Patient subset analyses of these randomised phase III trials or retrospective trials (Kaneda et al, 2004; Miller et al, 2004) clearly show the existence of populations that are more likely to respond to gefitinib and erlotinib, including women, patients with adenocarcinoma (especially with bronchial alveolar carcinoma (BAC)), nonsmokers and Asian patients (compared with Caucasians). Somatic mutations in specific regions of exons 18, 19 and 21 of the ATP-binding domain of EGFR have recently been shown to have strong associations with sensitivity to gefitinib or erlotinib (Lynch et al, 2004; Paez et al, 2004; Pao et al, 2004). Consistent with these findings, the frequencies of these EGFR mutations were higher in women, patients with adenocarcinoma, nonsmokers and Asians, all of whom are among the more frequent responders, as mentioned above (Shigematsu et al, 2005). There are two characteristic types of EGFR mutations. One is the presence of in-frame deletions, including the amino acids at codons 746–750 in exon 19, and the other is an amino-acid substitution at codon 858 (L858R) in exon 21. Recent analyses (Bell et al, 2005) of phase II and III trials for EGFR-TKI, in which patients were not selected based on their mutation status, have suggested that EGFR mutations are correlated with response to therapy but are not correlated with overall survival (OS). Furthermore, EGFR gene amplification/copy number (Cappuzzo et al, 2005; Hirsch et al, 2005) or overexpression (Hirsch et al, 2003) has been shown to be a more useful prognostic marker of response to gefitinib treatment. Patient selection according to EGFR mutation status may yield a superior survival rate by excluding patients who are unlikely to respond to gefitinib treatment. However, other populations that might obtain a clinical benefit from gefitinib treatment, even in the absence of EGFR mutation, may exist.

Three Japanese groups (Asahina et al, 2006; Inoue et al, 2006; Yoshida et al, 2007) have reported prospective phase II studies of gefitinib for advanced-stage NSCLC that were designed to consider the EGFR mutation status of the patients. All of these studies have reported a high response rate and extended progression-free survival (PFS) period, compared with historical controls. However, all of these studies had a relatively short observation period, making the data preliminary. Moreover, the original sample size was calculated after patient selection, and a critical consideration of the suitability of the assay used to detect the mutations (which was performed using small paraffin-embedded specimens obtained from bronchoscopic biopsies), and the estimated EGFR-positive rate were lacking. Additionally, all the trials were conducted at single institutions located in one small area of Japan. Thus, the published data may not be representative of the situation found in general clinical practice throughout Japan and therefore may not directly translate to the general feasibility of gefitinib treatment in Japan.

In view of this situation, we performed a multicentre prospective phase II trial of gefitinib for advanced NSCLC harbouring EGFR mutations. We prospectively registered patients from 15 different institutes in Japan at the beginning of EGFR mutation screening using a central database. Whether or not tissue was available from a bronchoscopic biopsy or surgery was not an inclusion criterion. All the clinical samples from the registered patients were delivered to a central laboratory that then determined the EGFR mutation status or the histological BAC features. The analysis of the survival data was based on a minimum observation period of at least 15 months from the time of entry of the last patient.

MATERIALS AND METHODS

Eligibility criteria

Eligible patients had histologically confirmed stage III NSCLC for which thoracic irradiation was not indicated or were stage IV. Chemotherapy-naive patients or those who had previously received up to two prior chemotherapy regimens, including those performed in an adjuvant setting, were eligible. Other eligibility criteria included an age $\geqslant 20$ years, measurable disease, the availability of sufficient amounts of tumour specimen for EGFR mutation analysis, an Eastern Cooperative Oncology Group performance status of 0–2, adequate organ function (WBC $\leqslant 3000\,\mu l^{-1}$, platelets $\geqslant 75\,000\,\mu l^{-1}$, AST and ALT $\leqslant 100\,IU\,l^{-1}$, serum creatinine $\leqslant$ twice the upper limit of the reference range; $P_{aO2} \geqslant 60\,mm\,Hg$). The exclusion criteria included pulmonary fibrosis, the presence of symptomatic brain metastasis, active concomitant malignancy, severe heart disease, active gastrointestinal bleeding and continuous diarrhoea. All the patients signed a written informed consent form. Approval of this study and the gene analyses were obtained from the Institutional Review Board and the Ethics Committee of each hospital.

EGFR gene analysis

Tumour specimens were obtained using bronchial fiberscope or surgical procedures. The specimens were fixed with formalin and embedded in paraffin. Four slices (4–5 μm) from the embedded block were sent to a central laboratory (Mitsubishi Chemical Safety Institute Ltd., Ibaraki, Japan) for genetic analysis. Most of the tumour specimens were available prior to the registration of this study. Genomic DNA was isolated from specimens using QIAamp Micro kits (QIAGEN KK, Tokyo, Japan). The EGFR mutations in exons 18, 19 and 21, as previously reported (Lynch et al, 2004; Paez et al, 2004), were determined using polymerase chain reaction (PCR) amplification and intron–exon boundary primers according to the published method. An EGFR registrant mutation in exon 20, which was reported by Pao et al (2005) was also examined using PCR and the previously reported primers. Polymerase chain reaction was performed using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), and the PCR products were confirmed using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA), then sequenced directly using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and were analysed using the Basic Local Alignment Search Tool (BLAST); all the electropherograms were reanalysed by visual inspection to check for mutations. The presence of an EGFR mutation was confirmed using at least three independent PCR.

All sequence data were sent from the central laboratory to Kinki University. A principle investigator then confirmed whether or not the EGFR mutation status was positive, and the results were sent to the West Japan Thoracic Oncology Group (WJTOG) data centre. The data centre then informed each participating centre of the results of the genetic analysis and requested that the eligibility criteria of the patients be rechecked to insure that only EGFR-positive subjects were registered in the trial. Each tumour was categorised according to histology by a pulmonary pathologist (JF). The percentage of area exhibiting a BAC pattern was also examined to determine the WHO pathological category.

Treatment plan

Gefitinib (250 mg day^{-1}) was administered once daily. Treatment was continued uninterrupted until disease progression or intolerable toxicity (grade 4 nonhaematological toxicities, any incidents of interstitial pneumonia or a treatment delay of more than 2 weeks because of adverse effects). Gefitinib administration was delayed if the patient's leukocyte and platelet counts were lower than 1500 and 5000 μl^{-1}, respectively, and was withheld until these counts had recovered. Gefitinib administration was also delayed if grade 3 or greater nonhaematological toxicities without nausea, vomiting or alopecia occurred and was withheld until recovery to grade 2.

Routine clinical and laboratory assessments and chest X-ray assessments were performed weekly or biweekly, where possible; CT examinations of the target lesion were performed every month, and magnetic resonance imaging of the whole brain and a bone scan were performed every 3 months. The objective responses of the patients were evaluated every month using the Response Evaluation Criteria in Solid Tumours (RECIST) guidelines (Therasse et al, 2000). Tumour response was centrally evaluated by independent reviewers at an extramural conference and was performed for the intent-to-treat population. All adverse effects that occurred during gefitinib treatment were reported, and the severity of the effects was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Statistical analyses

The primary end point of this study was the response rate. A one-stage design using the binominal probability was used to determine the sample size. Assuming that a response rate of 50% would indicate potential usefulness, whereas a rate of 25% would be the lower limit of interest, and with $\alpha = 0.10$ (two side) and $\beta = 0.20$, the estimated accrual number was 23 patients. Estimating that the EGFR-positive rate would be about 20%, the screening number required to accrue 23 EGFR-positive patients was 115. After assuming an inevaluability rate of < 10%, the final required screening number was 125.

The secondary end points of this study were toxicity, OS, PFS, 1-year survival (1Y-S) and the disease control rate (DCR). Survival analyses were conducted on the intent-to-treat population using follow-up data available as of 30 April 2007. The survival curves were estimated using Kaplan–Meier plots.

RESULTS

Patient characteristics

Between March 2005 and January 2006, 118 patients were prospectively screened from 15 institutions; 117 of them underwent EGFR mutation analysis (tumour tissue was not available for one patient). The median time required for the EGFR mutation analysis was 12 days (range: 7–28 days). Among the 117 patients, EGFR mutations were detected in 32 patients (27%), 14 of whom had a deletion in or near E746-A750 (including one del E746-T751 ins A, two del L747-T751 and one del L747-T753 ins S) in exon 19. A further 17 had L858R, and one had a L861Q point mutation in exon 21 (Table 1).

Tissue samples from 17 patients (53%) were obtained by transbronchial biopsy. The EGFR detection rates for the surgical specimens and the bronchoscopic biopsy specimens were similar (30 vs 25%). The EGFR mutations were significantly more frequent in women ($P \leqslant 0.02$), in patients with adenocarcinoma ($P = 0.001$) and in people who had never smoked ($P < 0.001$) (Table 2). Finally, 28 patients (14 with deletions in exons 19 and 14 with point mutations in exon 21) were actually registered and received treatment with gefitinib, whereas four patients were dropped from the study as they became ineligible because of tumour progression during the time required for the mutation analysis.

Patient characteristics are listed in Table 3. In the initial screening, there were 56 female patients (48%), 97 patients (83%) with adenocarcinoma and 53 (45%) who had never smoked. The frequency of these characteristics was higher among the patients with EGFR mutations who were actually registered; namely, 18 patients (64%) were women, 27 (96%) had adenocarcinoma and 19 (68%) had never smoked. The median age of the 28 actually registered patients was 68 years; 24 patients (86%) had a good performance status (0–1), 22 (79%) had stage IV diseases and 17

Table 1 Type of EGFR mutations ($n = 32$)

Characteristics	No. of patients	%
Exon 18	0	0
Exon 19	14	44
del E746-A750	10	32
del E746-T751 ins A	1	3
del L747-T751	2	6
del L747-T753 ins S	1	3
Exon 21	18	56
L858R	17	53
L861Q	1	3

EGFR = epidermal growth factor receptor.

Table 2 Relationship between patient characteristics and EGFR mutation status

Characteristics	EGFR mutation positive ($n = 32$)		EGFR mutation negative ($n = 85$)		
	No. of Patients	%	No. of Patients	%	P
Sex					
Male	11	34	50	59	
Female	21	66	35	41	<0.02
Histology					
Adenocarcinoma	31	97	66	78	
Nonadenocarcinoma	1	3	19	22	=0.001
Smoking status					
Never	21	66	31	36	
Current/former	11	34	54	64	<0.001

EGFR = epidermal growth factor receptor.

(61%) were chemotherapy naive. Thoracic irradiation was contra-indicated in one patient with stage IIIA disease because of the large irradiation field that would have been required. All five patients with stage IIIB diseases had malignant effusions. Four patients had received adjuvant therapies; five had received platinum doublets or a combination of gemcitabine and vinorelbine as their first-line therapy. Two patients had received two regimens of platinum doublets followed by docetaxel or pemetrexed. One patient had received local radiation for pain control.

Response and survival

The objective tumour responses are listed in Table 4. The overall response rate and DCR were 75% (95% CI: 57.6–91.0%) and 96% (95% CI: 87.0–96.4%), respectively. Five out of ten male patients (50%), six out of nine smokers (67%) and five out of eight male smokers with adenocarcinoma (63%) achieved a PR. One female nonsmoker with squamous cell carcinoma also achieved a PR. Among the registered patients with EGFR mutations, the response rate was no different between current/former smokers and those who had never smoked (67 vs 79%) or between chemotherapy-naive and postchemotherapy patients (77 vs 73%). Female and patients with a mutational deletion in exon 19 tended to have a higher response rate than male (89 vs 50%) and patients with a missense mutation in exon 21 (86 vs 64%), respectively.

The median follow-up time was 18.6 months (range: 13.8–23.4 months). The median PFS time was 11.5 months (95% CI: 7.3 months to -) (Figure 1A). The median OS has not yet been reached, and the 1Y-S was 79% (95% CI: 63.4–93.8%) (Figure 1B).

