Using circulating cell-free DNA to monitor personalized cancer therapy

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ABSTRACT

High-quality genomic analysis is critical for personalized pharmacotherapy in patients with cancer. Tumor-specific genomic alterations can be identified in cell-free DNA (cfDNA) from patient blood samples and can complement biopsies for real-time molecular monitoring of treatment, detection of recurrence, and tracking resistance. cfDNA can be especially useful when tumor tissue is unavailable or insufficient for testing. For blood-based genomic profiling, next-generation sequencing (NGS) and droplet digital PCR (ddPCR) have been successfully applied. The US Food and Drug Administration (FDA) recently approved the first such “liquid biopsy” test for EGFR mutations in patients with non-small cell lung cancer (NSCLC). Such non-invasive methods allow for the identification of specific resistance mutations selected by treatment, such as EGFR T790M, in patients with NSCLC treated with gefitinib. Chromosomal aberration pattern analysis by low coverage whole genome sequencing is a more universal approach based on genomic instability. Gains and losses of chromosomal regions have been detected in plasma tumor-specific cfDNA as copy number aberrations and can be used to compute a genomic copy number instability (CNI) score of cfDNA. A specific CNI index obtained by massive parallel sequencing discriminated those patients with prostate cancer from both healthy controls and men with benign prostatic disease. Furthermore, androgen receptor gene aberrations in cfDNA were associated with therapeutic resistance in metastatic castration resistant prostate cancer. Change in CNI score has been shown to serve as an early predictor of response to standard chemotherapy for various other cancer types (e.g. NSCLC, colorectal cancer, pancreatic ductal adenocarcinomas). CNI scores have also been shown to predict therapeutic responses to immunotherapy. Serial genomic profiling can detect resistance mutations up to 16 weeks before radiographic progression. There is a potential for cost savings when ineffective use of expensive new anticancer drugs is avoided or halted. Challenges for routine implementation of liquid biopsy tests include the necessity of specialized personnel, instrumentation, and software, as well as further development of quality management (e.g. external quality control). Validation of blood-based tumor genomic profiling in additional multicenter outcome studies is necessary; however, cfDNA monitoring can provide clinically important actionable information for precision oncology approaches.

Introduction

Progress in understanding the molecular pathways in tumors has encouraged attempts to move away from protocol-based approaches and toward more personalized cancer therapies. These new treatment concepts use information about a person’s genes, proteins, and environment to help diagnose, plan treatment, monitor treatment efficacy, or to prognosticate (National Cancer Institute 2016). Personalized cancer therapy allows more targeted treatment, which has been shown to be more effective for example in non-small cell lung cancer (NSCLC) harboring EGFR and ALK mutations as well as melanomas with BRAF mutations. KRAS mutations allow identification of cancers that will not respond to EGFR antibodies and EGFR kinase inhibitors (Oxnard et al. 2014). Identification of non-responders is helpful to avoid unsuccessful, unnecessary treatment and costs.

A prerequisite for the successful application of such personalized approaches to cancer care is the availability of practical diagnostic tools for identifying subsets of patients with solid tumors sensitive to targeted therapies, real-time molecular monitoring of treatment, the detection of recurrence, and tracking resistance (Oxnard et al. 2014). Advances in genome technologies within

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the past decade have been essential for monitoring genotype-directed cancer care. Next-generation sequencing (NGS) platforms have been developed that allow for the simultaneous sequencing of multiple areas of the genome (Gagan & Van Allen 2015). This technology is useful to develop more universal approaches for cancer detection. Droplet digital PCR (ddPCR) has been shown to be especially useful and practical for the quantification of target nucleic acids (Oxnard et al. 2014).

Comparative genomic hybridization, and more recently, NGS genomic profiling of tumor tissue have been used to identify cancer associated somatic genomic alterations (Gagan & Van Allen 2015). Genomic information about cancer is listed in several national and international cancer genome resources, including The Cancer Genome Atlas (Grade et al. 2015) and the International Cancer Genome Consortium (ICGC). The ICGC has mapped genomic, transcriptomic, and epigenetic changes in numerous types of tumors, which have been used to develop more precise clinical laboratory tests to detect and treat cancers (ICGC 2016). Figure 1 shows patterns of chromosomal aberrations in solid tumors (Grade et al. 2015). It shows the most common gains and losses in colon, rectal, cervical, bladder, NSCLC, and breast cancer. The prevalence of specific genomic imbalances is unique to each tumor type. Plasma DNA tissue mapping has become possible using genome-wide methylation sequencing. This general approach is useful to assess the tissue contribution to the circulating cell-free DNA (cfDNA) pool (Sun et al. 2015).

