One-Step Polymerase Chain Reaction-Free Nanowire-Based Plasma Cell-Free DNA Assay to Detect EML4-ALK Fusion and to Monitor Resistance in Lung Cancer

YOUNGJOO LEE,† YOUNGNAM CHO,† EUN YOUNG PARK, SEONG-YUN PARK, KUM HUI HWANG, JI-YOUN HAN

†Contributed equally.

Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Plasma • Circulating tumor DNA • Nanowire • Lung cancer • EML4-ALK

ABSTRACT

Background. Next-generation sequencing has mostly been used for genotyping cell-free DNA (cfDNA) in plasma. However, this assay has several clinical limitations. We evaluated the clinical utility of a novel polymerase chain reaction–free nanowire (NW)-based plasma cfDNA assay for detecting ALK fusion and mutations.

Patients, Materials, and Methods. We consecutively enrolled 99 patients with advanced non-small cell lung cancer undergoing a fluorescence in situ hybridization (FISH) test for ALK fusion; ALK-positive (n = 36). The NW-based assay was performed using 50–100 μL of plasma collected at pretreatment and every 8 weeks during ALK inhibitor treatment.

Results. There was high concordance between the NW-based assay and the FISH test for identification of ALK fusion (94.9% with a kappa coefficient value of 0.892, 95% confidence interval [CI], 0.799–0.984). There was no difference in the response rate to the first anaplastic lymphoma kinase inhibitor between the ALK-positive patients identified by the NW-based assay and by the FISH test (73.5% vs. 72.2%, p = .931). In the ALK variant analysis, variants 1 and 3 subgroups were detected in 27 (75.0%) and 8 (22.2%) patients, respectively. Among 24 patients treated with crizotinib, variant 3 subgroup was associated with worse median overall survival than variant 1 subgroup (36.5 months; 95% CI, 0.09–87.6 vs. 19.8 months; 95% CI, 9.9–not reached, p = .004). A serial assessment identified that ALK L1196M resistance mutation emerged before radiologic progression during crizotinib treatment.

Conclusion. The newly developed simple NW-based cfDNA assay may be clinically applicable for rapid diagnosis of ALK fusion with its variant forms and early detection of resistance.

INTRODUCTION

Small inversions on the short arm of chromosome 2 lead to the rearrangement of the echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK) genes and was first reported in 2007 as a transforming fusion oncogene [1]. Fusion of EML4 and ALK induces aberrant activation of downstream oncogenic pathways, such as the
The NW-based cfDNA analysis allows for a fast, cost-effective approach to detect gene fusions that can trigger cancer cell growth, proliferation, and apoptosis [2, 3]. Several kinase inhibitors that target this gene fusion were developed, tested in multiple clinical trials, and showed improved survival outcomes and quality of life in patients with NSCLC with this gene fusion [6]. Thus, an ALK-targeting treatment became the standard of care for patients with advanced NSCLC and EML4-ALK gene fusion. In addition, genotyping for this gene fusion became mandatory for patients newly diagnosed with advanced NSCLC to determine the appropriate treatment strategy [7]. Immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and reverse transcription polymerase chain reaction (PCR) analyses are commonly used to detect EML4-ALK fusion [8, 9]. These methods analyze tumor tissue from some biopsy procedures. Thus, the lack of sufficient tissue amount and the possible complication risk from biopsy procedure remain inherent limitations in using these standard diagnostic tests in patients with lung cancer. Moreover, because these methods use tissues obtained from a single lesion, the results may not represent the characteristics of the entire tumor because of sampling bias or inherent tumor heterogeneity [10].

