Realizing the Potential of Plasma Genotyping in an Age of Genotype-Directed Therapies


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Correspondence to: Bruce E. Johnson, MD, Dana-Farber Cancer Institute, Lowe Center for Thoracic Oncology, 450 Brookline Avenue, Boston, MA (e-mail: bejohnson@partners.org).

The identification of oncogenic driver mutations in cancer has led to the rapid rise of genotype-directed treatments such as EGFR and BRAF kinase inhibitors. Standard tumor biopsy remains a cumbersome and morbid procedure for patients, leading to a growing interest in noninvasive plasma genotyping approaches. Circulating tumor cells are of interest; however, the processing of specimens is complicated and time consuming. By comparison, cell-free DNA (cfDNA) genotyping has the potential to be convenient and relatively simple to process in a short time period. Several technologies are under development for cfDNA analysis, such as allele-specific polymerase chain reaction (PCR), coamplification at Lower Denaturation temperatures (COLD) PCR, emulsion PCR, and massively parallel sequencing. Broad clinical validity will need to be established for different assays, and clinical utility will need to be evaluated within prospective trials to determine which assays will best predict the efficacy of therapy and patient outcomes. In addition, assay standardization will be critical prior to widespread use in routine clinical practice. The Cell Free DNA Working Group, under the sponsorship of Transgenomic, was convened to evaluate the molecular assays in development and provide recommendations for application and interpretation of these tests in the context of future clinical research. The consensus commentary of the Cell Free DNA Working Group for the use of cfDNA plasma genotyping assays is presented here, including future steps in the development of these technologies.


The dramatic development over the past decade of genotype-directed, anti-cancer therapies has generated considerable interest in noninvasive strategies for cancer genotyping and monitoring of therapeutic response. In hematologic malignancies, monitoring of response through serial genotyping is standard of care, owing to the abundance of circulating malignant cells. In solid tumors, much enthusiasm has similarly been directed toward the study of circulating tumor cells (CTCs) and their association with prognosis. Technologies are now emerging to allow genotyping of cell-free plasma DNA (cfDNA) to detect genomic changes present in the tumor and to noninvasively monitor the development of acquired resistance. Indeed, recent years have witnessed several high profile publications demonstrating the potential power of cfDNA genotyping (1–5). In a study examining the mechanism of resistance to EGFR inhibition by monoclonal antibodies in colon cancer, investigators performed genotyping on cfDNA specimens from 24 patients demonstrating that clinical resistance arose via point mutations in KRAS that were detectable in the plasma prior to evidence of radiographic progression (1). In a second study, KRAS mutations were identified in two of two patient biopsy samples after progression on EGFR inhibitors with the corresponding KRAS mutant allele detectable in cfDNA up to 10 months prior to radiographic progression (2).

In breast cancer, studies have compared genotyping of cfDNA and the biopsy tumor specimens from breast cancers. In a well-designed experimental (34 patients) and validation (41 patients) study, cfDNA and appropriately matched tumor tissue had 100% concordance for mutational status of the PIK3CA gene (4). In comparison to standard circulating tumor markers, a study of 30 patients with breast cancer harboring somatic genomic alterations demonstrated that the quantification of mutant cfDNA every three weeks may be more readily measurable and be more closely associated with radiographic tumor burden than the serum tumor marker CA15-3 or CTCs (5). Cell-free DNA has also been used for whole genome sequencing of tumor-derived chromosomal alterations in patients with breast or colon cancer, showing the potential for cfDNA to act as a personalized cancer biomarker (6). In a study comparing 10 healthy individuals with 10 patients with colorectal carcinoma and 10 with breast carcinoma, a wide range of personalized chromosomal copy number changes and rearrangements were observed, including amplification of known cancer driver genes including ERBB2 and CDK6.

Finally, in lung cancer, cfDNA has been evaluated as a predictive biomarker of tumor aggressiveness and survival. In a study of 134 patients with non-small cell lung cancer, increased quantities of baseline plasma cfDNA were associated with overall tumor burden, decreased response to chemotherapy, and shorter survival (7). Additionally, preliminary analysis has suggested that cfDNA may be a reasonable alternative to tumor-based genomic testing for determining EGFR mutation status. For example, members of
our group have recently reported on the development of a digital droplet polymerase-chain reaction assay that demonstrated a 67% sensitivity and 100% specificity for mutant EGFR in 12 patient specimens (8).

