

Performance of Oncomine Fusion Transcript kit for formalin-fixed, paraffin-embedded lung cancer specimens

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Abstract

Gene fusions play an important role in the carcinogenesis of lung adenocarcinoma. The recent association of four oncogenic driver genes, *ALK*, *ROS1*, *RET*, and *NTRK1*, as lung tumor predictive biomarkers has increased the need for precision medicine. We used formalin-fixed, paraffin-embedded tissue samples of non-small cell lung cancer from 150 *EGFR* mutation-negative cases and 10 fusion status-known cases and compared the performance of the Oncomine Dx Fusion Transcript Test (ODxFT) with FISH break-apart for the detection of *ALK*, *RET*, and *ROS1* fusion genes. RNA was extracted from the paraffin-embedded tissue samples with or without macrodissection under hematoxylin and eosin staining, and the *ALK* fusion gene was independently determined using these assays. Fusion detection analyses were successfully carried out using ODxFT in 150 cases, with only one invalid case. *ALK* fusion genes were detected at a frequency of 7.3% (11/150) in the lung cancer specimens. Concordance rate between the ODxFT and *ALK*-FISH analyses was 99.3% (148/149). Sensitivity and specificity were 91.7% and 99.3%, respectively. All the samples with a known fusion status were accurately matched between the two assays. Our results show a high concordance rate between the ODxFT and *ALK*-FISH analyses. ODxFT was thus validated as an effective method for detecting clinically significant *ALK* fusion genes in paraffin-embedded tissue samples.

KEYWORDS

ALK fusion gene, companion diagnosis, FISH, next-generation sequencer, non-small cell lung cancer

1 | INTRODUCTION

Non-small cell lung cancer (NSCLC) is characterized by gene alterations. Activation of driver oncogenes such as *EGFR*, *BRAF*, and *ERBB2*¹⁻³ increases the sensitivity of tumors to tyrosine kinase inhibitors. Gene fusions involving tyrosine kinase receptor

genes such as *ALK*,⁴ *ROS1*,⁵ *RET*,^{6,7} and *NTRK1*⁸ have also been described. These fusion events, which can involve a variety of partner genes, result in the formation of chimeric fusion kinases capable of oncogenic transformation and the induction of oncogene dependency within neoplastic cells. The individual prevalence of each of these chromosomal rearrangements ranges from

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1% to 7% in NSCLC,^{4,6,9,10} and, together, these rearrangements can be identified in approximately 5%-9% of NSCLC.^{7,11,12} Lung cancers harboring these fusion genes show oncogene addiction to the respective fusion genes and are therefore sensitive to treatment with tyrosine kinase inhibitors (TKI), such as crizotinib, alectinib (CH5424802), and ceritinib (LDK378).

Fluorescence in situ hybridization, immunohistochemistry (IHC), and real-time PCR can be used to detect fusion genes in clinical tissue samples. The FISH break-apart assay is the gold standard for companion diagnosis. Development of the above-mentioned agents^{8,9,13} has driven a rapid need for systematic and sensitive assays to detect fusion genes. The massively parallel nature of next-generation sequencing (NGS) allows rapid characterization of point mutations, small insertions, and deletions as well as the detection of chromosome rearrangements in a large set of genes through the targeted sequencing of fusion junctions. In the present study, we validated the use of a new library kit, OncoPrint Dx Fusion Transcript Test (ODxFT), for the characterization of the most frequent fusion genes in lung adenocarcinoma using NGS. This library kit is based on the high-multiplexing capabilities of PCR and focuses on the identification of 72 different fusion genes. Herein, we report the clinical performance of this assay for the detection of fusion genes implicated in the occurrence of NSCLC.

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 160 lung cancer samples were collected from Tokyo Medical University Hospital and Kindai University Hospital. All the samples were from resections that had been formalin-fixed and paraffin-embedded (FFPE) and had the wild-type *EGFR* genotype. Ten of these samples had been previously tested for *ALK*, *RET*, or *ROS1* fusion genes using an Ion AmpliSeq RNA Lung Cancer Research Fusion Panel for research purposes. All the patients enrolled in the study provided written informed consent for the use of their resected tissue. This study was approved by the ethics committee of Kindai University Faculty of Medicine (Authorization Number: 27-228) and Tokyo Medical University (Authorization Number: 3380).

