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Figure 1. Histopathology of SQSTM1-ALK-positive large B-cell lymphoma.

(A) The pattern of tumor infiltration was diffuse. The lymphoma cells were large with abundant cytoplasm and had round, vesicular nuclei, each containing a centrally located large nucleolus. These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was extremely large compared with these typical cell types (40× objective). (B) Some lymphoma cells expressed cytokeratin (AE1/AE3) (20× objective). (C) Syndecan1/CD138 was strongly expressed (20× objective). (D) In anti-ALK immunohistochemistry, a diffuse cytoplasmic staining pattern with ill-demarcated spots was clearly shown (20× objective).

Figure 2. Discovery of *SQSTM-ALK* fusion gene.

(A) A chromosome translocation, t(2;5)(p23.1;q35.3), generates a cDNA fusion in which exon 5 of *SQSTM* is joined to the *ALK* cDNA for the intracellular region of its encoded protein (containing the tyrosine kinase domain). Numbers indicate amino acid positions of each protein. PB1: Phox and Bem1p; Z: atypical zinc finger; U: ubiquitin-associated. (B) A section of the specimen for the present case was subjected to FISH with an *SQSTM1-ALK* fusion assay. Nuclei are stained blue with DAPI. (C) Murine 3T3 fibroblasts were infected with retroviruses expressing SQSTM1-ALK. The cells were photographed after culture for 14 days. (D) A nude mouse was injected subcutaneously with 3T3 cells infected as in (C), and tumor formation was examined after 20 days.

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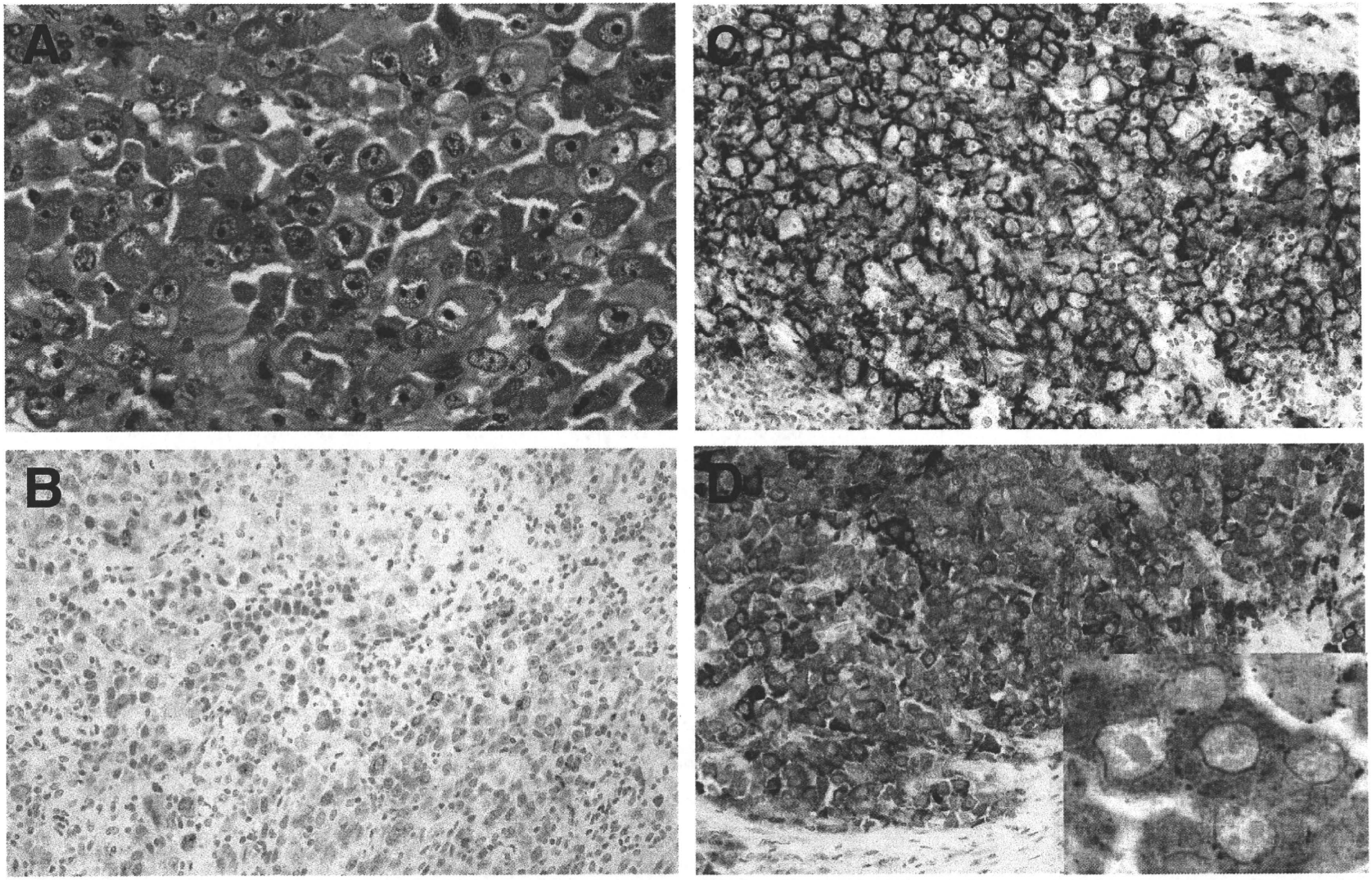

Figure 1

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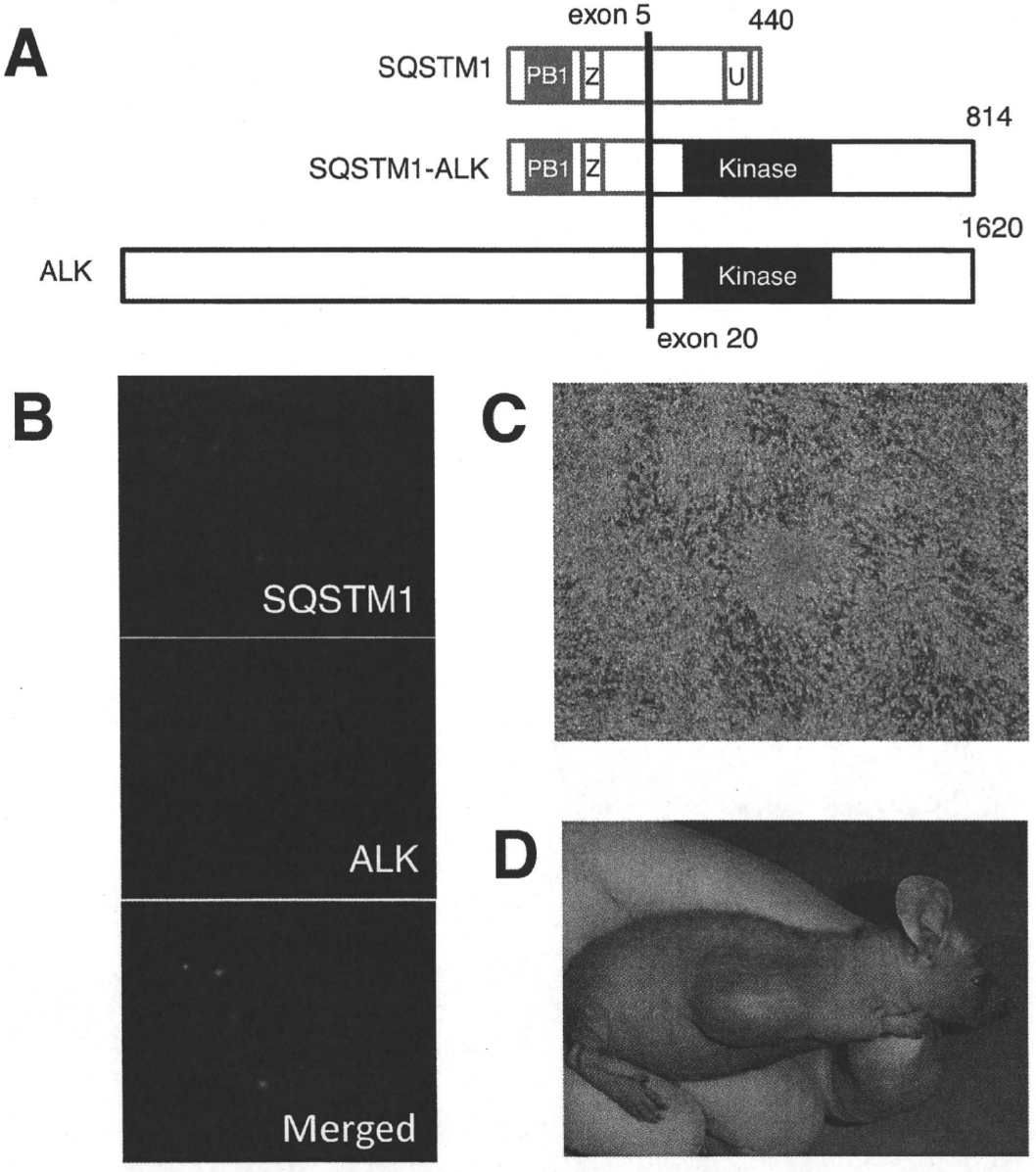

