A Novel Mass Spectrometry–Based Assay for Diagnosis of *EML4-ALK*–Positive Non–Small Cell Lung Cancer

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Introduction: The presence of the transforming fusion gene echinoderm microtubule-associated protein–like 4 (*EML4*)–anaplastic lymphoma kinase (*ALK*) in non–small-cell lung cancer (NSCLC) is a predictive marker for the efficacy of anaplastic lymphoma kinase inhibitors. However, the currently available assays for the detection of the different variants of *EML4-ALK* have limitations.

Methods: We developed an assay system for the detection of *EML4-ALK* variants 1, 2, 3a, 3b, 4, 5a, 5b, 6, or 7 transcripts in total RNA obtained from formalin-fixed, paraffin-embedded (FFPE) specimens of NSCLC tissue. The assay is based on region-specific polymerase chain reaction amplification of *EML4-ALK* complementary DNA followed by specific single-base primer extension and analysis of the extension products by matrix-assisted laser desorption/ionization–time of flight mass spectrometry. The assay was validated by fluorescence in situ hybridization and the results confirmed by subcloning and sequencing of polymerase chain reaction products.

Results: Evaluation of the analytic sensitivity of the assay with serial dilutions of plasmids containing *EML4-ALK* complementary DNA sequences revealed it to be capable of the reliable detection of one copy of each plasmid per reaction. The assay also detected *EML4-ALK* variants 1 or 3 in three FFPE samples of surgically resected NSCLC shown to be positive for anaplastic lymphoma kinase rearrangement by fluorescence in situ hybridization. Furthermore, the assay identified variant 1 of *EML4-ALK* in 3 of 20 FFPE biopsy samples from patients with advanced NSCLC. All positive samples were confirmed by subcloning and sequencing.

Conclusions: Our novel assay is highly sensitive and effective for the detection of *EML4-ALK* in FFPE specimens.

Key Words: EML4-ALK, Non–small cell lung cancer, Paraffin-embedded tissue, Mass spectrometry, Diagnosis.

(*J Thorac Oncol.* 2012;7: 913–918)

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Conflict of interest: The authors declare no conflict of interest.

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ISSN: 1556-0864/12/913-918

Echinoderm microtubule-associated protein–like 4 (EML4)–anaplastic lymphoma kinase (ALK) was recently identified as a transforming fusion protein in lung cancer.[1] Both *EML4* and *ALK* genes are located on the short arm of chromosome 2. Multiple variants of *EML4-ALK* have been identified, with all variants including the same cytoplasmic portion of ALK but different portions of EML4 (with truncations at exons 2, 6, 13, 14, 15, 18, and 20).[2–4] The most common *EML4-ALK* variants are 1 and 3, which together account for ~60% of *EML4-ALK*–positive cases of non–small cell lung cancer (NSCLC).[5] Crizotinib, an inhibitor of the tyrosine kinase activity of ALK and MET (hepatocyte growth factor receptor), has been shown to be effective for the treatment of lung cancer patients harboring *EML4-ALK*.[6] Indeed, crizotinib was recently approved by the U.S. Food and Drug Administration for the treatment of advanced NSCLC in patients with an abnormal *ALK* gene.

The *EML4-ALK* translocation occurs in 5 to 10% of lung cancer patients.[1,2,7–10] Several assays have been developed for the detection of this translocation in clinical samples.[2,4,11] However, all of these assays have limitations with regard to sensitivity or the reliance on specialized techniques, with the development of higher-sensitivity assays based on simple methods being warranted.

MATERIALS AND METHODS

Plasmid Construction

Plasmids based on pCR2.1 or pcDNA3.1(+) and containing *EML4-ALK* or *ALK* complementary DNA (cDNA) were constructed as positive controls by TA cloning (Invitrogen, Madison, WI) or In-Fusion PCR cloning (Clontech, Palo Alto, CA).[12] Details of plasmid construction and corresponding primer sequences are provided in Supplemental Digital Content 1 (http://links.lww.com/JTO/A219) and 2 (http://links.lww.com/JTO/A220).

