

Table 1
Laboratory features at the time of diagnosis in FAB-RA patients who could be classified according to the WHO classification 2008.

	RCUD	MDS-U	RCMD	5q- synd
(A) Japanese patients, n = 102				
Patients = n (%)	46(45)	28(29)	25(25)	3(3)
Gender (male/female)	28/18	12/16	11/14	2/1
Age (years)	57(16-86)	51(15-82)	63(16-88)	60(59-74)
Neutrophils ($\times 10^9/L$)	1.89 (0.44-4.69)	1.10 (0.26-1.77)	1.28 (0.05-10.24)	0.73 (0.50-2.54)
Hemoglobin (g/dL)	10.2 (3.0-14.3)	6.9 (4.2-9.1)	8.2 (2.9-14.0)	6.3 (4.6-10.8)
Platelets ($\times 10^9/L$)	41 (4-246)	29 (7-98)	50 (13-390)	207 (134-212)
Abnormal karyotype = n (%)	12(26)	6(21)	9(36)	3(100)
Hypoplastic bone marrow = n (%)	3(7)	3(11)	0(0)	0(0)
(B) German patients, n = 193				
Patients = n (%)	37(19)	6(3)	111(58)	39(20)
Gender (male/female)	20/17	1/5	80/31	14/25
Age (years)	62(20-80)	56(19-59)	63(15-86)	62(32-78)
Neutrophils ($\times 10^9/L$)	1.92 (0.36-8.72)	1.41 (0.48-1.50)	1.60 (0.21-19.40)	1.95 (0.61-6.78)
Hemoglobin (g/dL)	11.0 (5.2-15.4)	9.4 (5.5-9.8)	9.2 (5.1-16.9)	8.7 (3.0-12.2)
Platelets ($\times 10^9/L$)	128(2-840)	33(10-90)	102(9-999)	250(28-1540)
Abnormal karyotype = n (%)	12(32)	3(50)	47(42)	39(100)
Hypoplastic bone marrow = n (%)	3(8)	2(33)	13(12)	5(13)
Japan vs Germany				
(C) Comparison between Japanese and German patients				
(1) RCUD patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.532$			
Age (years)	$p = 0.150$			
Neutrophils ($\times 10^9/L$)	$p = 0.466$			
Hemoglobin (g/dL)	$p = 0.087$			
Platelets ($\times 10^9/L$)	$p < 0.001$			
Abnormal karyotype (%)	$p = 0.526$			
Hypoplastic bone marrow (%)	$p = 0.782$			
(2) MDS-U patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.239$			
Age (years)	$p = 0.557$			
Neutrophils ($\times 10^9/L$)	$p = 0.821$			
Hemoglobin (g/dL)	$p = 0.036$			
Platelets ($\times 10^9/L$)	$p = 0.752$			
Abnormal karyotype (%)	$p = 0.150$			
Hypoplastic bone marrow (%)	$p = 0.156$			
(3) RCMD patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.007$			
Age (years)	$p = 0.401$			
Neutrophils ($\times 10^9/L$)	$p = 0.494$			
Hemoglobin (g/dL)	$p = 0.016$			
Platelets ($\times 10^9/L$)	$p = 0.030$			
Abnormal karyotype (%)	$p = 0.561$			
Hypoplastic bone marrow (%)	$p = 0.072$			
(4) 5q- synd patients				
Frequency	$p < 0.001$			
Gender (male/female)	$p = 0.290$			
Age (years)	$p = 0.920$			
Neutrophils ($\times 10^9/L$)	$p = 0.144$			
Hemoglobin (g/dL)	$p = 0.370$			
Platelets ($\times 10^9/L$)	$p = 0.188$			
Abnormal karyotype (%)	N/A			
Hypoplastic bone marrow (%)	$p = 0.509$			

Values for presentation characteristics are given as median and range where applicable. N/A, not applicable; RCUD, refractory cytopenia with unilineage dysplasia; MDS-U, MDS-unclassified; RCMD, refractory cytopenia with multilineage dysplasia; 5q- synd, MDS with isolated del(5q).

and MDS-U subtypes were not significantly different between the two countries. The frequency of male patients in Japanese RCMD subgroup was significantly lower than that in German RCMD subtype. Japanese patients had significantly lower platelet counts than German patients in both the RCUD and RCMD subtypes. Japanese MDS-U (RCUD/pancytopenia type) and RCMD patients showed significantly lower Hb concentrations than German MDS-U (RCUD/pancytopenia type) and RCMD patients. Japanese RCUD patients showed a tendency towards lower Hb concentrations than German RCUD patients. The ANC did not

differ significantly between the two countries in RCUD, MDS-U (RCUD/pancytopenia type), and RCMD patients (Table 1). The frequency of cytogenetic abnormalities in the Japanese FAB-RA patients was significantly lower than in German patients ($p < 0.001$) (Tables 1 and 2). The frequencies of cytogenetic abnormalities in the RCUD, MDS-U (RCUD/pancytopenia type), and RCMD subtypes were not significantly different between the two countries (RCUD, $p = 0.526$; RCMD, $p = 0.561$; MDS-U (RCUD/pancytopenia type), $p = 0.150$). The frequency of isolated del(5q) in Japanese FAB-RA patients was significantly lower than in German patients

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Table 2
Cytogenetic findings at the time of diagnosis in FAB-RA patients who could be classified according to the WHO classification 2008.

	RCUD	MDS-U	RCMD	5q- synd	Total
(A) Japanese patients, n = 102					
Patients = n	46	28	25	3	102
Good	37 (80.4%)	23 (82.1%)	16 (64.0%)	3 (100%)	79 (77.5%)
Normal	34 (73.9%)	22 (78.6%)	16 (64.0%)	0 (0%)	70 (68.6%)
-Y	0	1	0	0	1
del(5q)	0	0	0	3	3
del(20q)	3	0	0	0	3
Intermediate	8 (17.4%)	3 (10.7%)	4 (16.0%)	0	15 (14.7%)
Poor	1 (0.2%)	2 (7.2%)	5 (20.0%)	0	8 (7.8%)
Complex (≥ 3 abnormalities)	0	1	4	0	5
Chromosome 7 anomalies	1	1	1	0	3
(B) German patients, n = 193					
Patients = n	37	6	111	39	193
Good	27 (73.0%)	3 (50.0%)	72 (64.9%)	39 (100%)	141 (73.1%)
Normal	25 (67.6%)	3 (50.0%)	64 (57.7%)	0 (0%)	92 (47.7%)
-Y	2	0	2	0	4
del(5q)	0	0	0	39	39
del(20q)	0	0	6	0	6
Intermediate	4 (10.8%)	2 (33.3%)	23 (20.7%)	0	29 (15.0%)
Poor	6 (16.2%)	1 (16.7%)	16 (14.4%)	0	23 (11.9%)
Complex (≥ 3 abnormalities)	5	0	9	0	14
Chromosome 7 anomalies	1	1	7	0	9

Good indicates normal, -Y, del(5q), del(20q); poor, complex (≥ 3 abnormalities) or chromosome 7 anomalies; intermediate, other abnormalities not listed in good and poor subgroups.

($p < 0.001$) (Table 2). The most frequent cytogenetic aberration in the intermediate cytogenetic risk according to the IPSS publication was trisomy 8 (4 German RCMD cases, 3 Japanese RCUD cases, 1 Japanese MDS-U case). The frequencies of hypoplastic BM were not significantly different between the two countries

in the RCUD and MDS-U (RCUD/pancytopenia type) subtypes. In the RCMD subtype, there were no Japanese patients presenting with findings concordant with hypoplastic BM. However, the frequency of German RCMD patients with hypoplastic BM was 12% (Table 1).

