





ORIGINAL ARTICLE

Highly sensitive fusion detection using plasma cell-free RNA in non-small-cell lung cancers

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Abstract

ALK, *ROS1*, and *RET* kinase fusions are important predictive biomarkers of tyrosine kinase inhibitors (TKIs) in non-small-cell lung cancer (NSCLC). Analysis of cell-free DNA (cfDNA) provides a noninvasive method to identify gene changes in tumor cells. The present study sought to use cfRNA and cfDNA for identifying fusion genes. A reliable protocol was established to detect fusion genes using cfRNA and assessed the analytical validity and clinical usefulness in 30 samples from 20 cases of fusion-positive NSCLC. The results of cfRNA-based assays were compared with tissue biopsy and cfDNA-based liquid biopsy (Guardant360 plasma next-generation sequencing [NGS] assay). The overall sensitivity of the cfRNA-based assay was 26.7% (8/30) and that of cfDNA-based assay was 16.7% (3/18). When analysis was limited to the samples collected at chemo-naïve or progressive disease status and available for both assays, the sensitivity of the cfRNA-based assay was 77.8% (7/9) and that of cfDNA-based assay was 33.3% (3/9). Fusion gene identification in cfRNA was correlated with treatment response. These results suggest that the proposed cfRNA assay is a useful diagnostic test for patients with insufficient tissues to facilitate effective administration of first-line treatment and is a useful tool to monitor the progression of NSCLC for consideration of second-line treatments.

KEYWORDS

cell-free DNA, cell-free RNA, fusion gene, liquid biopsy, non-small-cell lung cancer

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1 | INTRODUCTION

The discovery of gene rearrangements involving the receptor tyrosine kinase genes *ALK*, *ROS1*, and *RET* has revolutionized management of the subsets of non-small-cell lung cancer (NSCLC) with these changes.¹⁻³ Tyrosine kinase inhibitors (TKIs) targeting these oncogenic signaling pathways have achieved dramatic and durable responses in patients.^{4,5} As TKIs are currently available as first-line treatments for fusion-positive advanced NSCLC, reliable detection of these fusions is essential in clinical practice. Fluorescence in situ hybridization (FISH), immunohistochemical (IHC) analysis, and next-generation sequencing (NGS) are often used to detect fusions as companion diagnoses with the use of TKIs.^{6,7}

Liquid biopsy involves the analysis of tumor-derived materials obtained by minimally invasive or noninvasive methods, such as sampling of blood or other body fluids.⁸⁻¹⁰ Recently, several analytes have been established for liquid biopsy such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), and circulating microRNA.¹⁰⁻¹² Liquid biopsy has several potential advantages over tissue biopsy, including better resolution of spatial and temporal intratumoral heterogeneity,¹³ detection of acquired resistance to targeted therapies, and monitoring of disease progression.¹⁴

Many studies have reported the use of liquid biopsy for analysis of cfDNA in lung cancers.¹⁵⁻¹⁷ Detection of the *EGFR* exon 19 mutations p.L858R and p.T790M with cfDNA has been applied as a companion strategy to monitor the efficacy of *EGFR*-related therapies.¹⁸⁻²¹ In contrast, although studies have suggested that plasma genotyping is sufficient for detection of *ALK* or *ROS1* fusions and kinase domain mutations with a high degree of concordance with tissue genotyping,²²⁻²⁴ the feasibility of plasma genotyping of fusion-positive NSCLC has not been fully evaluated due to the limited number of cases.

Gene fusions, which usually arise from the inter-chromosomal or intra-chromosomal ligation of distinct introns, are difficult to detect by DNA sequence capture and NGS, especially when the corresponding introns are large and/or contain repetitive sequences.^{25,26} In addition, various breakpoints and fusion partners may pose unique barriers to DNA-based genotyping. In contrast, RNA sequencing (RNA-Seq) is not influenced by the complication of large introns and is reportedly superior to DNA sequencing for the identification of *ROS1* fusion genes in tissue biopsy samples.²⁷⁻²⁹

However, the feasibility and performance of cfRNA for fusion detection remain unclear. Therefore, we developed a cfRNA-based liquid biopsy protocol that may be superior to conventional liquid biopsy with cfDNA for the evaluation of gene fusions.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

The study cohort consisted of 20 patients with NSCLC who were treated at the National Cancer Center Hospital and Juntendo

University Hospital. Tumor tissue specimens were collected by either surgery or diagnostic biopsy and histologically confirmed as NSCLC. All tumors were positive for the *ALK*, *ROS1*, or *RET* fusion, as determined by IHC analysis, FISH, reverse-transcription polymerase chain reaction (RT-PCR), or NGS. Blood samples (10-15 mL) were collected into blood collection tubes (EDTA-2K) and assayed within 3 h. The study protocol was approved by the Ethics Committees of the National Cancer Center (approval no. 2015-202) and Juntendo University Hospital (approval no. 2020017). All patients underwent tumor imaging at baseline and subsequent tumor evaluation every 3-6 mo until disease progression. Tumor response to treatment was assessed according to the *Response Evaluation Criteria in Solid Tumors* (version 1.1). Written informed consent was obtained from all patients. In addition, whole blood was collected from healthy participants. All procedures involving human participants were performed in accordance with the ethical standards of the Institutional Research Committee and the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