Cancer genomics data have facilitated a revolution in oncology drug discovery by identifying candidate drug targets for use in trials that target clinically relevant driver mutations or molecular features. This has allowed the development of modern oncology treatments, which focus on identifying the therapy most appropriately matched to a particular patient’s genetic profile. So far, however, the success and clinical benefit of personalized cancer care have been limited (Tannock & Hickman 2016). A major challenge is the fact that there is only partial inhibition of signaling pathways by molecular-targeted agents. In addition, combinations of such agents may be too toxic (Tannock & Hickman 2016). An alternative promising approach is immunotherapy, particularly with checkpoint inhibitors (Maus et al. 2014). Some such immunotherapies can be combined with other anticancer agents (Zugazagoitia et al. 2016). Personalized combination strategies can be envisaged.

For routine application, non-invasive molecular monitoring tools, such as circulating cell-free tumor DNA (ctDNA), seem to be useful to assess the effectiveness of these new precision cancer treatment strategies. Blood-based tumor genomic profiling was therefore chosen to demonstrate the potential of these emerging technologies to support personalized cancer care.

**Genotype-directed cancer care**

Pharmacogenomics facilitates the identification of biomarkers that are useful to optimize drug selection, dose, and treatment duration and avoid adverse drug reactions (Wang et al. 2011).

Table 1 lists some pharmacogenomic biomarkers in US Food and Drug Administration (FDA) approved oncology drug labeling (FDA 2016). Two genomes are involved: the somatic genome of the tumor and the germline genome of the patient. The tumor genome plays a critical role in the variation in response to anti-neoplastic therapy. Biomarkers from the tumor genome include EGFR, KRAS, BRAF, and others that are already being routinely determined in biopsy specimens. There are also biomarkers, which are related to drug metabolism. For example, germline single nucleotide polymorphisms (SNPs) in the gene encoding the enzyme TPMT can result in increased sensitivity to mercaptopurines (Schütz et al. 1993). These pharmacogenetic tests are already considered part of routine oncologic care.

The first example of a targeted therapy, imatinib, is a tyrosine kinase inhibitor (TKI) that has been successfully used for the treatment of patients with chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). CML presents an estimated incidence of 1/100,000 cases per year, which accounts for 15–20% of leukemias (Koshiyama et al. 2013). The annual age-adjusted incidence of GIST averaged 6.8 per 1,000,000 (Coe et al. 2016). Imatinib is a potent selective inhibitor of the BCR-ABL tyrosine kinase present in CML. BCR-ABL tyrosine kinase is constitutively activated in the oncogenic fusion protein BCR-ABL (BCR = “Breakpoint Cluster Region”), causes anti-apoptotic effects and stimulates proliferation (McDermott et al. 2011). BCR-ABL is a clonal driver mutation, expressed at high levels only in leukemic cells. While pre-imatinib therapy was associated with a poor prognosis, the current overall five-year survival rate for newly diagnosed chronic-phase CML patients treated with imatinib is 89% (McDermott et al. 2011). For imatinib, the therapeutic target is BCR-ABL tyrosine kinase. A biomarker of major molecular response (MMR) is the BCR-ABL transcript concentration measured in peripheral blood cells to predict clinical response. A MMR is defined as a three or more log reduction of BCR-ABL1 transcript levels of a standardized baseline (<0.1 in the International Scale), which is
associated with a favorable progression-free survival (PFS) (Machado et al. 2011). Another biomarker of response is the complete cytogenetic response, which is defined as absence of Philadelphia-positive marrow cell metaphases (Baccarani et al. 2009). The Philadelphia chromosome is a changed chromosome 22, due to fusion of BCR region of chromosome 22 and ABL1 proto-oncogene of chromosome 9. In more than 90% of CML cases, the presence of the Philadelphia chromosome can be detected (Koshiyama et al. 2013). With
imatinib, there is the particular advantage in that the genetic biomarker is present in a high proportion of CML patients and, therefore, allows for the treatment of a group rather than only individual patients (Tannock & Hickman 2016). Through imatinib plasma concentrations determined by liquid chromatography tandem mass spectrometry (LC–MS/MS) can be used to rule out pharmacokinetic causes of treatment failure (Picard et al. 2007).

