RNA Analysis as a Tool to Determine Clinically Relevant Gene Fusions and Splice Variants

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- **Context.**—Technologic advances have contributed to the increasing relevance of RNA analysis in clinical oncology practice. The different genetic aberrations that can be screened with RNA include gene fusions and splice variants. Validated methods of identifying these alterations include fluorescence in situ hybridization, immunohistochemistry, reverse transcription–polymerase chain reaction, and next-generation sequencing, which can provide physicians valuable information on disease and treatment of cancer patients.

- **Objective.**—To discuss the standard techniques available and new approaches for the identification of gene fusions and splice variants in cancer, focusing on RNA analysis and how analytic methods have evolved in both tissue and liquid biopsies.

- **Data Sources.**—This is a narrative review based on PubMed searches and the authors’ own experiences.

- **Conclusions.**—Reliable RNA-based testing in tissue and liquid biopsies can inform the diagnostic process and guide physicians toward the best treatment options. Next-generation sequencing methodologies permit simultaneous assessment of molecular alterations and increase the number of treatment options available for cancer patients.

**Background.**

Since the days of James Watson, Francis Crick, Maurice Wilkins, and Rosalind Franklin, DNA analysis has opened up a new world in molecular biology and has outshadowed RNA to form the basis of modern biotechnology. DNA sequencing plays a pivotal role in assessing genetic susceptibility to specific diseases and identifying therapeutically actionable genomic aberrations.1 Recently, however, RNA-based investigation has also advanced, largely because of the new available methodologies that allow RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue. These new methods have increased both the quality and integrity of RNA obtained during its isolation and have made RNA analysis from FFPE material more reliable.2–4

Any molecular analysis with DNA or RNA for clinical utility has to be compatible with the routine processing and workflow of clinical samples. Cytologic samples, obtained through minimally invasive methods, often represent the only means to obtain tumor cells for diagnosis and molecular profiling. Although prone to poor cellularity, cytology samples are an attractive option to collect tumor material for serial biomarker analysis on recurrent or metastatic disease and are gaining importance in the clinic. Furthermore, the alcohol-based fixatives used in cytology allow the preservation of RNA integrity and, therefore, cytology specimens are potentially suitable for RNA-based molecular assays such as detection of gene fusions and splicing variant analysis.5,6

Thus, the ability to perform RNA analysis in both FFPE and cytologic specimens has opened the doors for the identification of therapeutically relevant cancer gene aberrations and, consequently, influence the decision on the possible therapeutic option. Herein we discuss the importance of RNA analysis to identify gene fusions and splice variants at the time of cancer diagnosis.

**GENE FUSIONS**

Gene fusions are chimeric genes resulting from joining parts of 2 previously independent different genes. They can...
occur within the same chromosome or between different chromosomes and result from different genetic alterations, including inversions, deletions, translocations, and duplications. At present, almost 10,000 individual gene fusions involving 8607 genes have been identified among different cancer types.

However, most of these fusion genes are likely passenger aberrations with little or no effect on tumorigenesis. On the other hand, there exist gene fusions associated with oncogenic properties that act as drivers in tumor onset or therapy resistance. The introduction of next-generation sequencing (NGS) has led to the identification of more than 90% of the gene fusions known to date.

The most relevant gene fusions detected in cancer are listed in Supplemental Table 1 (see supplemental digital content, containing 4 tables, at www.archivesofpathology.org in the April 2018 table of contents).

Detection of Gene Fusions in Cancer

The identification of gene fusions, by DNA-, RNA-, and protein-based methods, represents an important step to guide therapeutic decisions and determine prognosis in a subset of patients eligible for targeted therapies. Conventional methods of identifying rearrangements are fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and reverse transcription–polymerase chain reaction (RT-PCR).

Applications of the FISH Technique for Detection of Gene Alterations

FISH technique is a cytogenetic method developed in the 1980s that uses fluorescent probes to detect and localize specific DNA or RNA targets in chromosomes, circulating tumor cells, cytoplasm, organelles, or nuclei of tissue samples and other preparations.