Circulating tumor DNA (ctDNA) includes genetic fragments shed into the bloodstream through necrosis or apoptosis of malignant tumor cells. The average size of ctDNA fragments is ~167 base pairs, which represents the length of a chromatosome containing core histones and linker DNA [11, 12]. Accumulating evidence shows that ctDNA accurately reflects the molecular and genetic features of primary and metastatic tumors [13–15]. Thus, several clinical studies demonstrated that plasma ctDNA analysis is useful as a sensitive and noninvasive method for predicting and monitoring a response or resistance to anticancer treatments instead of the tissue DNA analysis [16, 17]. The next-generation sequencing (NGS) method has been mostly used as plasma ctDNA assay in these previous studies. With the advent of NGS, it is possible to simultaneously analyze a broad range of genetic alterations, allowing the detection of ctDNA levels qualitatively and quantitatively. However, the NGS-based mutation profiling of ctDNA comprises complex processing steps, including sample preparation, nucleic acid extraction and amplification, library preparation, sequencing, bioinformatic analysis of NGS data. In particular, due to the vast amount of raw data generated by NGS sequencing and its complexity, the role of bioinformatics experts is crucial for processing, interpreting, and analyzing complex bioinformatics data, ultimately obtaining biological specificity from sequenced samples. Recently, we developed a new genotyping assay that accurately and rapidly detects small amounts of tumor-derived cell-free DNA (cfdNA) in various body fluids in patients with lung cancer or cervical cancer [18–20]. This assay isolates cfdNA in body fluids using nanowires (NWs) and directly detects DNA variants or mutations without PCR amplification within 1 hour. Our previous studies demonstrated that this NW-based cfdNA assay can be used to detect low-abundance DNA variants in plasma cfdNA with high sensitivity and specificity [18–20]. The NW-based cfdNA analysis allows for a fast, cost-effective, and real-time multiplex analysis of target mutations, making it ideal for monitoring therapeutic responsiveness in a very simple manner.

In this study, we aimed to determine whether the novel PCR-free, NW-based plasma cfdNA assay could be clinically feasible to detect EML4-ALK fusion, thereby overcoming the limitations of the standard tissue-based tests and the current NGS-based cfdNA assay. In addition, we assessed the ability of this plasma cfdNA assay to classify fusion variants and to monitor secondary drug resistant ALK mutations.

Materials and Methods

Patients and Sample Collection

We consecutively enrolled 99 patients who were diagnosed with advanced NSCLC and underwent a standard IHC analysis and a dual-color break-apart FISH test for EML4-ALK fusion at the National Cancer Center Hospital (Goyang, Korea) from August 2017 to May 2019. Thirty-six (36.4%) patients were diagnosed with ALK-positive tumors, whereas 63 (63.6%) were diagnosed with ALK-negative tumors. The blood samples were collected at pretreatment and if any ALK inhibitor was given, it was repeated every 8 weeks. Evaluations of response to ALK inhibitor were performed with computerized tomography scans, magnetic resonance imaging, and positron emission tomography every 8 to 12 weeks, as appropriate. This study was performed with approval from the National Cancer Center Institutional Review Board (approval number NCC2016-0208). All patients provided written informed consent.

Colorimetric Detection of the EML4-ALK Fusion in Cell Lines

First, we used H2228, A549, H1993, PC9, RT4, SNU2535, and H596 cell lines to evaluate the performance of the NW-based cfdNA assay. Fragmented DNA (fDNA) was generated by mechanically shearing genomic DNA extracted from the cell lines into random DNA fragments of size <1 kb using ultrasonication. Positively charged polyethyleneimine-conjugated–NWs (PEI-NWs, 5 μg/ml, Genopsy Inc, Seoul) and 200 μL of blood samples spiked with 250 ng of fDNA from the cell lines were applied into filter paper discs in spin column (GeneAll, Biotechnology, Korea) and mixed for 20 minutes at room temperature (RT) to induce DNA-NW complex formation. The fDNAs captured on the PEI-NWs were incubated at 95°C for 1 minute. The biotinylated EML4-ALK probes (supplemental online Table 1) were designed using the University of California, Santa Cruz genome browser and synthesized by Macrogen (Seoul). The biotin-labeled probe mixture (1 pM) and 2 μg/mL of horseradish peroxidase-labeled streptavidin-conjugated nanoparticles (HRP-st NPs; Genopsy Inc, Seoul) were added to the DNA-NW complexes and incubated for 30 minutes. After centrifugation at 8,000 rpm for 15 minutes, 50 μL of 10 mM 3,3′,5,5′-tetramethylbenzidine, 50 μL of 0.1 M H2O2, and 200 μL of 0.2 M sodium acetate trihydrate buffer (pH 7.0) were added to the DNA-NW complexes. Optical density (OD) of the samples was measured using absorbance at 490–800 nm using an Epoch UV-Vis spectrophotometer (Biotek, Epoch, Winooski, VT). The signal associated with ALK

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fusion was determined by subtracting the absorbance at 500 nm from the absorbance at 650 nm (\(\Delta\text{OD}_{650-500}\)).