Table 3 Patient characteristics of all registered patients (n = 28)

Characteristics	No. of patients (%)
Age	
Median	68
Range	49–89
Performance status	
0	11 (39)
1	13 (47)
2	4 (14)
Sex	
Male	10 (36)
Female	18 (64)
Histology	
Adenocarcinoma	27 (96)
Squamous cell carcinoma	1 (4)
Large cell carcinoma	0 (0)
Adenosquamous carcinoma	0 (0)
Other	0 (0)
Smoking status	
Never	19 (68)
Current/former	9 (32)
Stage	
IIIA[a]	1 (3)
IIIB	5 (18)
IV	22 (79)
Prior cancer therapy	
Chemotherapy	
No	17 (61)
One regimen (adjuvant)	4 (14)
One regimen (not adjuvant)	5 (18)
Two regimens	2 (7)
Recurrence after surgery	11 (39)
Radiation	1 (4)

[a]Unresectable, no indication for thoracic radiation because of a large radiation field.

Table 4 Response rate (n = 28)

Response	No. of patients	Response rate (%)	95% CI
Complete response	1	3.6	
Partial response	20	71.4	
Stable disease	6	21.4	
Progressive disease	0	0.0	
Not evaluable[a]	1	3.6	
Overall response	21	75.0	57.6–91.0
Disease control rate	27	96.4	87.0–96.4

CI = confidence interval. [a]One patient was not evaluable because of a poor evaluation of efficacy.

Safety and toxicity

Toxicity was evaluated in all eligible patients (Table 5). The most frequent adverse events were rash, dry skin, diarrhoea, stomatitis and elevated AST/ALT levels. Two patients experienced grade 3 rash and one patient experienced grade 3 keratitis; however, these patients all achieved a PR, and the adverse effects subsided after pausing gefitinib treatment for around 2 weeks. Four patients experienced grade 3 hepatotoxicity; three of these patients had to discontinue treatment for this reason.

One patient developed interstitial lung disease (ILD) (Ando et al, 2006). Ground-glass opacity was detected in the right upper lobe 19 days after the start of gefitinib administration, resulting in the cessation of treatment. However, the lesion enlarged into bilateral

lung fields on day 25, and steroid therapy was initiated. Nonetheless, the patient died of respiratory failure on day 48. Two patients also experienced grade 1 ILD. They recovered without steroid administration.

Subsequent treatment after disease progression

Of the 14 patients who become refractory to gefitinib and exhibited disease progression, 10 received chemotherapy as their first treatment regimen after gefitinib (Table 6); 5 patients received platinum doublets and 1 patient received vinorelbine as a second-line treatment; and 3 received docetaxel and 1 received platinum doublet as a third-line treatment. In all, 4 out of the 10 patients (40%) had a PR. Of the nine patients who become refractory to the first treatment regimen after gefitinib, six received chemotherapy as their second regimen after gefitinib, including one who received gemcitabine, one who received docetaxel, and one who was re-treated with gefitinib as a third-line therapy; two other patients received docetaxel and one was re-treated with gefitinib as a fourth-line therapy. Two of the six patients (33%) had a PR. The two patients who received gefitinib re-treatment both had SD.

BAC features, EGFR amplification and T790M mutation in exon 20

A total of 110 tissue samples were available for pathological review, of which 90 were from adenocarcinoma; 33 of these specimens (37%) revealed proportional BAC components in the specimen. Among them, 15 were considered extensive and the remaining 18 were found to have minor BAC components. The 39 surgical specimens included 36 from adenocarcinomas. The EGFR mutations were detected in 12 out of the 36 adenocarcinoma specimens. None of the samples with a BAC component, micropapillary pattern or mucin production was associated with an EGFR mutation (Table 7).

Data on EGFR gene copy numbers were available in only 12 samples. We used the criteria for defining a high EGFR gene copy number (gene amplification or high polysomy, as determined using FISH) that were described in a previous report (Cappuzzo et al, 2005). A total of 7 out of the 12 samples had a high gene copy number (FISH positive), and 6 (3 with EGFR mutations) out of the 7 samples had proportional BAC components. In all, 5 out of the 12 samples were FISH negative, only 1 (with no EGFR mutation) of which had a BAC component. Two patients that were FISH negative, BAC negative and EGFR mutation positive had SD when treated with gefitinib.

Another EGFR mutation, T790M in exon 20, has been reported to be associated with resistance to gefitinib (Kobayashi et al, 2005; Pao et al, 2005). We checked for this mutation in six patients who did not respond to gefitinib; however, the mutation could not be identified in any of the patients.

DISCUSSION

We performed a multicentre phase II study examining the use of gefitinib for advanced NSCLC in patients with EGFR mutations, prospectively recruiting patients at the time of genetic screening and avoiding a selection bias. All patients were registered in a central database. All tissues were delivered from the local participants to the central facility, where they were reviewed by a pathology specialist and the EGFR mutation status was evaluated. The median time for the EGFR mutation detection analysis was 12 days, which is probably an acceptable time lag before the start of treatment for advanced NSCLC. However, a shorter period would clearly be desirable for routine clinical practice. Indeed, 4 out of the 32 EGFR-positive patients were dropped from the study because of disease progression before their actual registration
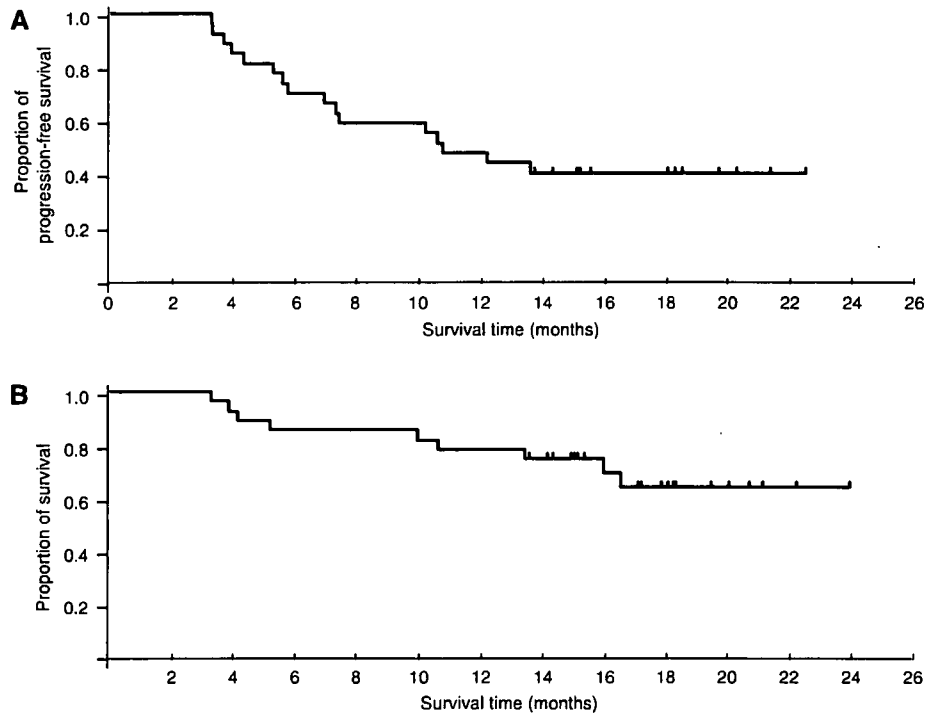
Figure I (**A**) Progression-free survival (PFS) and (**B**) overall survival (OS) of all eligible patients (*n* = 28). The median PFS was 11.5 months. The median OS has not yet been reached. The 1-year survival rate was 79%.

Table 5 Common adverse events (*n* = 28)

Adverse events	No. of patients (%)			
	Grade 1	Grade 2	Grade 3	Grade 4
Haematologic				
Anaemia	12 (43)	3 (11)	0 (0)	0 (0)
Leucopaenia	4 (14)	1 (4)	2 (7)	0 (0)
Neutropaenia	4 (14)	1 (4)	1 (4)	0 (0)
Thrombocytopaenia	3 (11)	0 (0)	0 (0)	0 (0)
Nonhaematologic				
Rash	10 (36)	11 (39)	2 (7)	0 (0)
Dry skin	9 (32)	10 (36)	0 (0)	0 (0)
Nail changes	5 (18)	2 (7)	0 (0)	0 (0)
Keratitis	0 (0)	0 (0)	1 (4)	0 (0)
Fever	0 (0)	1 (4)	0 (0)	0 (0)
Fatigue	3 (10)	3 (10)	3 (10)	0 (0)
Diarrhoea	7 (25)	1 (4)	0 (0)	0 (0)
Constipation	1 (4)	0 (0)	0 (0)	0 (0)
Stomatitis	8 (29)	1 (4)	0 (0)	0 (0)
Gastritis	1 (4)	0 (0)	0 (0)	0 (0)
Anorexia	2 (7)	1 (4)	0 (0)	0 (0)
Nausea	3 (11)	1 (4)	0 (0)	0 (0)
Vomiting	2 (7)	2 (7)	1 (4)	0 (0)
Dyspnoea	2 (7)	0 (0)	1 (4)	0 (0)
ILD	2 (7)	0 (0)	0 (0)	1 (4)[a]
Vertigo	1 (4)	1 (4)	0 (0)	0 (0)
Dysgeusia	0 (1)	1 (4)	0 (0)	0 (0)
Elevated AST/ALT	10 (36)	2 (7)	4 (14)	1 (4)[a]
Elevated creatinine	2 (7)	1 (4)	2 (7)	0 (0)

ALT = alanine transaminase; AST = aspartate transaminase; ILD = interstitial lung disease. [a]Same patient.

could occur. Yatabe *et al* (2006) has developed a rapid assay to detect *EGFR* mutations, and we have decided to use this assay in a phase III trial. The *EGFR* mutation rates in transbronchial biopsy

samples were found to be the same as those in surgical specimens, suggesting that this assay can also accommodate stage IV NSCLC. We detected the two characteristic types of *EGFR* mutations (in exons 19 and 21) in 44 and 56% of the patients, respectively (Table 1); these percentages are identical to those in previous reports from Japan (Shigematsu *et al*, 2005; Asahina *et al*, 2006; Inoue *et al*, 2006; Yatabe *et al*, 2006; Yoshida *et al*, 2007). In summary, we confirmed the feasibility of using the *EGFR* detection assay in daily practice.

The overall response rate was 75%, which was comparable to those of other phase II studies of gefitinib in patients with *EGFR* mutations (Asahina *et al*, 2006; Inoue *et al*, 2006), despite our study permitting the entry of patients who had previously received up to two chemotherapy regimens. The DCR of 96% was relatively high, and the median PFS of 11.5 months and 1Y-S of 79% were also very promising. In a Korean study, Lee *et al* (2006) also reported a very promising response rate (56%) and 1Y-S (76%) for gefitinib in a prospective study of selected NSCLC patients with adenocarcinoma and never/light smokers, defined as having smoked no more than 100 cigarettes during one's lifetime. In the screening process for the present study, *EGFR* mutations were significantly more frequent in women, patients with adenocarcinoma and those who had never smoked. However, among the patients who were selected according to their *EGFR* mutation status, no differences in response were observed between never smokers and current/former smokers or between chemotherapy-naive and postchemotherapy patients. In a retrospective study, Han *et al* (2006) directly compared clinical predictors (smoking history, gender and histology) and the *EGFR* mutation status for their ability to predict response and survival. They showed that female never smokers with adenocarcinoma (three clinical predictors) had a 33% response rate, whereas patients with a positive *EGFR* mutation status had a 62% response rate. Furthermore, in a multivariate analysis, only a positive *EGFR* mutation status was associated with an improved OS, suggesting that the *EGFR* mutation status should be analysed whenever possible to optimise response predictions based on clinical

Table 6 Subsequent treatments after failure to respond to gefitinib (n = 28)

Gefitinib treatment	No. of Patients	1st regimen after gefitinib	No. of patients	2nd regimen after gefitinib	No. of patients
1st line	17	Plt doublet	5	Gem or Doce	2
				Gefitinib[a]	1
		VNR	1	—	-
2nd line[b]	4	Doce	2	Doce	1
		Plt doublet	1	Doce	1
2nd line	5	Doce	1	Gefitinib[a]	1
3rd line	2	—	—	—	—
Total	28		10		
Response			4/10		2/6

Doce = docetaxel; Gem = gemcitabine; Plt = platinum; VNR = vinorelbine. [a]Both patients had an SD response after gefitinib re-treatment. [b]First regimen as systemic chemotherapy after adjuvant treatment.