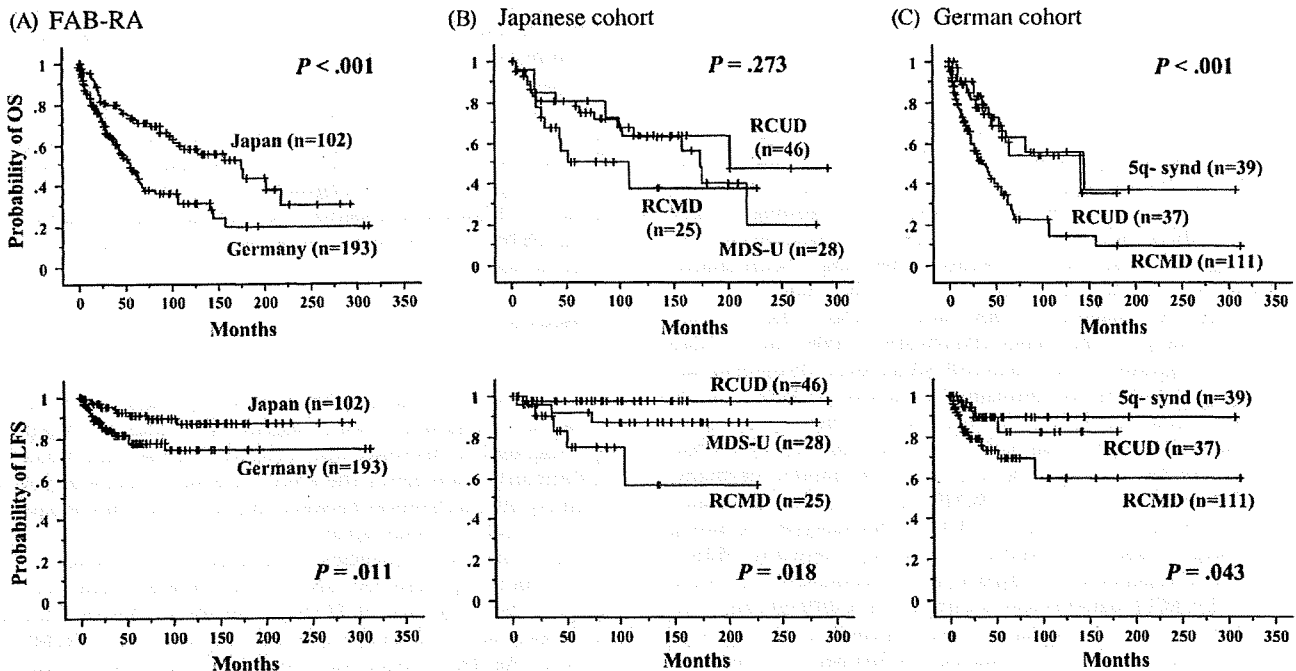


Fig. 1. Cumulative overall survival and leukemia free survival of FAB-RA patients who could be classified according to the WHO classification 2008. (Top) Overall survival (OS). (Bottom) Leukemia free survival (LFS). (A) In FAB-RA patients who could be classified according to the WHO classification 2008, Japanese patients had a more favorable OS than German patients ($p < 0.001$). Japanese patients had a more favorable LFS than German patients ($p = 0.011$). (B) In Japanese FAB-RA patients who could be classified according to the WHO classification 2008, RCMD patients showed the least favorable OS and LFS compared with the other subtypes excluding a rare subtype (5q- syndrome subtype). RCUD patients showed more favorable OS and LFS than RCMD patients (OS, $p = 0.128$; LFS, $p = 0.004$). MDS-U (RCUD/pancytopenia type) patients tended to show more favorable OS and LFS than RCMD patients (OS, $p = 0.218$; LFS, $p = 0.137$). (C) In German FAB-RA patients who could be classified according to the WHO classification 2008, RCMD patients showed the least favorable OS and LFS compared with the other subtypes excluding a rare subtype (MDS-U (RCUD/pancytopenia type) subtype). RCUD patients showed more favorable OS and LFS than RCMD patients (OS, $p = 0.003$; LFS, $p = 0.075$). 5q- syndrome patients showed more favorable OS and LFS than RCMD patients (OS, $p = 0.002$; LFS, $p = 0.043$).

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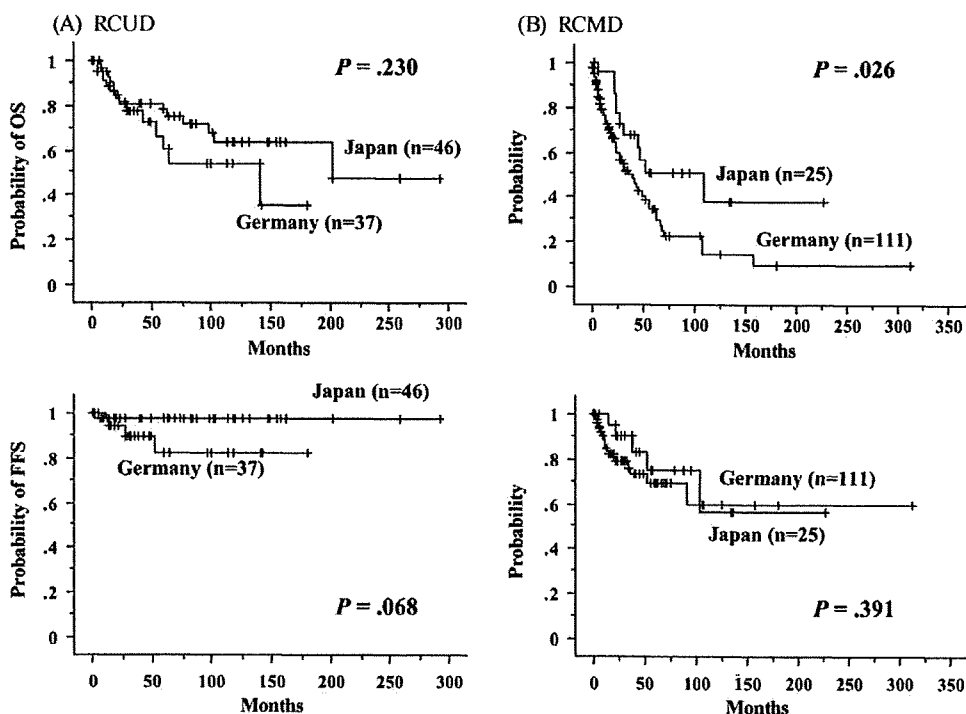


Fig. 2. Comparison of cumulative overall survival and leukemia free survival of RCUD and RCMD between Japanese and German patients. (Top) Overall survival (OS). (Bottom) Leukemia free survival (LFS). (A) The OS of RCUD patients was not significantly different between the two countries ($p=0.230$). Japanese RCUD patients tended to show a more favorable LFS than German RCUD patients ($p=0.068$). (B) Japanese RCMD patients showed a more favorable OS than German RCMD patients ($p=0.026$). The LFS of RCMD patients was not significantly different between the two countries ($p=0.391$).

3.3. Prognosis

Follow-up periods ranged from 1 to 292 months (median, 78 months) in Japanese FAB-RA patients who could be classified according to the WHO classification 2008. Follow-up periods in German patients ranged from 0 to 313 months (median, 23 months). During the follow-up period, 9 Japanese patients and 27 German patients progressed to acute myeloid leukemia (AML). Forty Japanese patients (9 AML, 15 infection, 7 bleeding, 1 heart failure, 2 others (non-hematological causes), 6 unknown) and 81 German patients (24 AML, 16 infection, 7 bleeding, 2 heart failure, 5 others (non-hematological cause), 27 unknown) died.