Many of the studies described above investigate both a qualitative and quantitative approach to the use of cfDNA. Conceptually, cfDNA has the ability to serve both as a measure of total tumor burden as well as a biomarker of treatment effect by measuring changes in the prevalence of specific mutations and other genomic changes. This technology can also be used to identify emerging resistant clones prior to radiographic progression. This would potentially allow for early modification of therapy directed against the patients’ mechanism of acquired resistance. Taken together, existing literature suggests that analysis of cfDNA is feasible and may be a useful approach for the noninvasive analysis of tumor DNA, potentially obviating the need for some tumor biopsies. Here, we lay a framework for how this technology can now be developed into a clinical biomarker for guiding patient care.

Applications of cfDNA Genotyping

The applications for cfDNA are varied; they include blood-based molecular diagnostics, monitoring of tumor burden or response to treatment, as well as potentially screening healthy patients for tumor DNA. The most likely initial use for the application of cfDNA genotyping is the possibility of replacing some types of tumor biopsies. Few believe that a blood test will replace histologic diagnosis of malignancy, because tissue biopsy is likely to remain essential for initial diagnosis. However, second biopsies are increasingly being performed specifically to facilitate tumor genotyping and selection of targeted therapies as well. This is true both on initial diagnosis as well as after the development of drug resistance, and potentially these invasive secondary diagnostic procedures could be replaced by a minimally invasive analysis of cfDNA.

A second potentially valuable role for cfDNA genotyping is the monitoring of advanced cancers undergoing treatment. Following levels of a specific genotype and being able to quantify the evolving genotypes may make cfDNA a reliable pharmacodynamic marker for efficacy of a targeted therapy. Serial monitoring of cfDNA following a response may show a decrease in the genotype being targeted and allow for early detection of resistance mutations prior to radiographic progression of disease. In this manner, the response and then the mechanism of resistance might be defined. This would allow therapy to be adjusted in more rapid fashion or for consideration of cycling strategies of different targeted treatments.

Lastly, some hope that cfDNA may play a role as a screening assay in patients without evidence of cancer. While this is an exciting possibility, expectations should be tempered given the large number of blood biomarkers that have failed to succeed as routine screening tests. Indeed, even the most widely adopted screening blood test, prostate-specific antigen, is now being questioned for its clinical utility (9). A more likely use might be surveillance for recurrence in patients with a known history of a cancer, in the way that tumor markers are used in the management of ovarian cancer and germ cell tumors. It remains to be determined, however, whether low volume recurrent disease will shed sufficient cfDNA to allow for effective genotyping.

Performance Characteristics of cfDNA Genotyping

One of the major challenges with cfDNA genotyping is the demonstration of adequate accuracy for use as a clinical biomarker. This same challenge has been encountered by highly sensitive tumor genotyping assays, which can detect mutations in scant biopsy specimens but can be vulnerable to false positive results (10). This trade-off of decreased specificity with increased sensitivity is a common problem in the development of diagnostic tests. When a tumor genotype is only detectable with a highly sensitive assay, the possibility of a false positive test increases—caused either by a biologically insignificant subclone or false detection of a mutation in tumor DNA that in fact has only wild-type sequence.

The need for reliable, highly specific assays grows even more important when analyzing cfDNA. Tumor DNA constitutes only a small proportion of the circulating DNA, with the majority representing germline DNA derived from ruptured leukocytes or other benign cells. For this reason, cfDNA genotyping must have high sensitivity to detect mutant alleles that make up less than 1% of the total DNA, but high specificity to avoid false-positive results in patients with wild-type cancers. This is essential to avoid the possibility of mistaken administration of inappropriate targeted therapies. It is well recognized that cancers with wild-type EGFR, ALK, and BRAF have minimal response to targeted EGFR, ALK, and BRAF kinase inhibitors. Therefore, selection of a targeted therapy based upon a false-positive cfDNA genotyping result does the patient a disservice because he or she is unlikely to respond to the treatments and may suffer from substantial toxicities. For this reason, only assays with a negligible false-positive rate (high specificity) will have a chance of being adopted clinically, and this may require acceptance of a slightly lower sensitivity in the assay.