2.2 | RNA extraction

RNA was extracted from each of the clinical research samples using the AllPrep DNA/RNA FFPE Kit (Qiagen, Valencia, CA, USA) based on the respective standard extraction procedures. RNA was quantified using the Nanodrop 2000 instrument (Thermo Fisher Scientific, Wilmington, DE, USA) and the RiboGreen RNA Assay Kit (Thermo Fisher Scientific).

2.3 | OncoPrint Solid Tumor Fusion Transcript kit design

Primers spanning 72 fusions (37 *ALK*, 9 *RET*, 15 *ROS1*, and 11 *NTRK1*) were designed in order to span all previously described fusions (as at time of development) of *ALK*, *ROS1*, *RET*, and *NTRK1* by a research team at Thermo Fisher Scientific. Sources used for the curation of all known fusions included the COSMIC and NCBI databases and a review of current medical literature. Targeted fusion genes and numbers of subtypes are shown in Tables 1 and 2. The multiplex primer mixes also included primers for the amplification of five housekeeping genes: *HMBS*, *ITGB7*, *LMNA*, *MYC*, and *TBP*. Additionally, primers designed to amplify the 5' and 3' regions of *ALK*, *RET*, *ROS1*, and *NTRK1* were included in the primer mix. Amplification of these regions for each gene of interest allowed for the comparison of expression levels between the 3' end of the gene, which was part of the resulting fusion, and the non-involved 5' end of the gene, but was not used for the determination of fusion gene detection.

2.4 | Detection of fusions

Total RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies Japan, Tokyo, Japan) followed by library preparation using ODxFT (Life Technologies Japan). Reverse-transcribed cDNA was amplified using the supplied reagent. The PCR product was digested and ligated with a barcode adaptor followed by purification (all from supplied reagent). The purified libraries were quantified using the Ion Library TaqMan Quantitation Kit (Life Technologies Japan), followed by a pooled and prepared sequencing template using the Ion OneTouch Dx Template

TABLE 1 Partners for *ALK*, *RET*, *ROS1*, and *NTRK1*

Target gene	<i>ALK</i>	<i>RET</i>	<i>ROS1</i>	<i>NTRK1</i>
Partner gene	<i>EML4</i>	<i>KIF5B</i>	<i>CD74</i>	<i>CEL</i>
	<i>KIF5B</i>	<i>CCDC6</i>	<i>SDC4</i>	<i>NFASC</i>
	<i>KLC1</i>	<i>CUX1</i>	<i>SLC34A2</i>	<i>IRF2BP2</i>
	<i>HIP1</i>		<i>EZR</i>	<i>TFG</i>
	<i>TPR</i>		<i>TPM3</i>	<i>SQSTM1</i>
			<i>LRIG3</i>	<i>SSBP2</i>
			<i>GOPC</i>	<i>CD74</i>
				<i>DYNC2H1</i>
				<i>MPRIIP</i>

TABLE 2 Constitution of subtypes

Fusion genes	No. of subtypes
<i>EML4-ALK</i>	18
<i>KIF5B-RET</i>	7
<i>KIF5B-ALK</i>	4
<i>SDC4-ROS1</i>	4
<i>SLC34A2-ROS1</i>	4
<i>CD74-ROS1</i>	2
<i>GOPC-ROS1</i>	2
<i>HIP1-ALK</i>	2
<i>MPRIP-NTRK1</i>	3
Other	1

Kit and an Ion OneTouch Dx Instrument (Life Technologies Japan). The sequencing template was purified using an Ion OneTouch ES Dx Instrument followed by sequencing on an Ion PGM Dx Sequencer using the Ion PGM Dx Sequencing Kit and the Ion 318 Dx Chip Kit (all from Life Technologies Japan).

Data analysis was carried out using an Ion Torrent T430 Server. This assay determined the fusion gene to be positive if the detection of the fused sequence was greater than the cutoff value.