2.2 | cfRNA extraction, cDNA synthesis, multiplex PCR, and sequencing

Total cfRNA was extracted from 4-5 mL of plasma using the Quick-cfRNA Serum and Plasma Kit (Zymo Research) and reverse-transcribed into cDNA using the GenNext RamDA-seq Single Cell Kit according to the manufacturers' protocols. The resulting template cDNA in a final volume of 15 μ L was subjected to multiplex PCR using Go Taq G2 Green Master Mix (Promega Corporation). The fusion genes were amplified with the primers shown in Table S1. The following primer sets were used to detect TKI-resistance mutations of *ALK* and *ROS1*: *ALK* set 1: 5'-GGATTTCTCATGGAAGCCCTG-3'/5'-GTCTCTCGGAGGAAGGACTTG-3'; *ROS1* set 1: 5'-GTGTATGAAGAACAGCAGTGGAC-3'/5'-GAAGGAGGCACATCTGATGAGC-3'; and set 2: 5'-GGAATCAATCTTCTCTCTGGTCTG-3'/5'-CATCCGGCTTTACGCAAATAAG-3'. The amplified products were subjected to Sanger sequencing with the use of a 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) or NGS with a MiSeq benchtop sequencer (Illumina) using the Reagent Nano Kit V2 (300 cycles) with the 150-bp paired-end option. Sanger sequencing was performed on 5 samples (cases 4, 5, 7, 8, and 9). A NGS library was generated using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) according to the manufacturer's instructions. The library quality was assessed using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and the Agilent 2200 TapeStation System (Agilent).

2.3 | NGS analytical pipeline for fusion detection and RNA mutation call

Reference sequences were constructed from the fusion junction data of 689 previously reported fusions retrieved from the annotation database of the University of California at Santa Cruz (refGene.txt.gz)

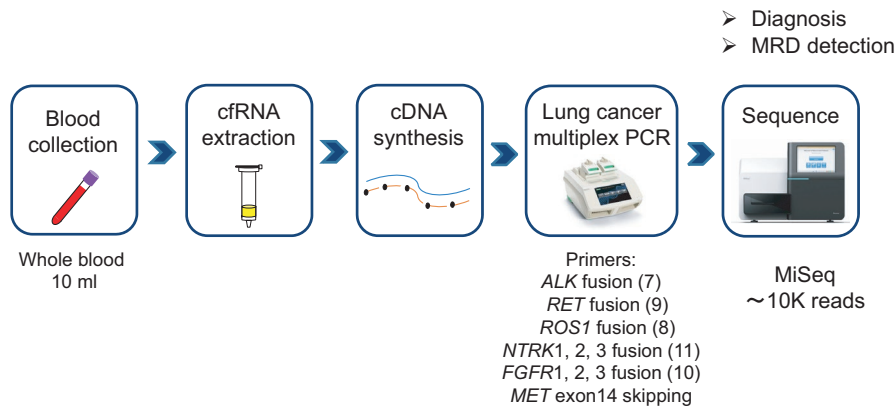


FIGURE 1 Protocol for cfRNA extraction, cDNA synthesis, multiplex PCR, and sequencing. An overview of the cfRNA-based assay in this study. Whole blood samples were obtained from NSCLC patients. cfRNA extracted from 4–5 mL of plasma was converted to cDNA by the RT-RamDA method and subsequently subjected to PCR with primers for the lung cancer-related fusion genes (7 ALK fusions, 9 RET fusions, 8 ROS1 fusions, 11 NTRK1/2/3 fusions, and 10 FGFR1/2/3 fusions) and MET exon 14 skipping. An NGS library was generated that contained c. 10 K reads on the MiSeq platform. (Image courtesy of Bio-Rad Laboratories and Illumina)

and the human reference genome (hg38). RNA-Seq reads for fusion detection were aligned to the created reference sequences using the Burrows-Wheeler Alignment Tool (BWA-MEM; <http://bio-bwa.sourceforge.net/>). The number of split reads to support the junction points spanning ≥ 60 mer was counted. RNA-Seq reads for mutation detection were mapped to hg38 using the Spliced Transcripts Alignment to a Reference (STAR) RNA-Seq read mapper (<https://github.com/alexdobin/STAR/>) and Gencode release 31 GTF (https://www.gencodegenes.org/human/release_31.html). Mutations were detected using the samtools “mpileup” function (<http://www.htslib.org/>) and discarded if (a) the read depth was < 50 or the variant allele frequency (VAF) was < 0.01 , (b) supported by only one strand of the genome, or (c) present in the 1000 Genomes Project dataset (<http://www.internationalgenome.org/>). Gene mutations were annotated with the SnpEff genomic variant annotations and functional effect prediction toolbox (<https://pcingola.github.io/SnpEff/>).

2.4 | Tissue NGS analysis

Formalin-fixed paraffin-embedded samples were subjected to the OncoPrint Comprehensive Assay v3 or OncoPrint Dx Target Test (Thermo Fisher Scientific). The OncoPrint Dx Target Test is a companion diagnostic test approved in Japan to evaluate patient tumor samples for multiple biomarkers associated with targeted therapies for NSCLC.

