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Cancer Diagnostics and Molecular Pathology

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

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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. ***The Oncologist*** 2021;26:e1683–e1692

Implications for Practice: The authors developed a novel one-step polymerase chain reaction–free nanowire (NW)-based plasma cell-free DNA (cfDNA) assay. This study evaluated the clinical utility of this novel method for the diagnosis of *EML4-ALK* fusion in advanced non-small cell lung cancer (NSCLC). The NW-based assay and FISH test showed high concordance rate in 99 patients with advanced NSCLC. Serial cfDNA assessment demonstrated this method provided early detection of resistance before radiologic progression during crizotinib treatment. Taken together, plasma cfDNA genotyping by the NW-based cfDNA assay may be useful for the rapid diagnosis of *ALK* fusion, classifying variants, 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

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Raf-MEK-ERK, *PI3K-Akt*, and *JAK3-STAT3* pathways and consequently triggers cancer cell growth, proliferation, and inhibition of apoptosis [2, 3]. This gene fusion has been identified in 2%–7% of non-small cell lung cancer (NSCLC) [4, 5]. 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, genotype testing 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 H_2O_2, 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*

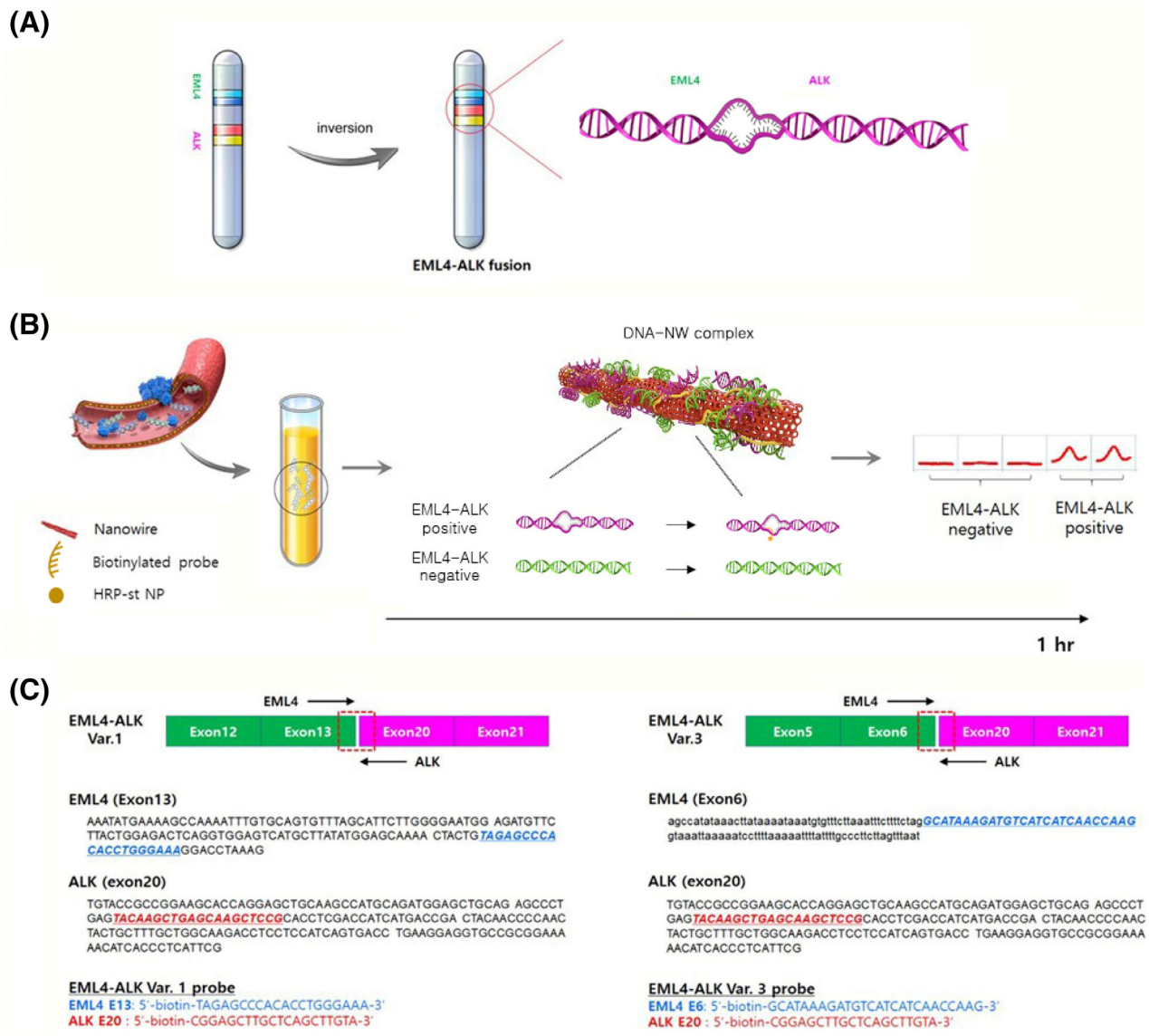

Figure 1. Schematic illustration of the NW-based assay for detection of the *EML4-ALK* fusion. **(A):** The *EML4-ALK* fusion is caused by a chromosomal rearrangement on the short arm of chromosome 2. We hypothesized that this inversion event would lead to chromosomal destabilization, thereby inducing 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.

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

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

To detect *EML4-ALK* fusion, PEI-NWs (5 μg/mL) and 150 μL 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 $\Delta OD_{650-500}$ of 0.007. The total experimental time for detecting *EML4-ALK* fusion by the NW-based cfDNA assay was ~60 minutes, and 3 μL 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)