Comparison With CTCs

Genotyping of cfDNA is not the first blood-based technology that has been studied. Since the introduction of microchip technologies to screen peripheral blood for tumor cells (11), investigators have studied strategies for CTC capture and analysis for guiding the use of personalized therapies. Several important differences exist between CTC analysis and cfDNA genotyping technologies. First, cfDNA genotyping can detect DNA alterations (point mutations, insertions/deletions, amplifications, rearrangements), but will be unable to detect changes in cancer morphology or protein expression. In addition, CTC analysis has the potential to allow analysis of morphology, immuno-histochemistry and fluorescent in situ hybridization, in contrast to cfDNA genotyping. Furthermore, cfDNA genotyping is only effective on cancers with identifiable genomic alterations; wild-type cancers are undetectable with cfDNA genotyping, but may be detected with CTC capture.

However, there are important advantages of cfDNA genotyping over CTCs for specimen processing. CTCs must be studied whole, meaning they must be separated from the much more abundant hematologic cells in the blood. Additionally, the purification steps require significant laboratory infrastructure to obtain a viable population of CTCs for study (12). Tumor cells in circulation demonstrate substantial apoptosis and fragility, both of which
introduce variability between different CTC assays (13,14). In contrast, cfDNA genotyping requires a minimum of special handling. Blood can potentially be collected in conventional ethylenediaminetetraacetic acid–containing tubes and processed in a standard laboratory centrifuge. Both CTCs and cfDNA do require some amount of immediate processing, as delays can impede sensitivity secondary to the death of CTCs and/or dilution of the cfDNA in the plasma specimen from lysis of leukocytes (15). Overall, cfDNA genotyping has the potential to be more widely used than many CTC capture technologies in development for specific purposes.

The prior development of the CellSearch platform, which is now US Food and Drug Administration (FDA) approved for quantification of CTCs and prognostic applications, may be a useful guide in consideration of next steps in the clinical application of cfDNA. An important developmental hurdle in quantitating CTCs was the lack of reproducibility of CTC capture and cell counts between different laboratories (16–18). In this regard, an important contribution was the study by Allard et al. in which standardization of CTC capture across multiple laboratories was demonstrated using the CellSearch system (Veridex LLC) (13). This study established the analytical accuracy, reproducibility, and linearity of CTC capture utilizing blood spiked with varying numbers of tumor cells from cell culture. These variables were investigated by plotting the expected number of tumor cells spiked into healthy donor samples against the actual observed tumor cells. Regression analysis of observed compared with expected tumor cells demonstrated a slope of 0.85 (95% confidence interval [CI] = 0.82 to 0.87) with an intercept of 5.6 (95% CI = 1.8 to 9.5), and correlation coefficient ($R^2$) of 0.99. The samples were compared following treatment with a preservative after sampling from patients with carcinomas, normal healthy volunteers, and patients with nonmalignant diseases to establish the dynamic range of the system and the reproducibility between different laboratories. Reproducibility of CTC counts across duplicate tubes and multiple operators were shown to have correlation coefficients ($R^2$) of 0.975 and 0.994, respectively. These approaches should provide a guide to the steps needed in standardizing cfDNA genotyping processes, including assay platform, cfDNA preservation, and time for plasma isolation.

### Specific Techniques for cfDNA Genotyping

Several different types of assays have been explored for cfDNA genotyping (Table 1), including allele-specific PCR, coamplification at lower denaturation temperatures (COLD) PCR, emulsion PCR, and massively parallel sequencing (or next-generation sequencing). Direct comparisons of different assays have not yet been undertaken because the techniques are in the early stage of development. More detailed information on the results of previous studies using these techniques can be gleaned from other comprehensive reviews (19–21). However, we believe it is valuable to review the technical characteristics of these existing technologies here.