NW-Based Detection of the EML4-ALK Fusion Using Plasma from Patients with NSCLC

To detect EML4-ALK fusion, PEI-NWs (5 \(\mu\)g/mL) and 150 \(\mu\)L of diluted plasma were combined and mixed for 20 minutes at RT to form DNA-NW complexes. The colorimetric assay was performed as described above. The limit of detection (LOD) was determined using fDNA from H2228 cells and plasma samples from healthy donors, and the cutoff value was a \(\Delta\text{OD}_{650-500}\) of 0.007. The total experimental time for detecting EML4-ALK fusion by the NW-based cfDNA assay was ~60 minutes, and 3 \(\mu\)L of plasma was used to identify each gene alteration.

Statistical Analysis

Areas under the curve (AUCs) with 95% confidence intervals (CIs) were used to evaluate the ability of the NW-based cfDNA assay to detect EML4-ALK fusion in plasma. The diagnostic performance results are presented as percentages with 95% CIs estimated by the exact method. The concordance was measured by Cohen’s unweighted kappa (K). Progression-free survival (PFS) was calculated from the start date of administration of the first ALK inhibitor to the documented date of disease progression or death. Overall survival (OS) was calculated from the start date of the first-line treatment for metastatic disease until the date of death. Survival rates were estimated using the Kaplan-Meier method, and differences in survival curves between groups were assessed using the log-rank test. All analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) and R project (version 3.6.1, R Foundation, Vienna).

Figure 1. Schematic illustration of the NW-based assay for detection of the EML4-ALK fusion. (A): The EML4-ALK fusion is caused by a chromosomal rearrangement on the short arm of chromosome 2. We hypothesized that this inversion event would lead to chromosomal destabilization, thereby inducing local DNA duplex opening. (B): The process of the NW-based assay. (C): Two pairs of biotinylated probes were designed for the identification of EML4-ALK variant 1 and EML4-ALK variant 3.

Abbreviations: HRP-st NP, horseradish peroxidase-labeled streptavidin-conjugated nanoparticle; NW, nanowire.
RESULTS

Assay Principle
Double-strand DNA molecule constantly and transiently undergoes DNA breathing, which leads to a local duplex opening, exposing single-stranded DNA sections [21]. Such DNA bubble formation plays a crucial role in many of the biological processes, making it deeper understanding of the activity of the DNA. In particular, intracellular events including mismatches, insertions, and deletions of base pairs have a significant impact on the stability of DNA and accelerate loose DNA and denaturation of bubbles in the DNA backbone [22, 23]. In this assay, we used local distortion or partial dehiscence of the DNA double helix around the fusion site because chromosomal translocation events destabilize the structure of DNA (Fig. 1A). Upon applying mild heat to cfDNA, regions containing the fusion are structurally and thermodynamically more unstable than other regions. Therefore, the DNA double helix at the fusion site behaves as single-stranded DNA such that DNA probes can access their corresponding target sequence (Fig. 1B). In brief description of the assay process, positively charged PEI-NWs capture negatively charged cfDNA in the plasma to create DNA-NW complexes. The DNA-NW complexes are incubated at 95°C for 1 minute and the ODs for each probe were measured. The experiment was performed in triplicate. (B): Average ΔOD₆₅₀₅₀₀ values for the EML4-ALK Variant 1 and Variant 3 probes were plotted to show relative expression. EML4-ALK relative expression was determined by subtracting the absorbance at 500 nm from the absorbance at 650 nm. (C): gDNA from H2228 cells was sonicated to obtain fDNA fragments of size <1 kb, plasma samples were spiked with H2228 fDNA, and PEI-NWs were added to form DNA-NW complexes. Various biotin-labeled probes (EML4(E4)-ALK(A20); exon 4 of EML4 and exon 20 of ALK, EML4(E5)-ALK(A20); exon 5 of EML4 and exon 20 of ALK, EML4(E6)-ALK(A20); exon 6 of EML4 and exon 20 of ALK, EML4(E7)-ALK(A20); exon 7 of EML4 and exon 20 of ALK, EML4(E8)-ALK(A20); exon 8 of EML4 and exon 20 of ALK, EML4(E6)-ALK(A19); exon 6 of EML4 and exon 19 of ALK, EML4(E6)-ALK(A21); exon 6 of EML4 and exon 21 of ALK) and HRP-st NPs were added to the DNA-NW complexes after short denaturation and ODs for each probe were measured. The experiment was performed in triplicate. (D): Average ΔOD₆₅₀₅₀₀ values for various EML4-ALK probes were plotted to show relative EML4-ALK expression.

