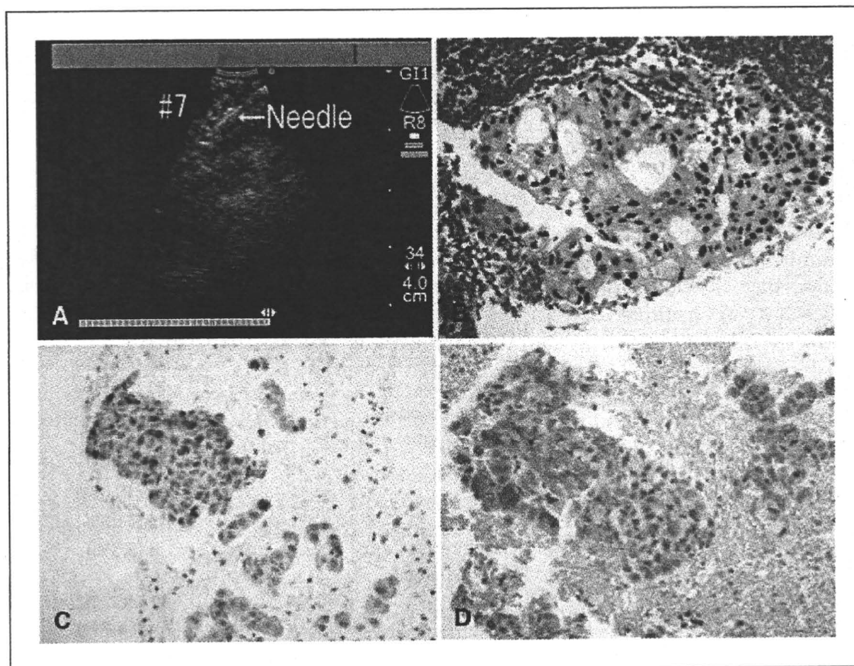

Fig. 1. Diagnosis of metastatic nodes by EBUS-TBNA. A, lymph node sampling by EBUS-TBNA. B, adenocarcinoma was revealed in the EBUS-TBNA sample. C, mucin production was observed by Alcian blue staining. D, immunohistochemistry with an anti-*ALK* antibody showed *ALK* fusion protein positivity in lung adenocarcinoma cells.

The samples obtained with EBUS-TBNA were small, paraffin-embedded biopsy specimens, which might limit the utility of immunohistochemistry. To avoid false-negative diagnosis, the first immunohistochemical procedure was used as a screening test to define three categories with which to judge the first run. Cancer cells were defined as "positive" if staining was as strongly positive as a positive control (clinical lung cancer tissues previously defined as positive by both molecular and immunohistochemistry analyses) and a fine, granular cytoplasmic staining pattern was observed. Cancer cells that showed no staining were classified as "negative." The "suspicious" classification was defined as the presence of weakly stained cells that were considered difficult to differentiate from background staining. While using these categories, we further subdivided the suspicious category into "probably positive" and "probably negative" categories. Probably positive meant that the tumor cells stained, but not strongly, whereas probably negative indicated very weak staining that was difficult to differentiate from background staining. After the screening immunohistochemistry, suspicious cases were re-tested by immunohistochemistry in addition to FISH by a second independent pathologist (K. Takeuchi).

Fluorescence *in situ* hybridization

To further confirm the *ALK* genomic rearrangement, two FISH assays were performed: an *ALK* split assay and an *EML4-ALK* fusion assay. Unstained sections were processed with a Histology FISH Accessory Kit (Dako), subjected to hybridization with fluorescently-labeled bacterial artificial chromosome clone probes for *EML4* and *ALK* (self-produced probes; *EML4* RP11-996L7, *ALK* RP11-984I21, and RP11-62B19) or for genomic regions upstream and downstream of the *ALK* breakpoint (Dako), stained with 4,6-diamidino-2-phenylindole, and examined with a fluorescence microscope (BX51; Olympus; ref. 7). FISH analysis was performed at the Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research (K. Takeuchi). The FISH positivity criteria for EBUS-TBNA samples were defined as "over 50% cancer cells." As EBUS-TBNA samples are small biopsy samples, entire tumor cells in the paraffin-embedded section were evaluated.

RT-PCR and direct sequencing

Frozen histologic cores obtained by EBUS-TBNA were used to extract RNA. All immunohistochemistry-positive or suspicious cases were subjected to direct sequencing of the fusion cDNAs. RNA was extracted from frozen samples using the AllPrep DNA/RNA mini kit (Qiagen), and cDNA cloning was performed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems). For RT-PCR analysis of *EML4-ALK*, we used primer sequences that have been described previously (2). After PCR amplification, PCR products were analyzed using agarose gel electrophoresis. RT-PCR products were extracted from gel slices using the QIAquick Gel Extraction Kit (Qiagen). Purified products were then sequenced with a capillary sequencer. Resultant nucleotide sequences were compared with previously reported sequences for determination of the *EML4-ALK* variant. *EGFR* mutation status was also examined using the peptide nucleic acid/locked nucleic acid PCR clamp method for samples obtained with EBUS-TBNA (8).

Ethics committee approval

This research was approved by the Ethics Committee of Chiba Cancer Center (nos. 20-21 and 21-10). Written consent was obtained from all patients. All samples were coded and managed independently.

Statistical analysis

For clinical characteristics and genetic factors, frequency analysis was performed with Fisher's exact test (dichotomous factors) and χ^2 test (multinomial factors). Mann-Whitney *U* test was applied to continuous data. General data analysis was conducted with StatView 5.0 (SAS Institute, Inc.). All *P* values were based on a two-sided hypothesis, $P < 0.05$ was considered to have statistical significance.

Results

Patient characteristics

The clinical characteristics of all 109 patients are listed in Table 1; 82 patients (75.2%) were male. The median age was 64.4 years (range, 38–90 y). Histologic examination was performed in all cases, leading to a diagnosis of adenocarcinoma (Fig. 1B) in 82 cases (75.2%), squamous cell carcinoma in 18 cases, and "other" in 9 cases. With respect to smoking status, 22 cases (20.4%) were never-smokers, 15 (13.9%) were light smokers (defined as a smoking index score <400), and 72 were heavy smokers (smoking index score ≥400). A total of 191 mediastinal lymph nodes and 84 hilar lymph nodes (2.52 lymph nodes/patient) were detected with EBUS, and 158 mediastinal lymph nodes and 71 hilar lymph nodes (2.10 lymph nodes/patient) were sampled. The median size of the sampled lymph nodes was 12.1 mm (range, 3.0–33.4 mm) in the short axis on ultrasound. According to criteria from the International Union Against Cancer, there were 9 stage II cases, 49 stage III cases, and 45 stage IV cases; the remaining 6 cases were defined as having recurrent lung cancer. *EGFR* gene mutations were detected in 25 cases (22.9%), which included 9 cases with in-frame deletions at exon 19, 9 cases with a point mutation at exon 21, 3 cases with a point mutation at exon 18, 2 cases with point mutations at exons 18 and 21, 1 case with a point mutation at exon 20, and 1 case with point mutations in exons 20 and 21.

Table 1. Clinical characteristics of patients with NSCLC

Parameter	Number of cases (%)
	109
Age	
Mean (y)	64.4 (range, 38–90)
Gender	
Male	82 (75.2%)
Female	27 (24.8%)
Pathology	
Adenocarcinoma	82 (75.2%)
Squamous cell	18 (16.5%)
Other histology	9 (8.3%)
Clinical stage	
II	9 (8.3%)
III	49 (45.0%)
IV	45 (41.3%)
Recurrence	6 (5.5%)
Bone metastasis	
Yes	22 (20.2%)
No	87 (79.8%)
Brain metastasis	
Yes	16 (14.7%)
No	93 (85.3%)
Smoking	
Never (SI = 0)	22 (20.4%)
Light (SI < 400)	15 (13.9%)
Heavy (SI ≥ 400)	70 (64.8%)
EGFR mutation status	25 (22.9%)
Exon 18	3
Exon 19	9
Exon 20	1
Exon 21	9
Exons 18 + 21	2
Exons 20 + 21	1

Abbreviation: SI, smoking index.

ALK fusion gene assessment

Out of 109 cases examined by immunohistochemistry using the iAEP method, 6 *ALK*-positive cases and 17 suspicious cases (1 probably positive and 16 probably negative) cases were detected. The staining of the small histologic core did not show any heterogeneity.

FISH confirmed the existence of an *ALK* fusion gene in all six *ALK*-positive cases (Figs. 1D, 2A and B), and there were no false-positive cases for immunohistochemistry. Sixteen probably negative cases were determined to be negative for the *ALK* fusion gene by re-testing with immunohistochemistry and FISH. One probably positive case had too few tumor cells to be used for FISH analysis; however, RT-PCR assessment confirmed the presence of *EML4-ALK* fusion cDNA. *EML4*, *ALK*, and fusion signals (arrows in Fig. 2A) are presented in the green, red, and merged image and a pair of split signals (arrow in Fig. 2B, downstream) shows rearrangement of *ALK*. In Fig. 2C, unique bands in each *ALK*-positive case reveal variant 1 and variant 3 *EML4-ALK* fusion genes. Thus, the *ALK* fusion gene was detected in a total of seven cases (6.4%). Direct sequencing of the PCR products revealed that four cases carried *EML4-ALK* variant 1, whereas three cases had variant 3. The fusion point of *ALK* and *EML4* is observed in the cDNA sequence (arrow in Fig. 2D).

Clinicopathologic characteristics of lung cancers possessing *ALK* fusion genes

Clinicopathologic characteristics were compared between the 7 *ALK*-positive cases and the 102 *ALK*-negative

cases (Table 2). All *ALK*-positive cases had an adenocarcinoma histology and lacked *EGFR* gene mutations. With respect to smoking habits, six out of the seven *ALK*-positive cases were either never-smokers or light smokers (smoking index score <400). No significant difference in gender was observed between *ALK*-positive and *ALK*-negative patients; however, *ALK*-positive patients were significantly younger than *ALK*-negative patients (55.4 versus 65.0 years; P = 0.0408). No significant differences in the incidence of bone metastasis (9.1% versus 5.7%; P = 0.64) or brain metastasis (12.5% versus 5.4%; P = 0.30) were observed. Overall, the mean primary tumor diameter was 40.4 mm; interestingly, the mean primary tumor diameter of *ALK*-positive cases was 28.6 mm, which was significantly smaller than that of *ALK*-negative cases (41.9 mm; $P < 0.05$). Mucin production was significantly more frequently observed in *ALK*-positive cases as shown by Alcian blue staining (Fig. 1C; $P < 0.01$). Finally, among the 84 cases expressing wild-type *EGFR*, 8.3% (7 of 84) were *ALK*-positive.