Figure 2

DOI: 10.3324/haematol.2010.033514

SQSTM1-ALK fusion cDNA

2499 bp (*SQSTM1* 760bp ~exon 5: *ALK* 1739bp exon 20~)

ORF: 7–2448

ctcgctATGGCGTCGCTCACCGTGAAGGCCTACCTTCTGGGCAAGGAGGACGCGGCGCGCGA
GATTCGCCGCTTCAGCTTCTGCTGCAGCCCCGAGCCTGAGGCGGAAGCCGAGGCTGCGGCGG
GTCCGGGACCCTGCGAGCGGCTGCTGAGCCGGGTGGCCGCCCTGTTCCCCGCGCTGCGGCCT
GGCGGCTTCCAGGCGCACTACCGCGATGAGGACGGGGACTTGGTTGCCTTTTCCAGTGACGA
GGAATTGACAATGGCCATGTCCTACGTGAAGGATGACATCTTCCGAATCTACATTAAAGAGA
AAAAAGAGTGCCGGCGGGACCACCGCCCACCGTGTGCTCAGGAGGCGCCCCGCAACATGGTG
CACCCCAATGTGATCTGCGATGGCTGCAATGGGCCTGTGGTAGGAACCCGCTACAAGTGCAG
CGTCTGCCCAGACTACGACTTGTGTAGCGTCTGCGAGGGAAAGGGCTTGCACCGGGGGCACA
CCAAGCTCGCATTCCCCAGCCCCTTCGGGCACCTGTCTGAGGGCTTCTCGCACAGCCGCTGG
CTCCGGAAGGTGAAACACGGACACTTCGGGTGGCCAGGATGGGAAATGGGTCCACCAGGAAA
CTGGAGCCCACGTCCTCCTCGTGCAGGGGAGGCCCGCCCTGGCCCCACGGCAGAATCAGCTT
CTGGTCCATCGGAGGATCCGAGTGTGAATTTCCTGAAGAACGTTGGGGAGAGTGTGGCAGCT
GCCCTTAGCCCTCTGGTGTACCGCCGGAAGCACCAGGAGCTGCAAGCCATGCAGATGGAGCT
GCAGAGCCCTGAGTACAAGCTGAGCAAGCTCCGCACCTCGACCATCATGACCGACTACAACC
CCAACTACTGCTTTGCTGGCAAGACCTCCTCCATCAGTGACCTGAAGGAGGTGCCACGGAAA
AACATCACCCTCATTCGGGGTCTGGGCCATGGAGCCTTTGGGGAGGTGTATGAAGGCCAGGT
GTCCGGAATGCCCAACGACCCAAGCCCCCTGCAAGTGGCTGTGAAGACGCTGCCTGAAGTGT
GCTCTGAACAGGACGAACTGGATTTCCTCATGGAAGCCCTGATCATCAGCAAATTCAACCAC
CAGAACATTGTTCGCTGCATTGGGGTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGA
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CCTCCTCCCTGGCCATGCTGGACCTTCTGCACGTGGCTCGGGACATTGCCTGTGGCTGTCAG
TATTTGGAGGAAAACCACTTCATCCACCGAGACATTGCTGCCAGAAACTGCCTCTTGACCTG
TCCAGGCCCTGGAAGAGTGGCCAAGATTGGAGACTTCGGGATGGCCCGAGACATCTACAGGG
CGAGCTACTATAGAAAGGGAGGCTGTGCCATGCTGCCAGTTAAGTGGATGCCCCCAGAGGCC
TTCATGGAAGGAATATTCACTTCTAAAACAGACACATGGTCCTTTGGAGTGCTGCTATGGGA
AATCTTTTCTCTTGGATATATGCCATACCCCAGCAAAAGCAACCAGGAAGTTCTGGAGTTTG
TCACCAGTGGAGGCCGGATGGACCCACCCAAGAACTGCCCTGGGCCTGTATACCGGATAATG
ACTCAGTGCTGGCAACATCAGCCTGAAGACAGGCCCAACTTTGCCATCATTTTGGAGAGGAT
TGAATACTGCACCCAGGACCCGGATGTAATCAACACCGCTTTGCCGATAGAATATGGTCCAC
TTGTGGAAGAGGAAGAGAAAGTGCCTGTGAGGCCCAAGGACCCTGAGGGGGTTCCTCCTCTC
CTGGTCTCTCAACAGGCAAAACGGGAGGAGGAGCGCAGCCCAGCTGCCCCACCACCTCTGCC

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TACCACCTCCTCTGGCAAGGCTGCAAAGAAACCCACAGCTGCAGAGGTCTCTGTTCGAGTCC
CTAGAGGGCCGGCCGTGGAAGGGGGACACGTGAATATGGCATTCTCTCAGTCCAACCCTCCT
TCGGAGTTGCACAGGGTCCACGGATCCAGAAACAAGCCCACCAGCTTGTGGAACCCAACGTA
CGGCTCCTGGTTTACAGAGAAACCCACCAAAAAGAATAATCCTATAGCAAAGAAGGAGCCAC
ACGAGAGGGGTAACCTGGGGCTGGAGGGAAGCTGTACTGTCCCACCTAACGTTGCAACTGGG
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ACCTCTGTTCAGGCTACGTCACTTCCCTTGTGGGAATGTCAATTACGGCTACCAGCAACAGG
GCTTGCCCTTAGAAGCCGCTACTGCCCCTGGAGCTGGTCATTACGAGGATACCATTCTGAAA
AGCAAGAATAGCATGAACCAGCCTGGGCCCtgagctcggtcgcacactcacttctcttcctt
gggatccctaagaccgtgg

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SQSTM1-ALK fusion protein

814 aa (SQSTM1 251aa; ALK 563aa)

MASLTVKAYLLGKEDAAREIRRFSFCCSPEPEAEAEAAAGPGPCERLLSRVAALFPALRPGG
FQAHYRDEDGDLVAFSSDEELTMAMSYVKDDIFRIYIKEKKECRRDHRPPCAQEAPRNMVHP
NVICDGCNGPVVGTRYKCSVCPDYDLCSVCEGKGLHRGHTKLAFPSPFGHLSEGFSHSRWLR
KVKHGHFGWPGWEMGPPGNWSPRPPRAGEARPGPTAESASGPSEDPSVNFLKNVGESVAAAL
SPLVYRRKHQELQAMQMELQSPEYKLSKLRTSTIMTDYNPNYCFAGKTSSISDLKEVPRKNI
TLIRGLGHGAFGEVYEGQVSGMPNDPSPLQVAVKTLPEVCSEQDELDFLMEALIISKFNHQN
IVRCIGVSLQSLPRFILLELMAGGDLKSFLRETRPRPSQPSSLAMLDLLHVARDIACGCQYL
EENHFIHRDIAARNCLLTCPGPGRVAKIGDFGMARDIYRASYYRKGGCAMLPVKWMPPEAFM
EGIFTSKTDTWSFGVLLWEIFSLGYMPYPSKSNQEVLEFVTSGGRMDPPKNCPGPVYRIMTQ
CWQHQPEDRPNFAIILERIEYCTQDPDVINTALPIEYGPLVEEEEKVPVRPKDPEGVPPLLV
SQQAKREEERSPAAPPPLPTTSSGKAAKKPTAAEVSVRVPRGPAVEGGHVNMAFSQSNPPSE
LHRVHGSRNKPTSLWNPTYGSWFTEKPTKKNNPIAKKEPHERGNLGLEGSCTVPPNVATGRL
PGASLLLEPSSLTANMKEVPLFRLRHFPCGNVNYGYQQQGLPLEAATAPGAGHYEDTILKSK
NSMNQPGP