Abbreviations: Avg. average; OD, optical density.

Assay Feasibility
To demonstrate proof-of-concept for the NW-based cfDNA assay, we first conducted ex vivo experiments with five cancer...
cell lines: the positive control cell line H2228 (lung cancer with EML4-ALK variant 3a/b) and four negative control cell lines A549 (lung cancer with KRAS G12S mutation), H1993 (lung cancer with MET amplification), PC9 (lung cancer with EGFR exon 19 deletion), and RT4 (bladder cancer). We spiked plasma from healthy donors with fDNA prepared from the various cancer cell lines (Fig. 2A). A distinct signal representing EML4-ALK fusion was only detected in H2228 cells using the variant 3 probe; no signal was detected in H2228 cells using the variant 1 probe or in the other cell lines using both probes. Upon quantification of expression of EML4-ALK fusion using the NW-based assay, ALK expression was significantly higher in H2228 cells using the variant 3 probe than in those using the variant 1 probe and in the other cell lines using both probes. Upon quantification of expression of EML4-ALK fusion using the NW-based assay, ALK expression was significantly higher in H2228 cells using the variant 3 probe than in those using the variant 1 probe and in the other cell lines using both probes. These results suggest that the signal detected by the NW-based cfDNA assay originated from the specific binding of the probe to the corresponding target DNA template.

On the other hand, we suspected that the DNA adjacent to the fusion may also be structurally and thermodynamically unstable. To address this, we evaluated the binding specificity of other EML4-ALK probes designed to bind to exons adjacent to the exon 6-EML4 and exon 20-ALK fusion. Interestingly, when we used the nearest adjacent probes to exon 5-EML4 and exon 20-ALK (EML4 [E5]-ALK [E20]) and exon 6-EML4 and exon 19-ALK (EML4 [E6]-ALK [E19]), we observed the same signal for EML4-ALK fusion that was observed when using the original exon 6-EML4 and exon 20-ALK (EML4 [E6]-ALK [E20]) probe (Fig. 2C). However, the expression of EML4-ALK fusion was significantly lower when using the adjacent exon probes than when using the original probe (Fig. 2D), indicating that the degree of structural loosening depends on the distance from the fusion site and can influence specific binding of the probe to target DNA in the cfDNA assay.

To determine the LOD for EML4-ALK fusion, we spiked a range of concentrations of fDNA from H2228 cells with EML4-ALK fusion variant 3 into plasma from healthy donors. We found that the NW-based cfDNA assay can detect EML4-ALK fusion at a concentration of 100 fg/mL of fDNA from H2228 cells based on a signal-to-noise ratio of 3 (supplemental online Fig. 1).

Acquired drug resistance is the most challenging problem in the treatment of patients with ALK-positive lung cancer with ALK tyrosine kinase inhibitors (ALK-TKIs). Secondary ALK point mutations are the most common resistance mechanism [24]. To examine the feasibility of the NW-based assay in monitoring for ALK resistance mutations, we conducted NW-based assay ex vivo by spiking plasma from healthy donors with fDNA prepared from five cancer cell lines: the positive control cell line SNU2535 (lung cancer with ALK G1269A mutation)
and four negative control cell lines (Fig. 3). The DNA-NW complexes derived from fDNA from SNU2535 cells were specifically targeted by ALK G1269A probe, but not by the variant 1a n dv a r i a n t3 EML4-ALK probes. There was also no signal for ALK G1269A mutation in four cancer cell lines without ALK G1269A mutation. Based on these results, in addition to identifying EML4-ALK fusion, the NW-based cfDNA assay can identify and monitor secondary ALK gene mutations.