For the OS, Japanese FAB-RA patients who could be classified according to the WHO classification 2008 had a more favorable prognosis than German FAB-RA patients (OS median survival: Japan, 117 months; Germany, 55 months; $p<0.001$). In LFS, Japanese FAB-RA patients who could be classified according to the WHO classification 2008 had a more favorable prognosis than German FAB-RA patients (10% LFS: Japan, 74 months; Germany, 14 months; $p=0.011$) (Fig. 1A). RCMD patients showed the least favorable OS and LFS compared with the other subtypes excluding rare subtypes (Japan, 5q-syndrome subgroup; Germany, MDS-U (RCUD/pancytopenia type) subgroup) in both countries (Fig. 1B and C). The OS of RCUD patients was not significantly different between the two countries (OS median survival: Japan, 202 months; Germany, 141 months; $p=0.230$). Japanese RCUD patients tended to show a more favorable LFS than German RCUD patients (LFS median survival: Japan, more than 292 months; Germany, 27 months; $p=0.068$) (Fig. 2A). Japanese RCMD patients showed a more favorable OS than German RCMD patients (OS median survival: Japan, 109 months; Germany, 36 months; $p=0.026$). The LFS of RCMD patients was not significantly different between the two countries (10% LFS: Japan, 38 months; Germany, 10 months; $p=0.391$) (Fig. 2B). Follow-up periods ranged from 1 to 282 months (median,

114 months) in Japanese MDS-U (pancytopenia type) patients. In contrast, follow-up periods ranged from 15 to 46 months (median, 31 months) in German MDS-U (RCUD/pancytopenia type) patients. In addition, there were only 6 German MDS-U (RCUD/pancytopenia type) patients. Because of the short follow-up periods and the small number of German patients, the comparison of OS and LFS between the two countries was not adequate in the MDS-U (RCUD/pancytopenia type) subgroup. For the same reasons as for the MDS-U (RCUD/pancytopenia type) subtype, the comparison of OS and LFS between the two countries was not adequate in the 5q-syndrome subtype.

4. Discussion

There was no centralized pathology review in this study. However, we previously reported that morphologic diagnosis between the German and Japanese hematologists was in line [17]. Morphologic diagnosis of this study was performed by the same Japanese and German hematologists. Therefore, we believe that there may be extremely little differences between the interpretations of pathologists in Germany versus Japan.

Concerning the frequencies of subtypes of the WHO classification 2008, Japanese FAB-RA patients differed from German patients. The frequency of RCUD in Japanese FAB-RA patients was higher than in German patients. The frequency of RCMD in Japanese FAB-RA patients was lower than in German patients. The frequency of RT of Japanese FAB-RA patients was higher than that of German patients. The frequency of 5q-syndrome in Japanese FAB-RA patients was lower than in German patients. Morel et al. [21] and Greenberg et al. [10] reported that the frequencies of isolated del(5q) in patients with all MDS subtypes were 4.7% and 5.9%, respectively. Several reports have already indicated that MDS with isolated del(5q) is rare in Japanese patients. Toyama et al. [5] and Matsushima et al. [6] (Toyama

et al., 2.0%; Matsushima et al., 1.5%) reported that Japanese MDS patients had a lower frequency of isolated del(5q) than patients in Western reports. Most interestingly, the frequency of MDS-U (RCUD/pancytopenia type) in Japanese FAB-RA patients was significantly higher than in German FAB-RA patients. It is suggested here that the frequencies of each MDS subtype cannot be solely judged by the results of the present study. However, in the previous consecutive dataset [17] of the present study including the patients classified according to the WHO classification 2008, the frequency of Japanese FAB-RA patients with pancytopenia (35.1%) was significantly higher than in German patients (13.1%) ($p < 0.001$). Therefore, it is very likely that the frequency of the MDS-U (RCUD/pancytopenia type) subtype in Japanese patients is higher than that in German patients. We believe that the different frequencies of RCUD and MDS-U (RCUD/pancytopenia type) between two countries are noticeable and important for discussing the differences in clinical features between these two countries.

Japanese FAB-RA patients were younger than German FAB-RA patients in our previous study [17]. In contrast, the age of Japanese patients was not significantly different from that of German patients in the RCUD, MDS-U and RCMD subgroups in the present study. However, the comparison of age in the present study is problematic. Cytogenetic findings are necessary for a diagnosis according to the WHO classification 2008. Therefore, patients in the previous data set without available cytogenetic data were excluded from the present study. In German patients with advanced age, the frequency of patients where cytogenetic examinations were performed was low. In German patients, the age of patients without available cytogenetic data (median, 74 years) was significantly higher than in patients with available cytogenetic data (median, 63 years) ($p < 0.001$). In contrast, the age of Japanese patients without available cytogenetic data (median, 60 years) was not significantly different from Japanese patients with available cytogenetic data (median, 56 years) ($p = 0.542$). The age of German patients without available cytogenetic data (median, 74 years) was significantly higher than that of Japanese patients without available cytogenetic data (median, 60 years) ($p < 0.001$). Therefore, it was considered that the age of German patients in the present study was not representative. MDS-U (RCUD/pancytopenia type) patients (median, 51 years) tended to be younger than FAB-RA patients excluding the MDS-U (RCUD/pancytopenia type) subtype (median, 58 years) in Japanese patients. The German MDS-U (RCUD/pancytopenia type) patients also tended to be younger than other subtypes.

We previously reported that Japanese FAB-RA patients showed more severe cytopenia(s) [17]. The MDS-U (RCUD/pancytopenia type) subtype showed more severe cytopenia(s) in the present study. The frequency of MDS-U (RCUD/pancytopenia type) in Japanese patients was higher than that in German patients. The high frequency of the MDS-U (RCUD/pancytopenia type) subtype in Japanese patients may largely influence the unique characteristics (younger age and more severe cytopenia(s)) of the Japanese FAB-RA patients that were clarified by our previous report [17].

We reported that the frequency of cytogenetic abnormalities in Japanese FAB-RA patients were lower than in German patients in previous study [17]. The cause of this finding was the low frequency of 5q- syndrome in Japanese FAB-RA patients.

We reported that Japanese FAB-RA patients presented with a favorable overall OS and LFS in previous study [17]. The OS and LFS of Japanese and German FAB-RA patients who could be classified according to the WHO classification 2008 in the present study were similar to our previous report. Several guidelines [22–24] have been published in Western countries. To adapt these Western guidelines to Asian patients, some modifications may be required, taking into account ethnic differences. Nevertheless, no difference

was found in LFS between Japanese and German RCMD patients, Japanese RCMD patients showed a more favorable OS than German RCMD patients. It was reported that transfusion dependency was an adverse prognostic factor in MDS patients [3]. Most Japanese patients with Hb concentrations lower than 7.0 g/dL had received red cell transfusion. In contrast, most German patients with Hb concentrations lower than 9.0 g/dL had received red cell transfusion. This difference in threshold for the induction of transfusion between the two countries may influence the different OS between the two countries. The frequency of German patients with Hb concentrations lower than 9.0 g/dL (41%) was higher than that of Japanese RCMD patients with Hb concentrations lower than 7.0 g/dL (28%). In fact, RCMD patients with Hb concentrations lower than 9.0 g/dL tended to show a more unfavorable OS than RCMD patients with Hb concentrations of 9.0 g/dL or more in German patients (OS median survival: Hb lower than 9.0 g/dL, 30 months; Hb at least 9.0 g/dL, 48 months; $p = 0.054$).

