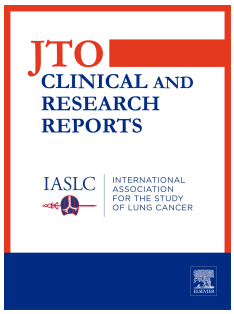

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Brief Report: Clinical Utility Validation of an Automated Ultra-Rapid Gene Fusion Assay for Non-Small Cell Lung Cancer

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Disclosures/Conflict of Interest

Mayo Clinic received sponsorship of Idylla™ GeneFusion Assay cartridges from Biocartis.

ABSTRACT

Introduction

Gene rearrangements are frequent oncological drivers in non-small cell lung cancer (NSCLC), and many are suitable for treatment with FDA-approved or experimental targeted therapies. We evaluated the accuracy, specimen acceptance profile, and limits of detection of a rapid fusion assay (Idylla™ GeneFusion Assay), a commercially available ultra-rapid molecular assay, for its clinical utility.

Methods

A collection of 97 specimens which had previously undergone NGS testing were analyzed using the rapid fusion assay. Accuracy was evaluated by sensitivity and specificity compared to the NGS results. The performance characteristics were tested by using a variety of different clinically relevant specimen types. Limits of detection were assessed by examining different input of tumor percentage and material amount.

Results

The rapid fusion assay demonstrated 100% sensitivity in detecting fusions of *ALK, ROS1, RET, NTRK1,* and *MET* exon 14 skipping; and 83% sensitivity for *NTRK2/3* fusions. There was 100% specificity in detecting fusions of *ROS1, RET, NTRK2/3,* and *MET* exon 14 skipping; and 98% specificity for *ALK.* Testing was successful with formalin-fixed paraffin-embedded biopsy and surgical tissues, cell blocks from fine needle aspiration and pleural fluid (down to 5% tumor content, 18 mm^2 tissue scraped), cytology smears (≥300 cells) and previously extracted RNA (minimal 20 ng).

Conclusions

The rapid fusion assay is quick, accurate and versatile, allowing reliable detection of *ALK*, *ROS1*, *RET* fusions, and *MET* exon 14 skipping in NSCLC, as well as *NTRK* fusions. Rapid molecular testing may expedite treatment with appropriate targeted therapies.

Introduction

Non-small cell lung cancer (NSCLC) represents the largest category of lung cancers, with adenocarcinoma being the most common[1, 2]. The discovery of oncologic drivers and development of targeted therapies have significantly improved patient outcomes [1, 3]. Several gene rearrangements and splicing alterations involving tyrosine kinase receptors are suitable for treatment with targeted therapies[4-7]. Indeed, multiple small molecules have been approved by the Food and Drug Administration targeting *ALK* (~4% of NSCLC), *ROS1* (~2%), *RET* (~2%), and *NTRK* (~0.1%) rearrangements, and *MET* exon 14 skipping (~3%)[1, 8]. Overall, these molecular alterations account for ~11% of potentially targetable NSCLC. As targeted therapies are primarily approved for the treatment of patients with metastatic disease, the rapid detection of these alterations can potentially expedite treatment decision making for patients who are often symptomatic and in need of the most effective therapy.

Accuracy and feasibility of detecting such alterations has been challenging. Routine testing by multiple stand-alone single gene assays, such as immunohistochemistry and fluorescence in situ hybridization (FISH), is not recommended due to concern for tissue exhaustion. Next-generation sequencing (NGS) panels, ideally including RNA-based component, are generally preferred since they may assess the presence of all relevant mutations and are more cost effective than multiple single gene tests [9-12]. However, the long turnaround time (2-4 weeks) dampens their clinical value in scenarios where timely decisions for patient management must be made. In these instances, a rapid and accurate assay with minimal tissue requirement is strongly desired.

Beta testing reports of an ultra-rapid gene fusion assay, using mainly prototype cartridges, showed encouraging accuracy results[13],[14];[15]. In the current study, we evaluated the clinical utility of this recently commercially available assay, using the manufactured kits. We tested its performance of accuracy, tissue acceptance profile, and limit of detection (LOD) for capturing clinically relevant gene fusions involving *ALK*, *ROS1*, *RET, NTRK1/2/3* genes and *MET* exon 14 skipping alterations.

Materials and Methods

This study was deemed exempt by the Mayo Clinic Institutional Review Board and patient's informed consent was obtained.