Sensitivity and Specificity
We used the NW-based cfDNA assay on 99 plasma samples from 36 ALK-positive patients and 66 ALK-negative patients (supplemental online Table 2). The rate of concordance for the detection of EML4-ALK fusion using the NW-based cfDNA assay with plasma and the FISH test with tissue samples was 94.9% with a kappa coefficient value of 0.892 (95% confidence interval, 0.799–0.984; supplemental online Table 3). The sensitivity, specificity, positive predictive value, and negative predictive value of EML4-ALK fusion detection by the NW-based cfDNA assay were 94.4% (95% CI, 81.3–99.3), 95.2% (95% CI, 86.7–99.0), 91.9% (95% CI, 78.1–98.3), and 96.8% (95% CI, 88.8–99.6), respectively. Absorbance values for EML4-ALK fusion in plasma from ALK-positive patients were higher than those for EML4-ALK fusion in plasma from ALK-negative patients (mean ± SD, 0.020 ± 0.012 vs. 0.001 ± 0.002; p < .0001; Fig. 4A). ROC analysis verified the ability of the assay to detect EML4-ALK fusion (AUC = 0.981;
Taken together, these results demonstrate that the NW-based cfDNA assay has high sensitivity and specificity for identifying EML4-ALK fusion in plasma from patients with lung cancer.

We further evaluated whether the NW-based cfDNA assay can distinguish between two variants of the EML4-ALK fusion in plasma from patients with ALK-positive lung cancer (Fig. 4C, 4D). Our data confirmed that the NW-based cfDNA assay successfully differentiates between the EML4-ALK variant 1 fusion and the EML4-ALK variant fusion using cfDNA in plasma.

Clinical Application
All patients with ALK-positive NSCLC were treated with ALK-TKIs. Twenty-three (63.9%) patients received ALK-TKIs as first-line treatment and the median number of ALK-TKIs administered was two (range, 1–5). Crizotinib (n = 25, 69.4%), alectinib (n = 9, 25.0%), or brigatinib (n = 2, 5.6%) were used as the first ALK inhibitor. There was no difference in the overall response rate (ORR) to the first ALK inhibitor between the ALK-positive patients identified by the FISH test and those identified by the NW-based cfDNA assay (72.2% vs. 73.5%; p = .931). The same

Figure 5. Kaplan-Meier curves of PFS (A) and OS (B) in patients with metastatic non-small cell lung cancer and EML4-ALK variants 1 and 3 who were treated with crizotinib as the first ALK inhibitor. Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

Figure 6. Assessment of ALK secondary resistant mutations using the NW-based assay. (A): The ALK G1202R mutation was detected by the NW-based assay and next-generation targeted sequencing using cfDNA and tissue, respectively, from a patient with ALK-positive lung adenocarcinoma treated with lorlatinib. (B): Serial assessment for the ALK L1196M mutation during crizotinib treatment followed by brigatinib treatment in a patient with ALK-positive lung adenocarcinoma. The ALK L1196M mutation emerged 4 weeks after the start of crizotinib treatment and before objective progression at 16 weeks. After starting brigatinib treatment, the level of resistance mutation DNA and tumor size decreased.

Abbreviations: cfDNA; cell-free DNA; CT, computed tomography; NW, nanowire.
trends were seen for PFS and OS values measured by the FISH and NW-based cfDNA assays, respectively (median PFS, 20.7 months; 95% CI, 9.7–31.7 vs. 20.7 months; 95% CI, 10.3–31.1; \( p = .991 \); median OS, 66.5 months; 95% CI, 40.9–92.0 vs. 66.5 months; 95% CI, 37.4–95.5; \( p = .998 \)).

The NW-based cfDNA assay showed that the frequencies of variant 1, variant 3, and nonvariant 1/3 among our patient samples were 75.0%, 22.2%, and 2.8%, respectively. There were no differences in the baseline characteristics between the variant 1 and variant 3 subgroups (supplemental online Table 4). For the 24 patients treated with crizotinib as the first-line treatment, the variant 3 subgroup \((n = 5)\) showed a lower ORR to crizotinib than that of the variant 1 subgroup \((n = 19); 50.0\% \text{ vs. } 84.2\%; \ p = .194 \) . Moreover, the variant 3 subgroup was associated with worse median PFS (1.2 months; 95% CI, 0.01–8.89 vs. 19.8 months; 95% CI, 8.9–30.8; \( p = .086 \)) and median OS (19.8 months; 95% CI, 9.9–not reached vs. 36.5 months; 95% CI, 0.09–87.6; \( p = .004 \)) than that of the variant 1 subgroup (Fig. 5).