2.5 | RT-PCR

RT-PCR to assess the expression of *GAPDH* was performed using the Power SYBR Green Master Mix (Thermo Fisher Scientific) and a 7500HT Fast Real-Time PCR System (Thermo Fisher Scientific). Each 20- μ L reaction volume contained 5 μ L of cDNA, 0.2 μ mol/L primers, and 10 μ L of SYBR Green Master Mix. *GAPDH* was amplified with the

primers (sense) 5'-CTGCCAACGTGTCTAGTGGTG-3' and (antisense) 5'-GTCGCTGTTGAAGTCAGAGGAG-3'. Thermal cycling was conducted according to the kit protocol.

2.6 | cfDNA analysis

cfDNA samples were examined with the Guardant360 plasma NGS assay (Guardant Health) as previously described.^{30–32} In brief, cfDNA was extracted from 2 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN), and 5–30 ng of the extracted cfDNA were labeled with nonrandom oligonucleotide barcodes for preparation of a sequencing library. The target regions of 74 genes and 90 microsatellite loci were then enriched using the hybrid capture method. The libraries were sequenced by paired synthesis using the NextSeq 500 or HiSeq 2500 Sequencing System (Illumina). Sequencing reads were mapped to the hg19/GRCh37 human reference sequence, and genomic changes were identified using a proprietary analysis pipeline (Guardant Health). The data reported single nucleotide variations, insertions/deletions, and fusions with each VAF, copy number amplification, and microsatellite instability status.

3 | RESULTS

3.1 | Establishment of cfRNA-based fusion gene detection assay

The sensitivity of cfRNA-based fusion gene analysis is dependent on the yield of cfRNA and efficiency of cDNA synthesis.³³ To maximize sensitivity, the whole process from cfRNA extraction to NGS analysis was optimized. For library preparation, an amplicon-based method was used to enrich target fusion points by multiplex PCR with specific primers, which requires less input cDNA. An overview

of the proposed cfRNA-based fusion gene detection assay is presented in Figure 1.

Reverse transcription with random displacement amplification (RT-RamDA) is reported to provide global cDNA amplification directly from RNA during RT.³⁴ First, the amplification efficiency of the RT-RamDA method was verified with the use of SeraSeq Fusion RNA Mix (SeraCare Life Sciences Inc, Milford, MA, USA), a reference standard of 16 clinically relevant fusion transcripts. Serially diluted RNA samples, equivalent to 1-600 fusion copies, were prepared for cDNA synthesis and subsequently subjected to PCR using primers for the fusion genes *EML4-ALK* and *KIF5B-RET*, which were successfully detected from a sample containing 3 copies of fusion RNA (Figure 2A).

Two commercially available kits for cfRNA extraction were compared: the MagMAX Cell-Free Total Nucleic Acid Isolation Kit (referred to as Kit A) (Thermo Fisher Scientific) and the Quick-cfRNA Serum and Plasma Kit (referred to as Kit B) (Zymo Research). Kit A uses magnetic beads for the purification of nucleic acids, while Kit B uses a spin column-based method. A pilot study was conducted using 4 mL of plasma collected from a healthy volunteer. The cfRNA was extracted with each kit and converted to cDNA using the RT-RamDA method. *GAPDH* mRNA expression was quantified by RT-PCR. Total RNA extracted from human lung carcinoma H2228 cells was prepared as a control. The amount of *GAPDH* mRNA extracted with Kit B was higher compared with that with Kit A (Figure 2B).

The cfRNA extracted from 1-ml plasma with Kit B was equivalent to 10 pg of H2228 total RNA.

To optimize the protocol for cfRNA extraction and cDNA synthesis, the ability of the assay to detect spiked RNA of fusion genes in cfRNA extracted from plasma was investigated. Approximately 3-500 copies of *EML4-ALK*, *CD74-ROS1*, and *KIF5B-RET* fusion RNA were spiked into cfRNA extracted from 500 μ L of plasma from a healthy volunteer. The samples were converted to cDNA and then amplified by PCR with multiplex primers for detection of the *ALK*, *ROS1*, and *RET* fusions. The PCR products were subjected to NGS to confirm the fusion reads. The number of obtained fusion reads was well correlated with the number of spiked fusion copies (Figure S1). Even less than 10 copies of each fusion spiked as the initial input were detected by NGS analysis, suggesting high sensitivity of the assay for multiplex detection of fusion genes. Similarly, analytical validation was conducted for the *TPM3-NTRK1*, *ETV6-NTRK3*, *FGFR3-TACC3*, and *LMNA-NTRK1* fusions and *MET* exon 14 skipping (Figure S2).

