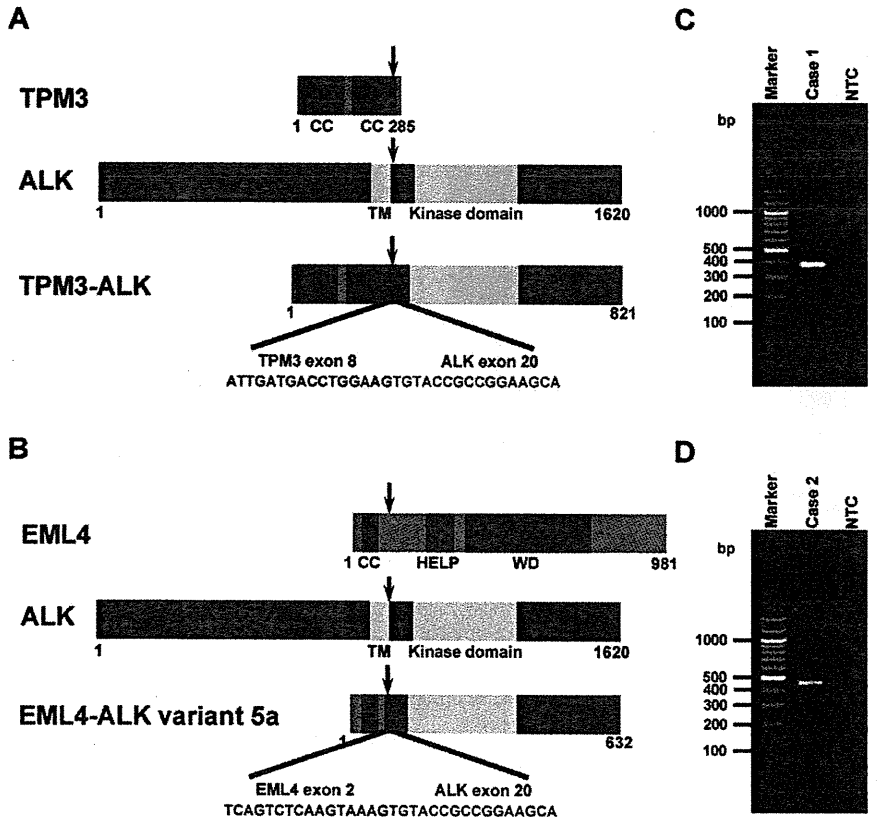

Figure 3. Identification of anaplastic lymphoma kinase (ALK) fusions. Tropomyosin 3 (TPM3) harbors 2 coiled-coil domains. (A) Case 1. A chromosome translocation generates a fusion protein in which the 2 coiled-coil domains of TPM3 and the intracellular region of ALK (containing the tyrosine kinase domain) are conserved. (B) Nucleotide sequencing of the polymerase chain reaction (PCR) products in case 2 revealed that exon 2 of echinoderm microtubule-associated protein like 4 (EML4), comprising a coiled-coil domain, was fused to exon 20 of ALK, generating the variant 5 complementary DNA (cDNA). In TPM3 and EML4 fusions, the region containing the coiled-coil domain is fused to the kinase domain of ALK. Numbers indicate amino acid positions of each protein. Arrow indicates the chromosomal breakpoint. The cDNA fragments of 385 base pairs (bp) and 454 bp were obtained by reverse transcription PCR, corresponding to (C) *TPM3-ALK* and (D) *EML4-ALK* variant 5, respectively. The left lane ("Marker") contains DNA size standards (100-bp ladder). CC indicates coiled-coil domain; HELP, hydrophobic echinoderm microtubule-associated protein; NTC, no-template control; TM, transmembrane domain; WD, WD repeats.

She underwent a translumbar left-radical nephrectomy and is currently alive and well without evidence of disease at 2 years of follow-up.

Case 2

A 53-year-old woman was found incidentally to have microscopic hematuria by medical check-up. Ultrasonography and magnetic resonance imaging showed a change in the left kidney, but the diagnosis was indefinite at that time. One year later, adenocarcinoma cells were detected by urinary cytology, and computed tomography revealed an isodence left renal mass (2.5 cm × 2.5 cm × 2.3 cm). The patient underwent a translumbar left-radical nephrectomy. She is currently alive and well at 7 years after surgery.

The patients had no episodes or family history indicative of sickle cell trait. To the best of our knowledge, there is no reported case of (genetically) Japanese individuals with sickle cell trait/disease.

Histopathological Examinations

The 2 ALK-positive renal cancers were papillary subtype and unclassified (with mixed features of papillary, mucinous cribriform, and solid patterns with rhabdoid cells). They comprised 2.3% of non–clear cell RCCs (2 of 88) and 3.7% of non–clear cell and nonchromophobe RCCs (2 of 54).

Case 1

Histologically, tumor cells were composed of papillary, tubular, or cribriform growth of cuboidal cells with

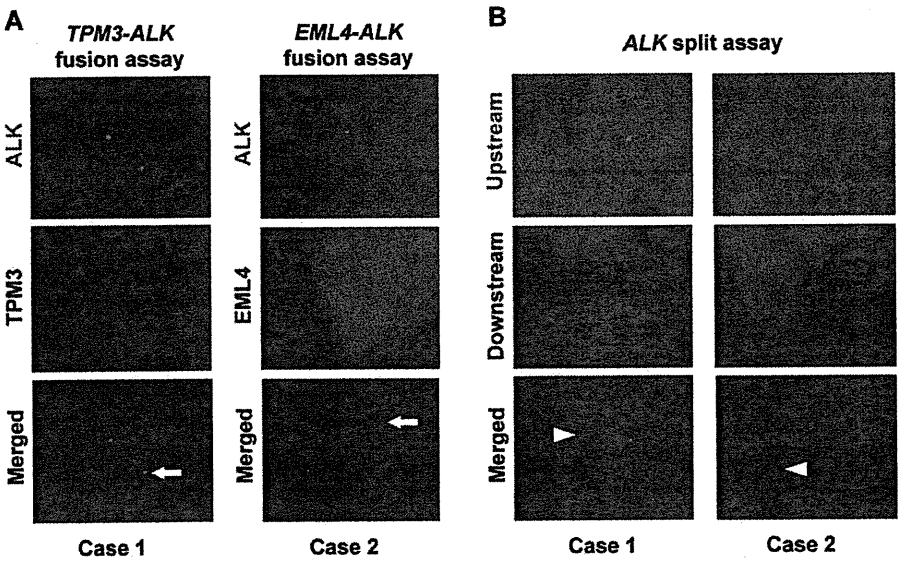

Figure 4. Fluorescence in situ hybridization analyses for *TPM3-ALK* (tropomyosin 3 fusion with anaplastic lymphoma kinase) and *EML4-ALK* (echinoderm microtubule-associated protein like 4 fusion with ALK). (A) In the *TPM3-ALK* and *EML4-ALK* fusion assays, the fusion genes are indicated by arrows. (B) The same clinical specimens as in (A) were subjected to fluorescence in situ hybridization analysis with differentially labeled probes for the upstream (green) or downstream (red) to the ALK breakpoint. In each case, the absence of 1 upstream signal indicated ALK rearrangement. Arrowhead indicates the rearranged ALK. The color of fluorescence for the bacterial artificial chromosome clones and the case numbers are indicated. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole.

eosinophilic cytoplasm. The cribriform morphology consisted of tubular structures with flattened epithelial cells, compressed by mucinous pool and inter- or intracytoplasmic vacuoles. Solid sheets of tumor cells with occasional deeply eosinophilic intracytoplasmic inclusions and eccentric nuclei, resulting in rhabdoid features, were focally identified. Nuclei were round to ovoid, and the nuclear size was basically uniform. Irregular nuclear membranes and nuclear grooves were occasionally observed. Mitotic figures were scant. The background stroma in the tumor area possessed abundant mucin. Frequent deposition of psammoma bodies and infiltration of numerous foamy macrophages were also seen. A large amount of mucinous matrix was highlighted with Alcian blue stain. These histological features resembled the mucinous cribriform pattern frequently observed in ALK-positive lung adenocarcinoma,[18,31] and also a representative case of unclassified RCC by Lopez-Beltran et al,[32] favoring a diagnosis of unclassified RCC. Immunohistochemically, neoplastic cells showed a diffuse and strong positivity for ALK (iAEP), vimentin, EMA, cytokeratin 7, AE1/AE3, cytokeratin CAM5.2, and cytokeratin 34βE12, and focally staining for PAX2, PAX8, AMACR, and CD10. TTF1 and RCC Ma were completely negative. Intracytoplasmic inclusions corresponded to aggregates of intermediate filaments of vimentin. The ALK-staining pattern appeared to be accentuated around the cell membrane of rhabdoid cells. The MIB1 (mindbomb homolog 1) labeling index was less than 1%.

Case 2

Histologically, the tumor consisted of papillary configuration of cuboidal or low columnar cells, with eosinophilic cytoplasm and small uniform round to oval nuclei. A clear cell change was focally seen. Nuclei showed a round to oval shape, and nuclear grooves were frequently observed. The size variation of nuclei was minimal, and the irregularity of the nuclear membrane was evident. Nuclear pseudoinclusions were seldom seen. Small nucleoli were occasionally identified, but mitoses were absent. The fibrovascular cores of papillary architecture contained numerous psammoma bodies and foamy macrophages. In addition, glandular lumens of tumor cells focally contained myxoid materials. These findings morphologically corresponded to papillary RCC, but did not fit to types 1 and 2 by the classification of Delahunt and Eble.[33] In contrast, the features resembled papillary RCC, type 2A, described by Yang et al.[34] Alcian blue stain highlighted a small amount of stromal-type mucin. Upon immunohistochemical analysis, neoplastic cells were diffusely and

strongly positive for ALK (iAEP), vimentin, EMA, cytokeratin 7, AE1/AE3, cytokeratin CAM5.2, cytokeratin 34βE12, and AMACR, and focally positive for PAX2 and PAX8, but negative for TTF1, CD10, and RCC Ma.