Reports of several Eastern countries showed consistently unique characteristics of Eastern MDS, like young age, and a low frequency of RARS and 5q- syndrome [5,8,9,15] and the absence of a prognostic impact of cytopenia [7,8,17], although environmental factors differ between the countries. Therefore, we consider that there are genetic differences between East and West, rather than environmental factors.

In conclusion, the frequency of RCUD and MDS-U (RCUD/pancytopenia type) in Japanese patients was higher than in German patients. In particular, MDS-U (RCUD/pancytopenia type) patients occupied approximately 30% among Japanese FAB-RA patients, but MDS-U was rare (3%) in German patients. Concerning the age at the time of diagnosis, the MDS-U (RCUD/pancytopenia type) subtype was apparently younger than other subgroups in Japanese patients. The cytopenia(s) of the MDS-U (RCUD/pancytopenia type) subtype were more severe than in the RCUD and RCMD subtypes in Japanese patients. RCMD patients showed the less favorable OS and LFS than the other subtypes in both countries. The frequency of RCMD in Japanese patients was lower than that in German patients. We believe that the different frequencies of MDS subtypes according to the WHO classification 2008 between Japanese and German FAB-RA patients underlie the different clinical characteristics of FAB-RA patients between the two countries.

Conflict of interest statement

The authors reported no potential conflict of interest.

Acknowledgements

Contributors. A.M. designed the research, performed morphological analyses, collected data, analyzed data and wrote the manuscript. U.G. and I.J. designed the research, performed morphological analyses, collected data and analyzed data. M.T. designed the research, performed morphological analyses and analyzed data. M.I. collected data, performed morphological analyses and analyzed data. M.B. designed the research and analyzed data. A.K., C.S. and N.G. performed morphological analyses and collected data. K.A., Y.M. and T.H. collected data.

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EML4-ALK Fusion in Lung

To the Editor-in-Chief:

The recent article by Martelli and colleagues¹ reports (i) the detection of *EML4-ALK* fusion cDNA² not only in non-small cell lung cancer (NSCLC) specimens but in non-tumor lung tissues, (ii) a very low proportion of FISH-positive cells for *ALK* rearrangements among *EML4-ALK*-positive specimens, and (iii) the failure to detect *EML4-ALK* protein by immunohistochemistry (IHC) and Western blotting. Based on these lines of observation, the authors questioned the clinical relevance of *EML4-ALK* in the carcinogenesis of NSCLC.

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

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

We speculate, therefore, that (i) if none of the fusion cDNA-positive cases reported by Martelli et al^{1,3} produce specific genomic PCR products, then the fusion cDNA

products likely arose from cDNA-contamination, (ii) if the fusion cDNA-positive cases yield identical genomic PCR products, then the fusion cDNAs likely arose from specimen-contamination, and (iii) if the fusion cDNA-positive cases display distinct genomic fusion points, then each specimen was truly positive for the *EML4-ALK* fusion gene. Without such careful examination, we have to conclude that their claims in the article have not as yet been clearly demonstrated.

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

It should be further noted that, in both our⁶ and other researchers' IHC analyses,⁷ almost all tumor cells in a given *EML4-ALK*-positive specimen were positively immunostained with anti-*ALK* antibodies, suggesting a homogenous presence of *EML4-ALK* within a tumor. Such observation is, however, in contrast to the FISH data by Martelli et al, which show that the *ALK* rearrangement was only positive in $\approx 2\%$ of tumor cells in a given *EML4-ALK*-positive specimen. On the contrary, FISH analyses of our *EML4-ALK*-positive samples clearly demonstrate that most of the tumor cells harbor rearranged *ALK* alleles, implying that the generation of the *EML4-ALK* fusion gene is an early event in NSCLC carcinogenesis. The homogenous presence of *EML4-ALK* in our fusion-positive tumors, as demonstrated by both FISH and IHC, further raises a concern about the "EML4-ALK-positive tumors" as defined by Martelli et al.

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

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

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Authors' reply:

In their letter, Mano and Takeuchi claim that our unstable PCR results in normal and cancerous lung tissues could be attributable to contamination. However, as clearly illustrated in our article,¹ serial dilution experiments in the H2228 cell

line demonstrate the specificity and sensitivity of our RT-PCR assay. Furthermore, the identification in our EML4-ALK fusion positive tissues of alternative isoforms of variant 3, rather than the described two isoforms coexpressed in the H2228 cell line, is indicative of exclusive events in tumors, making contamination unlikely. Lastly, our experiments were confirmed independently in two laboratories (Milan and Barcelona) and always contained appropriate negative PCR controls.

We disagree with Mano et al's claim that the results of genomic PCR could be used to prove a possible RT-PCR contamination in our samples, which can only be excluded by the use of appropriate controls and procedures, as outlined above. However, we used genomic PCR to amplify the sequence flanking the EML4-ALK variant 1 breakpoint in four positive NSCLC samples. Even though a strong amplification product had been obtained from the same DNA templates using primer sets amplifying a control genomic locus of similar size to that of the cases so far reported in literature, no amplification of the EML4-ALK variant 1 fusion product was identified, suggesting only a minority of cells carried the EML4-ALK gene. These findings concur with Maes et al² who reported that, in lymphoid tissues, high level detection of NPM-ALK and ATIC-ALK fusion transcripts coincided with ALK gene rearrangements (as detected by cytogenetics and FISH), whereas low-level detection was not supported by genomic evidence of rearrangements.

In our article,¹ we clearly stated that, unlike observations in ALK+ lymphomas, tumor cells from NSCLC specimens expressed such a low amount of the EML4-ALK fusion protein that immunoprecipitation and immunohistochemistry performed with the commercially available antibodies are unable to detect it. This is in keeping with the observation that the EML4-ALK fusion protein is detectable only using highly sensitive methods, such as mass spectrometry³ or the intercalated antibody-enhanced polymer (iAEP) method⁴ which, unfortunately, are not available in all pathology laboratories and are difficult to standardize. Therefore, the question of how best to detect the EML4-ALK fusion protein remains unanswered.

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

Frequency

We recently extended our FISH analysis to 173 surgically resected lung cancer specimens (mainly adenocarcinoma) from an unselected group of Caucasian patients. The incidence of truly positive cases (>50% FISH positive, fusion transcript, and protein positive) was only 0.6% (1/173 cases), which reinforces the results in our article and is in keeping with Rodig et al's⁵ recent report of 1/227 (0.45%) ALK rearranged case in a series of surgically treated Western adenocarcinoma.

Heterogeneity

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

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

Specificity

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

Finally, we wonder whether it is really appropriate to compare treatments such as ALK inhibitors in NSCLC with imatinib mesylate and gefitinib/erlotinib in other human neoplasms. In fact: i) the role of *EML4-ALK* in NSCLC is not as well established as that of *BCR/ABL* in chronic myeloid leukemia (CML); ii) NSCLC responses to ALK inhibitors⁹ are not as remarkable as the CML response to imatinib mesylate; and iii) patients with NSCLC were treated with a multitikinase, c-MET and ALK, inhibitor.⁹ Considering that about 20% of NSCLC have MET amplification and overexpression and that MET rearrangements are homogeneous in lung cancer,¹⁰ it may be possible that responses to the multitikinase inhibitor may be related to other coexisting oncogenic events, independently of ALK.

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

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KIF5B-ALK, a Novel Fusion Oncokinase Identified by an Immunohistochemistry-based Diagnostic System for ALK-positive Lung Cancer

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Abstract **Purpose:** EML4-ALK is a transforming fusion tyrosine kinase, several isoforms of which have been identified in lung cancer. Immunohistochemical detection of EML4-ALK has proved difficult, however, likely as a result of low transcriptional activity conferred by the promoter-enhancer region of *EML4*. The sensitivity of EML4-ALK detection by immunohistochemistry should be increased adequately.