The technique allows the simultaneous visualization of different sequences of interest and is used to diagnose hematologic malignancies and solid tumors, in preimplantation genetic screening, cytogenetics, karyotyping, as well as a research tool. Multicolor detection has improved the identification of unknown variants of already known gene fusions, as well as characterization of structural chromosomal aberrations (intrachromosomal and interchromosomal rearrangements) and the definition of spatiotemporal gene expression patterns.

Although the first specific translocation described in cancer, t(9;22)(q34;q11), was detected by Giemsa banding analyses in chronic myeloid leukemia and led to the identification of a short chromosome 22, the Philadelphia chromosome, it was not until years later that RNA analysis identified the chimeric protein encoded by the fusion of the BCR and ABL1 genes associated with this disease.

Currently, the detection of this and other fusions is performed by using commercially available FISH kits and is recommended for the diagnosis of certain types of malignancies. FISH can be performed on an FFPE tissue or cytologic specimen. Its testing requires a minimum of 50 to 100 tumor cells and, thus, can be mostly implemented in both material types.

On cytology samples (cytology smears, cytospins, and liquid-based cytology), nuclei are not truncated, allowing the detection of the true number of FISH signals in the nucleus.

Detection of fusion genes by FISH can help in determining the best choice of targeted treatment, such as the US Food and Drug Administration (FDA; Silver Spring, Maryland)–approved drugs crizotinib, ceritinib, and alectinib for ALK fusion gene and crizotinib for patients with ROS1 rearrangement–positive non–small cell lung cancer (NSCLC). ALK and ROS1 testing is usually performed on an FFPE or a cytology sample (cytology smear, cytospin, or liquid-based cytology) taken from a patient who has advanced disease (only ALK FISH has been FDA approved in FFPE tissue but not in conventional cytology). Nonetheless, in-house validation on non-FFPE tissue (smears, cytospins, and liquid-based cytology) has been performed in several laboratories and it is being used for clinical testing.

The algorithms for ALK testing in NSCLC depend on the ALK antibody clone used by IHC. Confirmatory test by FISH is only performed when an equivocal (2+) IHC result with ALK 5A4 clone is observed. ALK FISH testing is a reliable method for diagnosis of gene fusions in interphase cells but may produce equivocal or even erroneous results because the most common fusion partner is EML4, a gene located on the same chromosome and very proximal to ALK that fuses through intrachromosomal inversions. ROS1 FISH testing is performed in most laboratories as a confirmatory test when IHC (initial screening test) is positive at any intensity. Other drugs, targeting for example NTRK and others, are currently in clinical investigation with promising preliminary data in NSCLC, mammary analogue secretory carcinoma, and colorectal cancer.

Immunohistochemistry Technique

An alternative (and indirect) method for detection of gene rearrangements is IHC. Immunohistochemistry is a microscopy-based technique for routine diagnosis, prognosis, and research, and helps to stratify patients who can benefit from specific cancer therapies. The IHC assay uses antibodies against specific epitopes to evaluate the expression of the rearranged proteins as well as their localization in tissues or cells.

Immunohistochemistry was developed in 1941 by Coons and colleagues and 76 years of incremental technologic advances have made it the more affordable and faster immuno-based assay that can be performed by automatic staining systems. Immunohistochemistry can be performed on FFPE tissues as well as on cytology specimens, and several studies have been reported within the literature with both types of samples.

Immunohistochemistry can be used for detection of some oncogenic fusion genes owing to overexpression and hyperactivation of the kinase domain of one of the genes involved, which under normal conditions is relatively silent. For instance, this approach has been applied for the detection of the EWS-WT1, TMPRSS2-ERG, EWS-FLI1, and ALK fusion proteins, among others. The use of cytology specimens for diagnosis and/or for molecular analysis is very important in some pathologic processes. One example is NSCLC, whereby most patients receive a diagnosis at an advanced inoperable stage and need predictive marker analyses on cytology samples.ALK testing is being successfully performed in different institutions on cytologic samples.