Examinations of Other Gene Aberrations

For *MET*, a cDNA fragment with the predicted size was obtained by RT-PCR in case 1. In case 2, no products were identified, indicating that the tumor of the patient did not express *MET*. No mutations were identified in case 1 by sequencing. TFE3 split signals were not observed in either of the 2 cases by FISH.

DISCUSSION

Recently, 2 independent groups have reported vinculin-ALK (VCL-ALK) in renal cancer (Table 1).[35,36] These findings broaden the spectrum of ALK fusion–positive tumors. Interestingly, the 2 patients described in the reports share several uncommon backgrounds for renal cancer: very early onset (6- and 16-year-old boys), a history of sickle cell trait, and uncommon histopathological subtypes (medullary subtype and indeterminate subtype with mixed features of medullary, chromophobe, and transitional cell subtypes). In this study, we screened 355 renal tumors, including 343 RCCs, and identified ALK fusions in 2 RCCs. Significantly, we identified ALK fusions in adult patients (36- and 53-year-old females) without sickle cell trait. This finding will provide a key to ALK inhibitor therapy for more common renal cancers.

RCC associated with *TFE3* gene fusions is already a distinctive entity in the World Health Organization classification,[37,38] and *MET* mutation has been described in 13% of sporadic papillary RCCs.[39] In the present study, we identified neither *MET* nor *TFE3* aberrations in our ALK-positive renal cancer cases. *ALK* rearrangements are recognized as almost mutually exclusive to other mutations such as *EGFR* (epidermal growth factor receptor) and *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene) in lung cancer.[6,40] All of the tumor cells in the 2 ALK-positive renal cancers observed by immunohistochemistry expressed ALK fusion protein, suggesting that all tumor cells harbor one or more *ALK* fusion genes. Therefore, as well as other ALK-positive tumors, *ALK* rearrangement in renal cancer probably occurs at a very early phase of carcinogenesis, and is likely to be a driver mutation and mutually exclusive to other driver mutations. As in the case of ALK-positive ALCL, ALK-positive renal cancer will be a distinct molecular pathological entity.

TPM3-ALK was first identified in ALCL in 1999,[41] and subsequently found in IMT in 2000.[5] Therefore, RCC is the third type of cancer that may harbor TPM3-ALK. The organ distribution of EML4-ALK is somewhat controversial. Since its discovery, EML4-ALK has been reported to be identified in lung, breast, and colon cancers. Many research groups have reported the presence of EML4-ALK in a small subset of lung adenocarcinomas (2%-10%). Interestingly, a group in the United States reported the presence of EML4-ALK in breast (5 of 209) and colorectal (2 of 83) cancers, identified by RT-PCR optimized for variants 1, 2, and 3, without showing histopathological evidence.[42] In contrast, 2 Japanese groups examined these cancers (90 breast and 96 colon cancers by RT-PCR for EML4-ALK variants 1 and 2, and 48 breast and 50 colon cancers by multiplex RT-PCR for all possible fusions), but detected no positive cases.[30,43] One possible reason for this discrepancy may be differences in ethnicity. In the present study, we showed histopathological features of the 2 ALK-positive renal cancers. In addition to morphology, the positivity of PAX2 and PAX8 and the negativity of TTF1 strongly indicated that the ALK-positive cancers of the present cases were primary RCCs, and not metastatic lesions of ALK-positive lung cancer.

The oncogenic activities of TPM3-ALK and EML4-ALK have previously been documented,[30,44] and therefore we did not demonstrate them in the present study. As in the case of other ALK-positive tumors, ALK-positive renal cancer is a promising candidate disease for ALK inhibitor therapy. In the present study, we screened surgically removable cases; the prognoses for the 2 ALK-positive patients were good, without recurrence. To realize the full potential of ALK inhibitors in renal cancers, it is important to identify the detailed clinicopathological features of ALK-positive cases, especially those of advanced or recurrent cases, by large-scale screening. For this purpose, anti-ALK immunohistochemistry can most readily be carried out as a primary screening tool. However, caution is needed; the screening immunohistochemical assay should be appropriately sensitive, because our present findings indicate that renal cancer involves EML4-ALK, which is barely detectable by conventional immunohistochemistry methods.[13,45]

Is morphology a clue to the presence of ALK fusion in renal cancers? Almost all ALK-positive lung cancers are adenocarcinomas, and more frequently show mucinous cribriform patterns and signet-ring cells than do ALK-negative adenocarcinomas.[18,31,46] ALK fusion is probably very rare in clear cell RCC, which is the most common

Table 1. ALK-Positive Renal Cancers: Present Cases and Review of Literature

Characteristic	VCL-ALK (Debelenko et al[36])	VCL-ALK (Marino-Enriquez et al[35])	TPM3-ALK (Case 1)	EML4-ALK (Case 2)
Age, y	16	6	36	53
Sex	Male	Male	Female	Female
Ethnicity	African American	African American	Japanese	Japanese
Past history	Sickle cell trait	Sickle cell trait	Tuberculosis (22 y old)	Pleomorphic adenoma (50 y old)
Karyotype	Abnormal complex karyotype	46,XY,t(2;10)(p23;q22), add(14)(p11)	Not examined	Not examined
Symptom	Right flank pain, gross hematuria	Intermittent periumbilical pain, hematuria	Pyelonephritis	Microscopic hematuria
Stage	Stage III	Stage I	Stage I	Stage I
Follow-up	9 mo, alive. No evidence of disease	21 mo, alive. No evidence of disease	2 y, alive. No evidence of disease	3 y, alive. No evidence of disease
Gross findings	6.5-cm irregularly shaped solid tumor mass with infiltrative borders centered in the right renal medulla	4.5-cm irregularly spherical mass with lobulated, fleshy light tan appearance centered in the medulla	4.0 cm × 4.0 cm × 3.5 cm irregularly shaped solid tumor with expansive borders centered in the cortex	Double cancer. A: 2.5 cm × 2.5 cm ×2.3 cm solid yellow tumor in the cortex of the left intermediate pole. B: 0.6-cm yellow mass in the cortex of the left inferior pole
Microscopic findings	Diffuse sheet-like pattern; round, oval, and polygonal tumor cells; eosinophilic cytoplasm; moderately polymorphic and vesicular nuclei	Solid growth pattern; spindle-shaped cells with large vesicular nuclei; clear coarse chromatin and abundant eosinophilic cytoplasm	Papillary, tubular, or cribriform growth of cuboidal cells with eosinophilic cytoplasm. Nuclei round to ovoid; nuclear size basically uniform	A: Papillary structure of cuboidal or low columnar cells with eosinophilic cytoplasm and small uniform round to oval nuclei. B: Clear cell
Immunohistochemistry	Positive: AE1/AE3, CAM5.2, CK7, EMA, INI1, TFE3. Negative: CD10, S100, HMB45, WT1	Positive: AE1/AE3, CAM5.2, EMA	Positive: ALK, vimentin, EMA, cytokeratin 7, AE1/AE3, CAM5.2, 34βE12, AMACR (focal), CD10 (focal), PAX2 (focal), PAX8 (focal). Negative: TTF1, RCC Ma	A: Positive: ALK, vimentin, EMA, cytokeratin 7, AE1/AE3, CAM5.2, 34βE12, AMACR, PAX2 (focal), PAX8 (focal). Negative: CD10, TTF1, RCC Ma
Diagnosis	Renal cell carcinoma, indeterminate subtype (medullary, chromophobe, transitional cell carcinoma mixed)	Renal medullary carcinoma	Renal cell carcinoma, unclassified	A: Papillary renal cell carcinoma, type 2A. B: Clear cell renal cell carcinoma

ALK indicates anaplastic lymphoma kinase; EML4, echinoderm microtubule–associated protein like 4; TPM3, tropomyosin 3; VCL, vinculin.

subtype of renal cancer; 2 previously reported cases with VCL-ALK were not clear cell RCC,[35,36] and we identified no ALK-positive cases in 255 clear cell RCCs in this study. Interestingly, case 1 showed a mucinous cribriform pattern. This may be a characteristic feature of ALK-positive carcinomas, universally applicable to carcinomas of various organs. Further study with a larger number of cases is warranted.

Molecular-targeted therapy of advanced renal cancers is starting to realize its full potential. However, complete remission is rarely achieved, because no agent targets a key molecule associated with "oncogene addiction" of renal cancer. In this context, ALK fusion constitutes a promising advance in renal cancers, as has previously been demonstrated with various other types of cancer. In the present study, we identified 2 adult cases of ALK-positive renal cancer in patients without uncommon backgrounds. Our findings confirm the potential of ALK inhibitor therapy for RCC. More detailed clinicopathological features of ALK-positive renal cancers, especially at higher clinical stages, are desirable. Hunting the "ALKoma" in various types of carcinomas, as well as in lung and kidney cancer, will provide an answer to these pathological and clinical questions.

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CONFLICT OF INTEREST DISCLOSURE

Dr. Takeuchi is a scientific advisor for the anti-ALK iAEP immunohistochemistry kit (ALK Detection Kit, Nichirei Bioscience, Tokyo, Japan). All remaining authors have made no disclosures.

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ALK fusion gene positive lung cancer and 3 cases treated with an inhibitor for ALK kinase activity

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ABSTRACT

Background: Anaplastic lymphoma kinase (ALK) fusion gene-positive lung cancer accounts for 4–5% of non-small cell lung carcinoma. A clinical trial of the specific inhibitor of ALK fusion-type tyrosine kinase is currently under way.

Methods: ALK fusion gene products were analyzed immunohistochemically with the materials obtained by surgery or by endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA). The echinoderm microtubule-associated protein-like 4(EML4)-ALK or kinesin family member 5B (KIF5B)-ALK translocation was confirmed by the reverse transcription polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). After eligibility criteria were met and informed consent was obtained, 3 patients were enrolled for the Pfizer Study of Crizotinib (PF02341066), Clinical Trial A8081001, conducted at Seoul National University.