Discussion

This is the first attempt and report about using EBUS-TBNA samples in the detection of *ALK* fusion genes, and is expected to have a major effect on the management of patients with lung cancer. EBUS-TBNA is an established procedure for the evaluation of mediastinal and hilar adenopathy in patients with lung cancer. It is as safe, as highly diagnostic, and less invasive than other diagnostic modalities (9–11). Biopsy samples obtained with EBUS-TBNA can be subjected to histologic as well as cytologic evaluation. Nonsurgical modalities for obtaining tumor specimens are particularly critical in lung cancer because many patients have advanced disease at the time of first presentation, and are therefore not eligible for radical surgery. In addition to histologic diagnosis and stage definition, EBUS-TBNA enables molecular analysis of biopsy samples, the clinical significance of which is growing as molecularly targeted strategies for NSCLC are becoming increasingly important. We have previously reported that metastatic lymph node samples obtained by EBUS-TBNA can be applied to multidisciplinary analyses (5), and the present study is the first report of successful analysis of *ALK* fusion genes, a newly identified genetic abnormality in NSCLC, with such specimens (2). However, the small size of the paraffin-embedded biopsy samples obtained from EBUS-TBNA might limit the utility of this methodology; thus, multidirectional analysis will be critical for microsampling methods such as EBUS-TBNA.

The reliability of the newly developed immunohistochemistry (iAEP) method for the detection of *ALK* fusion

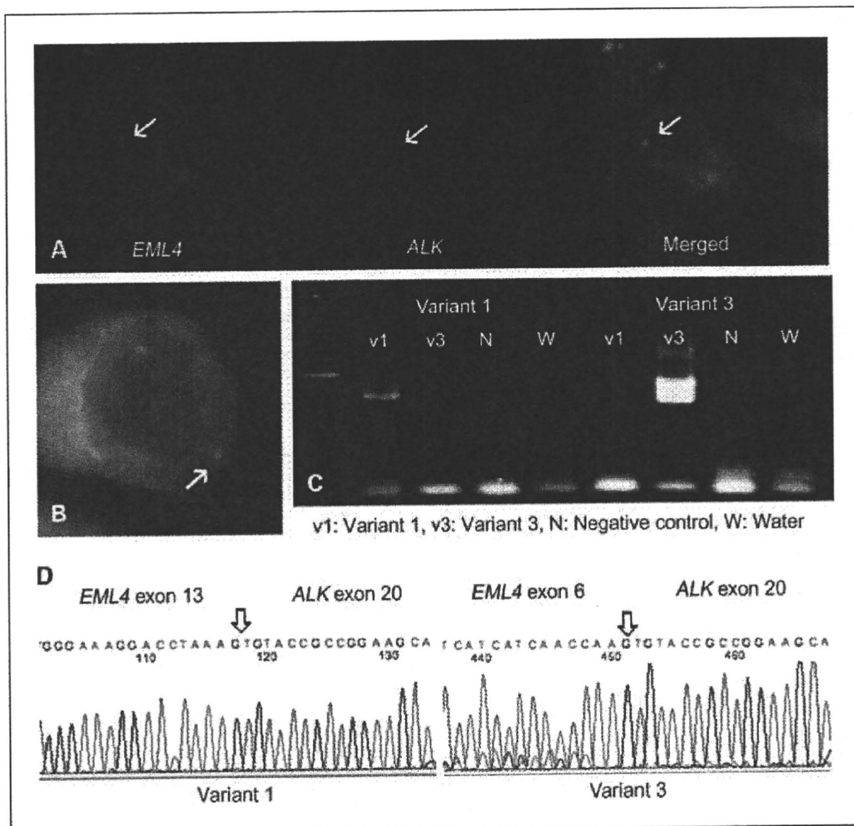

Fig. 2. Molecular analysis of *ALK* fusion genes. A, FISH *EML4-ALK* fusion assay with labeled probes for *EML4* (green, arrow) or *ALK* (red, arrow). The *EML4-ALK* fusion gene is observed (yellow, arrow). B, *EML4-ALK* split assay with labeled probes for the upstream (red) or downstream (green, arrow) region of the *ALK* locus. C, RT-PCR detection of the *EML4-ALK* fusion gene. D, direct cDNA sequence of *EML4-ALK* variants 1 and 3.

Table 2. Clinical, pathologic, and genetic analysis of *ALK*-positive NSCLC

Characteristic	*EML4-ALK* fusion			*P*
	NSCLC	+	–	
Female gender	27	4	23	0.062
Mean age (y)	64.4	55.4	65.0	0.0408
<60	29	5	24	0.0139
Bone metastasis	22	2	20	0.6396
Brain metastasis	16	2	14	0.2973
Mean tumor diameter (mm)	40.4	28.6	41.9	0.0478
Smoking index (n = 107)	784	161	827	0.0071
Never/light smoker	37	6	31	0.0056
Adenocarcinoma	82	7	75	0.1896
Mucin production	17	5	12	0.0009
EGFR wild-type	84	7	77	0.3317
ALK variant 1		4		
ALK variant 3		3		

NOTE: Two cases without primary tumors and six cases of recurrence were excluded from the tumor diameter analysis. Smoking history was recorded in 107 patients.

genes is very precise (4). This method is expected to be more practical for the detection of *ALK* fusion genes compared with FISH because FISH can sometimes be very difficult to perform for *ALK* fusion genes due to the close proximity of the two fusion gene components. We performed both fusion and split assays for FISH, and FISH was performed to confirm the immunohistochemical results. In addition, the *ALK* fusion genes are novel oncogenes in lung cancer. There is a possibility of existing unknown fusion pattern which cannot be detected by FISH or RT-PCR. Immunohistochemistry has an advantage of detecting novel unknown fusion patterns (4). In this study, we performed immunohistochemistry using the iAEP methodology and an Autostainer instrument. This technique is convenient, highly reproducible, and enables accurate diagnosis even if only a small amount of specimen is available. These features are well-suited for the screening of *ALK*-positive lung cancers using small biopsy samples. The Autostainer instrument also allows uniform immunohistochemical analysis, which may lead to consistent results among different institutions/hospitals; such uniformity is essential for the standardization of diagnostic procedures that assess the presence of *ALK* fusion genes. Recently, a highly sensitive antibody directed against *ALK* fusion products that can possibly be used for immunohistochemistry has been reported, therefore representing a novel candidate for *ALK* fusion detection (12).

The median age of *ALK*-positive cases in the present study was 55.4 years. Patients <60 years represent approximately 10% of all lung cancer deaths (6,655 of 63,255 deaths) according to the Japanese National Cancer Center Cancer Information Service Statistics published in 2008 (13). In the present study, a significant number of *ALK*-positive cases were <60 years of age (17.2%, 5 of 29; $P < 0.05$). *ALK*-positive cancer may therefore be more common in patients with early-onset NSCLC. However, it should be noted that two *ALK*-positive cases were >70 years of age (71 and 73 years); therefore, although patient age may become a predictor of *ALK* fusion gene positivity, *ALK* screening must also be performed in elderly individuals. The median diameter of primary lung tumors was significantly smaller in *ALK*-positive cases (28.6 versus 41.9 mm; $P < 0.05$), further emphasizing the importance of EBUS-TBNA because this technique does not require a large primary lesion. An additional advantage of EBUS-TBNA is that it can be used for lymph node sampling, which is relevant to the majority of advanced lung cancer cases. Although lung cancer is generally more common in smokers, most of the *ALK*-positive cases in this study (37 cases; 34.3%) were never-smokers or light smokers. The smoking index scores in the *ALK*-positive cohort were significantly lower than that of *ALK*-negative patients (161 versus 827; $P < 0.01$). Hence, being a never-smoker or light smoker seems to be a strong predictor of *ALK* positivity ($P < 0.01$).

Evaluation of the clinicopathologic characteristics of patients in our cohort indicated that *ALK*-positive lung cancer tends to have an adenocarcinoma histology, expresses wild-type *EGFR*, has an early age of onset (<60 y), manifests as a relatively small primary lesion, more frequently occurs in never-smokers or light smokers (smoking index score <400), and has a mucin-producing histology. However, as EBUS-TBNA samples are obtained from metastatic lymph nodes rather than the primary tumor, these clinical features are nearly compatible with previously reported features (14). Patients harboring one or more of these predictive factors may therefore derive the most benefit from *ALK* fusion gene screening.

Recently, *ALK*-positive NSCLC was reported to be a signet ring cell type adenocarcinoma (15, 16). We assume that this description also includes mucin production, i.e., mucin-producing tumors or tumors with >10% Alcian blue staining in the cytoplasm. Herein, we performed Alcian blue staining on suspected mucin-producing tumors as part of the histologic diagnosis. By this classification, 17 (15.6%) NSCLC cases were determined to be mucin-producing cancers. These cases were all adenocarcinomas and included five *ALK*-positive cases; thus, approximately 30% of the mucin-producing adenocarcinomas showed *ALK* positivity. This is a significantly high frequency compared with that of other NSCLCs ($P < 0.01$). This histologic feature, which can be assessed in cytologic samples, therefore seems to be useful for the prediction of *ALK* positivity.