Table 7 Bronchial alveolar carcinoma (BAC) features and EGFR mutation status

	EGFR mutation		
	+	−	P-value
Surgically resected adenocarcinoma case	12	24	
BAC component			
Yes	8	17	1.0
No	4	7	
Micropapillary pattern			
Yes	4	12	0.48
No	8	12	
Mucin production			
Yes	1	5	1.0
No	11	19	

EGFR = epidermal growth factor receptor.

background factors. In the present study, EGFR mutations were detected in 16 out of 40 (40%) female never smokers with adenocarcinoma who underwent the screening process, and 14 out of these 16 patients (88%) achieved a response after undergoing gefitinib therapy. We could not compare the predictive powers of clinical predictors and the EGFR mutation status with regard to the clinical benefits of gefitinib in this study. Thus, the need for EGFR mutation testing among clinically favourable patients remains uncertain. Decisions regarding the first-line therapy of choice for patients with EGFR mutations or a clinically favourable profile (nonsmoker with adenocarcinoma) must also await the results of an ongoing randomised phase III study in an Asian population (IPASS: Iressa Pan-Asian Study) comparing platinum doublets with gefitinib.

In contrast, 50% of the men, 67% of the smokers and 63% of the men who were smokers achieved a PR in this study. Furthermore, one female nonsmoker with squamous cell carcinoma also responded to gefitinib. The histological type of this tumour was reassigned by a pulmonary pathologist, and the tumour was finally confirmed to be a squamous cell carcinoma. Squamous cell carcinoma harbouring an EGFR mutation is rarely seen but has been previously reported (Asahina et al, 2006). In a Japanese phase II trial of gefitinib for unselected chemotherapy-naive patients (Niho et al, 2006), the response rates among smokers, men, and patients with nonadenocarcinoma were 19, 13 and 10%, respectively. Thus, NSCLC patients who are either smokers, men or have a nonadenocarcinoma histology are unlikely to receive gefitinib treatment as a first-line treatment instead of standard chemotherapies (platinum doublets), which yield a response rate of about 30% (Schiller et al, 2002). Therefore, EGFR mutation screening may

have a higher impact on the selection of responders to gefitinib treatment among these kinds of Asian patient subset (for example, smokers with adenocarcinoma, and nonsmoking men or women with nonadenocarcinoma).

The benefit of chemotherapy in general among patients with EGFR mutations, compared with EGFR mutation-negative patients, remains uncertain. Previous studies (Bell et al, 2005) have suggested that patients with EGFR mutations tend to be more sensitive to chemotherapy than those with wild-type EGFR. In the present study, 40 and 33% of the patients responded to first- and second-line chemotherapy regimens after gefitinib, respectively. These relatively high response rates for refractory NSCLC suggest that patients with an EGFR mutation-positive status are generally sensitive to chemotherapy. Large-scale multivariate analyses, using pooled data from prospective phase II or III trials in which the EGFR mutation status was clearly confirmed, are needed to clarify this point.

The toxicities observed in the present study were mostly tolerable. Most of the common adverse events, like rash, diarrhoea or hepatotoxicity, were mild and subsided after gefitinib administration was paused for a short period. One male smoker with adenocarcinoma died of ILD. Thus, even among patients who are selected based on their EGFR mutation status, men or smokers may still be at risk for developing ILD; therefore, biomarkers to predict ILD are needed.

Patients with exon 19 mutations tended to have a higher response rate than those with a missense mutation in exon 21, consistent with the findings of previous reports (Jackman et al, 2006; Riely et al, 2006). The Spanish Lung Cancer Group also reported on a prospective phase II study of erlotinib in advanced NSCLC patients with EGFR mutations (Paz-Ares et al, 2006). The overall response rate was 82%. They also showed a difference in response rates between patients with mutations in exons 19 and 21 (95 and 67%, respectively). Exon 11 c-kit mutations are more closely correlated with a good prognosis in patients with gastrointestinal stromal tumour, who may benefit from lower doses of imatinib, whereas patients with exon 9 mutations may require higher doses (Debiec-Rychter et al, 2006). In the case of EGFR, functional differences between mutation types may also exist.

We found no discernible associations between the EGFR mutation frequency and the presence of a BAC component. Several reports, including that of Hirsch et al (2005) suggest that a higher EGFR copy number is correlated with BAC histological features. We also found an association between a high EGFR copy number and the presence of a BAC component, even though the number of specimens examined was relatively small. In a study on erlotinib, the presence of a BAC component was clearly associated with EGFR amplification. As the EGFR mutation rate is lower in western populations than in Asian populations, the EGFR gene copy number might be a more useful biomarker in western populations, especially with regard to the use of erlotinib.

In conclusion, gefitinib treatment for patients with advanced NSCLC harbouring an EGFR mutation demonstrated a promising activity in patients with a good performance status. Patient screening according to EGFR mutation status may be a useful tool in daily practice and will likely have a great impact on the selection of patients who are likely to benefit from gefitinib treatment.

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ORIGINAL ARTICLE

Aberrant expression of Fra-2 promotes CCR4 expression and cell proliferation in adult T-cell leukemia

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Adult T-cell leukemia (ATL) is a mature CD4^{+} T-cell malignancy etiologically associated with human T-cell leukemia virus type 1 (HTLV-1). Primary ATL cells frequently express CCR4 at high levels. Since HTLV-1 Tax does not induce CCR4 expression, transcription factor(s) constitutively active in ATL may be responsible for its strong expression. We identified an activator protein-1 (AP-1) site in the CCR4 promoter as the major positive regulatory element in ATL cells. Among the AP-1 family members, Fra-2, JunB and JunD are highly expressed in fresh primary ATL cells. Consistently, the Fra-2/JunB and Fra-2/JunD heterodimers strongly activated the CCR4 promoter in Jurkat cells. Furthermore, Fra-2 small interfering RNA (siRNA) or JunD siRNA, but not JunB siRNA, effectively reduced CCR4 expression and cell growth in ATL cells. Conversely, Fra-2 or JunD overexpression promoted cell growth in Jurkat cells. We identified 49 genes, including c-Myb, BCL-6 and MDM2, which were downregulated by Fra-2 siRNA in ATL cells. c-Myb, BCL-6 and MDM2 were also downregulated by JunD siRNA. As Fra-2, these proto-oncogenes were highly expressed in primary ATL cells but not in normal CD4^{+} T cells. Collectively, aberrantly expressed Fra-2 in association with JunD may play a major role in CCR4 expression and oncogenesis in ATL.

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Introduction

Adult T-cell leukemia (ATL) is a highly aggressive malignancy of mature CD4^{+}CD25^{+} T cells etiologically associated with human T-cell leukemia virus type 1 (HTLV-1; Yamamoto and Hinuma, 1985). HTLV-1 encodes a potent viral transactivator Tax that activates the HTLV-1 long terminal repeat (LTR) and also induces the expression of various cellular target genes, including those encoding cytokines, cytokine receptors, chemokines, cell adhesion molecules and nuclear transcriptional factors, collectively leading to the strong promotion of cell proliferation (Yoshida, 2001; Grassmann et al., 2005). However, ATL develops after a long period of latency, usually several decades, during which oncogenic progression is considered to occur through the accumulation of multiple genetic and epigenetic changes (Matsuoka, 2003). Furthermore, circulating ATL cells usually do not express Tax and are considered to be independent of Tax (Matsuoka, 2003). Previously, Mori et al. have demonstrated the strong constitutive activation of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) in primary ATL cells (Mori et al., 1999, 2000). However, the molecular mechanisms of ATL oncogenesis still remain largely unknown.

CCR4 is a chemokine receptor known to be selectively expressed by Th2 cells, regulatory T cells (Treg) and skin-homing effector/memory T cells (Imai et al., 1999; Iellem et al., 2001; Yoshie et al., 2001). Previously, we and others showed that ATL cells in the majority of cases are strongly positive for surface CCR4 (Yoshie et al., 2002; Ishida et al., 2003; Nagakubo et al., 2007). Ishida et al. have also demonstrated a significant correlation of CCR4 expression with skin involvement and poor prognosis in ATL patients (Ishida et al., 2003). Furthermore, several groups have reported that FOXP3, a forkhead/winged helix transcription factor and a specific marker of Treg (Hori et al., 2003), is frequently expressed in ATL (Karube et al., 2004; Matsubara et al., 2005), supporting the notion that at least a fraction of ATL cases are derived from Treg.

It is also notable that primary ATL cells express CCR4 at levels much higher than normal resting CD4^{+}CD25^{+} T cells (Nagakubo et al., 2007). Given

that CCR4 is not inducible by Tax (Yoshie et al., 2002), transcription factor(s) constitutively active in ATL cells may be responsible for CCR4 expression. Here, we demonstrate that Fra-2, one of the AP-1 family members (Shaulian and Karin, 2002; Eferl and Wagner, 2003), is aberrantly expressed in primary ATL cells. We further demonstrate that the Fra-2/JunD heterodimer plays a major role in both CCR4 expression and cell proliferation in ATL cells. Furthermore, we demonstrate that the proto-oncogenes c-Myb, BCL-6 and MDM2 (Oh and Reddy, 1999; Pasqualucci et al., 2003; Vargas et al., 2003) are the downstream target genes of the Fra-2/JunD heterodimer and are highly expressed in primary ATL cells. Thus, aberrantly expressed Fra-2 in association with JunD may be involved in ATL oncogenesis.

Results

Analysis of CCR4 promoter activity in ATL-derived cell lines

To examine the transcriptional regulation of CCR4 expression in ATL, we constructed a reporter plasmid carrying the CCR4 promoter region from −983 to +25 bp (the major transcriptional initiation site, +1) fused with the luciferase reporter gene. As shown in Figure 1a, pGL3-CCR4 (−983/+25) showed much stronger promoter activities in ATL cell lines (HUT102 and ST1) than in control human T-cell lines (MOLT-4 and Jurkat). We therefore generated a series of 5'-truncated promoter plasmids and examined their activity in ATL cell lines. As shown in Figure 1b, the promoter region from −151 to −96 bp was the major positive regulatory region in both cell lines. The TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) revealed various potential transcriptional elements in this region (Figure 1c). To identify the actual regulatory elements, we introduced a mutation in each potential element and examined the promoter activity in ATL cell lines. As shown in Figure 1d, a mutation at the AP-1 site or the GATA-3 site significantly reduced the promoter activity. Moreover, double mutations targeting both sites further reduced the promoter activity.

Constitutive expression of Fra-2, JunB and JunD in primary ATL cells

AP-1 is known to be involved in tumorigenesis (Shaulian and Karin, 2002; Eferl and Wagner, 2003), while GATA-3 regulates Th2-type gene expression (Rengarajan et al., 2000). Therefore, we focused on AP-1 in the subsequent study. AP-1 constitutes a heterodimer of a member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and a member of the Jun family (c-Jun, JunB and JunD) or a homodimer of the Jun family (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Even though AP-1 was shown to be constitutively active in primary ATL cells (Mori et al., 2000), it has not been clarified which members of AP-1 are actually
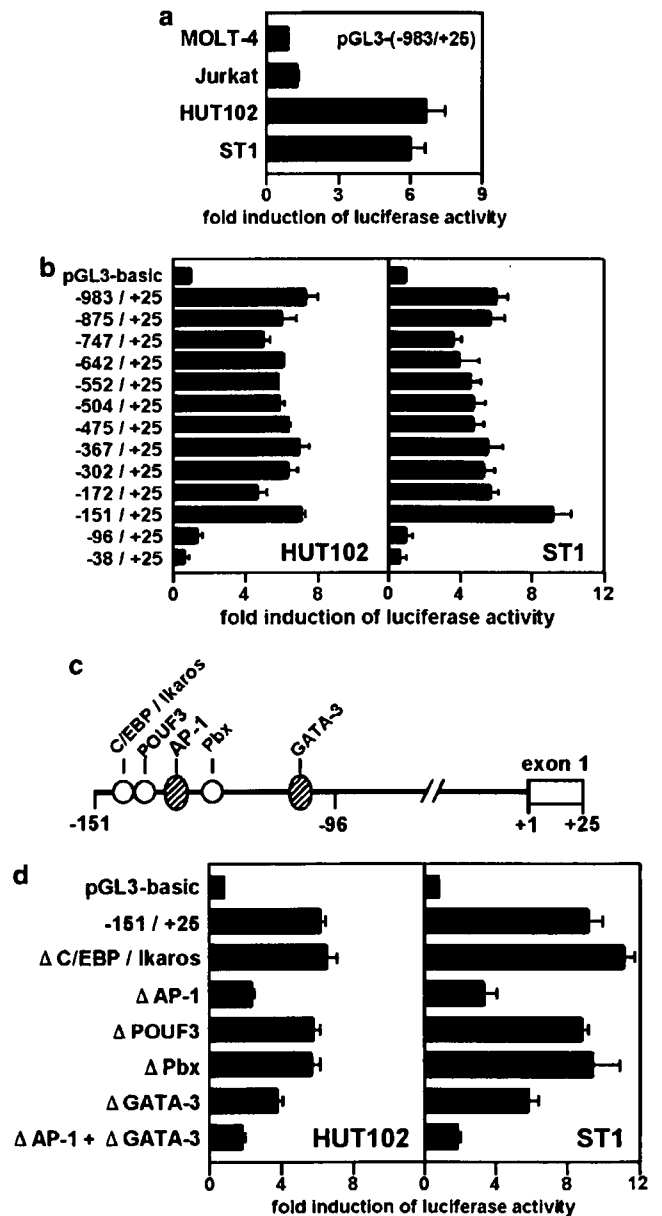

