specimens). Four cases of this cohort were positive for staining with both antibodies by the iAEP method. Again, most of the other specimens also showed a low level of background staining with ALK1, whereas only a few did so with 5A4. We therefore selected 5A4 to detect EML4-ALK with the iAEP method and included this approach in our routine diagnostic service at the pathology division of The Cancer Institute during the study period, thereby identifying four additional cases of lung adenocarcinoma positive for staining with 5A4.

Identification of variants 6 and 7 of EML4-ALK. The four specimens recognized by 5A4 in the validation set (IDs #24461, #24704, #26020, and #26422) were examined for the presence of *EML4-ALK* transcripts with our multiplex reverse transcription-PCR (RT-PCR) screening system, which was designed to capture all possible in-frame fusions between *EML4* and *ALK* at the cDNA level (8). Three cases (#24461, #26020, and #26422) were positive for *EML4-ALK* cDNAs (Fig. 1A), and nucleotide sequencing of the PCR products revealed that #24461 and #26422 tumors harbored variants 1 and 3 of *EML4-ALK*, respectively. The cDNA derived from tumor #26020, however, contained exon 13 of *EML4* as well as a portion of intron 19 and exon 20 of *ALK*, corresponding to a previously unidentified fusion variant (designated variant 6) of *EML4* and *ALK*

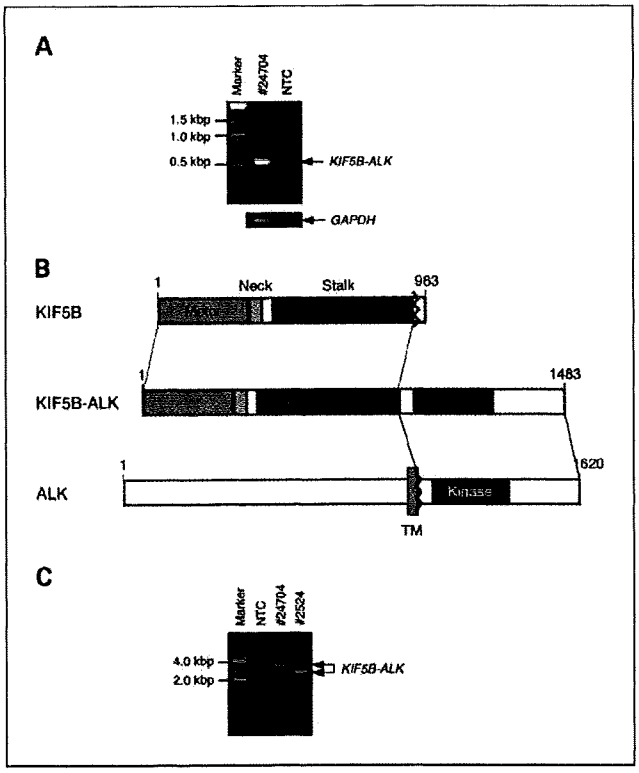

Fig. 2. Discovery of a *KIF5B-ALK* fusion gene associated with lung cancer. *A*, RT-PCR analysis of tumor ID #24704 with a forward primer targeted to exon 24 of *KIF5B* and a reverse primer targeted to exon 20 of *ALK*. Marker, 100-bp ladder. *B*, structure of KIF5B-ALK. KIF5B consists of an amino terminal ATP-dependent motor domain followed by a neck region and a stalk region containing seven coiled-coil domains. A chromosome translocation, t(2;10)(p23;p11), generates a fusion protein in which the entire motor domain and neck and stalk regions of KIF5B are joined to the intracellular region of ALK (containing the tyrosine kinase domain). Numbers indicate amino acid positions of each protein. *TM*, transmembrane domain. *C*, PCR analysis of genomic DNA from tumors #24704 and #2524 with primers flanking the putative fusion point of *KIF5B-ALK*. Marker, 500-bp ladder.

(Supplementary Fig. S3A; Fig. 1B). The fusion of exon 13 of *EML4* to a position 69 bp upstream of exon 20 of *ALK* in this fusion cDNA would be expected to constitute an in-frame fusion between the two genes. Although there were no reported mRNAs or expressed sequence tags containing intron 19 of *ALK* in the sequence databases, the genomic sequence surrounding the fusion point in this intron is AG-GA (Fig. 1B), which conforms to the consensus sequence for a splicing acceptor site, suggesting that this position of intron 19 may act as a cryptic acceptor site for RNA splicing.

Similar analysis by multiplex RT-PCR and sequence determination revealed that the additional 4 ALK-positive cases identified by our routine pathologic diagnostic service comprised one case (tumor ID #27265) with variant 2 of *EML4-ALK*, 2 cases (#26813 and #26953) with variant 3, and 1 case (#27998) with another novel variant (designated variant 7), in which exon 14 of *EML4* is fused to nucleotide 13 of exon 20 of *ALK* (Supplementary Fig. S3B; Fig. 1C and D). Genomic PCR analysis of the specimens positive for variants 6 and 7 of *EML4-ALK* cDNA confirmed the presence of genomic rearrangements responsible for the fusion events detected at the cDNA level (data not shown).

Identification of KIF5B-ALK *as a novel* ALK *fusion gene.* Whereas tumor #24704 of the validation cohort was strongly positive for ALK immunostaining by the iAEP method, multiplex RT-PCR analysis failed to amplify a specific product from this sample. We therefore examined the possibility that this tumor might harbor an *ALK* fusion gene other than *EML4-ALK*. We subjected the sample to an inverse RT-PCR analysis and obtained a PCR product containing both exon 24 of *KIF5B* and exon 20 of *ALK*. *KIF5B* is located on the short arm of human chromosome 10 and encodes member 5B of the kinesin family of proteins. To confirm the presence of a *KIF5B-ALK* fusion gene in this tumor, we directly amplified the fusion point of the *KIF5B-ALK* cDNA by RT-PCR with one primer targeted to exon 24 of *KIF5B* and the other to exon 22 of *ALK*. A single PCR product with the expected size of 546 bp was obtained (Fig. 2A). Nucleotide sequencing of the product further confirmed the fusion point of *KIF5B-ALK* at the cDNA level (data not shown).

KIF5B is a component of a motor protein complex that is associated with microtubules and mediates the transport of organelles within eukaryotic cells (15). It consists of an amino terminal motor domain followed by a neck region and a stalk region, the latter of which directly mediates homodimerization of KIF5B (Fig. 2B). Fusion of exons 1 to 24 of *KIF5B* to exon 20 of *ALK* would be expected to result in the production of a fusion protein consisting of almost the entire KIF5B sequence ligated to the intracellular region of ALK. It might therefore also be expected that KIF5B-ALK would undergo homodimerization mediated by the stalk region of KIF5B, with consequent activation of the kinase function of ALK, similar to the case of EML4-ALK, in which homo-oligomerization and activation are mediated by the amino terminal coiled-coil domain of EML4 (5, 8).

We next modified our multiplex RT-PCR method so that it could detect both *EML4-ALK* and *KIF5B-ALK* fusion mRNAs. In addition to a forward primer targeted to the boundary of exons 23 and 24 of *KIF5B* (to amplify the identified *KIF5B-ALK* fusion point), we included another forward primer targeted to exon 10 of *KIF5B* to detect potential novel fusion cDNAs for

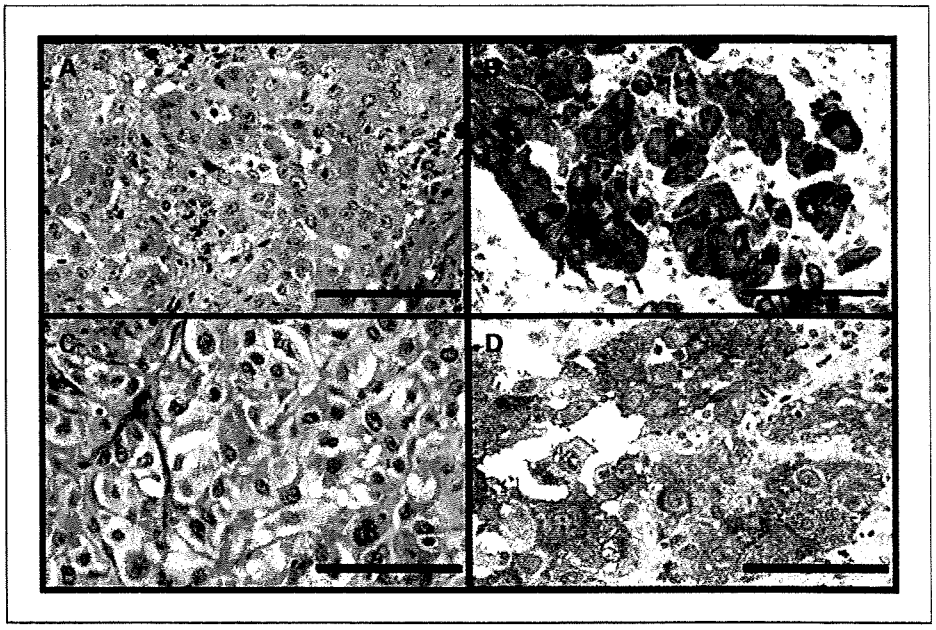

Fig. 3. Histopathology of KIF5B-ALK–positive lung adenocarcinoma. Sections of tumors #24704 (*A* and *B*) and #2524 (*C* and *D*) ware stained with H&E (*A* and *C*) or subjected to immunohistochemical analysis with 5A4 by the iAEP method (*B* and *D*). Some cancer cells of tumor #24704 contained intracytoplasmic macroglobular spots strongly positive for KIF5B-ALK (*B*). Some tumor cells showed a perinuclear halo positive for KIF5B-ALK (*D*). *Scale bars,* 100 μm.

KIF5B-ALK proteins containing a partial stalk region of KIF5B (given that the stalk region contains seven coiled-coil domains, a partial stalk region may still allow homodimerization of KIF5B-ALK). This newly designed multiplex RT-PCR assay was then applied both to the eight specimens found in this study to harbor *EML4-ALK* (7 cases) or *KIF5B-ALK* (#24704) and to the panel of cancer specimens including 253 samples of lung adenocarcinoma, 111 samples of other types of lung cancer, and 292 samples of tumors from 10 other organs, which was studied previously (8). Our modified multiplex RT-PCR method detected all 8 cases shown to be positive for *EML4-ALK* or *KIF5B-ALK* in the present study as well as 11 cases known to harbor various *EML4-ALK* fusion genes in the previous cohort (data not shown). The modified multiplex RT-PCR assay also identified one case (#2524) of lung adenocarcinoma harboring *KIF5B-ALK* among the previous cohort. We thus identified two cases positive for *KIF5B-ALK* among a total of 383 cases of lung adenocarcinoma (2 of 383 = 0.52%). Genomic rearrangement responsible for the identified *KIF5B-ALK* cDNAs was also confirmed in these two cases by genomic PCR analysis. The PCR products differed between the 2 cases, indicative of distinct breakpoints and fusion points within intron 24 of *KIF5B* and intron 19 of *ALK* (Fig. 2C).

Histopathology of* KIF5B-ALK–*positive lung adenocarcinoma. Histopathologic analysis of the two cases of *KIF5B-ALK*–positive lung adenocarcinoma revealed papillary structures, whereas the acinar pattern with prominent mucin production typically apparent in *EML4-ALK*–positive cases (7) was rarely observed. The individual cancer cells contained abundant eosinophilic cytoplasm and a large vesicular nucleus with one or two prominent nucleoli, and they were generally larger than those observed in *EML4-ALK*–positive cases (Supplementary Fig. S4A; Fig. 3A and C). Lymphatic invasion was prominent in tumor #24704, and the tumor cells in the lymphatic vessels contained an eccentric nucleus and a perinuclear eosinophilic globule (Supplementary Fig. S4A). Immunohistochemical detection of KIF5B-ALK with 5A4 by the iAEP method revealed a diffuse cytoplasmic staining in all of the cancer cells. Some cells manifested an uneven staining profile, with a perinuclear halo (Supplementary Fig. S4B; Fig. 3D) or macroglobular spots (Fig. 3B), neither of which was observed in tumors positive for EML4-ALK (8).

Fluorescence* in situ *hybridization analysis of* KIF5B-ALK–*positive tumors. To confirm further the genomic rearrangement in the two tumors positive for *KIF5B-ALK*, we did three fluorescence *in situ* hybridization assays: an *ALK* split assay, a *KIF5B* split assay, and a *KIF5B-ALK* fusion assay. The results for all three assays were consistent with the presence of a t(2;10)(p23;p11) responsible for the generation of *KIF5B-ALK* (Fig. 4). Cancer cells of tumor #24704 thus exhibited one isolated 3′-*ALK* signal and one isolated 5′-*KIF5B* signal in the *ALK* split assay and the *KIF5B* split assay, respectively, whereas they manifested one merged signal in the *KIF5B-ALK* fusion assay. Cancer cells of tumor #2524 exhibited at least two merged signals, indicative of possible amplification of the fusion gene. Neither an isolated 3′-*KIF5B* signal nor an isolated 5′-*ALK* signal was detected in the split assays for either case, suggesting that the derivative chromosome 2 harboring the *ALK-KIF5B* fusion gene may have been deleted after the balanced translocation between chromosomes 2 and 10.