3.2 | Clinical validation of the cfRNA-based assay for fusion gene detection

Samples were collected from fusion-positive NSCLC patients to validate the ability of the cfRNA-based assay to detect fusion genes in

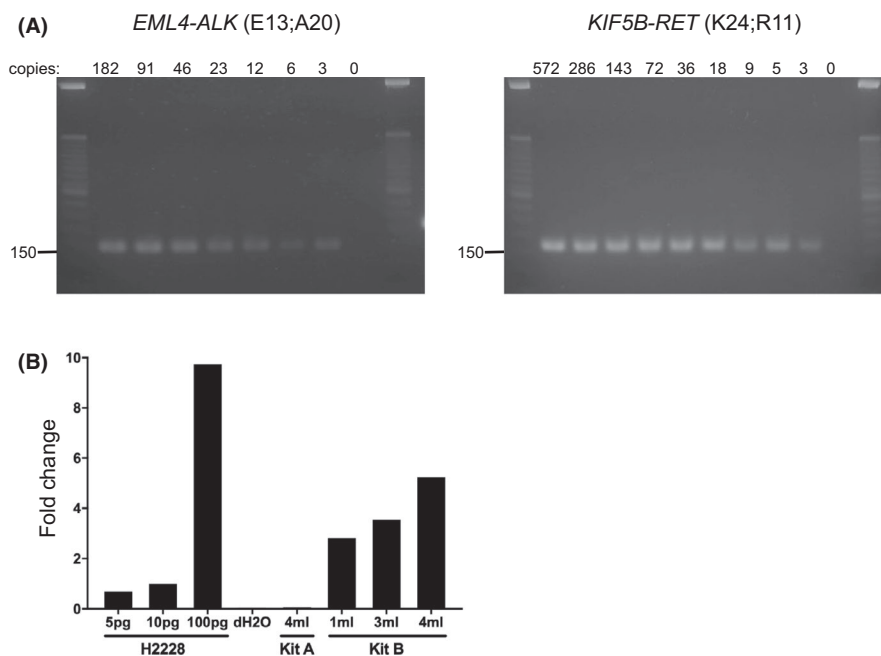


FIGURE 2 Validation of cDNA synthesis and cfRNA extraction. A, Agarose gel electrophoresis of PCR amplicons of *EML4-ALK* and *KIF5B-RET* gene-specific cDNA fragments from a standard RNA Mix. Estimated size bands were detected in all samples with indicated copy numbers of fusion genes. B, Real-time PCR of *GAPDH* mRNA expression. Total cfRNA was extracted from plasma using the MagMAX Cell-Free Total Nucleic Acid Isolation Kit (Kit A) or the Quick-cfRNA Serum and Plasma Kit (Kit B). As a control, total RNA of H2228 cells was extracted using the RNeasy Mini Kit. cfRNA and RNA of H2228 cells were converted to cDNA using the GenNext RamDA-seq Single Cell Kit. Real-time PCR was performed using the Power SYBR Green Master Mix on the 7500HT Fast Real-Time PCR System. Fold change of *GAPDH* mRNA expression was calculated by comparison with the value from 10 pg H2228 mRNA

the clinical setting. The demographic and clinicopathologic characteristics of the patients enrolled in this study are shown in Table 1. The numbers of males and females were equal and the *ALK* fusion was the most common (70%). Most patients had clinically advanced stages of disease (stage III, 20%; stage IV, 65%) and had received molecular-targeted therapies (75%) or cytotoxic chemotherapy (10%) as an initial treatment.

Plasma samples were collected from 11 patients (cases 1-11) who were chemotherapy-naïve or diagnosed with progressive disease (PD). The sensitivity of the cfRNA-based assay for detection of fusion genes in this cohort was 72.7% (8/11) (Table 2 and Figure S3). Among the 8 identified fusions, 4 were confirmed as identical to the variants (including the fusion partners) detected by tissue biopsies (cases 1, 4, 5, and 7). The fusion partner genes of the other 4 fusions were not identified by tissue biopsy analyses. The fusion partner genes of 3 patients classified as chemotherapy-naïve or diagnosed with PD, but negative for fusion genes by cfRNA-based assay (cases 2, 10, and 11), were also not identified (Table 2). Interestingly, the cfRNA-based assay failed to detect fusion genes in the samples from patients with disease status of complete response, partial response (PR), and stable disease.

In total, 18 cfDNA samples collected from 15 cases were subjected to the Guardant360 plasma NGS assay, a cfDNA-based liquid biopsy approved by the Food and Drug Administration for all advanced solid tumors. The sensitivity of the cfRNA- and cfDNA-based assays was compared with the use of 9 plasma samples collected from 9 patients who were chemotherapy-naïve or diagnosed with PD (cases 3-11) (Figure 3). The same plasma samples were used for both assays. Of the 9 plasma samples, fusions were detected in 7 (78%) with the cfRNA-based assay, but only 3 (33%) with the cfDNA-based assay. The sensitivity of *ROS1* fusion detection was 100% (2/2) and 0% (0/2) with the cfRNA- and cfDNA-based assay, respectively. All fusions identified with the cfDNA-based assay were also identified with the cfRNA-based assay.

Liquid biopsy was performed at multiple time points in 10 cases (Figures 4 and S4). In case 4, *EZR-ROS1* was detected only by the cfRNA-based assay, after the patient had advanced to PD following first-line treatment with crizotinib (Figure 4A). After 3 mo of second-line chemotherapy when the patient was diagnosed as PR, the cfRNA-based assay failed to detect the *EZR-ROS1* fusion. Similarly, in cases 5 and 6, the fusions detected before treatment initiation were negative after 3 mo of treatment when the patients achieved PR (Figure 4B, C).