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-

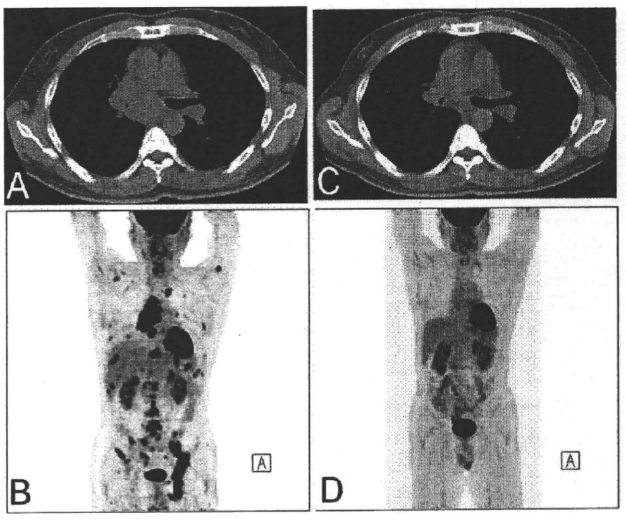

FIGURE 1. *A*, Chest computed tomography showed a narrowing of the right main bronchus due to massive lymphadenopathy. *B*, FDG-PET suggested multiple lymph node metastases and bone metastases. *C*, Six weeks after administration of the ALK inhibitor, the effect of the treatment was judged as partial 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.

*Division of Thoracic Diseases, Chiba Cancer Center, Chiba, Japan; †Division of Thoracic Surgery, Toronto General Hospital, University Health Network, Toronto, Canada; ‡Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research (JFCR), Koto-ku, Tokyo; and §Division of Functional Genomics, Jichi Medical University, Tochigi, Japan.

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Address for correspondence: Takahiro Nakajima, MD, PhD, Division of Thoracic Diseases, Chiba Cancer Center, 666-2 Nitona-cho, Chuo-ku, Chiba 260-8717, Japan. E-mail: nakajii@fc.med.miyazaki-u.ac.jp

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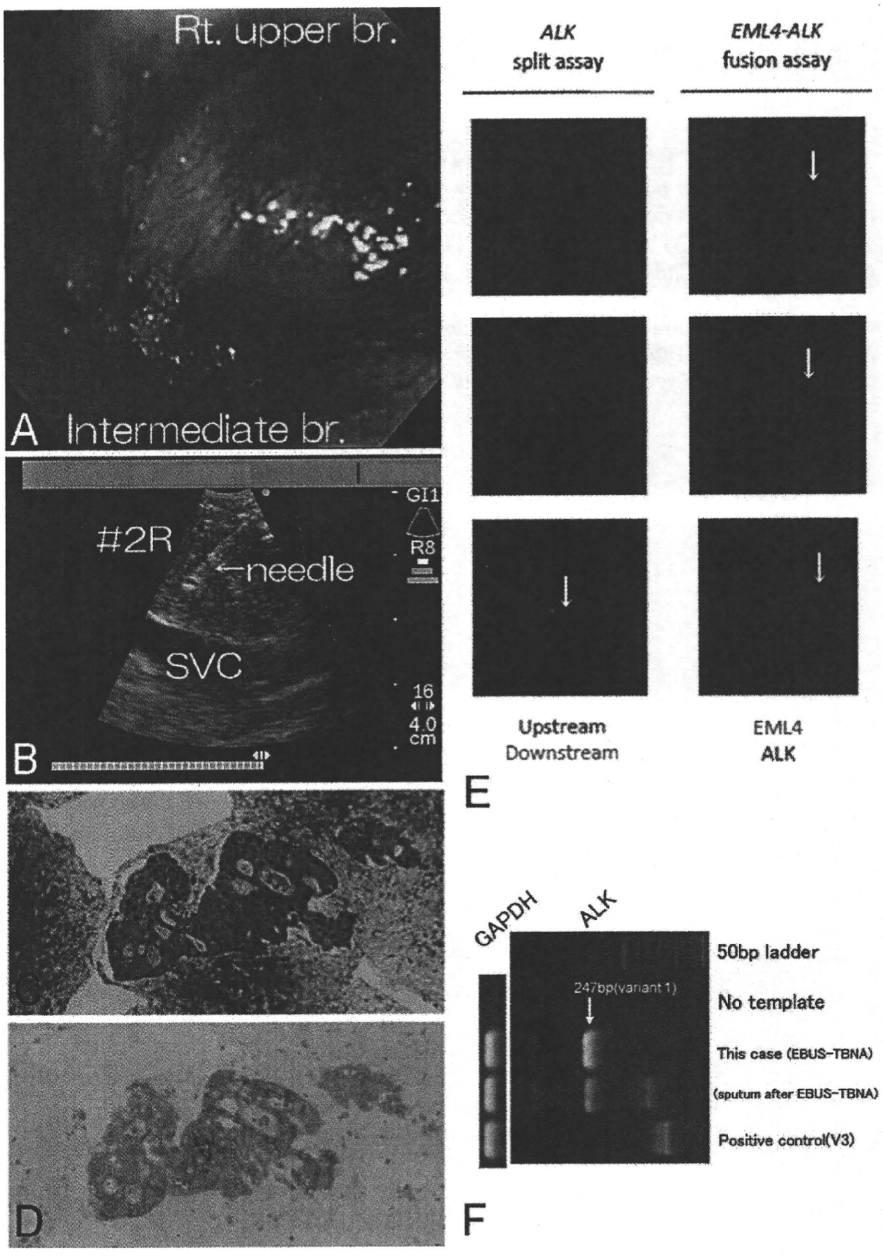

FIGURE 2. *A*, Bronchoscopic examination showed tumor compression of the right main bronchus, and the tumor had hyperplastic vessels on its surface. *B*, EBUS-TBNA was performed for a pretracheal lymph node (#2R). *C*, Histologic core revealed metastatic adenocarcinoma in #2R node. *D*, Immunohistochemistry 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

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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BRIEF REPORT

EML4-ALK Mutations in Lung Cancer That Confer Resistance to ALK Inhibitors

Young Lim Choi, M.D., Ph.D., Manabu Soda, M.D., Ph.D., Yoshihiro Yamashita, M.D., Ph.D., Toshihide Ueno, Ph.D., Junpei Takashima, M.D., Takahiro Nakajima, M.D., Ph.D., Yasushi Yatabe, M.D., Ph.D., Kengo Takeuchi, M.D., Ph.D., Toru Hamada, M.D., Hidenori Haruta, M.D., Ph.D., Yuichi Ishikawa, M.D., Ph.D., Hideki Kimura, M.D., Ph.D., Tetsuya Mitsudomi, M.D., Ph.D., Yoshiro Tanio, M.D., Ph.D., and Hiroyuki Mano, M.D., Ph.D., for the ALK Lung Cancer Study Group

SUMMARY

The EML4 (echinoderm microtubule-associated protein-like 4)–ALK (anaplastic lymphoma kinase) fusion-type tyrosine kinase is an oncoprotein found in 4 to 5% of non–small-cell lung cancers, and clinical trials of specific inhibitors of ALK for the treatment of such tumors are currently under way. Here, we report the discovery of two secondary mutations within the kinase domain of EML4-ALK in tumor cells isolated from a patient during the relapse phase of treatment with an ALK inhibitor. Each mutation developed independently in subclones of the tumor and conferred marked resistance to two different ALK inhibitors. (Funded by the Ministry of Health, Labor, and Welfare of Japan, and others.)

