

Figure 1

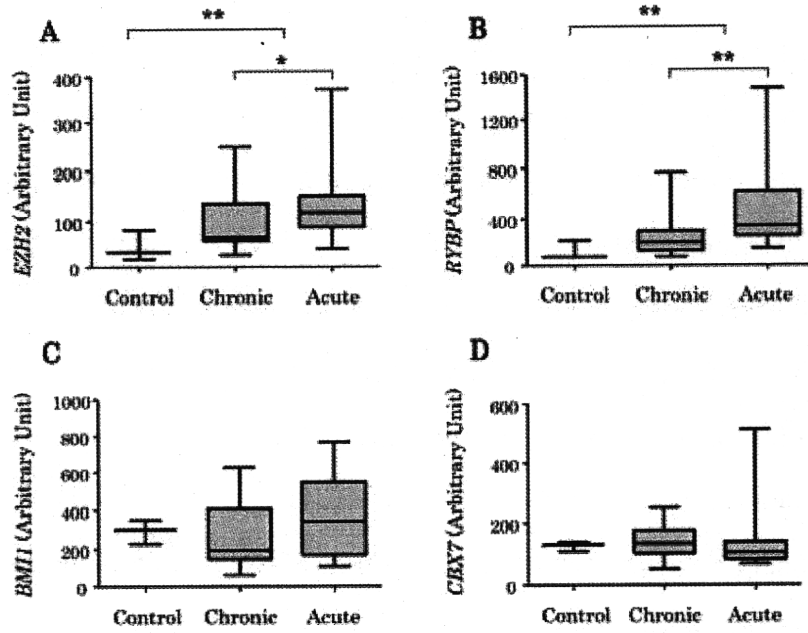


Figure 1

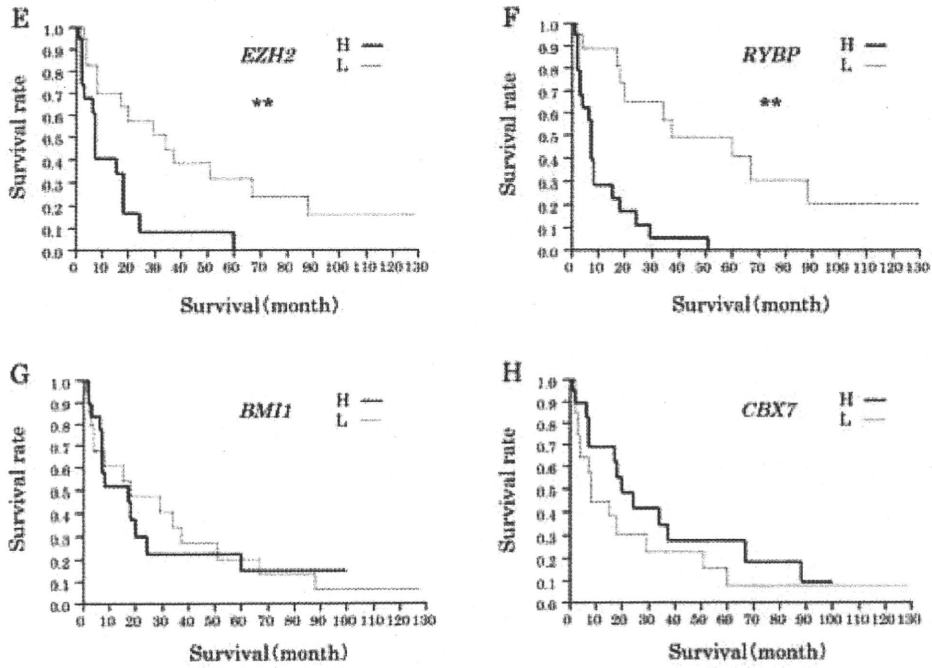