Clinical Samples

Formalin-fixed, paraffin-embedded (FFPE) samples were obtained from NSCLC patients who had undergone surgery or biopsy for the purpose of diagnosis at Kinki University Hospital. The study was approved by the Institutional Review Board with the conditions that samples

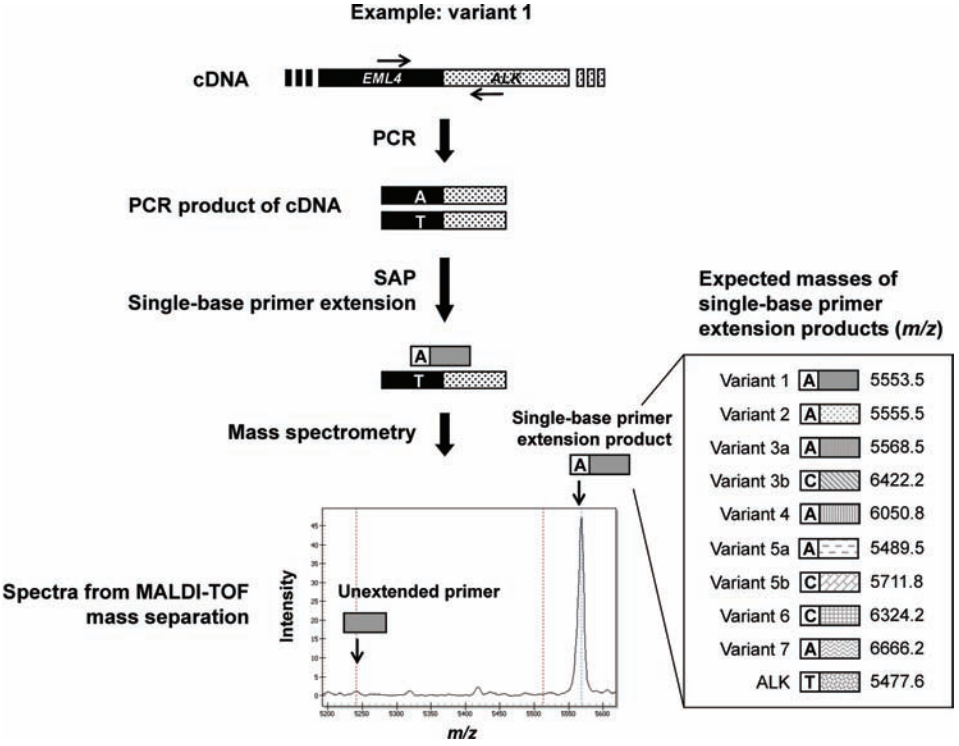

FIGURE 1. MassARRAY method for the detection of *EML4-ALK*. The region of *EML4-ALK* complementary DNA containing the fusion point is amplified by polymerase chain reaction (PCR), with the amplicons ranging in size from 70 to 130 bp. After dephosphorylation of the 5′ terminus of the PCR products with the use of shrimp alkaline phosphatase, a single-base primer extension reaction is performed. The primer extension products are then analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry . The single-base extension reaction occurs only when an *EML4-ALK* fusion product is present in the sample.

