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Detection of Gene Rearrangements in Targeted Clinical Next-Generation Sequencing

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The identification of recurrent gene rearrangements in the clinical laboratory is the cornerstone for risk stratification and treatment decisions in many malignant tumors. Studies have reported that targeted next-generation sequencing assays have the potential to identify such rearrangements; however, their utility in the clinical laboratory is unknown. We examine the sensitivity and specificity of *ALK* and *KMT2A (MLL)* rearrangement detection by next-generation sequencing in the clinical laboratory. We analyzed a series of seven *ALK* rearranged cancers, six *KMT2A* rearranged leukemias, and 77 *ALK/KMT2A* rearrangement–negative cancers, previously tested by fluorescence in situ hybridization (FISH). Rearrangement detection was tested using publicly available software tools, including Breakdancer, ClusterFAST, CREST, and Hydra. Using Breakdancer and ClusterFAST, we detected *ALK* rearrangements in seven of seven FISH-positive cases and *KMT2A* rearrangements in six of six FISH-positive cases. Among the 77 *ALK/KMT2A* FISH-negative cases, no false-positive identifications were made by Breakdancer or ClusterFAST. Further, we identified one *ALK* rearranged case with a noncanonical intron 16 breakpoint, which is likely to affect its response to targeted inhibitors. We report that clinically relevant chromosomal rearrangements can be detected from targeted gene panel–based next-generation sequencing with sensitivity and specificity equivalent to that of FISH while providing finer-scale information and increased efficiency for molecular oncology testing. *(J Mol Diagn 2014, 16: 405–417; http://dx.doi.org/10.1016/j.jmoldx.2014.03.006)*

The detection of recurrent chromosomal rearrangements by cytogenetics was one of the earliest clinical molecular oncology assays and continues to play a major role in cancer diagnosis and prognosis.[1,2] Although translocations in the clinical laboratory are generally detected by cytogenetics, fluorescence in situ hybridization (FISH), or RT-PCR, studies have demonstrated that they may also be detected by next-generation sequencing (NGS) of DNA or RNA.[3–5] DNA-level translocations can be detected in particular areas of interest by first performing hybrid capture enrichment to target one or both partner genes in a translocation, followed by NGS.[4,6] NGS-based translocation detection has several advantages over conventional clinical laboratory methods, such as the ability to precisely define the breakpoint region, detect cryptic rearrangements and unknown partner genes, and run in parallel with gene mutation detection.

Chromosomal rearrangements are detected in the clinical laboratory by routine cytogenetics, FISH, or RT-PCR; however, these methods have limitations. Cytogenetic studies, including chromosome analysis and metaphase FISH, require actively dividing cells, which can be especially difficult to obtain from solid tumors. In addition, chromosome analysis is of limited resolution, particularly in oncology specimens, and is therefore insensitive to cryptic and complex rearrangements.[5,7,8] Some rearrangements can be assayed via RNA-based RT-PCR methods, but this approach is less useful for translocations with a large number of partner genes or those with potentially diverse breakpoints.[9,10] FISH is

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among the most commonly used laboratory methods for the detection of chromosomal rearrangements and offers high sensitivity and the ability to test routine interphase, formalin-fixed, paraffin-embedded (FFPE) tissue sections. However, FISH relies on highly trained individuals to score rearrangements by fluorescent microcopy and is an inherently low-resolution method that may be confounded by complex, multiway rearrangements and may require numerous probes to fully elucidate translocation partners for promiscuous genes, such as *KMT2A*.[5,10] Finally, FISH results are generally difficult to validate by orthogonal methods, outside less sensitive cytogenetic assays.