Figure 2

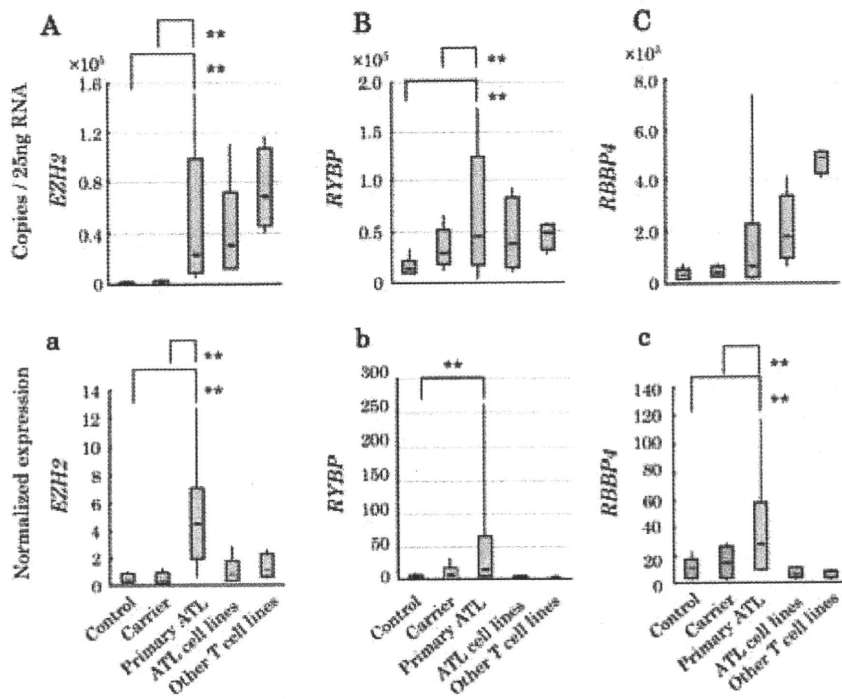


Figure 2

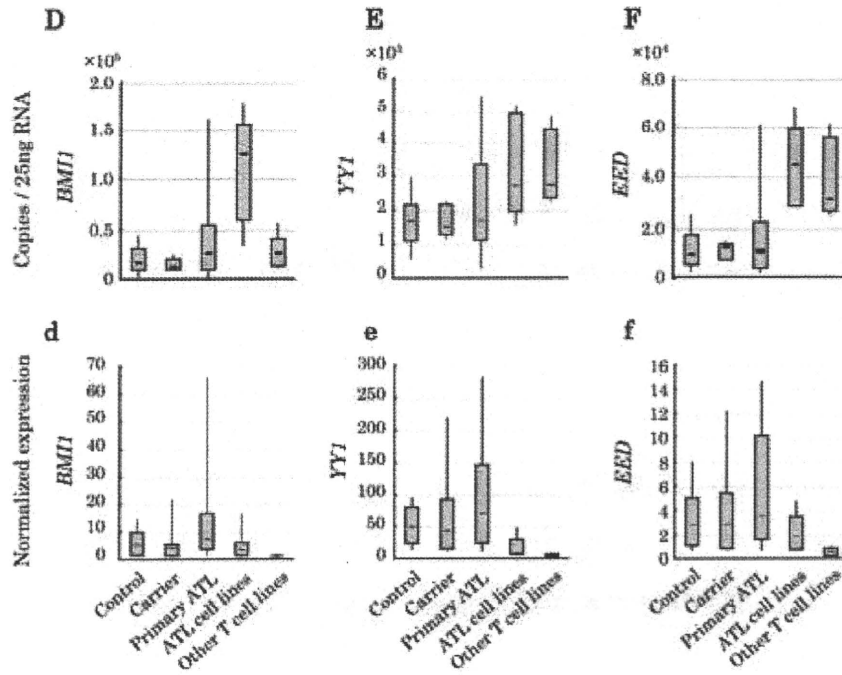