Figure 1 Identification of regulatory elements in the CCR4 promoter. Cells were transfected with pSV-β-galactosidase and pGL3-basic or pGL3-basic inserted with the CCR4 promoter regions as indicated. After 24–27 h, luciferase assays were performed. Promoter activation was expressed by the fold induction of luciferase activity in cells transfected with the CCR4 promoter–luciferase constructs versus cells transfected with the control pGL3-basic. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. (a) Selective activation of the CCR4 promoter in adult T-cell leukemia (ATL) cell lines. MOLT-4 and Jurkat: control human T-cell lines; HUT102 and ST1: ATL cell lines. (b) Deletion analysis. The promoter region from −151 to −96 bp is necessary and sufficient for reporter gene expression in the two ATL cell lines. (c) The schematic depiction of potential regulatory elements in the promoter region from −151 to −96 bp. (d) Mutation analysis. ΔC/EBP/Ikaros (from TCTTGGGAAA TGA to TCTTGCAAAATGA), ΔAP-1 (from AATGACTAAGA to AATGTCAAAGA), ΔPOUF3 (from CTTGGGAAATGA to CTTGGGAGGTGA), ΔPbx (from AAGAATCAT to AAGA CCCAT) and ΔGATA-3 (from TTCTATCAA to TTCTGACAA). The potential AP-1 and GATA-3 sites present within the −151 to −96 bp region are the major elements for CCR4 promoter activation in the two ATL cell lines.

expressed in primary ATL cells. We therefore first examined the mRNA expression of the AP-1 family members in primary ATL cells freshly isolated from patients in comparison with normal CD4$^+$ T cells in resting, activated and Th1/Th2-polarized conditions (Figure 2a). As reported previously (Yoshie et al., 2002; Nagakubo et al., 2007), primary ATL cells

consistently expressed CCR4 at levels much higher than various normal CD4$^+$ T-cell populations, including Th2-polarized cultured T cells. Furthermore, primary ATL cells consistently expressed Fra-2 in sharp contrast to various normal CD4$^+$ T-cell populations that were essentially negative for Fra-2 expression. Similar to various normal CD4$^+$ T-cell populations, primary ATL
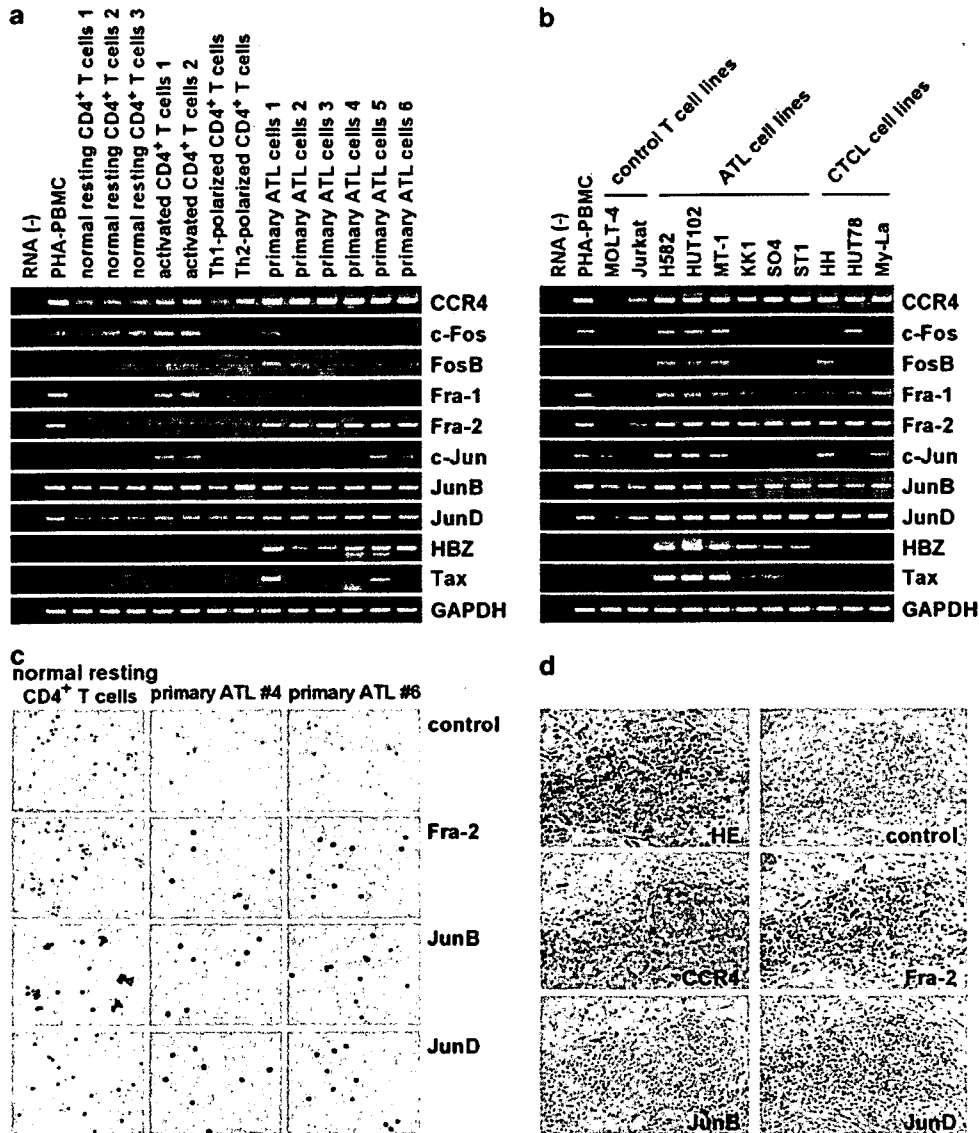

Figure 2 Constitutive expression of Fra-2, JunB and JunD in adult T-cell leukemia (ATL). (a) Reverse transcription (RT)–PCR analysis for the expression of the AP-1 family in normal T cells and primary ATL cells. Normal resting CD4$^+$ T cells (purity, >96%) from healthy donors (n = 3), activated CD4$^+$ T cells from normal donors (n = 2), Th1-polarized cultured CD4$^+$ T cells, Th2-polarized cultured CD4$^+$ T cells and freshly isolated primary ATL cells (>90% leukemic cells) from patients (n = 6) were examined as indicated. Normal peripheral blood mononuclear cells treated with phytohemagglutinin (PHA-PBMC) served as a positive control. GAPDH served as a loading control. The representative results from at least two separate experiments are shown. (b) RT–PCR analysis for the expression of the AP-1 family in human T-cell lines. Two control human T-cell lines, six ATL cell lines and three CTCL cell lines were examined as indicated. PHA-PBMC served as a positive control. GAPDH served as a loading control. The representative results from two separate experiments are shown. (c) Immunocytochemical staining for Fra-2, JunB and JunD in normal CD4$^+$ T cells and primary ATL cells. Normal CD4$^+$ T cells from healthy donors (purity, >96%) and primary ATL cells (leukemic cells, >90%) from two patients were stained with anti-Fra-2, anti-JunB or anti-JunD. Normal rabbit IgG was used as the negative control (control). The representative results from two separate experiments are shown. Original magnification: × 400. (d) Immunohistochemical staining of CCR4, Fra-2, JunB and JunD in ATL skin lesions. Tissue sections from ATL skin lesions (n = 6) were stained with anti-CCR4, anti-Fra-2, anti-JunB or anti-JunD. Mouse IgG$_1$ and normal rabbit IgG were used as the negative controls (control). Tissue sections were counterstained using Gill's hematoxylin. The representative results from a single donor are shown. Original magnification: × 400.

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cells also constitutively expressed JunD and JunB even though JunD expression appeared to be upregulated in primary ATL cells. Other members of the AP-1 family were mostly negative in primary ATL cells, while activated normal CD4$^+$ T cells expressed c-Fos, Fra-1 and c-Jun at high levels. There was no correlation in expression between Fra-2 and the virally encoded HTLV-1 basic leucine zipper factor HBZ or Tax in primary ATL cells. We also confirmed that Fra-2 is not inducible by Tax using JPX-9, a subline of Jurkat carrying the HTLV-1 Tax gene under the control of the metallothionein gene promoter (Nagata et al., 1989; data not shown). Thus, the constitutive expression of Fra-2 is highly unique for primary ATL cells.

We also examined expression of the same set of genes in various human T-cell lines. As shown in Figure 2b, compared to control T-cell lines, ATL cell lines consistently expressed CCR4 and Fra-2 at high levels. ATL cell lines also expressed JunB and JunD at high levels. HTLV-1 Tax has been shown to induce various AP-1 family members (Nagata et al., 1989; Iwai et al., 2001), which may be involved in HTLV-1 gene expression and cell proliferation (Jeang et al., 1991). Consistently, ATL cell lines expressing Tax (H582, HUT102 and MT-1) also expressed other AP-1 family members at low levels. Cutaneous T-cell lymphomas (CTCLs) are a subset of HTLV-1-negative T-cell lymphomas resembling ATL and known to be frequently positive for CCR4 (Kim et al., 2005). CTCL cell lines were also found to strongly express CCR4, Fra-2, JunB and JunD. Thus, the constitutive expressions of Fra-2, JunB and JunD were shared by CCR4-expressing ATL and CTCL cell lines.

We also examined the Fra-2, JunB and JunD protein expression in freshly isolated primary ATL cells and normal resting CD4$^+$ T cells. As shown in Figure 2c, primary ATL cells were indeed stained strongly positive for Fra-2, while normal CD4$^+$ T cells were totally negative for Fra-2. Primary ATL cells were also strongly positive for JunB and JunD, while normal CD4$^+$ T cells were variably positive for JunB and JunD at the single cell level. These results were highly consistent with the results from reverse transcription (RT)–PCR; Figure 2a). We also confirmed the CCR4, Fra-2, JunB and JunD protein expression in skin-infiltrating ATL cells (Figure 2d).

Activation of the CCR4 promoter by Fra-2/JunB and Fra-2/JunD heterodimers
AP-1 is known to function as a heterodimer of a member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and a member of the Jun family (c-Jun, JunB and JunD) or a homodimer of the Jun family (Shaulian and Karin, 2002; Eferl and Wagner, 2003). We, therefore, next examined the activation of the CCR4 promoter by individual AP-1 family members singly or in combination. As recipients, we used two T-cell lines, namely, MOLT-4 and Jurkat. The expression levels of AP-1 members, including Fra-2, JunB and JunD, were very low in these cell lines (Figure 2b). As shown in Figure 3a, only Fra-2/JunB

or Fra-2/JunD potently activated the CCR4 promoter in both cell lines. We confirmed that other members of the AP-1 family (c-Fos, FosB, Fra-1 and c-Jun) were transcriptionally active by using a synthetic promoter containing two tandem AP-1 consensus-binding sites (pGL3-2xAP-1; Figure 3b). Thus, among the AP-1 family members, only the Fra-2/JunB and Fra-2/JunD heterodimers are uniquely capable of activating the CCR4 promoter. This is highly consistent with their constitutive expression in primary ATL cells (Figure 2a).