3.3 | Analytical characterization of cfRNA and cfDNA

The values of RNA integrated number and DV200, cfRNA and cfDNA yield are summarized in Table S2. The relationship between the status of metastatic disease and the cfRNA yield was investigated. No significant difference in cfRNA yield was observed between samples

collected chemotherapy-naïve or PD status (9 samples) and those of CR, PR and stable disease (SD) status (14 samples, Wilcoxon rank-sum test $P = .34$; Figure S5).

The correlation between the yield of cfDNA and cfRNA was investigated in 10 cases. There was no positive correlation between the yields of cfDNA and cfRNA ($r = -0.58$; Figure S6).

3.4 | Evaluation of resistance mutations

Several studies have reported the use of cfDNA-based assays to identify TKI-resistance mutations within *ALK* or *ROS1* fusions.^{15,24} So, the ability of the cfRNA-based assay to detect such resistance mutations was evaluated. Plasma samples were collected from 3 patients (cases 3, 4, and 7) upon resistance to crizotinib or alectinib for analysis. Multiplex PCR was performed with cfRNA to account for known resistance mutations in the tyrosine kinase domains of *ALK* and *ROS1*. Subsequently, the PCR products were subjected to deep NGS. As a result, the cfRNA-based assay detected no resistance mutations (Figure S7). Although the *ALK* p.I1171T and *ROS1* p.S1986F mutations were present in cases 3 and 4 at VAFs of 0.21 and 0.14%, respectively, both were likely to be classified as sequence errors because each was also found at a VAF of 0.20% in the H2228 cells, which are known to be negative for these mutations. In contrast, the Guardant360 assay successfully detected the *ALK* p.I1171N mutation at a VAF of 0.54% in case 3 (Table 2). In addition, the results of the Guardant360 plasma NGS assay of 18 cfDNA samples from 13 cases are summarized in Table 2. Genomic changes were identified in 9 (50%) of 18 samples, including *TP53* mutations, which were detected in multiple cases (cases 3, 4, 5, 14, and 20).

4 | DISCUSSION

To the best of our knowledge, this is the first report to compare cfDNA-based and cfRNA-based assays for the detection of fusion genes in NSCLC. The key advantages of the proposed protocol are highly efficient isolation of cfRNA from plasma and subsequent cDNA conversion.

RT-RamDA with “not so random” primers (NSRs) improves sensitivity and reproducibility by eliminating the necessity of PCR amplification, which often results in amplification bias of other conventional methods.³⁴ NSRs contribute to high efficiency of capturing poly(A) and non-poly(A) RNA with multiple primers. Taking advantage of this cutting-edge technology, the proposed cfRNA-based assay is able to detect as low as a few copies of fusion transcripts in plasma.

Although cfDNA has emerged as the favored nucleic acid for analysis of liquid biopsy samples, in some situations this marker fails to provide necessary information. In such cases, cfRNA might help fill this void, which is particularly pertinent for *NTRK* fusions and translocations. DNA-based NGS fails to capture *NTRK* fusions with

TABLE 1 Demographic and clinicopathologic characteristics of the 20 patients enrolled in the study

Feature	No. of patients (N = 20)
Median age, y (range)	68 (29-86)
Sex, N (%)	
Male	10 (50)
Female	10 (50)
Smoking status, N (%)	
Ever	11 (55)
Never	9 (45)
T-stage, N (%)	
1	4 (20)
2	1 (5)
3	5 (25)
4	10 (50)
N-stage, N (%)	
0	1 (5)
1	3 (15)
2	2 (10)
3	14 (70)
M-stage, N (%)	
0	5 (25)
1	15 (75)
Clinical or pathologic stage, N (%)	
I	1 (5)
II	2 (10)
III	4 (20)
IV	13 (65)
Driver genes in tissue, N (%)	
ALK	14 (70)
ROS1	4 (20)
RET	2 (10)
Initial treatment, N (%)	
Operation	3 (15)
Chemotherapy	2 (10)
Alectinib	10 (50)
Crizotinib	3 (15)
Selpercatinib	2 (10)

breakpoints that involve long intron sequences, as these extended DNA stretches cannot be covered by hybridization-capture probes. However, this issue is avoided when analyzing fusion events using RNA.

MSK-IMPACT DNA sequencing analysis of driver-negative lung cancer was further refined with a custom RNA-Seq panel (MSK-Fusion) specifically focused on select RNA.²⁷ Of 254 samples that lacked driver mutations by DNA testing, 14% were subsequently

found by RNA testing to harbor fusion changes, with the great majority matched to targeted therapy.

Compared with the Guardant360 plasma NGS assay, the proposed cfRNA-based assay can sensitively detect *ALK* and *ROS1* fusion genes. Considering the technical difficulty of the cfDNA-based assay to capture *ROS1* gene rearrangements, which occur in *ROS1* gene introns with many repetitive sequences, it was reasonable to suggest that the cfRNA-based assay is superior to the Guardant360 plasma NGS assay for detection of *ROS1* fusions.²⁸ However, it was surprising that even the *ALK* fusion was more sensitively detected with the cfRNA-based assay.