The standard therapy for patients with advanced lung cancer at the time of presentation is chemotherapy and/or radiotherapy. However, standard platinum-based combined chemotherapy is not sufficient for disease eradication (17). Recently, lung cancer treatment strategies have become refined through the development of molecular markers and molecularly targeted agents. *ALK* inhibitors

have a high potential to become a definitive treatment for *ALK*-positive lung cancer, in a manner parallel to the exceptional therapeutic response of *EGFR*-positive lung cancers to *EGFR* tyrosine kinase inhibitors (18, 19). The efficacy of *ALK* inhibitors has been confirmed in cell lines (20, 21), and phase I clinical development of an oral *ALK* inhibitor for patients with lung cancer is currently under way (PF-02341066); two of the seven *ALK*-positive NSCLC cases from the present series have been enrolled in this trial (22, 23). As the background of *ALK*-positive lung cancer is similar to that of *EGFR*-positive lung cancer, and *ALK* tyrosine kinase inhibition is fundamentally similar to *EGFR* tyrosine kinase inhibition, *ALK* inhibitors might experience a similar progression of drug development and clinical and pathologic prediction of *ALK* positivity in lung cancer patients as *EGFR* tyrosine kinase inhibitors have for patients with *EGFR*-positive lung cancer. In this study, all *ALK*-positive lung cancers possessed wild-type *EGFR* and were therefore ineligible for *EGFR* tyrosine kinase inhibitor therapy (24). Therefore, *ALK* fusion gene assessment and administration of *ALK* inhibitors may become important for patients with *EGFR*-negative lung cancers.

Although some *ALK* inhibitors have already been developed and are currently being evaluated in clinical trials, it is important to establish a method for determining the existence of *ALK* fusion genes prior to the administration of *ALK* inhibitors. Both the presence of *ALK* fusion genes as well as *EGFR* gene mutations were successfully evaluated using histologic samples obtained by EBUS-TBNA of lung cancer regional lymph nodes. This diagnostic strategy allowed both pretreatment staging and evaluation of critical molecular markers to be definitively determined in a less invasive manner. There are some publications related with the genomic difference between primary tumor and metastatic site (25–29). EBUS-TBNA is a minimally invasive modality that allows the sampling of tumor cells from metastatic lymph node with a very low morbidity. The possibility of genetic differences should be considered whenever the biomarker information is used for the selection of patients for molecular target therapies. EBUS-TBNA is an ideal approach in this aspect.

In conclusion, EBUS-TBNA sampling is feasible for *ALK* fusion gene assessment by immunohistochemistry, FISH, and RT-PCR, as well as for pathologic diagnosis. The development of a safe and highly precise modality that enables the acquisition of a sufficient amount of high-quality tissue without surgery will become increasingly important in the molecularly targeted therapy era. EBUS-TBNA is one of the best candidates for such a methodology.

Disclosure of Potential Conflicts of Interest

K. Yasufuku, recipent of an unrestricted grant from Olympus Medical Corporation for Continuing Medical Education; H. Mano, member of the scientific advisory board, Pfizer Inc.

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INTERNAL MEDICINE

□ CASE REPORT □

Incidentally Proven Pulmonary "ALKoma"

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Abstract

Genetic alterations of echinoderm microtubule-associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*ALK*) inversion were recently found in lung cancer. A 39-year-old woman with multiple brain metastases and bulky mediastinal lymph node metastases was admitted. Biopsy from her supraclavicular lymph nodes was performed to differentiate the diagnosis between lymphoma and lung cancer. Pathologically, the lymph nodes had a feature of adenocarcinoma. On the other hand, the commercially available chromosomal fluorescent in situ hybridization (FISH) analysis showed split signals of *ALK*, which was confirmed to be the *EML4-ALK* inversion. The commercial-based ALK FISH is useful for screening pulmonary ALKoma.

Key words: EML4-ALK, lung cancer, oncogene addiction

(Inter Med 49: 603-606, 2010)
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Introduction

The echinoderm microtubule-associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*ALK*) inversion was recently detected in 6.7% of Japanese non-small cell lung cancer (NSCLC) patients (1). The fusion gene encodes a constitutive active oncoprotein with activated ALK kinase, resulting in the aberrant activation of the downstream signaling targets including Akt, signal transducer and activator of transcription (STAT) 3, and Ras-extracellular signal-regulated kinase (ERK) 1/2 (2).

The term ALKoma, coined by Benharroch et al, originally was used to represent anaplastic large cell lymphoma (ALCL) carrying the t(2 ; 5)(p23 ; q35) chromosome translocation (3). In 1994, Morris et al found that the t(2 ; 5) translocation fuses part of the nucleophosmin (*NPM*) gene on chromosome 5q35 to a portion of the ALK receptor tyrosine kinase gene on chromosome 2p23 (4). As with other fusion proteins found in hematological malignancies, ALKoma is also thought to become addicted to the ALK signaling pathway (3). Recently oncogene addiction has mainly been recognized among non-smoking NSCLC patients (5, 6). Just as epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) have become a mainstay of therapy for patients harboring *EGFR* mutation, patients with *EML4-ALK* inversion may benefit from therapy with ALK inhibitors. We herein report an incidentally proven *EML4-ALK* inversion in primary pulmonary adenocarcinoma.

Abbreviations: EML4: echinoderm microtubule-associated protein-like 4, ALK: anaplastic lymphoma kinase, NSCLC: non-small cell lung cancer, ALCL: anaplastic large cell lymphoma, NPM: nucleophosmin, EGFR: epidermal growth factor receptor

Case Report

A 39-year-old woman was admitted to hospital because of generalized seizures. An initial screening head and body CT showed multiple brain metastases and swelling lymph nodes throughout the thorax (Fig. 1A, B). Fiber optic bronchoscopy showed direct invasion of tumor to the carina (Fig. 1C). After a crisis of generalized seizures, neurological disorders were not obvious. The patient's performance status (PS) was graded as one, because of a dry cough, which had

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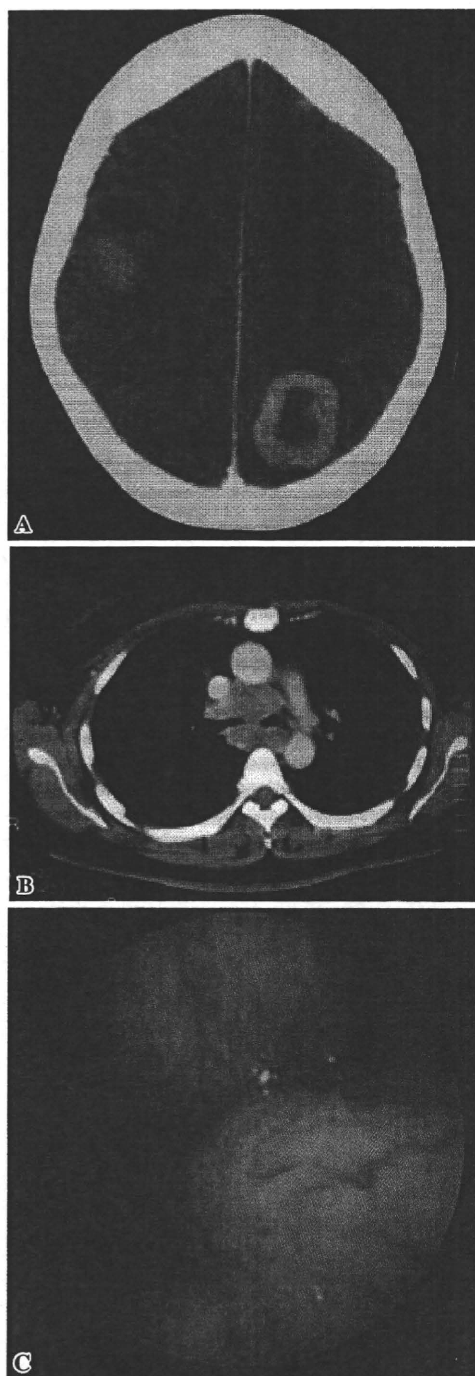

Figure 1. The imaging at presentation. Computed tomography presents multiple brain metastases (A) and mediastinal lymphadenopathy (B). Fiber optic bronchoscopy showed a direct invasion from metastatic lymph nodes to the carina (C). Both bronchi are too narrow to perform further examinations.

been apparent for the past 6 months before her admission to our hospital. Fine needle aspiration was performed from her right supraclaviclar lymph nodes, and malignancy at any origin was detected. As the patient was suspected to have malignant lymphoma or lung neoplasm, she was transferred to our institution for further examinations and therapies.

The laboratory data, including the tumor markers (carcinoembrionic antigen and soluble interleukin 2 receptor) were normal. The white blood cell count was 11,770/μL, probably due to the prophylactic use of corticosteroids against seizures. As the patient was thought to be in need of immediate therapy, an open biopsy from her right supraclavicular lymph nodes under local anesthesia was performed on the day of the transfer. The frozen samples were subjected to pathological examination, to *EGFR* mutation analysis (the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PCR) clamp method (7), Mitsubishi Chemical Medience, Tokyo, Japan), and to a comprehensive analysis for malignant lymphoma. Pathologically, the lymph nodes had a feature of moderately to poorly differentiated adenocarcinoma (Fig. 2A), with positive immunohistochemical staining for thyroid transcription factor-1 and epithelial markers (CAM5.2 and AE1/AE3). Immunohistochemical staining for lymphocyte markers was negative (CD20, CD45 RO, and CD30). Finally, her clinical diagnosis was determined to be cTxN3M1(BRA), clinical stage IV, adenocarcinoma of the lung. Although she was a young, never-smoking Japanese woman (8), she was found to be negative for *EGFR* mutations.

Meanwhile, the results of a comprehensive analysis for malignant lymphoma were reported. These analyses consisted of flow cytometric analyses with CD45 gating and a chromosomal G-banding analysis. In addition, the chromosomal fluorescent in situ hybridization (FISH) analyses were performed to detect the transition of *ALK* (2p23), *BCL6* (3q27), *IGH/BCL1* t(11 ; 14)(q13 ; q32), *IGH/BCL2* t(14 ; 18)(q32 ; q21), and *IGH/CMYC* t(8 ; 14)(q24 ; q32), based on a pathologist's decision ("ML-NET", SRL, Tokyo). In this case, the FISH analyses were added because the sample was not adequate for G-banding. Surprisingly, the FISH analysis of *ALK*, using 5′-(green) and 3′-(red) sequences for hybridization probes, showed the split signals of *ALK*, in up to 96% of the cells counted (total 100 cells) (Fig. 2B). In order to analyze the counterpart of transition for *ALK*, multiplex reverse transcription PCR of the EML4-ALK fusion transcripts was performed by YLC, MS and HM, and the transition was found to be EML4-ALK inversion, variant 2 (Fig. 2C).