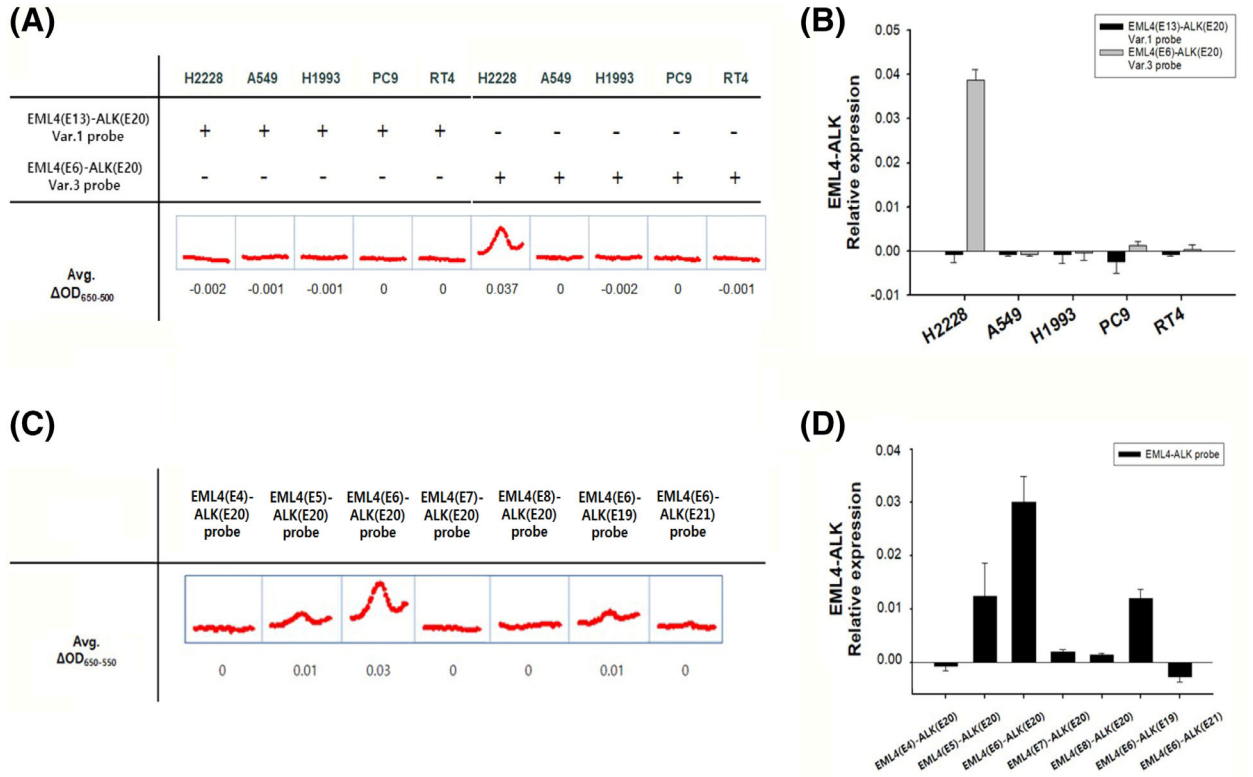

Figure 2. Validation of the nanowire (NW)-based assay for detecting *EML4-ALK* variants 1 and 3 in human plasma samples spiked with fragmented DNA (fDNA) from various cancer cell lines and EML4-ALK expression. **(A):** fDNA was prepared by sonication of genomic DNA (gDNA) from H2228, A549, H1993, and PC9 lung cancer and RT4 bladder cancer cell lines. Polyethyleneimine(PEI)-conjugated–NWs were added to plasma samples from healthy donors spiked with fDNA from various cell lines to form DNA-NW complexes. Subsequently, biotin-labeled probes (*EML4-ALK* Variant 1: exon 13 of *EML4* and exon 20 of *ALK* and *EML4-ALK*; Variant 3: exon 6 of *EML4* and exon 20 of *ALK*) and horseradish peroxidase-labeled streptavidin-conjugated nanoparticles (HRP-st NPs) were added to the DNA-NW complexes after short thermal denaturation at 95°C for 1 minute and the ODs for each probe were measured. The experiment was performed in triplicate. **(B):** Average $\Delta OD_{650-500}$ 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 $\Delta OD_{650-500}$ values for various EML4-ALK probes were plotted to show relative EML4-ALK expression.
Abbreviations: Avg. average; OD, optical density.

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 to induce local duplex opening near the fusion site, the relevant probes and HRP-st NPs are added to the DNA-NW complexes, and the absorption at 490–800 nm is measured. We designed two pairs of biotinylated probes for the two most common variants of *EML4-ALK* fusion: variant 1, exon 13 of *EML4* fused to exon 20 of *ALK*, and variant 3a/b, exon 6a/b of *EML4* fused to exon 20 of *ALK* (Fig. 1C).

Assay Feasibility

To demonstrate proof-of-concept for the NW-based cfDNA assay, we first conducted ex vivo experiments with five cancer