Eighteen patients provided serial plasma samples during ALK-TKI treatment, and the following mutations were detected by the NW-based cfDNA assay: ALK G1202R \((n = 1)\), L1171T \((n = 1)\), L1152R \((n = 1)\), G1269A \((n = 1)\), and L1196M \((n = 1)\). One case involved a 52-year-old male patient with stage IV ALK-positive adenocarcinoma of the lung (Fig. 6A) who received lorlatinib after disease progression with ceritinib. He showed a partial response to lorlatinib with disease progression after nine cycles. At the time of disease progression, the tissue from right upper lung mass was biopsied and ALK G1202R mutation was identified by targeted sequencing. In concordance with the tissue biopsy result, the NW-based assay detected the new ALK G1202R mutation, which did not exist before lorlatinib treatment. The other case was a 47-year-old female patient with recurrent ALK-positive adenocarcinoma of the lung (Fig. 6B) who received crizotinib after disease progression to definite concurrent chemoradiotherapy. She showed a partial response to crizotinib with disease progression after 16 weeks. Four weeks after crizotinib treatment, the ALK L1196M mutation, which is the most common secondary resistance ALK mutation, was detected, and the level of the mutation in the plasma increased until the time of objective radiologic progression. After receiving treatment withbrigatinib, the level of the ALK L1196M mutation in the plasma decreased and the tumor showed shrinkage. The patient continued to be treated withbrigatinib.

**Discussion**

This study demonstrated a new NW-based cfDNA assay optimized for identifying the gene fusion in plasma. By using several fusion-negative cancer cell lines carrying nonoverlapping oncogenes and probes specific to different fusion variants, we demonstrated that the NW-based cfDNA assay has high sensitivity and specificity for detection of EML4-ALK fusion. These findings were clinically validated with plasma from ALK-negative patients and patients with ALK-positive lung cancer receiving ALK-targeted inhibitors. In addition, this study demonstrated that the ALK fusion variants and ALK gene mutations identified by the NW-based cfDNA assay were correlated with the clinical outcomes.

A FISH analysis has been considered the gold standard for detecting EML4-ALK fusion in clinical practice. However, it can be technically difficult to interpret the fusion signals generated with a dual-color break-apart probe because the EML4 and ALK genes are located close together (~12 mega-bases apart) on the same chromosome [1]. Moreover, the FISH analysis is costly and requires high-level expertise and labor-intensive work. Thus, there has been a clinical need for a new genotyping technique with high feasibility, sensitivity, and specificity. The NW-based cfDNA assay uses only 3 μl of plasma per gene and takes only 60 minutes to test for EML4-ALK fusion. Moreover, this assay directly uses the abnormal DNA structures present within the fusion without the need for PCR amplification, making it highly sensitive and specific in contrast to the current NGS-based cfDNA assay [25]. The key feature of this assay involves using a lower denaturation temperature similar to that used in the coamplification at lower denaturation temperature (COLD)-PCR method proposed by Milbury et al., which showed that PCR performed at a lower denaturation temperature leads to highly-selective amplification and enrichment of target gene mutations [26]. Milbury et al. suggested that COLD-PCR can overcome the limitations of conventional molecular assays such as NGS methods for the detection of low-abundance DNA variants. Considering that the majority of plasma cfDNA originates from ruptured benign cells, tumor-derived mutant-containing cfDNA is intrinsically present at a low level. Thus, this NW-based cfDNA assay is appropriate for the detection of low-abundance cfDNA variants in plasma.

To date, at least 15 variants of EML4-ALK fusion have been reported [27–29]. All of the variants have different parts of EML4 gene fused with the intracellular kinase domain (encoded by exons 20–29) of the ALK gene. The fusion variants differ in the lengths of the linker region and the tandem atypical propeller (TAPE) domain of EML4 and therefore, EML4-ALK variants have different biological and molecular features, which affect protein stability and the clinical response to ALK-TKIs [30, 31]. EML4-ALK variants 1 and 3a/b are the most common in NSCLC and account for 70%–80% of all variants [30–32]. Several retrospective studies showed that crizotinib is less effective against variants 3a/b and 5a that lack the TAPE domain of EML4 than against variants 1 and 2 that contain a partial TAPE domain [31, 33]. Similar to other studies, our study confirmed that the ALK variant 3, which was identified by the NW-based cfDNA assay, was associated with worse outcomes when crizotinib was used as first-line treatment than the ALK variant 1. Taken together, these findings highlight the importance of identifying the variant to determine the best treatment strategy for ALK-positive NSCLC [20]. The NW-based cfDNA assay showed better feasibility for the detection of specific ALK fusion variants than genotyping methods used in other studies such as targeted next-generation sequencing, Sanger sequencing, and whole-exome sequencing.