Transforming activities of EML4-ALK variants 6 and 7 and of KIF5B-ALK. To isolate full-length cDNAs for the new variants of *EML4-ALK*, we did RT-PCR analysis with a forward primer targeted to the 5′ untranslated region of *EML4* cDNA and a reverse primer targeted to the 3′ untranslated region of *ALK* cDNA as described previously (6, 8). From oligo(dT)-primed cDNA preparations of tumor IDs #26020 or #27998, we isolated cDNAs of 3365 and 3435 bp, corresponding to variants 6 and 7 of *EML4-ALK*, respectively (data not shown). Similarly, a full-length cDNA of 4479 bp for *KIF5B-ALK* was obtained by RT-PCR analysis from tumor ID #2524. Nucleotide sequencing of these cDNAs confirmed that each of them would be expected to produce a functional PTK, with a predicted molecular size of 119,380 Da for EML4-ALK variant 6 (Supplementary Fig. S3A),

122,220 Da for EML4-ALK variant 7 (Supplementary Fig. S3B), and 167,903 Da for KIF5B-ALK (Supplementary Fig. S5).

Recombinant retroviruses encoding each of these fusion PTKs were generated and used to infect cultured 3T3 fibroblasts. Infection with the viruses encoding EML-ALK variant 6, EML4-ALK variant 7, or KIF5B-ALK, but not that with the empty virus, resulted in the formation of dozens of transformed foci *in vitro* (Fig. 5). As positive controls for focus formation, EML4-ALK variant 1 and NPM-ALK each yielded a similar number of transformed foci.

The same set of 3T3 cells was injected into nude mice for an *in vivo* tumorigenicity assay. All fusion PTKs induced s.c. tumors at all injection sites within an observation period of 20 days (Fig. 5), confirming the transforming potential of the novel variants of EML4-ALK as well as that of KIF5B-ALK.

Discussion

Immunohistochemical detection of ALK fusion proteins has been applied successfully to analysis of anaplastic large cell lymphoma and inflammatory myofibroblastic tumors, with the mouse monoclonal antibody ALK1 being most widely used for this purpose. However, many researchers were not able to reliably detect EML4-ALK in NSCLC specimens with this same immunohistochemical technique (14). Even if NSCLC specimens were positive for such staining, its intensity was usually low and varied substantially among sections of the same tumor, rendering the current standard technique unsuitable for screening of NSCLC specimens. This low sensitivity for the detection of EML4-ALK may be attributable to the low level of *EML4* transcriptional activity (see, for example, a public database for serial analysis of gene expression)[5] or to instability of EML4-ALK in cells.

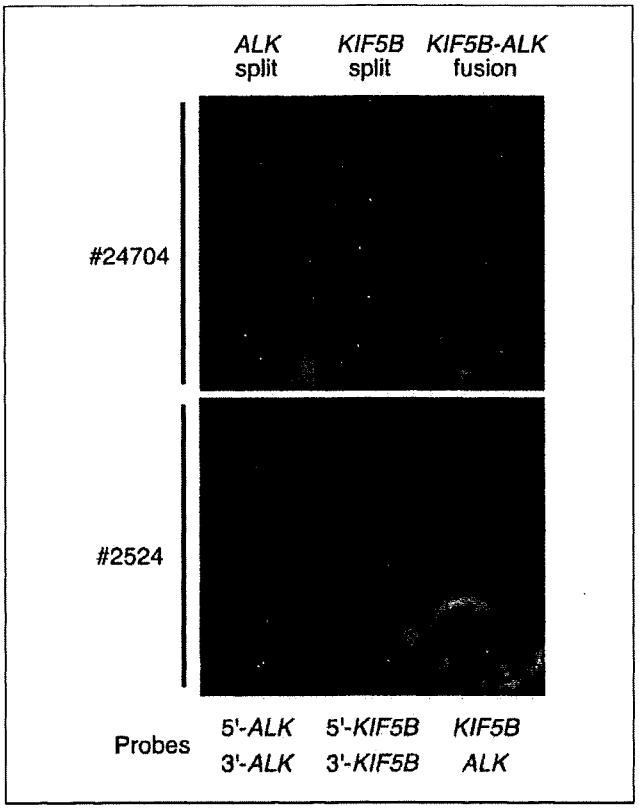

Fig. 4. Fluorescence *in situ* hybridization analysis of *KIF5B-ALK*. Sections of tumors positive for *KIF5B-ALK* (IDs #24704 and #2524) were subjected to fluorescence *in situ* hybridization with an *ALK* split assay (*left*), a *KIF5B* split assay (*middle*), or a *KIF5B-ALK* fusion assay (*right*). *Bottom*, the color of fluorescence for the BAC clones in each hybridization. Nuclei are stained blue with 4',6-diamidino-2-phenylindole.

However, given that immunohistochemical analysis is a convenient means to detect a protein of interest in pathology laboratories, it is desirable to establish a sensitive and accurate screening system for ALK fusion proteins based on this approach. Several candidate techniques with improved sensitivity, such as tyramide signal amplification (16), have been recently proposed. These techniques generally require multiple steps, however, which can compromise reproducibility and render them unsuitable for screening in routine pathologic diagnosis.

We have now achieved a moderate increase in the sensitivity of immunohistochemical detection of ALK fusion proteins by including antibodies to mouse or rabbit immunoglobulin as an intercalating reagent between the primary antibody and the EnVision+DAB polymer detection system. This iAEP method allowed the detection of EML4-ALK fusion proteins in all 11 specimens positive for *EML4-ALK* in our test cohort. This simple method can be readily done in ordinary diagnostic pathology laboratories. Although we selected the mouse monoclonal antibody 5A4 for immunohistochemistry by the iAEP method, other antibodies may be more suitable for routine diagnostic analysis with a modified version of this approach.

All antibodies used in the present study are specific for the intracellular region of ALK and so would be expected to detect both EML4-ALK and wild-type ALK. It is therefore possible that positive staining with 5A4 by the iAEP method does not reflect only the presence of ALK fusion proteins. To address this issue, we determined the amount of *ALK* mRNA with primers targeted to the 5′ or 3′ regions of *ALK* cDNA separately (whereas the latter would be expected to amplify cDNAs for both wild-type *ALK* and *ALK* fusion genes, the former would be expected to amplify only that of wild-type *ALK*). None of the *EML4-ALK*–positive specimens in the test set of samples yielded a substantial amount of wild-type *ALK* cDNA (although tumor #9968 may express the wild-type gene at a low level), suggesting that our iAEP method with 5A4 detected EML4-ALK proteins rather than wild-type ALK in the positive specimens. For ALK-rich tissues such as the brain or spinal cord (17), however, it would be important to determine which proteins are recognized by 5A4 in iAEP analysis.

We identified 8 tumors positive for staining with 5A4 by the iAEP method among the validation set of samples (n = 130) and the fresh cases subjected to routine diagnostic testing. Although 5 of these specimens harbored known EML4-ALK variants, the remaining three were found to express novel ALK fusion proteins, including EML4-ALK variants 6 (#26020) and 7 (#27998) and KIF5B-ALK (#24704). These results thus showed that sensitive immunohistochemical analysis was superior to PCR-based methods for detecting novel ALK fusion constructs among tumor specimens. This conclusion was further supported by the fact that neither *EML4-ALK* nor *KIF5B-ALK* was identified in the iAEP-negative cases by our modified multiplex RT-PCR assay (data not shown).

[5] http://cgap.nci.nih.gov/sage/anatomicviewer

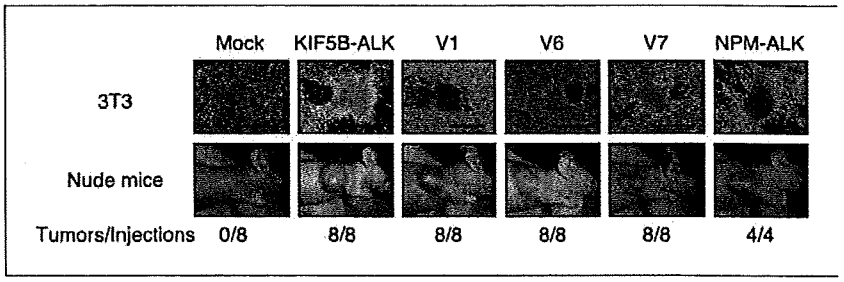

Fig. 5. Transforming activities of EML4-ALK and KIF5B-ALK. *A*, mouse 3T3 fibroblasts were infected with retroviruses encoding KIF5B-ALK, NPM-ALK, or variant 1, 6, or 7 of EML4-ALK or with the corresponding empty virus (*mock*). The cells were photographed after culture for 14 d. *Scale bars*, 500 μm. *B*, nude mice were injected s.c. with 3T3 cells infected as in *A*, and tumor formation was examined after 20 d. *Bottom*, the number of tumors formed per eight injections.

A fusion protein containing most of KIF5B and the intracellular (kinase) domain of the platelet-derived growth factor receptor A has been detected in idiopathic hypereosinophilic syndrome (18). The genome of some patients with this condition exhibits a chromosome translocation, t(4;10)(q12;p11), which results in the production of a *KIF5B-PDGFRA* fusion mRNA in which exon 23 of *KIF5B* is ligated to exon 12 of *PDGFRA*. Given that the KIF5B portion of KIF5B–platelet-derived growth factor receptor A contains six of the seven coiled-coil domains within the stalk region, the fusion protein likely dimerizes constitutively and thereby possesses transforming potential. KIF5B-ALK is thus the second example of an oncogenic KIF5B fusion to a PTK.

The subcellular localization of ALK fusion proteins likely depends on the fusion partner. For instance, whereas NPM-ALK, which is associated with anaplastic large cell lymphoma, is present in both the nucleus and cytoplasm, nuclear localization has not been detected for other ALK fusion proteins including CLTC-ALK, TPM3-ALK, TFG-ALK, ATIC-ALK (19), and EML4-ALK (5). The pattern of immunohistochemical staining for KIF5B-ALK did not resemble that of any of these other ALK fusion proteins. The observed perinuclear halo of KIF5B-ALK staining may indicate accumulation of the fusion protein at the periphery of the cytoplasm (subcell membrane region), possibly reflecting transport of KIF5B-ALK along microtubules. Signaling downstream of KIF5B-ALK may thus differ substantially from that of other ALK fusion proteins, as exemplified by the differential phosphorylation of STAT proteins associated with these fusion proteins (19).

In conclusion, we have developed a modified immunohistochemical staining procedure for the detection of ALK and ALK fusion proteins in lung cancer that may prove suitable for screening purposes in pathology laboratories. Our identification of a second ALK fusion gene, *KIF5B-ALK*, in NSCLC further supports the clinical relevance of *ALK* in the pathogenesis of this disease. Given the recent development of several ALK inhibitors and their potential therapeutic efficacy for tumors positive for ALK fusion proteins (6, 10, 20), accurate diagnosis of tumors expressing activated ALK or ALK fusion proteins (5, 21, 22) will be essential to identify subgroups of patients who are suitable for treatment with such drugs.

Disclosure of Potential Conflicts of Interest

K.T. serves as a consultant to Dako.

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References

1. Mitelman F. Recurrent chromosome aberrations in cancer. Mutat Res 2000;462:247–53.
2. Bartram CR, de Klein A, Hagemeijer A, et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1983;306:277–80.
3. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer 2007;7:233–45.
4. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. Nat Rev Cancer 2008;8:497–511.
5. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561–6.
6. Choi YL, Takeuchi K, Soda M, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 2008;68:4971–6.
7. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol 2008;3:13–7.
8. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–24.
9. Soda M, Takada S, Takeuchi K, et al. A mouse model for EML4-ALK-positive lung cancer. Proc Natl Acad Sci U S A 2008;105:19893–7.
10. McDermott U, Sharma SV, Dowell L, et al. Identification of genotype-correlated sensitivity to selective kinase inhibitors by using high-throughput tumor cell line profiling. Proc Natl Acad Sci U S A 2007;104:19936–41.
11. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 2007;131:1190–203.
12. Koivunen JP, Mermel C, Zejnullahu K, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008;14:4275–83.
13. Cataldo KA, Jalal SM, Law ME, et al. Detection of t(2;5) in anaplastic large cell lymphoma: comparison of immunohistochemical studies, FISH, and RT-PCR in paraffin-embedded tissue. Am J Surg Pathol 1999;23:1386–92.
14. Martelli MP, Sozzi G, Hernandez L, et al. EML4-ALK rearrangement in non-small-cell lung cancer and non-tumor lung tissues. Am J Pathol 2009;174:661–70.
15. Sablin EP. Kinesins and microtubules: their structures and motor mechanisms. Curr Opin Cell Biol 2000;12:35–41.
16. McLachlan CS, Jusuf PR, Rummery N, et al. Tyramide signal amplification enhances the detectable distribution of connexin-43 positive gap junctions across the ventricular wall of the rabbit heart. Arch Histol Cytol 2003;66:359–65.
17. Pulford K, Morris SW, Turturro F. Anaplastic lymphoma kinase proteins in growth control and cancer. J Cell Physiol 2004;199:330–58.
18. Score J, Curtis C, Waghorn K, et al. Identification of a novel imatinib responsive KIF5B-PDGFRA fusion gene following screening for PDGFRA overexpression in patients with hypereosinophilia. Leukemia 2006;20:827–32.
19. Armstrong F, Duplantier MM, Trempat P, et al. Differential effects of X-ALK fusion proteins on proliferation, transformation, and invasion properties of NIH3T3 cells. Oncogene 2004;23:6071–82.
20. McDermott U, Iafrate AJ, Gray NS, et al. Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. Cancer Res 2008;68:3389–95.
21. Morris SW, Kirstein MN, Valentine MB, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1994;263:1281–4.
22. Chen Y, Takita J, Choi YL, et al. Oncogenic mutations of ALK kinase in neuroblastoma. Nature 2008;455:971–4.