In lung cancer, new antibody clones (D5F3 and D9E4) were developed and optimized, as earlier antibodies for detection of anaplastic large cell lymphoma (ALK1 clone) demonstrate poor sensitivity. These protocols include signal amplification with linked-polymer methods. This occurs because NSCLC ALK-rearranged tumors have lower expression of protein product than lymphoma. ALK IHC assays are validated clinical tools for screening for the
presence of ALK gene fusions in NSCLC, where normal lung does not display immunoreactivity for ALK protein. Among the 2 ALK Cell Signaling Technology (Danvers, Massachusetts) clones, D5F3 has slightly stronger staining intensity than D9E4, which was chosen for subsequent development.38 Furthermore, Ventana (Tucson, Arizona) ALK (D5F3) CDx Assay has been approved by the FDA and by several other regulatory authorities worldwide as a stand-alone ALK diagnostic test. This assay is more widely used clinically and its algorithm for patient selection is based on a definitive IHC result (positive or negative) where patients with positive results are eligible for treatment with an ALK inhibitor without need of verification by FISH. One caveat of IHC technique, however, is the intrinsic lack of antibody specificity for all proteins including detection of gene fusions events, as for example RET rearrangements.30

Reverse Transcription–Polymerase Chain Reaction

RT-PCR analysis is an RNA-based technique and is commonly used to detect fusion genes in the clinical practice. Fusion transcript detection by RT-PCR involves a specific pair of primers at opposite sides of the fusion breakpoint regions of the 2 independent genes. Therefore, the PCR product includes the juxtaposed transcript sequences. Although very sensitive, this technique requires prior knowledge of both fusion genes partners, so it does not detect unknown fusion partners. This approach is widely used for accurate, specific, fast, and reliable detection of tumors containing fusion genes.31 Gene fusions can occur anywhere in the genome (coding and noncoding regions), but those that we are interested in detecting are those that are expressed and potentially oncogenic. DNA PCR is not performed in this scenario because in most known gene fusions the breakpoint fusion regions are spread over 10 kilobases or more because there are intronic regions, distances which are difficult to cover with this DNA-based method. On the other hand, RNA is the ideal analyte to search for rearrangements because it is the intermediate product of gene expression.

Different laboratory groups have reported discordant results when using FISH versus RT-PCR, and/or IHC for detection of fusion genes. RT-PCR is a suitable method for detecting rearrangements on cytologic samples, such as ALK fusions, but is not recommended as an alternative to FISH or IHC either in FFPE or cytologic samples for selecting patients for ALK tyrosine-kinase inhibitor therapy.32,33 This occurs because of risk of false negatives, owing to variability in the EML4-ALK fusion structure and the existence of multiple ALK variants. In our experience, RT-PCR–based detection of variants 1, 2, and 3 of the EML4-ALK fusion gene in patients with lung cancer is a very sensitive and reliable approach. We have successfully identified patients with the 3 EML4-ALK fusion gene variants, where the most detected was variant 1 followed by variants 3 and 2.34 The inconvenience of this technique is the material need for high-quality RNA.

To solve this problem, alternative methods can be used, such as multiplex NGS assays, which can detect all fusion genes without prior knowledge of their breakpoint regions. This is detailed further below in the section on "Next-Generation Sequencing Approaches in Cancer.”

**SPLICE VARIANTS**

Splice variants represent another class of potential diagnostic biomarkers. Alternative splicing occurs in up to 90% (16 810 of 17 866) of human genes and provides for extensive protein diversity without increasing genome size.35 Although the specific functions of most isoforms generated by alternative splicing are unknown, it is accepted that different isoforms of a gene can lead to different biological properties and functions. Thus, alterations of the splicing process are known to cause human diseases and may play an important role in carcinogenesis.