Another clinical advantage of plasma cfDNA genotyping is the ability to perform serial noninvasive assessments for response and resistance to response to anticancer treatment [16]. In our study, the NW-based cfDNA assay successfully detected secondary acquired resistant mutations, such as ALK
G1202R, in the plasma of patients treated with ALK-TKIs. Moreover, serial assessment by the NW-based cfDNA assay demonstrated that the ALK L1196M mutation, which is the most common secondary mutation conferring resistance to crizotinib, emerged at the beginning of crizotinib treatment before the objective progression developed [34]. These findings suggest that noninvasive assessment of cfDNA using the NW-based cfDNA assay allows for early detection of drug resistance to ALK-TKIs and guidance for subsequent treatment.

NGS using cfDNA has proved to be useful for detecting EML4-ALK fusions with high sensitivity and specificity of 80% and 100%, respectively [35]. However, the needs for specialized equipment, slow processing times (2 to 3 weeks), and bioinformatics support for data interpretation often limit the wide application. Although we did not directly compare the NW-based test with NGS using cfDNA, our data also demonstrated high levels of sensitivity and specificity of NW-based test for detecting ALK fusions in patients with lung cancer. Furthermore, the NW-based test is an emerging technology that allows not only isolation of the cfDNA from the plasma but also rapid detection of gene mutations or rearrangements in small volumes without use of PCR amplification. As an optical sensing platform, the NW-based method makes it an attractive alternative for clinical sample analysis because of its relatively low cost, small sample volume, and fast turnaround time (1 hour). Nevertheless, ALK fusion testing using cfDNA is rather challenging because genomic breakpoints are usually unknown, and the rearrangement usually involves thousands of base pairs [35]. Thus, further large-scale study is required to find an appropriate and optimal balance of threshold levels between false positives and false negatives.

REFERENCES

1. Soda M, Choi YL, Enomoto M et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448: 561–566.
2. Palmer RH, Vernerson E, Grabbe C et al. Anaplastic lymphoma kinase: Signalling in development and disease. Biochem J 2009;420:345–361.
3. Roskoski R. Anaplastic lymphoma kinase (ALK): Structure, oncogenic activation, and pharmacological inhibition. Pharmacol Res 2013;68:68–94.
4. Kivunen JP, Mermel C, Zejnullahu K et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008;14:4275–4283.
5. Lee Y, Jeon JH, Goh SH et al. The clinical impact of family history of cancer in female never-smoker lung. Lung Cancer 2019;136:15–22.
6. Kwak EL, Bang Y, Camidge DR et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 2010;363:1693–1703.
7. Ahmadzada T, Kao S, Reid G et al. An update on predictive biomarkers for treatment selection in non-small cell lung cancer. J Clin Med 2018;7:153.
8. Wu Y, Chang I, Wang C et al. Comparison of IHC, FISH and RT-PCR methods for detection of ALK rearrangements in 312 non-small cell lung cancer patients in Taiwan. PLoS One 2013;8: e70839.
9. Wallander ML, Geiersbach KB, Tripp SR et al. Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion-positive non-small cell lung carcinoma: Implications for optimal clinical testing. Arch Pathol Lab Med 2012;136:796–803.
10. Fleischhacker M, Schmidt B. Cell-free DNA resuscitated for tumor testing. Nat Med 2008;14:914–915.
11. Moulere F, Robert B, Arnaud Peyrotte E et al. High fragmentation characterizes tumour-derived circulating DNA. PLoS One 2011;6:e23418.
12. Jiang P, Chan CW, Cheng SH et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci USA 2015;112:E1317–E1325.
13. Diaz LA, Bardelli A. Liquid biopsies: Genotyping circulating tumor DNA. J Clin Oncol 2014;32:579–586.
14. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–437.
15. Wan JCM, Massie C, Garcia Corbacho J et al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;17:223–238.
16. Oxnard GR, Paveletz CP, Kuang Y et al. Non-invasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 2014;20:1698–1705.
17. Cohen JD, Javed A, Thoburn C et al. Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. Proc Natl Acad Sci USA 2017;114:10202–10207.
18. Lee H, Choi M, Jo M et al. Assessment of clinical performance of an ultrasensitive nanowire assay for detecting human papillomavirus DNA in urine. Gynecol Oncol 2020;120:461–466.
19. Lee H, Choi M, Lim J et al. Magnetic nanowire networks for dual-isolation and detection of tumor-associated circulating biomarkers. Theranostics 2018;8:505–517.
20. Lee H, Choi M, Hwang S et al. A versatile nanowire platform for highly efficient isolation and direct PCR-free colorimetric detection of human papillomavirus DNA from unprocessed urine. Theranostics 2018;8:399–409.
21. Sicard F, Destainville N, Rousseau P et al. Dynamical control of denaturation bubble nucleation in supercoiled DNA minicircles. Phys Rev E 2020;101:024403.
22. Paul T, Bera SC, Mishra P. Direct observation of breathing dynamics at the mismatch induced DNA bubble with nanometre