Assay

The Idylla™ GeneFusion Assay (Biocartis, Mechelen, Belgium) is a rapid and automated cartridge-based system optimized for formalin-fixed, paraffin embedded (FFPE) tumor tissue[13, 14]. RNA extraction, amplification, multiplex quantitative reverse transcription PCR (RT-qPCR), and data analysis are performed within a single cartridge-based workflow. It detects fusions by two methods: (1) fusion specific (FS) detection, and (2) expression imbalance (EI) analysis. FS detection includes RT-qPCR primers designed specifically for 17 *ALK*, 13 *ROS1*, and 7 *RET* rearrangements, and primers designed for the *MET* exon 13-exon 15 junction. The EI method detects fusion events by analyzing expression difference between the 3' (kinase domain) and 5' ends of the mRNA of *ALK*, *ROS1*, *RET*, and *NTRK1/2/3*.

Samples

A total of 97 tumor samples from 87 unique patients with prior clinical NGS profiling were included. Sixty-four cases were NSCLC, including 44 samples with either *ALK*, *ROS1* or *RET* fusion, or *MET* exon 14 skipping alterations. Twenty NSCLC samples were negative for gene rearrangements. An additional 33 samples had a fusion involving *NTRK1/2/3*, predominantly representing sarcomas and central nervous system tumors. Accuracy was evaluated for each individual gene tested, compared to the NGS results. The reference NGS testing included 5 NGS panels currently used at Mayo Clinic, including: Lung Panel with Tumor Rearrangement (LNGPR), MayoComplete Solid Tumor Panel (MCSTP), Sarcoma Targeted Gene Fusion Panel (SARCP), NTRK Gene Fusion Panel (NTRK), and Neuro-Oncology Expanded Panel (NONCP). Detailed information can be found in supplementary table 1.

Specimen Type Acceptance Studies

For NSCLC samples, a wide range of material types were included: 33 FFPE surgical specimens, 10 fine needle aspiration (FNA) or pleural fluid in cell blocks (also FFPE), 10 FNA smears stained with Diff Quik or Pap, and 11 cases of previously extracted RNA. Samples positive for *NTRK1/2/3* fusions were all FFPE specimens.

Limit Of Detection (LOD) Studies, Variable Parameters

To assess minimum tumor percentage input requirement, a pathologist used an H&E slide to mark areas with approximately 10% or 5% tumor cell content for 3 different samples. Unstained FFPE sections were scraped accordingly and tested.

For minimal size of tumor tissue required, 4 FFPE samples were each scraped at areas of 72 mm^2, 36 mm^2, and 18 mm^2 and tested. To evaluate the cellularity requirement for smears, 2 samples with high cellularity (>5,000 cells on a slide), 4 samples with medium cellularity (3,000-5,000 cells), and 4 samples with low cellularity (300-3,000 cells) were run, all containing at least 5% tumor cell content. To determine the lowest acceptance limit of nucleic acid, 3 different samples were each tested at RNA amounts of 20, 10 and 5 ng.

Results

Accuracy

All 12 samples (100%) with known *ALK* fusion were correctly detected by this assay, including 11 by FS detection and 1 by EI analysis (Table 1). Ten samples with *ROS1* fusion (100%) were correctly detected, all by FS method. All 12 *RET*-rearranged cases (100%) were successfully detected (11 FS, 1 EI). Ten samples with *MET* exon 14 skipping were all (100%) detected. The sensitivities of detecting *ALK*, *ROS1*, *RET* fusions and *MET* exon 14 skipping in the tested NSCLC samples are all 100%. All results were available in approximately 3 hours.

Of the 10 *NTRK1* fusion-positive samples, all (100%) were successfully detected by this assay. Among the 10 samples positive for *NTRK2* fusion, only 1 (10%) produced a *NTRK2* fusion detected result. Surprisingly, 5 samples were classified as detection of

NTRK3 fusion, while 4 samples showed no fusion detected. All 13 (100%) *NTRK3* fusion-positive samples were accurately detected.