Cancer-specific splice variants have been reported in different tumors including breast, colorectal, and prostate tumors. Supplemental Table 2 summarizes alternative splice variants associated with cancer. Specific splice variants could be considered as drivers, as they provide tumors with a selective advantage and could be potential targets for therapy. As a consequence, their detection may have diagnostic and therapeutic implications.36 Splice variants can be detected by IHC, RT-PCR, and microarrays, but only RT-PCR technique has been used in cytologic samples (cytology smear, cytospin, or liquid-based cytology).37–39

At the mRNA level, single variants can be detected by classical RT-PCR by using primers that specifically amplify the splicing regions, and their relative abundance can be determined by RT quantitative PCR (RT-qPCR). RT-qPCR was introduced by Vandenbroucke and collaborators40 using a boundary-spanning primer and producing false-positive results due to similarities between exon-exon junction sequences. Improvements in the method used to identify both wild-type and variant isoforms have been made since then, avoiding the need for an external reference gene or standard curves. Thanks to these improvements, RT-qPCR is now the gold standard technique for detection of splice variants.39 Siddiqui et al41 collected and analyzed cytologic samples (fine-needle aspirates [FNAs]) for the expression of osteopontin (OPN) isoforms by RT-PCR from patients with pancreatic ductal adenocarcinoma and found a strong association between OPN variant c and presence of metastasis in pancreatic ductal adenocarcinoma, and OPN variant c and poor survival. Recently, Ma and colleagues42 discovered MET exon 14 splice variants in lung cancer. These MET exon 14 skipping alterations result in the deletion of juxtamembrane domain of MET, which activates the MET signaling pathway43 and primes for response to MET inhibitors such as capmatinib, cabozantinib, or crizotinib.44,45 More than 100 mutations have been identified, resulting in splice variants (MET exon 14 skipping) and, collectively, are present with a frequency of 3% to 4%.46

The detection of this splice variant by RT-PCR in RNA isolated from FFPE tissue from patients with lung cancer is now standard in our institution and we are now setting up this technique in cytology smears.46 Nevertheless, RT-PCR does have limitations in terms of quantification of individual splicing changes, does not allow simultaneous quantification of multiple splicing isoforms within 1 primary transcript, and requires stably expressed reference genes. These RT-PCR analyses have clinical significance but their quantification is laborious and error-prone. Therefore, this has favored the development of microarray analysis for detection of splice variants. Despite the fact that yield of RNA is an important consideration for microarray technology (requires large amounts) and RNA recovery is low in cytologic samples, it has been successfully used for reliable distinction between benign and malignant lesions of thyroid nodule FNA but not for detection of alternative splicing.47

Since NGS appeared on the scene, some studies have suggested these techniques have had their day, as NGS
NEXT-GENERATION SEQUENCING APPROACHES IN CANCER

During recent years, NGS, also known as “massively parallel” or “deep” sequencing technologies, has played an essential role in elucidating the genetic and epigenetic aberrations involved in human cancer. NGS-based assays use DNA or RNA (from both tumor and normal tissue) and include DNA-sequencing, RNA-sequencing, chromatin immunoprecipitation sequencing, DNA immunoprecipitation-sequencing, and methyl-sequencing analysis, among others.

NGS is a high-throughput method that allows massively parallel sequencing of DNA or RNA fragments and produces large amounts of data that normally require interpretation by a trained bioinformatician. This bioinformatician is no longer needed with the introduction of smaller-scale low-cost platforms such as Ion Torrent (Thermo Fisher Scientific, Waltham, Massachusetts) and Misex (Illumina, San Diego, California).

NGS has numerous advantages over traditional sequencing technology (Sanger sequencing, developed by Frederick Sanger49 in 1977). One of the most important is the increased sensitivity and determination of the limit of detection, the lowest concentration at which 95% of positive samples are detected. Thus, diagnostic assays require a careful validation and statistically reproducible limit of detection for clinical applicability. NGS is able to detect 2% to 10% allele frequency compared to 15% to 25% by Sanger sequencing.50 NGS is able to accurately and simultaneously detect a variety of genomic aberrations and concomitant mutations, including splice variants and fusion transcripts. Moreover, NGS reduces turnaround time, costs, and amount of input DNA/RNA required. However, NGS does not have the same limitations in terms of the size and tools for the infrastructure required.