2.5 | Fluorescence in situ hybridization

Formalin-fixed and paraffin-embedded tissues sectioned at a thickness of 4 μ m and placed on glass slides were subjected to FISH using a break-apart probe for the *ALK* gene (Vysis *ALK* break-apart FISH probe; Abbott Molecular, Des Plaines, IL, USA), *RET* gene (*RET* Split Dual Color FISH probe; GSP Research Inc.), or *ROS1* gene (*ROS1* Split Dual Color FISH probe; GSP Research, Inc., Kobe, Japan). FISH positivity was defined as the presence of >15% split signals in the tumor cells.

2.6 | Statistical analysis

The kappa statistic and associated 95% confidence intervals were used to measure agreement among the assays.

3 | RESULTS

3.1 | Method correlation agreement analysis for the detection of *ALK* fusion transcripts

Of the 150 clinical research samples tested, 149 (99.3%) passed the quality control requirement. One failure case could not be analyzed using ODxFT because of the low yield of library.

ALK fusion genes were detected in 11/149 (7.4%) and 12/150 (8.0%) samples using ODxFT and *ALK*-FISH, respectively (Table 3). The ODxFT results were 99.3% concordant (148/149 samples) with the FISH analysis. The invalid test rate for ODxFT was 0.7% (1/150). Positive predictive value and negative predictive value for ODxFT

TABLE 3 Concordance between ODxFT and FISH analysis for *ALK* fusion gene

	ALK FISH			Total
	Positive	Negative	Invalid	
ODxFT				
Positive	11	0	0	11
Negative	1	137	0	138
Invalid	0	1	0	1
Total	12	138	0	150

ODxFT, Oncomine Dx Fusion Transcript Test.

were 91.7% and 99.3% when the FISH results were assumed to be true. No statistically significant difference was observed between the correlations of ODxFT and *ALK*-FISH ($\kappa = 0.95$, 95% CI 0.79-1.11). Detailed findings for all the clinical samples are shown in Table S1. Representative FISH data and Integrative Genomics Viewer (IGV) diagram from *ALK*-positive samples are shown in Figure 1A.

3.2 | Detection of *ROS1* and *RET* fusion transcripts

Oncomine Dx Fusion Transcript Test is a hotspot panel designed to detect *ALK*, *ROS1*, *RET*, and *NTRK1* fusion transcripts. ODxFT detected five *RET* and three *ROS1* fusions. Panel results for *ROS1* and *RET* were concordant in 5/5 samples (100%) for *ROS1* and in 3/3 samples (100%) for *RET* when compared with the respective FISH findings (Figure 1B,C). No *NTRK1* fusion transcripts were detected using ODxFT.

3.3 | Detection of fusion genes in 10 fusion status-known cases

Because *ALK*, *RET*, or *ROS1* fusion genes are not common oncogenic events in patients with NSCLC, we examined an additional 10 positive samples that had been previously tested using an Ion AmpliSeq RNA Lung Cancer Research Fusion Panel for research purposes (8 *EML4-ALK*, 1 *KIF5B-RET*, and 1 *SLC34A2-ROS1* cases). All the gene fusions were detected by ODxFT and FISH. The results of ODxFT and FISH matched exactly.

4 | DISCUSSION

In the present study, we showed the feasibility of using the ODxFT assay to detect fusion genes in lung cancer tissues with a high rate of success. Discordance between *ALK* FISH and other methods has been previously reported.^{14,15} A recent study using the Ion AmpliSeq RNA Lung Cancer Research Fusion Panel for fusion detection reported 100% concordance between this method and other methodologies.¹⁶ This study and ours confirm the clinical performance of inhouse NGS tests using RNA samples obtained from small tissue samples.