Recently, the mRNA of HTLV-1 HBZ has been shown to be expressed in primary ATL cells (Satou et al., 2006). We indeed observed the expression of HBZ in some primary ATL samples (Figure 2a). HBZ has been shown to activate JunB homodimer- or JunD homodimer-dependent transcription (Basbous et al., 2003; Thebault et al., 2004). Therefore, we also examined the effects of HBZ as well as Tax on the CCR4 promoter in MOLT-4 and Jurkat cells. As shown in Figure 3c, HBZ alone or in combination with Fra-2, JunB, JunD, Fra-2/JunB or Fra-2/JunD showed no effect on the activation of the CCR4 promoter. Similarly, Tax had no significant effect on the CCR4 promoter either alone or in combination with Fra-2, JunB, JunD, Fra-2/JunB or Fra-2/JunD. Thus, HTLV-1 encoded HBZ or Tax neither activates the CCR4 promoter nor affects its activation by Fra-2/JunB or Fra-2/JunD.

We have also confirmed that GATA-3 is constitutively expressed in primary ATL cells and activates the CCR4 promoter (data not shown). In normal CD4$^+$ T cells, GATA-3 may be responsible for the selective expression of CCR4 in Th2 cells (Imai et al., 1999; Rengarajan et al., 2000).

Specific binding of Fra-2, JunB and JunD to the AP-1 site in the CCR4 promoter
We next examined the specific binding of AP-1 family members to the AP-1 site in the CCR4 promoter using the NoShift transcription factor assay, an enzyme-linked immunosorbent assay (ELISA)-like colorimetric assay that is an alternative to the electrophoretic mobility shift assay. As shown in Figure 4a, when the nuclear extracts of two control T-cell lines (MOLT-4 and Jurkat) were used, the specific binding of any AP-1 family members to the AP-1 site of the CCR4 promoter was hardly observed. On the other hand, when the nuclear extracts of two ATL cell lines (HUT102 and ST1) were used, we detected a high level of specific binding of Fra-2, JunB and JunD to the AP-1 site. These results are highly consistent with the results from RT–PCR analyses (Figure 2b) and the luciferase reporter assays (Figure 3a).

By using the chromatin immunoprecipitation (ChIP) assay, we further examined the binding of Fra-2, JunB and JunD to the AP-1 site of the CCR4 promoter *in vivo*. As shown in Figure 4b, we detected specific binding of Fra-2, JunB and JunD to the AP-1 site of the endogenous CCR4 promoter in primary ATL cells but not in normal CD4$^+$ T cells. These results further

support the hypothesis that the CCR4 gene is a direct target gene of Fra-2/JunB and Fra-2/JunD heterodimers in primary ATL cells.

Effects of Fra-2, JunB and JunD small interfering RNAs on CCR4 expression and cell proliferation

To examine the role of Fra-2, JunB and JunD in CCR4 expression and cell proliferation in ATL cells, we next employed the small interfering RNA (siRNA) knock-down technique. As shown in Figure 5a, Fra-2 siRNA, JunB siRNA and JunD siRNA specifically reduced Fra-2 mRNA, JunB mRNA and JunD mRNA, respectively, in two ATL cell lines. On the other hand, control siRNA showed no such effect. Under these

conditions, we examined the effects of these siRNAs on CCR4 expression and cell growth. As shown in Figure 5b, Fra-2 siRNA and JunD siRNA reduced CCR4 expression by approximately 50% in both cell lines, whereas JunB siRNA had hardly any inhibitory effect and control siRNA showed no inhibitory effect. Furthermore, as shown in Figure 5c, Fra-2 siRNA and JunD siRNA significantly reduced cell proliferation in both cell lines, whereas JunB siRNA or control siRNA did not. None of the siRNAs affected the growth of the control T-cell lines MOLT-4 and Jurkat. We also compared the effects of single and double knockdown of Fra-2 and JunD on cell growth in two ATL cell lines (Figure 5d). Compared to the effect of single knock-down of Fra-2 or JunD, no additive effect was observed by double knockdown of Fra-2 and JunD in both cell lines. These results may be consistent with the notion that Fra-2 and JunD promote growth in ATL cell lines by functioning as a heterodimer.

To further demonstrate the growth-promoting effects of Fra-2 and JunD, we performed stable transfection of Fra-2 and JunD in the control T-cell line Jurkat. As shown in Figure 5e, Jurkat cells overexpressing Fra-2 or JunD (see inset) indeed showed enhanced growth compared to those transfected with the vector alone. We were, however, unable to isolate Fra-2/JunD double transfectants in Jurkat, probably because of some adverse effects on Jurkat cells by the overexpression of both Fra-2 and JunD.
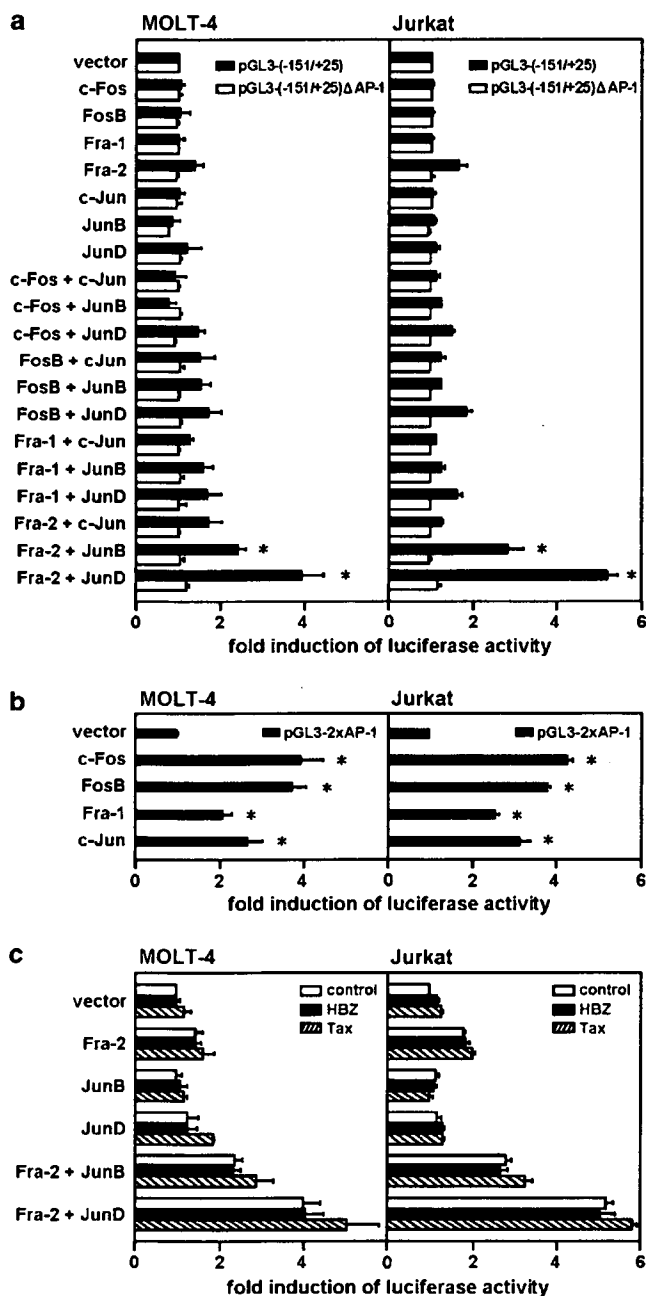

Figure 3 Transactivation of the CCR4 promoter by Fra-2/JunD and Fra-2/JunB. (a) Transactivation of the CCR4 promoter with or without the AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-CCR4 (−151/+25) or pGL3-CCR4 (−151/+25)ΔAP-1 and an expression vector for c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD or a control vector as indicated. After 24–27 h, luciferase assays were performed in triplicate. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated AP-1 expression vector versus cells transfected with the vector alone. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. *P < 0.05. (b) Transactivation of a synthetic promoter with two copies of the consensus AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-2xAP-1 and an expression vector for c-Fos, FosB, Fra-1, c-Jun or the vector alone as indicated. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated expression vector versus cells transfected with a control vector. After 24–27 h, luciferase assays were performed in triplicate. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. *P < 0.05. (c) Effect of HBZ or Tax on the activation of the CCR4 promoter. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and the pGL3-basic vector or pGL3-CCR4 (−151/+25) and an expression vector for Fra-2, JunB, JunD or a control vector and an expression vector for HBZ, Tax or a control vector as indicated. After 24–27 h, luciferase assays were performed in triplicate. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated expression vector versus cells transfected with a control vector. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments.
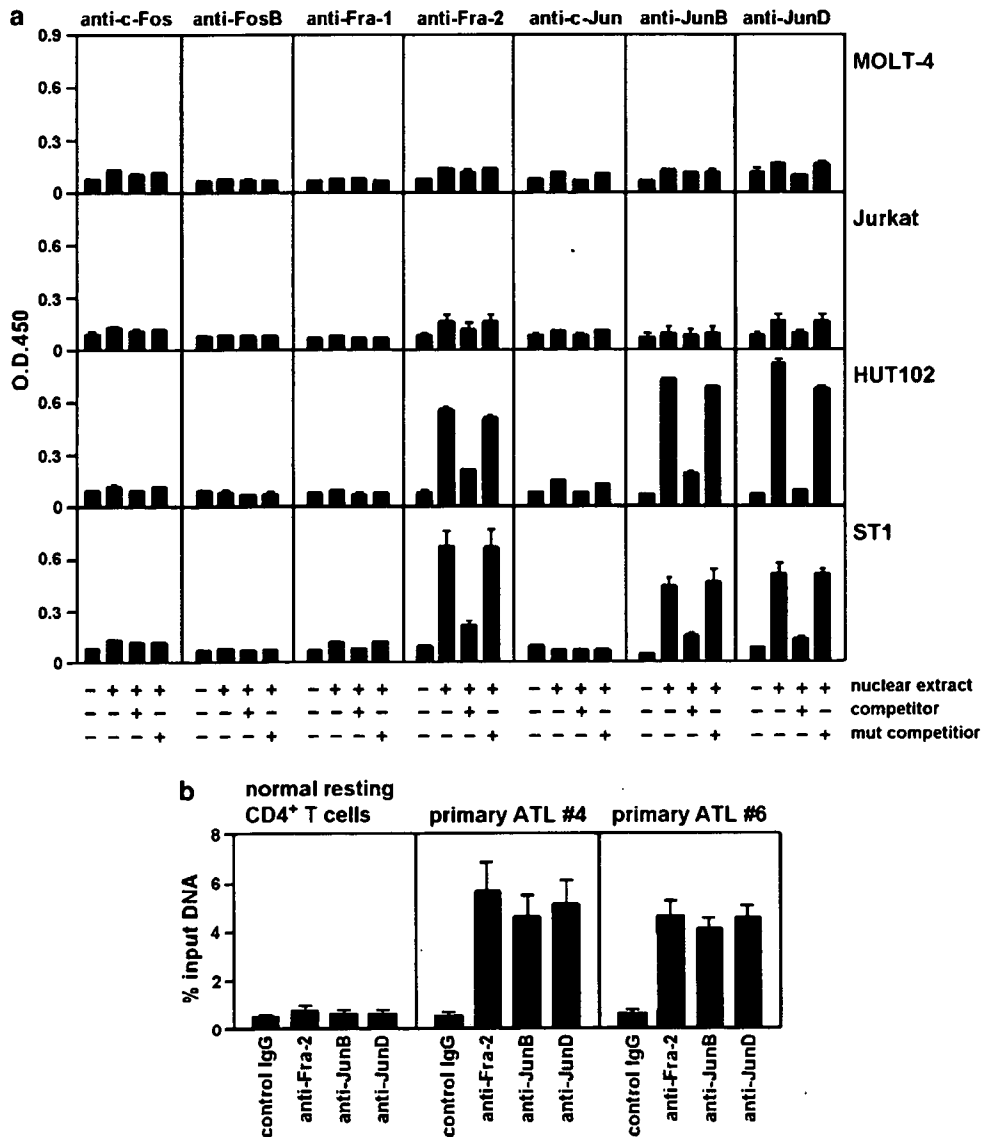
Figure 4 Specific binding of Fra-2, JunB and JunD to the AP-1 site in the CCR4 promoter. (a) NoShift assay. Nuclear extracts were prepared from two control T-cell lines (MOLT-4 and Jurkat) and two adult T-cell leukemia (ATL) cell lines (HUT102 and ST1). Nuclear proteins that bound to the biotinylated AP-1 site oligonucleotide (TGGGAAATGACTAAGAATCAT) were captured on an avidin-coated plate and detected by anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2, anti-c-Jun, anti-JunB or anti-JunD, as indicated. Specificity was determined by adding unlabeled probe (competitor; TGGGAAATGACTAAGAATCAT) or mutant probe (mut competitor; TGGGAAATGTCAAAGAATCAT; differences underlined). Each bar represents the mean ± s.e.m. from three separate experiments. (b) Chromatin immunoprecipitation (ChIP) assay. Chromatins from normal CD4$^+$ T cells from healthy donors (purity, >96%) and primary ATL cells from two patients (leukemic cells, >90%) were immunoprecipitated with anti-Fra-2, anti-JunD or control IgG. The amounts of precipitated DNA relative to total input DNA were quantified by real-time PCR for the CCR4 promoter region containing the AP-1 site. Each bar represents the mean ± s.e.m. from three separate experiments.

