Since the seminal reports of the utility of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) in evaluating a cancer patient’s response to therapy (1,2), the field of blood-based nucleic acid testing (also referred to as “liquid biopsies”) has intensified and accelerated. A recent, comprehensive review of ctDNA and CTCs (3) summarizes research and technical advances in both areas. The goal of this editorial is to discuss how these technologies can be used in the development of non-invasive companion diagnostics that can be used in patient stratification and in monitoring of drug response and of drug resistance. These blood-based diagnostics can obviate the need for tissue biopsy, providing an attractive solution when tissue is limiting or in instances where biopsies are not routinely performed, and may provide a more accurate representation of tumor heterogeneity and evolution. However, there are several technical, regulatory and commercial issues that must be considered when developing this next generation of companion diagnostics.

**ctDNA**

Despite the short (approximately two hours) half-life of ctDNA in the blood (4) and the fact that it can represent less than 1% of the total circulating free DNA (1,5), improved capture and amplification methodologies and computational and bioinformatics approaches have drastically improved the detection of ctDNA. Many methods have been developed to detect ctDNA levels and genomic aberrations in ctDNA, differing in their sensitivities, multiplexing capability, ability to discover new aberrations, cost, throughput, and regulatory/commercialization paths (Table 1). Two salient points regarding ctDNA were raised by the group at Johns Hopkins in their landmark article (6). First, both the percent of cancer patients having detectable ctDNA and the number of circulating mutant fragments can vary across tumor types. Second, the majority of ctDNA is not derived directly from CTCs.

Several approaches use PCR or digital PCR for high sensitivity mutation detection. One approach involves the use of blocking primers, as is done with competitive allele specific TaqMan PCR (CAST PCR) (7) or modified blockers (8). Variations of such approaches are also in development at several companies. Another approach employs optimizing denaturing temperatures to selectively enrich for minority alleles, as is done with co-amplification at lower denaturation temperature PCR (COLD PCR) (9). These methods can deliver a sensitivity as low as 0.01-0.1%, although some assays are less sensitive than others. Droplet digital PCR (ddPCR) using either the Bio-Rad QX100 reader (10) or the RainDance Technologies system (11) can generate a sensitivity of 0.01%. BEAMing (beads, emulsification, amplification and magnets) , which is also based on digital PCR, has also demonstrated a sensitivity of 0.01% (4). BEAMing has been extensively employed in ctDNA studies. Taniguchi et al. (12) showed that BEAMing could be employed in a quantitative manner, detecting the fraction of T790M-positive alleles from the alleles with activating EGFR mutations. Diaz et al. (13) demonstrated that mutant KRAS ctDNA could be detected in nine (out of 24) patients being treated with panitumumab, and these patients’ tumors were initially KRAS wild-type.
The KRAS mutant ctDNA was detected 21 weeks (on average) before radiographic progression in three of the nine cases. Similarly, BEAMing was used to detect, in the blood of patients being treated with cetuximab, KRAS mutant alleles up to ten months before radiographic progression (14). In another study using the ddPCR approach, Oxnard et al. (10) found the EGFR T790M resistance mutation in plasma samples up to sixteen weeks prior to radiographic evidence of disease progression in NSCLC patients who were being treated with erlotinib. These investigators recently demonstrated, using both next generation sequencing of ctDNA and Biorad’s ddPCR, that a previously undocumented C797S mutation arose in six of the fifteen subjects who developed resistance to AZD9291 (15). Interestingly, four cases lost the T790M mutation. Again, the levels of activating EGFR mutation correlated well with disease progression. Similarly, Piotrowska et al. (16) found, using BEAMing, that ctDNA levels of the T790M mutation and the EGFR del19 correlated with radiographic progression and with the tumor biopsy in some patients. Importantly, in other cases the authors demonstrated that levels of activating mutations rose at progression but levels of T790M remained low and their tumor biopsy was wild-type for T790, consistent with the findings of Thress et al. (15). Most recently, Siravegna et al. (17) found, using ddPCR, a 97% concordance in RAS pathway mutations in the tumor and blood.