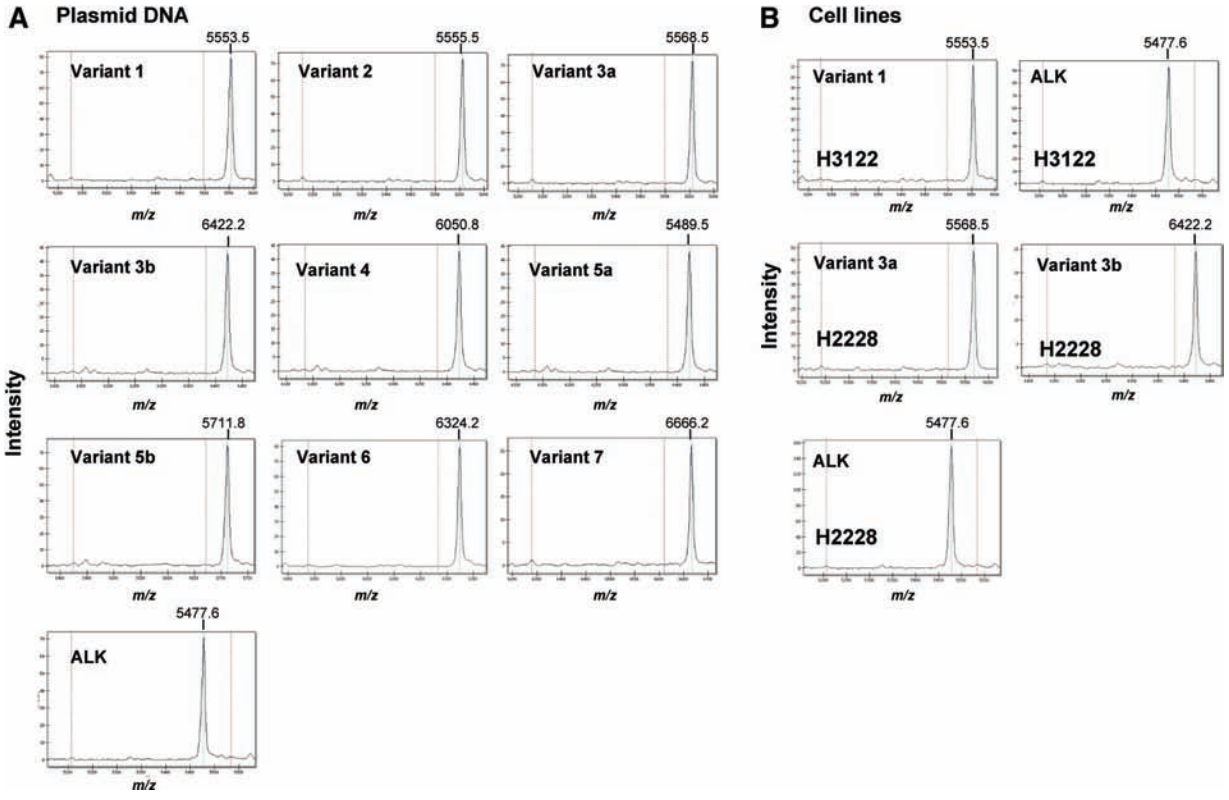

FIGURE 2. Representative spectra for *EML4-ALK* variants. Plasmid DNA (1 ng per reaction) containing the indicated variants of *EML4-ALK* (*A*) or total complementary DNA (5 ng per reaction) prepared from H3122 or H2228 cell lines (which harbor *EML4-ALK* variant 1 and variants 3a and 3b, respectively) (*B*) was subjected to the MassARRAY assay. Representative spectra for extension products between ~5100 and ~6800 Da are shown. The expected *m/z* values for each single-base extension product are as indicated in Figure 1. Three independent experiments were performed in triplicate with identical results.