Another example of successful genotype-directed cancer care is the use of gefitinib (another TKI) in patients with NSCLC (Wang et al. 2011). Lung cancer is a leading cause of cancer related death, with 1.6 million cases worldwide and 85% of all lung cancer cases have NSCLC (Sandelin et al. 2015). The tumor genome using biopsy specimens was shown to play an important role in this differential response to approved EGFR TKIs. Tumor EGFR encoding activating mutations within the kinase domain result in enhanced tumor sensitivity to gefitinib. The presence of the EGFR sensitizing mutations (L858R or exon 19 deletion) is a selection criterion for the use of EGFR TKIs including gefitinib, erlotinib, and afatinib. The initial patients treated with gefitinib and erlotinib identified with these mutations in their tumor biopsies were found only retrospectively to have better clinical responses than did patients without these mutations. Studies involving afatinib in EGFR mutant NSCLC had prospective tumor assessment for these sensitizing mutations. Adenocarcinoma (ADC), which is among the most common of the NSCLC histological subtypes, has been shown to be associated with activating mutations in the EGFR gene in 13% of European and 47% of Japanese patients (corresponding rates for non-ADC are 5% and 7%, respectively) (Reck et al. 2016). Additionally, patients who had these mutations but who more slowly metabolized gefitinib were shown to likely have better PFS (Widmer et al. 2014).

The use of imatinib and EGFR TKI gefitinib is two examples of successful genotype-directed cancer care. Another example comes from a study by the Lung Cancer Mutation Consortium testing for driver mutations in metastatic lung ADCs at 14 centers (Kris et al. 2014). In 64% of lung ADCs, actionable drivers were detected. Individuals with oncologic drivers receiving a matched targeted agent lived longer (median survival 3.5 years), compared to patients who did not receive genotype-directed therapy (median survival 2.4 years).

### Liquid biopsy tumor genomic profiling and tissue biopsy-based approaches

While both fresh and archived biopsy material was and still is commonly used for solid tumor genomic profiling, there are many limitations to biopsies including their limited availability, repeatability, and high failure rates (Mao et al. 2015); 10–50% of all biopsies fail to obtain sufficient tumor tissue. One often underestimated problem is tumor heterogeneity. There are also often real or perceived risks prohibiting patients from undergoing biopsies. Tumors can be located in difficult or dangerous locations to access, but even when in accessible locations, biopsies are associated with risks, inconvenience, and major costs; especially when they need to be done repeatedly such as to monitor for the development of resistance to targeted therapies. Recently, less invasive, blood-based tumor genomic profiling methods using either circulating tumor cells (CTCs) or ctDNA are being investigated as a way to overcome these limitations (Oxnard et al. 2014). For example, in a systematic review of 25 studies it was concluded that blood is a good substitute when tissue

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**Table 1. Anticancer drugs and their pharmacogenomic biomarkers.**

<table>
<thead>
<tr>
<th>Biomarker with pharmacodynamic effect (drug target)</th>
<th>Associated drug</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Somatic genome of the tumor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Erlotinib, gefitinib, afatinib, osimertinib</td>
<td>NSCLC</td>
</tr>
<tr>
<td>ALK</td>
<td>Crizotinib, ceritinib, alectinib</td>
<td>NSCLC</td>
</tr>
<tr>
<td>ROS-1</td>
<td>Crizotinib</td>
<td>NSCLC</td>
</tr>
<tr>
<td>HER2 (ERBB2)</td>
<td>Lapatinib, trastuzumab, ado-trastuzumab, emtansine</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>ESR1</td>
<td>Exemestane, anastrozole, letrozole, tamoxifen</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>AR</td>
<td>Enzalutamide</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>BRAF</td>
<td>Vemurafenib, dabrafenib, trametinib, cobimetinib, pembrolizumab, nivolumab</td>
<td>Melanoma</td>
</tr>
<tr>
<td>BCR-ABL1</td>
<td>Imatinib, dasatinib, nilotinib</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>c-KIT</td>
<td>Imatinib</td>
<td>Gastrointestinal stromal tumor</td>
</tr>
<tr>
<td><strong>Germline genome of the patient</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPMT</td>
<td>Mercaptopurine, thioguanine</td>
<td>Acute lymphatic leukemia (ALL)</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Irinotecan, nilotinib</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>DPYD</td>
<td>Fluorouracil</td>
<td>Colorectal, gastric, pancreatic, breast, esophageal, head and neck cancer</td>
</tr>
</tbody>
</table>

is insufficient for testing EGFR mutations to guide EGFR TKIs treatment (Mao et al. 2015), where cfDNA seems more sensitive compared to CTCs (Freidin et al. 2015).

Serial ctDNA measurements are being used as a form of “liquid biopsy” to monitor responses and to detect both relapses as well as drug resistance during treatment (McDermott et al. 2011). The methods involve as the first step identification and quantification of the specific somatic genomic alterations by sequencing either tumor tissue or the ctDNA isolated from plasma. Repeated measurement of these mutations in ctDNA can then be used to monitor response and early detection of cancer relapse (Figure 2).