Identification of downstream target genes of the Fra-2/ JunD heterodimer in ATL cells

To identify the target genes of Fra-2 in ATL cells, we compared the gene expression profiles of ATL-derived ST1 cells transfected with Fra-2 siRNA or control siRNA using the Affymetrix high-density oligonucleotide microarray. As summarized in Figure 6a, at least 49 genes were downregulated more than threefold by Fra-2 siRNA. The classification of these genes according to their biological functions shows that Fra-2 promotes the expression of genes involved in signal transduction (10 genes), protein biosynthesis and modification

(8 genes) and transcription (6 genes); it also stimulates the expression of 10 genes of unknown function. Most notably, the list includes the proto-oncogenes c-Myb, BCL-6 and MDM2 (Oh and Reddy, 1999; Pasqualucci et al., 2003; Vargas et al., 2003). As shown in Figure 6b, RT–PCR analysis verified that not only Fra-2 siRNA but also JunD siRNA downregulated these proto-oncogenes in two ATL cell lines. Therefore, c-Myb, BCL-6 and MDM2 are the downstream target genes of the Fra-2/JunD heterodimer in both cell lines. This prompted us to examine the expression of c-Myb, BCL-6 and MDM2 in freshly isolated primary ATL cells by
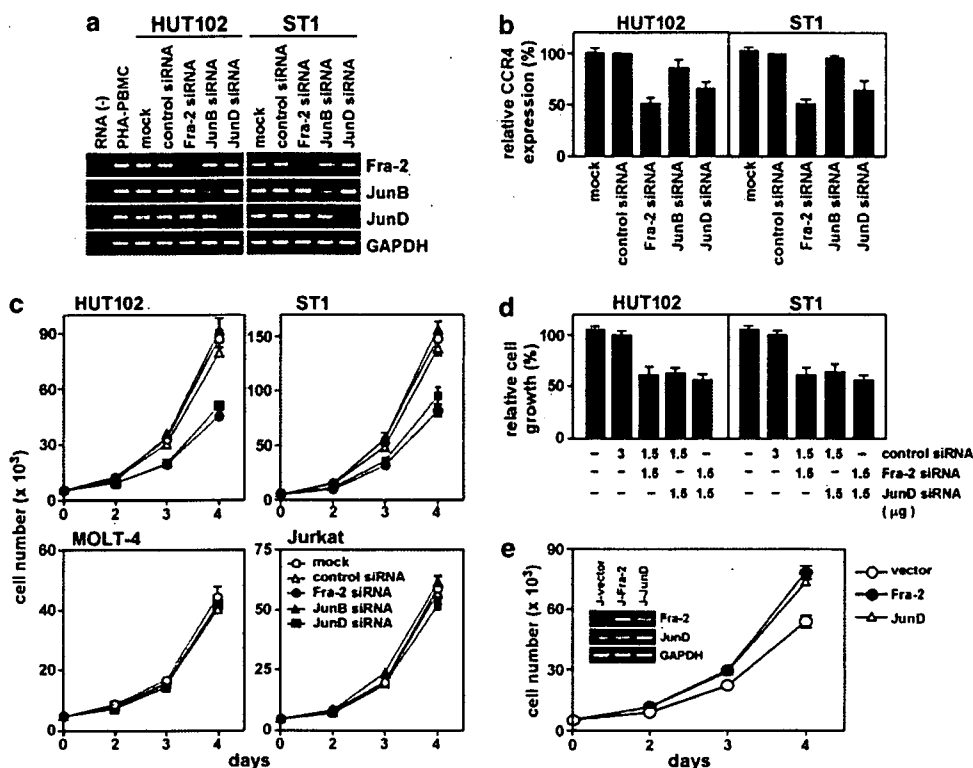
Figure 5 Dominant role of Fra-2/JunD in CCR4 expression and cell proliferation in adult T-cell leukemia (ATL). (a) Reverse transcription (RT)–PCR analysis to determine the effect of siRNAs. HUT102 and ST1 were transfected with control siRNA or siRNA for Fra-2, JunB or JunD. After 48 h, total RNA was prepared. The representative results from three separate experiments are shown. (b) Real-time RT–PCR analysis for CCR4 expression. HUT102 and ST1 were transfected with control siRNA or siRNA for Fra-2, JunB or JunD. After 48 h, total RNA was prepared and real-time RT–PCR was performed for CCR4 and 18S ribosomal RNA (an internal control). Data are presented as the mean ± s.e.m. of three separate experiments. (c) Effect of siRNAs on cell growth. HUT102, ST1, MOLT-4 and Jurkat were transfected with control, Fra-2, JunB and JunD siRNAs and cultured in a 96-well plate at 0.5×10^4 cells per well. At the indicated time points, viable cell numbers were determined using a FACSCalibur by gating out cells stained with propidium iodide. Data are shown as the mean ± s.e.m. of three separate experiments. (d) Effect of double knockdown of Fra-2 and JunD on cell growth. HUT102 and ST1 were transfected with control, Fra-2 and JunD siRNAs as indicated and cultured in a 96-well plate at 0.5×10^4 cells per well. At 4 days, viable cell numbers were determined on a FACSCalibur by gating out dead cells stained with propidium iodide. Data are shown as the mean ± s.e.m. of three separate experiments. (e) Effect of stable expression of Fra-2 and JunD on cell growth. Jurkat cells were transfected with a control IRES-EGFP expression vector or an IRES-EGFP expression vector for Fra-2 or JunD. Stable transfectants expressing green fluorescence protein were sorted and cultured in a 96-well plate at 0.5×10^4 cells per well. At the indicated time points, viable cell numbers were determined on a FACSCalibur by gating out dead cells stained with propidium iodide. Data are shown as the mean ± s.e.m. of three separate experiments.

RT–PCR. As shown in Figure 6c, we indeed detected the constitutive expression of c-Myb, BCL-6 and MDM2 at high levels in primary ATL cells. In sharp contrast, normal resting CD4$^+$ T cells hardly expressed these proto-oncogenes.

Discussion

The AP-1 transcription factors function as homodimers or heterodimers formed by Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2) and the ATF family proteins (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Most of them are rapidly and transiently induced by extracellular stimuli that trigger the activation of the Janus kinase (JNK), extracellular signal regulated protein kinases 1 and 2 (ERK1/2) or p38 mitogen-activated protein (MAP) kinase pathways (Shaulian and Karin, 2002; Eferl and Wagner, 2003). The AP-1 family

is known to be involved in cellular proliferation, oncogenesis and even tumor suppression, depending on the combination of AP-1 proteins and the cellular context (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Previously, by using the AP-1 site of the IL-8 promoter, Mori et al. demonstrated a strong Tax-independent expression of JunD in primary ATL cells (Mori et al., 2000). In the present study, we have shown that Fra-2 is constitutively expressed at high levels in primary ATL cells (Figure 2a). Furthermore, except for JunB and JunD, other members of the AP-1 family are mostly negative in primary ATL cells (Figure 2a). Therefore, as demonstrated in the present study, the Fra-2/JunD and Fra-2/JunB heterodimers may be the major AP-1 factors constitutively active in primary ATL cells.

It has been shown that HTLV-1 Tax induces the expression of various AP-1 family members such as c-Fos, Fra-1, c-Jun and JunD (Nagata et al., 1989; Iwai et al., 2001). We indeed observed the expression of

a siRNA

Gene symbol	Description	Probe set	p-value	Fold change
MYB	v-myb myeloblastosis viral oncogene homolog (avian)	215152_at	p < 1e-07	9.3
TAGAP	T-cell activation GTPase activating protein	1552542_s_at	p < 1e-07	7.5
PLK2	polo-like kinase 2 (Drosophila)	201939_at	1E-07	6.7
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	200664_s_at	p < 1e-07	6.1
TFPI	tissue factor pathway inhibitor	210665_at	2E-07	6.1
STK17B	serine/threonine kinase 17b (apoptosis-inducing)	243797_at	1E-07	5.5
RGS2	regulator of G-protein signalling 2, 24kDa	202388_at	p < 1e-07	5.5
KLHL24	kelch-like 24 (Drosophila)	221985_at	1.4E-06	5.3
CHN1	chimerin (chimaerin) 1	212624_s_at	1E-07	5.2
MT1F	metallothionein 1F (functional)	217165_x_at	1E-07	4.7
BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	203140_at	3E-07	4.7
NR1D2	nuclear receptor subfamily 1, group D, member 2	209750_at	p < 1e-07	4.5
SPSB3	splA/ryanodine receptor domain and SOCS box containing 3	46256_at	1E-07	4.5
CLEC2B	C-type lectin domain family 2, member B	209732_at	6E-07	4.5
F2R	coagulation factor II (thrombin) receptor	1569642_at	1E-07	4.4
LOC285513	hypothetical protein LOC285513	1554697_at	6E-07	4.4
FBXO32	F-box protein 32	225803_at	6E-07	4.3
PAQR3	Progestin and adipoQ receptor family member III	240622_at	5E-07	4.2
SLC30A1	solute carrier family 30 (zinc transporter), member 1	228181_at	p < 1e-07	4.1
PRR8	proline rich 8	1554096_a_at	2.4E-06	4.0
C8orf166	Chromosome 8 open reading frame 166	227366_at	1E-07	3.9
RPL35	Similar to 60S ribosomal protein L35	211456_x_at	1.7E-06	3.8
MKRN1	makorin, ring finger protein, 1	209845_at	3.3E-06	3.8
DUSP4	dual specificity phosphatase 4	204015_s_at	p < 1e-07	3.6
GPR18	G protein-coupled receptor 18	210279_at	1E-07	3.6
MGC16635	Kelch domain containing 7B	236285_at	4E-07	3.6
WASPIP	Wiskott-Aldrich syndrome protein interacting protein	231182_at	6.3E-06	3.6
LGR4	leucine-rich repeat-containing G protein-coupled receptor 4	218326_s_at	p < 1e-07	3.5
SOX4	SRY (sex determining region Y)-box 4	201416_at	1E-07	3.5
LOC153222	adult retina protein	1554229_at	3E-07	3.5
PAN3	poly A-specific ribonuclease subunit PAN3	239393_at	2.1E-06	3.5
SQSTM1	sequestosome 1	201471_s_at	2E-07	3.4
LGMN	legumain	201212_at	9E-07	3.4
MT2A	metallothionein 2A	212185_x_at	3.4E-06	3.3
DBP	D site of albumin promoter (albumin D-box) binding protein	209782_s_at	5.2E-06	3.3
CKAP2	cytoskeleton associated protein 2	1554264_at	0.000001	3.3
YTHDF3	YTH domain family, member 3	1564053_s_at	1E-07	3.2
ESTs	ESTs	241743_s_at	3.8E-06	3.2
ESTs	ESTs	230494_at	2E-07	3.1
HSPA1B	heat shock 70kDa protein 1B	202581_at	8E-07	3.1
CTTN	cortactin	214073_at	0.000001	3.1
DUSP10	dual specificity phosphatase 10	216501_s_at	1.7E-06	3.1
ZA20D2	zinc finger, A20 domain containing 2	217741_a_at	p < 1e-07	3.1
ESTs	CDNA clone IMAGE:4814292	1569852_x_at	2.1E-06	3.0
ESTs	CDNA FLJ31683 fis, clone NT2RI2005353	228315_at	4.4E-06	3.0
ING3	inhibitor of growth family, member 3	231663_at	1.7E-06	3.0
SGK	serum/glucocorticoid regulated kinase	201738_at	7.9E-06	3.0
EIF4G3	eukaryotic translation initiation factor 4 gamma, 3	1554309_at	1E-07	3.0
MDM2	Mdm2, p53 binding protein (mouse)	217373_x_at	7.2E-06	3.0