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Tec protein tyrosine kinase inhibits CD25 expression in human T-lymphocyte

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ABSTRACT

The Tec protein tyrosine kinase (PTK) belongs to a group of structurally related nonreceptor PTKs that also includes Btk, Itk, Rlk, and Bmx. Previous studies have suggested that these kinases play important roles in hematopoiesis and in the lymphocyte signaling pathway. Despite evidence suggesting the involvement of Tec in the T-lymphocyte activation pathway via T-cell receptor (TCR) and CD28, Tec's role in T-lymphocytes remains unclear because of the lack of apparent defects in T-lymphocyte function in Tec-deficient mice. In this study, we investigated the role of Tec in human T-lymphocyte using the Jurkat T-lymphoid cell line stably transfected with a cDNA encoding Tec. We found that the expression of wild-type Tec inhibited the expression of CD25 induced by TCR cross-linking. Second, we observed that LFM-A13, a selective inhibitor of Tec family PTK, rescued the suppression of TCR-induced CD25 expression observed in wild-type Tec-expressing Jurkat cells. In addition, expression of kinase-deleted Tec did not alter the expression level of CD25 after TCR ligation. We conclude that Tec PTK mediates signals that negatively regulate CD25 expression induced by TCR cross-linking. This, in turn, implies that this PTK plays a role in the attenuation of IL-2 activity in human T-lymphocytes.

1. Introduction

The activation and development of lymphocytes are regulated by the engagement of cell surface immune cell antigen receptors. Following receptor engagement, these receptors transmit signals by the activation of cytoplasmic protein tyrosine kinases (PTKs), such as Src, Syk, and Tec families [1,2]. The Tec family PTKs are nonreceptor PTKs including Tec, Btk, Itk (Emt/Tsk), Rlk (Txk), and Bmx (Etk). They are typically characterized by a pleckstrin-homology domain, a Tec-homology domain, Src homology domains (SH2 and SH3), and a kinase domain [3,4]. The biological importance of the Tec PTK subfamily was first confirmed in B-lymphocytes by the finding that Btk is essential for B-cell development [5,6] and that mutations in Btk cause X-linked agammaglobulinemia (XLA) in humans and B-cell defects in *xid* mice [7–10]. For T-cells, mice lacking Itk exhibited decreased numbers of mature thymocytes and reduced proliferative responses to both allogeneic major histocompatibility complex stimulation and T-cell receptor (TCR) cross-linking [11]. In addition, TCR-induced phosphorylation and activation of PLC-γ are reduced in T-cells lacking Itk [12]. According to early observations, it has been speculated that the functions of Btk and Itk are essentially related to B- and T-lymphoid development and activation, respectively, while Tec participates mainly in signaling pathways regulating myeloid cell growth and differentiation.

In our previous studies, we revealed Tec's contribution to antigen receptor signaling in B-lymphoid cells. Ligation of the B-cell receptor (BCR), CD19, and CD38 caused tyrosine phosphorylation of Tec and increased Tec PTK activity [13]. Tec's important role in B-cells was further confirmed by the generation of Tec/Btk double-deficient mice exhibiting an early block in B-cell development and a severe reduction in peripheral B-cell numbers [14]. In T-cells, TCR stimulation induces the activation of Itk [15], Rlk [16], and Tec [17]. In addition, the ligation of T-cell costimulatory receptor CD28 also activates Itk [18] and Tec [17]. In primary splenocytes from 5C.C7 TCR-transgenic mice, depletion of Tec using an antisense oligonucleotide treatment reduces IL-2 production in response to TCR ligation [19]. Studies using the Tec-transfected Jurkat human T-lymphoid cell line proposed the unique roles of Tec in T-cell activation [17,20]. However, purified T-cells from Tec-deficient mice were reported to have no apparent defects in TCR or CD28 signaling [14]. Thus, it is still an open question whether or not Tec is essential in the signaling pathway of T-lymphoid cells.

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In the present study we investigated Tec's role in human T-lymphoid cells using a Jurkat cell line stably transfected with a cDNA encoding Tec. We found that the expression of wild-type Tec inhibited the expression of CD25 induced by TCR cross-linking. Second, we observed that LFM-A13, a selective inhibitor of Tec family PTK, rescued the suppression of TCR-induced CD25 expression observed in wild-type Tec-expressing Jurkat cells. In addition, expression of kinase-deleted Tec did not alter the CD25 expression level after TCR ligation. We conclude that Tec PTK activity mediates signals that negatively regulate CD25 expression induced by TCR cross-linking in human T-lymphocytes.

2. Materials and methods

2.1. Reagents and cells

The rabbit polyclonal anti-Tec antibodies were previously described [13]. A monoclonal antibody to phosphotyrosine (PY99) and goat antisera to Tec were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-CD3 antibody (OKT3) was obtained from Janssen Pharmaceutical (Tokyo, Japan). Monoclonal anti-CD28 antibody was from Immunotech (Marseille, France). PE-conjugated anti-CD25 antibody and FITC-conjugated anti-CD69 antibody were from Dako (Glostrup, Denmark). LFM-A13 (a-cyano-b-hydroxy-b-methyl-N-(2,5-dibromophenyl) propenamide) was from Calbiochem (San Diego, CA). LFM-A13 was dissolved in dimethyl sulfoxide (DMSO) and aliquots were stored at −30 °C. The final concentration of DMSO was less than 0.5% for all experiments. DMSO at this concentration had no discernible effect on cell growth or surface marker expression profiles, including CD3 and CD25 expression (data not shown). All other agents were purchased from commercial sources.

The Jurkat human T-lymphoid cell line was a generous gift from Dr. D. Campana (St. Jude Children's Research Hospital, Memphis, TN). Jurkat cells were maintained in RPMI-1640 (Sigma, St. Louis, MO) with 10% fetal calf serum, L-glutamine, and antibiotics.

2.2. Immunoprecipitation, electrophoresis, and Western blotting

The cells were lysed in lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% [v/v] Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethyl-sulfonyl fluoride, 5 μg/ml aprotinin, 1 mM EDTA–2Na). Immunoprecipitation and Western blotting analysis were performed as described previously [13]. The experiments were repeated independently at least three times.

2.3. DNA constructs and electroporation conditions

The construction of pSR expression vector containing cDNA of wild-type Tec (TecWT) and kinase-deleted Tec (TecKD) has been described elsewhere [21]. Jurkat cells (5×10^6/experiment) were subjected to electroporation with 30 μg of pSR or pSR containing TecWT or TecKD, as described previously [22]. Transfected cells were selected after 2 weeks' culture in the presence of 5 μg/ml of blasticidin S hydrochloride (Funakoshi, Tokyo, Japan). Blasticidin-resistant clones were expanded and screened for Tec expression by means of immunoprecipitation and Western blotting. Individual clones were cultured and were analyzed as a mixture of clones to avoid clonal variations.

2.4. Stimulation of T cells

Anti-CD3 antibody (2 μg/ml) was incubated in 24-well flat-bottom plates at 4 °C for 16 h for immobilization to the bottoms of the plates. The plates were washed twice to remove excess antibodies. Cells were incubated in each well of anti-CD3-coated plates at 37 °C in 5% CO_2 with 90% humidity for indicated periods. At the termination of the cultures, the cells were harvested, suspended in PBS, and subjected to further analysis. The experiments were repeated independently at least three times.

2.5. Flow cytometric analysis

The surface phenotypes of the cells were examined by flow cytometry as described previously [23]. Briefly, collected cells were incubated with a specific fluorescent-conjugated monoclonal antibody or control mouse IgG on ice for 30 min. After two washes with PBS, cells were analyzed with an EPICS XL flow cytometry system equipped with EXPO32 ADC software (Beckman Coulter, Miami, FL). The experiments were repeated independently three times.

2.6. Quantification of IL-2

To measure IL-2 production, Jurkat cells were cultured in 24-well plates at 1×10^6 cells/ml, 1 ml/well and stimulated with 2 μg/ml anti-CD3 plus 2 μg/ml anti-CD28 monoclonal antibodies, or 50 ng/ml PMA and 1 μM ionomycin for the positive control cultures. After 24 h culture, IL-2 secreted in the culture supernatant was measured using Quantiflow Human IL-2 Immunoassay kits (BioE, St. Paul, MN) according to the manufacturer's instructions. The experiments were repeated independently at least three times.

2.7. RT-PCR analysis

RT-PCR analysis was performed as described previously [24]. For amplification of the cDNA products, the following oligodeoxynucleotide primers were used: CD25 primers, 5′-GGGATACAGGGCTCTACACAG-3′ (sense) and 5′-ACCTGGAAACTGACTGGTCTC-3′ (antisense); β-actin primers, 5′-ATCATGTTTGAGACCTTCAA-3′ (sense) and 5′-GATGTCCACGTCACACTTCA-3′ (antisense). The PCR product was resolved by agarose gel electrophoresis and analyzed by means of densitometric analysis, and the fold increase in the CD25 cDNA level was normalized to the β-actin product. The experiments were repeated independently at least three times.

2.8. Statistical analysis

Data were analyzed by Student's *t*-test; $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Ectopic expression and activation of Tec in Jurkat cells

As we reported previously, Jurkat cells lack endogenous Tec expression [13,25], making this cell line a useful model for studying the role of Tec in human T-cell biology. To investigate the role of Tec in human T-lymphoid cells, we introduced Tec cDNA to Jurkat cells. Clonal Jurkat cells expressing Tec protein (Jurkat-TecWT cells) were obtained after transfection and a subsequent series of limiting dilution procedures (Fig. 1a). In contrast, proteins in the anti-Tec immunoprecipitates from mock-transfected Jurkat cells (Jurkat-Mock cells) did not react with anti-Tec antibody (Fig. 1a). Ligation of TCR or CD28 is known to induce tyrosine phosphorylation of intracellular proteins in T-lymphoid cells, including Jurkat cell lines [1]. To determine whether or not the signaling pathways triggered by TCR or CD28 ligation were affected by the presence of Tec, intracellular protein tyrosine phosphorylation was analyzed by Western blotting using anti-phosphotyrosine antibody. As shown in Fig. 1b, in Jurkat-TecWT cells the ligation of CD3 or CD28 induced tyrosine phosphorylation with molecular weights and intensities

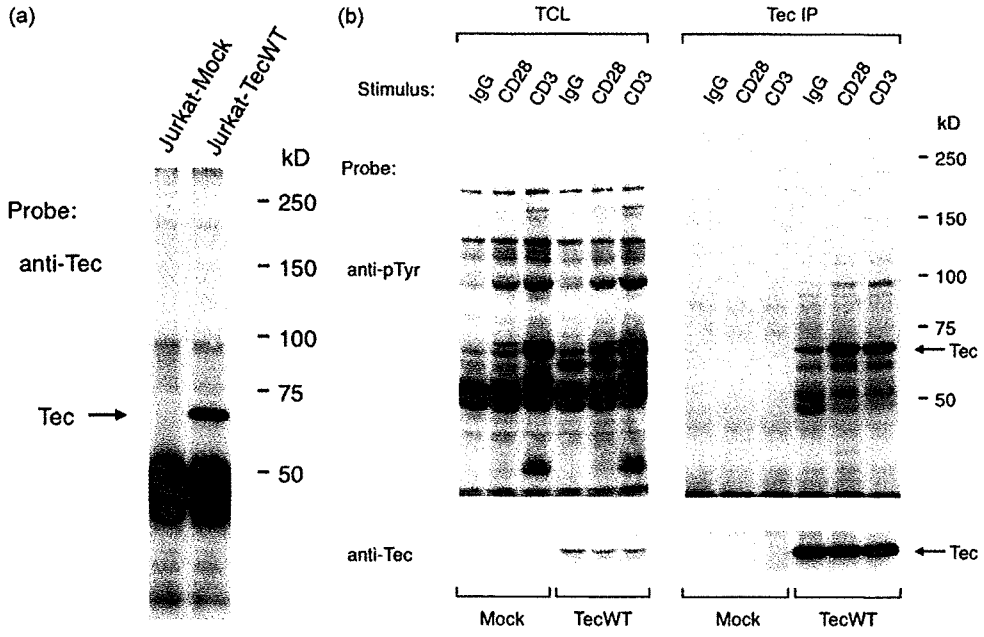

Fig. 1. Ectopically expressed Tec is activated following cell surface receptor cross-linking in Jurkat cells. (a) Cell lysates of Jurkat-Mock cells and Jurkat-TecWT cells were subjected to immunoprecipitation with anti-Tec antibody. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with anti-Tec polyclonal antibody. The positions of Tec and molecular mass markers (in kDa) are indicated. The intense band of approximately 50 kDa corresponds to the Ig heavy chain of the antibody used for immunoprecipitation. (b) Jurkat-Mock cells and Jurkat-TecWT cells were stimulated with control IgG, anti-CD28, or anti-CD3 for 5 min. Total cell lysates (TCLs) and proteins immunoprecipitated with anti-Tec (Tec IP) from these lysates were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with anti-phosphotyrosine antibody (anti-pTyr; upper panel), then stripped and reprobed with anti-Tec polyclonal antibody (lower panel). The positions of Tec and molecular mass markers (in kDa) are indicated.

similar to those seen in Jurkat-Mock cells. Thus, the ectopic expression of Tec did not affect the overall profile and magnitude of the tyrosine-phosphorylated proteins, at least to an extent detectable by Western blotting. To determine whether or not TCR signaling activated transfected Tec in Jurkat cells, we examined Tec tyrosine phosphorylation after cross-linking the TCR with an anti-CD3 antibody. In contrast to the lack of a significant effect of Tec expression on the overall pattern of tyrosine phosphorylation, exposure to anti-CD3 antibody markedly increased tyrosine phosphorylation of Tec in Jurkat-TecWT cells (Fig. 1b). Stimulation of cells with anti-CD28 also triggered the tyrosine phosphorylation of Tec. Thus, activation of transfected Tec by ligation of T-cell-specific surface molecules was confirmed in Jurkat-TecWT cells. No tyrosine phosphorylation signal was detected in anti-Tec immunoprecipitates obtained from Jurkat-Mock cells (Fig. 1b).