Experimental Design: We developed an intercalated antibody-enhanced polymer (iAEP) method that incorporates an intercalating antibody between the primary antibody to ALK and the dextran polymer-based detection reagents.

Results: Our iAEP method discriminated between tumors positive or negative for *EML4-ALK* in a test set of specimens. Four tumors were also found to be positive for ALK in an archive of lung adenocarcinoma ($n = 130$) and another 4 among fresh cases analyzed in a diagnostic laboratory. These 8 tumors were found to include 1 with *EML4-ALK* variant 1, 1 with variant 2, 3 with variant 3, and 2 with previously unidentified variants (designated variants 6 and 7). Inverse reverse transcription-PCR analysis revealed that the remaining tumor harbored a novel fusion in which intron 24 of *KIF5B* was ligated to intron 19 of *ALK*. Multiplex reverse transcription-PCR analysis of additional archival tumor specimens identified another case of lung adenocarcinoma positive for *KIF5B-ALK*.

Conclusions: The iAEP method should prove suitable for immunohistochemical screening of tumors positive for ALK or ALK fusion proteins among pathologic archives. Coupling of PCR-based detection to the iAEP method should further facilitate the rapid identification of novel ALK fusion genes such as *KIF5B-ALK*.

Gene fusion is a major mechanism of carcinogenesis in hematologic malignancies and sarcomas (1). Identification of the BCR-ABL fusion kinase, which is generated as a result of the balanced chromosome anomaly t(9;22)(q34;q11) in chronic myelogenous leukemia (2), has thus been followed by the discovery of many fusion-type oncogenes (3). In contrast, it has remained unclear whether such translocation-dependent fusion-type oncogenes also play a major role in the pathogenesis of epithelial tumors. Recently, however, almost 50% of prostate cancer cases have been suggested to harbor gene fusions involving ETS transcription factor loci

(4), and we have discovered a recurrent chromosome translocation, inv(2)(p21p23), in non-small cell lung cancer (NSCLC) that results in the production of an EML4-ALK fusion-type protein tyrosine kinase (PTK; refs. 5–8).

Forced expression of EML4-ALK in lung epithelial cells induced the rapid development of hundreds of lung cancer nodules in mice, and peroral administration of inhibitors of the PTK activity of EML4-ALK was shown to clear such tumors from the lungs, demonstrating the pivotal role of EML4-ALK in the pathogenesis of NSCLC positive for this fusion kinase (9). This latter observation also supports the clinical application of ALK

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Translational Relevance

Immunohistochemistry (IHC) is a reliable and relatively easy diagnostic tool to detect pathologic proteins in paraffin-embedded tissues. We have recently discovered an oncogenic fusion tyrosine kinase EML4-ALK in ~5% of non-small cell lung cancer cases. In contrast to the sensitive detection of other ALK fusions, such as NPM-ALK in anaplastic large cell lymphoma, however, IHC-mediated identification of EML4-ALK has been difficult, probably owing to the low expression level of the protein. To overcome such limitation, we here developed an intercalated antibody-enhanced polymer (iAEP) method, which is simple, yet provides high sensitivity in the IHC-mediated detection of EML4-ALK. With iAEP, in addition to the faithful detection of all non-small cell lung cancer specimens known to be positive for EML4-ALK, we have further identified specimens carrying novel variants of EML4-ALK or an unknown oncogenic fusion, KIF5B-ALK. Therefore, iAEP would provide a reliable and sensitive means to detect ALK fusions in human cancers.

all in-frame fusions between EML4 and ALK cDNAs, as exemplified by our multiplex reverse transcription- and PCR-based detection system for EML4-ALK (8).

Given that, in many pathology laboratories, most specimens submitted for histopathologic diagnosis are stored as formalin-fixed, paraffin-embedded tissue, the DNA or RNA of which may be substantially degraded, it is desirable to develop a suitable and sensitive means to detect EML4-ALK in such samples. An immunohistochemistry-based diagnostic system is one potential approach to such screening. In contrast to the efficient detection of NPM-ALK fusion proteins in anaplastic large cell lymphoma specimens with such an approach (13), however, many researchers have encountered difficulty in detecting ALK fusion proteins in lung tissue by immunohistochemical analysis (14), possibly as a result of weak transcriptional activity of the promoter-enhancer region of EML4 that drives the expression of EML4-ALK compared with that of the NPM promoter. We have now attempted to establish a sensitive screening system for ALK fusion protein-positive tumors with an immunohistochemical approach. Furthermore, with such an approach, we unexpectedly discovered a novel ALK fusion gene, KIF5B-ALK, in NSCLC.

inhibitors (6, 10) to treat EML4-ALK-positive lung cancer in humans. It should be noted, however, that multiple isoforms of EML4-ALK, generated mainly as a result of diversity in the breakpoint-fusion point within EML4 (6, 8, 11, 12), have been identified in NSCLC specimens. The accurate diagnosis of EML4-ALK-positive tumors will therefore require detection of

Materials and Methods

Samples. As a test set of samples for the development of sensitive immunohistochemical detection of EML4-ALK, we examined specimens from 11 patients with NSCLC positive for EML4-ALK (previously analyzed in ref. 8) and 10 patients with NSCLC negative for the fusion

Table 1. Immunohistochemical staining of EML4-ALK-positive or EML4-ALK-negative NSCLC specimens and quantitation of ALK mRNA

Tumor ID	EML4-ALK variant	Staining intensity						ALK mRNA level	
		ALK1	ALK1 with iAEP	5A4	5A4 with iAEP	SP8	SP8 with iAEP	5' region	3' region
#4808	V1	-	+	-	+	-	+	1.3	57.3
#9034	V1	-	++	+	++	-/+	++	0	83.3
#9968	V1	-	+	-	+	-	+	15.9	150.1
#2374	V2	-	++	-/+	++	-/+	++	1	182.3
#3121	V2	-	++	-/+	++	-/+	++	0	118.6
#4180	V2	-	++	-/+	++	-/+	++	1.4	124.5
#2075	V3	-	++	-/+	++	-/+	++	7	72
#7969	V3	+	+++	++	+++	+	++	3.6	52.7
#9616	V3	-/+	++	+	++	+	++	5.7	33.8
#8398	V4	-	++	-/+	++	-/+	++	0	118.6
#8993	V5	-	++	-/+	++	-/+	++	1.1	61.4
NC #1	NA	-	-	-	-	-	-/+	1	1
NC #2	NA	-	-	-	-	-	-/+	1.2	5.5
NC #3	NA	-	-	-	-	-/+	+	0.9	8.8
NC #4	NA	-	-	-	-/+	-/+	++	4.1	1.4
NC #5	NA	-	-	-	-	-	+	0.6	2.2
NC #6	NA	-	-	-	-	-	+	0.3	1.1
NC #7	NA	-	-	-	-	-/+	+	4.8	2.7
NC #8	NA	-	-	-	-	-	+	1.7	3.8
NC #9	NA	-	-	-	-	-	+	1.5	3.3
NC #10	NA	-	-	-	-/+	-/+	++	2.5	3

NOTE: Tumor specimens of the test cohort were subjected to immunohistochemical staining with the antibody preparations ALK1, 5A4, or SP8 according to the standard protocol or by the iAEP method. Staining intensity is represented as follows: +++, strongly positive; ++, positive; +, weakly but definitely positive; -/+, indeterminate; -, negative. The abundance of ALK mRNA in the specimens was determined by real-time RT-PCR analysis with the primers targeted to the 5' or 3' regions, which correspond to the extracellular and intracellular portions, respectively, of ALK; data are normalized relative to the NC (negative control) #1 specimen. Abbreviation: NA, not applicable.