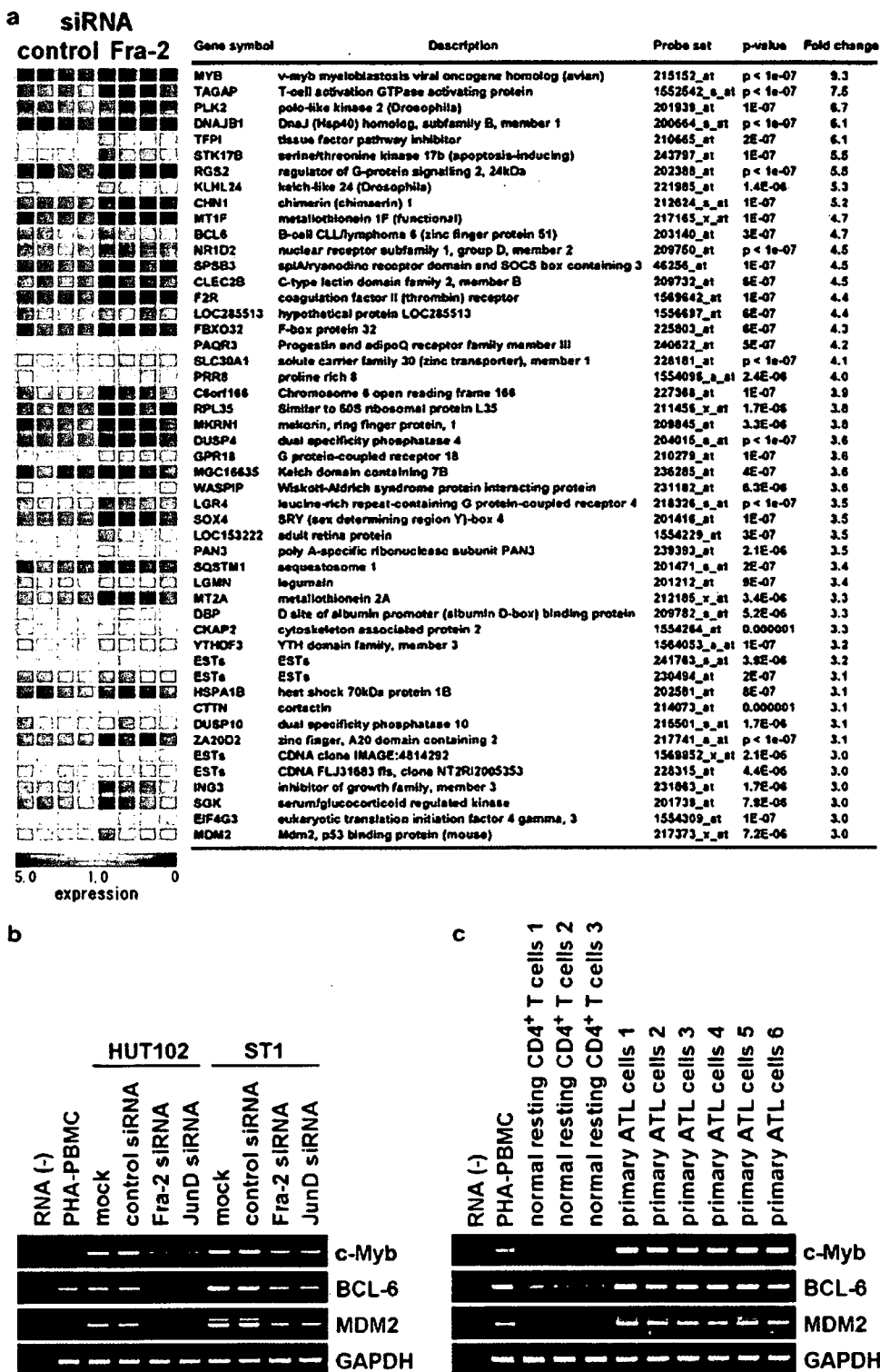5.0   1.0   0
expression

b

c

Figure 6   Identification of downstream target genes of Fra-2 in adult T-cell leukemia (ATL). (a) Microarray analysis. ST1 cells were transfected with control siRNA or Fra-2 siRNA. After 48 h, microarray analysis was performed using the Affymetrix GeneChip HG-U133 Plus 2.0 array. Four independent transfection samples were analysed for each group. Each column represents the expression level of a given gene in an individual sample. Red represents increased expression and blue represents decreased expression relative to the normalized expression of the gene across all samples. We computed the statistical significance level for each gene between the Fra-2-knockdown group and the control group with a mean fold change of > 3 by the t-test ($P < 10^{-5}$). (b) Reverse transcription (RT)–PCR analysis. HUT102 and ST1 cells were transfected with control siRNA or siRNA for Fra-2 or JunD. After 48 h, the expression of c-Myb, BCL-6, MDM2 and GAPDH was determined by RT–PCR. The representative results from three separate experiments are shown. (c) RT–PCR analysis. Normal CD4+ T cells from healthy donors ($n = 3$; purity, > 96%) and PBMC from ATL patients ($n = 6$; leukemic cells, > 90%) were examined for the expression of c-Myb, BCL-6 and MDM2 by RT–PCR. The representative results from two separate experiments are shown.

various AP-1 family members in primary ATL cells (patient nos. 1 and 5) and in some ATL cell lines expressing Tax (Figures 2a and b). However, the constitutive expression of Fra-2, JunD and JunB in freshly isolated primary ATL cells and ATL cell lines is apparently independent from Tax expression (Figures 2a and b). This is further supported by the finding that CCR4-expressing HTLV-1-negative CTCL cell lines also constitutively express Fra-2, JunB and JunD at high levels (Figure 2). By using JPX-9, which is a subline of Jurkat carrying the HTLV-1 Tax gene under the control of the metallothionein gene promoter (Nagata et al., 1989), we have also confirmed that Fra-2 is not inducible by Tax (data not shown).

The CCR4 promoter was potently activated by the Fra-2/JunB and Fra-2/JunD heterodimers (Figure 3a). Fra-2, JunB and JunD were also shown to bind specifically to the AP-1 site in the CCR4 promoter in vitro by the NoShift binding assays and in vivo by the ChIP assays (Figures 4a and b). By using the siRNA knockdown technique, however, only Fra-2 siRNA and JunD siRNA efficiently suppressed CCR4 expression and cell growth in ATL cell lines (Figure 5). On the other hand, JunB siRNA showed little such effect (Figure 5). Therefore, it is likely that, at least in terms of CCR4 expression and cell proliferation, the Fra-2/JunD heterodimer plays a more dominant role than the Fra-2/JunB heterodimer in ATL cells. It thus remains to be determined whether the Fra-2/JunB heterodimer has any specific functions in ATL.

The most striking finding in the present study is the aberrant expression of Fra-2 in primary ATL cells. Fra-2 expression is essentially absent in normal CD4^{+} T cells under various conditions thus far examined (Figures 2a and c). Physiologically, Fra-2 is known to be expressed by various epithelial cells and in cartilaginous structures and has been shown to be required for efficient cartilage development (Karreth et al., 2004). With regard to lymphoid cells, developing murine thymocytes were reported to express Fra-2 (Chen et al., 1999). Previous studies have shown that individual homodimeric and heterodimeric AP-1 proteins have different functional properties and target genes (Shaulian and Karin, 2002; Eferl and Wagner, 2003). However, little is known about the target genes of Fra-2 and even less is known about the oncogenic role of Fra-2 in human malignancies. In this study, we have shown that CCR4 is the direct target gene of Fra-2 in association with JunD in ATL cells. Furthermore, we have shown that at least 49 genes are downregulated more than threefold in the ATL cell line ST-1 by Fra-2 siRNA (Figure 6). Among these genes, the proto-oncogenes c-Myb, BCL-6 and MDM2 (Oh and Reddy, 1999; Pasqualucci et al., 2003; Vargas et al., 2003) are further confirmed to be dependent on the Fra-2/JunD heterodimer and to be expressed at high levels in primary ATL cells (Figure 6). It remains to be seen whether the Fra-2/JunD heterodimer directly induces these proto-oncogenes or indirectly maintains their expression by promoting cell growth.

c-Myb is the genomic homologue of the avian myeloblastosis virus oncogene v-Myb. c-Myb is widely expressed in immature hematopoietic cells and also in various leukemias and carcinomas (Oh and Reddy, 1999; Shetzline et al., 2004; Hess et al., 2006). The target genes of c-Myb include the anti-apoptotic genes BCL-2 and BCL-X$_L$ and also c-Myc (Ramsay et al., 2003). Thus, c-Myb may promote the survival of ATL cells via BCL-2 and BCL-X$_L$ (Galonek and Hardwick, 2006) and also cell cycle progression via c-Myc (Dang, 1999). BCL-6 was originally identified as the target gene of recurrent chromosomal translocations affecting 3q27 in non-Hodgkin's lymphoma. The expression of BCL-6 is frequently upregulated in diffuse large-cell lymphoma and follicular lymphoma through promoter substitution or somatic promoter point mutations (Ye et al., 1993; Migliazza et al., 1995; Chang et al., 1996). Frequent expression of BCL-6 has also been reported in some T-cell lymphomas (Kerl et al., 2001). The BCL-6 protein has been shown to exert cell-immortalizing and anti-senescence activities (Shvarts et al., 2002; Pasqualucci et al., 2003). Thus, BCL-6 may also inhibit apoptosis and promote cell cycle progression in ATL. The MDM2 protein is a negative regulator of p53 and suppresses p53-mediated cell cycle arrest and apoptosis (Vargas et al., 2003). Elevated expression of MDM2 has been demonstrated in various types of human cancer (Rayburn et al., 2005). Given that only a minor fraction of ATL cases have mutations affecting p53 (Cesarman et al., 1992), the elevated expression of MDM2 may contribute to the functional downregulation of p53 in the majority of ATL cases.

CTCLs are a group of T-cell lymphomas derived from skin-homing memory T cells. CTCLs are not associated with HTLV-1 infection but resemble ATL and frequently express CCR4 (Ferenczi et al., 2002; Kim et al., 2005). Furthermore, CCR4 expression has been shown to be a consistent feature of the large-cell transformation of mycosis fungoides (Jones et al., 2000). In the present study, we have shown that CTCL cell lines also express Fra-2, JunB and JunD at high levels (Figure 2b). Therefore, it is likely that aberrantly expressed Fra-2 in association with Jun proteins, particularly JunD, is also involved in CCR4-expression and cell proliferation in CTCLs.

In conclusion, we have shown that aberrantly expressed Fra-2 in association with JunD is responsible for CCR4 expression in ATL and is also likely to play an important role in ATL oncogenesis in part by inducing the expression of the proto-oncogenes c-Myb, BCL-6 and MDM2. Future studies are necessary to elucidate how the Fra-2/JunD heterodimer induces the expression of these proto-oncogenes and their individual roles in ATL oncogenesis. It also remains to be seen how ATL cells aberrantly express Fra-2 at high levels. Furthermore, the expression and function of Fra-2 in CTCLs remain to be determined.

Materials and methods

Cells
All the human T-cell lines used were described previously (Nagata et al., 1989; Yamada et al., 1996; Hata et al., 1999;

Yoshie et al., 2002). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples obtained from healthy adult donors and acute ATL patients with a high leukemic cell count (>90%) by using Ficoll-Paque (Amersham Biosciences Corp, Piscataway, NJ, USA). Normal CD4$^+$ T cells (purity, >96%) were further prepared from PBMC by negative selection using an IMagnet system (BD Pharmingen, San Diego, CA, USA). Activated CD4$^+$ T cells were prepared by stimulating CD4$^+$ T cells with anti-CD3 (clone HIT3a; BD Pharmingen) and anti-CD28 (clone CD28.2; BD Pharmingen) for 24 h. The preparation of naive CD4$^+$CD45RA$^+$ T cells and their polarization into Th1 and Th2 cells were performed as described previously (Imai et al., 1999). Primary ATL cells and normal resting CD4$^+$ T cells were used without culture for the experiments. This study was approved by the local ethical committee and written informed consent was obtained from each patient.