We next examined the effect of Tec expression on Jurkat cell surface marker expression. The cell surface antigenic phenotype of Jurkat-TecWT cells was investigated by flow cytometry and compared with that of Jurkat-Mock cells. No apparent differences were observed in the expression of T-lymphoid cell markers and the activation markers examined, such as, CD1, CD2, CD3, CD4, CD8, CD25, CD28, and CD69, indicating that Tec expression had a minimal effect on the basal expression of representative T-cell surface proteins (data not shown).

3.2. Effect of Tec on IL-2 production

Because Tec overexpression in Jurkat cells has been reported to enhance IL-2 production and can induce TCR-mediated phospholipase Cγ (PLC-γ) phosphorylation and NFAT (nuclear factor of activated T-cells) activation [17,19,20,26,27], we attempted to replicate those findings with Jurkat cells stably transfected with Tec. Unexpectedly, exposure of Jurkat-TecWT cells to anti-CD3 plus anti-CD28 resulted in low levels of IL-2 production in both Jurkat-Mock cells and Jurkat-TecWT cells, without significant differences between the two cell types. In one experiment, after 24 h of incubation, 36 pg/ml IL-2 with anti-CD3 plus anti-CD28 stimulation versus 845 pg/ml IL-2 in control cultures with 50 ng/ml of PMA and 1 μM ionomycin were detected in the supernatant of the Jurkat-Mock cell culture, while 10 pg/ml IL-2 versus 850 pg/ml IL-2 was detected in the Jurkat-TecWT cell culture. Low IL-2 secretion in response to TCR stimulation was reproduced in both cell lines in repeated experiments. The addition of IL-2 at concentrations below 100 pg/ml had no influence on CD25 expression in either Jurkat-Mock cells or Jurkat-TecWT cells (data not shown).

3.3. Tec downregulates CD25 expression

CD25 is an essential component of high-affinity IL-2 receptors [28,29]. Although several investigators have proposed the possibility that Tec is involved in the IL-2-producing machinery [4,17,19,20,26,27,31,32,35], little is known about the relationship between Tec family PTK and CD25 expression, except the downregulation of CD25 observed in stimulated T-cells from Itk-deficient mice [12]. We evaluated the effect of Tec expression in Jurkat cells on CD25 expression. The membrane expression of CD25 increases after T-lymphocyte activation [28,29]. To examine whether or not Tec expression modifies TCR-mediated signaling, we examined changes in CD25 surface expression on Jurkat-derived clones activated for 24 h with TCR cross-linking using flow cytometry. As shown in Fig. 2a , enhanced CD25 expression was observed in Jurkat-Mock cells after the 24 h incubation with plate-bound anti-CD3. In contrast, the expression of CD25 after TCR cross-linking was markedly suppressed in Jurkat-TecWT cells (Fig. 2a). The percentage of CD25-expressing cells after TCR cross-linking was 39.5% in Jurkat-Mock cells and 9.9% in Jurkat-TecWT cells. These findings suggest that activation of Tec kinase results in the downregulation of CD25 expression induced by TCR cross-linking. CD69 (an activation-inducer molecule) is also known to be upregulated upon T-cell activation [1,12]. Next, we examined the effect

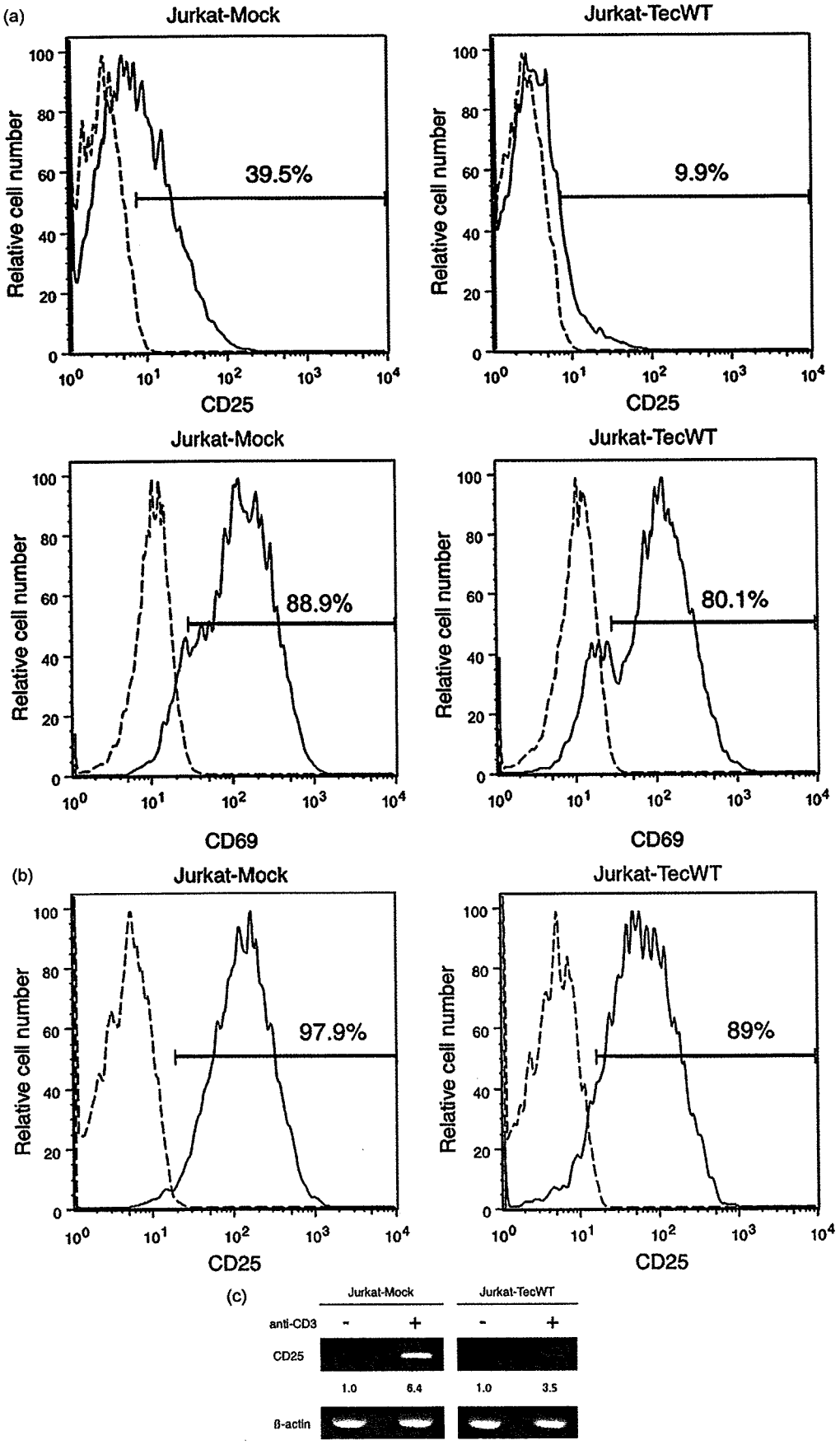

Fig. 2. Expression of Tec inhibits upregulation of CD25 but not that of CD69 induced by TCR cross-linking. (a) Jurkat-Mock cells and Jurkat-TecWT cells after TCR cross-linking were incubated with anti-CD25 (upper panels) and anti-CD69 (lower panels) antibodies. Flow cytometric histograms show the intensity of staining with the indicated antibody (solid line) compared with that of an isotype-matched nonreactive control antibody (broken line). (b) Jurkat-Mock cells and Jurkat-TecWT cells cultured

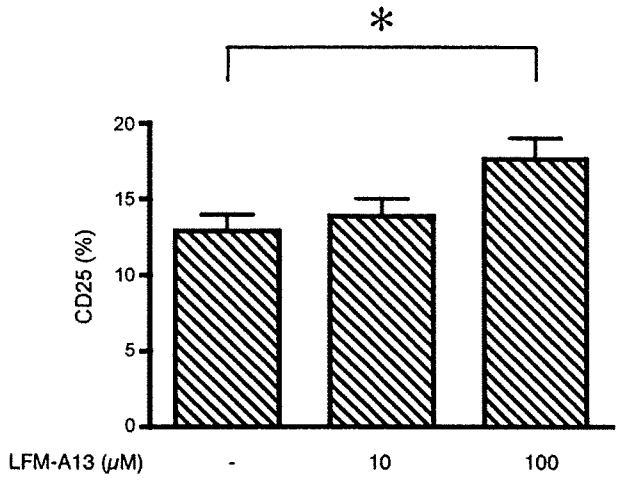

Fig. 3. Tec selective inhibitor LFM-A13 increases the CD25 expression on Jurkat-TecWT cells after TCR cross-linking. Jurkat-TecWT cells were treated with the indicated concentrations of LFM-A13 or DMSO (vehicle) for 1 h. The cells were then stimulated with TCR cross-linking for 24 h. CD25 expression was evaluated by means of flow cytometric analysis. Bars (mean ± SD of quadruplicate tests) represent the percentage of cells expressing CD25. *$P < 0.05$.

of Tec expression on the induction of CD69 caused by TCR cross-linking. Although CD25 expression was markedly suppressed after TCR stimulation in Jurkat-TecWT cells, no apparent difference was observed on the CD69 expression between Jurkat-Mock cells and Jurkat-TecWT cells (Fig. 2a). Thus, Tec expression inhibited CD25 expression after TCR cross-linking, without affecting CD69 induction. The defect in the signal seems to be adjacent to TCR, as Tec expression does not affect the CD25 expression level in Jurkat cells after PMA plus ionomycin activation, which bypasses the early stage signals induced by TCR cross-linking (Fig. 2b).

CD25 gene expression is tightly regulated at the transcriptional level [28,29]. Therefore, we next investigated the expression of CD25 mRNA in Jurkat-derived clones. Using RT-PCR, we examined the effect of TCR cross-linking on CD25 mRNA expression in Jurkat clones. As shown in Fig. 2c, CD25 mRNA expression in Jurkat-Mock cells was increased after 24 h stimulation with TCR cross-linking. In contrast, the increase in CD25 mRNA expression in Jurkat-TecWT cells after TCR cross-linking was markedly suppressed. The densitometric analysis of the relative intensities (means ± S.E.) of three independent experiments showed significant inhibition of the CD25 mRNA expression in Jurkat-TecWT cells after TCR cross-linking ($P < 0.05$) (data not shown). These results imply the importance of Tec PTK on the downregulation of CD25 expression after TCR cross-linking.

To further elucidate the contribution of Tec PTK activity on the results obtained by comparing Jurkat clones with or without Tec, we took advantage of LFM-A13, a compound that preferentially inhibits the enzymatic activity of Tec family PTKs both *in vitro* and *in vivo* [30] in order to investigate Tec's role in the regulation of CD25 expression. We examined LFM-A13's effect on CD25 surface expression in Jurkat-TecWT cells after TCR cross-linking. LFM-A13 dose-dependently increased CD25 expression in Jurkat-TecWT cells after TCR cross-linking (Fig. 3). After 24 h of culture, 17.6 ± 2.8% of cells incubated with 100 μM LFM-A13 expressed CD25, versus 12.9 ± 2.1% of cells in control cultures. CD3 surface expression was not altered when measured after 1 or 24 h incubation of Jurkat-TecWT cells with LFM-A13 (data not shown). Thus, LFM-A13's effect was not due to the modulation of cell-surface CD3 expression. In Jurkat-Mock cells, CD25 surface expression induced by TCR cross-linking was not affected by the presence of LFM-A13 (data not shown).

To corroborate the results obtained using LFM-A13, we established stable transfectants of Jurkat cells expressing a kinase domain-deleted Tec (Jurkat-TecKD) (Fig. 4a). Although rapid and transient tyrosine phosphorylation of Tec was observed after ligation of TCR in Jurkat-TecWT cells, no detectable tyrosine phosphorylation was observed in TecKD protein obtained from Jurkat-TecKD cells throughout the time course examined (Fig. 4b). In Jurkat-TecKD cells, CD25 expression after TCR cross-linking was comparable to that of Jurkat-Mock cells (Fig. 4c and d). These results indicate that Tec PTK activity contributes to the downregulation of CD25 observed in TCR-stimulated Jurkat-TecWT cells.