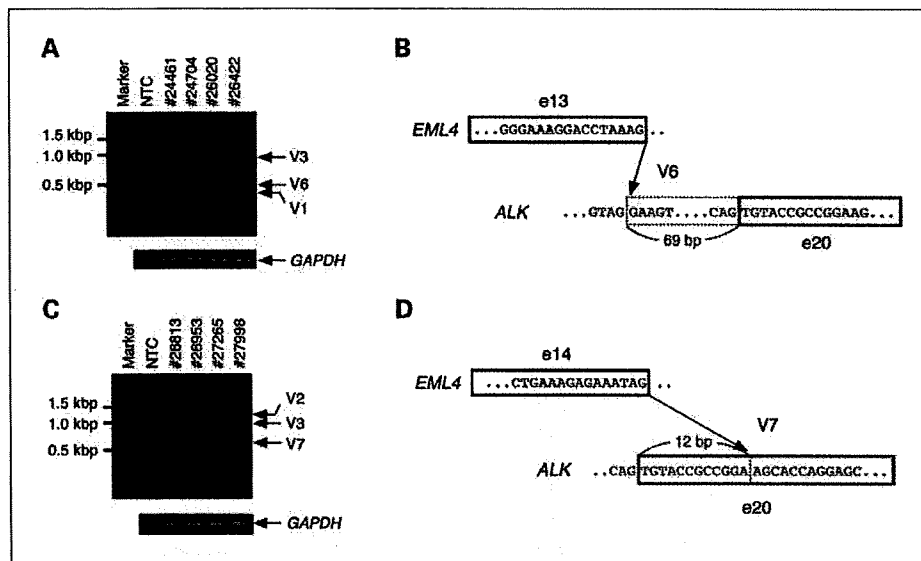


Fig. 1. Identification of novel variants of *EML4-ALK*. **A**, multiplex RT-PCR analysis of all possible in-frame fusions between *EML4* and *ALK* was done with the four specimens of lung adenocarcinoma in the validation cohort that were positive for immunostaining with 5A4 by the iAEP method (top). In addition to the detection of *EML4-ALK* variants (V) 1 and 3 in tumor IDs #24461 and #26422, respectively, a novel PCR product (variant 6) was obtained with tumor ID #26020, whereas no product was obtained with tumor ID #24704. The *GAPDH* cDNA was also amplified as a control for each specimen (bottom). Tumor IDs (top) and the size of DNA markers (bottom; 100-bp ladder) are shown. Right, the positions of *EML4-ALK* variants and the PCR product for *GAPDH*. **B**, fusion point for *EML4-ALK* variant 6 cDNA. Exon (e) 13 of *EML4* is fused to intron 19 of *ALK* at a position 69 bp upstream of exon 20. **C**, multiplex RT-PCR analysis as in **A** for the 4 specimens of lung adenocarcinoma that were identified as positive for immunostaining with 5A4 by the iAEP method in routine diagnostic screening. Tumor IDs #26813 and #26953 were shown to be positive for variant 3 of *EML4-ALK*, whereas #27265 was positive for variant 2. Tumor ID #27988 yielded a PCR product corresponding to a novel variant (variant 7) of *EML4-ALK*. **D**, fusion point of *EML4-ALK* variant 7 cDNA. Exon 14 of *EML4* is fused to nucleotide 13 of exon 20 of *ALK*.

gene. The former cohort comprised 3 cases each for *EML4-ALK* variants 1, 2, and 3 as well as one case each for variants 4 and 5. As a validation set of samples, we examined specimens from 130 consecutive patients with lung adenocarcinoma, from whom written informed consent was obtained. All specimens were collected with the approval of the ethics committee at the Cancer Institute Hospital (Tokyo, Japan), and the study was approved by the institutional review board of the Japanese Foundation for Cancer Research. Surgically removed cancer specimens were routinely fixed in 20% neutralized formalin and embedded in paraffin. Total RNA was extracted from the corresponding snap-frozen specimens and purified with the use of an RNeasy Mini kit (Qiagen).

Intercalated antibody-enhanced polymer method. Formalin-fixed, paraffin-embedded tissue was sliced at a thickness of 4 μ m, and the sections were placed on silane-coated slides. Five antibody preparations specific for the intracellular region of *ALK* (*ALK1* from Dako, 5A4 and SP8 from Abcam, ZAL4 from Zymed, and p80 from Nichirei) were evaluated for immunohistochemical staining according to standard protocols with the use of a dextran polymer reagent (anti-rabbit or anti-mouse immunoglobulin EnVision+DAB system; Dako). On the basis of their reactivity in such experiments, three antibodies (*ALK1*, 5A4, and SP8) were selected for development of the intercalated antibody-enhanced polymer (iAEP) method as follows. For antigen retrieval, the slides were heated for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako). They were then incubated at room temperature first with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 min and then with antibodies to *ALK* for 30 min. To increase the sensitivity of detection, we included an incubation step of 15 min at room temperature with rabbit polyclonal antibodies to mouse immunoglobulin (Dako) or mouse antibodies to rabbit immunoglobulin (Dako), as appropriate. The immune complexes were then detected with the dextran polymer reagent and an AutoStainer instrument (Dako).

Detection of *EML4-ALK* and *KIF5B-ALK* cDNAs and characterization of their protein products is described in Supplementary Methods.

Results

Development of the iAEP method. A specimen of NPM-*ALK*-positive anaplastic large cell lymphoma was subjected to immunohistochemical staining with 5 different antibody preparations specific for *ALK* (*ALK1* at 1:20 dilution, 5A4 at 1:50, SP8 at 1:100, ZAL4 at 1:200, or p80 at 1:100) by the EnVision+DAB polymer method. All antibody preparations stained both the nucleus and cytoplasm of the lymphoma cells, whereas ZAL4 also reacted with normal mesenchymal cells (data not shown). In addition, the staining intensity with p80 was relatively low. We therefore selected *ALK1*, 5A4, and SP8 for initial development of a detection system for *EML4-ALK*.

Immunohistochemical analysis of a test set of samples (11 specimens of *EML4-ALK*-positive NSCLC and 10 specimens of *EML4-ALK*-negative NSCLC) with these 3 antibody preparations revealed negative to marginally positive reactivity with *EML4-ALK* by a conventional staining protocol based on the EnVision+DAB system (Supplementary Fig. S1; Table 1). We therefore incorporated an intercalating antibody before the EnVision+DAB system and applied this iAEP method to the same set of specimens. All three antibody preparations detected *EML4-ALK* in all *EML4-ALK*-positive cases in the test cohort (Supplementary Fig. S1; Table 1). However, SP8 also reacted with most of the *EML4-ALK*-negative specimens (Supplementary Fig. S2; Table 1), rendering it unsuitable for large-scale screening. Furthermore, a low level of nonspecific background staining of nontumor cells was apparent in all sections stained with *ALK1*.