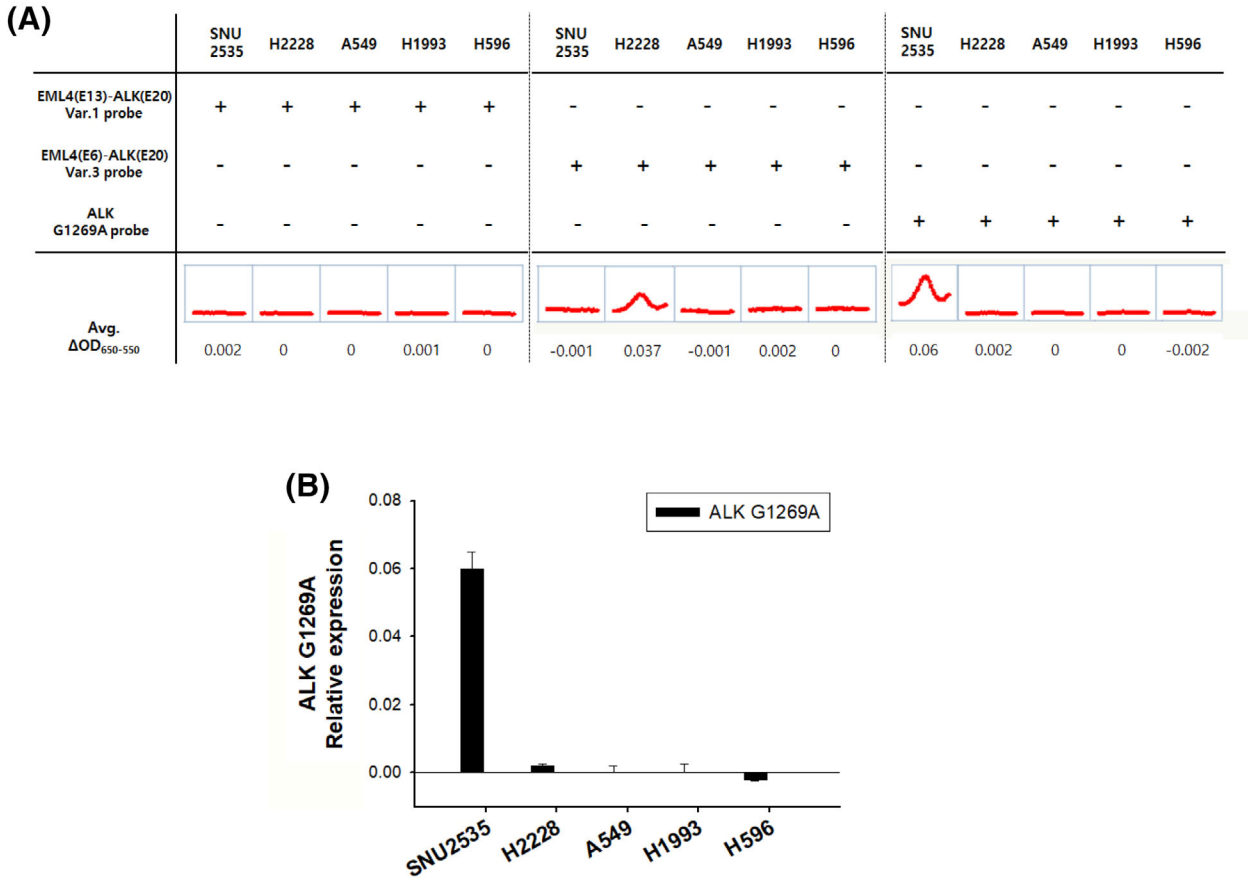

Figure 3. Identification of the resistance mutation ALK G1269A using the nanowire (NW)-based assay. **(A):** We prepared SNU2535 fragmented DNA (fDNA) by mechanically shearing genomic DNA obtained from SNU2535 lung cancer cells. Polyethyleneimine(PEI)-conjugated–NWs were added to plasma samples from healthy donors spiked with fDNA from various cell lines to form DNA-NW complexes. The experiment was performed in triplicate. **(B):** Average $\Delta OD_{650\text{-}500}$ values for the ALK G1269A probe were plotted to show relative EML4-ALK expression.
Abbreviation: OD, optical density.

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 (Fig. 2B). 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)