Figure 3

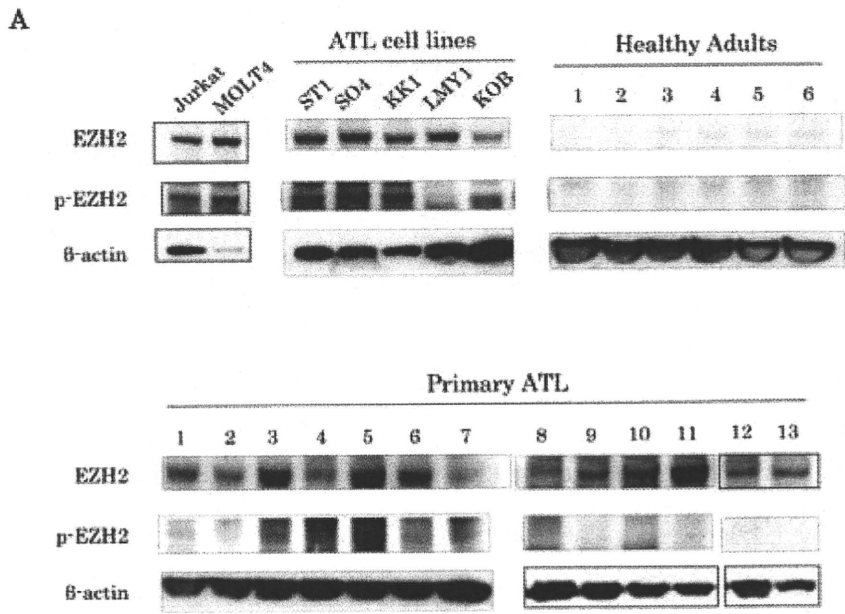


Figure 3

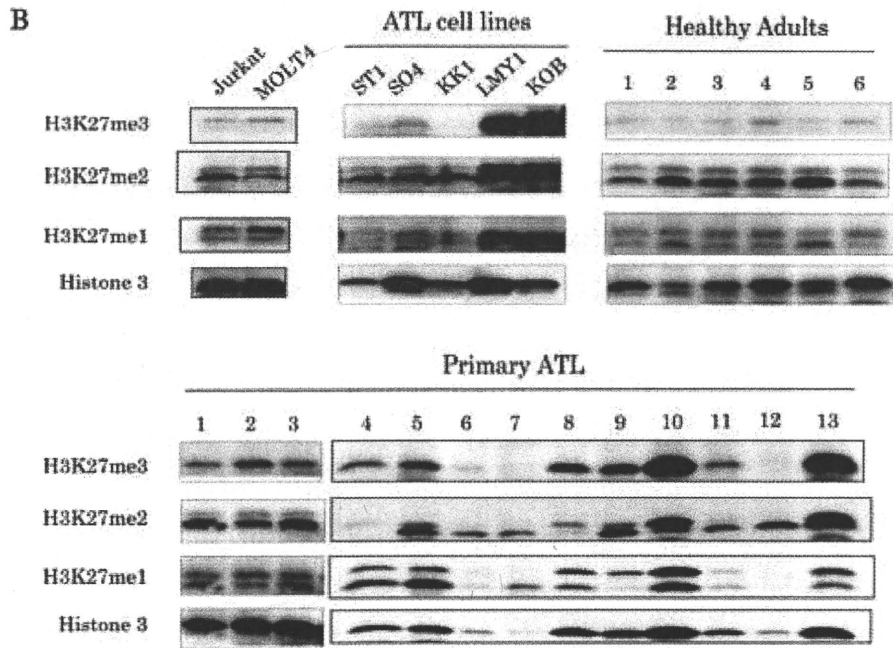