Next generation sequencing of plasma DNA has been demonstrated by several groups. For example, Leary et al. (18) demonstrated sensitive detection of chromosomal copy number changes and rearrangements. Not surprisingly, sensitivity was dependent on the total number of reads generated. Consistent with that report, Chan et al. (5) calculated that, for a fractional concentration of tumor-derived plasma DNA of 10%, 2500 and 12,000 molecules per 1 Mb window would need to be analyzed to detect two-copy and one-copy gains, respectively. Forshew et al. (19) developed tagged-amplicon deep sequencing (Tam-Seq) for the noninvasive detection of somatic mutations in plasma DNA. The technique can detect circulating mutations, down to an allele frequency of 2% (depending on sequencing depth). The authors demonstrated a high concordance (in allele frequency) with digital PCR: a Pearson correlation coefficient of 0.90 when p53 mutations in 40 plasma samples from ovarian cancer patients were examined. Importantly, Tam-Seq was able to monitor changes in mutant allele fractions and correlated well with radiographic evidence of partial response, stable disease and progressive disease. In a follow up study, these authors found, for two patients, a good correlation between mutant allele fractions in the plasma and tumor biopsy and showed the emergence of resistance mutations in the plasma correlated with specific therapeutic regimens (20).

Interestingly, in a study focusing on metastatic breast cancer patients (21), these investigators also demonstrated that, in 50 samples in which no CTCs were detected, 33 samples had detectable levels of ctDNA. Conversely, in the 20 samples in which no ctDNA was detected, three samples had one or more CTCs. Overall, ctDNA and CTC sensitivity were reported to be 84% and 60%, respectively, and a moderately strong correlation ($R^2 = 0.61$) was found between ctDNA levels and number of CTCs. These data are similar to those from other investigators who found that, in thirteen cases where no CTCs were found, ctDNA was found in all thirteen cases and that 80% of patients with metastatic disease had detectable ctDNA levels (6). Other groups have developed high sensitivity sequencing-based approaches, achieving sensitivities of 0.02% (22) and 0.01% (23). The latter study showed, once again, that detection

Table 1. Examples of companies offering plasma-based diagnostic testing.

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sensitivity was a function of the read error rate and depth of sequencing. These two studies also showed a 100% and 86% concordance between mutations found in the plasma and tissue biopsy, respectively. Another group of investigators developed cancer personalized profiling by deep sequencing (CAPP-Seq), demonstrated detection of mutant allele fractions of 0.02%, and detected ctDNA in 100% of patients with stage II-IV NSCLC (24). Significantly, only 50% of stage I patients had detectable ctDNA levels, nearly identical to the 55% value reported by Bettegowda et al. (6) in localized disease across malignancies. CAPP-Seq was also found to correlate well with tumor volume both pretreatment ($R^2 = 0.89$) and during various treatment regimens ($R^2$ of 0.85 and 0.95 for two patients) and was capable of detecting both mutations and fusions. These data demonstrate that analysis of ctDNA can serve as a surrogate for tumor biopsy for patient stratification and can be used to non-invasively and longitudinally monitor tumor evolution and the growth of drug resistant clones before radiographic evidence of disease progression.

CTCs

CTCs can be used as a template for analysis of DNA, RNA and proteins. Early work demonstrated that CTCs could be used to evaluate copy number variations and rearrangements, using FISH, in patients with castration-resistant prostate cancer (25). A study examining copy number variation demonstrated, using whole genome amplification and sequencing of single CTCs, that every CTC from the same patient showed reproducible copy number variations (26). A recent study examining ALK rearrangements found that all eighteen patients whose tumors exhibited ALK rearrangement also showed ALK rearrangements in their CTCs (27). Interestingly, the CTCs displayed a unique split pattern while the tumors demonstrated heterogeneous split patterns, and the CTCs displayed a mesenchymal phenotype (positive for N-cadherin and vimentin) and were cytokeratin-negative while the tumors displayed both epithelial and mesenchymal phenotypes. Thus, the ability to analyze CTCs having novel phenotypes, especially mesenchymal phenotypes, at the molecular level could be informative for patient stratification (28,29).

Mutational status can also be interrogated in CTCs. A seminal study examining EGFR mutations in CTCs from lung cancer patients found, using a highly sensitive genotyping assay, activating and resistance (T790M) mutations in CTCs from 11 of 12 (92%) patients (30). Importantly, the number of CTCs correlated well with tumor diameter, and the appearance of the T790M mutation appeared to correlate with tumor progression. A later study, using ultra-deep next generation sequencing of CTCs from NSCLC patients, found EGFR mutations in CTCs in 84% of patients whose tumors exhibited EGFR mutations (31). Whole-exome sequencing of CTCs from patients with metastatic prostate cancer was used to demonstrate high levels of concordance of mutations in these CTCs with those in the tumor (32). Mutations in the androgen receptor gene have also been found in CTCs from patients with castration-resistant prostate cancer (33). In that study, 57% of patients exhibited AR mutations. PIK3CA mutational status in CTCs from patients with breast cancer was found to be 53% and 75% concordant for mutations at nucleotides 1633 and 3140, respectively, compared to the primary tumor (34).