Two of the most commonly tested translocations in the clinical laboratory are for rearrangements of the anaplastic lymphoma kinase gene, *ALK*, in non−small cell lung cancer and of the mixed-lineage leukemia gene, *KMT2A* (formerly known as *MLL*), in acute leukemia. The *EML4-ALK* fusion results from an inversion event on chromosome 2p that generally causes an in-frame fusion of *EML4* exons 1 to 13 to *ALK* exons 20 to 29, producing an aberrant fusion gene with constitutive kinase activity, sensitive to crizotinib.[11−14] The occurrence of *ALK* fusions and other common lung cancer gene mutations in *KRAS* and *EGFR* are generally considered to be mutually exclusive, arguing that these tumors represent a distinct subset of lung cancers.[15] Although not pharmacologically targetable, *KMT2A* rearrangements are of diagnostic and prognostic significance in acute leukemias, including both acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL).[16,17] *KMT2A* rearrangements can be readily detected by FISH using break-apart probes; however, elucidation of the translocation partner gene may be difficult because >100 have been identified.[10,18]

NGS has had a tremendous effect on cancer discovery and is now becoming routine in the clinical molecular oncology laboratory.[3,19−21] NGS allows for the cost-effective, simultaneous evaluation of numerous sequence variants as part of focused clinical oncology panels or whole exomes. We and other groups have previously found that a range of DNA variants, including translocations, insertions or deletions, and copy number variants, can be detected from targeted NGS data and that it is possible to identify DNA-level breakpoints with single-nucleotide precision.[4,22,23] However, to be useful in the clinical setting, a thorough evaluation of the sensitivity and specificity of structural variation (SV) detection by NGS compared with standard methods is required. Given that numerous potential translocations can be evaluated by NGS simultaneously as part of a larger NGS cancer panel, for little to no additional cost, such methods could provide a significant savings for laboratories that perform multiple single-gene tests and multiple FISH assays on oncology specimens.

We present a comprehensive evaluation of targeted translocation detection by NGS in the clinical laboratory by comparing four publicly available translocation detection tools (including the laboratory derived ClusterFAST) on targeted NGS data from 13 cases with *ALK* or *KMT2A* rearrangements (six lung carcinomas and one anaplastic large cell carcinoma with *ALK* rearrangements; six leukemias with *KMT2A* rearrangements) and 77 cancers negative for *ALK* and *KMT2A* rearrangements by FISH. We found that translocations can be reliably detected at the DNA level by targeted NGS panels and that such methods offer sensitivity and specificity similar to that of routine FISH with the advantage of single-nucleotide breakpoint resolution. Further, we examine approaches to designing capture probes for targeted NGS evaluation, evaluate the minimal coverage levels necessary to detect translocations, and explore methods to reduce false-positive translocation reports.

Materials and Methods

Sample Selection

A total of six FFPE lung adenocarcinomas and one anaplastic large cell lymphoma that had previously tested positive for *ALK* rearrangements by FISH and six *KMT2A* rearranged acute leukemias were used as positive controls. The *ALK* rearranged cases were selected from the Washington University Cytogenomics and Molecular Pathology Laboratory (two cases), ARUP References Laboratories (three cases), and the University of Washington (two cases). *ALK* rearranged positive controls were selected on the basis of remaining tissue available for sequence analysis; cases were not excluded based on FISH results (ie, the percentage of positive nuclei or the presence of complex rearrangements). The mean tumor cellularity of *ALK* rearranged cases was 40% (range, 30% to 50%) by morphologic estimate (slides for five of seven cases were available for morphologic review). The *KMT2A* rearranged acute leukemias were selected from the Washington University Cytogenomics and Molecular Pathology Laboratory based on availability of the remaining specimen in the form of a fixed cell pellet derived from a bone marrow aspirate from which DNA was obtained. The mean tumor cellularity of KMT2A rearranged cases was 90% (range, 75% to 100%) based on FISH or cytogenetics. A total of 77 consecutive control samples negative by FISH for *ALK* and *KMT2A* rearrangements were identified from the Washington University Genomics and Pathology Services (GPS) Laboratory. All negative control cases were evaluated by *KMT2A* and *ALK* break-apart FISH probes and by the same panel-based NGS as FISH-positive cases in a College of American Pathologists and Clinical Laboratory Improvement Amendments accredited laboratory (GPS). This study was approved by the Human Studies Committee of Washington University School of Medicine (institutional review board approval 201101733).

Evaluation by FISH

Locus-specific FISH for *ALK* (2p23) and *KMT2A* (11q23) was performed on FFPE solid tumors or fixed cell pellets derived from hematologic specimens. The *ALK* and *KMT2A*

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