Results: Out of 404 cases, there were 14 of EML4-ALK non-small cell carcinoma (NSCLC) and one KIF5B-ALK NSCLC case (8 men, 7 women; mean age, 61.9 years, range 48–82). Except for 2 light smokers, all patients were non-smokers. All cases were of adenocarcinoma with papillary or acinar subtypes. Three were of stage IA, 5 of stage IIIA, 1 of stage IIIB and 6 of stage IV. Ten patients underwent thoracotomy, 3 received chemotherapy and 2 only best supportive care (BSC). One BSC and 2 chemotherapy cases were enrolled for the clinical trial. Patients with advanced stages who received chemotherapy or best supportive care were younger (54.0 ± 6.3) than those who were surgically treated (65.8 ± 10.1) ($p < 0.05$).

The powerful effect of ALK inhibitor on EML4-ALK NSCLC was observed. Soon after its administration, almost all the multiple bone and lymph node metastases quickly disappeared. Nausea, diarrhea and the persistence of a light image were the main side effects, but they diminished within a few months.

Conclusion: ALK-fusion gene was found in 3.7% (15/404) NSCLC cases and advanced disease with this fusion gene was correlated with younger generation. The ALK inhibitor presented in this study is effective in EML4-ALK NSCLC cases. A further study will be necessary to evaluate the clinical effectiveness of this drug.

1. Introduction

As the mechanisms of carcinogenesis become clearer, the target of cancer treatment is shifting from non-specific cytotoxic agents to specific agents that block key molecular events in the carcinogenesis of malignancy such as EGFR-TKI and anti-HER2 antibody (trastuzumab) [1–3]. Recently, Mano et al. [4–6] reported that a small inversion within chromosome 2p results in the formation of a fusion gene comprising portions of the

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echinoderm microtubule-associated protein-like 4 (EML4) gene and the anaplastic lymphoma kinase (ALK) gene in non-small-cell lung cancer. Transgenic mice that express EML4-ALK specifically in lung epithelial cells develop multiple foci of adenocarcinoma in the lung soon after birth, and the oral administration of a specific inhibitor of ALK tyrosine kinase activity eradicated completely the foci of adenocarcinoma. Clinical trials of specific inhibitors of EML4-ALK tumors are currently underway [7–11]. Kwak et al. [11] reported the effect of crizotinib in Clinical Trial A8081001 on the 82 patients with advanced ALK-positive disease. Over a mean treatment duration of 6.4 months, the overall response rate was 57% and the estimated probability of 6-month progression-free survival was 72%. We report 15 cases of ALK fusion gene-positive NSCLC cases and 3 cases in our experience with ALK inhibitor in the Pfizer Study of crizotinib (PF02341066), Clinical Trial A8081001, which was conducted at Seoul National University.

2. Materials and methods

Out of 404 patients who had undergone surgical resection (295 cases) or bronchoscopy (109 cases) in Chiba Cancer Center, Japan, from 2007 to 2009, 15 ALK fusion gene-positive NSCLC patients were initially screened by immunohistochemical procedures. Diagnoses were confirmed by RT-PCR and/or FISH for their molecular translocation.

2.1. ALK fusion protein detection by immunohistochemical methods

The intercalated antibody-enhanced polymer method of Takeuchi et al. [12,13] was used to detect ALK proteins. Formalin-fixed paraffin-embedded tissue was sliced at a thickness of 4 μm and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 40 min at 97 °C in target Retrieval Solution (pH 9.0; Dako). They were then incubated at room temperature, first with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 min, and then with an anti-ALK antibody (5A4, Abcam) for 30 min. To increase the sensitivity of detection, we included an incubation step of 15 min at room temperature with rabbit polyclonal antibodies to mouse immunoglobulin (Dako). The immune complexes were then detected with the dextran polymer reagent and an AutoStainer instrument (Dako).

2.2. Confirmation of EML4-ALK fusion gene by RT-PCR and FISH

We confirmed the existence of ALK fusion gene expression by fluorescence in situ hybridization (FISH) and/or by the reverse transcription-polymerase chain reaction (RT-PCR).

2.3. Fluorescence in situ hybridization (FISH)

An EML4-ALK fusion assay was performed [10–12]. Unstained sections were processed with a Histology FISH Accessory Kit (Dako), subjected to hybridization with fluorescence-labeled bacterial artificial chromosome clone probes for EML4 and ALK (self-produced probes; EML4: RP11-996L7, ALK: RP11-984I21 and RP11-62B19), stained with 4,6-diamidino-2-phenylindole, and examined with a fluorescence microscope (BX51; Olympus). The FISH positivity criteria specified "over 50% cancer cells" for EBUS-TBNA samples.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

The multiplex PCR method proposed by the Japanese ALK lung cancer study group (ALCAS) was used to confirm the expression of ALK fusion gene [4–6].

Table 1
Characteristics of ALK fusion gene positive lung cancer patients.

Patient no	Sex	Age	SI	Histology	Variant	p Stage	Therapy	Recurrence	Distant meta	Survival (M)	Prognosis	ALK inhibitor case no
1	f	64	0	Ad: papillary	3	IIIA	Surgery	Positive	Bone, brain	21	Dead	
2	m	82	0	Ad: solid	2	IIIA	Surgery	Positive	Ascites	36	Alive	
3	f	68	0	Ad: papillary	3	IIIB	Surgery	Positive	Brain	34	Alive	
4	f	60	0	Ad: solid	3	IIIA	Surgery	Negative	None	29	Alive	
5	m	73	0	Ad: acinar	3	IA	Surgery	Negative	None	21	Alive	
6	m	66	0	Ad: papillary	KIF5B	IA	Surgery	Negative	None	15	Alive	
7	m	56	300	Ad: papillary	1	IA	Surgery	Negative	None	13	Alive	
8	m	46	0	Ad: acinar	5	IIIA	Surgery	Negative	None	22	Alive	
9	m	71	0	Ad: papillary	1	IIIA	Surgery	Negative		17	Alive	
10	f	73	0	Ad: acinar	1	IV	Surgery	Negative		14	Alive	
11	m	55	100	Ad: muc+	3	IV	BSC		Bone, brain	5	Dead	
12	m	48	0	Ad: muc+	1	IV	Chemo		Bone, brain	29	Dead	Case 1
13	f	49	0	Ad: muc+	3	IV	BSC		Bone, brain	15	Alive	Case 2
14	f	54	0	Ad: muc+	1	IV	Chemo		Bone, brain, pul	22	Alive	Case 3
15	f	64	0	Ad: acinar	3	IV	Chemo		Pul	2	Alive	

SI, smoking index; f, female; m, male; Ad, adenocarcinoma; muc+, mucin production; Distant meta, at the recurrence (surgery group) at the diagnosis (non-surgery group); pul, pulmonary metastasis; Case 1 was already reported by Nakajima et al. [16].

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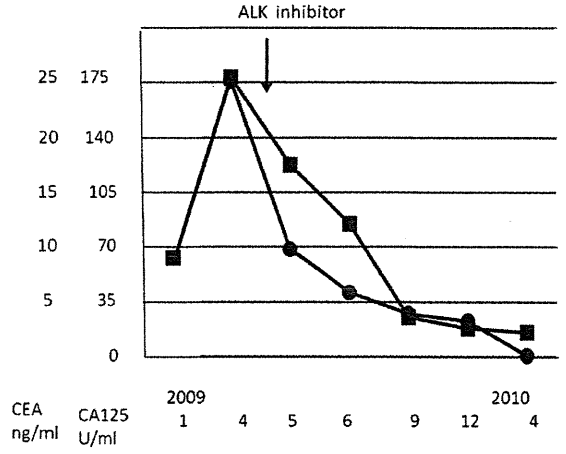

Fig. 1. Changes of tumor markers before and during the treatment with ALK inhibitor (Case 1) CEA (■), CA125 (●). Marked reduction of tumor markers was observed.

Total RNA was isolated from EBUS-TBNA or surgical samples using AllPrep DNA/RNA Mini Kit (Qiagen) and was reverse-transcribed into single strand cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). To detect a fusion cDNA derived from EML4 or KIF5B and ALK, PCR analysis was performed with the AmpliTaq Gold PCR Master Mix (Applied Biosystems), the forward primers derived from EML4, EA-F-cDNA-S (5′-GTGCAGTGTTTAGCATTCTTGGGG-3′), EA-F-2-g-S (5′-AGCTACATCACACACCTTGACTGG-3′), EA-F-cDNA-v3-S-2 (5′-TACCAGTGCTGTCTCAATTGCAGG-3″) and EA-W-cDNA-in-S (5′-GCTTTCCCCGCAAGATGGACGG-3′) and the forward primers derived from KIF5B, KA-F-cDNA-S-e24 (5′-CAGCTGAGAGAGTGAAAGCTTTGG-3′), KA-F-cDNA-S-e17 (5′-GACAGTTGGAGGAATCTGTCGATG-3′), KA-F-cDNA-S-e11 (5′-ATCCTGCGGAACACTATTCAGTGG-3′), and KA-cDNA-S-e2 (5′-TCAAGCACATCTCAAGAGCAAGTG-3′) and the reverse primer derived from ALK, EA-F-cDNA-A (5′-TCTTGCCAGCAAAG-CAGTAGTTGG-3′). PCR products were purified from gel bands using QIAquick Gel Extraction Kit (Qiagen) and confirmed by direct sequencing analysis.

2.5. Enrolment of patients for the Clinical Trial A8081001

Informed consent was obtained from each patient to be enrolled for the study [10]. Eligibility criteria for the enrolment of ALK translocation positive patients into the ALK TKI PI Trial were as required by the Committee of Clinical Trials A8081001.

3. Results

There were 15 ALK fusion gene-positive cases which were screened immunohistochemically and confirmed by RT-PCR and FISH [14,15]. Eight patients were men and 7 women, of mean age

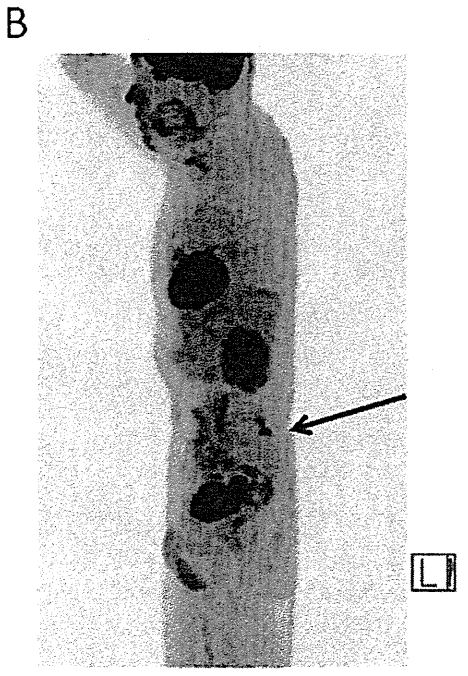

Fig. 2. FDG-PET scan of Case 1 performed at the same time (09/28/2009) as the previously reported Fig. 1D (Nakajima et al. [16]) shows bone metastasis of the left vertebral arch of L5 (arrow) in a sagittal view.