Figure 4

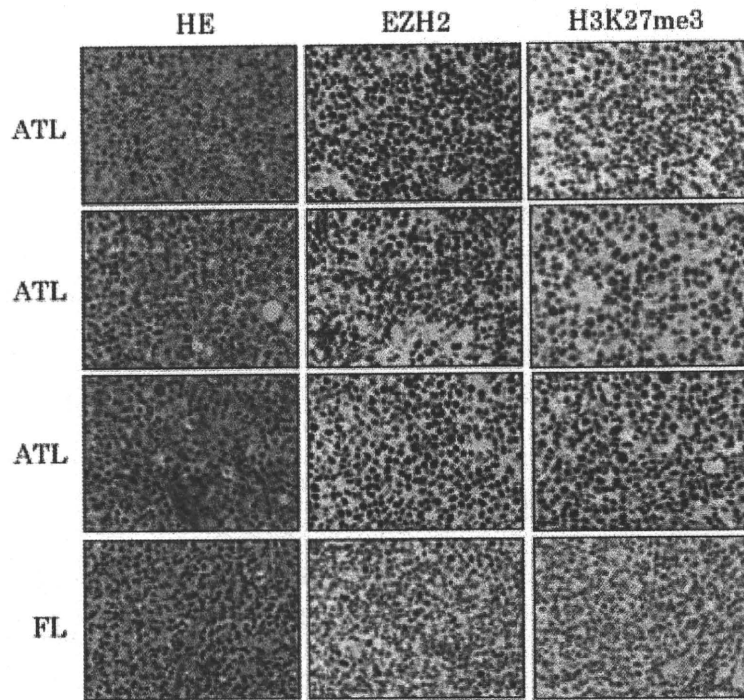


Figure 5

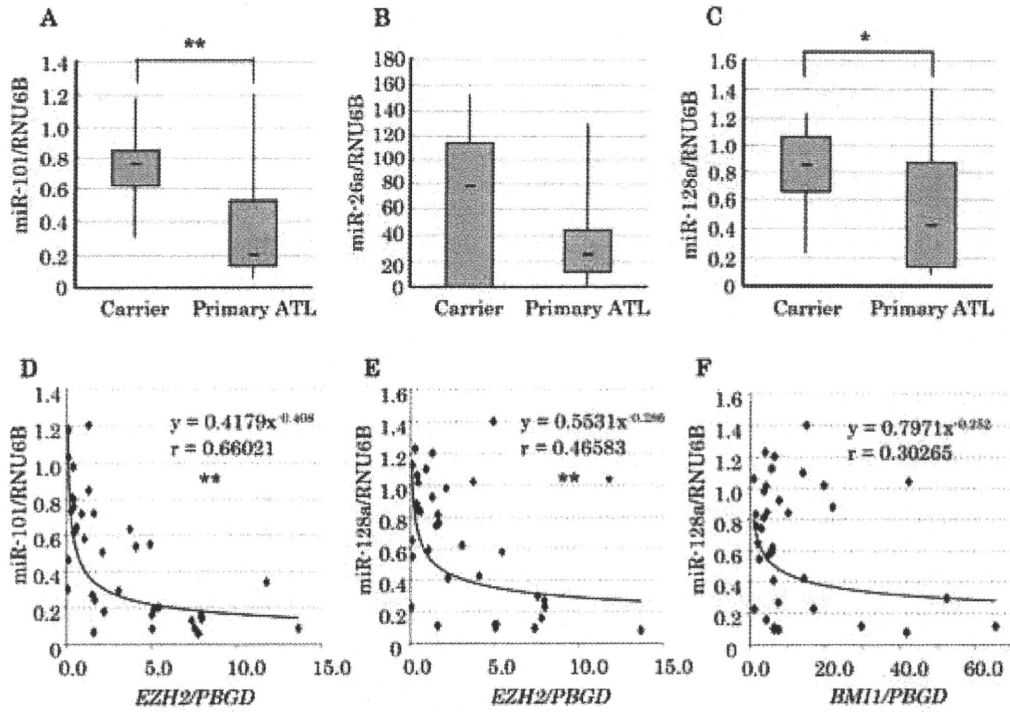