From the Division of Functional Genomics, Jichi Medical University, Tochigi (Y.L.C., M.S., Y. Yamashita, T.U., T.H., H.H., H.M.); the Department of Medical Genomics, Graduate School of Medicine, University of Tokyo, Tokyo (Y.L.C., H.M.); the Department of Internal Medicine, Osaka General Medical Center, Osaka (J.T., Y.T.); the Division of Thoracic Diseases, Chiba Cancer Center, Chiba (T.N., H.K.); the Departments of Pathology (Y. Yatabe) and Thoracic Surgery (T.M.), Aichi Cancer Center Hospital, Aichi; the Pathology Project for Molecular Targets (K.T.) and Division of Pathology (Y.I.), the Cancer Institute, Japanese Foundation for Cancer Research, Tokyo; and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama (H.M.) — all in Japan. Address reprint requests to Dr. Mano at the Division of Functional Genomics, Jichi Medical University, 3311-1 Yakushiji, Shimotsukeshi, Tochigi 329-0498, Japan, or at hmano@jichi.ac.jp.

N Engl J Med 2010;363:1734-9.

EML4-ALK IS A FUSION-TYPE PROTEIN TYROSINE KINASE THAT IS PRESENT in 4 to 5% of cases of non–small-cell lung cancer and is generated as a result of a small inversion within the short arm of human chromosome 2.[1-3] EML4-ALK undergoes constitutive dimerization through interaction between the coiled-coil domain within the EML4 region of each monomer, thereby activating ALK and generating oncogenic activity. In transgenic mice that express EML4-ALK specifically in lung epithelial cells, hundreds of adenocarcinoma nodules develop in both lungs soon after birth, and oral administration of a specific inhibitor of ALK tyrosine kinase activity rapidly eradicates such nodules from the lungs.[4] These observations reveal the essential role of EML4-ALK in the carcinogenesis of non–small-cell lung cancer harboring this fusion kinase. Furthermore, clinical trials are investigating crizotinib (PF-02341066), an inhibitor of the tyrosine kinase activity of both ALK and the met proto-oncogene (MET), for the treatment of EML4-ALK–positive non–small-cell lung cancer.

In addition to crizotinib, other tyrosine kinase inhibitors have been shown to have pronounced therapeutic activity in patients with cancer. For instance, imatinib mesylate and gefitinib, tyrosine kinase inhibitors for the c-abl oncogene 1 nonreceptor tyrosine kinase (ABL) and epidermal growth factor receptor (EGFR), improve the outcome for patients who have chronic myeloid leukemia that is positive for the BCR (breakpoint cluster region protein)–ABL fusion kinase[5] and patients who have non–small-cell lung cancer that is associated with EGFR activation,[6]

respectively. Unfortunately, however, a fraction of the target tumors are either refractory to corresponding tyrosine kinase inhibitors from the start of treatment or become resistant after an initial response.

In a case of EML4-ALK–positive non–small-cell lung cancer that became resistant to crizotinib after successful treatment for 5 months, we have discovered two de novo mutations in EML4-ALK, each of which confers resistance to the drug.

CASE REPORT

The patient was a 28-year-old man without a history of smoking who had received a diagnosis of lung adenocarcinoma, at a tumor–node–metastasis (TNM) clinical stage of T4N3M1, in April 2008. Given that the tumor did not harbor any EGFR mutations, the patient was treated with conventional chemotherapy. However, his tumor progressed after six cycles of three two-drug combinations. In November 2008, the presence of *EML4-ALK* variant 1 messenger RNA (mRNA)[1] in the tumor was confirmed by means of reverse transcription–polymerase-chain-reaction (PCR) analysis of a sputum sample. At this stage, the patient had large tumor nodules in the hilum of the right lung, multiple enlarged lymph nodes in the mediastinum, atelectasis in the right lung, and a massive effusion in the right pleural cavity (Fig. 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

The patient was enrolled in the A8081001 study of crizotinib (ClinicalTrials.gov number, NCT00585195) on November 28, 2008, with oral administration of the drug at a dose of 250 mg twice per day. Within 1 week after the start of crizotinib treatment, his symptoms improved markedly. Although he had a partial response to the treatment, his pleural effusion was not completely eradicated (Fig. 1 in the Supplementary Appendix). After 5 months of treatment, however, the tumor abruptly started to grow again, resulting in a rapid expansion of the pleural effusion and in the development of tumors in both lungs (Fig. 1 in the Supplementary Appendix). The patient was withdrawn from the trial on May 25, 2009, and a sample of the pleural effusion in the right lung was then obtained for molecular analysis.

METHODS

DNA sequencing and characterization of the EML4-ALK mutants are described in detail in the Supplementary Appendix.

RESULTS

Because our patient's tumor resumed growth despite sustained administration of the ALK inhibitor crizotinib, we speculated that it might have acquired secondary genetic changes that confer resistance to the drug. Furthermore, given that resistance to tyrosine kinase inhibitors often results from acquired mutations within the target kinases,[7-9] we first examined the possibility that EML4-ALK itself had undergone amino acid changes.

Molecular analysis was performed on sputum specimens obtained before crizotinib treatment and pleural-effusion specimens obtained after relapse when treatment was stopped. Given that the proportion of tumor cells in the two types of specimens may have differed, we performed deep (high-coverage) sequencing of *EML4-ALK* complementary DNA (cDNA) derived from the specimens, using a high-throughput sequencer (Genome Analyzer II, Illumina) (Fig. 2 in the Supplementary Appendix). The sensitivity of our sequencing system, examined with the use of cDNA corresponding to the Janus kinase 3 (JAK3) amino acid mutation V674A[10] as a control, revealed that the maximum detection sensitivity was no more than one mismatched read per 6.50×10^5 total reads (Table 1 in the Supplementary Appendix).

Using deep sequencing, we detected a known single-nucleotide polymorphism, rs3795850, in the cDNA from the four specimens that were positive for *EML4-ALK* (Table 2 and Fig. 3 in the Supplementary Appendix). In addition, a T→C change at a position corresponding to nucleotide 4230 of human wild-type *ALK* cDNA (GenBank accession number, NM_004304) was detected at a low frequency (8.9%) in the sputum cDNA from our patient. Furthermore, two new alterations, G→A and C→A changes at positions corresponding to nucleotides 4374 and 4493 of wild-type *ALK* cDNA, were detected at frequencies of 41.8% and 14.0%, respectively, in the patient's pleural-effusion cDNA. There were no other recurrent alterations (present in 5% of reads) in the kinase-domain cDNA derived from any of the specimens.

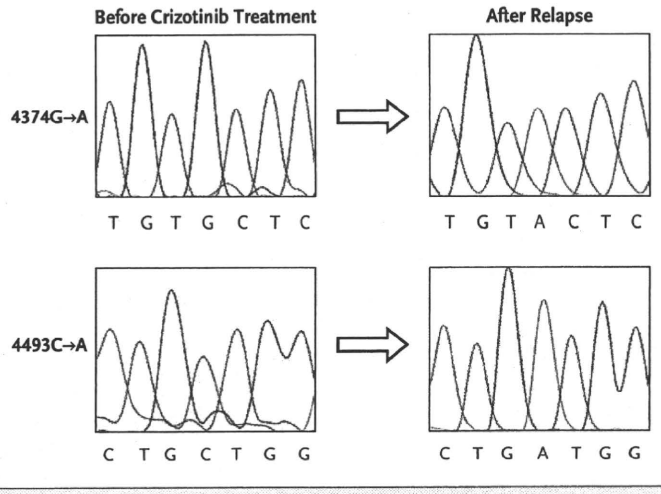

Figure 1. Secondary Mutations within *EML4-ALK*.
Electrophoretograms are shown for *EML4-ALK* cDNA clones prepared from sputum specimens obtained from our patient before crizotinib treatment and from pleural-effusion specimens obtained after relapse. The 4374G→A and 4493C→A mutations are present in the specimens obtained after relapse.