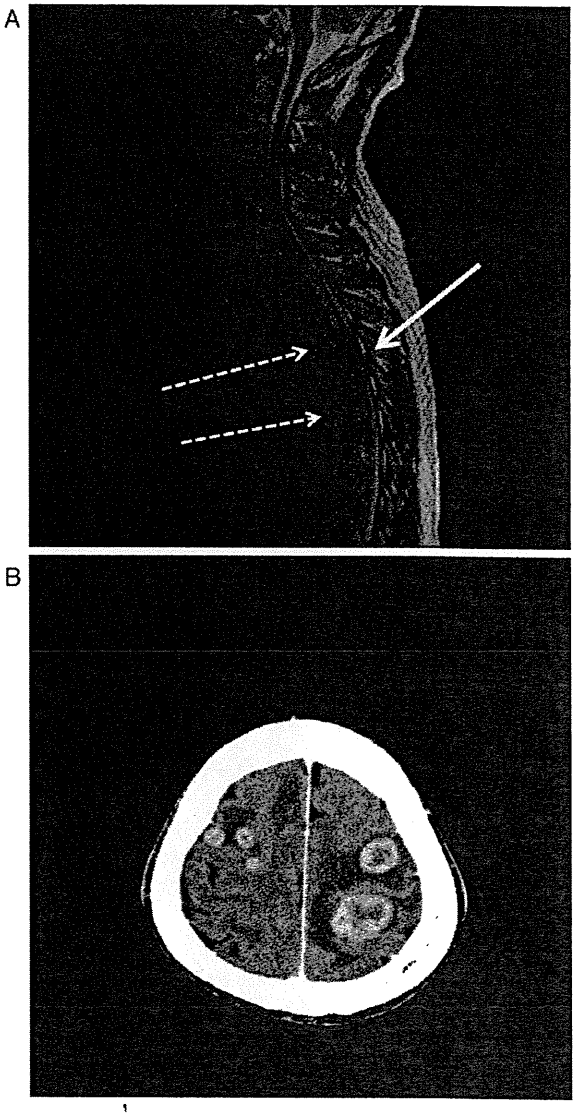

Fig. 3. MRI (Case 1) of the spinal cord on 04/05/2010 shows the metastases to the spinal cord (straight allow) and the spinal column (Th 4,6 dotted allow). B. CT scan (Case 1) of the brain on 04/05/2010 shows multiple brain metastases.

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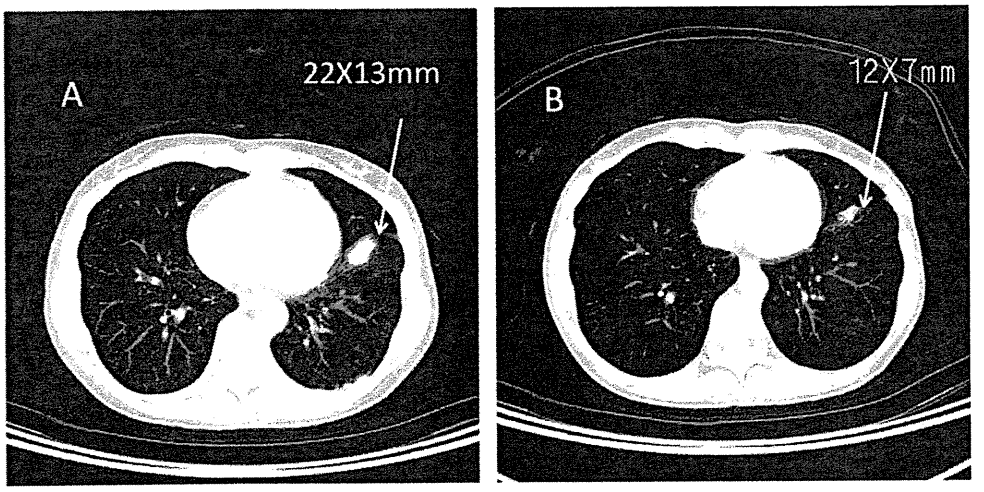

Fig. 4. CT scan (Case 2): A, 07/22/2009 (before ALK inhibitor) and B, 09/02/2009 (5 weeks after the initiation of the therapy). Left S8 tumor (arrow) decreased in size from 22X13 mm to 12X7 mm (PR).

61.9 years (range 48–82). Most were non-smokers, but 2 smoked lightly (Table 1). All tumors were adenocarcinomas with a papillary pattern predominant (5 cases), an acinar pattern predominant (3 cases), with mucin production (4 cases), etc. There were fourteen cases of fusion with EML4 and one KIF5B gene. There were 7 variant 3, 5 variant 1, and 1 each of variants 2 and 5. There were 3 stage IA, 5 stage IIIA, 1 stage IIIB and 6 stage IV cases. Ten cases were diagnosed after surgical resection, and 5, by tissue samples obtained with EBUS-TBNA. Ten cases underwent thoracotomy, 3 cases, chemotherapy, and 2 cases, only best supportive care. Of 5 cases diagnosed by EBUS-TBNA, 2 cases receiving chemotherapy and one receiving best supportive care were enrolled for the clinical trial. The mean age of the surgically treated group was 65.8 ± 10.1, and that of chemotherapy and BSC group was 54.0 ± 6.3. The difference was found by Student's t test to be statistically significant ($p < 0.05$), indicating that younger patients tend to have advanced cancer.

Out of 10 surgically treated cases, seven survived without a sign of recurrence, 3 had recurrence in both bone and brain tissue, and one died of bone and lymph node metastasis.

3.1. Case 1

Case 1 has already been reported in a case report (Nakajima et al.) [16] but without precise descriptions of the response to crizotinib, the adverse effects, the pattern of recurrence or the metastatic

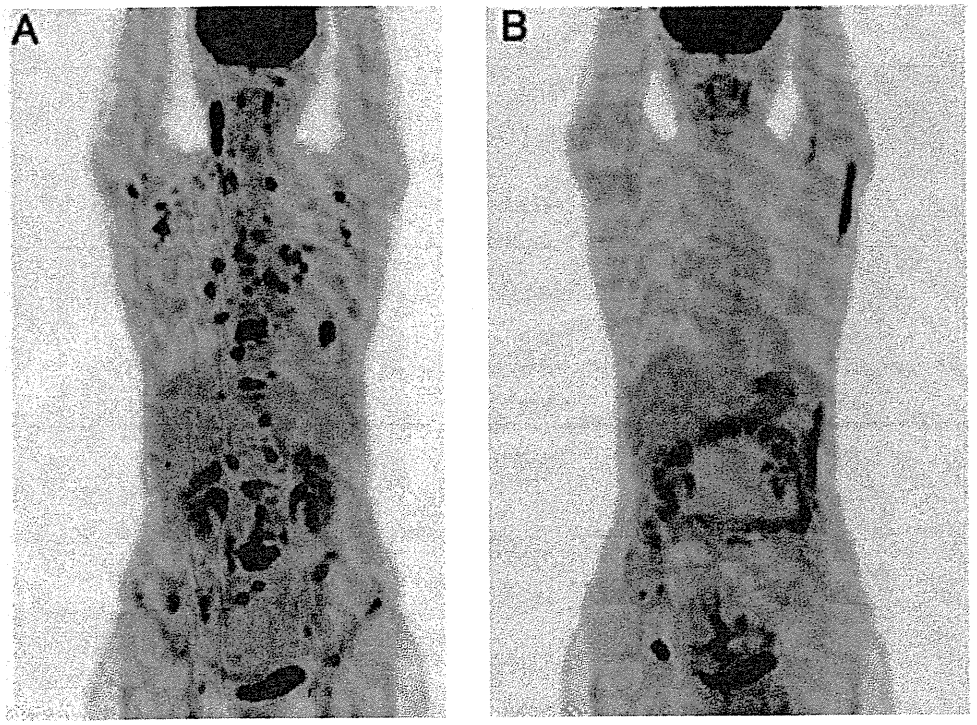

Fig. 5. FDG-PET scan (Case 2): A, 07/22/2009 (before ALK inhibitor) and B, 03/10/2010 FDG-PET scan shows marked reduction of accumulation in multiple bone and lymph node metastases 7 months after the initiation of the treatment.

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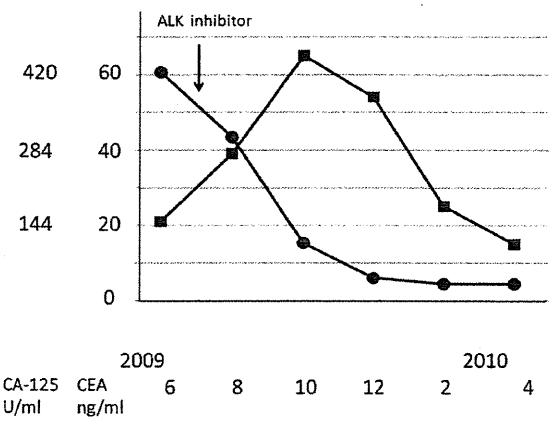

Fig. 6. Changes of tumor markers before and during the treatment with ALK inhibitor in case 2. CA125 (●) gradually decreased along with the treatment, but CEA (■) increased soon after the initiation of the therapy. The value of CEA then gradually decreased to 15.2 ng/ml in April 2010 (after 10 months).

