IN THE SPOTLIGHT

Tackling Formalin-Fixed, Paraffin-Embedded Tumor Tissue with Next-Generation Sequencing

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Summary: Most tumor samples available for clinical genotyping are formalin-fixed and paraffin-embedded (FFPE), but there has been relatively little published on the suitability of such samples for next-generation sequencing approaches. A new study by Wagle and colleagues shows that a combination of hybridization-capture and deep sequencing yields high-quality data from FFPE specimens. *Cancer Discovery; 2(1); 23–4. ©2012 AACR.*

Commentary on Wagle et al., p. 82 (8).

Identification of clinically actionable gene alterations in cancers is of increasing importance in the era of targeted therapeutics, and these alterations keep growing in number. However, researchers in most clinical (i.e., Clinical Laboratory Improvement Amendments–licensed) laboratories are using single-gene or single-mutation assays to screen for treatment targets. Multiplexed panels for mutation profiling that use mass spectrometry or allele-specific PCR are being offered by a few laboratories (1–4) and allow for the detection of common hotspot mutations in genes relevant to the treatment of colon cancer (*KRAS*), non–small cell lung cancer (*EGFR*), and melanoma (*BRAF*), among others. However, these panels provide little coverage for tumor suppressor genes and are not suited for the determination of gene copy number or the identification of gene fusions. For these reasons, many laboratories are turning to massively parallel sequencing to analyze clinical tumor samples, which may circumvent all of these concerns. As an example, such efforts recently uncovered a fusion gene in a case of acute leukemia, leading to an important change in patient management (5).

Tumor biopsies are rarely frozen in the fresh state for specific assays. Instead, most of the clinical samples available for molecular studies are formalin-fixed, paraffin-embedded (FFPE) specimens. Fortunately, the relatively short fragments of DNA recoverable from FFPE tissue still work with next-generation sequencing. Indeed, Schweiger and colleagues (6, 7) have shown very good correlations between matched fresh-frozen and FFPE tumor samples in both whole-genome and targeted exome sequencing. DNA derived from FFPE tissue can cause problems with single-nucleotide variant calls (false positives and negatives) and also with the interpretation of insertions and deletions at read depths below 30×, but these issues are largely negated by increasing read depths to ≥80× (7). Perhaps surprisingly, there appears to be a negligible decrease in sequence quality from FFPE samples more than a decade old.

In this issue of *Cancer Discovery*, Wagle and colleagues (8) further explore the use of next-generation sequencing in the identification of actionable mutations in samples of FFPE tumor. They describe a hybridization-capture approach based on 7,021 biotinylated RNA baits designed to pull down 433 Kbp of DNA representing 137 cancer genes, as well as 79 pharmacogenetically important polymorphisms in 34 additional genes. Captured libraries prepared from 8 tumor samples were bar-coded and sequenced together on a single lane of an Illumina HiSeq instrument. When 100-bp paired-end sequencing was used, the average target coverage ranged from 116× to 532× per sample; with the exception of one sample with lower-quality DNA, 94% of the target sequences had at least 30 reads. Thus, this multiplexed approach achieves the twin goals of maximizing sequence yield while minimizing cost.

Protocols for the analysis of clinical samples must be robust, and Wagle and colleagues (8) demonstrated the reproducibility of their hybrid-capture approach using DNA from tumor cell lines. Correlations between repeat sequencing runs performed on the same library were excellent (r = 0.96–0.97) and between separately prepared libraries from the same cell line were also very good (r = 0.86). In analyses of FFPE cancer samples, single-nucleotide variants previously identified via a mass spectroscopy-based genotyping panel were all readily apparent in the Illumina output.

Among the single-nucleotide variants identified in the FFPE samples were common mutations in *KRAS* and *PIK3CA*, which can be readily detected by current clinical assays. However, several of these mutations were observed in samples containing only 10% to 20% tumor cells, demonstrating the power of deeper read depths. Gene copy number variations also were examined. Copy number variation is routinely assessed in the whole-genome sequences of fresh-frozen tumors, but the extent to which it can be predicted from a hybridization-capture approach is less well established. On the basis of DNA from tumor cell lines, Wagle and colleagues (8) showed that the copy number variations identified by their hybrid-capture method compared very favorably with those from Affymetrix SNP 6.0 platform arrays (r = 0.89–0.98). However, the performance on FFPE tumor samples was less impressive. A colon cancer sample

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that contained 80% tumor cells showed amplification of *FGFR1* and *CCND1* (confirmed by quantitative PCR), but no copy number variations were identified in the other tumors, which ranged from 80% down to 10% tumor cell content. Although it is possible that algorithms focused on allelic ratios could increase the sensitivity for copy number variations, use of this approach on clinical samples will nevertheless require close attention to tumor cellularity. No gene fusions were detected in the study, but these were not targeted as part of the capture.

Other practical considerations in the application of next-generation sequencing to clinical tumor samples include turnaround time, the amount of input DNA required, the need for secondary verification, and cost. Wagle and colleagues (8) estimated that their approach could be performed in 2 weeks, which is within the time frame of many molecular tests currently being performed in clinical laboratories. The turnaround might be shortened by a few days, but it would be difficult to deliver results in anything less than 1 week. The hybridization capture protocol used 1 μg of tumor DNA, which is an amount that can be recovered from most, but not all, clinical samples; however, there is some evidence that it may be possible to reduce the input to 500 ng (7). Whether secondary confirmation of key results by Sanger sequencing is necessary remains an open question. It would provide an extra measure of quality control against any problems created during sample bar-coding and would also be prudent for single-nucleotide variants called at lower read depths. Copy number variations will certainly require corroborative testing until more experience has been gained with this approach. Finally, by multiplexing the sequencing on a single lane, Wagle and colleagues (8) minimized the costs of the assay.

A separate important issue is how the results of a large cancer gene panel should be reported. Wagle and colleagues (8) distinguished findings that were "actionable in principle" from those that were "variants of unclear significance." The former were further divided into those that predict response to a Food and Drug Administration–approved drug (tier 1) and those for which an experimental therapeutic might be appropriate (tier 2). Although the classification of tier 1 mutations seems straightforward, making treatment recommendations on the basis of tier 2 alterations treads into the very frontier of translational medicine. What would be the best treatment for an advanced, chemotherapy-resistant colorectal cancer that harbors both a *KRAS* and a *PIK3CA* mutation (a combination seen in approximately 5% of cases)? A mitogen-activated protein/extracellular-regulated kinase kinase inhibitor, a phosphoinositide 3-kinase inhibitor, or both? Soliciting input from a multidisciplinary team, including oncologists, pathologists, and cancer biologists, might be an appropriate approach to deciding which gene alterations should be classified as tier 2 and what treatment recommendations should be made.

Whole-genome and whole-exome sequencing of cancer specimens is becoming commonplace in research laboratories, and many are asking whether the application of either (or both) of these approaches to clinical samples might offer still more than targeted panels. In theory the answer is yes, but currently these methods would cost more and generate greater "noise" in the form of variants of unknown significance. Still, the introduction of targeted panels via the use of next-generation sequencing is a step in this direction, and the time when a "sequence everything" approach is regarded as appropriate and necessary may not be very far off.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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