In contrast to ctDNA, CTCs have also been used to evaluate protein levels (Figure 1). For example, Punnoose and coworkers found that, in 11% of cases, Her2 status in CTCs differed from that found in the primary tumor from patients with metastatic breast cancer (35). This finding is consistent with the observation that Her2 expression may differ between the primary and metastatic tumor (36), underscoring the importance of determining Her2 status in metastatic biopsies. However, access to metastatic tumors may be challenging in certain cases. Her2 levels have recently been evaluated in CTCs and found to be 69% and 74% concordant to the primary tumor and the metastatic tumor, respectively (37). Could Her2 levels in CTCs be used to direct therapy? The DETECT study (NCT01619111) is enrolling patients who have a Her2-negative primary tumor but Her2-positive CTCs and randomizing them to standard of care alone or in combination with lapatinib. Other studies examining protein levels in CTCs are also noteworthy. One study has evaluated

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**Figure 1. Molecular characterization of liquid biopsies.**
estrogen receptor levels in CTCs from patients with metastatic breast cancer whose primary tumors were ER-positive (38). In that study, ER-positive CTCs only were found in 5 of 16 patients, ER-negative CTCs only were found in 3 of 16 patients and ER-positive and −negative were found in 8 of 16 patients, demonstrating intra-patient heterogeneity. Consistent with those findings, Paolletti and coworkers (39) have found, in metastatic breast cancer patients, discordance between protein levels in CTCs and tumor tissue. For example, 47% of patients whose primary tumor was ER-positive had CTCs which were ER-negative, and two of ten patients whose primary tumors were Her2-negative had positive Her2 staining in at least 10% of their CTCs. These authors have developed an endocrine therapy index, using ER, Bcl-2, Her2, and Ki67 staining on CTCs, which may predict resistance to endocrine therapy among patients with hormone receptor-positive metastatic breast cancer. Prospective trials (COMETI P2 and SWOG S1222) will determine the clinical validity of the index. Androgen receptor signaling has also been investigated in CTCs from castration sensitive and castration resistant prostate cancer (CRPC) patients (40).

That study demonstrated an “AR-on” phenotype (positive for PSA staining but negative for PSMA staining in CTCs) in untreated patients. Interestingly, after androgen deprivation therapy, these patients then displayed an “AR-off” phenotype (negative for PSA staining but positive for PSMA staining) within one month, followed by the complete loss of CTCs within three months of therapy initiation. Importantly, patients with CRPC showed heterogeneity in CTC phenotypes, and patients who had more than a 10% fraction of an “AR-mixed” phenotype (positive staining for both proteins) had a statistically significant reduced overall survival. PD-L1 expression on CTCs from hormone receptor positive, Her2 negative, breast cancer patients has recently been examined (41). In that study, 69% of patients had PD-L1 positive CTCs. Validation of these findings in other tumor types and their relevance to therapy response will require further investigation.

Transcript levels have also been examined in CTCs. RNA in situ hybridization of epithelial and mesenchymal transcripts have been examined from CTCs from metastatic breast cancer patients (29). In one patient, the fractions of epithelial or mesenchymal CTC populations varied during the course of therapy response. Quantitative RTPCR has also been used on RNA from CTCs obtained from patients with advanced prostate cancer (42). The authors demonstrated that patients who, at baseline, were AR-V7 positive had statistically significant lower PSA response rates, shorter progression free survival, and shorter overall survival compared to patients who were AR-V7 negative, regardless of whether they were treated with enzalutamide or abiraterone. Interestingly, six patients (out of 42) who were AR-V7 negative at baseline converted to AR-V7 positive during treatment and these patients had intermediate levels of response and survival.