4. Discussion

Studies of Tec family PTKs have begun to reveal the crucial roles of these kinases in transducing stimuli triggered by immune cell antigen receptors, such as TCR and BCR, regulating lymphoid cell development and activation [31,32]. Targeted disruption of Tec family PTK genes has revealed the unique roles of individual PTKs in lymphocyte signal transduction. In T-cells, Itk and Rlk play important roles in the TCR-mediated signaling pathway, which leads to the phosphorylation and activation of PLC-γ, an essential step in lymphoid cell activation [4,33–35]. Despite evidence suggesting Tec's involvement in TCR and CD28 signaling, Tec's role in T-lymphocyte remains unclear because of the lack of an overt defect in T-lymphocyte function in Tec-deficient mice [14]. Recent findings indicating that Itk and Rlk have nonessential roles in pre-TCR signaling in the thymus [36] may suggest that Tec has a compensatory effect on the lack of these kinases in T-cell development. In the present study, we attempted to address Tec's role in human T-lymphocyte function using Jurkat cells stably transfected with Tec-based constructs. We have demonstrated that Tec PTK activation results in the suppression of TCR-induced CD25 expression, implying that this PTK transmits signals attenuating IL-2 activity in human T-lymphocytes.

IL-2 transmits its effects via a high-affinity IL-2 receptor, which is composed of three transmembrane proteins (α, β, γc subunits) [28,29]. The binding of CD25 (α subunit) to the low-affinity IL-2R (β, γc subunits) increases affinity to IL-2, enhancing the cellular responses to the low concentration of IL-2. A very small population of circulating mononuclear cells expresses CD25 in normal human peripheral blood. After antigen-induced activation, CD25 was strongly expressed in human T-lymphocytes [28,29]. CD25 expression is induced not only by antigen-induced activation, but also by various mitogenic stimulations including cytokines such as IL-1, IL-2, IL-7, IL-12, IL-15, IL-16, TNF-α, TGF-β, and IFN-α [28,29]. There have been extensive studies of how CD25 expression is regulated in response to these stimuli. CD25 expression is believed to be controlled mostly at the stage of transcription regulation. Therefore, the promoter lesions of CD25 have been analyzed in detail, and multiple molecules regulating its transcriptional level have been identified [28,29]. In contrast, relatively little effort has been made to identify the PTK that plays a key role in CD25 expression after T-cell activation. Although a higher degree of CD25 upreg-

with 50 ng/ml of PMA and 1 μM ionomycin were incubated with anti-CD25 antibody. Flow cytometric histograms show the intensity of staining with anti-CD25 antibody (solid line) compared with that of an isotype-matched nonreactive control antibody (broken line). (c) Total RNA was isolated from Jurkat-Mock cells and Jurkat-TecWT cells with or without TCR cross-linking using anti-CD3 antibody. The expression of CD25 mRNA in the cells was analyzed by means of RT-PCR using specific primers as described in Section 2. The expression of β-actin was used as a control. The intensity of the CD25 mRNA band was measured by scanning densitometry and normalized to β-actin. The fold change in CD25 mRNA after TCR cross-linking is shown in comparison with the level in the unstimulated cells as the average of three independent experiments.

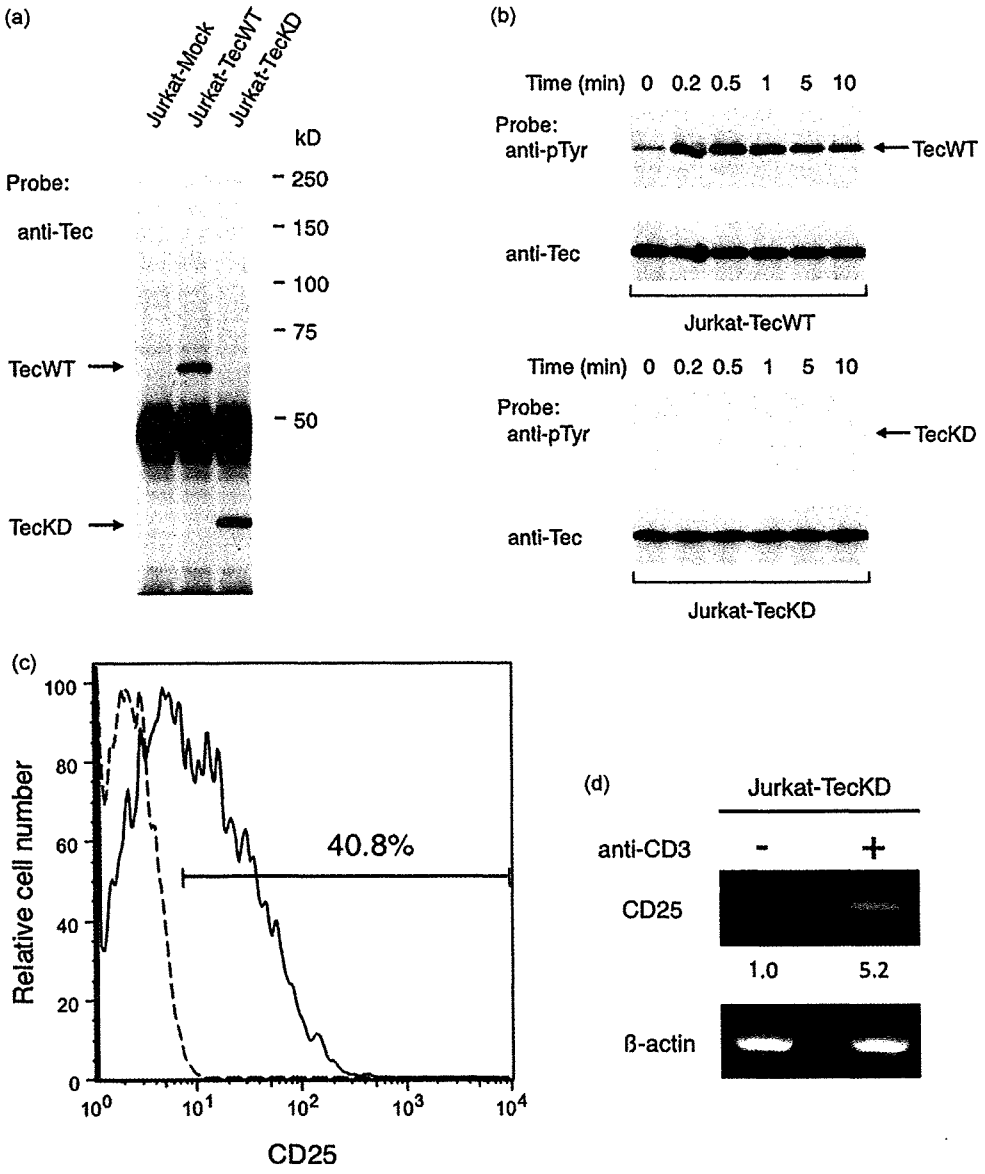

Fig. 4. Expression of Tec that lacks a kinase domain does not alter CD25 expression induced by TCR cross-linking. (a) Cell lysates of Jurkat-Mock cells, Jurkat-TecWT cells, and Jurkat-TecKD cells were subjected to immunoprecipitation with anti-Tec antibody and analyzed by Western blotting using anti-Tec antibody. The positions of TecWT and TecKD and molecular mass markers (in kDa) are indicated. The intense band of approximately 50 kDa corresponds to the Ig heavy chain of the antibody used for immunoprecipitation. (b) Jurkat-TecWT cells and Jurkat-TecKD cells were incubated with anti-CD3 antibody for the times indicated. Cell lysates were prepared and subjected to immunoprecipitation with anti-Tec antibody. The membrane was probed with anti-phosphotyrosine antibody (anti-pTyr; upper panel), then stripped and reprobed with anti-Tec polyclonal antibody (lower panel). The positions of TecWT and TecKD are indicated. (c) Jurkat-TecKD cells after TCR cross-linking were incubated with anti-CD25 antibody (solid line) or nonreactive control antibody (broken line), both conjugated to PE, and the fluorescence intensity was analyzed by flow cytometry. (d) Total RNA was isolated from Jurkat-TecKD cells with or without TCR cross-linking. The expression of CD25 mRNA in the cells was analyzed by means of RT-PCR using specific primers as described in Section 2. The expression of β-actin was used as a control. The intensity of the CD25 mRNA band was measured by scanning densitometry and normalized to β-actin. The fold change in CD25 mRNA after TCR cross-linking is shown in comparison with the level in the unstimulated cells as the average of three independent experiments.

ulation on wild-type T-cells compared with Itk-deficient T-cells was observed after TCR cross-linking, this difference is attributed to the IL-2-induced increase in CD25 expression, which is absent in Itk-deficient T-cells [12]. In our Jurkat system, the effect of Tec expression on IL-2 production was too small to alter CD25 expression level. The inefficient expression of CD25 in Jurkat-TecWT cells upon TCR stimulation seems to be dependent on the Tec PTK activity. Thus, the induction and activation of Tec in TCR-stimulated T-cells may impair the regulation of CD25 expression, resulting in the attenuation of IL-2-induced biological effects accomplished by autocrine and paracrine mechanisms. Prolonged upregulation of Tec relative to that of Itk in primary T-cells following anti-CD3 plus anti-CD28 stimulation [20] may imply that Tec has a negative regulatory role in the latter phase of the TCR-mediated signaling pathway. In human CD4+ T-cells, the Tec expression 24 h after TCR cross-linking was not altered (Susaki and Kitanaka, unpublished observation). Due to the difficulty of maintaining cell viability after sustained cell culture, we failed to examine the Tec expression level within the long time course in TCR-stimulated human CD4+ T-cells.

Previous studies using Jurkat cells have revealed that Tec overexpression enhances IL-2 promoter activity [17,19,26,27]. In our study, IL-2 production did not differ significantly between Jurkat-TecWT cells and Jurkat-Mock cells after anti-CD3 plus anti-CD28 stimulation. There is an apparent discrepancy between our find-

ings and those of previous studies. This may simply reflect clonal variations of individual Jurkat cell lines maintained in individual laboratories. Another possible explanation for the conflicting results is that these studies employed different gene transfer methods. Our experiment was performed using Jurkat cells stably transfected with Tec cDNA, whereas others carried out experiments with Jurkat cells transiently transfected with Tec. In most of the experimental conditions, transient transfection of cDNA results in higher levels of protein expression than those observed in the stable transformants. The differences in Tec expression levels among the experiments may have had diverse cellular effects.

In Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines from XLA patients, Fluckiger et al. [37] showed that the ectopic expression not only of Btk but also of Tec or Itk restored deficient extracellular calcium influx after BCR cross-linking in Btk-deficient cells. We, as well as Fluckiger et al. [13,37], have found that these XLA-derived Btk-deficient cell lines express endogenous Tec. The difference in the expressed amount of protein is considered the cause of the endogenous Tec's inability to compensate for Btk deficiencies. Interestingly, the overexpression of other PTK family members, such as Src (Lyn or Fyn) and Syk, failed to restore Btk-mediated signaling in XLA cells, suggesting the presence of strict kinase-substrate relationships between different PTK families regardless of the expression level [37]. These observations suggest that the expression of excess amounts of proteins may overcome the substrate specificity among individual Tec family PTKs that are present under physiological protein expression levels. This hypothesis is supported by our failure to detect any alteration of CD25 expression after TCR ligation in human primary CD4+ T-cells transiently transfected with Tec cDNA (Susaki and Kitanaka, unpublished observation). To reproduce findings obtained using the Jurkat cell line in human primary T-cells, it may be essential to establish a more sophisticated method to regulate the expression of introduced genes.

Tomlinson et al. [20] quantitated individual Tec family PTK protein levels in murine lymphoid cells. They found substantially lower Tec expression in murine primary T- and B-cells relative to Itk and Btk, respectively. They speculated that the lack of an obvious phenotype in the immune systems of Tec-deficient mice reflected the small amounts of Tec in murine lymphoid cells. Although there is not enough quantitative information on Tec expression relative to other Tec family PTKs in human lymphoid cells, our previous study revealed that EBV-transformed human B-lymphoblastoid cell lines expressed Tec levels similar to those observed in the K562 human erythroleukemia cell line [13]. In this regard, it is clear that human B-lymphoid cells express an amount of Tec comparable to the amounts in the representative human myeloid cell line. Therefore, the inability of a physiological amount of Tec to compensate for Btk in human lymphoid cells may be the reason why defective Btk function results in more severe consequences in humans than in mice [14,38]. Thus, the expression profiles and/or functional redundancies of individual Tec family PTK in lymphoid cells may differ among species. To clarify this issue, the Tec expression level should be compared against Tec's biological significance in human lymphoid cells. It is necessary to assess Tec expression in human lymphoid cells at different stages of development using quantitative methods such as flow cytometric analysis. To date, such analysis has not yet been accomplished because of the lack of a good anti-Tec antibody applicable to flow cytometric analysis (Kitanaka, unpublished observations).

In summary, we have found that the expression and activation of Tec in Jurkat cells inhibited the expression of CD25 induced by TCR cross-linking, suggesting that this PTK plays a negative regulatory role in the TCR-mediated signaling pathway. Our results imply that Tec participates in signaling that suppresses IL-2-mediated signaling by downregulating its receptor expression. Future studies should clarify the role of Tec expression and activation in the IL-2/IL-2 receptor system-mediated human T-lymphocyte activation pathway.