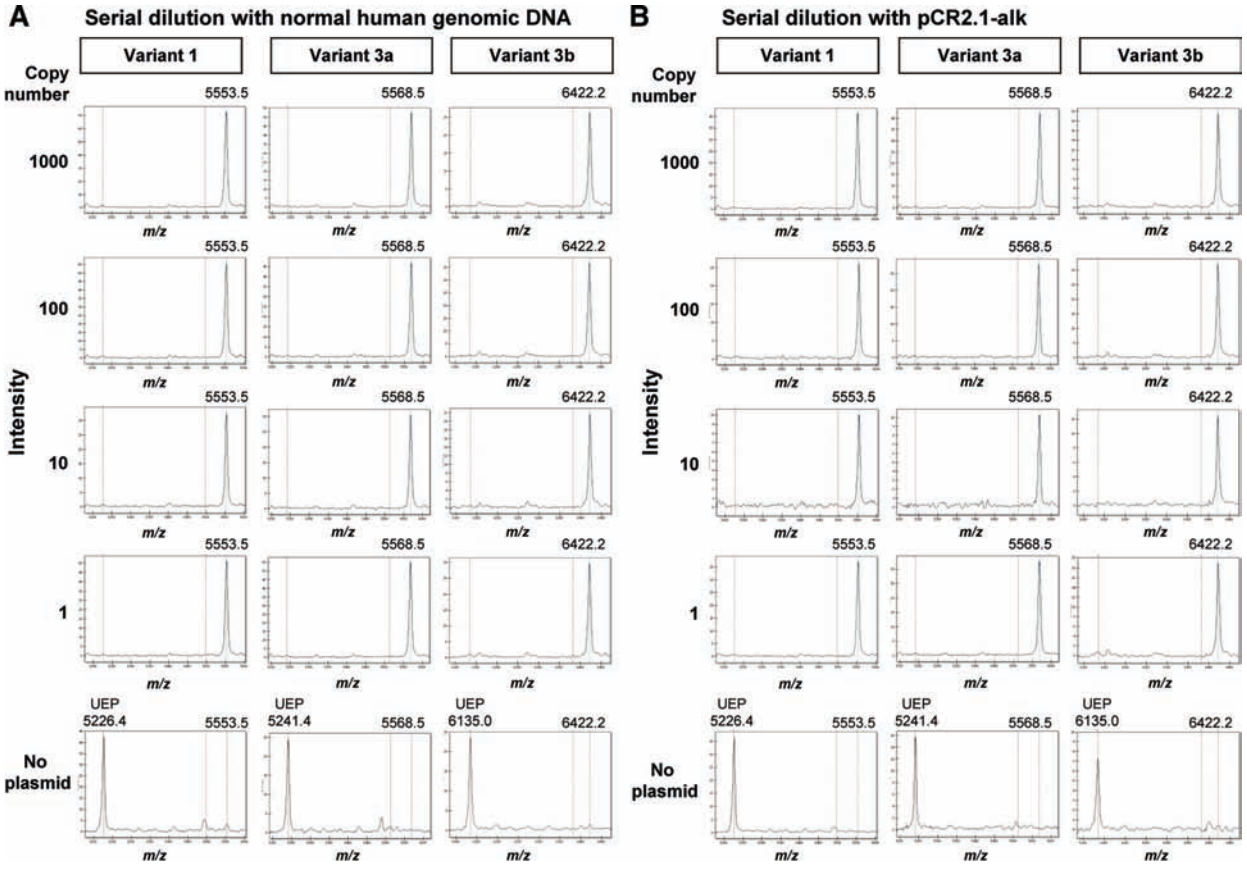

FIGURE 3. Sensitivity of *EML4-ALK* detection in plasmid DNA. The indicated copy numbers of pcDNA3.1(+) containing variants 1, 3a, or 3b of *EML4-ALK* were assayed in the presence of 1 ng of normal human genomic DNA (*A*) or 10 pg of pCR2.1-alk plasmid DNA (*B*). The copy number of plasmid DNA per reaction was calculated according to the formula: $6.022 \times 10^{23} \times$ [mass of DNA (g)]/[plasmid size (bp) $\times$ 660]. Three independent experiments were performed in triplicate with identical results. UEP, unextended primer.

be processed anonymously and analyzed for gene expression and that the study be disclosed publicly, according to the Ethical Guidelines for Human Genome Research published by the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare, and the Ministry of Economy, Trade, and Industry of Japan. The study also conforms to the provisions of the Declaration of Helsinki.

Cell Lines

The human NSCLC cell lines H2228 and H3122 were kindly provided by Dr. P. A. Jänne (Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA). Short tandem repeat analysis was performed with genomic DNA isolated from H2228 and H3122 cells. The DNA profile of H2228 cells matched that in the American Type Culture Collection short tandem repeat database. The cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Kerrville, TX).

Sample Processing

Total RNA was extracted from cell lines and FFPE samples with the use of an RNeasy Mini Kit (Qiagen, Valencia, CA) and an RNeasy FFPE Kit (Qiagen), respectively. The percentage of tumor cells in each FFPE specimen was evaluated by hematoxylin-eosin staining and found to be >10%. The isolated RNA was subjected to reverse transcription (RT) with the use of a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and the resulting cDNA was stored at –80°C until analysis.

