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 for molecular studies are formalin-fixed, paraffin-embedded (FFPE) specimens. Fortunately, the relatively short fragments of DNA recoverable from FFPE tissue still work 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 identified by their hybrid-capture method compared very favorably with those from Affymetrix SNP 6.0 platform arrays (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) 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).
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 phosphoinositol 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.
Tackling Formalin-Fixed, Paraffin-Embedded Tumor Tissue with Next-Generation Sequencing

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