Published OnlineFirst June 3, 2019; DOI: 10.1158/1078-0432.CCR-19-1361

CCR Translations

Clinical Cancer Research

Wake Up and Smell the Fusions: Single-Modality Molecular Testing Misses Drivers

Kurtis D. Davies and Dara L. Aisner

Multitarget assays have become common in clinical molecular diagnostic laboratories. However, all assays, no matter how well designed, have inherent gaps due to technical and biological limitations. In some clinical cases, testing by multiple methodologies is needed to address these gaps and ensure the most accurate molecular diagnoses.

See related article by Benayed et al., p. 4712

In this issue of *Clinical Cancer Research*, Benayed and colleagues illustrate the growing need to consider multiple molecular testing methodologies for certain clinical specimens (1). The rapidly expanding list of actionable molecular alterations across cancer types has resulted in the wide adoption of multitarget testing approaches, particularly those based on next-generation sequencing (NGS). NGS-based assays are commonly viewed as "one-stop shops" to detect a vast array of molecular variants. However, as Benayed and colleagues discuss, even well-designed and highly vetted NGS assays have inherent gaps that, under certain circumstances, are ideally addressed by analyzing the sample using an alternative approach.

In the article, the authors examined a cohort of lung adenocarcinoma patient samples that had been deemed "driver-negative" via MSK-IMPACT, an FDA-cleared test that is widely considered by experts in the field to be one of the best examples of a DNA-based large gene panel NGS assay (2). Of 589 driver-negative cases, 254 had additional material amenable for a different approach: RNA-based NGS designed specifically for gene fusion and oncogenic gene isoform detection. After accounting for quality control failures, 232 samples were successfully sequenced, and, among these, 36 samples (representing an astonishing 15.5% of tested cases) were found to be positive for a driver gene fusion or oncogenic isoform that had not been detected by DNA-based NGS. The real-world value derived from this orthogonal testing schema was more than theoretical, with 8 of 10 (80%) patients demonstrating clinical benefit when treated according to the alteration identified via the RNA-based approach.

To detect gene rearrangements that lead to oncogenic gene fusions (and to detect mutations and insertions/deletions that lead to *MET* exon 14 skipping), MSK-IMPACT employs hybrid capture-based enrichment of selected intronic regions from genomic DNA. While this approach has proven to be successful in a variety of settings, there are associated limitations that were determined in this study to underlie the discrepancies between MSK-IMPACT and the RNA-based assay. First, some introns that are involved in clinically actionable rearrangement events are very large, thus requiring substantial sequencing capital that can represent a disproportionate fraction of the assay. Despite the ability via NGS to perform sequencing at a large scale, this sequencing capacity is still finite, and thus decisions must be made to sacrifice coverage of certain large genomic regions to ensure sufficient sequencing depth for other desired genomic targets. In the case of MSK-IMPACT (and most other DNA-based NGS assays), certain important introns in *NTRK3* and *NRG1* are not included in covered content, simply because they are too large (>90 Kb each). The second primary problem with DNA-based analysis of introns is that they often contain highly repetitive elements that are extremely difficult to assess via NGS due to their recurring presence across the genome. Attempts to sequence these regions are largely unfruitful because any sequencing data obtained cannot be specifically aligned/mapped to the desired targeted region of the genome (3). This is particularly true for intron 31 of *ROS1*, because it contains two repetitive long interspersed nuclear elements, and many DNA-based assays, including MSK-IMPACT, poorly cover this intron (4). In this study by Benayed and colleagues, the most common discrepant alteration was fusion involving *ROS1*, which accounted for 10 of 36 (28%) cases. At least six of these, those that demonstrated fusion to *ROS1* exon 32, were likely directly explained by incomplete intron 31 sequencing. RNA-based analysis is able to overcome the above described limitations owing to the simple fact that sequencing is focused on exons post-splicing and the need to sequence introns is entirely avoided (Fig. 1).

Lack of sufficient intronic coverage could not account for all of the discrepancies between DNA-based and RNA-based analysis however. Six samples in the cohort were found to be positive for *MET* exon 14 skipping based on RNA. In five of these, genomic alterations in *MET* introns 13 or 14 were observed, however they did not conform to canonical splice site alterations and thus were not initially called (although this was addressed by bioinformatics updates). In RNA-based testing, however, determination of exon skipping is simplified such that, regardless of the specific genomic alteration that interferes with splicing, absence of the exon in the transcript is directly observed (5). In another two of the discrepant cases, tumor purity was observed to be low in the sample, meaning that the expected variant allele frequency (VAF) for a genomic event would also likely be low, potentially below detectable levels. However, overexpression of the fusions at the transcript

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Clin Cancer Res 2019;25:4586–8