In contrast to detection of activating mutations for patient stratification, detection of drug-resistance mutations are a more complicated situation from a clinical utility/actionability/regulatory perspective. Stuart and coworkers have shown that a discontinuous dosing regimen can prevent the emergence of lethal drug resistance if the drug-resistant clone has a fitness disadvantage in the absence of the drug (43). Analogously, Chmielecki et al. (44) used modeling to predict that using continuous low dose treatment combined with simultaneous high dose pulsed dosing of erlotinib would delay the onset of T790M-mediated drug resistance. Thus, sensitive, early detection of drug-resistant mutations, indicating emergence of a drug-resistant clone, via plasma-based diagnostics, could enable dosing holidays or alternative dosing strategies and longer therapeutic effects. Prospective clinical trials employing such a strategy will be required to determine the effects on progression-free and overall survival.
It should be noted that evidence is emerging that such a paradigm may work in patients. For example, Sequist et al. (45) found that, in three patients who developed resistance mutations to erlotinib, after a dosing holiday the patients’ tumors responded to rechallenge with erlotinib and this corresponded to a loss of the resistance mutation. Similarly, Hata and coworkers (46) demonstrated, in one patient, that disappearance of the T790M resistance mutation was predictive for response to a rechallenge with gefitinib. Most recently, Siravegna et al. (17) found that mutated KRAS levels, in the plasma of colorectal cancer patients undergoing treatment with cetuximab or panitumumab, declined after the therapeutic antibodies were no longer being given. Furthermore, the authors found levels of mutant KRAS, in the plasma in patients who responded to multiple challenges with the therapeutic antibodies, increased during each rechallenge session. Thus, levels of the mutated KRAS in the plasma mirrored the efficacy of the rechallenge periods. Thus, the clonal heterogeneity of tumors and the differential fitness of these clones could be exploited in new treatment strategies. Clearly, the use of noninvasive diagnostics will play a key role in such a paradigm.

Applications to immuno-oncology

A challenge in immune-oncology regimens is the kinetics of tumor response wherein the typical Response Evaluation Criteria in Solid Tumors (RECIST) guidelines may not accurately reflect tumor response at early timepoints. Various recommendations have been generated, one of which is to be able to better identify relevant response patterns since the sum of perpendicular diameters may actually increase in some patients and show a decrease only after many months (47). Can liquid biopsies be used as intermediate markers of response to determine the likelihood that patients undergoing immunotherapy regimens will eventually respond? Diaz and coworkers (48) analyzed plasma from a very small cohort of melanoma patients undergoing treatment with ipilimumab. The ctDNA levels were found to correlate with clinical and radiologic outcomes. Interestingly, in one patient ctDNA levels dropped below detectable levels three weeks prior to clinical improvement. A similar study in melanoma patients found nearly identical results (49). Specifically, for two patients being treated with immunotherapy regimens, ctDNA levels dropped to undetectable levels three or four weeks prior to radiographic evidence of tumor response. Changes in CTC levels may also serve as early biomarkers of response. Earlier work from Hayes and coworkers (50) demonstrated that CTCs provided an earlier, more reproducible measure of disease status and correlated more strongly with overall survival than did imaging in metastatic breast cancer patients, although this study did not employ an immunotherapy regimen. Punnoose et al. (51) also demonstrated decreases in CTC levels correlated with response.

Diagnostic, regulatory, and commercial considerations.

Which template, ctDNA or CTCs, should be used in the companion diagnostic? The answer will require consideration of cost, ease of use, clinical context, analyte stability and access, and regulatory and commercial considerations. From a technical perspective, ctDNA may be more amenable to interrogation of simple mutations and translocations while CTCs may be better suited for interrogation of RNA, protein and small copy number variations. However, in cases where resistance may occur via multiple mechanisms (e.g., resistance to EGFR tyrosine kinase inhibitors in NSCLC can arise due to the T790M mutation or amplification or upregulation or oncogenes (52)), CTCs may provide a more comprehensive picture of drug resistance. A better understanding of the complementarity of ctDNA and CTC approaches will require further investigation. Studies such as TRACERx, which will collect ctDNA and CTCs at numerous time points, will be instructive in that regard (53). In addition, investigation and optimization of pre-analytical variables will have to be performed to understand the impact on sensitivity and precision for these various methods since storage temperature and time, freeze thaw cycles and other parameters can affect ctDNA results (54). Nevertheless, the sensitivity of these approaches suggests that plasma-based companion diagnostics can be developed for patient stratification, obviating the need for tumor tissue. Indeed, detection of activating mutations in EGFR in plasma using ctDNA has been utilized as the basis for a recent companion diagnostic approval (www.qiagen.com) (55). From a regulatory perspective, bridging from a mutation assay in ctDNA to a mutation assay in CTCs may generate considerable risk so it is advisable to carefully consider which template and platform will be used prior to enrollment in clinical trials.

In summary, the field of blood based diagnostics holds substantial promise for cancer therapeutics, and the coming years should see the realization of these methodologies and their utility in precision medicine.

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References