Allele-specific PCR is a variation of standard PCR that allows for the detection of prespecified mutations in DNA. Allele-specific PCR takes advantage of the transcription initiation requirement of DNA polymerase for homology between the 3′ sequence of an oligonucleotide primer and the DNA sequence to be transcribed. In situations of sequence mismatch, such as a wild-type

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**Table 1. Advantages and disadvantages of cfDNA analysis methodologies**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Allele-specific</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Sensitivity</th>
<th>cfDNA studies (tumor type and gene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele-specific PCR</td>
<td>Yes</td>
<td>Can easily be multiplexed Inexpensive Requires no special equipment</td>
<td>Mutations must be known in advance Results are, at best, semiquantitative</td>
<td>0.5%–0.1%</td>
<td>NSCLC: EGFR [29–32] Breast: PIK3CA [33] Melanoma: BRAF [34]</td>
</tr>
<tr>
<td>COLD-PCR</td>
<td>No</td>
<td>No additional reagents or instruments required Inexpensive Can enrich for known and unknown alleles irrespective of mutation type</td>
<td>Mutant allele Tc is unknown for most mutations and need to be empirically determined. Results are, at best, semiquantitative Vulnerable to polymerase induced errors</td>
<td>0.1%–0.01%</td>
<td>NSCLC: EGFR [23] Colorectal: KRAS and BRAF [35]</td>
</tr>
<tr>
<td>Emulsion PCR</td>
<td>Yes</td>
<td>Inherently quantitative Highly sensitive Consumables are inexpensive</td>
<td>Mutation must be known in advance Consistent droplet generation can be time consuming Requires specialized instrumentation</td>
<td>0.01%–0.005%</td>
<td>Colorectal: KRAS [1, 2, 36, 37] Breast: PIK3CA [4, 5] Lung: EGFR [8]</td>
</tr>
<tr>
<td>NGS</td>
<td>No</td>
<td>Identifies known and unknown alleles irrespective of mutation type Quantitative</td>
<td>Requires specialized instrumentation Requires dedicated bioinformatics Slow turnaround time Requires large amounts of sample input</td>
<td>1%</td>
<td>Breast: PIK3CA and MEDI [38], CDK6 [6] Ovarian: PIK3C and RB1 [38, 39] NSCLC: EGFR [38] Colorectal: ERBB2 [6]</td>
</tr>
</tbody>
</table>

* Cost can be reduced via a batched analysis with bar coded samples. cfDNA = cell-free deoxyribonucleic acid; COLD-PCR = coamplification at lower denaturation temperature-PCR; NGS = next-generation sequencing; NSCLC = non-small cell lung cancer; PCR = polymerase chain reaction; Tc = critical temperature.
primer and a point mutation in the DNA, DNA polymerase will not initiate base pair extension. As such, specific primers can be designed for the mutant DNA sequence, and these primers can then be fluorescently tagged and detected by a variety of methods. Performance of allele-specific PCR can be multiplexed to allow for the detection of multiple prespecified mutations at a time (22). Advantages of this approach include low background signal and high sensitivity. Disadvantages include a requirement for larger amounts of tumor DNA and prespecification of each mutation of interest.

Coamplification at lower denaturation temperatures (COLD) PCR is a handling technique that leads to enrichment and preferential expansion of mutant sequences relative to wild type. This five-step process includes DNA denaturation at high temperature, a reannealing stage that allows for cross-hybridization and development of heteroduplex DNA strands, a second selective denaturing stage at intermediate temperature that allows denaturing of any strands with DNA base mismatches, followed by a primer annealing stage and finally sequence extension (23). The Improved and Complete Enrichment (ICE)-COLD PCR technique further optimizes COLD PCR in allowing evaluation for all mutations within a reference DNA sequence while still maintaining rapid PCR cycle length (24). The key to this approach is the introduction of a reference sequence to the wild-type, antisense DNA strand. When this reference sequence is added to the PCR reaction at high concentration, homoduplexes form between the heat-stable reference strand and wild type DNA, facilitating near-complete amplification of mutant DNA only. Advantages to ICE-COLD PCR include its simplicity, reliability in detecting low-level mutations from mixed samples, and identification of any DNA mutation within the sequence of interest.

Emulsion PCR represents a family of techniques that involve dilution of DNA molecules into an aqueous/oil suspension containing the necessary reagents for PCR (25). After disturbance of the suspension to allow formation of thousands of aqueous compartments (or “droplets”) within the oil emulsion, PCR reactions are cycled. DNA variations can be detected using fluorescently tagged primers, and then quantified using flow cytometry. With one particular technique, referred to as BEAMing (beads, emulsion, amplification, and magnets), DNA variants within each aqueous compartment are attached to magnetic beads so they can subsequently be recovered and studied (25). While the quantitative nature of emulsion PCR is attractive due to the potential for a high signal-to-noise ratio, the generation of droplets can be time consuming and can slow turnaround time.