Abbreviations: PCR: polymerase chain reaction, FISH: fluorescent in situ hybridization

Discussion

EML4-ALK inversion was first identified by Soda et al, from a lung adenocarcinoma specimen that was surgically resected from a 62-year-old male with a history of smoking. They made a cDNA library from the specimen, inserted cDNAs into the plasmid clones, and then infected them into mouse 3T3 fibroblasts with recombinant retrovirus to assess its ability to transform the foci. *The EML4-ALK* inversion transcripts were found in one of the transformed foci (1).

ALK, as well as leukocyte tyrosine kinase (*LTK*), is a re-

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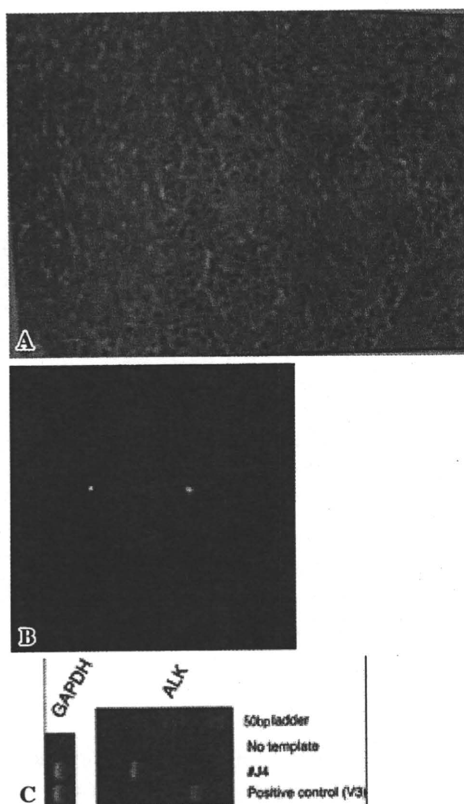

Figure 2. Histopathology and genetic analyses. Moderately to poorly differentiated adenocarcinoma is recognized with acinar patterns (A). A genomic FISH analysis showed that 96% of the cells which were analyzed had the split signal of ALK. A representative cell is shown (B). Multiplex RT-PCR to capture all in-frame fusions between *EML4* and *ALK* messages was conducted with the following primers;
5′-GTGCAGTGTTTAGCATTCTTGGGG-3′,
5′AGCTACATCACACACCTTGACTGG-3′,
5′-TACCAGTGCTGTCTCAATTGCAGG-3′,
5′-GCTTTCCCCGCAAGATGGACGG-3′,
5′-CAGCTGAGAGAGTGAAAGCTTTGG-3′,
5′-GACAGTTGGAGGAATCTGTCGATG-3′,
5′-ATCCTGCGGAACACTATTCAGTGG-3′,
5′-TCAAGCACATCTCAAGAGCAAGTG-3′ and
5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′.
Examination of an enlarged lymph node revealed the successful amplification for the *EML4-ALK* variant 2 transcript (indicated as #J4).

ceptor tyrosine kinase similar to the insulin receptor subfamily of kinases. *LTK* is found in murine B lymphocyte precursors and in forebrain neurons. *ALK* is usually found in the nervous system, where it serves the normal neural differentiation and construction. By transfusing its kinase domain with an activating counterpart with coiled-coil domain, like *NPM*, TRK-fused gene (*TFG*), *EML4* and so on, *ALK* gains oncogenic potential via a constitutional dimerization (2).

Since the receptor tyrosine kinases are one of the main targets of therapy in malignancy, Rikova et al performed a global survey of phosphotyrosine using lung cancer cell lines and clinical samples (9). Along with the well-known phosphorylation of EGFR and MET, the tyrosine phosphorylation of ALK was found in one cell line and in seven patients. A further analysis revealed three *EML4-ALK* inversions and one *TFG-ALK* fusion in 103 NSCLC patients, thus resulting in an overall frequency of ALK fusion of 4% in the Chinese population (9). A NSCLC cell line, H3122, which harbored an *EML4-ALK* inversion, showed massive apoptosis with an ALK kinase inhibitor, TAE-684 (10). Furthermore, transgenic mice expressing *EML4-ALK* conditionally in lung alveolar epithelial cells, which developed innumerable lung adenocarcinomas within a few weeks after birth, responded greatly with the oral administration of small-molecule inhibitors of the ALK kinase (11). Therefore, ALK is a novel therapeutic target in NSCLC. A phase I trial using PF02341066, TKI for MET and ALK, is ongoing for NPM-ALK-positive lymphoma (NCT00585195).

The clinicopathological background in patients with *EML4-ALK* inversion has been previously well described in two series. Inamura et al described that *EML4-ALK* positive lung cancers are characterized by an acinar histology, harboring neither *EGFR* mutation nor *KRAS* mutation, a non- or light smoking background and a young onset (12). Another group reported similar findings in which patients with *EML4-ALK* inversion were younger, more likely to be never/light smokers. They have also shown mutations of *KRAS*, *EGFR* and the rearrangement of *EML4-ALK* to be mutually exclusive (13). Furthermore, the latter group focused on the higher incidence of metastatic diseases in patients with an *EML4-ALK* inversion or *EGFR* mutation compared to patients without those alterations. The present case closely matches these findings. It is therefore suggested that an ALK FISH analysis should be recommended in the case of a never/light smoker, of younger onset, who is negative for *EGFR* mutation and advanced diseases.

Although several counterparts of *ALK* transition have been reported in some kind of tumors (2), the *EML4-ALK* inversion occurs most frequently in NSCLC (14). The identification of fusion transcripts is somewhat difficult because of the variation in the breakpoints of inversion. Soda et al are in the process of establishing multiplex RT-PCR for detecting fusion transcripts (1, 15). However, taking into consideration that there are a few other types of *ALK*-fusion (*TFG* and *KIF5B* (16)), the commercially used, previously established ALK FISH analysis is more useful in the screening of *ALK* altered NSCLC.

Since the incidence of patients demonstrating NSCLC with ALK transition who may benefit from a timely diagnosis and appropriate therapy exceeds the incidence of ALCL, the commercially-based ALK FISH is therefore considered to be a promising diagnostic modality for determining NSCLC patients with *ALK* transition.

Abbreviations: TKI: tyrosine kinase inhibitor, TRK-fused gene

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CASE REPORT

Treatment of Lung Cancer with an ALK Inhibitor After *EML4-ALK* Fusion Gene Detection Using Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration

Takahiro Nakajima, MD, PhD,† Hideki Kimura, MD, PhD,* Kengo Takeuchi, MD, PhD,‡ Manabu Soda, MD, PhD,§ Hiroyuki Mano, MD, PhD,§ Kazuhiro Yasufuku, MD, PhD,† and Toshihiko Iizasa, MD, PhD**

A 40-year-old man who had complained of bloody sputum was referred to our hospital for workup. Chest computed tomography showed a significant mediastinal lymphadenopathy (Figure 1*A*). Bronchoscopic examination revealed a tumor compressing the right mainstem bronchus (Figure 2*A*). Massive bleeding from the tumor was caused by passage of the bronchoscope. Therefore, a diagnosis of pulmonary adenocarcinoma was made by sputum cytology. The patient first received conventional chemotherapy in the form of four courses of cisplatin plus vinorelbine (CDDP + VNR), two cycles of cisplatin plus gemcitabine (CDDP + GEM), and four cycles of carboplatin plus gemcitabine (CBDCA + GEM). However, both the size of the tumor and the serum carcinoembryonic antigen level continued to increase. Fluorodeoxyglucose positron emission tomography suggested systemic metastasis in hilar and mediastinal lymph nodes and bone (Figure 1*B*).

We performed endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) to avoid bleeding from the tumor. Metastatic adenocarcinoma was revealed in an upper paratracheal lymph node (#2R) (Figures 2*B*, *C*). Because the epidermal growth factor receptor gene was wild type, we examined the presence of ALK fusion genes. Immunohistochemistry by the intercalated antibody-enhanced polymer (iAEP) method[1] showed an expression of ALK protein in the samples obtained by EBUS-TBNA (Figure 2*D*). *EML4-ALK* fusion gene was also confirmed by both fluorescence in situ hybridization (Figure 2*E*) and reverse transcriptase-polymerase chain reaction (Figure 2*F*). Direct sequencing of the PCR product revealed the presence of *EML4-ALK* variant 1. Thus, we referred the patient for enrollment in a clinical trial with crizotinib (PF-02341066).[2] Six weeks after administration of the crizotinib (250 mg twice a day, oral administration), the bloody sputum disappeared, and the tumor size decreased on chest computed tomography (Figure 1*C*). The carcinoembryonic antigen level also normalized. Five months after administration, an abnormal accumula-

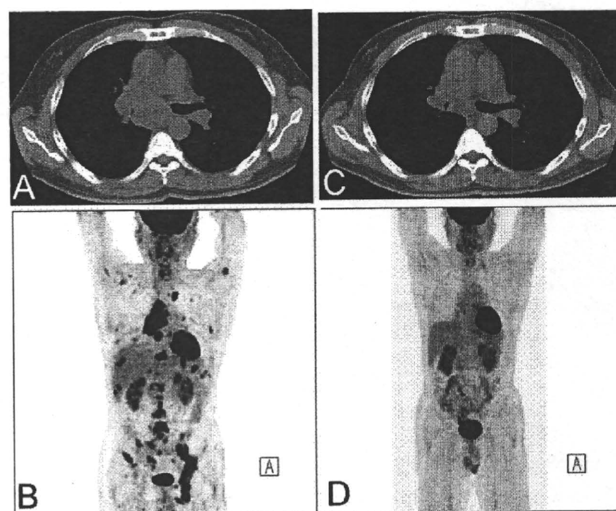

FIGURE 1. *A*, Chest computed tomography showed a narrowing of the right main bronchus due to massive lymphadenopathy. *B*, FDG-PET suggested multiple lymph node metastases and bone metastases. *C*, Six weeks after administration of the ALK inhibitor, the effect of the treatment was judged as partial response based on RECIST. *D*, Five months after administration of the ALK inhibitor, abnormal accumulation on FDG-PET had disappeared. FDG-PET, fluorodeoxyglucose positron emission tomography.