Figure 6

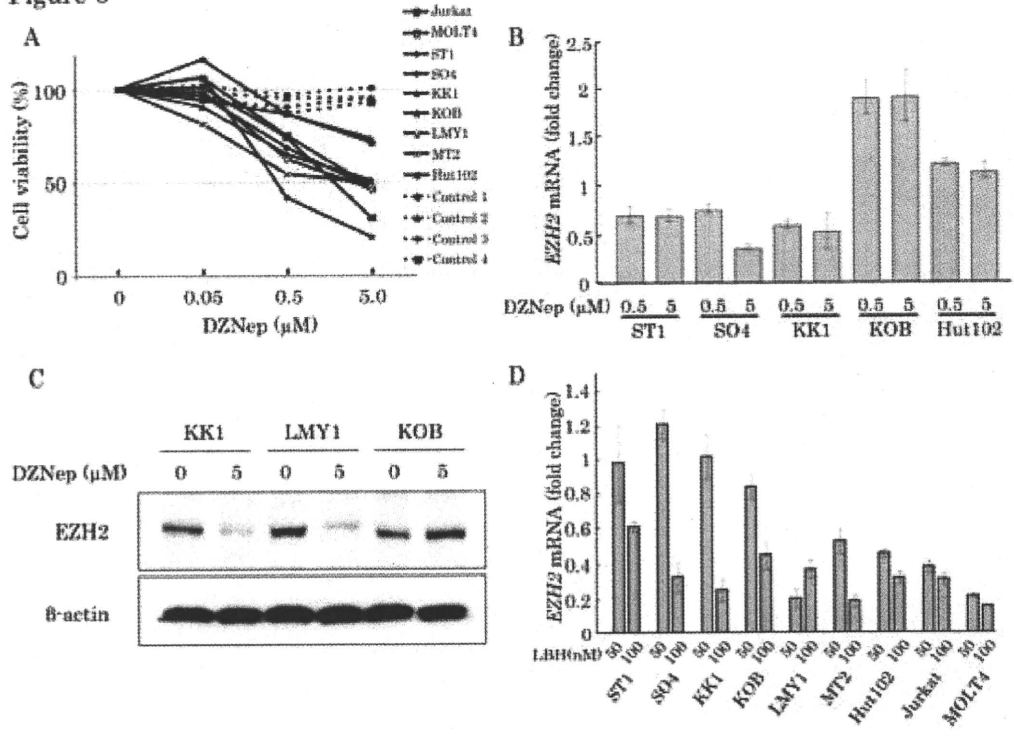
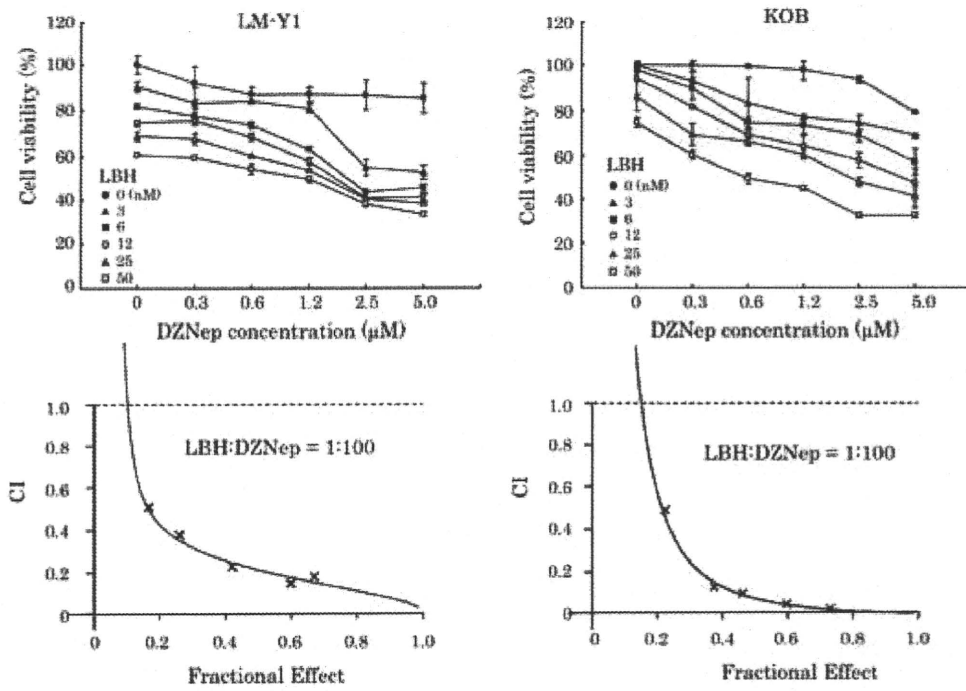