In an additional study, repeated ctDNA sampling was used as a form of “liquid biopsy” to detect resistance mutations up to 16 weeks before radiographic evidence of tumor progression (Oxnard et al. 2014). Another indication of the potential value of ctDNA monitoring is the fact that in May 2016 the US FDA approved the first such “liquid biopsy” test (Cobas® EGFR Mutation Test v2) for patients with NSCLC.

**Origin of ctDNA and quantification techniques**

Various techniques that have been described for the quantification of ctDNA (Diehl et al. 2005; Oxnard et al. 2014; Freidin et al. 2015; Sandelin et al. 2015; Zugazagoitia et al. 2016) are summarized in Table 2. In the nucleus of a cell, the DNA is wound around histones, forming nucleosomes (Jorde et al. 2010). As cells undergo apoptosis or necrosis, these nucleosomes are released into the blood stream and circulate freely in the plasma. The DNA seems to be present in the circulation largely in the form of nucleosomes, or in apoptotic vesicles (Mouliere et al. 2013). Because apoptosis leads to high inter-nucleosomal DNA fragmentation, fragments as small as 158–200 base pairs (the length of a mononucleosome DNA) may be found following apoptosis. Necrosis on the other hand results in larger ctDNA fragments, larger than 10,000 base pairs (bp). Estimation of ctDNA fragmentation might be able to provide evidence as to the cfDNA release mechanism involved. The half-life of ctDNA in plasma is very short, less than 1.5 h (Lo et al. 1999). The liver, spleen, and kidney are presumably involved in this rapid cfDNA removal (Lo et al. 1999). As tumors become more aggressive, the degree of necrosis increases and the absolute amount of circulating mutant DNA correspondingly increases.

**Clinical studies on ctDNA tumor genomic profiling**

**Detection of EGFR acquired resistance mutation**

One of the most promising uses of ctDNA as a liquid biopsy is monitoring for the development of acquired resistance. Genetically acquired resistance commonly occurs even with the most successful, personalized targeted therapies as a result of solid tumor heterogeneity that selects for resistant tumor cells. ctDNA has been shown to be especially useful for detection of resistance because of the many previously mentioned limitations of biopsies (Murtaza et al. 2013). A schematic presentation of the methods used is shown in Figure 3.

Non-invasive detection of acquired resistance to cancer therapy has been shown to be possible by repeated sampling of plasma ctDNA, representing a non-invasive “liquid biopsy”. In a study published by Murtaza et al. (Murtaza et al. 2013), an analysis of mutations in circulating plasma cfDNA over time using exome sequencing allowed for the identification of specific resistance mutations. For example, the mutant allele frequency significantly increased following treatment in a NSCLC patient. Of particular interest was a resistance conferring mutation in EGFR (T790M) that had an increase of 13% in allele frequency following treatment with gefitinib. This mutation inhibits binding of gefitinib to EGFR, causing resistance to gefitinib (Murtaza et al. 2013). Now, in such cases, the patient can be switched to an alternative therapy with osimertinib (AZD 9291). This is a newer oral, irreversible, mutant-selective EGFR TKI with potency against NSCLC cells harboring the EGFR T790M resistance mutation (Thress et al. 2015).

Such serial analysis of plasma ctDNA can complement or even replace the invasive biopsy approaches currently being used to identify acquired drug resistance mutations in patients with advanced cancers. This would have major implications both for patients’ survival and quality of life, as well as for the costs of care (Tie & Gibbs 2016). In Germany, TKI treatment costs per day are 96 EUR for erlotinib and 150 EUR for gefitinib. The costs per plasma ctEGFR PCR test is about 200 EUR and the costs per tumor biopsy tissue EGFR testing varies between 200 and 400 EUR, depending on the method used. It is evident that the diagnostic costs are comparatively small compared to the treatment costs. Ineffective use of these expensive TKIs can be avoided by ctDNA testing, resulting in substantial cost savings. Being able to identify non-responders would avoid unnecessary further treatment of such patients, thereby avoiding treatment-related toxicity, unnecessary costs, and potentially allowing a switch to an alternative effective therapy.