We next attempted to confirm these nucleotide changes by using Sanger sequencing. To rule out the possibility that the mutations had occurred in endogenous wild-type *ALK* rather than in *EML4-ALK*, we performed PCR with a forward primer targeted to *EML4* cDNA so that only the fusion cDNA would be amplified (Fig. 2 in the Supplementary Appendix). We did not detect the 4230T→C change among the 256 fusion cDNA clones derived from the patient's sputum specimens (data not shown), indicating that it was an artifact of the initial PCR or the deep-sequencing step. We did, however, readily confirm both 4374G→A and 4493C→A changes. Among 73 *EML4-ALK* cDNA clones from the patient's pleural-effusion specimens, 34 (46.6%) were positive for 4374G→A and 11 (15.1%) were positive for 4493C→A (Fig. 1). (The remaining 28 [38.4%] were negative for both point mutations.) These rates of detection are similar to those from the deep sequencing of *ALK*, indicating that wild-type *ALK* mRNA was present at a low level in lung tissue, as reported previously.[1]

The PCR analyses covered both nucleotide positions, yet none of the patient's specimens contained both mutations, indicating that each mutation occurred independently. Genomic fragments encompassing the 4374G and 4493C positions were also amplified by means of a PCR assay and were then subjected to nucleotide sequencing, which confirmed the presence of each of the two mutations in the tumor genome (Fig. 4 in the Supplementary Appendix).

The 4374G→A and 4493C→A substitutions result in cysteine→tyrosine (C→Y) and leucine→methionine (L→M) changes at the positions corresponding to amino acids 1156 and 1196, respectively, of wild-type human ALK (Fig. 2 in the Supplementary Appendix). We examined whether such amino acid changes affect the sensitivity of EML4-ALK to ALK inhibitors.

Cells of the mouse interleukin-3–dependent cell line BA/F3 that were made to individually express primary EML4-ALK and secondary mutant EML4-ALK (with the C1156Y or L1196M mutation) were exposed to ALK inhibitors. Crizotinib inhibited the growth of BA/F3 cells expressing primary EML4-ALK, in a concentration-dependent manner (Fig. 2A). In contrast, cells expressing either the C1156Y or L1196M mutant form manifested a markedly reduced sensitivity to the drug. Cells expressing the L1196M mutant form of EML4-ALK were more resistant to crizotinib than were those expressing the C1156Y mutant form (Fig. 2A, and Fig. 5 in the Supplementary Appendix).

We also examined whether cells expressing these EML4-ALK mutants are also refractory to other ALK inhibitors. A 2,4-pyrimidinediamine derivative (PDD) has a median inhibitory concentration for ALK of less than 10 nM,[11] and oral administration of PDD has been shown to eradicate lung-cancer nodules in transgenic mice with EML4-ALK expression.[4] BA/F3 cells expressing EML4-ALK with either the C1156Y or L1196M mutation were markedly less sensitive to PDD than were those expressing the primary EML4-ALK (Fig. 2A). Thus, although these mutations appear to develop during clinical treatment with crizotinib, their generation probably renders EML4-ALK resistant not only to crizotinib but also to other ALK inhibitors. In contrast to the resistance profile for crizotinib, BA/F3 cells expressing the EML4-ALK C1156Y mutant form were slightly more resistant to PDD than were those expressing the L1196M mutant form (Fig. 2A, and Fig. 6 in the Supplementary Appendix), indicating that the resistance profiles for the two mutations may be, in part, inhibitor-dependent, as was previously shown for BCR-ABL mutants.[12]

We examined tyrosine phosphorylation of

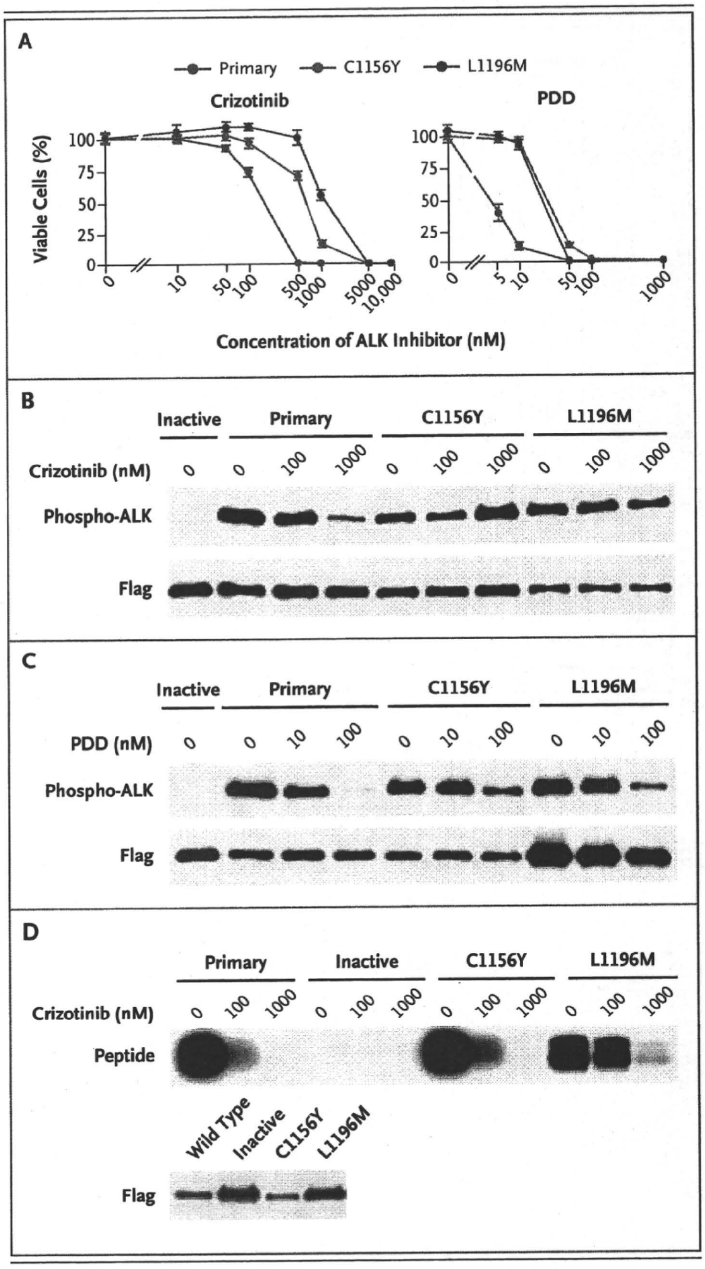

Figure 2. Properties of EML4-ALK with Secondary Mutations.

Panel A shows the percentage of viable BA/F3 cells expressing primary EML4-ALK, EML4-ALK with the C1156Y mutation, or EML4-ALK with the L1196M mutation, after 5×10^5 cells were incubated for 48 hours with the indicated concentration of crizotinib (left) or 2,4-pyrimidinediamine derivative (PDD) (right). Data are expressed as the mean value, from three separate experiments, for the percentage of cells expressing primary EML4-ALK after incubation in the vehicle (dimethyl sulfoxide) only. The I bars indicate standard deviations. Because primary EML4-ALK, EML4-ALK with the C1156Y mutation, and EML4-ALK with the L1196M mutation each abrogate the interleukin-3 dependence of BA/F3 cells, the assays were performed in the absence of the interleukin. Panels B and C show the effect of ALK inhibitors on EML4-ALK and its secondary mutant forms, tagged with the Flag epitope, in BA/F3 cells. Panel B shows the results of exposure to various concentrations of crizotinib for 15 hours, after which EML4-ALK was immunoprecipitated from cell lysates with antibodies against the Flag epitope and the immunoprecipitate was subjected to immunoblot analysis with the use of antibodies specific for ALK phosphorylated at the tyrosine at position 1604 (Phospho-ALK) or for the Flag epitope. Cells expressing an inactive mutant form of EML4-ALK were examined as a negative control. Panel C shows the results of a similar experiment, involving PDD instead of crizotinib. Panel D shows the results of an in vitro kinase assay for Flag-tagged EML4-ALK or its secondary mutants immunoprecipitated from BA/F3 cells with antibodies against the Flag epitope. The immunoprecipitates were incubated with [γ-^{32}P]ATP, a synthetic peptide, and various concentrations of crizotinib (top). Separate immunoprecipitate samples were subjected to immunoblot analysis with antibodies against the Flag epitope (bottom).