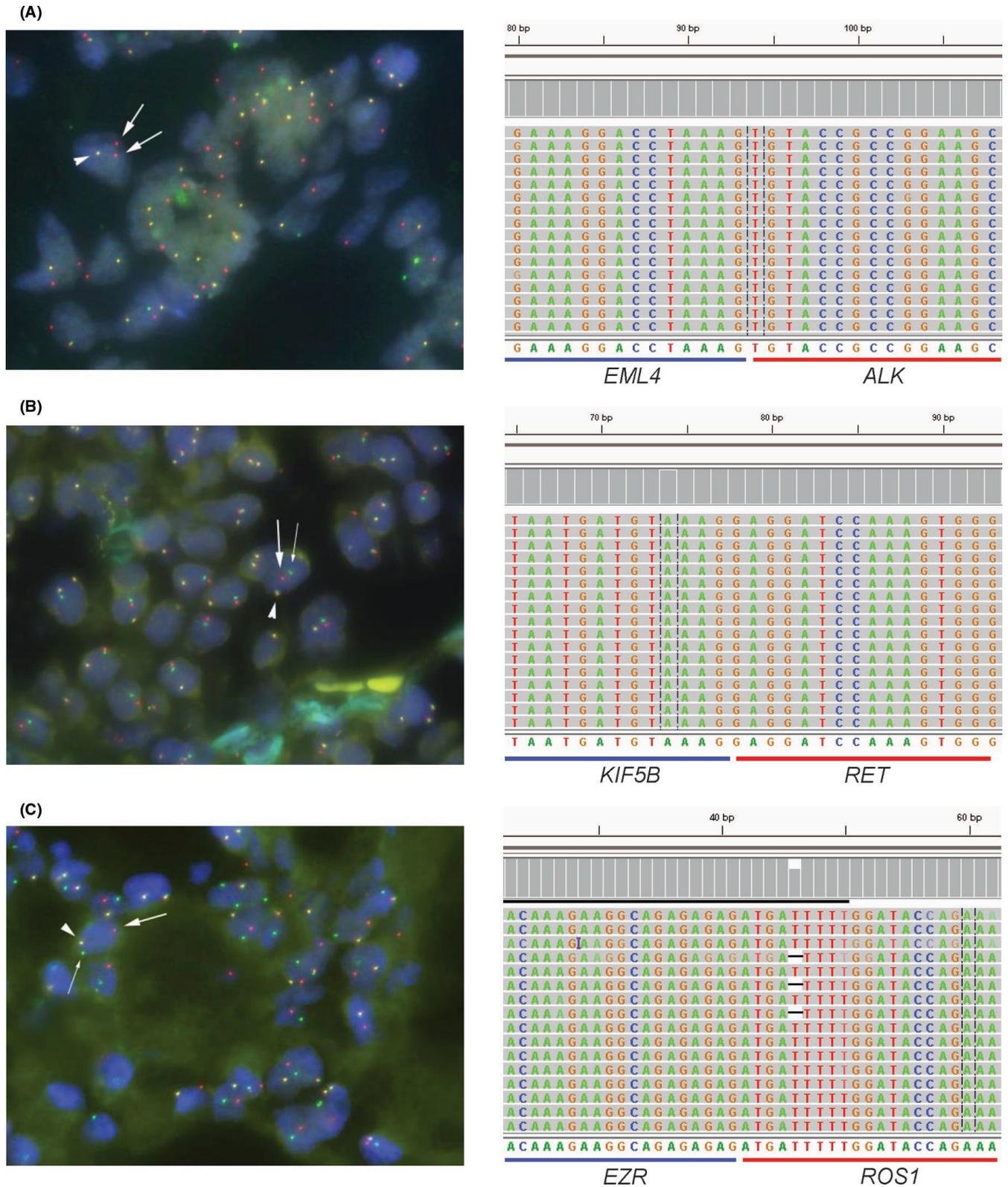


FIGURE 1 FISH analysis (left) and Integrative Genomics Viewer (IGV) diagram (right) of *ALK*, *RET*, and *ROS1* fusion cases. A-C, Representative FISH images for an *ALK* fusion-positive specimen (A), a *RET* fusion-positive specimen (B), and a *ROS1* fusion-positive specimen (C). Arrowhead, bold rightwards arrow, and the fine arrow show the pseudocolor signals of the 5'/3' probe, the 5' probe, and the 3' probe, respectively