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References

[1] Kane LP, Lin J, Weiss A. Signal transduction by the TCR for antigen. Curr Opin Immunol 2000;12:242–9.
[2] Wang LD, Clark MR. B-cell antigen-receptor signalling in lymphocyte development. Immunology 2003;110:411–20.
[3] Kawakami Y, Yao L, Han W, Kawakami T. Tec family protein-tyrosine kinases and pleckstrin homology domains in mast cells. Immunol Lett 1996;54:113–7.
[4] Miller AT, Berg LJ. New insights into the regulation and functions of Tec family tyrosine kinases in the immune system. Curr Opin Immunol 2002;14:331–40.
[5] Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, et al. Defective B cell development and function in Btk-deficient mice. Immunity 1995;3:283–99.
[6] Kerner JD, Appleby MW, Mohr RN, Chien S, Rawlings DJ, Maliszewski CR, et al. Impaired expansion of mouse B cell progenitors lacking Btk. Immunity 1995;3:301–12.
[7] Vetrie D, Vorechovský I, Sideras P, Holland J, Davies A, Flinter F, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature 1993;361:226–33.
[8] Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell 1993;72:279–90.
[9] Thomas JD, Sideras P, Smith CIE, Vorechovsky I, Chapman V, Paul WE. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science 1993;261:355–8.
[10] Rawlings DJ, Saffran DC, Tsukada S, Largaespada DA, Grimaldi JC, Cohen L, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science 1993;261:358–61.
[11] Liao XC, Littman DR. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity 1995;3:757–69.
[12] Liu KQ, Bunnell SC, Gurniak CB, Berg LJ. T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J Exp Med 1998;187:1721–7.
[13] Kitanaka A, Mano H, Conley ME, Campana D. Expression and activation of the nonreceptor tyrosine kinase Tec in human B cells. Blood 1998;91:940–8.
[14] Ellmeier W, Jung S, Sunshine MJ, Hatam F, Xu Y, Baltimore D, et al. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. J Exp Med 2000;192:1611–24.
[15] Tanaka N, Abe H, Yagita H, Okumura K, Nakamura M, Sugamura K. Itk, a T cell-specific tyrosine kinase, is required for CD2-mediated interleukin-2 promoter activation in the human T cell line Jurkat. Eur J Immunol 1997;27:834–41.
[16] Debnath J, Chamorro M, Czar MJ, Schaeffer EM, Lenardo MJ, Varmus HE, et al. rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. Mol Cell Biol 1999;19:1498–507.
[17] Yang WC, Ghiotto M, Barbarat B, Olive D. The role of Tec protein-tyrosine kinase in T cell signaling. J Biol Chem 1999;274:607–17.
[18] August A, Gibson S, Kawakami Y, Kawakami T, Mills GB, Dupont B. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. Proc Natl Acad Sci USA 1994;91:9347–51.
[19] Yang WC, Ching KA, Tsoukas CD, Berg LJ. Tec kinase signaling in T cells is regulated by phosphatidylinositol 3-kinase and the Tec pleckstrin homology domain. J Immunol 2001;166:387–95.
[20] Tomlinson MG, Kane LP, Su J, Kadlecek TA, Mollenauer MN, Weiss A. Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec. Mol Cell Biol 2004;24:2455–66.
[21] Yamashita Y, Watanabe S, Miyazato A, Ohya K, Ikeda U, Shimada K, et al. Tec and Jak2 kinases cooperate to mediate cytokine-driven activation of c-fos transcription. Blood 1998;91:1496–507.
[22] Kitanaka A, Suzuki T, Ito C, Nishigaki H, Coustan-Smith E, Tanaka T, et al. CD38-mediated signaling events in murine pro-B cells expressing human CD38 with or without its cytoplasmic domain. J Immunol 1999;162:1952–8.
[23] Dobashi H, Sato M, Tanaka T, Tokuda M, Ishida T. Growth hormone restores glucocorticoid-induced T cell suppression. FASEB J 2001;15:1861–3.
[24] Kubota Y, Tanaka T, Kitanaka A, Ohnishi H, Okutani Y, Waki M, et al. Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase. EMBO J 2001;20:5666–77.
[25] Sato K, Mano H, Ariyama T, Inazawa J, Yazaki Y, Hirai H. Molecular cloning and analysis of the human Tec protein-tyrosine kinase. Leukemia 1994;8:1663–72.
[26] Yang WC, Ghiotto M, Castellano R, Collette Y, Auphan N, Nunès JA, et al. Role of Tec kinase in nuclear factor of activated T cells signaling. Int Immunol 2000;12:1547–52.
[27] Yang WC, Olive D. Tec kinase is involved in transcriptional regulation of IL-2 and IL-4 in the CD28 pathway. Eur J Immunol 1999;29:1842–9.

[28] Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell 1993;73:5–8.
[29] Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev 2006;17:349–66.
[30] Mahajan S, Ghosh S, Sudbeck EA, Zheng Y, Downs S, Hupke M, et al. Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl)propenamide]. J Biol Chem 1999;274:9587–99.
[31] Yang WC, Collette Y, Nunès JA, Olive D. Tec kinases: a family with multiple roles in immunity. Immunity 2000;12:373–82.
[32] Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. Tec family kinases in T lymphocyte development and function. Annu Rev Immunol 2005;23:549–600.
[33] Schaeffer EM, Debnath J, Yap G, McVicar D, Liao XC, Littman DR, et al. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science 1999;284:638–41.
[34] Kurosaki T. Regulation of B-cell signal transduction by adaptor proteins. Nat Rev Immunol 2002;2:354–63.
[35] Takesono A, Finkelstein LD, Schwartzberg PL. Beyond calcium: new signaling pathways for Tec family kinases. J Cell Sci 2002;115:3039–48.
[36] Lucas JA, Felices M, Evans JW, Berg LJ. Subtle defects in pre-TCR signaling in the absence of the Tec kinase Itk. J Immunol 2007;179:7561–7.
[37] Fluckiger AC, Li Z, Kato RM, Wahl MI, Ochs HD, Longnecker R, et al. Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. EMBO J 1998;17:1973–85.
[38] Conley ME, Rohrer J, Rapalus L, Boylin EC, Minegishi Y. Defects in early B-cell development: comparing the consequences of abnormalities in pre-BCR signaling in the human and the mouse. Immunol Rev 2000;178:75–90.

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EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset

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A subset of lung cancers harbors a small inversion within chromosome 2p, giving rise to a transforming fusion gene, *EML4-ALK* (*echinoderm microtubule-associated protein-like 4 gene* and the *anaplastic lymphoma kinase gene*), which encodes an activated tyrosine kinase. We have earlier examined the presence of *EML4-ALK* by multiplex reverse transcription-polymerase chain reaction in 363 specimens of lung cancer, identifying 11 adenocarcinoma cases featuring the fusion gene. In this study, we clinicopathologically examined the characteristics of the *EML4-ALK*-positive cases, including the mutation status of *EGFR*, *KRAS*, and *TP53*, and whether they were of thyroid transcription factor-1 (TTF-1) cell lineage or not. Of 11 patients, 4 (36%) with *EML4-ALK*-positive lung adenocarcinomas who were below 50 years of age were affected by these diseases, as compared with 12 of 242 patients (5.0%) with *EML4-ALK*-negative lung adenocarcinomas ($P=0.00038$). *EML4-ALK*-positive lung adenocarcinomas were characterized by less-differentiated grade ($P=0.0082$) and acinar-predominant structure ($P<0.0001$) in histology. Furthermore, the presence of *EML4-ALK* appears to be mutually exclusive for *EGFR* and *KRAS* mutations ($P=0.00018$), whereas coexisting with *TP53* mutations at a low frequency (1/11 = 9.1%), and correlating with non- or light smoking ($P=0.040$), in line with the TTF-1 immunoreactivity. Thus, *EML4-ALK*-positive tumors may form a distinct entity among lung adenocarcinomas, characterized by young onset, acinar histology, no or rare mutations in *EGFR*, *KRAS*, and *TP53*, and a TTF-1 cell lineage, all in agreement with the prevalence in non- or light smokers.
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Lung cancer is one of the leading causes of cancer death in both men and women worldwide. Activating mutations within *epidermal growth factor receptor* (*EGFR*) have been identified in lung cancers,[1,2] and chemical inhibitors for the kinase activity of EGFR have been found effective in treatment of a subset of lung cancer patients harboring such mutations. Interestingly, the tumors for which EGFR inhibitors are effective develop preferentially in populations of Asian ethnicity and non-smokers, and generally lack *KRAS* mutations.[2–4] Furthermore, such tumors have low rates of smoking-specific *TP53* mutations, such as G/C to T/A transversions.[5,6]

Recently, we have found a novel transforming fusion gene joining the *echinoderm microtubule-associated protein-like 4 gene* (*EML4*) and the *anaplastic lymphoma kinase gene* (*ALK*) in four lung adenocarcinomas and one squamous cell carcinoma.[7] The *EML4-ALK* fusion gene is formed by a small inversion within chromosome 2p. The encoded protein contains the N-terminal part of *EML4* and the intracellular catalytic domain of *ALK*. Replacement of the extracellular and transmembrane domain of ALK with a region of EML4 results in constitutive dimerization of the kinase domain and thereby a consequent increase in its catalytic activity.[7]

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More recently, we have identified novel variants for *EML4-ALK* fusion gene with cDNA screening and multiplex reverse transcription-polymerase chain reaction (RT-PCR), capturing all possible in-frame fusions of *EML4* to exon 20 of *ALK*. By carrying out cDNA screening, we identified variant 3,[8] and using multiplex RT-PCR assays, we identified variants 4 and 5[9] after the first identification of variants 1 and 2. In variant 3, exon 6 of *EML4* is joined to exon 20 of *ALK*. cDNA from variant 4 ligates exon 14 of *EML4* to a position within exon 20 of *ALK*, whereas another cDNA from a variant 5 tumor connects exon 2 of *EML4* to exon 20 of *ALK*. All the new three isoforms of *EML4-ALK* have a marked oncogenic activity *in vitro* as well as *in vivo*.[8,9] The variant 3 was also identified by Rikova *et al*[10] and another new variant, in which exon 15 of *EML4* is jointed to a position within exon 20 of *ALK*, was identified by Koivunen *et al*.[11]

Earlier we conducted the first large scale-study to detect *EML4-ALK* (3.4%) in lung adenocarcinomas and found five fusion-positive cases (two variant 1 and three variant 2) in 149 adenocarcinoma samples.[12] At that point in time, only two variants were recognized, and we investigated their clinicopathological characteristics. However, with development of multiplex RT-PCR for detecting all possible in-frame variants, we captured theoretically all *EML4-ALK* variants and found 11 *EML4-ALK*-positive cases among 363 lung cancers.[9] In this study, we examined the clinicopathological and genetic features of the 11 tumors, and found *EML4-ALK* lung cancers to be characterized by a lack of *EGFR* and *KRAS* mutations, a low rate for *TP53* mutations, a thyroid transcription factor-1 (TTF-1)-positive cell lineage, an acinar histology, and young onset.

Materials and methods

Clinical Samples and Pathological Review

The clinical specimens for this study were 11 lung tumors detected in our earlier study, using multiplex RT-PCR and fluorescent *in situ* hybridization.[9] Briefly, samples were obtained from 363 individuals who underwent surgery at the Cancer Institute Hospital (Tokyo, Japan) between May 1997 and February 2004. The 363 lung cancers comprised 253 adenocarcinomas, 7 adenosquamous carcinomas, 72 squamous cell carcinomas, 7 large-cell carcinomas (including 4 large-cell neuroendocrine carcinomas), 2 pleomorphic carcinomas, and 22 small-cell carcinomas. This project was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research, and informed consent was obtained from each study subject. Histological diagnoses were made based on the World Health Organization (WHO) classification.[13] However, with its subdivision of lung adenocarcinomas, more than 80% tumors fell into the mixed subtype category. We therefore additionally used a predominance classification of invasive components, which is mostly based on the WHO classification except for the mixed subtype, such as papillary predominant, acinar predominant, etc. In the predominance classification of invasive components, we diagnose by a component that makes up the predominant portion of invasive lesion even if it is <50%. In addition, we used a differentiation grading that was basically according to the former version of the Japanese Lung Cancer Society criteria,[14] as performed earlier.[15]

Immunohistochemical Analysis

Unstained paraffin-embedded sections were depleted of paraffin with xylene, rehydrated through a graded series of ethanol solutions, and subjected to immunohistochemical staining with a mouse monoclonal antibody (ALK1, 1:20, Dako, Carpinteria, CA, USA). Heat-induced antigen retrieval pretreatment was performed with Target Retrieval Solution pH 9.0 (Dako). Immune complexes were detected with the EnVision+DAB system (Dako) with minor modifications.[16] TTF-1 was also immunostained using a mouse monoclonal antibody (clone 8G7G3/1, 1:100, Dako), as described earlier.[15] Tumors were considered negative if staining was found in <5% of neoplastic cells, partly positive if present in 5–50%, and positive if in more than 50%. The results of immunostaining with TTF-1 were based on nuclear staining of neoplastic cells.