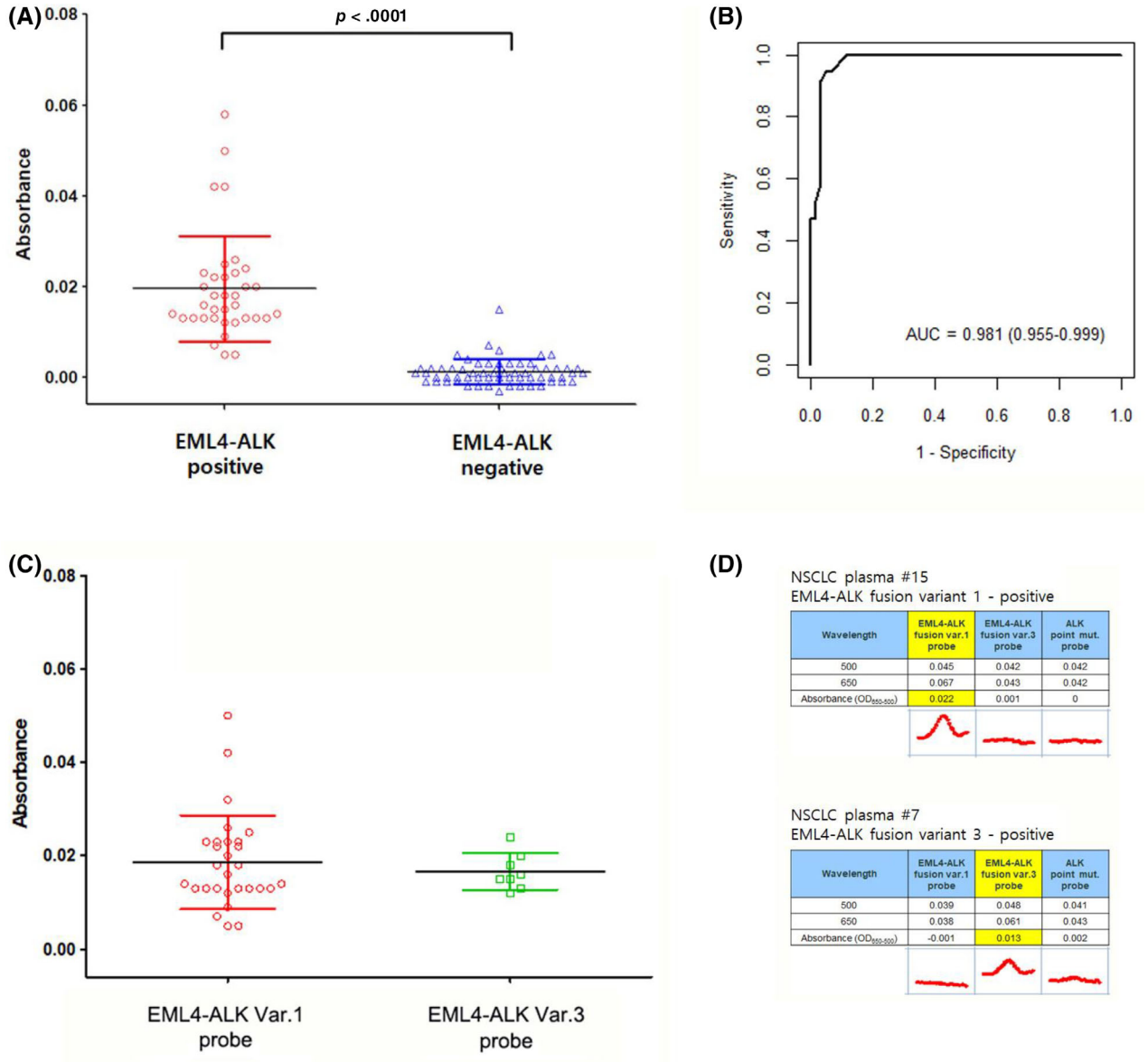

Figure 4. Nanowire (NW)-based detection of the EML4-ALK fusion in plasma from patients with NSCLC and differentiation of *EML4-ALK* variants. **(A):** The NW-based assay showed that the $\Delta OD_{650\text{-}500}$ signal for the *EML4-ALK*–positive group (average $\Delta OD_{650\text{-}500} = 0.020 \pm .012$) was higher ($p < .001$) than in the *EML4-ALK*–negative group ($\Delta OD_{650\text{-}500} = 0.001 \pm .002$). **(B):** ROC curves for the detection of *EML4-ALK* indicated that the AUC values for the *EML4-ALK* probes were 0.981 (95% confidence interval, 0.95–0.99). **(C):** Cell-free DNA was isolated from the plasma of patients with EML4-ALK fusion–positive lung adenocarcinoma ($n = 36$) to identify probe-dependent signals for the *EML4-ALK* variants. **(D):** The NW-based assay was able to detect *ALK* variants; patient #15 with NSCLC had the *EML4-ALK* variant 1 (E13:A20), whereas patient #7 had the *EML4-ALK* variant 3.
Abbreviations: AUC, area under the curve; NSCLC, non-small cell lung cancer; OD, optical density; var, variant.

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 1 and variant 3 *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% CI, 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 $\pm$ 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;