EML4-ALK by means of immunoblot analysis, using antibodies specific for ALK phosphorylated at the tyrosine at position 1604. The exposure of BA/F3 cells to crizotinib markedly inhibited the tyrosine phosphorylation of EML4-ALK but did not substantially affect that of the C1156Y and L1196M mutants (Fig. 2B). Exposure to PDD also inhibited the tyrosine phosphorylation of EML4-ALK, in a concentration-dependent manner, with a lesser effect on the mutants (Fig. 2C). The results of an in vitro kinase assay were consistent with these findings, showing pronounced inhibition of the enzymatic activity of primary EML4-ALK with crizotinib, whereas the effect on the C1156Y mutant was less pronounced and the effect on the L1196M mutant was much less pronounced (Fig. 2D).

Figure 3 shows the cysteine at position 1156 (C1156) and the leucine at position 1196 (L1196) of the kinase domain of ALK.[13] C1156 is positioned adjacent to the N-terminal of the predicted helix αC as well as close to the upper edge of the ATP-binding pocket. No activating mutations have been reported at this position in other tyrosine kinases in cancer specimens. L1196 of ALK corresponds to the threonine at position 315 in ABL and at position 790 in EGFR, each of which is the site of the most fre-

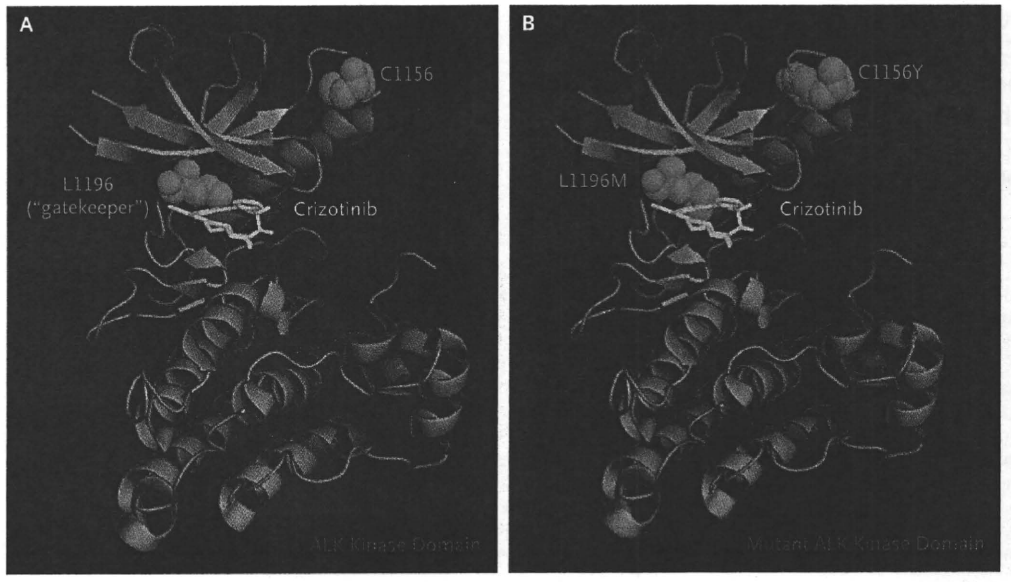

Figure 3. Predicted Crystal Structure of the Kinase Domain of ALK.

Panel A shows the nonmutant kinase domain and the site of binding of crizotinib. Panel B shows the EML4-ALK secondary mutations at positions 1156 and 1196. Adapted from Bossi and colleagues.[13]

quently acquired mutations that confer resistance to tyrosine kinase inhibitors in these kinases (Fig. 7 in the Supplementary Appendix).[14,15] This site is located at the bottom of the ATP-binding pocket (Fig. 3), and the presence of an amino acid with a bulky side chain at this "gatekeeper" position may interfere with the binding of many tyrosine kinase inhibitors.[7,16]

DISCUSSION

We identified two de novo mutations within the kinase domain of EML4-ALK from the tumor of a single patient that confer resistance to multiple ALK inhibitors. Given that we did not detect any *EML4-ALK* cDNA harboring both mutations, we propose that each mutation developed independently in distinct subclones of the tumor. Because we were not able to examine pleural-effusion specimens from the patient before he received crizotinib treatment, we do not know whether the resistant clones were present initially or developed secondarily, during the treatment.

Amino acid substitutions at the gatekeeper position of several tyrosine kinases have been detected in tumors treated with tyrosine kinase inhibitors (Fig. 7 in the Supplementary Appendix).[7-9,17,18] Whereas no mutations at this site have previously been reported for EML4-ALK or ALK, the effects of various artificial amino acid substitutions at the gatekeeper position of nucleophosmin (NPM)–ALK, another fusion-type "oncokinase" form of ALK, were recently examined.[19] The findings were consistent with the results of our analysis of tumor cells in vivo: the introduction of methionine at this position rendered NPM-ALK resistant to ALK inhibitors. It is therefore likely that gatekeeper alterations constitute a universal mechanism for the acquisition of tyrosine kinase–inhibitor resistance in oncogenic tyrosine kinases.

In contrast to gatekeeper substitutions, activating mutations at the position adjacent, on the N-terminal side, to the αC helix (e.g., C1156 in ALK) have not been confirmed for other tyrosine kinases in cancer specimens. Though a T→I change at the corresponding position of EGFR was described in one case of non–small-cell lung cancer, its relevance to drug sensitivity was not examined.[16] The importance of helix αC for allosteric regulation of enzymatic activity has been shown, however, for serine–threonine kinases.[20] A change at C1156 of ALK might therefore interfere allosterically with the binding of tyrosine

kinase inhibitors. Determination of the crystal structure of the ALK kinase domain with the C1156Y or L1196M mutation should shed light on these matters, as well as provide a basis for the development of next-generation ALK inhibitors that may effectively eradicate tumors harboring EML4-ALK with the acquired mutations.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Original Article

Cancer of Unknown Primary Site: A Review of 28 Cases and the Efficacy of Cisplatin/Docetaxel Therapy at a Single Institute in Japan

Hisakazu Nishimori[a,b]*, Shunji Takahashi[a], Katsuyuki Kiura[b], Daisuke Ennishi[a,b], Takayuki Kobayashi[a], Koji Sano[a], Eiji Shinozaki[a], Masahiro Yokoyama[a], Yuko Mishima[a], Yasuhito Terui[a], Keisho Chin[a], Nobuyuki Mizunuma[a], Yoshinori Ito[a], Seiichiro Nishimura[c], Kengo Takeuchi[d], Yuichi Ishikawa[d], Masahiko Oguchi[e], Mitsune Tanimoto[b], and Kiyohiko Hatake[a]

[a]Division of Medical Oncology and Hematology, Cancer Institute Hospital, Koto-ku, Tokyo 135-8550, Japan,
[b]Department of Hematology, Oncology, Allergy, and Respiratory Medicine, Okayama University Hospital, Okayama 700-8558, Japan,
Divisions of [c]Breast Surgery, [e]Radiology, Cancer Institute Hospital, and
[d]Division of Pathology, Cancer Institute of the Japanese Foundation for Cancer Research, Koto-ku, Tokyo 135-8550, Japan

We evaluated the efficacy and toxicity of cisplatin/docetaxel (CDDP/TXT) chemotherapy and identified prognostic factors in Japanese patients with cancer of unknown primary site (CUP). Twenty-eight consecutive patients seen at a single institute were reviewed retrospectively. Sixteen patients were treated with TXT 80 mg/m^2, followed by CDDP 75 mg/m^2. The overall response rate to CDDP/TXT treatment was 62.5%, with a median survival time (MST) of 22.7 months. Common adverse reactions were myelosuppression and hyponatremia. The MST of all 28 patients with CUP was 8.3 months, and the 1-year overall survival rate was 45.6%. Univariate analysis identified 5 prognostic factors:performance status, liver involvement, bone involvement, pleural involvement, and lymph node involvement. In conclusion, CDDP/TXT chemotherapy is effective with tolerable toxicity in patients with CUP. Japanese patients with CUP might be chemosensitive and may survive longer.