doi: 10.1158/1078-0432.CCR-19-1361

Published OnlineFirst June 3, 2019; DOI: 10.1158/1078-0432.CCR-19-1361

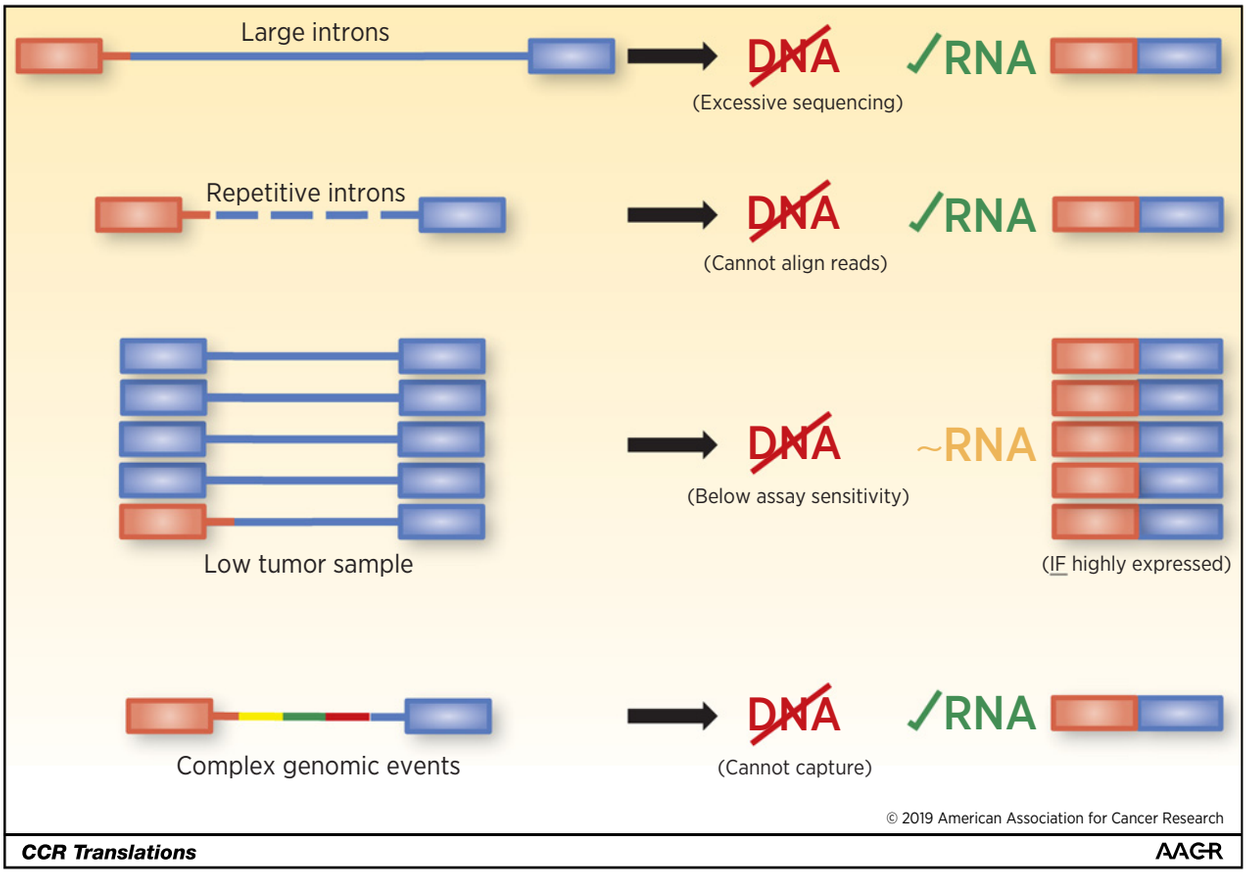

Figure 1.
Schematic representation of underlying genomic complexities that can lead to false-negative gene fusion results in DNA-based NGS analysis. In some cases, RNA-based approaches may overcome the limitations of DNA-based testing.

level was theorized to compensate for low VAF (Fig. 1). Additional explanations for discordant findings between the assays included sample-specific poor sequencing in selected introns and complex rearrangements that hindered proper capture (Fig. 1).

The take home message from Benayed and colleagues is simply this: there is no perfect assay that will detect 100% of the potential actionable alterations in patient samples. Even an extremely well designed, thoroughly vetted, and FDA-cleared assay such as MSK-IMPACT will have inherent and unavoidable "holes" due to intrinsic limitations. The solution to this dilemma, as adeptly described by Benayed and colleagues, is additional testing using a different approach. While in an ideal world every clinical tumor sample would be tested by multiple modalities to ensure the most comprehensive clinical assessment, the reality is that these samples are often scant and testing is fiscally burdensome (and often not reimbursed). Therefore, algorithms to determine which samples should be reflexed to secondary assays after testing with a primary assay are critical for maximizing benefit. In this study, the first algorithmic step was lack of an identified driver (because activated oncogenic drivers tend to exist exclusively of each other), which amounted to 23% of samples tested with the primary assay. In addition, the authors found a significantly higher rate of actionable gene fusions in samples with a low (<5 mut/Mb) tumor mutational burden, meaning that this metric, which was derived from the primary assay, could also be used to help inform decision making regarding additional testing. While this scenario is somewhat specific to lung cancer, similar approaches could be prescribed on a cancer type–specific basis.

These findings should be considered a "wake-up call" for oncologists in regard to the ordering and interpretation of molecular testing. It is clear from these and other published findings that advanced molecular analysis has limitations that require nuanced technical understanding. As this arena evolves, it is critical for oncologists (and trainees) to gain an increased comprehension of how to identify when the "gaps" in a test might be most clinically relevant. This requires a level of technical cognizance that has been previously unexpected of clinical practitioners, yet is underscored by the reality that opportunities for effective targeted therapy can and will be missed if the treating oncologist is unaware of how to best identify patients for whom additional testing is warranted. This study also highlights the mantra of "no test is perfect" regardless of prestige of the testing institution, number of past tests performed, or regulatory status. NGS, despite its benefits, does not mean all-encompassing. It is only through the adaptability of laboratories to utilize knowledge such as is provided by Benayed and colleagues that advances in laboratory medicine can be quickly deployed to maximize benefits for oncology patients.

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Disclosure of Potential Conflicts of Interest

K.D. Davies has received sponsored travel from ArcherDx. D.L. Aisner is a consultant/advisory board member for Bayer Oncology, Genentech, Loxo Oncology, AbbVie, and Bristol-Myers Squibb.

Received May 15, 2019; revised May 20, 2019; accepted May 29, 2019; published first June 3, 2019.

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