The use of ctDNA to sequentially monitor specific tumor SNP mutations associated with resistance has been described in a study of patients with EGFR mutant NSCLC receiving erlotinib (Oxnard et al. 2014). An increase in plasma ctDNA containing both the L858R
and T790M mutations was already observed in one patient before new brain metastases were detected (Oxnard et al. 2014). Later, decreased plasma ctDNA levels were seen when treatment was initiated with a new medication as part of a clinical trial. A simultaneous decrease in the plasma tumor genotype was associated with objective tumor shrinkage. In a second patient, the plasma L858R mutation decreased when the patient’s pleural drainage resolved and a computed tomography (CT) scan indicated stable disease.

Figure 2. ctDNA as a form of liquid biopsy. (From McDermott et al. 2011. Copyright © 2011 Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society.)
Another two patients from this study (Oxnard et al. 2014) exhibited a plasma response on first-line erlotinib, but later increasing plasma levels of the EGFR sensitizing mutation were detected; about 12–25 weeks before progression was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST). In each of these two patients, the plasma T790M mutation could also be identified at progression, generally at somewhat lower levels than the EGFR sensitizing mutation.

Targeted NGS and ddPCR have also been successfully used for the analysis of acquired resistance to newer EGFR TKIs. Osimertinib is an oral, irreversible, mutant selective EGFR TKI with potency against NSCLC cells harboring the EGFR T790M resistance mutation (Thress et al. 2015). The estimated PFS after use of this drug is about 10 months in patients with the T790M mutation. Patients may, however, acquire the EGFR C797S mutation which

**Table 2. Techniques for quantification of ctDNA.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Next-generation sequencing (NGS)</td>
<td></td>
</tr>
<tr>
<td>- NGS gene panels (e.g. hybrid capture-based)</td>
<td></td>
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<tr>
<td>- Whole genome sequencing (low coverage)</td>
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<tr>
<td>Droplet digital PCR (ddPCR)</td>
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<tr>
<td>Quantitative allele-specific, real-time PCR</td>
<td></td>
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<tr>
<td>Idylla</td>
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<tr>
<td>Competitive allele-specific TaqMan PCR (cast-PCR)</td>
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<tr>
<td>Allele-specific PCR</td>
<td></td>
</tr>
<tr>
<td>Coamplification at lower denaturation</td>
<td></td>
</tr>
<tr>
<td>temperature (COLD)-PCR</td>
<td></td>
</tr>
<tr>
<td>BEAMing assay</td>
<td></td>
</tr>
</tbody>
</table>

Oxnard et al. 2014
Zugazagoitia et al. 2016
Schütz et al. 2015
Oxnard et al. 2014
López et al. 2016
Thress et al. 2015
Gao et al. 2010

Janku et al. 2016
Ashida et al. 2016
Sandelin et al. 2015
Freidin et al. 2015

Thress et al. 2015
Gao et al. 2010

mediates resistance to osimertinib (Thress et al. 2015).

Another example of the ability of targeted plasma NGS to detect resistance mutations at the time of systemic progression is illustrated by results obtained in a patient who acquired resistance to osimertinib mediated by the EGFR C797S mutation (Figure 4) (Thress et al. 2015). In this patient targeted NGS identified an acquired mutation (in 1.3% of reads) encoding EGFR C797S at the time of progression. The T790M mutation had decreased from 3.3% baseline to 1.8% during progression.

In this study of 15 patients treated with osimertinib, who were all positive for the T790M mutation, three molecular subtypes emerged: 6 patients acquired the C797S mutation, 5 maintained the T790M mutation but did not acquire the C797S mutation, and 4 lost the T790M mutation despite the presence of the underlying EGFR activating mutation (Thress et al. 2015). This tumor genome monitoring study illustrates both the diversity of resistance mechanisms and the potential of ctDNA serial analysis to identify and monitor for the presence of such mechanisms.

cDNA monitoring in cancer patients with KRAS mutation

KRAS mutations are responsible for resistance to EGFR-targeted antibody therapy with cetuximab or panitumumab (López et al. 2016). A recent study (Freidin et al. 2015) reported that ctDNA outperformed the use of CTCs for the detection of the KRAS mutation in patients with thoracic malignancies. This study included 82 patients with lung cancer, and 11 with benign diseases of the lung. Various molecular tests were used, including custom designed co-amplification at lower denaturation temperature (COLD)-PCR assays, high-resolution melt analysis (HRM), and commercial assays including the Cobas KRAS mutation test and the Therascreen pyrosequencing KRAS kit. The diagnostic sensitivity and specificity of KRAS mutation detection achieved with ctDNA was 0.96 and 0.95, respectively, which was higher than for CTC DNA (0.52 for sensitivity and 0.88 for specificity).