Key words: cancer of unknown primary site (CUP), cisplatin, docetaxel, prognosis

Cancer of unknown primary site (CUP) is defined as the presence of metastatic cancer documented in the absence of an identifiable primary tumor site. These tumors are not rare; they represent 3-5% of all malignancies diagnosed in oncology practice [1, 2]. CUP occurs in a heterogeneous group of patients, and subgroups with treatment-responsive diseases exist that may achieve long-term, disease-free survival [1]. Generally, however, the prognosis of CUP is poor, with median survival times of 6-12 months, and the benefits of chemotherapy compared with best supportive care remain unclear [3].

Chemotherapy for patients with CUP is improving, but no chemotherapy regimen has been established as a standard first-line therapy for these patients [2]. Recent clinical reports have shown that cisplatin (CDDP)-containing regimens have good response rates of 32-55% in patients with CUP and are relatively well-tolerated [3, 4]. Docetaxel (TXT) has definite antitumor activity in various solid tumors and seems to

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*Corresponding author. Phone:+81-86-235-7227; Fax:+81-86-232-8226
E-mail:n-mori@md.okayama-u.ac.jp (H. Nishimori)

be a good candidate for inclusion in a chemotherapy regimen for patients with CUP [5]. A CDDP plus TXT phase II study revealed a 26% response rate with 42% 1-year survival [6].

This paper presents a retrospective analysis of 28 consecutive Japanese patients with CUP to clarify the disease course and prognostic factors. We also report an excellent response rate and survival of Japanese patients with CUP who were treated using a combination regimen of CDDP/TXT.

Patients and Methods

Patients. Twenty-eight consecutive patients referred to the Division of Medical Oncology and Hematology at the Cancer Institute Hospital between April 1, 2000 and September 30, 2004 were reviewed retrospectively. Patients referred with a presumed diagnosis of CUP were identified and registered in the database at the time of their initial clinical evaluation. All patients diagnosed with CUP during this period were registered; however, 2 female patients with adenocarcinoma involving only the axillary lymph nodes were treated for occult breast cancer and were excluded from this analysis. The medical records of the patients were reviewed for the results of diagnostic studies and pathologic and cytologic diagnosis before referral, the results of subsequent radiographic evaluations, pathology review, involved disease sites, treatment, and survival.

Clinical evaluation. All patients with CUP underwent a basic evaluation consisting of a complete medical history, a physical examination (including careful palpation of the thyroid, breasts, lymph nodes, and prostate), general laboratory studies, chest radiography, and computed tomography from the neck to pelvis. If possible, gastrointestinal endoscopy, nose and pharyngeal endoscopy, and bronchoscopy were conducted. Positron emission tomography (PET) was performed in some patients when all other tests were inconclusive. In some cases, an extensive immunohistochemical study was carried out with the biopsied specimen to minimize the possibility of a misdiagnosis of other malignancies such as non-Hodgkin's lymphoma, extragonadal germ cell tumor, malignant melanoma, or undifferentiated sarcoma [2]. The most commonly used markers were the leukocyte common antigen, cytokeratins, neuron-specific enolase or chromogranin, S-100 protein, vimentin, thyroid transcription factor-1 (TTF-1), estrogen receptors, HMB45, and prostate-specific antigen (PSA). The blood concentrations of CA19-9, CA15-3, CA125, and carcinoembryonic antigen (CEA) were assessed in most cases.

Decision-making in the 'cancer board meeting'. Determining whether the primary site is unknown or whether it will be possible to detect with further evaluation is difficult. In the present study, members of the cancer board, including medical oncologists, hematologists, surgeons, pathologists, and radiation oncologists, evaluated the diagnosis and treatment strategies for the patients with CUP.

Treatment schedule for CDDP/TXT therapy. Eligible patients with CUP were treated with CDDP/TXT combination chemotherapy. All patients gave written informed consent. TXT 80 mg/m^2 in 300 mL of normal saline was administered over 2 h, followed by CDDP 75 mg/m^2, which was administered via a 120-min intravenous infusion. Premedication included intravenous administration of 4 mg of dexamethasone 24 h before treatment, 30 min before starting the docetaxel infusion, and 24 h after the infusion. A single 3-mg intravenous dose of granisetron was given to all patients as an antiemetic. Concurrent radiotherapy for symptom control in the absence of disease progression was allowed, but the drugs were held for at least 2 weeks after irradiation. Chemotherapy cycles were repeated every 3 weeks. Doses were modified for some patients mainly due to hematological toxicity.

Assessment of response and toxicity. Responses were defined according to the World Health Organization criteria [7]. Briefly, complete response (CR) was defined as the entire disappearance of all assessable lesions and signs of disease for at least 4 weeks. Partial response (PR) was defined as a reduction of 50% or more in the sum of the products of the perpendicular dimensions of measurable lesions and the appearance of no new lesion for at least 4 weeks. No change (NC) was defined as a decrease of less than 50% or an increase of less than 25% in the 2 greatest dimensions of measurable lesions and the appearance of no new lesions. Progressive disease (PD) was defined as any evidence of disease progression of 25% or more, or the appearance of a new lesion. Chemotherapy-related adverse events were recorded according to the National Cancer Institute's Common

Terminology Criteria, version 3.0 [8].

Statistical methods. Survival was calculated from the first day of pathologically or cytologically diagnosed malignancy. Survival following CDDP/TXT therapy was calculated from the first day of treatment. Survival curves were estimated using the Kaplan-Meier method [9] and compared using the Cox-Mantel log-rank test [10]. StatView 5.0 (SAS Institute, Cary, NC, USA) was used for the statistical analyses.

Results

General patient characteristics. The characteristics of our 28 patients (19 men and 9 women) are listed in Tables 1 and 2. One female patient was excluded from the survival analysis because she was postoperatively diagnosed with ovarian cancer. The median age at diagnosis was 58.5 years (range 32–76 years). Performance status according to the Eastern Cooperative Oncology Group (ECOG) [11] was 0–1 in 17 patients (60.7%). The sites of metastasis documented pathologically, cytologically, or radiographically are listed in Table 3. Lymph nodes were involved most frequently (64.3%), and visceral metastases including bone, lung, or liver were also common. The lymph node involvement was further subclassified by anatomic site. Of the 18 patients with nodal metastases, 11 had retroperitoneal, 8 had supraclavicular or cervical, 7 had mediastinal, 4 had axillary, and 4 had inguinal lymph nodes.

Table 1 Characteristics of 28 CUP patients

Characteristic	No. of Patients
Sex	
Female	9
Male	19
Age, years	
0–40	1
41–50	6
51–60	8
61–70	10
71–80	3
Median	58.5
Range	32–76
Performance status	
0–1	17
2–3	11

The pathological diagnoses of the patients are also listed in Table 2. Thirteen patients (46.4%) were diagnosed with adenocarcinoma, 9 (32.1%) with poorly differentiated carcinoma, 2 (7.1%) with squamous cell carcinoma, and 4 with unknown or other diagnoses. One patient was diagnosed based only on the cytology of ascites. No patients with neuroendocrine carcinoma were included in this study. No patients appeared to belong to subgroups with a favorable prognosis [1].

Twelve patients (42.9%) had a single metastatic organ site, 4 (14.3%) had 2, 7 (25.0%) had 3, and 5 (17.9%) had 4 or more. Serum tumor markers at baseline were assessed in all 16 patients who underwent CDDP/TXT therapy.

Twenty-five patients (89.3%) were treated with chemotherapy with or without concurrent radiotherapy for symptom control. One patient was treated with radiotherapy only. Two patients were treated with supportive care alone.