Of the 11 cases classified as chemotherapy-naïve or PD, the cfRNA-based assay failed to detect fusion genes in 3. Case 10 had carcinomatous pleurisy but no metastasis to distant organs. Therefore, relatively small amounts of ctDNA were shed into the bloodstream. The tumors of the other 2 cases had metastasized to distant organs at the time of blood collection. Case 2 had a chemotherapy-naïve primary tumor that had metastasized to the bone and lymph node. In case 11, the blood sample was collected following metastasis to the brain during third-line treatment with lorlatinib. Considering that cases 2 and 11 were also negative by the Guardant360 plasma NGS assay, tissue biopsy is recommended if no driver mutation is identified by liquid biopsy.

Theoretically, the proposed protocol may be applicable for the isolation of any type of fusion gene. The cfRNA genotyping system, therefore, can potentially help with an initial diagnosis in patients with insufficient tissue specimens for tumor genotyping, which can facilitate the administration of effective first-line therapy.

Moreover, cfRNA-based assays may be applicable for the identification of fusion genes acquired during TKI treatments. Tyrosine kinase and *BRAF* fusions are reportedly rare events that mediate resistance to TKIs in patients with advanced EGFR-mutated NSCLC.³⁵ So far, *ALK*, *ROS1*, *RET*, *BRAF*, *FGFR3*, and *NTRK1* have been identified as fusions that are potentially responsible for acquired resistance to first-line or second-line treatment with osimertinib.³⁶⁻³⁸ Notably, in some cases, combination therapies of osimertinib and fusion-targeting TKIs were effective at overcoming resistance in the presence of fusions.^{39,40} Recently, *ST7-MET* rearrangements were identified as potential mechanisms of acquired resistance to *ALK* TKIs. Considering that repeated biopsy of a tumor at the same site may not reflect intratumoral heterogeneity, cfRNA-based fusion detection should be considered to evaluate fusion genes in patients with PD.

For cases in which blood was collected at 2 points (before and after treatment), the detection of cfRNA was consistent with the therapeutic effect, suggesting that cfRNA might also be a useful predictor of metastases and the treatment response of NSCLC.

This study has some limitations. First, the sample size was small due to the rarity of fusions among patients with NSCLC. However, the main scope of this study is to develop a new application for liquid biopsy and provide novel information about cfRNA focusing analytical and clinical validity of the assay. Therefore, future studies with larger number of cases will be needed to clarify the

TABLE 2 Summary of 20 NSCLC cases analyzed for fusion detection by cRNA-based assay

Case #	Sex	Age	Stage	Tissue analysis			Treatment			Treatment at sample collection	Status at sample collection	Liquid fusion detection		Gene alteration cDNA
				Fusion	Method	Metastasis	1st	2nd	3rd			cfRNA	cfDNA	
#1	F	30	IVB	EML4-ALK	NGS	Bo, Li	Alectinib	Chemo	-	1st	PD	EML4-ALK	NA	NA
#2	F	85	IVB	ALK	IHC	Bo, Ly	Alectinib	-	-	Pre	Naïve	ND	NA	NA
#3	F	55	IVB	ALK	IHC	Bo, Li	Alectinib	Chemo	-	1st	PD	EML4-ALK	EML4-ALK	TP53 G266E (9.07%) ALK I1171N (0.54%)
#4	M	53	IVB	EZR-ROS1	NGS	Pa, Ly	Crizotinib	Chemo	-	1st	PD	EZR-ROS1	ND	TP53 E204* (0.14%)
#5	F	36	IIIC	CD74-ROS1	NGS	Ly	Crizotinib	-	-	Pre	Naïve	CD74-ROS1	ND	TP53 L145P (0.3%)
#6	F	36	IVB	RET	NGS	Ly	Selpercatinib	-	-	Pre	Naïve	KIF5B-RET	KIF5B-RET	ND
#7	M	57	IIIA	EML4-ALK	RT-PCR	Ly	Operation	Chemo	Alectinib	3rd	PD	EML4-ALK	ND	ND
#8	F	29	IVB	ALK	NGS	Br	Alectinib	-	-	Pre	Naïve	EML4-ALK	ND	BRCA1 S1212P (0.56%) FBXW7 T536R (0.12%)
#9	F	70	IVA	RET	NGS	PI	Selpercatinib	-	-	Pre	Naïve	KIF5B-RET	KIF5B-RET	ND
#10	M	49	IVA	ALK	NGS	PI	Alectinib	-	-	Pre	Naïve	ND	ND	ND
#11	M	44	IV	ALK	IHC	Br, Bo, Ly	Alectinib	Chemo	Lorlatinib	3rd	PD	ND	ND	ND
#12	F	65	IIIB	EML4-ALK	RT-PCR	Ly	Operation	Alectinib	-	2nd	PR	ND	ND	ND
#13	M	61	IIA	ALK	IHC	Ly	Operation	Alectinib	-	2nd	PR	ND	ND	AR G300A (1.89%)
#14	F	66	IIIB	ALK	IHC	Ly	Chemo	Alectinib	-	2nd	CR	ND	NA	NA
#15	M	64	IV	ALK	IHC	Br	Alectinib	Chemo	Lorlatinib	3rd	PR	ND	NA	TP53 V274A (0.18%)
#16	M	58	IVB	ALK	FISH	Br, Bo	Alectinib	Pembro	Lorlatinib	3rd	SD	ND	NA	NA
										3rd	SD	ND	NA	NA