DNA Extraction and Mutation Analysis of *EGFR*, *KRAS*, and *TP53*

Of 253 patients with adenocarcinomas, both *EGFR* and *KRAS* data were available for 68 patients and *EGFR* data alone for further 12 patients, including all the patients with *EML4-ALK*-positve cases.[12,17] DNA extraction and mutation analysis of *EGFR* and *KRAS* were performed as described earlier.[17] Mutation analysis of *TP53* was also performed as described earlier.[18] Genomic DNAs from fresh tumor samples were prepared and exons 4–8 and 10 of the *TP53* gene were analyzed by the PCR – single-strand conformation polymorphism and DNA sequencing. For case #4808, *TP53* mutation analysis was performed using DNA extracted from formalin-fixed paraffin-embedded tissue and a method based on direct sequencing, because no fresh sample was available for this study at the time of the current study.

Results

Histologically, the 253 adenocarcinomas comprised 213 mixed subtypes, 19 acinar, 9 papillary, 4 solid, 1 other, and 7 bronchioloalveolar carcinomas based on the WHO classification. According to the subdivision

of lung adenocarcinomas with the WHO criteria, more than 80% of tumors fell into the mixed subtype category. However, this contains very varied lesions; for example, a tumor comprising solid and acinar adenocarcinoma elements would be expected to have a very different prognosis from one composed of bronchioloalveolar carcinoma, with only a small amount of papillary adenocarcinoma. We therefore additionally used a predominance classification and also paid attention to the minor components. According to the predominance subtypes in adenocarcinomas, 6 of 11 *EML4-ALK*-positive lung cancers (54.5%) were subclassified as acinar adenocarcinomas ($P=0.000044$, Table 1), as compared with 4 based on the WHO classification (36.4%, $P=0.0018$, Table 2). In other words, 6 of 34 (18%) acinar-predominant adenocarcinomas, as well as 4 of 19 (21%) acinar adenocarcinomas based on the WHO classification, have *EML4-ALK* fusion. In adenocarcinomas not subclassified as acinar adenocarcinomas based on the WHO criteria, acinar structures were also frequently observed (Figure 1). In differentiation grading, *EML4-ALK* lung cancers were less differentiated (Table 3, $P=0.0082$, 10/11). In addition, they often featured mucin production, as proven by Alcian Blue staining (Figure 1b) with acinar structures. As for the cell types originally proposed by Hashimoto *et al*,[18] the columnar cell type was characteristic of *EML4-ALK* lung cancers (Figure 1).

EML4-ALK-positive lung adenocarcinomas were also found to be significantly smaller than other lung adenocarcinomas (Table 3, $P=0.031$), in line with the lack of bronchioloalveolar components.

Patients with *EML4-ALK* lung cancers tended to be young (56 *vs* 64 years for other tumor types, $P=0.0062$). We defined early-onset lung cancers by classifying patients as below or above 50 years of age. In 253 patients with lung adenocarcinomas, 16 patients were affected by the disease at below 50 years of age. Four of 11 patients (36%) with *EML4-ALK*-positive lung cancers were affected by these diseases at below 50 years of age, as compared with 12 of 242 patients (5.0%) with *EML4-ALK*-negative lung cancers ($P=0.00038$).

It is true that the *EML4-ALK* translocation was first found in a smoker's lung cancer, but overall there was no significant difference between smokers' and non-smokers' tumors with regard to *EML4-ALK* fusion ($P=0.37$). Smoking habits can be classified into the following two grades of cumulative smoking based on the smoking index (SI), a product of the number of cigarettes per day, and the duration in years: (a) non-smokers and light smokers (SI <400); and (b) heavy smokers (SI $=400$ or above). Ten of the 11 (91%) *EML4-ALK*-positive lung cancer patients had SI <400, as compared with 109 of 241 (45%) *EML4-ALK*-negative lung cancer patients ($P=0.040$). In this study, *EML4-ALK* fusion was detected in only one heavy smoker's lung cancer (1/11 = 9.1%).

EGFR and *KRAS* mutations are mutually exclusive in usual cases while being two major oncogenic drivers of lung adenocarcinoma development. *EML4-ALK*-positive lung cancers lacked both *EGFR* and *KRAS* mutations ($P=0.00018$), and only 1 of 11 (9.1%) harbored a *TP53* mutation (Table 2). It is noteworthy that the single mutation was a G/A transition (GTG→ATG) (V→M) in codon 273, exon 8. This is known to be a spontaneous rather than a tobacco-carcinogen-induced mutation, usually seen in non-smokers' lung cancers.

In the *EML4-ALK*-positive 11 cases, immunohistochemical assays with the anti-ALK antibody ALK1 consistently showed definite staining. As illustrated

Table 1 *EML4-ALK* fusion and histology of adenocarcinomas classified by predominant subtypes

Histology	*Total (363)*	*EML4-ALK(+)*	*EML4-ALK(−)*
Adenocarcinoma	253	11 (4.3%)	242 (96%)
Subtype by predominance classification			
Invasive carcinoma			
Papillary adenocarcinoma	206	5 (5/11 = 45%)	201 (201/242 = 83%)
Acinar adenocarcinoma	34	6 (6/11 = 55%)[a]	28 (28/242 = 12%)
Solid adenocarcinoma with mucin	5	0 (0%)	5 (5/242 = 2.1%)
Others	1	0 (0%)	1 (1/242 = 0.41%)
Noninvasive carcinoma			
Bronchioloalveolar carcinoma	7	0 (0%)	7 (7/242 = 2.9%)
Adenosquamous carcinoma	7	0 (0%)	7 (100%)
Squamous cell carcinoma	72	0 (0%)	72 (100%)
Large-cell carcinoma	7	0 (0%)	7 (100%)
Large-cell neuroendocrine carcinoma	4	0 (0%)	4 (100%)
Pleomorphic carcinoma	2	0 (0%)	2 (100%)
Small-cell carcinoma	22	0 (0%)	22 (100%)

Acinar-predominant adenocarcinomas *vs* the other adenocarcinomas.
[a]Fisher's exact test, $P<0.0001$ ($P=0.000044$).

Table 2 *EML4-ALK* variants detected by multiplex RT-PCR analysis and clinicopathologic and genetic data

V	*Tumor ID*	*Sex*	*Age (years)*	*p-Stage*	*LKD*	*Survival (days)*	*Size (mm)*	*SI*	*WHO subtype*	*Pred-subtype*	*diff.*	*Histological components*	*ALK IHC*	*TTF-1 IHC*	*KRAS mut*	*EGFR mut*	*TP53 mut*
1	#9034	F	43	IA	Alive	1714	12	10	Acinar	Acinar	Por		+	P+	–	–	–
1	#4808	F	58	IA	Alive	2246	27	0	Mixed	Pap	Well	Papillary adenocarcinoma with BAC	+	+	–	–	–
1	#9968	F	66	IIIA	Alive	1036	33	0	Mixed	Pap	Mod	Papillary adenocarcinoma with BAC	+	+	–	–	–
2	#4180	M	43	IV	Dead	527	23	160	Acinar	Acinar	Por		+	P+	–	–	–
2	#3121	M	64	IIIB	Alive	2673	18	220	Acinar	Acinar	Por		+	+	–	–	–
2	#2374	F	66	IA	Alive	1632	28	0	Acinar	Acinar	Mod		+	P+	–	–	–
3	#7969	M	47	IIIA	Alive	1328	17	540	Mixed	Pap	Por	Papillary adenocarcinoma with BAC	+	+	–	–	–
3	#2075	F	62	IIA	Dead	522	19	0	Mixed	Acinar	Mod	Acinar+papillary+solid adenocarcinoma	+	P+	–	–	+[a]
3	#9616	M	73	IA	Alive	1465	13	300	Pap	Pap	Mod		+	+	–	–	–
4	#8398	F	52	IA	Alive	1834	24	0	Mixed	Acinar	Mod	Acinar+papillary adenocarcinoma with BAC	+	P+	–	–	–
5	#8993	M	44	IA	Alive	1730	15	0	Pap	Pap	Mod		+	+	–	–	–

acinar, acinar adenocarcinoma; BAC, bronchioloalveolar carcinoma; diff., differentiation; *EGFR* mut, *EGFR* mutation; IHC, immunohistochemistry; *KRAS* mut, *KRAS* mutation; LKD, lung cancer death; mixed, adenocarcinoma with mixed subtype; P+, Partly +; pap, papillary adenocarcinoma; Pred-subtype, predominance subtype; p-Stage, pathological-Stage; SI, smoking index; *TP53* mut, *TP53* mutation; V, *EML4-ALK* variant.

[a]G/A transition (GTG→ATG) (V→M) in codon 273, exon 8.

in Figure 2a, the cytoplasm of tumor cells harboring the variant 2 (tumor ID #2374) was strongly stained with fine granular accentuation. Although we performed the immunostaining of 88 *EML4-ALK*-negative lung adenocarcinoma specimens, we could discriminate all the fusion-negative specimens from the fusion-positive ones by our refined immunohistochemical condition.[16] All the 11 cases were also positive (six cases) or partly positive (five cases) for TTF-1 immunohistochemistry (Figure 2b), a characteristic of alveolar type II cells, which is featured in non-smokers' cancers.

Discussion

With the present large-scale screen for *EML4-ALK* fusion in lung cancers, we detected 11 adenocarcinomas with an *EML4-ALK* translocation. In the current study, we revealed a relatively young occurrence and a typically less-differentiated acinar histology, which might be used as clinical pointers. It is of great interest that *EML4-ALK* translocation is associated with young onset, whereas *EGFR* mutation status is not associated with the patient's age at diagnosis.[4]

Currently, anaplastic large-cell lymphomas (ALCLs) are divided into three entities, namely primary systemic *ALK* (+) ALCL, primary systemic *ALK* (−) ALCL, and primary cutaneous ALCL. The ALK expression is caused most commonly t(2;5) by chromosomal translocations, and *ALK* (+) ALCL predominantly affects young male patients and, if treated with chemotherapy, has a favorable prognosis.[19] This might similarly be applicable to *EML4-ALK* lung cancers. Presently, the primary treatment for lung cancers is surgery where possible. However, for *EML4-ALK* lung cancers, chemotherapy or a targeted therapy with an ALK inhibitor might be effective, given that *EML4-ALK*-dependent cells are known to undergo apoptosis in response.[7–9,11]

Here, *EML4-ALK* fusion was found to be mutually exclusive for *EGFR* or *KRAS* mutations, thus pointing to a distinct genetic subtype of lung adenocarcinoma. The possibility of a genetic classification of lung adenocarcinomas based on oncogene mutations has already been considered. In fact, one-third to nearly half of Japanese adenocarcinomas harbor *EGFR* mutations,[4,20] about 10% have *KRAS* mutations[21–23] and about 4% have *EML4-ALK* translocations, implying that two-thirds of adenocarcinomas feature mutually exclusive oncogenic mutations. The mutation rate of *TP53* (1/11 = 9.1%) was also low compared with that of lung adenocarcinomas in general (41%),[18] and the single mutation found was G to A transition, which was not related to smoking. Strong *in vitro* as well as *in vivo* oncogenic activity of *EML4-ALK* fusion products[8,9] might account for the lack of other genetic alterations.

All 11 *EML4-ALK* lung cancers were positive or partly positive for TTF-1 immunostaining. TTF-1

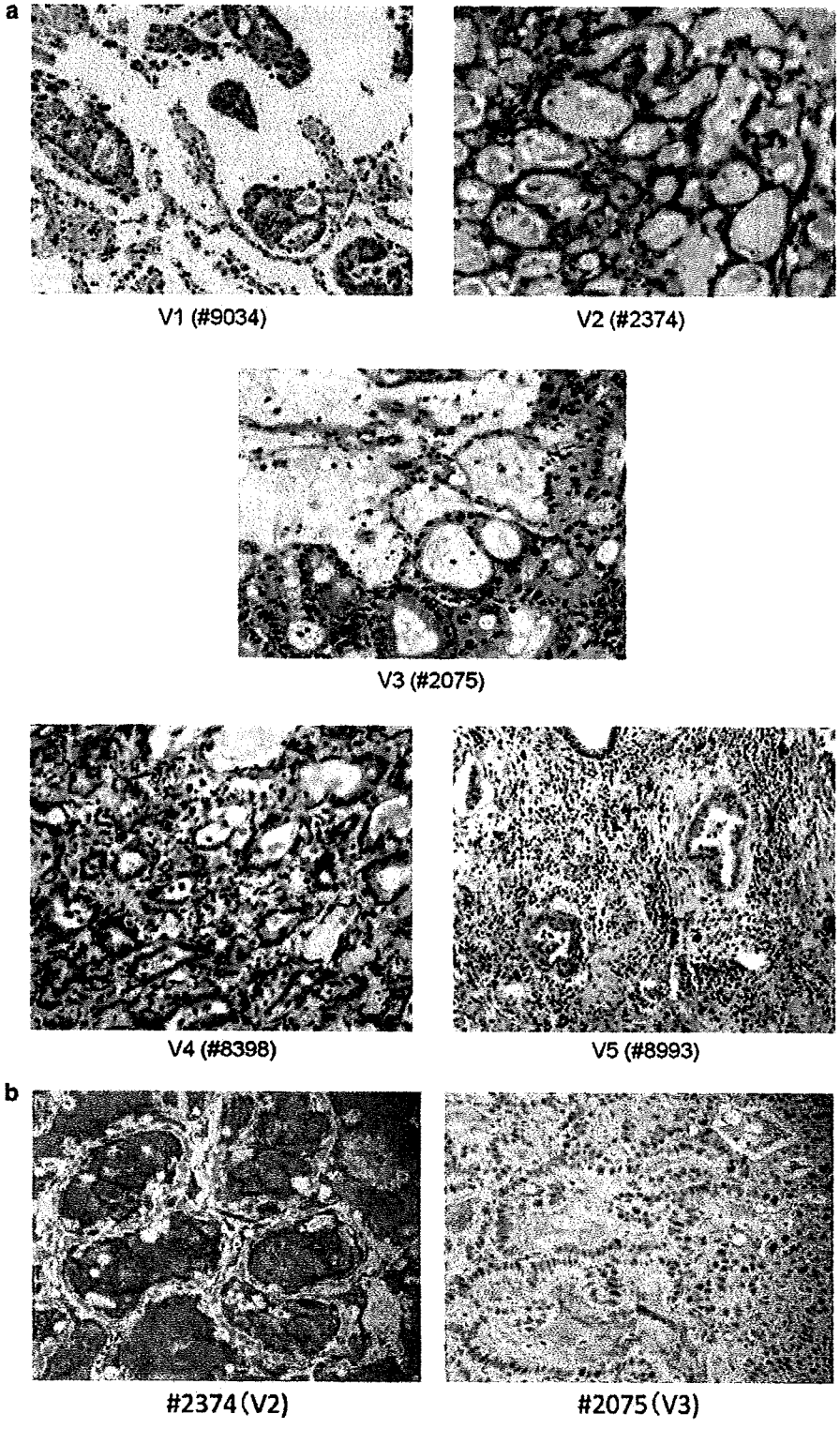

Figure 1 (**a**) Representative appearance of all the five variants of the *EML4-ALK* lung cancers (hematoxylin and eosin staining). Histologically, acinar structures with some mucin production are characteristic. (**b**) Alcian Blue staining shows the abundant mucin production.