Detection of *EML4-ALK*

We used the MassARRAY iPLEX platform (Sequenom, San Diego, CA) for detection of *EML4-ALK*. The MassARRAY system involves a three-step process consisting of polymerase chain reaction (PCR), single-base primer extension, and separation of the products on a matrix-loaded silicon chip by matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry (MS) (Fig. 1). PCR and the single-base primer extension reactions were performed in a thermal cycler (ABI-9700 instrument, Applied Biosystems), and the extension products were analyzed using the MALDI–TOF MS

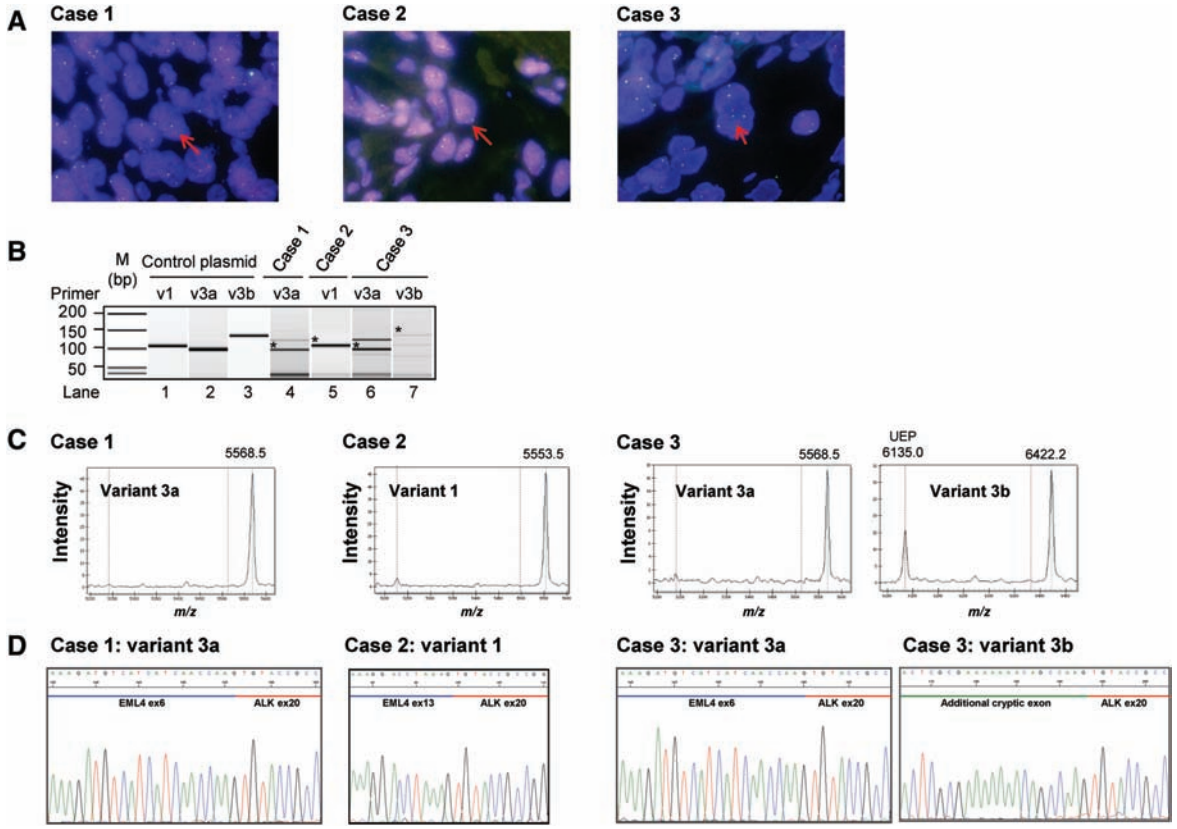

FIGURE 4. Detection of *EML4-ALK* in formalin-fixed, paraffin-embedded (FFPE) samples of surgically resected non–small cell lung cancer tissue. (*A*) Clinical specimens were subjected to fluorescence in situ hybridization analysis with differentially labeled probes for the 5′ (green) or 3′ (red) regions of the *ALK* locus. A pair of split signals (arrows) indicates the rearranged *ALK* locus. (*B*) polymerase chain reaction (PCR) products derived with PCR primer sets in the MassARRAY assay were separated with the use of an Agilent 2100 Bioanalyzer and a DNA 1000 LabChip Kit (Agilent Technologies, Inc., Palo Alto, CA). The PCR products are from pcDNA3.1(+)-v1 (lane 1), pcDNA3.1(+)-v3a (lane 2), pcDNA3.1(+)-v3b (lane 3), case 1 (lane 4), case 2 (lane 5), and case 3 (lanes 6 and 7). The asterisk indicates the corresponding band in each case. (*C*) Total RNA (10 ng) isolated from the FFPE samples in (*A*) was analyzed by the MassARRAY assay. Representative spectra are shown. The experiment was repeated twice with identical results. (*D*) The PCR products from (*C*) were also subcloned and sequenced. ex, exon.

(Sequenom). Detailed procedures and corresponding primer sequences are provided in Supplemental Digital Content 1 (http://links.lww.com/JTO/A219) and 3 (http://links.lww.com/JTO/A221), respectively.

Sequencing Analysis

PCR products were subcloned into the TOPO TA pCR2.1 vector (Invitrogen) and sequenced with an automated sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) with the use of M13 universal primers.

Fluorescence In Situ Hybridization Analysis