Figure 6 F



Supplementary Appendix

Figure legends for Supplementary Figures

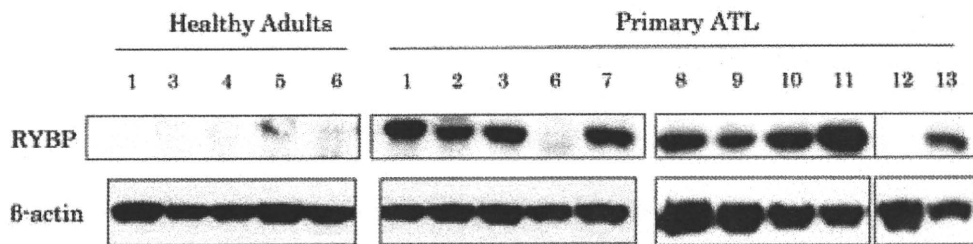
Supplementary Figure 1. RYBP protein expression. Western blot analysis for RYBP protein was performed on primary ATL cells and cells from healthy adults. Most primary ATL samples showed a clear band for RYBP. In contrast, cells from healthy adults lacked the band.

Supplementary Figure 2. Quantitative genomic PCR for miR-101. PCR was performed in two loci, miR-101-1 (chromosome 1p31) and miR-101-2 (chromosome 9p24), in 10 primary ATL samples and cells from 10 HTLV-1 carriers as a control. Both loci were preserved in ATL cells, refuting the possibility that downregulation of miR-101 is caused by genomic loss of the gene.

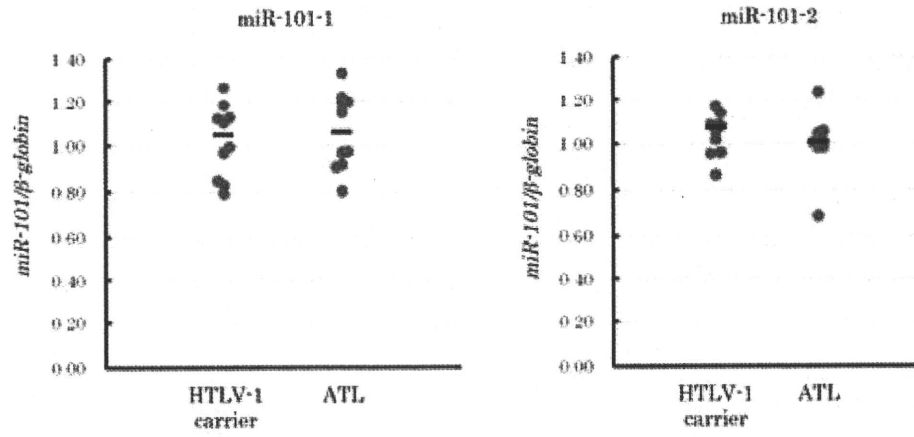
Supplementary Figure 3. Analysis of 3'-UTR sequence of EZH2 to predict potential target sites for miRNA. In addition to the target sites for miR-101 and miR-26a, there is also a potential target site for miR-128a in the 3'-UTR of EZH2 near one of the miR-101 target sites.

Supplementary Figure 4. Sequence analysis of EZH2. Pyrosequence analysis of EZH2 Try641 was performed in 10 ATL patients and 10 HTLV-1 carriers. Pyrograms of 6 ATL patients are shown. There were no mutations in the examined samples.

Supplementary Figure 1.



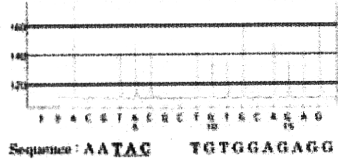
Supplementary Figure 2.



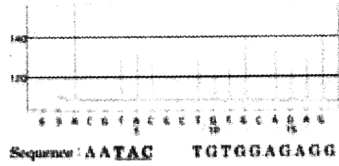
Supplementary Figure 4

Wild-type sequence: **AATACTGTGGAGAGG**

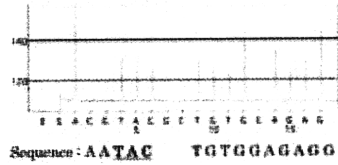
ATL patient 1: wild type



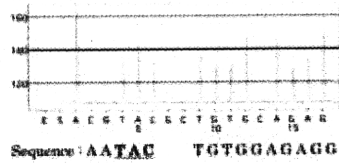
ATL patient 4: wild type



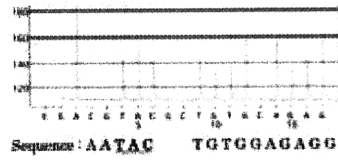
ATL patient 2: wild type



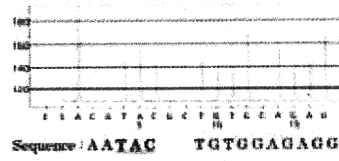
ATL patient 5: wild type



ATL patient 3: wild type



ATL patient 6: wild type



BRIEF REPORT

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

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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.)