This assay did not detect fusions in the 20 fusion-negative NSCLC samples. Since each sample run contained multiplex RT-qPCR reactions evaluating multiple gene rearrangements, a sole detection of one gene fusion implied negative results in other genes. One false positive detection of *ALK* fusion was called by EI analysis only in a *RET*-rearranged sample. For this sample, break apart *ALK* FISH showed no *ALK* gene rearrangement but 80% of nuclei had 3-5 copies of the *ALK* gene. In the tested NSCLC samples, the overall specificities for *ALK*, *ROS1*, *RET* fusions and *MET* exon 14 skipping alteration detection were 98% (51/52), 100% (54/54), 100% (52/52), and 100% (54/54), respectively.

No false positive calls of *NTRK1* or *NTRK2* fusion were noted in tested samples. The individual specificities of *NTRK1*, *NTRK2* and *NTRK3* fusion detection were 100% (87/87), 100% (36/36), and 86% (32/37), respectively. If reported in combination as *NTRK2/3* fusion, the detection specificity was 100% (26/26) with the sensitivity of 83% (19/23).

Specimen Type Acceptance

Various specimen types were validated. For the 64 NSCLC samples, the concordances were 100% across every material type examined (Table 2), except for a false positive *ALK* fusion call on a *RET*-rearranged case from an FFPE tissue block. Most samples

were less than 4 years old. Rearrangement events were concordantly detected in 2 samples older than 5 years, and in 4 samples collected between 4 and 5 years ago.

Limits Of Detection

All 3 cases produced concordant results when areas of either 10% or 5% tumor content were scraped and tested. Tumor size LOD studies revealed that all 4 samples were concordant when 72, 36, or 18 mm^2 of tissue were scraped. All cytology smears yielded concordant results, including one *ROS1*-rearranged case detected at 5% tumor content and low cellularity (300-3,000 cells). Finally, 3/3 RNA samples were concordant at 20 ng, 1/3 was concordant at 10 ng, but all 3 samples were invalid when 5 ng was used. These findings indicated that 20 ng of RNA input is required when using pre-extracted RNA (Table 3).

Discussion

In clinical settings where a timely therapeutic decision must be made, rapid molecular testing is desired. The ultra-quick turnaround time of the rapid fusion assay, yielding results in roughly 3 hours, can have impactful clinical utility. For patients with NSCLC who have rapid disease progression, quick molecular testing for gene fusions, *EGFR* mutation status and PD-L1 IHC can provide valuable information for immediate treatment options. Besides, surgical candidates considered for neoadjuvant

immunotherapy[16] would benefit from timely exclusion of oncogenic mutations and fusions.

We found that the rapid fusion assay has robust sensitivities and specificities in detecting *ALK*, *ROS1*, *RET* fusions, and *MET* exon 14 skipping in NSCLC samples. The detection of *ALK*, *ROS1*, *RET* fusions includes dual FS and EI methods in this assay. The FS detection covers common fusion partners (for example *EML4::ALK* and *CD74::ROS1*). Using FS method only, regardless of the findings by EI, 94% (32/34) positive cases were successfully detected by this method with 100% (52/52) specificity. The EI analysis provided additional detection covering fusions with uncommon partners or exon combinations. Determining fusion detection by either FS or EI, as suggested by the manufacturer, increases the sensitivity to 100% (34/34) while slightly decreasing the specificity to 98% (51/52). According to the manufacturer, the EI algorithm was designed with a higher threshold; indeed, among 32 samples with fusion detection by FS method, only 22 were called positive by the EI method. Nevertheless, using EI method to complement the FS method successfully increases the sensitivity, as it was designed to identify novel fusions. However, one false positive case was revealed in our study. Thus, in clinical practice, we recommend confirmatory testing (i.e., FISH or NGS) when fusions are detected by EI method only.

This assay detects *NTRK1/2/3* fusions by EI method only without FS method. It demonstrated an acceptable accuracy in detecting *NTRK* fusions. Using non-NSCLC samples, it showed excellent detection rate for *NTRK1* and *NTRK3* fusions but not for detection of *NTRK2* fusions. Among the 10 samples known to have *NTRK2* fusion, only

1 produced a positive result by this assay, while 5 were misidentified as *NTRK3* fusion detected. Importantly, the assay was developed with a limited number of *NTRK2* rearranged specimens[13-15]. Also, it has been recently reported that baseline tyrosin kinase gene expression differs among cancer types: (1) *NTRK3* displayed higher expression in glioblastoma and colorectal cancer compared to other kinase receptors, such as *ALK*, *ROS1*, *RET*, and *NTRK1*; and (2) *NTRK2* demonstrated strong difference in baseline expression between adenocarcinoma and squamous cell carcinoma of the lung[15]. These factors could have affected the *NTRK2* and *NTRK3* EI method calling algorithm. Since *NTRK* fusions are rare in NSCLC (~0.1-0.3%)[7] and treatments among *NTRK1/2/3* fusions are the same, we suggest reporting *NTRK1* and combined *NTRK2/3* findings, with confirmatory NGS or FISH testing.