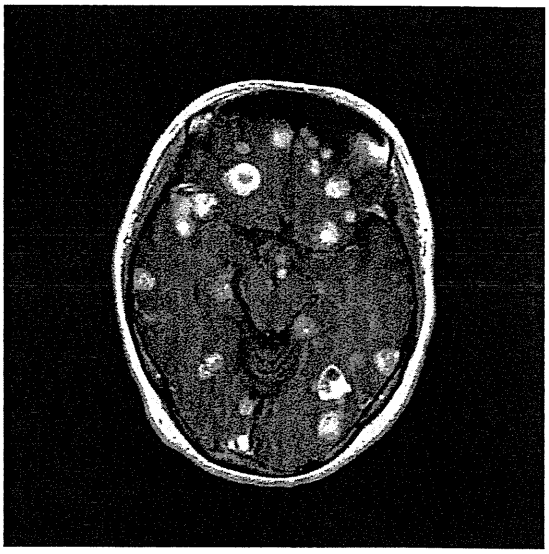

Fig. 7. Brain MRI of case 2 on 7/30/2010 showing multiple metastases.

tumor lesions. Such descriptions may contribute to a better understanding of the other cases, and so case 1 is described briefly below.

A 48-year-old non-smoking male patient had lung adenocarcinoma in the right lower lobe and multiple bone and lymph node metastases (T3N2M1 stage IV) at his first medical examination in November 2007. After several courses of chemotherapy, the patient was enrolled in a trial of crizotinib (PF02341066) from May 5th 2009 at Seoul National University, in which the drug was orally administered at 500 mg/day.

The effect of ALK inhibitor appeared rapidly. The patient's dyspnea improved within one week after drug administration. PS improved from 2 to 0 and a marked reduction in the tumor markers was observed (Fig. 1). Within 3 months after the start of therapy, almost all metastases disappeared except for those at the left vertebral arch of L5 (Fig. 2, arrow). The patient had severe adverse effects: diarrhea, nausea and persistence of light images started soon after the administration of the drug, but these gradually diminished over a 3-week period.

The control of the primary and metastatic tumors continued for 11 months until the patient visited Seoul University in April 2010, when he was hospitalized for paralysis of the lower extremities. MRI revealed spinal column (Th4-6) and spinal cord metastases (Fig. 3A). Soon after his hospitalization in our Cancer Center in April 2010, multiple brain metastases (Fig. 3B) were found, so the drug administration was stopped and he was transferred to a palliative care unit.

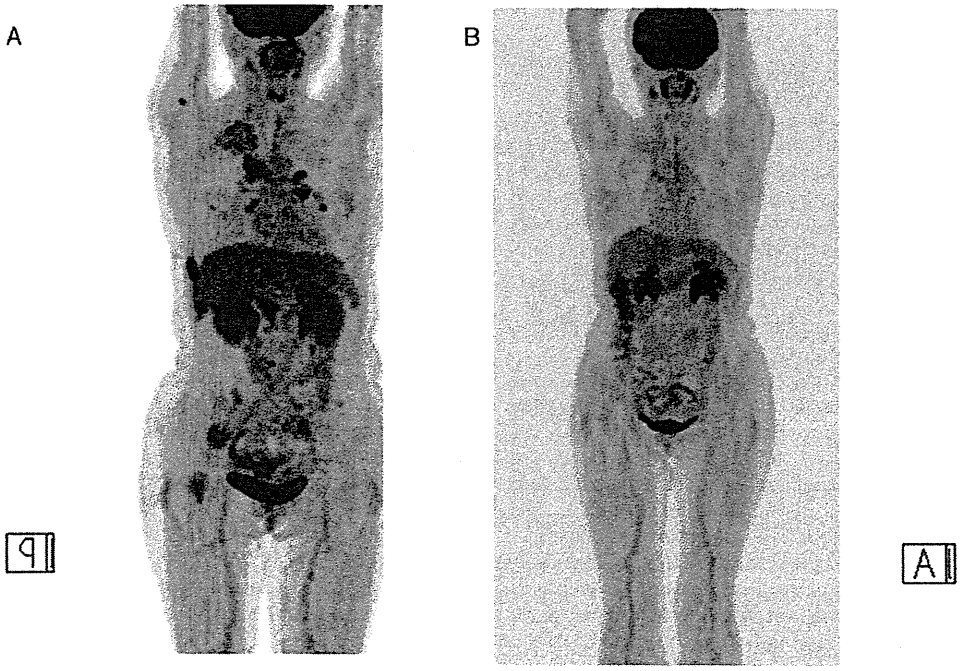

Fig. 8. FDG-PET scan: A, 09/08/2009 (before ALK inhibitor) and B, 07/05/2010 FDG-PET scan follow-up for 10 months indicated complete control of primary and distant metastases in case 3.

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3.2. Case 2

A 49-year-old woman, a non-smoker with no history of illness, PS0, was introduced to the Orthopedics Department of our Center in April 2009 for back pain and multiple osteoplastic changes in the bones. Systematic examination revealed an abnormal shadow 22X13 mm in size in the left lower lobe (Fig. 4A). Bronchoscopy and a PET scan indicated left S8 adenocarcinoma with cervical, axial, mediastinal, hilar, pancreatic and retroperitoneal lymph node metastases, as well as cranial, thoracic (Th1-12), lumbar (L1-5), rib (1–12) pelvis, humerus, and femur metastases (Fig. 5A).

She refused any therapy except for best supportive care. One month after the examination, an additional immunohistochemical examination for EML4-ALK fusion protein was performed, and found to be positive. The presence of mRNA for EML4-ALK gene was also confirmed by RT-PCR and FISH from the mediastinal #4R lymph nodes obtained with EBUS-TBNA, which was performed 2 months later. EGFR mutation was negative, but the direct sequence of the EML4-ALK mRNA indicated that the translocation was variant 3 [9]. She decided to be enrolled to the crizotinib study (PF02341066) at a dosage of 500 mg/day at Seoul National University from July 2009.

She had nausea, diarrhea and light image persistence as in case 1, but her gastrointestinal symptoms were severer than those in case 1. Two weeks after the administration of ALK inhibitor, her back pain disappeared. A PET scan performed 5 weeks after the initiation of the therapy showed marked reduction of bone and lymph node metastases, and the primary tumor had decreased in size from 22X13 mm to 12X7 mm (Fig. 4A and B). Also, the SUV max dropped from 10.7 to 2.42. Changes of tumor markers were not parallel with the clinical course since the measured value of CA-125 dropped from 424 to 107 U/ml, but that of CEA increased from 21.5 to 65.4 ng/ml 4 months later. The value of CEA then gradually decreased to 15.2 ng/ml in April 2010 (10 months after that: Fig. 6). The PET scan conducted after 7 months indicated a partial response to multiple bone and lymph node metastases (Fig. 5B). The patient continued to take the drug until the end of July 2010, when brain metastases (Fig. 7) were found.

3.3. Case 3

A fifty-four-year-old woman, also a non-smoker, PS0, visited a doctor because of back pain in August 2008. Chest X-ray and CT scan showed an S3 59X22 mm tumor in the right upper lobe, combined with #4R, #2R mediastinal lymph nodes and intrapulmonary metastases. The tumor had invaded the SVC and the azygos vein. She had undergone bronchoscopy and EBUS-TBNA in October 2008. A diagnosis of lung adenocarcinoma was obtained with TBNA samples from #7 lymph nodes. Bone scans indicated cranial, costal, vertebral, scapular, pelvic and femoral metastases (T4N2M1 stage IV). She received 2 courses of CBDCA + GEM (1000 mg/m^2) and 7 courses of docetaxel (TXTL: 60 mg/m^2) from November 2008 to June 2009, but the effect was minimal.

EML4-ALK fusion gene was suggested immunohistochemically in August 2009 and confirmed by RT-PCR obtained by EBUS-TBNA samples from the primary tumor in September 2009. She was enrolled for the clinical trial from November 2009 with an oral administration of crizotinib 500 mg/day. Dyspnea and cough were alleviated within 2 weeks, and she complained of severe diarrhea, nausea, vomiting, light image persistence and perceived changes of taste. A PET scan one month after the start of the treatment demonstrated complete disappearance of the primary tumor as well as all the metastases except for a bone metastasis to the right 8th rib. A PET scan follow-up 8 months later indicated complete control of primary and metastatic tumors (Fig. 8A and B). CEA declined slowly from 1764 ng/ml to 79 ng/ml 6 months after the start of administration (Fig. 9). The patient had 12 brain metastases from 5 mm^3 to 309 mm^3 in volume and underwent gamma knife irradiation in August 2009, 2 months before the start of ALK inhibitor treatment. The irradiated field still showed little change for 5 months, but small new lesions appeared in the left occipital area 6 months after the start of the trial. Brain metastases grew very slowly, so we have maintained our observation until October 2010.

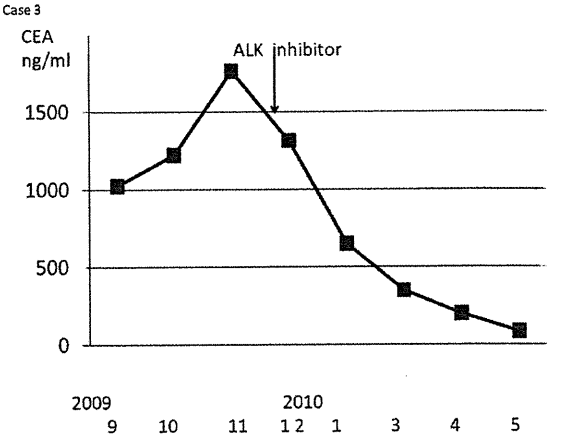

Fig. 9. CEA (■) declined slowly from 1764 ng/ml to 79 ng/ml 6 months after the start of the therapy in case 3.

4. Discussion

Above, we have reported the far-reaching effects of an ALK inhibitor on *EML4-ALK*-positive lung cancer patients. Soon after the administration of crizotinib, almost all metastases to bone and lymph nodes rapidly disappeared, followed by a marked reduction in the level of tumor markers in the sera. These observations clearly support the pivotal role of EML4-ALK oncokinase for the growth/survival of not only primary tumors but of the metastases. Such profound effects were rare among the patients when treated with conventional cytotoxic anticancer drugs.