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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 non-receptor 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 kinase⁵ 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)⁴ 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

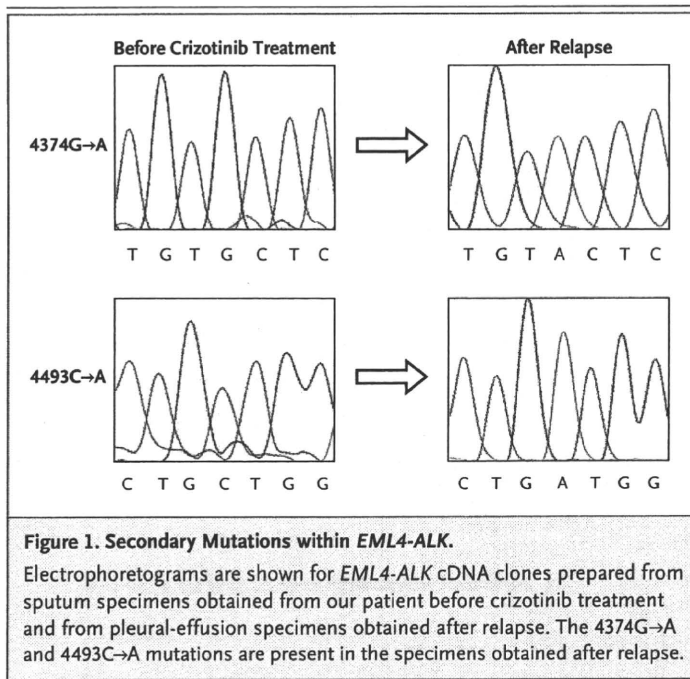
DNA sequencing and characterization of the EML4-ALK mutants are described in detail in the Supplementary Appendix.

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 V674A¹⁰ 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.