(Continues)

TABLE 2 (Continued)

Case #	Sex	Age	Stage	Tissue analysis			Treatment			Treatment at sample collection	Status at sample collection	Liquid fusion detection		Gene alteration
				Fusion	Method	Metastasis	1st	2nd	3rd			cfRNA	cfDNA	
#17	M	78	IV	ALK	FISH	PI	Alectinib	Lorlatinib	Chemo	3rd	PR	ND	NA	NA
										3rd	PR	ND	NA	NA
#18	M	63	IVB	ALK	IHC	Br, Bo	Alectinib	-	-	1st	PR	ND	NA	NA
										1st	PR	ND	ND	ND
#19	F	86	IV	EZR-ROS1	RT-PCR	PI	Chemo	Pembro	Crizotinib	3rd	PR	ND	NA	NA
										3rd	PR	ND	ND	ND
#20	F	84	IVA	CD74-ROS1	RT-PCR	PI	Crizotinib	-	-	1st	PR	ND	NA	NA
										1st	PR	ND	ND	TP53 R213P (0.14%) RAF1 S26N (0.11%) ERBB2 (CN = 2.19)

Note: Variant allele frequencies detected with the cfDNA-based assay are shown in parentheses. *, stop codon.

Abbreviations: Bo, bone; Br, brain; Chemo, cytotoxic chemotherapy; CN, copy number; FISH, fluorescence in situ hybridization; IHC, immunohistochemical; Li, liver; Ly, lymph node; NA, not analyzed; ND, not detected; NGS, next-generation sequencing; Pa, pancreas; PD, progressive disease; PI, pleura; PR, partial response; RT-PCR, reverse-transcription PCR; SD, stable disease.

clinical utility of the assay, including studies to compare the clinical utility of cfRNA-based vs. cfDNA-based assays such as Gurdant 360 and FoundationOne liquid. Especially, as this study did not include fusion negative cases, the specificities of the assays were not evaluated. Second, there is still room to improve the sensitivity of the proposed cfRNA-based assays for detection of fusion genes. A possible reason for the less than optimal sensitivity may be due to the quality and quantity of the assayed cfRNA. Cryopreserved plasma was used in this study and the freeze-thaw event may have affected the cfRNA quality. The cfRNA yield could be further improved by attempting other nucleic extraction methods that minimize cfRNA decay. Third, the sensitivity of cfRNA-based assays for detection of single nucleotide variations or insertions/deletions is not as high as that of cfDNA-based assays. The use of unique molecular identifiers in library construction may improve the limit of detection of mutational analysis with cfRNA-based assays. Fourth, the current assay does not have high quantitation. One applicable way to obtain quantitative data in amplicon-based NGS analysis is the compensation of fusion read number using the expression of housekeeping genes. Finally, although this method was highly sensitive for detection of known fusions, novel fusions theoretically cannot be detected, which is a major trade-off with the use of amplicon-based sequencing.

Overall, this study highlights new applications for liquid biopsy and novel information about RNA shed from tumor cells. The accuracy of the cfRNA-based assay for fusion gene detection was markedly improved. The evaluation of cfRNA by liquid biopsy can be useful for diagnostic, treatment prediction, and even prognostic purposes. Future laboratory testing is needed to apply this protocol for molecular profiling and monitoring recurrence and therapeutic effects, which will increase opportunities for treatment and eventually improve patient prognosis.

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DISCLOSURE

The authors certify that no actual or potential conflict of interest in relation to this article exists.

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FIGURE 3 Fusion detection sensitivities of the cRNA-based and cfDNA-based assays for patients who were untreated or with PD. The sensitivities of the cRNA-based and cfDNA-based assays were compared with the use of 9 plasma samples collected from 9 patients who were chemotherapy-naïve or diagnosed with PD (cases 3-11). The same plasma was used for both assays