FFPE tissue sectioned at a thickness of 4 μm and placed on glass slides was subjected to fluorescence in situ hybridization (FISH) with a break-apart probe for the *ALK* gene (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Des Plaines, IL). FISH positivity was defined as the presence of >15% split signals in tumor cells.

RESULTS

Detection of *EML4-ALK* Variants

We developed an assay system based on the MassARRAY platform for the detection of *EML4-ALK* variants in NSCLC tissue (Fig. 1). The MassARRAY assay detected plasmid DNA corresponding to nine different *EML4-ALK* variants and wild-type *ALK* (Fig. 2*A*). Appropriate mass spectra for the single-base extension products were thus obtained (Figs. 1 and 2*A*). We next attempted to detect *EML4-ALK* in the human NSCLC cell lines H3122 and H2228, which harbor *EML4-ALK* variants 1 and 3, respectively.[7] The RT-PCR products of total RNA isolated from the cell lines were subjected to the MassARRAY assay. *EML4-ALK* variant 1 was detected in H3122 cells, whereas variants 3a and 3b were detected in H2228 cells (Fig. 2*B*). Wild-type *ALK* was also detected in both cell lines.

Sensitivity of the Assay

The major variant forms of *EML4-ALK* are variants 1, 3a, and 3b, which account for ~60% of all *EML4-ALK* fusions.[7]

TABLE 1. Characteristics of Patients Screened for *EML4-ALK*

No.	Age (years)	Sex	Histology	Procedure	Smoking status	*EML4-ALK*
1	63	F	Ad	TLB	Never	Negative
2	62	M	Ad	TBLB	Current	Negative
3	74	M	Ad	TLB	Current	Negative
4	71	M	Ad	TBLB	Current	Negative
5	63	F	Ad	TBLB	Never	Variant 1
6	75	M	Ad	TBLB	Former	Negative
7	59	M	Ad	Needle biopsy	Current	Negative
8	85	M	Ad	TBLB	Current	Negative
9	73	F	Ad	Lymph node biopsy	Never	Negative
10	68	F	Ad	TBLB	Current	Negative
11	65	M	Ad	TBLB	Current	Variant 1
12	57	M	Ad	TBLB	Current	Negative
13	68	F	Ad	TLB	Never	Negative
14	73	M	Ad	TBLB	Former	Negative
15	67	F	Ad	TBLB	Never	Negative
16	67	M	Ad	TBLB	Former	Negative
17	57	M	Ad	TBLB	Former	Negative
18	56	M	Large	TBLB	Current	Negative
19	65	M	Ad	TBLB	Current	Negative
20	60	M	Ad	TBLB	Current	Variant 1

Ad, adenocarcinoma; Large, large cell carcinoma; TLB, thoracoscopic lung biopsy; TBLB, transbronchial lung biopsy.