The three cases which were enrolled for the study had surprisingly similar biological characteristics. They had multiple bone and lymph node metastases at the first medical examination, and were non-smokers at younger ages (48–54) who were resistant to chemotherapy. Adverse effects with crizotinib were also similar among them, including transient diarrhea, nausea, light image persistence, and subjective changes of taste. In addition, their response to ALK inhibitor was similar. Bone and lymph node metastases had disappeared within one month after the initiation of the therapy. The response of the primary tumor in case 2 was relatively slow compared with those of the metastases. The difference between the response of primary tumor and metastases to the ALK inhibitor in this case seems to indicate that the similar subclones of tumor cells in the primary tumors that were highly responsive to ALK inhibitor metastasized to distant organs and may give some explanation for the discrepancy in the time-course between CEA and CA125.

Molecular and immunohistochemical analyses in this cohort were conducted on the basis of the specimens obtained through EBUS-TBNA. Originally, EBUS-TBNA had been proposed useful for the pathological diagnosis of mediastinal involvement (N2 disease) of lung cancer [17–20]. However, we have already reported that EBUS-TBNA is also a versatile way of obtaining histological samples for the molecular analyses of cancer-related genes, such as *EGFR*, p53 et al. [21,22]. For those who have advanced NSCLC, it is often difficult to conduct surgery to obtain specimens from patients. Among such cases, however, EBUS-TBNA can usually be safely carried out to obtain specimens from enlarged mediastinal

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lymph nodes or parathracheal tumors. We carried out EBUS-TBNA procedure for the reasons of its advantage in obtaining high quality core samples adequate for this purpose as well as its safety. We do not disregard the importance of TBB for the diagnosis of lung cancer; however, we needed histological samples to examine the immunohistochemistry and FISH for enrolment in a trial of crizotinib. Our experience with the three cases clearly demonstrates the importance and clinical relevance of obtaining such specimens for molecular analyses.

Although the initial effects of crizotinib are substantial in our cases, as well as in those reported by Bang et al. [10,11], such efficacy may not always last long. There was, for instance, development (case 1 and 2) and recurrence (case 3) of brain metastases while favorable control was maintained outside the brain. Given that the primary tumors and lymph node metastases were under control of crizotinib even at the appearance of brain metastases, the tumor cells outside the brain did not lose sensitivity to crizotinib. Relapses in the brain only may indicate either (i) subclones of the tumor acquired both the homing ability to the brain and resistance to crizotinib, or (ii) crizotinib may not penetrate the blood-brain barrier, leading to insufficient concentrations of crizotinib in the brain. It is thus highly important to examine in detail the molecular basis that would account for such acquired resistance to crizotinib, which may be secondary mutations within EML4-ALK itself or mutations/gene amplification of other genes, as demonstrated in the cases of acquired resistance of NSCLC to gefitinib/erlotinib [23–26].

Conflict of interest

None declared.

Acknowledgements

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KLC1-ALK: A Novel Fusion in Lung Cancer Identified Using a Formalin-Fixed Paraffin-Embedded Tissue Only

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Abstract

The promising results of anaplastic lymphoma kinase (ALK) inhibitors have changed the significance of ALK fusions in several types of cancer. These fusions are no longer mere research targets or diagnostic markers, but they are now directly linked to the therapeutic benefit of patients. However, most available tumor tissues in clinical settings are formalin-fixed and paraffin-embedded (FFPE), and this significantly limits detailed genetic studies in many clinical cases. Although recent technical improvements have allowed the analysis of some known mutations in FFPE tissues, identifying unknown fusion genes by using only FFPE tissues remains difficult. We developed a 5′-rapid amplification of cDNA ends-based system optimized for FFPE tissues and evaluated this system on a lung cancer tissue with *ALK* rearrangement and without the 2 known ALK fusions EML4-ALK and KIF5B-ALK. With this system, we successfully identified a novel ALK fusion, KLC1-ALK. The result was confirmed by reverse transcription-polymerase chain reaction and fluorescence *in situ* hybridization. Then, we synthesized the putative full-length cDNA of *KLC1-ALK* and demonstrated the transforming potential of the fusion kinase with assays using mouse 3T3 cells. To the best of our knowledge, KLC1-ALK is the first novel oncogenic fusion identified using only FFPE tissues. This finding will broaden the potential value of archival FFPE tissues and provide further biological and clinical insights into ALK-positive lung cancer.

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Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that was discovered in anaplastic large-cell lymphoma (ALCL) in the form of a fusion protein, NPM-ALK [1,2]. The formation of a fusion protein with a partner through chromosomal translocations is the most common mechanism of ALK overexpression and ALK kinase domain activation. Recent promising results of clinical trials with an ALK inhibitor, crizotinib, have changed the significance of ALK fusions in lung cancer [3,4,5,6], inflammatory myofibroblastic tumors (IMTs) [7], and ALCL [8]. ALK fusions are no longer mere research targets or diagnostic markers and are now directly linked to the therapeutic benefit of patients.

In lung cancer, 3 fusion partners of ALK have been reported—EML4, TFG, and KIF5B—although the presence of TFG-ALK in lung cancer has not yet been proven with histopathological evidence [9,10,11]. In addition to lung cancer, ALK has further been found to generate fusions in ALCL (fused to NPM, TPM3, TPM4, ATIC, TFG, CLTC, MSN, MYH9, or ALO17) [1,2,12,13,14,15,16,17,18,19], IMT (TPM3, TPM4, CLTC, CARS, RANBP2, ATIC, or SEC31A) [19,20,21,22,23,24], ALK-positive large B-cell lymphoma (CLTC, NPM, SEC31A, or SQSTM1) [25,26,27,28], and renal cancer (VCL, TPM3 or EML4) (Table 1) [29,30]. In addition to TFG-ALK in lung cancer, some ALK fusions have been reported without histopathological evidence: TPM4-ALK in esophageal squamous cell carcinoma [31,32] and EML4-ALK in colon and breast carcinomas [33].

Anti-ALK immunohistochemistry played an important role in identifying these ALK fusion partners. Several ALK fusions exhibit a characteristic staining pattern in anti-ALK immunohistochemistry because the subcellular localization of ALK fusion proteins depends on the fusion partner. For example, NPM-ALK, which is the most common fusion in ALK-positive ALCL (85%), exhibits a nuclear and cytoplasmic staining pattern because the heterodimer of NPM and NPM-ALK localizes in the nucleus and the homodimer of NPM-ALK in the cytoplasm; CLTC-ALK exhibits a cytoplasmic granular pattern because it localizes in the small vesicles. If a tumor exhibits an unrecognized anti-ALK staining pattern, the patient may have a novel fusion partner. In addition to the difference in subcellular localization, the difference in staining intensity is a key to identifying novel partners. EML4-ALK is hardly stained by conventional anti-ALK immunohistochemistry [11,34]. To overcome this limitation, we developed the intercalated antibody-enhanced polymer (iAEP) method, which moderately increases

Table 1. ALK fusion partners.

Reported year	Partner	Locus	ALK+ALCL	ALK+LBCL	IMT	NSCLC	RCC
1994	NPM	5q35.1	+	+			
1999	TPM3	1p23	+		+		+
1999	TFG	3q12.2	+			+*	
2000	ATIC	2q35	+		+		
2000	TPM4	19p13	+		+		
2001	CLTC	17q23	+	+	+		
2001	MSN	Xp11.1	+				
2002	ALO17	17q25.3	+				
2003	MYH9	22q13.1	+				
2003	RANBP2	2q13			+		
2003	CARS	11p15			+		
2006	SEC31A	4q41		+	+		
2007	EML4	2p21				+	+
2009	KIF5B	10p11.22				+	
2011	SQSTM1	5q35.3		+			
2011	PPFIBP1	12p11			+		
2011	VCL	10q22.2					+
Present study	KLC1	14q32.1				+	

*Histopathological evidence is lacking. Abbreviations: ALCL, anaplastic large cell lymphoma; LBCL, large B-cell lymphoma; IMT, inflammatory myofibroblastic tumor; NSCLC, non-small cell lung carcinoma; RCC, renal cell carcinoma.

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sensitivity in the immunohistochemical detection system, and EML4-ALK was consistently stained with this method [11]. This indicated that a tumor that is positively immunostained for ALK only by a sensitive immunohistochemistry method but not by conventional methods may harbor a novel ALK fusion. Based on this hypothesis, we successfully identified PPFIBP1-ALK in 2 IMT cases that were positive in anti-ALK immunohistochemistry only when stained by the iAEP method [35].

Anti-ALK immunohistochemistry may thus be useful to detect candidate tumors for a novel ALK fusion. However, to identify the fusion partner, other molecular techniques are usually required such as 5′-rapid amplification of cDNA ends (5′-RACE) or inverse reverse-transcription polymerase chain reaction (RT-PCR). To the best of our knowledge, no novel oncogenic fusions have been discovered using formalin-fixed paraffin-embedded (FFPE) tissues only because nucleic acids extracted from FFPE tissues are severely degraded during the fixation process. In the present study, we developed a 5′-RACE method optimized for *ALK* fusion partner detection that was applicable to FFPE tissues and identified a novel fusion, kinesin light chain 1 (KLC1)-ALK, in lung cancer by using only an FFPE tissue.

Methods

Materials

A FFPE tissue block of pulmonary adenocarcinoma in situ, nonmucinous (formerly called bronchioloalveolar carcinoma) [36], which was excised from a 47-year-old female patient was used [37]. This carcinoma was negative for EML4-ALK and KIF5B-ALK, although the presence of *ALK* rearrangement was confirmed by anti-ALK iAEP immunohistochemistry and a split fluorescence in situ hybridization (FISH) assay for ALK (hereafter referred to as the unknown ALK fusion-positive case) (Figure 1) [37]. Two FFPE tissue blocks of ALK-positive tumor cases were also employed, for which the presence of EML4-ALK or KIF5B-ALK had already been confirmed. Total RNA was extracted from each FFPE tissue with the use of the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems Japan, Tokyo, Japan). The ages of the 3 FFPE blocks used (time from FFPE tissue production to RNA extraction) were 65, 40, and 51 months for the unknown ALK fusion-positive case, EML4-ALK, and KIF5B-ALK, respectively. Written informed consent was obtained from each patient. The study was approved by the institutional review board of the Shizuoka Cancer Center (approval ID 22-J132-22-1) and the Japanese Foundation for Cancer Research (approval ID 2010-1011).