Plasma ctDNA monitoring has also been studied in colorectal cancer patients with the KRAS G12V mutation (López et al. 2016). Measurement of ctDNA using ddPCR was used to look for the KRAS G12V mutation in 10 colorectal cancer patients and 6 healthy controls. The mutation was detected in 9 of 10 patients and the median number of copies per ml was significantly elevated in the colorectal cancer patients; with the highest values seen in patients with metastatic disease. While demonstrating that the KRAS G12V mutation was detectable in the plasma of the majority (9/10) of these colorectal cancer patients using ddPCR, the one false negative raises the possibility that there may not be sufficient cfDNA to be detected in some colorectal cancer patients; possibly those at the earliest stages of the disease.

Pancreatic ductal adenocarcinoma (PDAC) has one of the lowest survival rates of all cancers and there is a lack of personalized treatment options because biopsies are often inadequate for molecular characterization. Zill et al. (2015) studied ctDNA by sequencing in 17 patients with PDAC or biliary carcinoma and compared matched sequencing tests from tumor biopsies and plasma cfDNA. These authors showed that 90.3% of mutations detected in biopsies were also detected in ctDNA. Across five informative genes (KRAS, TP53, APC, FBXW7, SMAD4), the diagnostic accuracy of ctDNA was 97.7% with a sensitivity of 92.3% and a specificity of 100%. They concluded that ctDNA sequencing reliably detected tumor-derived mutations and could pave the way for precision oncology approaches to the treatment of PDAC.

cDNA monitoring in melanoma patients with BRAF mutations

The prognostic value of BRAF mutation-positive cfDNA was also investigated in patients with melanoma (Santiago-Walker et al. 2016). Although it comprises less than 2% of skin cancers, melanoma is responsible for the largest number of skin cancer-related deaths (9940 annually in the USA). BRAF mutations are observed in approximately 50% of melanoma tumor tissue samples. In this study (Santiago-Walker et al. 2016), 732 patients with BRAF V600 mutation-positive melanoma, most
treated with dabrafenib were evaluated. A comparison between plasma ctDNA determined by BEAMing (Beads, Emulsion, Amplification, and Magnetic) technology and analysis of tumor PCR BRAF mutation status showed that the BRAF mutation was detectable using ctDNA in 76% and 81% of late-stage patients with BRAF V600E- and V600K-positive tumors, respectively. Also, patients with BRAF mutation-positive tumors, but negative for BRAF-mutant ctDNA at baseline, had longer PFS and overall survival compared to patients with a positive ctDNA result. These results suggest that ctDNA alone, while of some prognostic value, may not be suitable as the principal screening method for patients with unknown BRAF mutation status.

Another recent study (Ashida et al. 2016) investigated the relationship between BRAF V600E ctDNA and melanoma progression in a 46-year old patient who had no recurrence for five years after radical surgery. In order to quantify small amounts of the mutant allele, both BRAF V600E ctDNA copies per ml and the ctDNA fraction were determined using competitive allele-specific TaqMan PCR with allele-specific blockers added to suppress amplification of the wild type (WT) allele. Between day 0 and day 91 of the study period, ctDNA was not detected and a CT scan on day 54 revealed no metastases. After day 91, there was a major increase of ctDNA. Multiple metastases were detected in the pleural cavity at day 180 and the patient died on day 343. The increase in BRAF V600E ctDNA preceded the CT diagnosis, suggesting that ctDNA may be useful for monitoring such patients.

**ctDNA monitoring in patients with metastatic breast cancer**

A trial at the Cambridge Cancer Research Center compared the use of ctDNA, CA 15–3 (Cancer-Antigen 15–3) and CTCs to monitor 30 metastatic breast cancer patients (Dawson et al. 2013). Detection rates in women with somatic genomic alterations were 97% with ctDNA, 78% with CA 15–3, and 87% with CTCs. The ctDNA also showed a greater correlation with tumor burden and was the earliest measure of treatment response.

In this same study (Dawson et al. 2013), these authors sequentially measured six separate point mutations; all of which showed similar but somewhat different dynamic patterns. There was a decrease in mutations detected in patients with stable disease on epirubicin treatment, but with disease progression there was a strong increase in mutations that persisted after the patient was switched to paclitaxel.

The comparison of the ctDNA, CA 15–3, and CTCs results in one patient from this study (Figure 5) illustrates the ability of these three methods to monitor individual tumor dynamics. The patient was treated with capecitabine, vinorelbine, and epirubicin. The ctDNA changes reflected partial response, progressive disease, and stable disease very well. The number of CTCs also decreased significantly during stable disease and both ctDNA and CTCs had significant increases with disease progression. On the other hand, CA 15–3 showed no consistent serial changes and had only a low correlation ($r^2 = .36$) with ctDNA. Finally, increasing ctDNA levels were significantly associated with poor overall survival in this study (Dawson et al. 2013).