We selected *ALK1* and 5A4 for examination of a validation set of samples (a consecutive series of 130 lung adenocarcinoma

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

(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

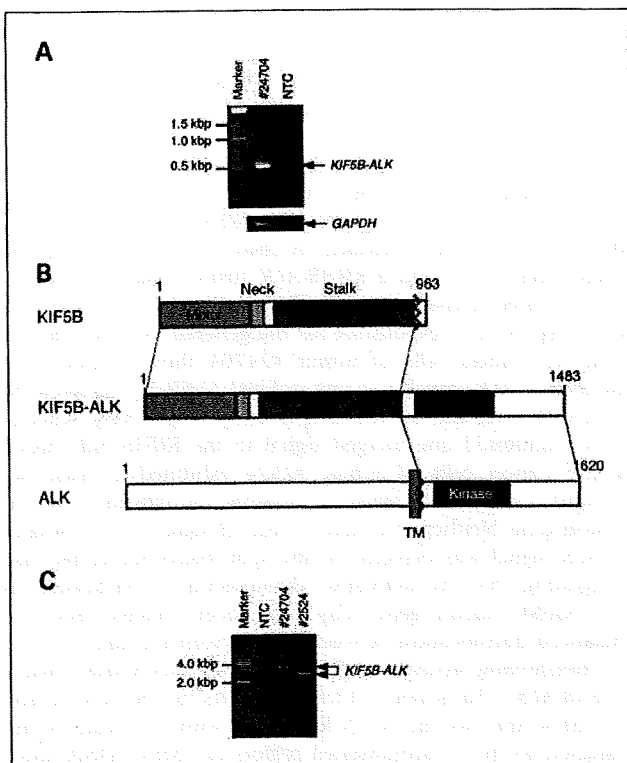
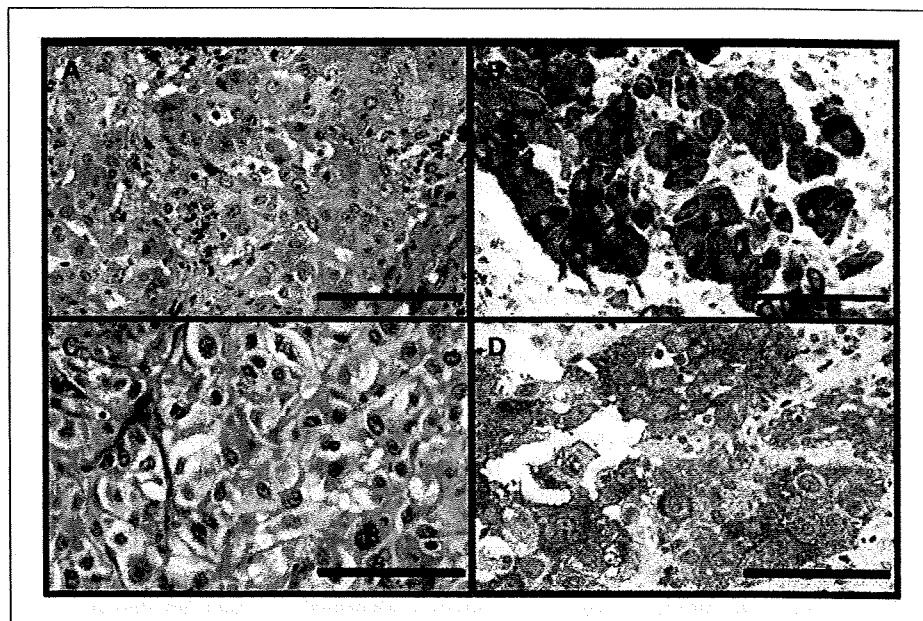


Fig. 2. Discovery of a KIF5B-ALK fusion gene associated with lung cancer. **A**, RT-PCR analysis of tumor ID #24704 with a forward primer targeted to exon 24 of KIF5B and a reverse primer targeted to exon 20 of ALK. Marker, 100-bp ladder. **B**, structure of KIF5B-ALK. KIF5B consists of an amino terminal 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.

Fig. 3. Histopathology of KIF5B-ALK-positive lung adenocarcinoma. Sections of tumors #24704 (A and B) and #2524 (C and D) were 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 μ m.



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.

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).

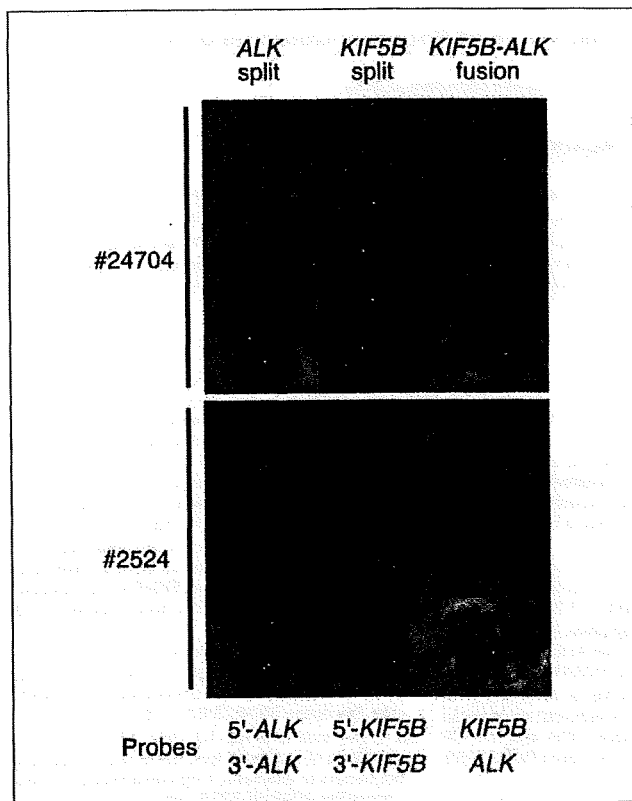
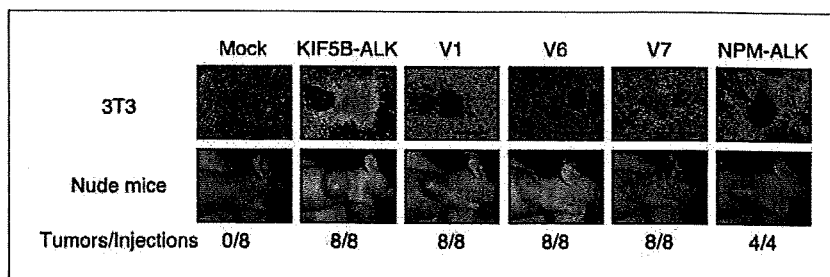


Fig. 4. Fluorescence *in situ* hybridization analysis of *KIF5B-ALK*. Sections of tumors positive for *KIF5B-ALK* (IDs #24704 and #2524) were subjected to fluorescence *in situ* hybridization with an *ALK* split assay (left), a *KIF5B* split assay (middle), or a *KIF5B-ALK* fusion assay (right). Bottom,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

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 μ m. **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 hyper-eosinophilic 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-PDGFR*A fusion mRNA in which exon 23 of *KIF5B* is ligated to exon 12 of *PDGFR*A. 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 substan-

tially 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

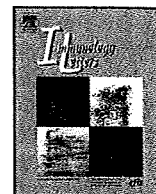
K.T. serves as a consultant to Dako.

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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.