Modified 5′-RACE for ALK fusions applicable to FFPE tissues

5′-RACE was performed with the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instruction with minor modifications. In brief, instead of the primers included in the kit, ALK-3242R (5′-CTCAGCTTG-TACTCAGGGC-3′) was used for cDNA synthesis. The cDNA was subjected to 5′-RACE PCR using PrimeSTAR HS DNA Polymerase (TaKaRa) and the following primers: Universal Primer A Mix of the kit and ALK-3206R (5′-ATGGCTTG-CAGCTCCTGGTGCTT-3′). The PCR condition consisted of 5 cycles at 94°C for 30 s and 72°C for 3 min; 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min; and 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min.

FISH

FISH analysis of fusion genes was performed with DNA probes for KLC1 and ALK. Unstained sections (4-μm thick) were subjected to hybridization with an ALK-split probe set (Dako, Tokyo, Japan) or with bacterial artificial chromosome (BAC) clone-derived probes for ALK (RP11-984I21 and RP11-62B19)

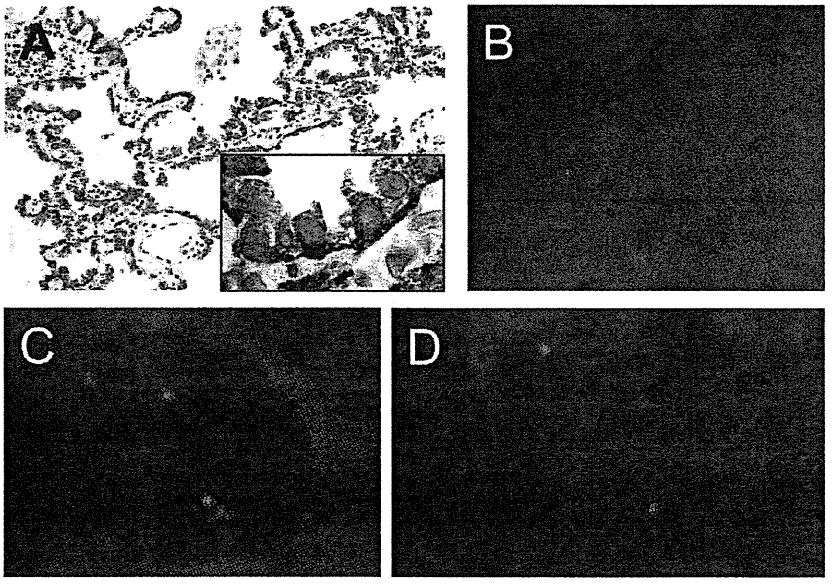

Figure 1. ALK-rearranged lung adenocarcinoma without EML4-ALK and KIF5B-ALK. Panel A shows the results of anti-ALK immunohistochemistry with the iAEP method on pulmonary adenocarcinoma in situ, nonmucinous. The staining pattern was diffusely cytoplasmic. The basal side of tumor cells was more strongly stained, indicating an uneven subcellular localization of KLC1-ALK protein. FISH analyses revealed that this case was positive in the split assay for *ALK* (Panel B: individual 5′- and 3′-signals are observed) and negative in *EML4-ALK* and *KIF5B-ALK* fusion assays (Panel C: *EML4*, red; *ALK*, green; Panel D: *KIF5B*, green; *ALK*, red).
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and KLC1 (RP11-186F6). Hybridized slides were then stained with DAPI and examined using a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Synthesis of the putative cDNA of *KLC1-ALK*

Two independent PCRs were performed using cDNA synthesized from a tumor tissue expressing KIF5B-ALK with the following primer sets: KLC1-NheI-M (5′-GCGCTAGCGAATGTATGACAACATGTCCAC-3′) and KLC1-bpR (5′-GTGCTTCCGGCGGTACACATCTACAGAACCAAACTC-3′), and ALK-bpF (5′-GGGAGTTTGGTTCTGTAGATGTGTACCGCCGGAAGC-3′) and ALK-EcoRI (5′-GATAGAATTCTCAGGGCCCAGGCT-3′). Then, the second PCR was performed using a 1/100 dilution of a mixture of the first PCR products as a template with the KLC1-NheI-M and ALK-EcoRI primers (Figure 2).

Transformation assay for *KLC1-ALK*

Analysis of the transforming activity of kinase fusions was performed as described previously [9,38,39]. A pMXS-based expression plasmid for each fusion was used to generate recombinant ecotropic retroviruses [40], which were then used individually to infect mouse 3T3 fibroblasts. The formation of transformed foci was evaluated after culturing the cells for 4 days. The same set of 3T3 cells was injected subcutaneously into nu/nu mice, and tumor formation was examined after 14 days. The animal experiments were approved by the animal ethics committee of Jichi Medical University (approval ID 1135).

Results

Identification of *KLC1-ALK* as a novel *ALK* fusion gene

Our modified 5′-RACE faithfully isolated cDNA fragments for *EML4-ALK* or *KIF5B-ALK* from known ALK- positive tumors (Supplementary Figure S1A and B). We then attempted to isolate cDNA fragments encompassing the fusion points from the unknown ALK fusion-positive case. Nucleotide sequencing of such 5′-RACE products revealed that 2 of 10 clones contained the 3′-terminus of exon 9 of *KLC1* (ENST00000348520) fused to the first nucleotide of exon 20 of *ALK* (ENST00000389048), indicating the presence of a novel fusion between *KLC1* and *ALK*. As this rearrangement constituted an in-frame fusion between the 2 genes, the full-length *KLC1-ALK* cDNA probably produces a protein of 984 amino acids containing an amino-terminal two-thirds of KLC1 and an intracellular region of ALK (Figure 3A). RT-PCR-mediated isolation of a fusion point successfully confirmed the in-frame fusion between the 2 messages (Figure 3A and B). Further, to confirm the genomic rearrangement responsible for the fusion, a fusion FISH assay was performed (Figure 3C). These results were consistent with the presence of t(2;14)(p23;q32.3), leading to the generation of *KLC1-ALK*.

Transforming potential of *KLC1-ALK*

The putative full-length cDNA of *KLC1-ALK* was synthesized from the frozen tissue with KIF5B-ALK fusion expression (Figure 2, Supplementary Figure S2), and was used to generate a recombinant retrovirus expressing the fusion protein with an amino-terminal FLAG epitope tag. Infection of 3T3 cells with the virus expressing KLC1-ALK readily produced multiple transformed foci in culture and subcutaneous tumors in a nude mouse tumorigenicity assay (Figure 4), confirming the potent transforming ability of KLC1-ALK.

Discussion

Here, by analyzing the FFPE tissues only, we successfully discovered a novel ALK fusion, KLC1-ALK. While snap-frozen materials sampled from biopsied or surgically removed specimens

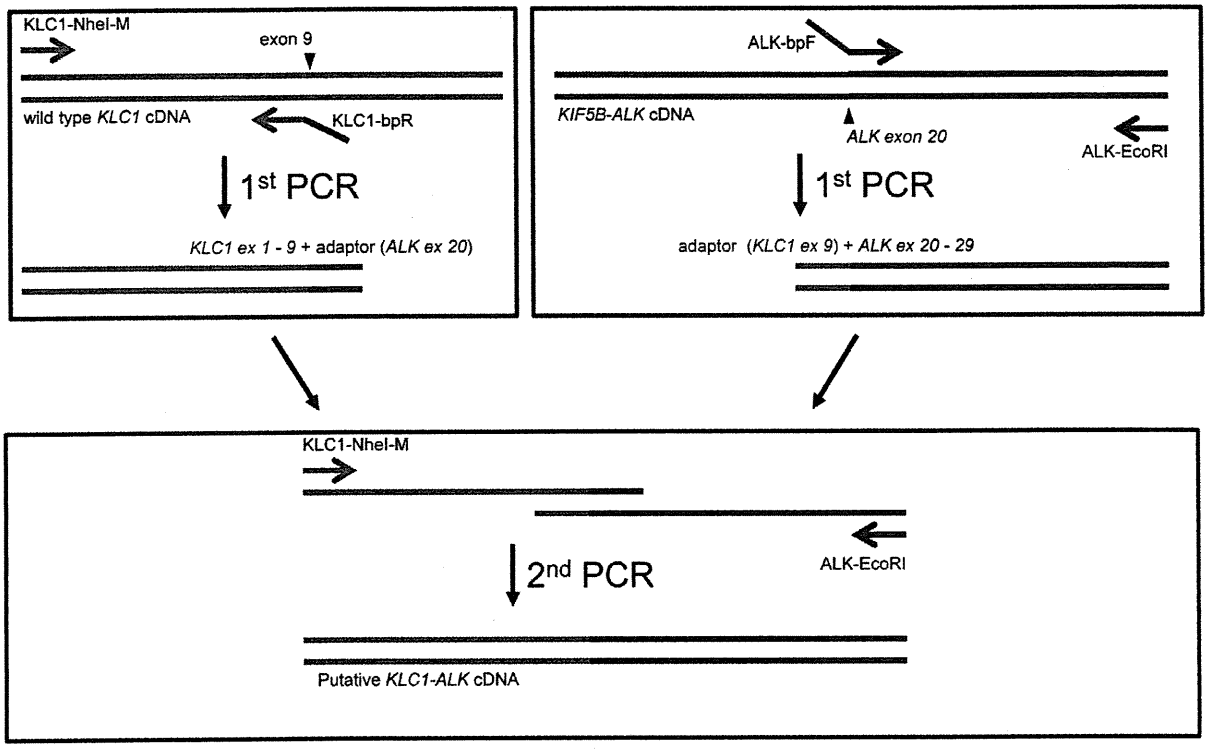

Figure 2. Synthesis of the putative KLC1-ALK full-length cDNA. Two first-round PCRs were performed separately using cDNA synthesized from a tumor tissue expressing KIF5B-ALK with the following primer sets: KLC1-NheI-M and KLC1-bpR, and ALK-bpF and ALK-EcoRI. KLC1-bpR and ALK-bpF had sequences downstream of the ALK break point (exon 20) and upstream of the KLC1 break point (exon 9) as adopter sequences, respectively. Then, the second PCR was performed using a 1/100 dilution of the mixture of the first PCR products as a template with primers KLC1-NheI-M and ALK-EcoRI. The first PCR products were annealed, extended with each other, and then amplified with the primers.
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can be used for various types of molecular analyses, they are not routinely sampled in most clinical settings. In contrast, FFPE specimens are usually produced, and histopathology diagnostic archives are an extremely large resource of FFPE tissues in ordinary diagnostic pathology laboratories. However, DNA and RNA extracted from FFPE tissues are severely degraded during formalin fixation and are usually not suitable for assays that need long DNA/RNA of high quality. Recent technical advances have allowed some analyses for known point mutations and known fusion genes, but it is still difficult to identify an unrecognized gene aberration using only an FFPE tissue.