Table 3 Clinicopathologic and genetic comparisons between *EML4-ALK* fusion-positive and -negative lung adenocarcinomas

Variables category	*No. of samples (%)*	*EML4-ALK fusion*		P-*value*
		(+) (n = *11)*	*(−)* (n = *242)*	
Age (years; mean ± s.d.)	253	56 ± 11	64 ± 9	0.0062[a] 0.00038[b]
<50	16 (6.3)	4 (36)	12 (5.0)	
≤50	237 (94)	7 (64)	230 (95)	
Sex				0.61[b]
Males	134 (53)	5 (45)	129 (53)	
Females	119 (47)	6 (55)	113 (47)	
Smoking habit				0.37[b]
Never smokers	105 (41)	6 (55)	99 (41)	
Ever smokers	147 (59)	5 (45)	142 (59)	
Heavy smokers or not				0.040[b]
Heavy smokers	110 (44)	1 (9.1)	109 (45)	
Not heavy smokers	142 (56)	10 (91)	132 (55)	
Tumor size (mm)		20.8 ± 6.7	31.8 ± 16.7	0.031[a] 0.039[b]
<30	142 (56)	10 (80)	132 (55)	
≥30	111 (44)	1 (20)	110 (45)	
Differentiation grading				0.0082[b]
Well	98 (39)	1 (9.1)	97 (40)	
Less	155 (39)	10 (91)	145 (60)	
EGFR mutation				0.00085[b]
Mutation(+)	41 (52)	0 (0)	41 (60)	
Mutation(−)	39 (48)	11 (100)	28 (40)	
KRAS mutation				0.49[b]
Mutation(+)	7 (10)	0 (0)	7 (12)	
Mutation(−)	61 (90)	11 (100)	50 (88)	
EGFR or *KRAS mutation*				0.00018[b]
Mutation(+)	38 (59)	0 (0)	38 (67)	
Mutation(−)	30 (41)	11 (100)	19 (33)	
p-Stage				0.89[b]
I	143 (57)	6 (55)	137 (57)	
II–IV	110 (43)	5 (45)	105 (43)	

Percentages may not total 100, because of rounding.
We have no smoking history of one patient.
Smoking habits were classified into the following two grades based on the smoking index: (a) non-smokers and light smokers (smoking index <400); and (b) heavy smokers (smoking index = 400 or above).
[a]Student's *t*-test.
[b]Fisher's exact test.

has a decisive role as a master regulatory transcription factor in lung development and in maintenance of the functions of terminal respiratory unit (TRU) cells.[24] The TTF-1 positivity of *EML4-ALK* lung cancers suggests that this subtype might have a TRU histogenesis. TRU-type lung cancers with a TTF-1-positive cell lineage often occur in non- or light smokers, which frequently harbor *EGFR* mutations (61%) and have less-frequent *TP53* mutations (36%) as compared with non-TRU-types (57%).[22] *EML4-ALK* lung cancers also occur in non- or light smokers but do not harbor *EGFR* mutations. The low frequency of *TP53* mutations (9.1%) not only indicates strong oncogenic activity for *EML4-ALK* fusion products but also suggests an independence from smoking, because smoker's adenocarcinomas very frequently harbor *TP53* mutations.[18]

Histologically, less-differentiated acinar structures composed of columnar cells appear characteristic of *EML4-ALK* lung adenocarcinomas. Generally, the columnar cell type is also found in smoker's lung adenocarcinomas, whereas the hobnail cell type, characterized by cytoplasmic protrusions and with a tadpole shape, is often observed in non-smoker's lung adenocarcinomas.[18] Although *EML4-ALK* lung cancers are TTF-1-positive, their histology is

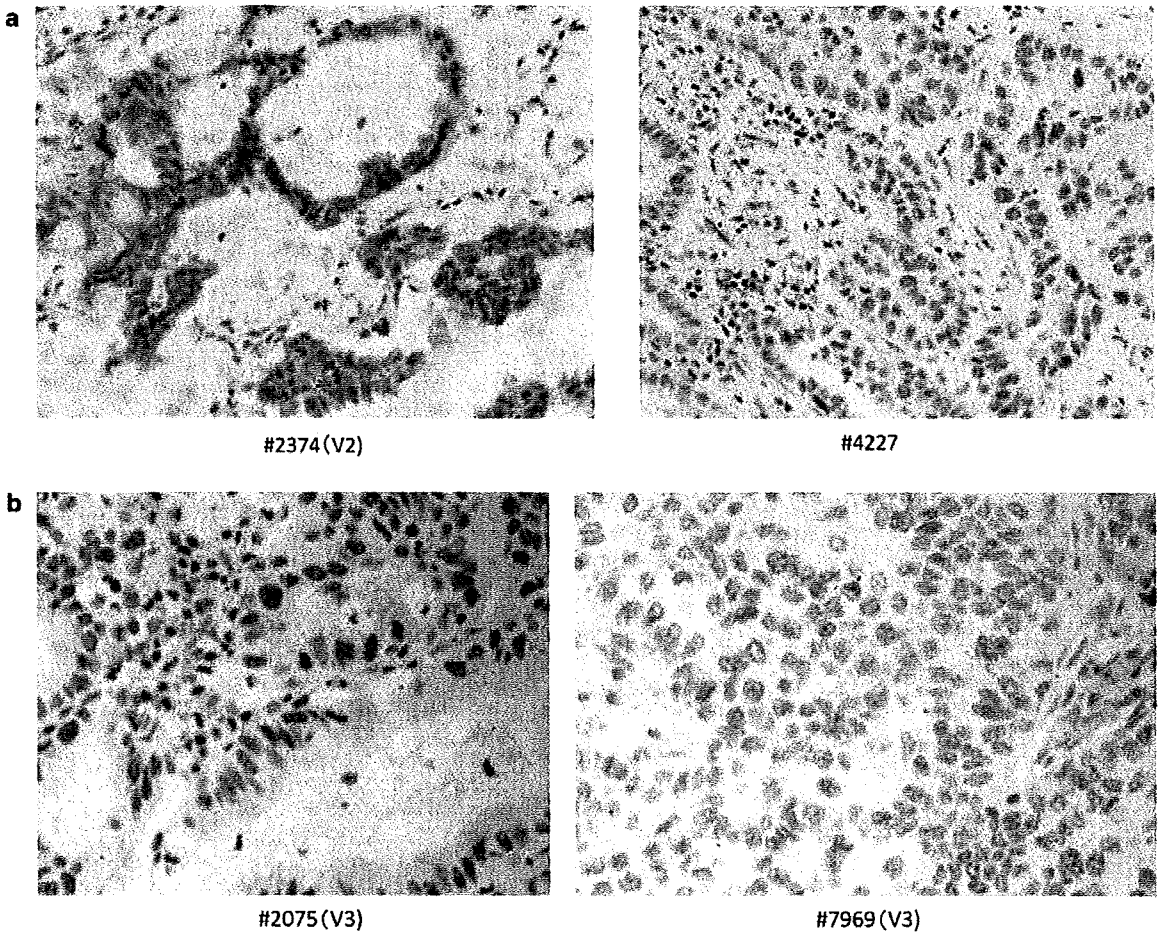

Figure 2 (**a**) Immunohistochemical analysis with a monoclonal anti-ALK antibody of lung adenocarcinoma specimens with (tumor ID #2374) and without (tumor ID #4227) *EML4-ALK* fusion. Note the diffuse staining in the cytoplasm with fine granular accentuation apparent for the *EML4-ALK*-positive tumor. (**b**) Immunohistochemical analysis of lung adenocarcinoma specimens with *EML4-ALK* fusion using a monoclonal anti-TTF-1 antibody. The *EML4-ALK*-positive tumors are partly (ID #2075) or diffusely (ID #7969) positive.

similar to lung cancers developing in smokers, which is interesting in the view of histology–etiology relationships.

Presently, lung adenocarcinomas may be genetically divided into *EGFR*-mutated, *KRAS*-mutated, and *EML4-ALK*-related subtypes. We here elucidated the clinicopathologic, histologic, and genetic characteristics of *EML4-ALK* lung cancers, bearing etiologic implications in mind. Just as some *EGFR*-mutated lung cancers can be successfully treated with EGFR inhibitors, *EML4-ALK* lung cancers may respond to a specific inhibitor treatment, allowing a good prognosis.

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Conflict of interest

K Takeuchi is a consultant providing advisory services to Dako for their antibodies.

References

1 Lynch TJ, Bell DW, Sordella R, *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–2139.

2 Paez JG, Janne PA, Lee JC, *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–1500.
3 Pao W, Miller V, Zakowski M, *et al.* EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004;101:13306–13311.
4 Shigematsu H, Lin L, Takahashi T, *et al.* Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–346.
5 Pfeifer GP, Denissenko MF, Olivier M, *et al.* Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21:7435–7451.
6 Suzuki H, Takahashi T, Kuroishi T, *et al.* p53 mutations in non-small cell lung cancer in Japan: association between mutations and smoking. Cancer Res 1992;52:734–736.
7 Soda M, Choi YL, Enomoto M, *et al.* Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561–566.
8 Choi YL, Takeuchi K, Soda M, *et al.* Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 2008;68:4971–4976.
9 Takeuchi K, Choi YL, Soda M, *et al.* Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–6624.
10 Rikova K, Guo A, Zeng Q, *et al.* Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 2007;131:1190–1203.
11 Koivunen JP, Mermel C, Zejnullahu K, *et al.* EML4–ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008;14:4275–4283.
12 Inamura K, Takeuchi K, Togashi Y, *et al.* EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol 2008;3:13–17.
13 Travis WD, Brambilla E, Muller-Hermelink HK, *et al.* World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Lung, Pleural, Thymus and Heart. Springer: Berlin, 2004.
14 Japan Lung Cancer Society. General Rules for Clinical and Pathologic Record of Lung Cancer [in Japanese], 5th edn. Kanahara: Tokyo, 1999.
15 Inamura K, Satoh Y, Okumura S, *et al.* Pulmonary adenocarcinomas with enteric differentiation: histologic and immunohistochemical characteristics compared with metastatic colorectal cancers and usual pulmonary adenocarcinomas. Am J Surg Pathol 2005;29:660–665.
16 Takeuchi K, Choi YL, Togashi Y, *et al.* KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 2009 (in press).
17 Inamura K, Togashi Y, Nomura K, *et al.* Up-regulation of PTEN at the transcriptional level is an adverse prognostic factor in female lung adenocarcinomas. Lung Cancer 2007;57:201–206.
18 Hashimoto T, Tokuchi Y, Hayashi M, *et al.* Different subtypes of human lung adenocarcinoma caused by different etiological factors. Evidence from p53 mutational spectra. Am J Pathol 2000;157: 2133–2141.
19 Stein H, Foss HD, Durkop H, *et al.* CD30(+) anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. Blood 2000;96:3681–3695.
20 Kosaka T, Yatabe Y, Endoh H, *et al.* Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004;64:8919–8923.
21 Noda N, Matsuzoe D, Konno T, *et al.* K-ras gene mutations in non-small cell lung cancer in Japanese. Oncol Rep 2001;8:889–892.
22 Yatabe Y, Kosaka T, Takahashi T, *et al.* EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. Am J Surg Pathol 2005;29:633–639.
23 Shibata T, Hanada S, Kokubu A, *et al.* Gene expression profiling of epidermal growth factor receptor/KRAS pathway activation in lung adenocarcinoma. Cancer Sci 2007;98:985–991.
24 Tanaka H, Yanagisawa K, Shinjo K, *et al.* Lineage-specific dependency of lung adenocarcinomas on the lung development regulator TTF-1. Cancer Res 2007;67:6007–6011.