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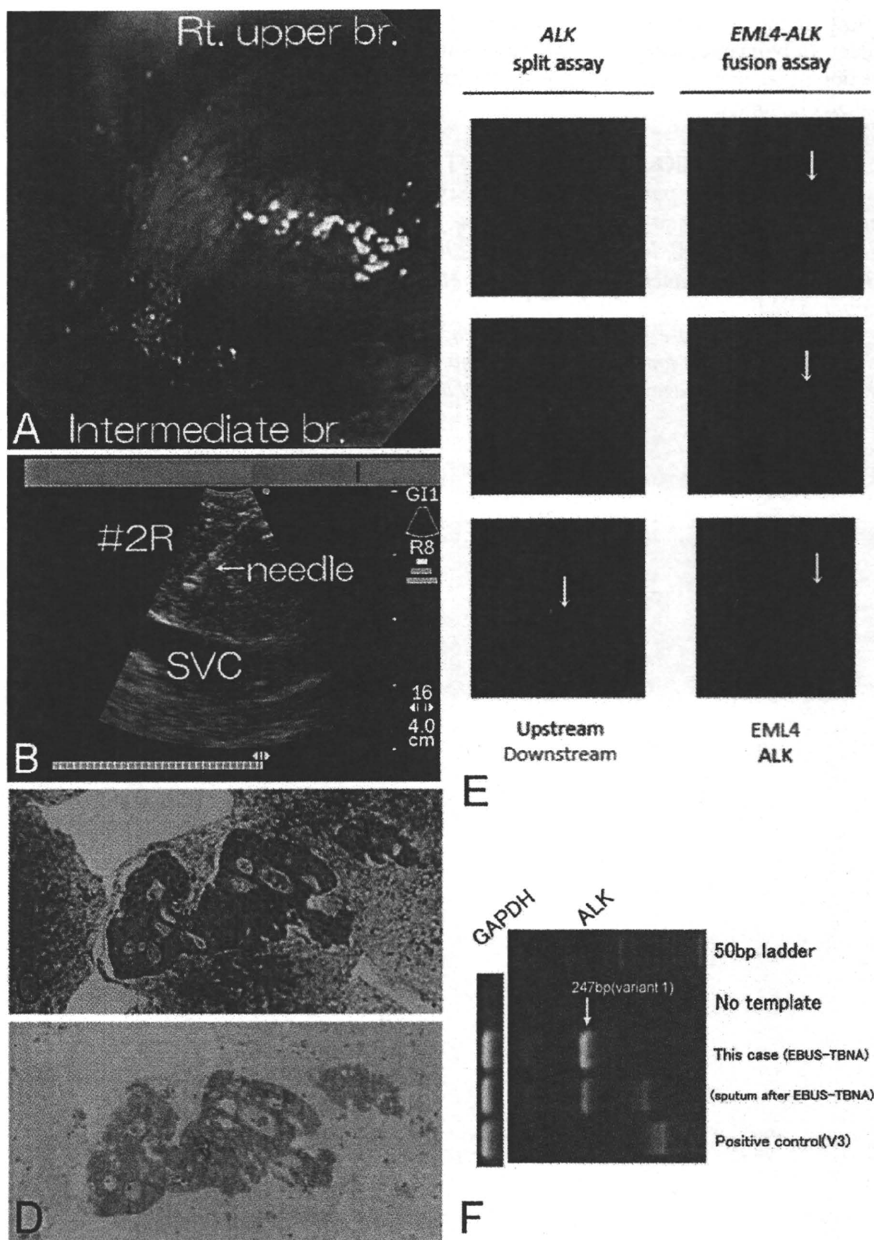

FIGURE 2. *A*, Bronchoscopic examination showed tumor compression of the right main bronchus, and the tumor had hyperplastic vessels on its surface. *B*, EBUS-TBNA was performed for a pretracheal lymph node (#2R). *C*, Histologic core revealed metastatic adenocarcinoma in #2R node. *D*, Immunohistochemistry was positive for ALK protein using the iAEP method. *E*, FISH revealed the EML4-ALK fusion gene. EML4-ALK split assay with labeled probes for the upstream (red) and downstream (green, arrow) region of the ALK locus. EML4-ALK fusion assay with labeled probes for EML4 (green, arrow) or ALK (red, arrow). Fusion gene showed EML4-ALK (arrow). *F*, RT-PCR using specific primer set for each variant also confirmed the presence of EML4-ALK variant 1 (274bp). The presence of variant 1 type fusion was also confirmed by direct sequence of the RT-PCR product (data not shown). RT-PCR, reverse transcriptase-polymerase chain reaction; FISH, fluorescence in situ hybridization.

tion almost disappeared on fluorodeoxyglucose positron emission tomography scan (Figure 1*D*). The observed side effects were only slight nausea during the early period of administration. The patient remains in good condition without tumor relapse for 10 months. The patient suddenly complained bilateral lower extremities paralysis, and the spinal cord metastasis was revealed. The patient was discontinued treatment during the trial in April 2010 because of disease progression.

DISCUSSION

Fusion of *ALK* with *EML4* gives rise to a highly potent oncogene in non-small cell lung cancer,[3] being detected in ~5% of all non-small cell lung cancer cases.[1,3,4] Presence of the ALK fusions can be detected by immunohistochemical screening[4] and can be also confirmed by fluorescence in situ hybridization and reverse transcriptase-polymerase chain reaction.[4] Recently, with progress in chemotherapeutic research, molecular targeted therapeutic agents have been developed, including ALK kinase inhibitors that are now being clinically tested.[2] Ideally, ALK fusion gene assessment should be performed using minimally invasive means to obtain biopsy samples sufficient for genetic analysis for subsequent targeted molecular therapy. Histologic as well as cytologic samples can be obtained by EBUS-TBNA, and we have previously reported that high-quality cores are adequate for molecular analyses for biomarkers.[5] The dramatic

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effect of the ALK inhibitor in this patient demonstrates that adequate biomarker assessment contributes to the optimum selection of reagents in targeted molecular therapy and in individualized treatment.

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Correspondence

EML4-ALK Fusion in Lung

To the Editor-in-Chief:

The recent article by Martelli and colleagues[1] reports (i) the detection of *EML4-ALK* fusion cDNA[2] not only in non-small cell lung cancer (NSCLC) specimens but in non-tumor lung tissues, (ii) a very low proportion of FISH-positive cells for *ALK* rearrangements among *EML4-ALK*–positive specimens, and (iii) the failure to detect EML4-ALK protein by immunohistochemistry (IHC) and Western blotting. Based on these lines of observation, the authors questioned the clinical relevance of *EML4-ALK* in the carcinogenesis of NSCLC.

Although detection of fusion kinases in normal tissues is a potentially interesting observation, caution is warranted in the interpretation of their results.[1,3] They replicated thrice the reverse transcription-polymerase chain reaction (RT-PCR) for *EML4-ALK* and noted that "In half of the (positive) cases, one replicate experiment did not confirm the fusion transcript was present." They then suggested that the fusion gene was "expressed at very low level." It is, however, also quite possible that such unstable PCR results may simply represent contaminated experiments. If this is the case, a discussion on FISH and protein analyses would become irrelevant. In their report, the presence of the *EML4-ALK* fusion gene was only evidenced by unstable RT-PCR results and a small proportion of FISH-positive cells among specimens.

In this regard, it was surprising that the authors had not tried genomic PCR to exclude the possibility of PCR contamination.[1,3] In most of their fusion-positive cases, they found the *EML4-ALK* variant 1 cDNA, in which exon 13 of *EML4* cDNA is connected to exon 20 of *ALK* cDNA. Because the length of intron 14 of *EML4* gene and intron 19 of *ALK* gene is 5724 bp and 1932 bp, respectively, the maximum size of the genomic PCR to detect the gene fusion should be ≈7.7 kbp, which is within the scope of current long-range PCR systems. Indeed, we have been able to detect genomic PCR products among >50% of the fusion cDNA-positive cases. Interestingly, the break/fusion points in the genome vary substantially among NSCLC specimens,[2,4,5] and we have not obtained, to date, any pairs of NSCLC specimens carrying identical break/fusion points in their genome (even among those positive for the same *EML4-ALK* variants).

We speculate, therefore, that (i) if none of the fusion cDNA-positive cases reported by Martelli et al[1,3] produce specific genomic PCR products, then the fusion cDNA products likely arose from cDNA-contamination, (ii) if the fusion cDNA–positive cases yield identical genomic PCR products, then the fusion cDNAs likely arose from specimen-contamination, and (iii) if the fusion cDNA-positive cases display distinct genomic fusion points, then each specimen was truly positive for the *EML4-ALK* fusion gene. Without such careful examination, we have to conclude that their claims in the article have not as yet been clearly demonstrated.

As described previously,[6] immunohistochemical detection of the EML4-ALK protein is highly difficult, probably owing to the weak activity of the *EML4* promoter that drives the expression of *EML4-ALK* messages. We have thus examined the suitability of commercially available antibodies to ALK for IHC and successfully developed the intercalated antibody–enhanced polymer (iAEP) method, which enables reliable detection of EML4-ALK among formalin-fixed and paraffin-embedded specimens.[6] The same specimen positive for *EML4-ALK* RT-PCR can be, for instance, readily stained to be positive with iAEP, but negative with conventional IHC methods (see Supplemental Figure S1 in ref. 6). We thus agree with Martelli et al that screening of NSCLC specimens with conventional IHC methods will not detect EML4-ALK protein, but strongly argue that such failure does not simply indicate the absence of EML4-ALK. For such screening, we recommend iAEP or other sensitive techniques.[7]

It should be further noted that, in both our[6] and other researchers' IHC analyses,[7] almost all tumor cells in a given *EML4-ALK*–positive specimen were positively immunostained with anti-ALK antibodies, suggesting a homogenous presence of *EML4-ALK* within a tumor. Such observation is, however, in contrast to the FISH data by Martelli et al, which show that the *ALK* rearrangement was only positive in ≈2% of tumor cells in a given *EML4-ALK*–positive specimen. On the contrary, FISH analyses of our *EML4-ALK*–positive samples clearly demonstrate that most of the tumor cells harbor rearranged *ALK* alleles, implying that the generation of the *EML4-ALK* fusion gene is an early event in NSCLC carcinogenesis. The homogenous presence of EML4-ALK in our fusion-positive tumors, as demonstrated by both FISH and IHC, further raises a concern about the "EML4-ALK–positive tumors" as defined by Martelli et al.

