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

Kazuko Sakai, PhD,* Isamu Okamoto, MD, PhD,† Ken Takezawa, MD, PhD,† Tomonori Hirashima, MD,‡ Hiroyasu Kaneda, MD, PhD,† Masayuki Takeda, MD, PhD,† Kazuko Matsumoto, MD, PhD,* Hideharu Kimura, MD, PhD,* Yoshihiko Fujita, PhD,* Kazuhiko Nakagawa, MD, PhD,† Tokuzo Arao, MD, PhD,* and Kazuto Nishio, MD, PhD*

**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, 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)

**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). 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.
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% CO₂ 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 RNaseasy 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.
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. 2A). Appropriate mass spectra for the single-base extension products were thus obtained (Figs. 1 and 2A). 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. 2B). 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
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. 3A). 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 × 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. 3B). 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. 4A). 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. 4B). 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. 4C). The presence of EML4-ALK variants in these three cases was confirmed by subcloning and sequencing of PCR products (Fig. 4D). 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.

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

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. 3A). 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 × 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. 3B). 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. 4A). 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. 4B). 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. 4C). The presence of EML4-ALK variants in these three cases was confirmed by subcloning and sequencing of PCR products (Fig. 4D). 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. 5A). The presence of EML4-ALK variant 1 in these three cases was confirmed by subcloning and sequencing of PCR products (Fig. 5B). 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].

REFERENCES