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

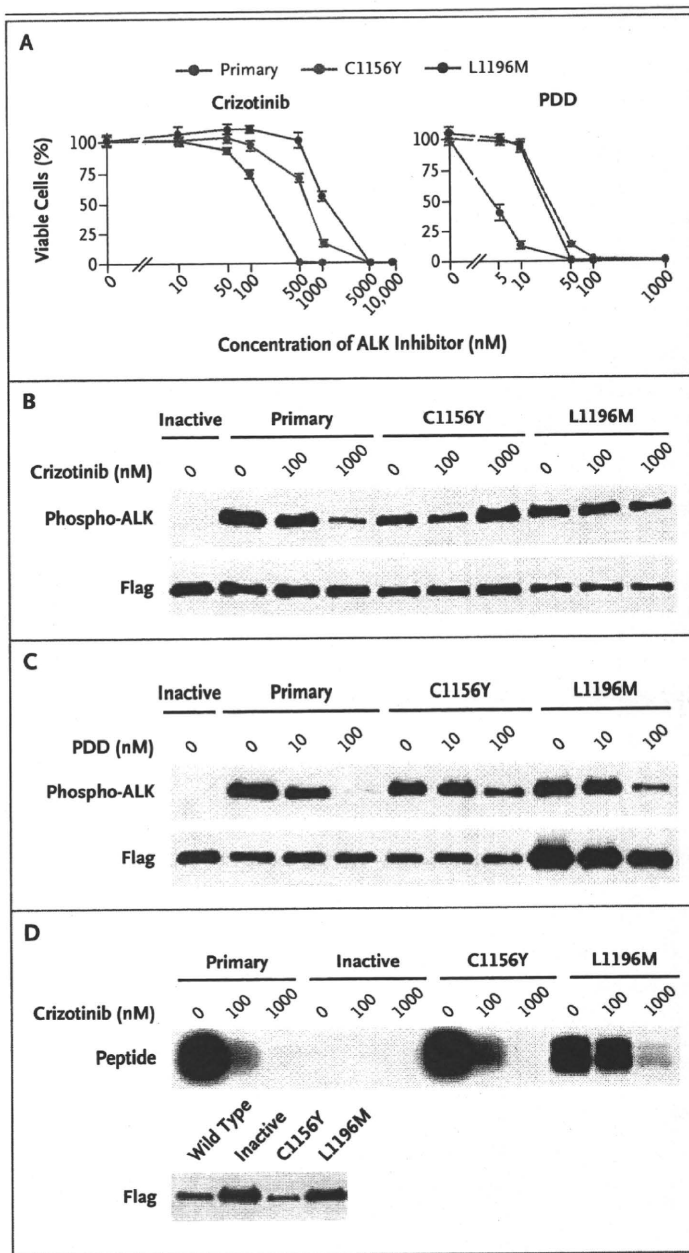
We examined tyrosine phosphorylation of

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

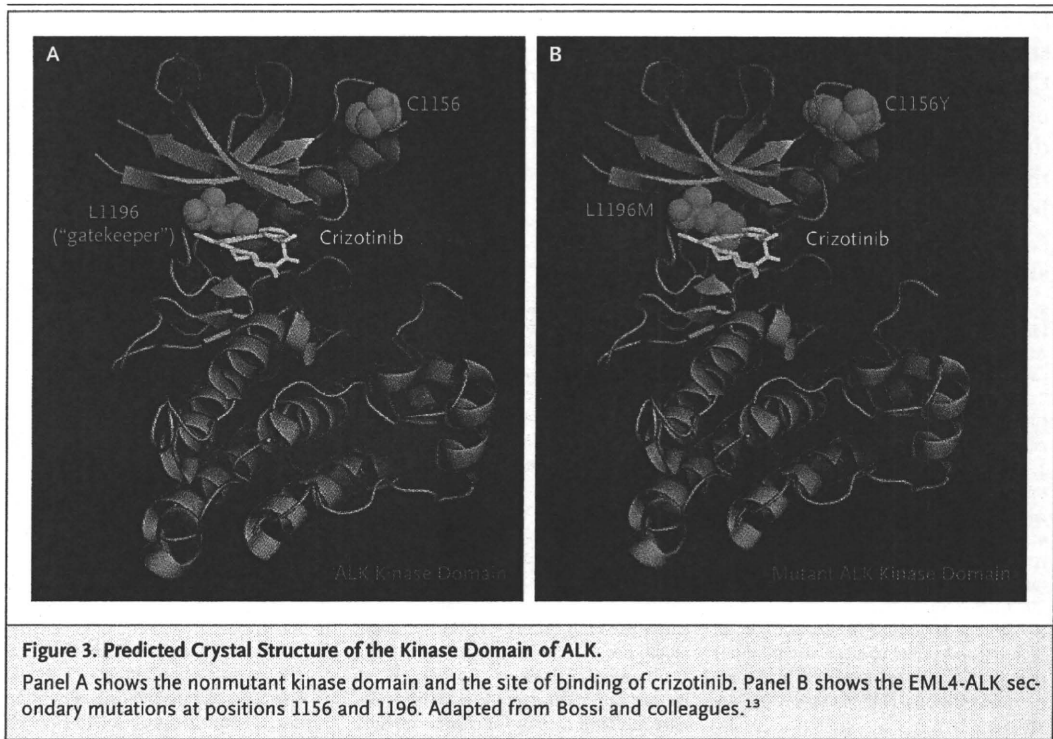
Panel A shows the percentage of viable BA/F3 cells expressing primary EML4-ALK, EML4-ALK with the C1156Y mutation, or EML4-ALK with the L1196M mutation, after 5×10^5 cells were incubated for 48 hours with the indicated concentration of crizotinib (left) or 2,4-pyrimidinediamine derivative (PDD) (right). 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 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 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-



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 Appen-

dix).^{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 α C 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 α C 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