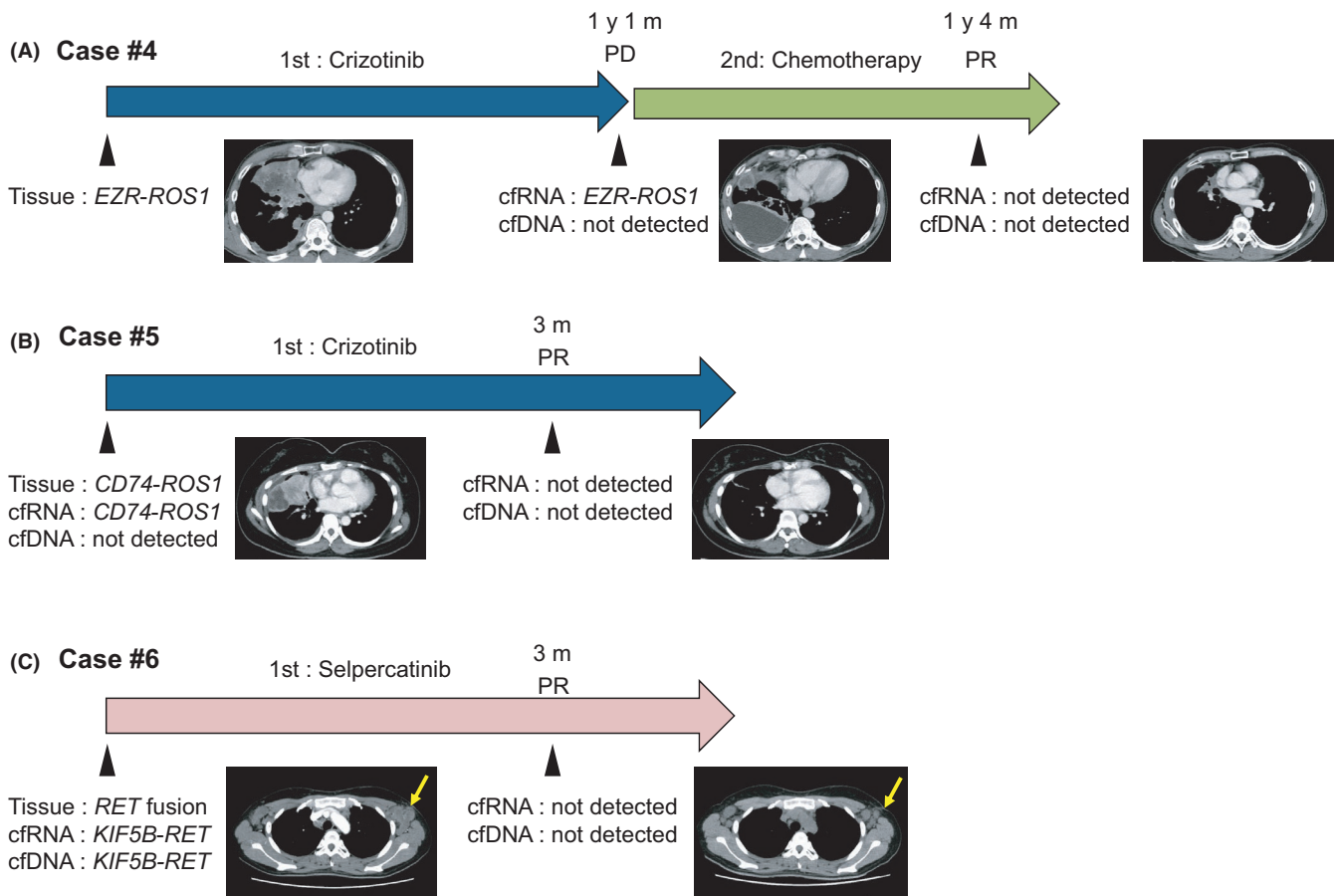
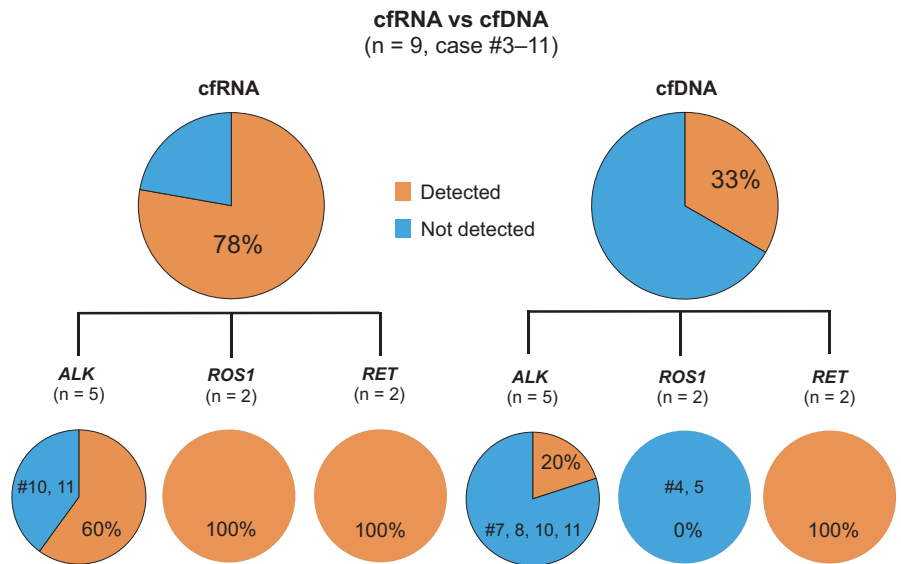


FIGURE 4 Representative cases of liquid biopsy at multiple time points. The cRNA-based assay was performed during treatment to monitor disease status. The arrows indicate the treatment courses and the arrowheads indicate the timings of sample collection and computed tomography scanning. (A), *EZR-ROS1* fusion was detected in cRNA but not cfDNA when the patient exhibited PD to first-line treatment with crizotinib. After 3 mo of second-line chemotherapy, when the patient exhibited PR, the *EZR-ROS1* fusion gene was not detected with the RNA-based assay. (B), The *CD74-ROS1* fusion was detected with the cRNA-based assay before treatment initiation and then was negative after 3 mo of treatment when the patients exhibited PR. (C), The *KIF5B-RET* fusion was detected by both cRNA-based and cfDNA-based assays before treatment initiation and switching to negative after 3 mo of treatment when the patients exhibited PR. The yellow arrows indicate tumor shrinkage

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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