This automated system demonstrated wide specimen acceptance profile and a simple workflow. It is common for thoracic oncology molecular testing to encounter low tumor percentage and/or limited size tissues from bronchoscopy biopsies and FNA specimens. While NGS RNA panel assay generally requires at least 20% of tumor content and 10-15 unstained slides, this assay can reliably detect gene fusions on samples with as little as 5% tumor content with only 1-3 unstained slides (Table 3). We successfully validated a wide range of tissue types, including tissue FFPE, FNA and pleural fluid cell block FFPE, cytology smears, and extracted RNA, expanding its practical clinical utility. In the current practice, a significant number of non-small cell carcinoma diagnoses are made by bronchoscopy procedures. However, cytology specimens have not been thoroughly validated in molecular tests nor in immunohistochemistry studies. Here we provided validation data to support the reliable

clinical utility of this assay to accept various cytology specimens, including FNA, smears, and fluid cell block preparation. Finally, the automated workflow of this assay requires minimal hands-on time, saving labor cost for molecular laboratories.

In conclusion, our study reveals the rapid fusion assay as a fast and reliable alternative assay for detecting targetable *ALK*, *ROS1*, *RET* fusions, and *MET* exon 14 skipping in the setting of NSCLC, and detection of the less common *NTRK1/2/3* fusions. In the clinical setting, this assay offers timely and impactful molecular information for managing patients with NSCLC with targeted therapy and selecting patients for neo-adjuvant therapy or clinical trials. Rapid molecular testing could be considered prior to larger comprehensive gene panel testing to expedite patient care.

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Tables

Table 1: Accuracy summary of fusion detection of each gene

Gene	Number of positive samples	Number of samples detected by FS method	Number of samples detected by EI only	Sensitivity	Specificity #
ALK	12	11	1	12/12 (100%)	51/52 (98%)
ROS1	10	10	0	10/10 (100%)	54/54 (100%)
RET	12	11	1	12/12 (100%)	52/52 (100%)
***MET* exon 14 skipping**	10	10	N/A	10/10 (100%)	54/54 (100%)
20 samples negative for fusion or *MET* exon 14 skipping					
NTRK1	10	NA	10	10/10 (100%)	87/87 (100%)*
NTRK2	10	NA	1	1/10 (10%)	36/36 (100%)*
NTRK3	13	NA	13	13/13 (100%)	32/37 (86%)*
NTRK2/3	23	NA	19	19/23 (83%)	26/26 (100%)*

Different NGS panels (served as gold standard) have different gene coverage.

* Including NSCLC samples.

Key

NSCLC specimens

Non-NSCLC specimens

Table 2: Summary of testing results of samples with different specimen types

Specimen type	Number of samples	Positive cases by NGS	Positive cases by Idylla	Negative cases by NGS	Negative cases by Idylla	Concordance of Idylla vs NGS
FFPE tissue block	33	23	23*	10	10	33/33 (100%)
FNA or pleural fluid in cell block (FFPE)	10	7	7	3	3	10/10 (100%)
Cytology/FNA smear	10	7	7	3	3	10/10 (100%)
Pre-extracted RNA	11	7	7	4	4	10/10 (100%)

* A *RET*-rearranged sample showed both *RET* and *ALK* fusions detected by Idylla.

Table 3: Limit of detection of Idylla GeneFusion Assay and comparison with large panel NGS assays

Characteristic	Suggested requirement for NGS*	Minimum requirement for NGS*	Suggestion requirement by manufacturer for Idylla	Minimum requirement for Idylla after verification
Tissue size (in mm^2)	72 mm^2	36 mm^2	20 mm^2	18 mm^2
Tumor %	40%	20%	10%	5%
Total cells on smear	At least 5,000 cells	3,000 cells	NA	3,000 cells, possible >300 cells
RNA, total (ng)	80 ng	80 ng	NA	20 ng

* Using clinically validated MayoComplete Solid Tumor Panel for reference.