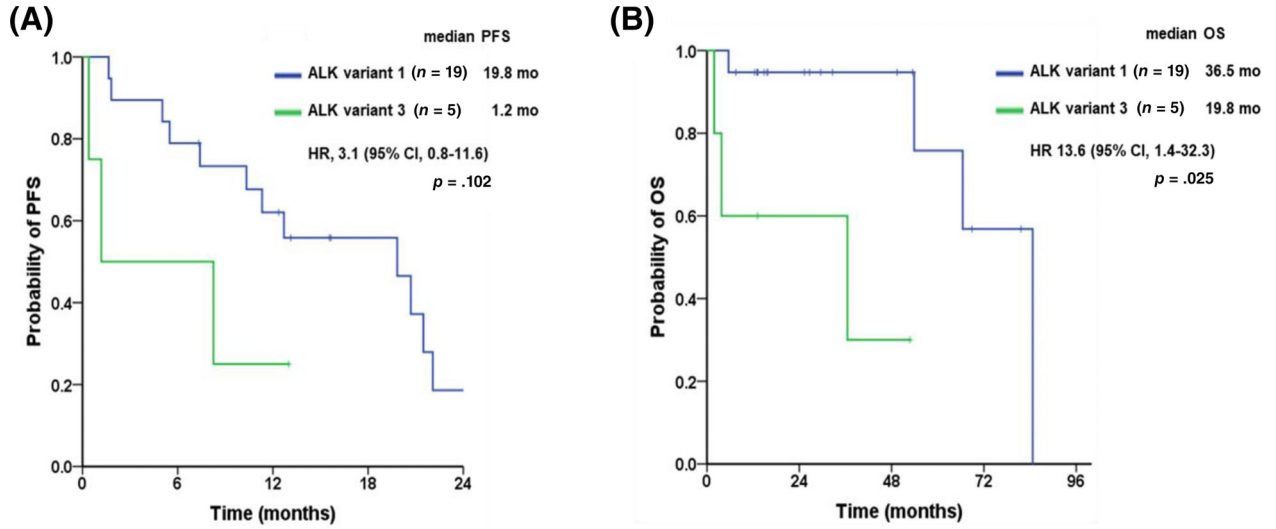

Figure 5. Kaplan-Meier curves of PFS **(A)** and OS **(B)** in patients with metastatic non-small cell lung cancer and *EML4-ALK* variants 1 and 3 who were treated with crizotinib as the first ALK inhibitor.
Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free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

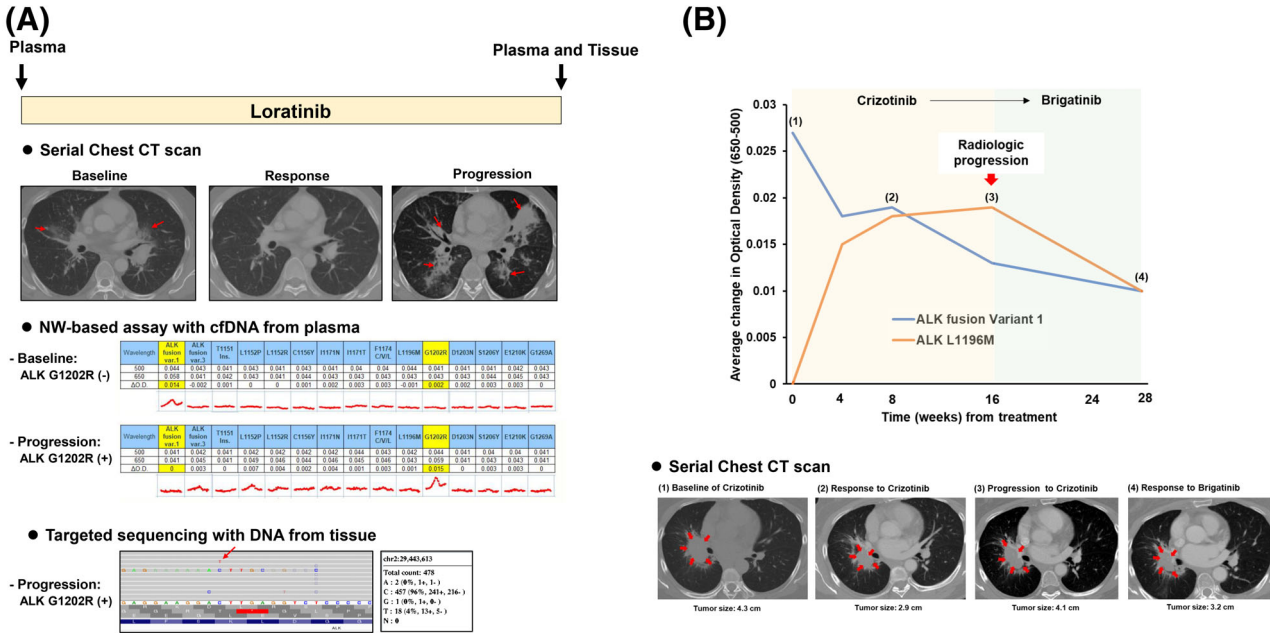

Figure 6. Assessment of *ALK* secondary resistant mutations using the NW-based assay. **(A):** The ALK G1202R mutation was detected by the NW-based assay and next-generation targeted sequencing using cfDNA and tissue, respectively, from a patient with ALK-positive lung adenocarcinoma treated with loratinib. **(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.

95% CI, 0.955–0.999; Fig. 4B). 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

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% 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 loratinib after disease progression with ceritinib. He showed a partial response to loratinib 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 loratinib 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 with brigatinib, the level of the *ALK* L1196M mutation in the plasma decreased and the tumor showed shrinkage. The patient continued to be treated with brigatinib.

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 megabases 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.

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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Disclosures

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