One potential benefit of using CTCs is their DNA integrity; their DNA can be extracted from viable intact cells, whereas ctDNA is fragmented and partially degraded. However, tumor heterogeneity may be a major drawback to the use of CTCs because the CTCs isolated may not adequately represent the entire tumor population. The higher sensitivity and specificity of the ctDNA found in this study suggests that it may be more representative of primary tumor tissue than DNA extracted from CTCs. If so, ctDNA would be the preferred substrate for “liquid biopsy” tumor monitoring. While not yet a conventional clinical practice, many completed and ongoing trials have and are examining this possibility.

Garcia-Murillas et al. (Garcia-Murillas et al. 2015) speculated in a recent article that monitoring ctDNA serially, over multiple time-points, may be superior to a single time-point at judging risk of relapse. This possibility may have an impact on how future ctDNA studies are designed. Overall, given the high likelihood of relapse when ctDNA is detected, these findings lay the groundwork for future clinical studies that can be designed to test the intensification of adjuvant therapy options in breast cancer with detectable ctDNA at the
exposed to AIs. Patients with ESR1 mutations are a major mechanism of resistance to aromatase inhibitors (AIs). Schiavon et al. (Schiavon et al. 2015) developed ultra-high sensitivity multiplex digital PCR assays for the detection of ESR1 mutations in ctDNA and investigated the clinical relevance of these mutations in 171 women with advanced breast cancer. ESR1 mutations were found only in estrogen receptor-positive breast cancer patients previously exposed to AIs. Patients with ESR1 mutations had a substantially shorter PFS on subsequent AI-based therapy. ESR1 mutations can be identified with ctDNA analysis and predict resistance to subsequent AI therapy (Schiavon et al. 2015).

**Chromosomal aberration pattern analysis by low coverage whole genome sequencing**

In contrast to SNP-based applications discussed so far, chromosomal aberration pattern analysis is a more universal approach based on genomic instability (Schütz et al. 2015). With the availability of NGS, it was recently shown for the first time that this technology can be applied to cfDNA for quantitative analyses of genomic patterns (Beck et al. 2009). After having defined the cfDNA genomic profile in healthy individuals, the same group showed for the first time that the method can be used to detect differences between the cfDNA profile in cancer patients compared to apparently healthy individuals (Beck et al. 2010). The first step in this method is to construct a whole genome sequencing library from ctDNA. After sequencing, the mapped reads are counted in windows along the chromosomes (25,000 reads per window, 700 windows total). The copy number instability (CNI) score is generated by statistical comparisons to the normal population and is a general measure of genomic instability directly related to the regional chromosomal DNA ploidy. Genomic instabilities reflected as CNI often occur in malignant tumors.

In a recent study (Schütz et al. 2015), it was shown that ctDNA can be used as a diagnostic tool in prostate cancer. Patients with prostate cancer could be discriminated with a diagnostic accuracy of 83% from controls and 90% from patients with benign hyperplasia or prostate. Prostate cancer patients showed significantly more regional copy number imbalances (DNA ploidy heterogeneity) compared to healthy controls. Figure 6 shows a genome wheel with regions of genomic instability of cfDNA identified in prostate cancer. The outer circle depicts all preselected regions. The inner circle depicts the 20 regions selected for calculating the CNI index. The authors recommended adjusting CNI for copy number variations of the individual patient’s white blood cells. The CNI index (determined in cfDNA by ploidy heterogeneity) in this study discriminated well between 204 patients with prostate cancer (Gleason score 2–10), 207 male controls, 10 patients with benign hyperplasia, and 10 patients with prostatitis. However, perhaps surprisingly, no separation was observed in the CNI index on the basis of the Gleason score, which would be required to allow differentiation between aggressive and indolent prostate cancer. It appears that cfDNA sequencing may better reflect the genomes of all cancer sub-clones present in a patient. While these are still preliminary results, it appears that the CNI index using liquid biopsy analysis could provide information of substantial value in the future for the selection of therapeutic regimens. One hypothesis that needs to be examined further is whether repeated CNI determinations or sequencing would improve the diagnostic discrimination of such testing.

Azad et al. (2015), in a study in 62 patients with metastatic castration-resistant prostate cancer (mCRPC) progressing on abiraterone and enzalutamide treatment, demonstrated that androgen receptor (AR) gene aberrations in cfDNA were associated with therapeutic resistance. Chromosome copy number analysis and AR exon 8 sequencing was performed on cfDNA. AR gene aberrations were seen in 50% of patients. Enzalutamide resistance was linked to AR amplification and pretreatment AR aberration was predictive of adverse outcomes.