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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 non-receptor 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 activa-

tion 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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$) 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 $\mu\text{g}/\text{ml}$ anti-CD3 plus 2 $\mu\text{g}/\text{ml}$ 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'-ACCTGGAACTGACTGGTCTC-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

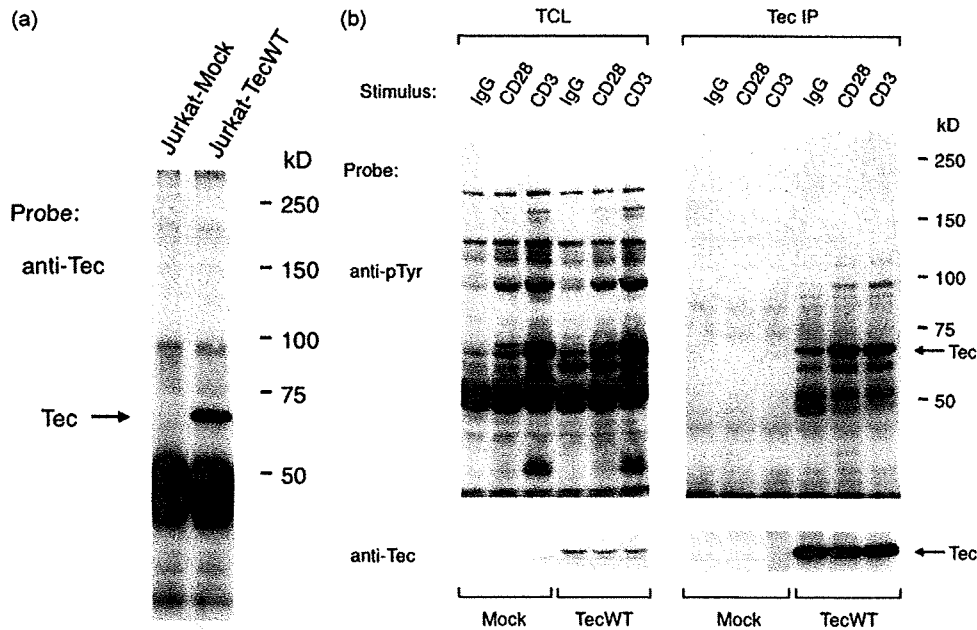


Fig. 1. Ectopically expressed Tec is activated following cell surface receptor cross-linking in Jurkat cells. (a) Cell lysates of Jurkat-Mock cells and Jurkat-TecWT cells were subjected to immunoprecipitation with anti-Tec antibody. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. 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 C γ (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 μ M 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

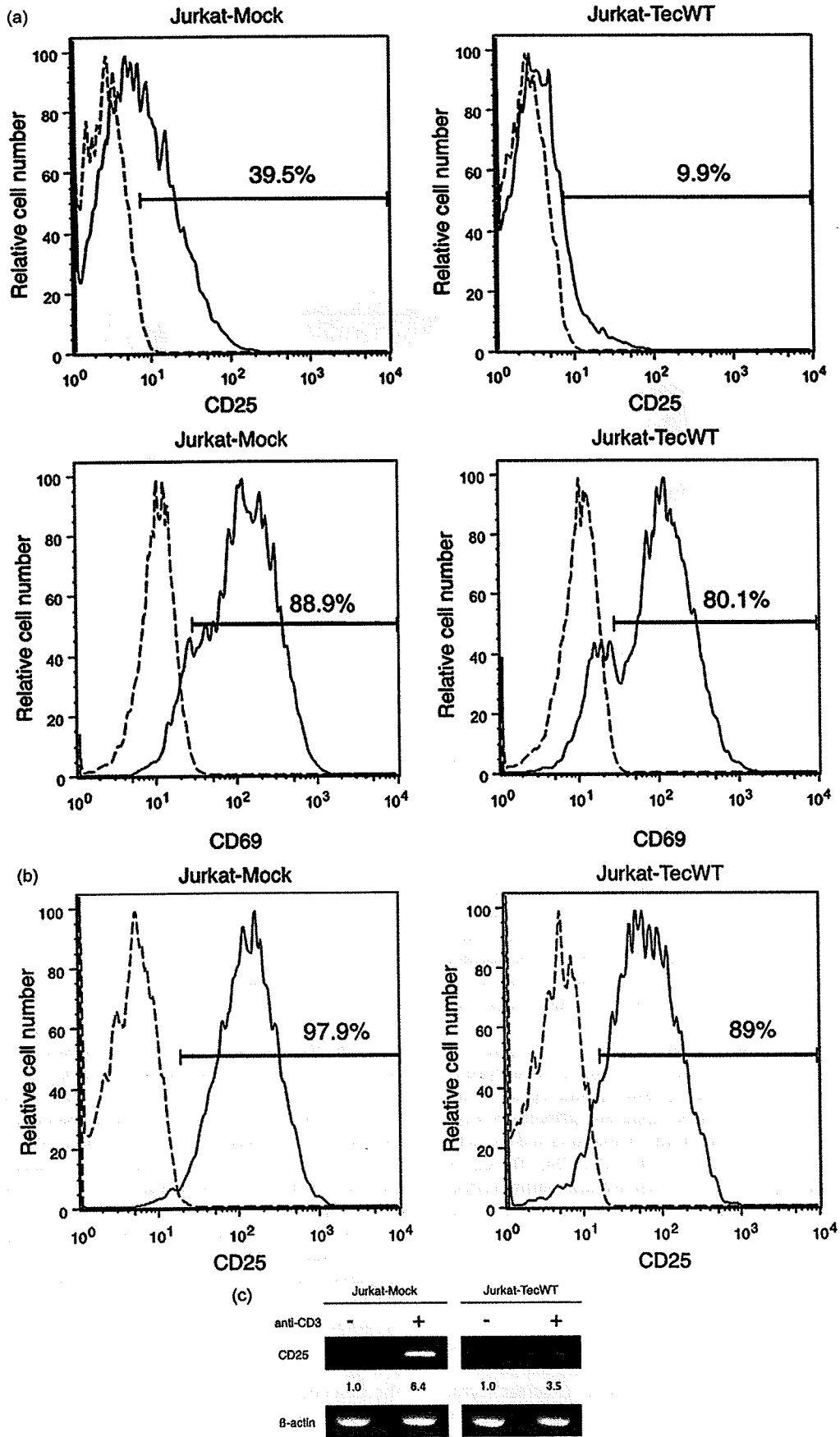


Fig. 2. Expression of Tec inhibits upregulation of CD25 but not that of CD69 induced by TCR cross-linking. (a) Jurkat-Mock cells and Jurkat-TecWT cells after TCR cross-linking were incubated with anti-CD25 (upper panels) and anti-CD69 (lower panels) antibodies. Flow cytometric histograms show the intensity 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

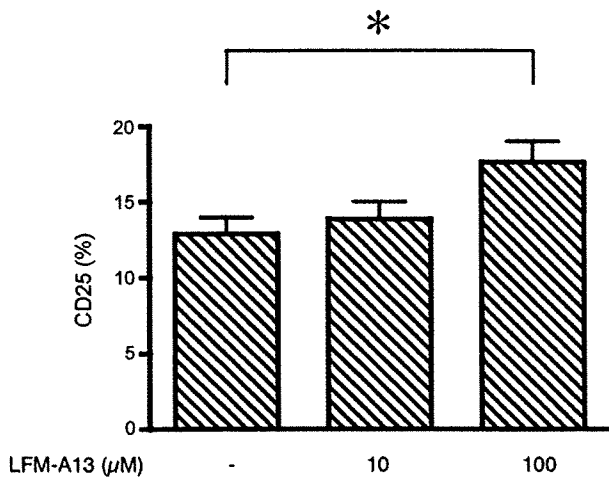


Fig. 3. Tec selective inhibitor LFM-A13 increases the CD25 expression on Jurkat-TecWT cells after TCR cross-linking. Jurkat-TecWT cells were treated with the indicated concentrations of LFM-A13 or DMSO (vehicle) for 1 h. The cells were then stimulated with TCR cross-linking for 24 h. CD25 expression was evaluated by means of flow cytometric analysis. Bars (mean \pm 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 \pm 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, $17.6 \pm 2.8\%$ of cells incubated with $100 \mu\text{M}$ LFM-A13 expressed CD25, versus $12.9 \pm 2.1\%$ 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 , γ subunits) [28,29]. The binding of CD25 (α subunit) to the low-affinity IL-2R (β , γ 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 upregulation

with 50 ng/ml of PMA and $1 \mu\text{M}$ 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.