Massively parallel sequencing (MPS) is a collection of technologies that allow for the rapid amplification and sequencing of DNA with multiple simultaneous sequencing reactions. Whereas Sanger sequencing utilizes capillary-based DNA chain-termination strategies for nucleotide sequence detection (26), MPS allows for determination of DNA sequence as nucleotides are added prospectively. Techniques for MPS vary in approach but all share certain elements, including the generation of spatially separated, amplified DNA fragments as well as simultaneous DNA sequencing of these amplified products by prospective base pair addition and signal detection. The main advantage of MPS is the ability to evaluate all types of DNA alterations (point mutations, insertions/deletion, copy number alterations, and rearrangements) in a multiplexed fashion. Disadvantages to this technique include the requirement for considerable technical and analytical expertise, as well as a long experimental/result reporting time. While the low cost per base sequenced makes this an attractive technology for discovery, the cost of this technology could still be quite high when testing for a handful of actionable alterations in an individual patient’s specimen.

**Next Steps**

In a clinical setting where cfDNA genotyping is to be employed as a diagnostic test, it will be important to determine preanalytical variables and their impact on sensitivity, accuracy, and predictive value. The concept of “fit-for-purpose” clinical biomarker qualification for decision making has been extensively described in previous publications (27), and the FDA has recently released guidance for pharmaceutical and diagnostic companies (28). Aligned with these concepts and guidance documents, we envision that cfDNA assay parameters will include identification of a suitable sample preparation strategy, optimizing of assay performance, and appropriate assay validation assessment (sensitivity, specificity, linearity, and reproducibility).

Several different types of assays are currently being explored for clinical validity relative to the concordance between cfDNA measurements, the tumor genotype, and clinical outcomes (Table 1). This is similar to the state of affairs in the development of CTCs from their discovery until 2004, prior to the rigorous evaluation of the CellSearch system. It is notable that despite the efforts of many groups, the CellSearch system continues to be the only CTC technology approved by the FDA as a diagnostic test reliable enough for clinical use. This highlights the need for standardization and validation of potential cfDNA technologies. It also draws attention to the potential timeframe for clinical realization of a new biomarker assay in the clinical realm.

In this context, it will be of great importance that the day-to-day, laboratory-to-laboratory, and assay-to-assay variance introduced by sample handling and DNA isolation be evaluated and understood prior to attempting to evaluate the true clinical validity and utility of these cfDNA assays themselves. At the current time, clinical validation is primarily taking place within small groups working on individualized assays and approaches to assess associations between specific analytes, tumor genotype, and a specific clinical outcome. Once requirements for analytic validity are defined and shown to be associated with clinical outcomes, multi-institutional, adequately powered studies utilizing common methods, assay materials, and data analyses will be performed. Under this model, a blinded, systematic head-to-head comparison of well-characterized standards for distinct cfDNA genotyping platforms and processes to determine the appropriate state of the technology is highly desirable.

Furthermore, if cfDNA genotyping is to be further developed into a clinical disease monitoring biomarker; a key need will be well-annotated plasma specimens from prospective clinical trials. Tissue collection as part of prospective trials is now routine, to
allow for biomarker discovery upon trial completion. Similarly, we encourage investigators to consider routine collection of plasma specimens given the potentially powerful role of cfDNA genotyping in the near future.

There is no question that cfDNA genotyping has the potential to be a powerful and widely applied technology in this age of cancer genomics. The time is ripe to now transform cfDNA genotyping from a research tool into a clinical biomarker.

References


Notes

BEJ, DBS, DRC and JVH participated in an advisory board for Transgenomics. BEJ also reports consulting for GE Healthcare. Other authors disclose no relevant conflicts of interest.

Affiliations of authors: Dana-Farber Cancer Institute and Brigham and Women’s Hospital, Boston, MA (JUL, GRO, CPP, BEJ); Memorial Sloan-Kettering Cancer Center, New York, NY (DBS); University of Colorado Cancer Center, Denver, CO (DRC); The University of Texas MD Anderson Cancer Center, Houston, TX (JVH).