomarkers may also be used to define nonresponsive patient populations in drug labels, with a notable example being *KRAS* mutations in the case of colorectal cancer patients treated with cetuximab or panitumumab. Again, clinical studies of ctDNA have suggested that baseline KRAS status can be determined with high sensitivity and specificity using ctDNA. The example of KRAS in colorectal cancer has also demonstrated the utility of ctDNA as a potential monitoring technology in the clinic. Specifically, the idea that as tumors become resistant to therapies, somatic mutations in key oncogenes can emerge as acquired resistance mechanisms or alternatively that were present at low allele frequencies before treatment, which become more abundant during the pressure of drug selection. As tissue biopsies can be challenging due to anatomical location and other factors, recent technological advancements have unleashed the power of ctDNA to potentially provide critical insights into biomarker status both at the time of initial treatment and, moreover, to enable dynamic monitoring for the appearance of resistance mechanisms during the course of therapy. Of course, such dynamic monitoring will only be useful if it identifies an actionable alteration that allows a physician to switch to a potentially more efficacious therapy or therapeutic combination. Another attractive application for ctDNA analysis would be in patients with early stage disease, especially monitoring patients following surgical resection of their primary tumor for early detection of disease relapse prior to symptomatic detection. Indeed, comprehensive profiling of such residual DNA could theoretically be used to identify actionable alterations to guide subsequent therapy choices upon relapse. In the past few years, the field has seen ever improving sensitivities and specificities of assay platforms that have been demonstrated to accurately represent biomarker status in tissue, with some impressive demonstrations of potential clinical utility. Unfortunately, such studies have thus far been retrospective and anecdotal in nature, and there is a pressing need in the field for the careful design and execution of prospective clinical studies to validate ctDNA-based biomarker assessments for enabling patient treatment decisions. As predictive biomarkers become increasingly common in patient management, having an up-to-date snapshot of the genetic landscape of the tumor will be imperative for the treating physician not only to tailor an individual's anticancer regimen, but also to monitor and infer sequential therapies – a potential unparalleled advantage for ctDNA in guiding clinical biomarker assessment.

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