CDDP/TXT treatment. Sixteen patients who received the CDDP/TXT combination according to the protocol were assessable for response. The patient

Table 2 Sites of tumor involvement and histologic diagnoses in 28 patients with CUP

Site of involvement	No. of patients
Lymph nodes	18
Bone	10
Lung	7
Liver	6
Pleura/pleural space	4
Peritoneum	4
Skin	3
Adrenal	2
Others	9
Histologic Diagnosis	**No. of patients**
Adenocarcinoma	13
Poorly differentiated	4
Papillary	1
No descriptor/other	8
Poorly differentiated carcinoma	9
Squamous cell carcinoma	2
Unknown/other	4
No. of involved organ sites	**No. of patients**
1	12
2	4
3	7
4 or more	5

Table 3 Characteristics of 16 patients treated with the CDDP/TXT regimen

Characteristic	No. of Patients
Sex	
Female	6
Male	10
Age, years	
Median	62.5
Range	41–76
PS	
0–1	12
2–3	4
Histology	
Adonocarcinoma	10
Poorly differentiated	2
Papillary	1
No descriptor/other	7
Poorly differentiated Carcinoma	4
Squamous cell carcinoma	0
Unknown/other	2
Metastatic sites at presentation	
Lymph nodes	10
Bone	6
Lung	2
Liver	1
Pleura/pleural space	1
Peritoneum	2
Skin	1
Adrenal	1
Others	4
No. of involved organ sites	
Single site	9
Multiple (≥2) sites	7
No. of courses given	
Median	3
Range	1–6

characteristics were similar to those of all 28 patients (Table 3): 10 men and 6 women, median age 62.5 years (range 41–76 years). However, performance status (PS) and the number of metastatic sites were lower, with PS 0–1 in 12 patients and single-site involvement in 9. In 8 patients (50%), more than 2 tumor markers had increased at diagnosis. The median duration from the day of the pathological diagnosis of metastatic carcinoma to the first day of CDDP/TXT therapy was 43 days (range 0–154 days). A total of 44 cycles of therapy was given, and the patients underwent a median of 3 treatment cycles (range 1–6 cycles). Doses were modified mainly because of hematological toxicity; 4 patients had a 20% dose reduction.

The overall response rate was 62.5% (95% CI 8.6–81.5%), with CR in one patient and PR in nine patients. Six of 10 patients with adenocarcinoma responded, and all 4 patients with poorly differentiated carcinoma responded. Tumor markers decreased in most responding patients. Fig. 1 shows the survival curve for these patients. The median follow-up was 20.4 months (range 1.7–60.2 months), the median disease-free survival (DFS) was 8.7 months, the 1-year overall survival (OS) rate was 68.8% (95% CI 40.6–91.5%), and the median OS was 22.7 months. The median hospitalization stay of the 16 patients treated with CDDP/TXT therapy was 65.5 days (range 26–162 days).

Toxicity data are listed in Table 4. Grade 3–4 neutropenia was frequent (14 patients, 87.5%). One patient who had multiple lung, liver, and bone metastases died of bacterial pneumonia due to neutropenia. Hyponatremia occurred in 14 patients (87.5%) and was grade 3–4 in 3 patients; however, all patients were able to continue the treatment. The hyponatremia was caused by a loss of sodium due to renal tubule damage caused by cisplatin or the syndrome of inappropriate secretion of antidiuretic hormone (SIADH). Grade 3 allergic reactions due to docetaxel occurred in 2 patients during the first course. These 2 patients were given hydrocortisone and treated with cisplatin-only beginning with the next course. Other recorded toxicities were mild to moderate.

Prognostic factors for survival in the 28 patients with CUP. Fig. 2 shows the survival

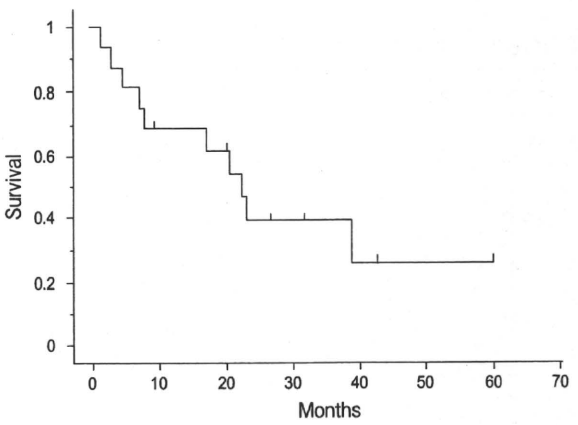

Fig. 1 Kaplan-Meier survival curve for the CUP patients treated with CDDP/TXT chemotherapy (n = 16).

curve for the 28 patients with CUP calculated from the day of diagnosis. The 1-year OS rate for all 28 patients was 45.6% (95% CI 26.8–64.4%), and the median survival was 8.3 months.

Table 5 lists the median survival of the CUP patient subgroups according to various factors. The univariate analysis revealed that 4 factors were deleterious: performance status 2–4, liver involvement, bone involvement, and pleural involvement. The advantageous clinical feature was lymph node involvement.

Discussion

In this study, we obtained an excellent response rate and survival with CDDP/TXT therapy for patients with CUP. An overall response rate of 62.5% was seen in patients with CUP who were given the CDDP/TXT combination once every 3 weeks. The median disease-free survival was 8.7 months. The 1-year OS was 68.8%, and the median OS was 22.7 months. The response rate and survival were superior to those obtained in the reported phase II trials of platinum plus taxane-based chemotherapy [2, 5, 6, 12]. Greco *et al.* reported a prospective phase II study of the CDDP/TXT regimen with a response rate of 22% and a 1-year survival of 40% [6]. The patient characteristics and dose intensity were similar to those in our study, although they had fewer patients with a single metastasis.

Yakushiji *et al.* have reported that 35 Japanese patients received a median of four cycles of CDDP and TXT, and had a response rate of 57.1%. The median survival time was 13.2 months [13]. These results in Japanese patients together with ours in the present study seem to be better than those reported from other countries. Although the prognostic factors in our reports are similar to those for other countries, Japanese patients with CUP might be chemosensitive and thus survive longer.

The treatment-related toxicity of the CDDP/TXT regimen mainly involved myelosuppression; in particular, grade 3–4 neutropenia was severe. Non-prophylactic G-CSF seemed to be the cause of this severity. One patient died of bacterial pneumonia due to neutropenia on day 12 of the first course. This patient was a 62-year-old man with PS 3. He had multiple metastases to lung, liver, and bone. He was therefore at high risk of pneumonia and had a very poor prognosis. Although hyponatremia occurred in 87.5% of our patients, it has not been reported in other studies that treated patients with CUP using platinum-containing regimens. Greco *et al.* have reported the toxicities of CDDP/TXT therapy to consist primarily of gastrointestinal events, with myelosuppression being moderate [6]. Based on urinalysis and the serum osmolarity, SIADH was the main cause of hyponatremia in the present study (data not shown). Since 1990, many Japanese researchers have reported SIADH following platinum administration for solid tumors [14, 15]. Collectively, Japanese patients seem to be more sensitive to platinum in terms of developing SIADH. Other recorded toxicities are mild to moderate. Overall, the regimen is generally tolerated in the majority of patients.

Table 4 Toxicity of CDDP/TXT therapy as worst grade per patient (n = 16)

Toxicity	No. of patients (%)			
	Grade 1	Grade 2	Grade 3	Grade 4
Anemia	6 (37.5)	6 (37.5)	1	0
Thrombocytopenia	4 (25.0)	1	0	1
Leukocytopenia	0	5 (31.3)	7 (43.8)	3 (18.8)
Granulocytopenia	0	1	3 (18.8)	11 (68.8)
Diarrhea	2 (12.5)	0	0	0
Nausea	3 (18.8)	5 (31.3)	4 (25.0)	0
Hyponatremia	11 (68.8)	0	3 (18.8)	0
High serum bilirubin	2 (12.5)	0	1	0
High serum creatinine	7 (43.8)	0	0	0
Allergic reaction	0	0	2 (12.5)	0

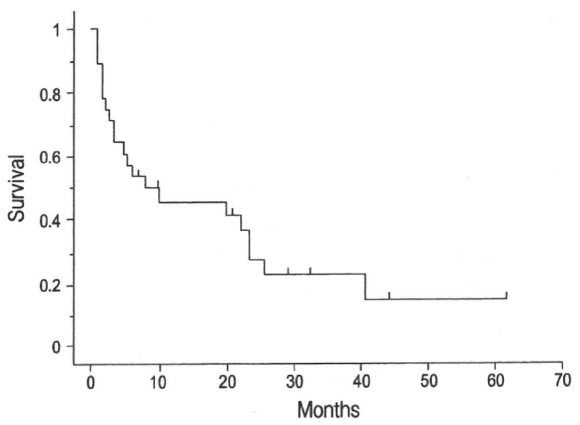

Fig. 2 Kaplan-Meier survival curve of all 28 CUP patients.