In addition to AR gene copy gains, point mutations occur and recently it was recognized that exon deletions during transcription (splice variants) lead to various truncated isoforms of AR. Notably and most importantly: defective transcription of the C-terminal ligand binding domain of AR resulted in a constitutive AR activation. Variant 7 or AR-V7 were detectable by the presence of their plasma-derived exosomal RNA using ddPCR. The truncated AR is resistant to all AR inhibitors, and its occurrence can be detected in patients’ plasma (Del Re et al. 2017).

Changes in CNI score were also found to predict therapeutic response in 24 patients with a number of metastatic cancers (Figure 7) including advanced esophageal cancer (N = 2), colorectal cancer (N = 3), non-Hodgkin lymphoma (N = 3), PDAC (N = 4), and NSCLC (N = 12). Treatment response to chemotherapy was recorded by RECIST 1.1 (Eisenhauer et al. 2009) or EORTC (European Organization for Research and Treatment of Cancer) PET/CT criteria. CNI changes were considered predictive of either response or stable completion of definitive treatment for non-metastatic disease. This may be complementary to other prognostic features, such as residual cancer in the resected specimen following neoadjuvant chemotherapy.
disease when there was a reduction of CNI score ≥50% relative to baseline. For at least 15 patients, CNI change predicted response about 3–8 weeks prior to scan results demonstrating response or progressive disease. In responders, significantly lower CNI values were observed compared to patients with disease progression. It was concluded that CNI change may serve as a predictor (potentially early predictor) of therapeutic response to standard chemotherapy for the investigated cancer types (Weiss et al. 2016).

Immunotherapy is an especially exciting new area for personalized therapy and diagnostics (Maus et al. 2014; Zugazagoitia et al. 2016). Immune checkpoint antibodies targeting negative regulatory molecules such as Programmed Cell Death Protein 1 (PD1) and Cytotoxic T-cell Lymphocyte/associate antigen 4 (CTL4) can be used to “release the brakes on natural T-cells responsive to tumor” (Maus et al. 2014). In a recent study (Weiss et al. 2016), it was shown that CNI scores predicted therapeutic responses to immunotherapy in 24 patients with various types of cancer. Patients were treated with either interleukin-2 alone or with a PD-1 inhibitor with or without chemotherapy. The CNI change from baseline is shown in Figure 8. Patients with stable disease or partial response by RECIST 1.1 showed a decreased CNI score, whereas the CNI score was distinctly increased in patients with disease progression. Meanwhile, other independent studies have used a similar approach of exploring copy number alteration in plasma (Chan et al. 2013; Heitzer et al. 2013; Heitzer et al. 2013).

**Remaining challenges for routine clinical application of ctDNA monitoring**

For the routine use of ctDNA, specialized personnel and specific instrumentation (NGS, ddPCR) is necessary. External quality control programs also have to be established for quality management and specific laboratory software and bioinformatics are necessary for the use of NGS. For SNP-based assays, recent findings indicate that the output from genetic testing can differ markedly, depending on which genetic test is applied (Kuderer...
et al. 2016). Significant discordance has also been noted between reports comparing tissue-based NGS tests. Further in-depth comparisons of NGS tests across larger numbers of patients with cancer are warranted to improve concordance and clinical utility. For proper interpretation of genomic results and their use to guide clinical decisions, expert multidisciplinary cooperation is essential. Further validation in multicenter outcome studies is important as the clinical benefit of ctDNA testing regarding better patient outcomes remains to be proven (Tie & Gibbs 2016).

Conclusions

Advances in both the understanding of tumor biology and the availability of advanced but practical technology have made it possible to use tumor genomic information to rapidly and reliably identify effective treatment targets. The recent progress in this field also allows for both prediction and monitoring of individual responses to specific treatments or drug concentrations, as well as to detect and even target some of the causes of drug resistance. The initial proof of concept studies used tumor biopsy material, but the many limitations of biopsies prompted the successful, and in some ways superior, use of less invasively obtained sources of tumor DNA including ctDNA. The use of ctDNA has been validated as a useful, non-invasive source of genetic material that can be used as a diagnostic tool for guiding personalized “precision” cancer therapy. Both specific SNP-based and a more universal approach, chromosomal aberration pattern analysis, have been shown to be predictive of treatment response, tumor recurrence, and the development of resistance. It is to be expected that ctDNA-based assays will be helpful to develop new diagnostic strategies to overcome some of the current limitations of personalized cancer medicine.

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