Specific inhibitors to ALK enzymatic activity are already in clinical trial, as reported at the 2009 annual meeting of America Society of Clinical Oncology and the European Cancer Organization and Congress of the European Soci-

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ety for Medical Oncology.[8] Such reports reveal only modest and transient side effects (nausea, vomiting, and diarrhea) with their ALK inhibitor, but without severe damage in hematopoiesis or renal function. On the other hand, the marked therapeutic efficacy of their compound against EML4-ALK–positive NSCLC makes it one of the rare, highly successful molecular targeted therapies against human cancer, in line with imatinib mesylate and gefitinib/erlotinib. These data further reinforce the essential role of EML4-ALK in the carcinogenesis of NSCLC, and question the validity of the conclusions led by Martelli et al.[1,3]

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Authors' reply:

In their letter, Mano and Takeuchi claim that our unstable PCR results in normal and cancerous lung tissues could be attribtuable to contamination. However, as clearly illustrated in our article,[1] serial dilution experiments in the H2228 cell line demonstrate the specificity and sensitivity of our RT-PCR assay. Furthermore, the identification in our EML4-ALK fusion positive tissues of alternative isoforms of variant 3, rather than the described two isoforms coexpressed in the H2228 cell line, is indicative of exclusive events in tumors, making contamination unlikely. Lastly, our experiments were confirmed independently in two laboratories (Milan and Barcelona) and always contained appropriate negative PCR controls.

We disagree with Mano et al's claim that the results of genomic PCR could be used to prove a possible RT-PCR contamination in our samples, which can only be excluded by the use of appropriate controls and procedures, as outlined above. However, we used genomic PCR to amplify the sequence flanking the *EML4-ALK* variant 1 breakpoint in four positive NSCLC samples. Even though a strong amplification product had been obtained from the same DNA templates using primer sets amplifying a control genomic locus of similar size to that of the cases so far reported in literature, no amplification of the *EML4-ALK* variant 1 fusion product was identified, suggesting only a minority of cells carried the *EML4-ALK* gene. These findings concur with Maes et al[2] who reported that, in lymphoid tissues, high level detection of *NPM-ALK* and *ATIC-ALK* fusion transcripts coincided with *ALK* gene rearrangements (as detected by cytogenetics and FISH), whereas low-level detection was not supported by genomic evidence of rearrangements.

In our article,[1] we clearly stated that, unlike observations in ALK+ lymphomas, tumor cells from NSCLC specimens expressed such a low amount of the EML4-ALK fusion protein that immunoprecipitation and immunohistochemistry performed with the commercially available antibodies are unable to detect it. This is in keeping with the observation that the EML4-ALK fusion protein is detectable only using highly sensitive methods, such as mass spectrometry[3] or the intercalated antibody-enhanced polymer (iAEP) method[4] which, unfortunately, are not available in all pathology laboratories and are difficult to standardize. Therefore, the question of how best to detect the EML4-ALK fusion protein remains unanswered.

Issues concerning the frequency, heterogeneity, and tissue specificity of the *EML4-ALK* rearrangement must also be addressed carefully.

Frequency

We recently extended our FISH analysis to 173 surgically resected lung cancer specimens (mainly adenocarcinoma) from an unselected group of Caucasian patients. The incidence of truly positive cases (>50% FISH positive, fusion transcript, and protein positive) was only 0.6% (1/173 cases), which reinforces the results in our artile and is in keeping with Rodig et al's[5] recent report of 1/227 (0.45%) ALK rearranged case in a series of surgically treated Western adenocarcinoma.

Heterogeneity

The heterogeneity of the *EML4-ALK* rearrangement we detected by FISH was confirmed by others in primary tumors

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and cell lines[6,7] and is supported by functional studies showing that the magnitude of growth inhibition by siRNA-mediated silencing did not correlate with the number of cells harboring the rearrangement and the lack of growth inhibition in 50% of *EML4-ALK*–positive cell lines. These observations suggest that additional signaling mechanisms independent of ALK may regulate growth and cell proliferation.

Specificity

Claims from Mano's group that the *EML4-ALK* product is specific for NSCLC is contradicted by our findings in normal tissues[1,8] and by a recent study from Lin E. et al,[6] who found *EML4-ALK* fusions in breast (2.4%) and colorectal (2.4%) cancer, in addition to NSCLC.

Finally, we wonder whether it is really appropriate to compare treatments such as ALK inhibitors in NSCLC with imatinib mesylate and gefinitinib/erlotinib in other human neoplasms. In fact: i) the role of EML4-ALK in NSCLC is not as well established as that of BCR/ABL in chronic myeloid leukemia (CML); ii) NSCLC responses to ALK inhibitors[9] are not as remarkable as the CML response to imatinib mesylate; and iii) patients with NSCLC were treated with a multikinase, c-MET and ALK, inhibitor.[9] Considering that about 20% of NSCLC have MET amplification and overexpression and that MET rearrangements are homogeneous in lung cancer,[10] it may be possible that responses to the multikinase inhibitor may be related to other coexisting oncogenic events, independently of ALK.

In conclusion, although we fully acknowledge the importance of Soda et al's discovery,[11] we believe that additional studies are required to elucidate the concurrent genetic events and cellular settings necessary for *EML4-ALK* to exert an oncogenic function and to better define the role of *EML4-ALK* in diagnosis and targeted therapy of NSCLC.

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Identification of transforming activity of free fatty acid receptor 2 by retroviral expression screening

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Gallbladder cancer (GBC) is a highly fatal malignancy in humans. Genetic alterations in *KRAS* or *TP53* as well as overexpression of *ERBB2* have been shown to contribute to the development of certain types of GBC. However, many cases of GBC do not harbor such genetic changes, with other transforming events awaiting discovery. We here tried to identify novel cancer-promoting genes in GBC, with the use of a retroviral cDNA expression library. A retroviral cDNA expression library was constructed from a surgically resected clinical specimen of GBC, and was used to infect 3T3 fibroblasts in a focus formation assay. cDNA incorporated into the transformed foci was rescued by PCR. One such cDNA was found to encode free fatty acid receptor 2 (FFAR2), a G protein-coupled receptor for short-chain fatty acids. The oncogenic potential of *FFAR2* was confirmed both *in vitro* with the focus formation assay and by evaluation of cell growth in soft agar as well as *in vivo* with a tumorigenicity assay in nude mice. The isolated *FFAR2* cDNA had no sequence alterations, suggesting that upregulation of *FFAR2* expression may contribute to malignant transformation. Indeed, all of quantitative RT-PCR, *in situ* hybridization, and immunohistochemical analyses showed that the amount of *FFAR2* mRNA and its protein product was increased in digestive tract cancer specimens. Furthermore, short-chain fatty acids potentiated the mitogenic action of FFAR2 in 3T3 cells. Our data thus, for the first time, implicate FFAR2 in carcinogenesis of the digestive tract. (*Cancer Sci* 2010; 101: 54–59)

Gallbladder cancer (GBC) is a highly fatal malignancy in humans, being most prevalent in South America and Asia. In most cases, GBC is not diagnosed until it has reached an advanced stage, when the 5-year survival rate is ~10%.[1,2] In the USA, ~8000 new cases of biliary tract cancer (BTC) are diagnosed each year, with ~4000 of the affected individuals subsequently dying of GBC.[3] Several risk factors have been identified for GBC, including cholelithiasis[4] and anomalous pancreaticobiliary duct junction.[5] Genetic alterations in *KRAS* or *TP53* as well as overexpression of *ERBB2* have been shown to contribute to the development of certain types of GBC. However, many cases of GBC do not harbor such genetic changes, with other transforming events awaiting discovery.

The focus formation assay with 3T3 or RAT1 fibroblasts has been used extensively to screen for transforming genes in various carcinomas.[6] In such screening, genomic DNA is isolated from cancer specimens and used to transfect fibroblasts, potentially resulting in the development of transformed cell foci. However, given that expression of the introduced genes is controlled by their own promoters or enhancers, oncogenes in cancer cells may exert effects in fibroblasts only when their control regions are active in these cells, which is not guaranteed.

Adequate expression of cDNA in fibroblasts can be achieved by placing them under the control of an exogenous promoter fragment. Toward this goal, we have recently established a retroviral cDNA expression library system that is sensitive enough to generate libraries with a high complexity even from small amounts of materials such as clinical specimens.[7–9] With this system, we have successfully discovered a fusion-type protein tyrosine kinase EML4–ALK in non-small cell lung cancer.[7]

In this manuscript, we have applied this technology to a surgically resected clinical specimen of GBC, and used this library to screen for transforming genes in GBC. Unexpectedly, transforming ability has been discovered for free fatty acid receptor 2 (FFAR2, also known as GPR43), which functions as a cellular receptor for short-chain fatty acids (SCFA).[10] Further, tumor-specific expression of FFAR2 has been proven among a panel of clinical specimens for GBC, gastric cancer, and colorectal cancer (CRC) by *in situ* hybridization and immunohistochemical analyses, indicating tumor-promoting activity among digestive tract cancers.

Materials and Methods

Clinical specimens and cells lines. Resected clinical materials were obtained from individuals who underwent surgery at Jichi Medical University Hospital. Written informed consent was obtained from each subject according to the protocols approved by the ethics committees of Jichi Medical University. Mouse 3T3 and BOSC23 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA), and maintained in Dulbecco's modified Eagle medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine.

Construction of retroviral cDNA expression library. The retroviral cDNA library was constructed as described previously.[7–9,11] Briefly, first-strand cDNA was synthesized from the RNA with the use of PowerScript reverse transcriptase, the SMART IIA oligonucleotide, and CDS primer IIA (all from Clontech, Mountain View, CA, USA). The resulting cDNA was then amplified by PCR with 5′-PCR primer IIA (Clontech) and PrimeSTAR HS DNA polymerase (Takara Bio, Otsu, Shiga, Japan) for 17 cycles of 98°C for 10 s and 68°C for 6 min. The PCR products were ligated to a BstXI adapter (Invitrogen) and then incorporated into the pMXS retroviral plasmid (kindly provided by T. Kitamura of the Institute of Medical Science, University of Tokyo).