There are currently a number of different platforms available for analysis of tumor and liquid biopsies, and more are being developed.51 Supplemental Table 3 summarizes the different platforms available on the market. The main NGS technologies are from Illumina, Thermo Fisher Scientific (Ion Torrent), and NanoString Technologies (Seattle, Washington). Illumina and Ion Torrent sequencing platforms for RNA-based assays need construction of cDNA (complementary DNA) libraries, the most difficult part of the NGS workflow, whereas NanoString Technologies’ platform does not. NanoString nCounter gene expression system is a direct digital counting method whereby barcode-labeled probes hybridize directly to the target molecule in solution. It is important to emphasize that each platform has a different strategy for gene target selection. Although Illumina can use a hybridization capture–based (SureSelect [Agilent, Santa Clara, California]) and SeqCap EZ (Roche NimbleGen, Madison, Wisconsin) method or the amplicon-based (HaloPlex [Agilent]) approach, usually the hybridization capture system is preferred, which has better uniformity. On the other hand, Ion Torrent only uses the amplicon-based method and, compared to the other approach, has higher on-target rates.52 One limitation for the Ion Torrent platform is the incidence of errors in sequencing homopolymer regions although it can generate libraries from low RNA quality and input (5 ng from FFPE samples), whereas Illumina requires higher RNA amounts (20 ng from FFPE samples).

NGS can be performed in cytology smears, and better results have been obtained with Papanicolaou smears than Diff-Quik smears in lung cancer on the Ion Torrent platform.53 Furthermore, NGS has led not only to better understanding of cancer, but also to important discoveries for cancer diagnosis through disease-targeted tests.50 It is being incorporated into routine clinical practice and NGS tumor panels have been developed, such as Archer FusionPlex kits (Boulder, Colorado) for gene fusion detection using NGS on Illumina and Ion Torrent platforms.

nCounter Technology

The nCounter platform (NanoString Technologies) allows multiplexed simultaneous identification of aberrant transcripts. The nCounter Elements (Nanostring Technologies) system uses a nonenzymatic protocol based on digital color-coded barcode probes that enables simultaneous counting for multiple genetic drivers in a single tube, without any enzymatic reaction by direct, digital transcript profiling using RNA purified from tumor materials; results can be available in 3 days. Despite its potential, the performance of the nCounter technique has mostly been tested in tissue samples, most of them fresh-frozen but it has also been validated in FFPE and cytologic smears.54,55

For gene fusion detection the technology uses a dual approach: the fusion target-specific assay that detects specific gene fusion partners and the 5’-3’ imbalance assay that can also identify novel aberrant transcripts and those not covered by the specific set of probes. From our experience, we have validated an ALK/ROS1/RET nCounter multiplexed assay for screening of FFPE samples in the clinical setting that compares favorably with FISH and IHC.56

Currently, we are testing 2 new panels: an in-house custom set with nCounter Elements chemistry consisting of 5’ and 3’ probes and/or fusion-specific probes to detect 4 gene-fusion drivers (ALK, ROS1, RET, NTRK1) and MET exon 14 skipping mutation, and the nCounter Vantage 3D single nucleotide variant (SNV):Fusions Lung Assay (Nanostring Technologies), which enables simultaneous assay of different analytes, DNA and RNA, from a single sample with 3D Biology (Nanostring Technologies) technology. Probes of SNV:Fusions Lung Assay were designed to target 25 genes for SNVs (104 different point and indel mutations) as well as 4 genes for fusion transcripts (ALK, ROS1, RET, NTRK1) including 33 specific variants.

Once these assays are validated in cytology samples, they can be used to test non-FFPE cytologic preparations such as smears.

RNA-Sequencing Analysis

RNA sequencing allows the quantification of mRNAs, and the identification and discovery of gene fusions, alternative gene-splicing, transcript modifications, and disease-associated single nucleotide polymorphisms that can occur across the transcriptome, even in noncoding RNA.56 Although the sample being sequenced is limited to the genes expressed at the time of RNA extraction, this method has several advantages over other approaches. For instance, a lower number of sequencing reads is required to cover the representation of the genome, as compared to DNA sequencing, and prior knowledge is not required. Other
advantages for incorporating RNA sequencing analysis in the clinical setting are its fast sequencing process, high throughput, and low RNA amount requirements.