In most *ALK* fusions, the break point of *ALK* is located within intron 19, and the fusion point in mRNA is typically the first nucleotide of exon 20. Therefore, if the primers for 5′-RACE are placed immediately downstream of the first nucleotide of *ALK* exon 20, such 5′-RACE may successfully isolate PCR products containing the partner gene sequence even using FFPE tissues. Based on this hypothesis, we established a 5′-RACE system for *ALK* fusions optimized for FFPE tissues. With this system, we identified a novel *ALK* fusion, *KLC1-ALK*. To the best of our knowledge, this is the first novel oncogenic fusion identified using only an FFPE tissue.

Caution, however, is needed. In some rare cases with *ALK* fusion, the break point of *ALK* fusion mRNA may not be at the 5′-end of exon 20. For example, in variant 4 of *EML4-ALK*, exon 14 of *EML4* is fused to an unknown sequence of 11 bp, which in turn is connected to nucleotide 50 of *ALK* exon 20 (E14;ins11;del49A20) [38]. Our 5′-RACE system would not work on such a case because the reverse primer ALK-3206R corresponds to nucleotides 12–34 of *ALK* exon 20. Therefore, if our modified 5′-RACE fails to isolate fusion cDNAs from cases with a confirmed ALK rearrangement, other primer settings may be attempted.

Kinesin is a heterotetramer of 2 kinesin heavy chains and 2 kinesin light chains, and it moves on the microtubules towards their plus ends carrying various cargos. The heavy chains harbor the motor activity, whereas the light chains play roles in cargo binding and in modulating the activity and subcellular localization of the heavy chains. KLC1 binds to the kinesin heavy chains with an N-terminal domain and to various cargos via the tetratricopeptide repeat domains [41,42]. Of the 3 histopathologically confirmed ALK fusion partners in lung cancer, EML4 colocalizes with microtubules and may contribute to the stabilization of microtubules [43], KIF5B moves on the microtubules as a kinesin heavy chain [44], and KLC1 binds to kinesin heavy chains as a kinesin light chain. Therefore, it is interesting that all the 3 ALK fusions in lung cancer are likely to colocalize with microtubules.

The most frequent ALK fusion in lung cancer is EML4-ALK (4–7%) [9,38], and the second is KIF5B-ALK (0.5%) [11]. One case with TFG-ALK is reported [10]. KLC1-ALK may be rare but exists in lung adenocarcinoma, and the patients with this fusion are highly likely to benefit from ALK inhibitor therapy as do patients with other ALK fusions. The incidence may be low, but the significance of this fusion is very high from the perspective

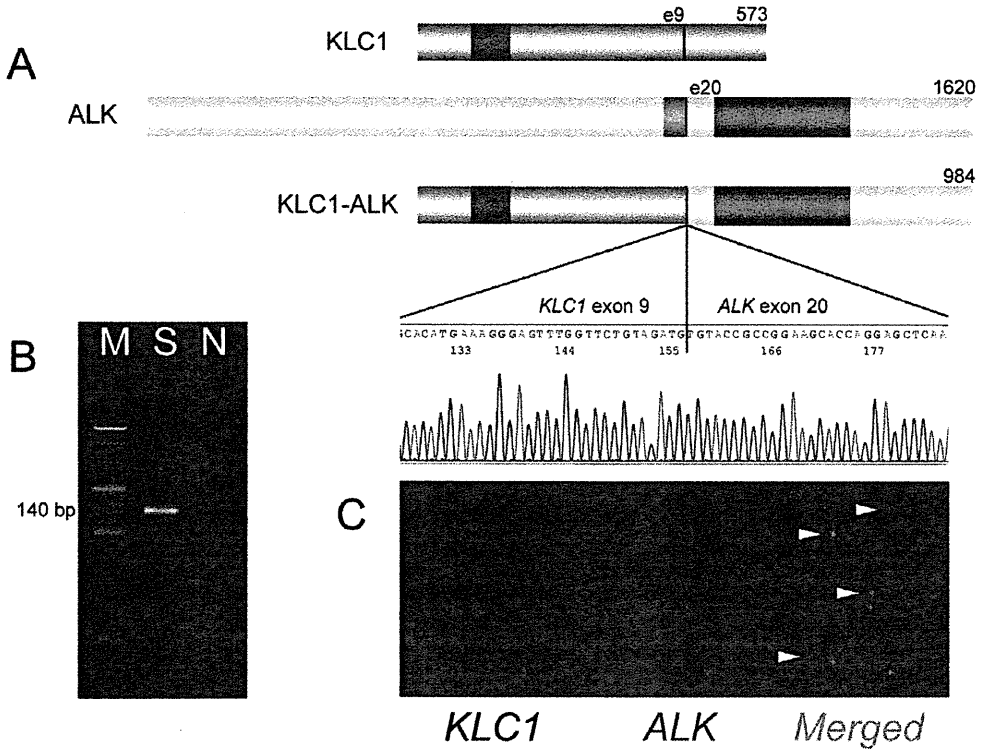

Figure 3. Identification of KLC1-ALK. Panel A shows the schematic structure of KLC1, ALK, and KLC1-ALK proteins and the cDNA sequence around the fusion point. Dark blue, orange, and red parts represent coiled-coil, transmembrane, and kinase domains, respectively. The break point exons and the number of amino acids are indicated. KLC1-ALK-specific RT-PCR using RNA extracted from the FFPE tissue of the unknown ALK fusion-positive case amplified a fragment of the expected product size (140 bp, Panel B) with the consistent fusion sequence (Panel A). A fusion FISH assay for *KLC1-ALK* revealed a fusion signal (yellow) in multiple tumor cells (Panel C). M, marker (100-bp ladder); S, sample (the unknown ALK fusion-positive case); N, no template control.
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of a tailor-made therapeutic option for the patient. Another important point is that KLC1-ALK was found in adenocarcinoma in situ, nonmucinous (formerly called bronchioloalveolar carcinoma, BAC). BAC is recognized to rarely harbor ALK fusions, although a small number of BAC cases has been examined for ALK fusion compared with invasive adenocarcinoma. It would be interesting from a pathobiological perspective to examine a large-scale cohort of BAC and other premalignant conditions for ALK fusion.

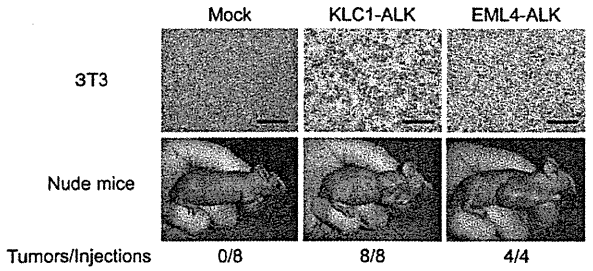

Figure 4. Transforming potential of KLC1-ALK. Upper panels: Mouse 3T3 fibroblasts were infected with retroviruses encoding KLC1-ALK or EML4-ALK or with the corresponding empty virus (Mock). The cells were photographed after 4 days of culture. Scale bar, 1 mm. Lower panels: Nude mice were injected subcutaneously with the corresponding 3T3 cells, and tumor formation was examined after 14 days. The number of tumors formed per injections is indicated at the bottom.
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There are 3 methods for the detection of ALK fusions: RT-PCR, ALK split FISH, and high-sensitivity anti-ALK immunohistochemistry. For RT-PCR, the 5′ partner gene must be known. Our findings in this study identified one more partner gene that should be targeted in ALK-fusion detection using RT-PCR in lung cancer. The other 2 methods can detect all ALK fusions regardless of fusion partner and, therefore, are suitable for ALK-fusion screening. In other words, these 2 methods cannot identify the fusion partner and need to be succeeded by partner-specific RT-PCR and/or fusion FISH for this purpose. If it is revealed that the partner gene in the tested case is unknown, a novel partner gene is highly likely to be discovered, as was shown in the present study. In fact, using high-sensitivity anti-ALK immunohistochemistry (iAEP method) as screening, we have identified several novel ALK fusions in various types of cancers including lung adenocarcinoma [11], lymphoma [28], sarcoma [35], and renal cell carcinoma [30].

Many efficient tools have been established for the detection of ALK fusion-positive cases using FFPE tissues, including anti-ALK immunohistochemistry and FISH. Our findings will further expand the potential value of archival FFPE tissues and provide further biological and clinical insights into ALK-positive cancers in the forthcoming era of ALK inhibitor therapy.

Supporting Information

Figure S1 5′-RACE products using FFPE tissues. Our modified 5′-RACE faithfully isolated cDNA fragments for *EML4-ALK* (A) or *KIF5B-ALK* (B) from known ALK- positive tumors.
(TIF)

Figure S2 Putative cDNA sequence of KLC1-ALK. The putative full-length cDNA of *KLC1-ALK* was synthesized from the frozen tissue with KIF5B-ALK fusion expression.
(PDF)

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Author Contributions

Conceived and designed the experiments: KT HM. Performed the experiments: YT MS SS ES SH RA. Analyzed the data: YT MS HM KT. Contributed reagents/materials/analysis tools: RA TN. Wrote the paper: KT HM.

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