Recombinant retroviruses were produced by introduction of the plasmid library into the packaging cell line BOSC23[12] and were used to infect 3T3 cells in the presence of polybrene (4 μg/mL; Sigma, St Louis, MO, USA). The cells were cultured for 2 weeks, after which transformed foci were isolated, expanded, and subjected to extraction of genomic DNA. Insert

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cDNA was recovered from the genomic DNA by PCR with 5′-PCR primer IIA and PrimeSTAR HS DNA polymerase. Amplified products were then ligated to the plasmid pT7Blue-2 (Novagen, Madison, WI, USA) and subjected to nucleotide sequencing.

Transformation assay. For a focus formation assay, recombinant retrovirus was used to infect 3T3 cells for 48 h. The culture medium of 3T3 cells was then changed to DMEM/F12 supplemented with 5% calf serum and 2 mM L-glutamine, and incubated for 2 weeks. To examine anchorage-independent growth in soft agar, 3T3 cells infected with retrovirus were resuspended in culture medium containing 0.4% agar (SeaPlaque GTG agarose; Cambrex, East Rutherford, NJ, USA), and seeded onto a base layer of complete medium containing 0.5% agar. Cell growth was assessed after 3 weeks of incubation.

For an *in vivo* tumorigenicity assay, 3T3 cells (2×10^6) infected with the retrovirus expressing FFAR2 were resuspended in 500 μL PBS, and injected into each shoulder of *nu/nu* BAL-Bc mice (6 weeks old). Tumor formation was assessed after 3 weeks.

Quantitation with real-time RT-PCR. Oligo(dT)-primed cDNA was synthesized from the clinical specimens with PowerScript reverse transcriptase, and subjected to quantitative PCR with a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and an amplification protocol consisting of incubations at 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s. Incorporation of the SYBR Green dye into PCR products was monitored in real time with an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), thereby allowing determination of the threshold cycle (C_T) at which exponential amplification of products begins. The C_T values for cDNA corresponding to the β-actin gene (*ACTB*) and *FFAR2* were used to calculate the abundance of the latter mRNA relative to that of the former. The oligonucleotide primers used for PCR were 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GTCCGCCTAGAAGCATTTGCG-3′ for *ACTB* and 5′-CACTCAACGCCAGTCTGGAC-3′ and 5′-TGGCATCCCTTCTCCTTGAC-3′ for *FFAR2*.

In situ hybridization with sense or antisense riboprobes corresponding to the 3′ region (nucleotides 867–1229) of the *FFAR2* cDNA isolated in this study was conducted as described previously.[13]

Immunohistochemistry. Human tissues were fixed in 4% formaldehyde in PBS at room temperature overnight, embedded in paraffin, and sectioned at a thickness of 3 μm. Sections were mounted on glass slides, deparaffinized through three changes of xylene for 4 min each, and rehydrated in distilled water through a series of graded alcohols. For histological evaluation, sections were stained with hematoxylin–eosin solutions. For immunohistochemical experiments, antigenicity was enhanced by boiling the sections in 10 mM citrate buffer (pH 6.0) in a microwave oven for 15 min, and the endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H_2O_2 for 30 min. After two washes with PBS containing 1% Triton X-100, the sections were preincubated with the blocking buffer (#X0909; Dako, Glostrup, Denmark) in a humidified chamber for 20 min at room temperature, and then incubated with anti-FFAR2 antibody (SP4226P; Acris Antibodies, Schillerstaβe, Herford, Germany) at 4°C overnight. Next, the sections were washed in PBS and incubated with horseradish peroxidase (HRP)-labeled polymers conjugated to goat antirabbit immunoglobulin (#K4003; Dako) at 37°C for 30 min. Color development was carried out by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) as a chromogenic substrate. Finally, the sections were lightly counterstained with hematoxylin, mounted, and viewed under a light microscope.

Cell proliferation assay. Mouse 3T3 cells expressing FFAR2 or not expressing FFAR2 were seeded into 96-well plates at a concentration of 4×10^3 cells/well, and incubated for 24 h with DMEM-F12 medium and 1% charcoal-treated fetal bovine serum (Invitrogen). Cells were further cultured for 48 h with 100 mM sodium acetate or 1 mM sodium butyrate, and were subjected to the cell proliferation assay with the WST-1 reagent (Clontech).

Results

Focus formation assay with a GBC library. To screen for transforming genes in digestive tract cancers, we constructed a retroviral cDNA expression library from a surgically resected GBC specimen, and obtained a total of 3.2×10^5 colony-forming units of independent plasmid clones, from which we randomly selected 20 clones and examined the incorporated cDNA. An insert of ≥500 bp was present in 16 (80%) of the plasmid clones, and the average size of these inserts was 1.48 kbp (data not shown). Infection of mouse NIH 3T3 fibroblasts with the recombinant retroviral library generated a total of 89 transformed foci (Fig. 1a). No foci were obtained for cells infected with the empty virus, whereas numerous foci were readily apparent for cells infected with a virus encoding the v-Ras oncoprotein.

Each focus obtained with the cDNA expression library was isolated, expanded independently, and used to prepare genomic DNA for recovery of retroviral inserts by PCR with the primers used originally to amplify the cDNA in construction of the library.[7] We recovered a total of 45 cDNA fragments by PCR, each of which was subjected to nucleotide sequencing in both directions. Screening of the 45 cDNA sequences against the public nucleotide sequence databases revealed that they corresponded to 19 independent genes (Supporting Information Table S1). To confirm the transforming potential of the isolated cDNA, we ligated each cDNA clone to pMXS and used the resulting retroviruses to infect 3T3 cells. The focus formation assay was carried out for cDNA corresponding to 19 independent genes, revealing reproducible transforming activity for: clone ID #2, corresponding to *ARHGEF1* (GenBank accession number NM_004706); clone ID #6, corresponding to *TBC1D3* (GenBank accession number NM_032258); clone ID #7, corresponding to *FGF4* (GenBank accession number NM_002007); and clone ID #14, corresponding to *FFAR2* (GenBank accession number NM_005306) (Fig. 1b).

FFAR2 as an oncogene. FFAR2 functions as a cellular receptor for SCFA,[10] and is expressed in the digestive tract.[14] It is thought to respond to fatty acids released in the digestive tract, but has not previously been shown to possess transforming potential. We therefore focused on FFAR2 in our subsequent analyses. Given that nucleotide sequencing of clone ID #14 did not reveal any sequence alterations compared to the published cDNA sequence of *FFAR2* (GenBank accession number NM_005306), we hypothesized that overexpression of *FFAR2* might contribute to malignant transformation.

We then assessed the transforming activity of FFAR2 in 3T3 cells with a soft-agar assay. Whereas cells infected with the empty virus did not grow in soft agar, those infected with a virus encoding v-Ras grew readily (Fig. 1b). Cells infected with a virus encoding FFAR2 also formed multiple foci in repeated experiments, indicative of the ability of FFAR2 to confer the property of anchorage-independent growth on 3T3 cells. We further tested the activity of FFAR2 in an *in vivo* tumorigenicity assay with athymic nude mice. 3T3 cells infected with the empty virus or with retroviruses encoding FFAR2 or v-Ras were thus injected subcutaneously into the mice. Tumor formation was readily apparent for the cells expressing FFAR2 or v-Ras (Fig. 1b).

Overexpression of FFAR2 in digestive tract cancers. Given that our data revealed an unexpected transforming potential of FFAR2 (at least, when it is abundantly expressed), we examined

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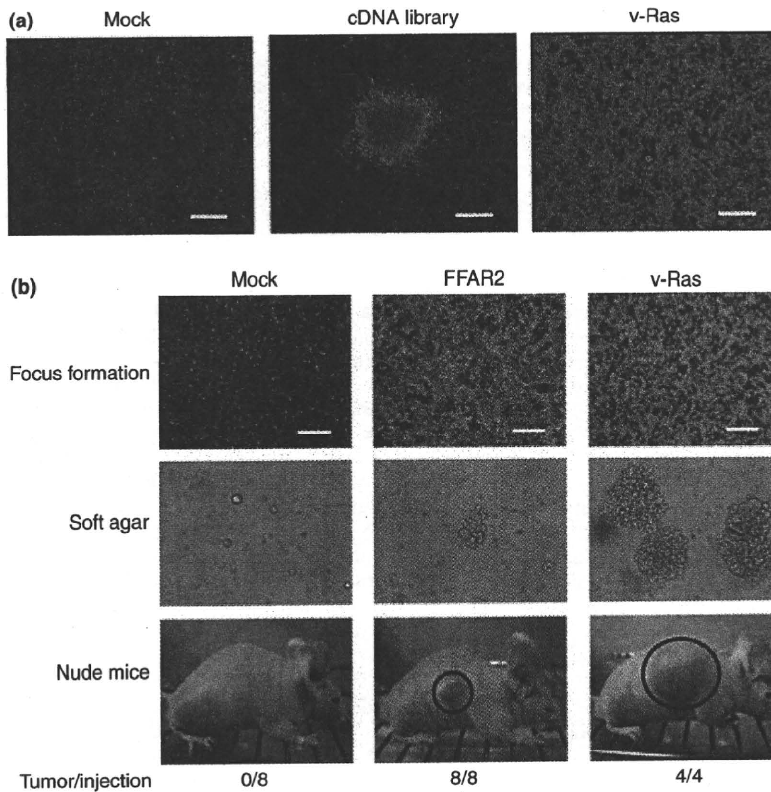

Fig. 1. Transforming activity of free fatty acid receptor 2 (FFAR2). (a) A retroviral cDNA expression library was constructed from a gallbladder cancer specimen isolated from a 64-year-old man. Mouse 3T3 cells were infected with the retroviral cDNA library, a virus encoding v-Ras, or the empty virus (Mock), and were photographed after culture for 2 weeks for the analysis of focus formation. Scale bars = 1 mm. (b) 3T3 cells were infected with viruses encoding FFAR2 or v-Ras or with the empty virus (Mock) and were then cultured for 5 days for analysis of focus formation (top panels: scale bars = 1 mm). The same batches of 3T3 cells were also assayed for anchorage-independent growth in soft agar over 17 days (middle panels) and for tumorigenicity in nude mice over 3 weeks (bottom panels). Tumors formed in the shoulders of mice injected subcutaneously with 1×10^5 cells are indicated by red circles. The frequency of tumor formation (tumor/injection) is also indicated.

whether FFAR2 might be overexpressed in human cancer specimens. We prepared oligo(dT)-primed cDNA from seven specimens of BTC, 89 specimens of gastric cancer, and 80 specimens of CRC by reverse transcription and then subjected the cDNA preparations to quantitative PCR analysis in order to measure the amount of *FFAR2* cDNA. For comparison, we also analyzed specimens of normal gallbladder ($n = 6$) and biliary duct ($n = 1$) as well as paired noncancerous tissue for all specimens of gastric cancer and CRC. Whereas the mean expression level of *FFAR2* seemed higher in BTC compared to normal gallbladder/biliary duct, a large standard deviation in the expression level made the difference insignificant ($P > 0.05$) (Fig. 2a). However, the *FFAR2* level was significantly increased ($P < 0.05$) in gastric cancer (Fig. 2b) and CRC (Fig. 2c) compared with the corresponding paired normal tissue specimens.