We therefore examined the sensitivity of our assay for detection of these variants. Samples containing 1000, 100, 10, or 1 copy of plasmid DNA [pcDNA3.1(+) for variants 1, 3a, or 3b] and 1 ng of normal human genomic DNA (Promega, Madison, WI) were prepared by 10-fold serial dilution. MS spectra revealed a clear peak at the expected size for variants 1, 3a, or 3b in all samples, including those containing only one copy of the variant DNA (Fig. 3*A*). We also examined assay sensitivity when the samples were diluted with pCR2.1 containing *ALK* cDNA (pCR2.1-alk). Samples containing 1000, 100, 10, or 1 copy of the variant plasmids were thus diluted with 10 pg (~2 $\times$ 10^6 copies) of pCR2.1-alk. A clear peak at the expected size was again detected in all the samples including those containing only one copy of the variant DNA (Fig. 3*B*). These results therefore suggested that the sensitivity of the assay for the detection of *EML4-ALK* variants is high.

Detection of *EML4-ALK* Variants in FFPE Samples

We next evaluated the ability of our assay to detect *EML4-ALK* variants in three surgically resected FFPE samples of NSCLC shown to be positive for *ALK* rearrangements by FISH analysis (Fig. 4*A*). The primary PCR products for the assay were also analyzed by gel electrophoresis, with the expected sizes of the amplification products being 115, 103, and 119 bp for variants 1, 3a, and 3b, respectively. Bands corresponding to *EML4-ALK* variant 1 (case 2), variant 3a (cases 1 and 3), and variant 3b (case 3) were detected (Fig. 4*B*). Similarly, we detected MS peaks corresponding to *EML4-ALK* variant 1 and to variants 3a or 3b in case 2 and in cases 1 and 3, respectively (Fig. 4*C*). The presence of *EML4-ALK* variants in these three cases was confirmed by subcloning and sequencing of PCR products (Fig. 4*D*). We further analyzed several NSCLC specimens shown to be negative for *EML4-ALK* by FISH analysis; no mass peaks for *EML4-ALK* variants were detected by the MassARRAY assay (data not shown). These results thus suggested that the MassARRAY assay is able to detect *EML4-ALK* variants in FFPE samples.

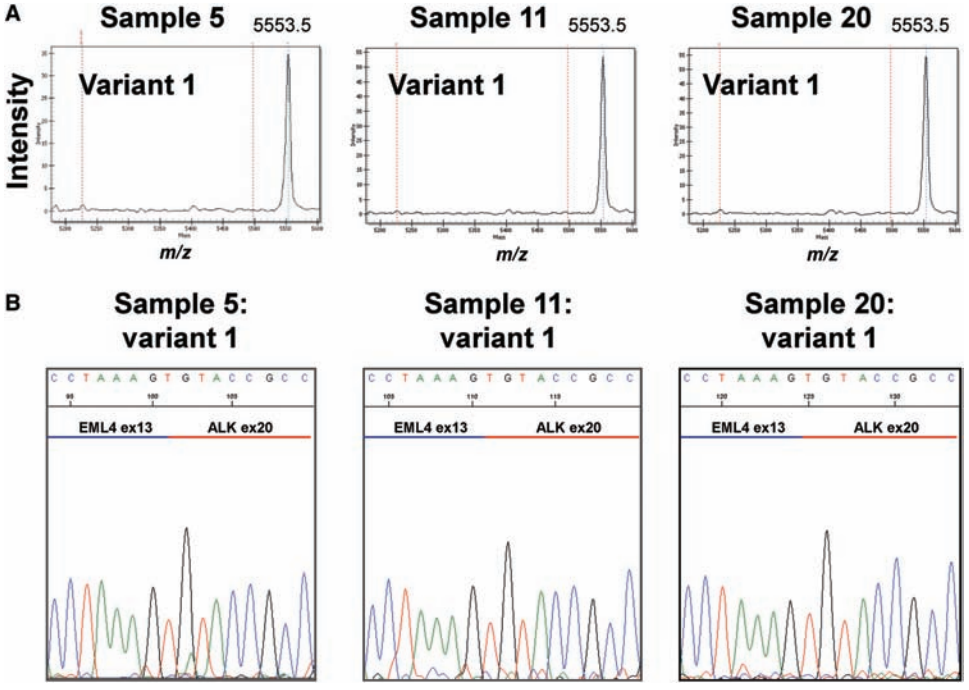

FIGURE 5. Detection of *EML4-ALK* in formalin-fixed, paraffin-embedded samples of non–small cell lung cancer obtained by transbronchial lung biopsy. (*A*) Representative spectra of *EML4-ALK*–positive samples. The experiments were repeated twice with identical results. (*B*) The polymerase chain reaction products from (*A*) were subcloned and sequenced.