Furthermore, single-cell RNA sequencing has recently emerged as a tool for understanding many diseases and complex tissues, since it enables cell-to-cell profiling and thus the study of tumor heterogeneity, clonal evolution, and drug resistance.57 Thus, robust, inexpensive, and customized RNA sequencing analysis might replace microarrays or RT-PCR techniques as a clinical test in the future.

Other gene fusion panels on the market are summarized in Supplemental Table 4.

L I C K Y D B I O P S Y–B A S E D A S S Y S

Challenges associated with obtaining serial tumor biopsy samples, the unavoidable intrinsic intratumor or intertumor heterogeneity, and the lack of sufficient material to perform sequential gene analysis have heightened the need to use liquid biopsies to perform different RNA analyses. This minimally invasive procedure can complement diagnostic tools or even serve as an alternative to tissue biopsy when tissue is unavailable. The most important advantage of liquid biopsy is the ability to take frequent, repeated samples for continuous genotyping of cancer patients, monitoring response to treatment, and/or detecting therapy resistance.58

Different biological materials such as cells, circulating tumor RNA, tumor-educated platelets, exosomes, and circulating tumor cells can be isolated from liquid biopsies to perform RNA analysis. Circulating tumor RNA is present in the plasma of cancer patients and its use in the laboratory presents difficulties owing to its fast degradation. Platelets can sequester RNA released as a product of apoptosis and necrosis from tumor cells and thus are an attractive source for noninvasive assessment of biomarkers.59 Exosomes are specialized small extracellular vesicles, more difficult to isolate than platelets, but are present in various body fluids, including plasma and saliva.60 Enumeration of circulating tumor cells in the peripheral blood of cancer patients has a prognostic value in metastatic breast, prostate, and colorectal cancer60–63 and gene fusions can be identified in all these materials, whereas certain splice variants can be detected in circulating tumor RNA, platelets, and exosomes.64,65

Several studies have demonstrated that it is possible to obtain a good RNA yield for molecular analysis from cytologic samples and these can be used in a way similar to liquid biopsies, as it is easy to follow up on patients for response to therapy and resistance monitoring through serial sampling.66

In our institution, we have been able to successfully detect relevant gene fusions and splice variants in liquid biopsies and incorporate some of these analyses into routine diagnostics. However, we are still learning how to best use these assays as we are applying the same technologies used for FFPE to liquid biopsy. Our laboratory has demonstrated that EML4-ALK rearrangements can be tested in liquid biopsies, either in FNA cytology samples, or in plasma and platelets from patients with NSCLC by RT-PCR. Moreover, we have found that ALK-positive platelet samples are correlated with clinical outcome to crizotinib treatment.58 Furthermore, we detected the aberration in platelet-derived RNA of a patient with NSCLC who harbored the exon 14 MET splice variant in tissue biopsy.46

CONCLUSIONS AND FUTURE DIRECTIONS

A new era of RNA-based testing and multiplex analysis has started in the clinical practice. RNA-based testing can now be used for conducting biomarker-driven investigations in tissue and liquid biopsies. Several clinically validated methods, including FISH, IHC, RT-PCR, and NGS, are available for the detection of fusion genes and splice variants. All these methods can provide physicians with valuable information to inform on the diagnosis and treatment of cancer patients. Furthermore, a large number of cancer-related genes that exhibit alternative splicing and fusion events have been characterized mainly through RNA sequencing analysis during the last decade. With the advent of new and powerful NGS tools that enable the detection of these alterations, this proportion is likely to increase substantially in the near future, although most will be rare and infrequent. These molecular events are bona fide therapeutic targets and can serve as prognostic biomarkers. Their identification represents an important step to individualize therapy and improve clinical management of patients. However, the scarcity of material for diagnosis or molecular classification is a common limitation, making multiplexing the best option for evaluation. We have had very positive experiences with nCounter Elements and recommend its use in clinical samples to interrogate genes of interest (fusions genes and/or splice variants) owing to the reduced cost, time to obtain results, and increased sensitivity and reproducibility. On the other hand, targeted genetic panels are currently used as diagnostic and prognostic tools in clinical practice, and more developed and wider panels, including more relevant aberrations in cancer, seem likely to be developed in the near future.

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