CONCLUSION

We demonstrated the clinical application of the novel PCR-free NW-based plasma cfDNA assay for detection of EML4-ALK fusion, classification of the variants, and for monitoring drug resistant mutations in patients with ALK-positive lung cancer. The NW-based cfDNA assay is highly sensitive and specific for plasma cfDNA genotyping and is feasible in terms of cost, time, and skill required; however, these findings need to be validated in larger prospective studies.

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AUTHOR CONTRIBUTIONS

Conception/design: Youngjoo Lee, Youngnam Cho, Ji-Youn Han
 Provision of study material or patients: Youngjoo Lee, Seong-Yun Park, Ji-Youn Han
 Collection and/or assembly of data: Youngnam Cho, Kum Hui Hwang
 Data analysis and interpretation: Youngjoo Lee, Youngnam Cho, Eun Young Park, Ji-Youn Han
 Manuscript writing: Youngjoo Lee, Youngnam Cho
 Final approval of manuscript: Youngjoo Lee, Youngnam Cho, Eun Young Park, Seong-Yun Park, Kum Hui Hwang, Ji-Youn Han

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accuracy: A smFRET study. Nanoscale 2017;9:5835–5842.

23. Gahlon HL, Walker AR, Cisneros GA et al. Reduced structural flexibility for an exonuclease deficient DNA polymerase III mutant. Phys Chem Chem Phys 2018;20:26892–26902.

24. Gainor JF, Dardaei L, Yoda S et al. Molecular mechanisms of resistance to first- and second-generation ALK inhibitors in ALK-rearranged lung cancer. Cancer Discov 2016;6:1118–1133.

25. Dehghannasiri R, Freeman DE, Jordanski M et al. Improved detection of gene fusions by applying statistical methods reveals oncogenic RNA cancer drivers. Proc Natl Acad Sci USA 2019;116:15524–15533.

26. Milbury CA, Li J, Liu P et al. Cold-PCR: Improving the sensitivity of molecular diagnostics assays. Expert Rev Mol Diagn 2011;11:159–169.

27. Sanders HR, Li H, Bruey J et al. Exon scanning by reverse transcriptase-polymerase chain reaction for detection of known and novel EML4-ALK fusion variants in non-small cell lung cancer. Cancer Genet 2011;204:45–52.

28. Takeuchi K, Choi YL, Soda M et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–6624.

29. Choi YL, Takeuchi K, Soda M et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 2008;68:4971–4976.

30. Sabir SR, Yeoh S, Jackson G et al. EML4-ALK variants: Biological and molecular properties, and the implications for patients. Cancers (Basel) 2017;9:118.

31. Woo CG, Seo S, Kim SW et al. Differential protein stability and clinical responses of EML4-ALK fusion variants to various ALK inhibitors in advanced ALK-rearranged non-small cell lung cancer. Ann Oncol 2017;28:791–797.

32. Lin J, Zhu VW, Yoda S et al. Impact of EML4-ALK variant on resistance mechanisms and clinical outcomes in ALK-positive lung cancer. J Clin Oncol 2018;36:1199–1206.

33. Yoshida T, Oya Y, Tanaka K et al. Differential crizotinib response duration among ALK fusion variants in ALK-positive non-small-cell lung cancer. J Clin Oncol 2016;34:3383–3389.

34. Dagogo Jack I, Rooney M, Lin J et al. Treatment with next-generation ALK inhibitors fuels plasma ALK mutation diversity. Clin Cancer Res 2019;25:6662–6670.

35. Sanchez-Herrero E, Provencio, Romero A. Clinical utility of liquid biopsy for the diagnosis and monitoring of EML4-ALK NSCLC patients. Adv Lab Med 2020;1:20190019.

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