To examine further the site and extent of *FFAR2* expression, we carried out *in situ* hybridization analysis with a series of cancer specimens. First, a section of a CRC specimen was subjected to hybridization with sense or antisense probe for *FFAR2* mRNA. Only the antisense probe yielded clear signals in the cytoplasm and nucleus of the cancer cells (Fig. 3a), thus confirming the specificity of this probe. A series of cancer specimens was then subjected to hybridization with the antisense probe for *FFAR2* mRNA. GBC cells exhibited an increased level of hybridization compared with the normal cells in the same section (Fig. 3b). However, epithelial cells of normal gall-

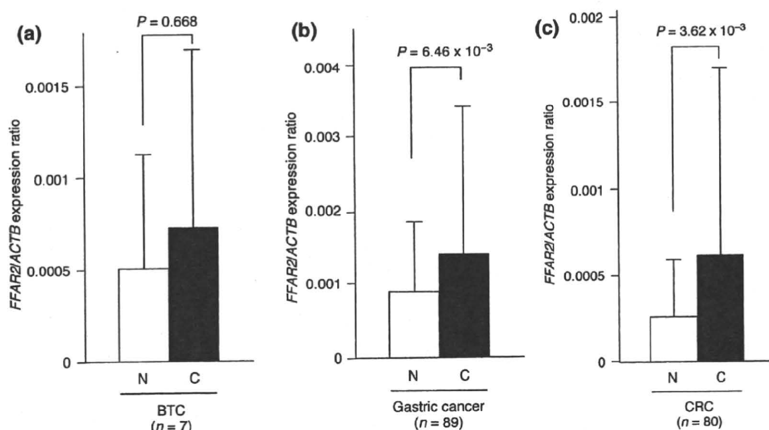

Fig. 2. Expression of free fatty acid receptor 2 (*FFAR2*) in digestive tract cancers. Oligo(dT)-primed cDNA was synthesized from (a) clinical specimens of biliary tract cancer (C) or normal gallbladder and biliary tract tissue (N), or from paired cancerous (C) and noncancerous (N) tissue specimens from patients with (b) gastric cancer or (c) colorectal cancer. The resultant cDNA was subjected to quantitative PCR analysis. Data are means + SD for the indicated n values, and P-values for the indicated comparisons were determined by Student's t-test. ACTB, β-actin.

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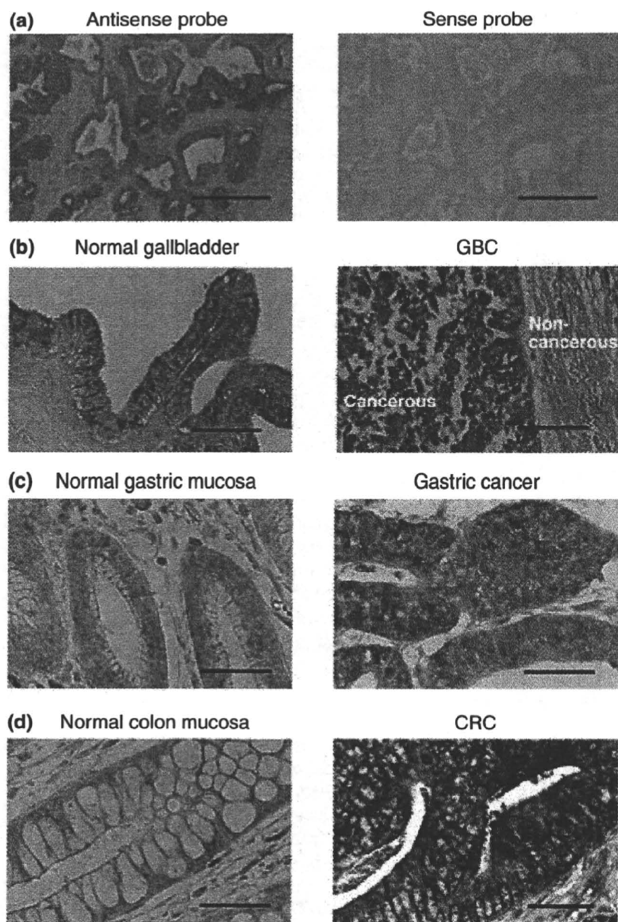

Fig. 3. *In situ* hybridization analysis of free fatty acid receptor 2 (*FFAR2*) expression. (a) A section of colorectal cancer (CRC) was subjected to *in situ* hybridization with sense or antisense riboprobes corresponding to the 3′ region (nucleotides 867–1229) of the *FFAR2* cDNA isolated in this study. (b–d) Sections of (b) normal gallbladder and gallbladder cancer (GBC), (c) paired normal gastric mucosa and gastric cancer, and (d) paired normal colon mucosa and CRC were also subjected to *in situ* hybridization with the antisense probe for *FFAR2* mRNA. Scale bars = 1 mm (a), 100 μm (b), or 50 μm (c,d).

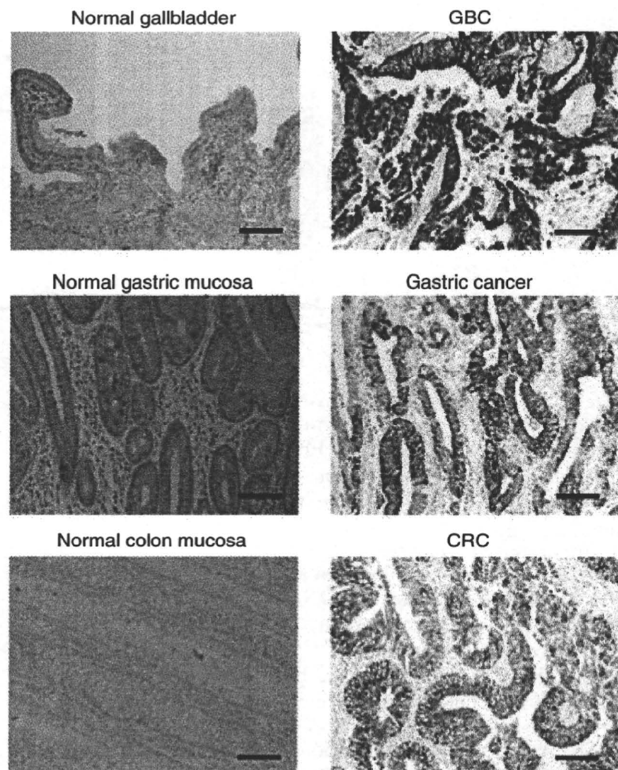

Fig. 4. Immunohistochemical analysis of free fatty acid receptor 2 (FFAR2) expression. Sections of normal gallbladder and gallbladder cancer (GBC) (upper panel), of paired normal gastric mucosa and gastric cancer (middle panel), and of paired normal colon mucosa and colorectal cancer (CRC) (lower panel) were subjected to immunohistochemical staining with antibody to FFAR2. Scale bars = 100 μm.

bladder were also stained with the probe, possibly explaining why the amount of *FFAR2* mRNA did not differ significantly between GBC and normal tissue by quantitative RT-PCR analysis (Fig. 2a). In contrast, the hybridization signal for *FFAR2* mRNA was markedly greater both in gastric cancer cells in eight of 10 specimens examined than in gland cells of the normal stomach (Fig. 3c), as well as in CRC cells in 13 of 14 specimens examined compared with the corresponding normal cells (Fig. 3d), consistent with the data obtained by quantitative RT-PCR analysis (Fig. 2b,c).

Additionally, we further examined the FFAR2 protein level by immunohistochemistry with anti-FFAR2 antibody among digestive tract cancers. As shown in Figure 4, FFAR2 was apparently induced in a GBC specimen (from which the cDNA library was generated) compared to normal gallbladder, in a gastric cancer specimen compared to its paired normal mucosa, and in a CRC specimen compared to the paired normal mucosa.

Ligand-mediated mitogenic signals of FFAR2. Given that SCFA are the presumptive ligands for FFAR2, we next examined whether the transforming activity of FFAR2 might be stimulated by its binding of such ligands. Toward this end, we incubated 3T3 cells expressing *FFAR2* cDNA in the absence or presence of the SCFA sodium acetate or sodium butyrate. Forced expression of FFAR2 induced a small increase in the growth rate of 3T3 cells even in the absence of the SCFA, whereas the SCFA had no effect on the growth of cells not expressing FFAR2. In contrast, sodium acetate (100 mM) induced a pronounced increase in the growth rate of cells expressing FFAR2 (Fig. 5a). A smaller but still significant increase in the growth rate of cells expressing FFAR2 was also induced by the addition of 1 mM sodium butyrate (Fig. 5b).

Discussion

In this study, we constructed a retroviral cDNA expression library for a GBC specimen and thereby identified the transforming potential of FFAR2. In response to its activation by ligand, FFAR2 regulates lipogenesis,[14] neutrophil migration,[15] and intestinal motility.[16] Although SCFA activate the p38 mitogen-activated protein kinase and heat shock protein 27 signaling pathway via FFAR2 in MCF-7 human breast cancer cells,[17] a relationship between FFAR2 and carcinogenesis has not previously been described.

The *FFAR2* gene has been shown to be preferentially expressed in stomach, small intestine, colon, spleen, and adipose tissue of mice.[14] A substantial amount of *FFAR2* mRNA was also detected in the rat gut, with the highest levels apparent in the colon and lower levels observed in esophagus and stom-

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