The simultaneous detection of fusions has important clinical implications for lung cancer patients in terms of turnaround time and cost. This method requires a very small amount of input RNA

(10 ng). This advantage is particularly attractive for assays targeting lung cancers, as these samples are often obtained by biopsy and contained a limited amount of tissue. In clinical practice, lung cancer

fusions have been detected using FISH, IHC, or RT-PCR. Although FISH is considered to be the gold standard, especially for ALK testing because of the availability of an FDA-approved ALK FISH assay, FISH analysis for multiple targets per sample can be costly and may potentially extend the time needed to rule out all relevant fusion genes. IHC staining offers a cheaper alternative; however, this methodology is subjective, and accurate interpretations are sometimes difficult.¹⁷ An RT-PCR kit to detect *ROS1* fusions is available as a companion diagnosis. The main limitation of traditional RT-PCR is that this method typically focuses on only the most common fusion events and is thus limited when it comes to detecting rarer exon combinations and is unable to detect fusions involving novel 5' partners.¹⁸ In contrast to FISH or IHC, detection of *ALK*, *RET*, *ROS1*, and *NTRK1* fusions can be combined into a single assay using ODxFT. This allows the detection of fusions that might otherwise remain undetected.

In the present study, we had one case with sequencing failure (K013) and one case discordant with FISH analysis (T060). Sample K013 had a sufficient amount of RNA (10 ng RNA) for the library preparation, but the library concentration was low. Hence, we speculate that the NGS analysis of K013 failed as a result of poor-quality RNA. One discordant case was shown to be ALK fusion negative in ODxFT but was positive in FISH. There are several fusion partners besides the partners contained in the ODxFT panel such as *GCC2*, *DCTN1*, and *CLIP1*.¹⁹ As the number of sequencing reads and alignment quality were sufficiently valid, it is possible that the partner gene was not included in the ODxFT panel.

Oncomine Dx Fusion Transcript Test includes a multiplexing of primers for 72 different fusion combinations and is thus not limited to only the most common fusions. For traditional RT-PCR, one must have previous knowledge of all possible relevant fusions. The ODxFT assay addresses this problem in two ways. First, during the analysis of the sequenced reads, all reads that are initially unaligned to the reference sequence are split in half and allowed to realign. This step fosters the detection of novel fusions involving existing primers. Second, the assay includes a method for detecting fusions involving unknown partners using a 3'/5' imbalance calculation. This step analyzes the expression levels of the 3' and 5' ends of each driver gene. For genes involved in a fusion event, the 3' end of the gene is now under different regulatory control and typically shows overexpression relative to the 5' end of the gene. Another recently described methodology using NanoString technology also exploits this phenomenon of 3' overexpression.²⁰ They found that looking at the imbalance between 3' and 5' expression works relatively well for *ALK* and *RET*, which are normally not expressed in lung tissue, but this calculation was more difficult for *ROS1*, as this gene is normally expressed at high levels. Therefore, in the process of developing this panel as an IVD (in vitro diagnostics), the use of 3'/5' expression imbalance was resigned

Oncomine Dx Fusion Transcript Test is focused on fusion genes using RNA materials obtained from FFPE tissue. In the present study, only samples that did not contain an epidermal growth factor receptor (*EGFR*) gene mutation were examined. In clinical practice, for Japanese lung cancer patients, *EGFR* gene mutation

testing is the first gene screening test that is carried out because the prevalence of *EGFR* gene mutation positivity is relatively high (40%-50%). Therefore, it is reasonable to screen for fusion genes in a single assay using ODxFT after the confirmation of a wild-type *EGFR* genotype.

In conclusion, the RT-PCR NGS assay described here offers many advantages for laboratory testing in lung adenocarcinoma samples. The single-assay format potentially allows for a faster turnaround time and a lower cost than carrying out the assays separately. Furthermore, the small amount of input RNA that is required is very advantageous for this type of sample. However, ODxFT primarily targets known fusion genes. Last, efforts to periodically update the primer pool as additional partner genes for *ALK*, *ROS1*, *RET*, and *NTRK1* fusions are identified would aid in the continuing utility of this assay.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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