Screening for *EML4-ALK* Variants in FFPE Biopsy Samples of Advanced NSCLC

Molecular analysis of biopsy samples from patients with advanced NSCLC is often difficult because of the small amount of tissue available. We therefore examined the feasibility of detection of *EML4-ALK* variants with the MassARRAY assay in FFPE biopsy specimens obtained from 20 patients with advanced NSCLC, the characteristics of whom are shown in Table 1. Three specimens (samples 5, 11, and 20) were found to be positive for variant 1 of *EML4-ALK*, with none of the other variants being detected (Fig. 5*A*). The presence of *EML4-ALK* variant 1 in these three cases was confirmed by subcloning and sequencing of PCR products (Fig. 5*B*). We also performed FISH analysis on the 20 FFPE specimens. Of the three *EML4-ALK*–positive cases detected by the MassARRAY assay, two (samples 5 and 20) were positive by FISH whereas the third (sample 11) could not be evaluated because of poor tissue quality (data not shown). None of the cases that tested negative by the MassARRAY assay was positive by FISH. These results thus suggested that our assay is effective for *EML4-ALK* screening with FFPE biopsy specimens of advanced NSCLC.

DISCUSSION

We have developed a MassARRAY assay for screening of *EML4-ALK* in FFPE samples of NSCLC tissue. This novel assay is capable of detecting nine different *EML4-ALK* variants with a high sensitivity.

Dual-color split-signal FISH analysis has been considered the gold standard in screening for *ALK* rearrangement. However, given that *EML4* and *ALK* loci are located in relatively close proximity on chromosome 2p, the detection of the *EML4-ALK* fusion gene on the basis of the gap between the probes is sometimes difficult. The identification and counting of signals on tissue sections is impeded by factors such as cutting artifacts and nuclear overlap related to the thickness and homogeneity of the tissue sections and the size of the nuclei. The MassARRAY system is a nucleic acid analysis platform which utilizes a three-step process composed of PCR amplification, single-base primer extension, and MALDI–TOF MS analysis. The presence of *EML4-ALK* in our MassARRAY assay is detected on the basis of the presence of products of predicted mass.

The amount of tumor tissue available for molecular analysis is often limited in patients with advanced NSCLC. Furthermore, RNA extracted from FFPE samples is highly degraded and less amenable to RT-PCR analysis compared with that isolated from nonfixed, freshly frozen tissue. We succeeded in amplifying *EML4-ALK* cDNA by PCR from FFPE tissue with primers designed to yield short amplicons (70–130 bp). The design of such short amplicons can also be applied to quantitative PCR systems such as amplification-refractory mutation system for allele-specific PCR assays. In our system, PCR amplification was performed with a high number of cycles (45 cycles), and increased accumulation of PCR products conferred higher sensitivity. We tested 20 FFPE biopsy samples from patients with NSCLC with our assay and detected a clear mass peak for *EML4-ALK* variant 1 in 3 of these samples, each of which was confirmed positive for this variant by subcloning and sequencing of the PCR products. In contrast to FISH, our assay is able to distinguish between the different *EML4-ALK* variants in a small amount of FFPE NSCLC tissue, and it should prove to be a useful tool for the detection of *EML4-ALK* variants in diagnostic testing for this fusion gene.

ACKNOWLEDGMENTS

Supported by the Third-Term Comprehensive 10-Year Strategy for Cancer Control of the Ministry of Education, Culture, Sports, Science, and Technology of Japan as well as by Health and Labor Scientific Research Grants [20-9].

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