Transfection and luciferase assay

The major transcriptional start site (+1) of the human CCR4 gene was determined by the method of rapid amplification of cDNA 5'-ends and was found to be located 1797 bases upstream from the translation start codon (data not shown). To generate a promoter–reporter construct, the 1-kb promoter region of the human CCR4 gene (−983 to +25) was amplified from the genomic DNA by PCR using primers based on a GenBank genomic DNA sequence (accession no. NC_000003) and inserted into the reporter plasmid pGL3-Basic (Promega, Madison, WI, USA). Deletions and site-directed mutations were also performed using PCR. pGL3-2xAP-1 was constructed by introducing a sequence containing two copies of the AP-1 consensus binding site (TGATGACTCAGCCG-GAATGATGACTCAGCC) in front of a minimal CCR4 promoter pGL3 (−96/+25; Figure 1b). The coding regions of human FosB and GATA-3 were amplified from a cDNA library generated from phytohemagglutinin (PHA)-stimulated PBMC by PCR and cloned into the expression vector pSG5 (Stratagene, La Jolla, CA, USA). The coding region of HTLV-1 HBZ was amplified from a cDNA library generated from the HTLV-1$^+$ T-cell line C8166 by PCR and cloned into the expression vector pEF4/myc-His A (Invitrogen, Carlsbad, CA, USA). The expression vectors for c-Fos, Fra-1, Fra-2, c-Jun, JunB, JunD and Tax were described previously (Iwai et al., 2001). Cells (5 × 10^5) were transfected with 2 μg of reporter plasmid, 0.5 μg of expression plasmids for various transcription factors and 1 μg of pSV-β-galactosidase using DMRIE-C (Invitrogen). After 24–27 h, luciferase assays were performed using a Luciferase Assay kit (Promega). Luciferase activity was normalized by β-galactosidase activity that served as an internal control for transfection efficiency.

RT–PCR

RT–PCR was carried out as described previously (Yoshie et al., 2002). The primers used were as follows: +5'-AAGAA GAACAAGGCGGTGAAGATG-3' and −5'-AGGCCCC TGCAGGTTTTGAAG-3' for CCR4; +5'-TACTACCACTC ACCCGCAGACTC-3' and −5'-CTTTTCCCTTCGGATTCT CCTTTT-3' for c-Fos; +5'-TAGCAGCAGCTAAATGC AGGAAC-3' and −5'-CCAGCTGAAGCCATCTTCCTT AG-3' for FosB; +5'-CAGTGGATGGTACAGCCTCA TTT-3' and −5'-GCCCAGATTTCTCATCTTCCAGT-3' for Fra-1; +5'-CCAGCAGAAATTCCGGGTAGATA-3' and −5'-TCTCCTCCTCTTCAGGAGACAGC-3' for Fra-2; +5'-AAACAGAGCATGACCCTGAACCT-3' and −5'-CTC CTGCTCATCTGTCACGTTCT-3' for c-Jun; +5'-AAAAT GGAACAGCCCTTCTACCA-3' and −5'-AGCCCTGACCA

GAAAAGTAGCTG-3' for JunB; +5'-AACACCCTTCT ACGGCGATGAG-3' and −5'-GGGTAGAGGAACTGTG AGCTCGT-3' for JunD; +5'-GAATTGGTGGACGGG CTATTATC-3' and −5'-TAGCACTATGCTGTTTCGCCT TC-3' for HBZ; +5'-CCGGCGCTGCTCTCATCCCGGT-3' and −5'-GGCCGAACATAGTCCCCCAGAG-3' for Tax; +5'-AAGGCATCCAGACCAGAAACCG-3' and −5'-AGC ATCGAGCAGGGCTCTAACC-3' for GATA-3; +5'-CAGT GACGAGGATGATGAGGACT-3' and −5'-AACGTTTCG GACCGTATTTCTGT-3' for c-Myb; +5'-ATTCCAGCTT CGGAACAAGAGAC-3' and −5'-GTCCTTTTGATCAC TCCCACCTT-3' for MDM2; +5'-CAAGAAGTTTCTAGG AAAGGCCGG-3' and −5'-GATTGATCACACTAA GGTTGCATT-3' for BCL-6 and +5'-GCCAAGGTCATCC ATGACAACTTTGG-3' and −5'-GCCTGCTTCACCA CCTTCTTGATGTC-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification conditions were denaturation at 94 °C for 30 s (5 min for the first cycle), annealing at 60 °C for 30 s and extension at 72 °C for 30 s (5 min for the last cycle) for 34 cycles for CCR4; 35 cycles for c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD, HBZ, Tax, c-Myb, BCL-6 and MDM2; 29 cycles for GATA-3 and 27 cycles for GAPDH. Amplification products were electrophoretically run on a 2% agarose gel and stained with ethidium bromide.

Quantitative real-time PCR was carried out using the TaqMan assay and a 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The conditions for PCR were 50 °C for 2 min, 95 °C for 10 min and then 50 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing extension). The primers and fluorogenic probes for CCR4 and 18S ribosomal RNA were obtained from a TaqMan kit (Applied Biosystems). Quantification of CCR4 expression was performed using the Sequence Detector System Software (Applied Biosystems).

NoShift transcription factor assay

Anti-c-Fos (sc-52), anti-FosB (sc-7203), anti-Fra-1 (sc-22794), anti-Fra-2 (sc-604), anti-c-Jun (sc-1694), anti-JunB (sc-73) and anti-JunD (sc-74) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transcription factors bound to specific DNA sequences were identified using the NoShift Transcription Factor Assay Kit (EMD Biosciences, Madison, WI, USA). Nuclear extracts were prepared from human T-cell lines by using the NucBuster Protein Extraction Kit (EMD Biosciences). The oligonucleotides used' were as follows (differences underlined): TGGGAAATGACTAAGAATCAT for the biotinylated probe and unlabeled competitor of the AP-1 site and TGGGAAATGTCAAAGAATCAT for the mutated AP-1 site.

ChIP assay

This assay was performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) following the manufacturer's instructions. In brief, cells (1 × 10^6) were cross-linked with 1% formaldehyde for 10 min at room temperature. The cell pellets were lysed with sodium dodecyl sulfate (SDS) lysis buffer and sonicated to shear DNA to a size range between 200 and 1000 bp. After centrifugation, the supernatant was diluted 10-fold in ChIP dilution buffer and incubated overnight at 4 °C with 4 μg of anti-Fra-2 (sc-604), anti-JunB (sc-73), anti-JunD (sc-74) or normal rabbit IgG (DAKO, Kyoto, Japan). Immunocomplexes were collected by adding protein A-agarose beads. The immune complexes were incubated at 65 °C for 4 h to reverse the protein/DNA cross-links. DNAs were then purified by phenol/chloroform extraction and used as templates for quantitative real-time PCR. The primers and

the fluorogenic probe for the AP-1 site of the CCR4 promoter were as follows: primers: + 5'-GGTCTTGGGAAATGACT AAGAATCA-3' and −5'-TCTCCCTCACCCAACTGTACT AAGT-3'; probe: 5'-TCTGCTTCCTACTTCTATCAAA AAACCCCACTTG-3'.

Immunological staining

Cells were spotted on a glass slide and fixed with 4% paraformaldehyde. Tissue sections were prepared from formalin-fixed and paraffin-embedded biopsy tissue samples and subjected to microwave irradiation for 5 min three times in Target Retrieval Solution (DAKO). Slides and tissue sections were incubated for 1 h at room temperature with anti-Fra-2 (sc-604), anti-JunB (sc-73), anti-JunD (sc-74) or mouse monoclonal anti-CCR4 (KM-2160; Kyowa Hakko, Tokyo, Japan). Normal rabbit IgG and control mouse IgG_1 (DAKO) were used as negative controls. After washing, the slides and tissue sections were incubated with biotin-labeled goat anti-rabbit IgG or biotin-labeled horse anti-mouse IgG followed by detection using the Vectastain ABC/HRP kit (Vector Laboratories, Burlingame, CA, USA). Finally, cells and sections were counterstained with Gill's hematoxylin (Polysciences, Warrington, PA, USA), dehydrated and mounted.

Transfection of siRNA

siRNAs for Fra-2 (SI00420455), JunB (SI03077445), JunD (SI00075985) and the negative control (1022064) were obtained from Qiagen (Hilden, Germany). Transfection experiments were performed using Amaxa Nucleofector (Amaxa, Cologne, Germany). Cells (1×10^6) were resuspended in 100 μl of Nucleofector solution (T solution for MOLT-4, HUT102 and ST1 and V solution for Jurkat) and transfected with 2.5 μg of siRNA using program O-17 for MOLT-4, HUT102 and ST1 and program S-18 for Jurkat. The transfection efficiency was ~95% as determined using fluorescent siRNA (Qiagen).

Cell proliferation assay

Cells were seeded in a 96-well plate at a density of 0.5×10^4 per well and cultured. The number of viable cells was determined

every 24 h on a FACSCalibur system (Becton Dickinson, Mountain View, CA, USA) by gating out cells stained with propidium iodide. To prepare stable transfectants of Fra-2 and JunD, the coding regions of human Fra-2 and JunD were inserted into the pIRES2-EGFP vector (BD Biosciences, San Diego, CA, USA). Jurkat cells were transfected with the plasmids using DMRIE-C (Invitrogen). Stable transfectants expressing green fluorescence protein were sorted by flow cytometry using FACSVantage (Becton Dickinson).

Oligonucleotide microarray

Microarray analysis was performed as described previously (Igarashi et al., 2007) using the Affymetrix GeneChip HG-U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, USA). In brief, the ATL-derived cell line ST1 was transfected with control siRNA or Fra-2 siRNA. Four independent transfections were performed for each group. After 48 h, total RNA samples were prepared and confirmed to be of good quality with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). All microarray data have been submitted to the Gene Expression Omnibus (GEO; http:// www.ncbi.nlm.nih.gov/geo; accession no. GSE6379). The analysis was performed using the BRB Array Tools software version 3.3.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed by Richard Simon and Amy Peng.

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ORIGINAL ARTICLE

Development and validation of diagnostic prediction model for solitary pulmonary nodules

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Development and validation of diagnostic prediction model for solitary pulmonary nodules
YONEMORI K, TATEISHI U, UNO H, YONEMORI Y, TSUTA K, TAKEUCHI M, MATSUNO Y, FUJIWARA Y, ASAMURA H, KUSUMOTO M. *Respirology* 2007; **12**: 856–862
Background and objective: The aim of this study was to develop a simple prediction model for the underlying diagnosis of solitary pulmonary nodules (SPN) based on clinical characteristics and thin-section CT findings.
Methods: Retrospective analysis was carried out on 452 patients with SPN (113 benign and 339 malignant) smaller than 30 mm, who underwent thin-section CT followed by surgical resection and histological diagnosis. The clinical characteristics were collected from medical records, and radiographic characteristics from thin-section CT findings. The prediction model was determined using multivariate logistic analysis. The prediction model was validated in 148 consecutive patients with undiagnosed SPN, and the diagnostic accuracy of the model was compared with that of an experienced chest radiologist.
Results: The prediction model comprised the level of serum CRP, the level of carcinoembryonic antigen, the presence or absence of calcification, spiculation and CT bronchus sign. The areas under the receiver-operating characteristic curve in training and validation sets were 0.966 and 0.840, respectively. The diagnostic accuracies of the prediction model and the experienced chest radiologist for the validation set were 0.858 and 0.905, respectively.
Conclusion: The simple prediction model consisted of two biochemical and three radiographic characteristics. The diagnostic accuracy of an experienced chest radiologist was higher compared with the prediction model.

Key words: benign, CT, malignant, prediction model, solitary pulmonary nodule.

INTRODUCTION

The prevalence of a solitary pulmonary nodule (SPN), discovered on a CXR or CT scan, is reportedly 0.09 to 0.20% for all chest radiographs.[1,2] Most SPNs are benign, but malignancy accounts for approximately 20% of SPN, with a range of 3–80%.[3–5]

Although the diagnosis of an SPN may require resection, physicians should, as far as possible, minimize the risk of unnecessary surgery, especially in patients with benign diseases. Most physicians attempt to distinguish benign from malignant nodules using CXR, CT scanning, PET scans and fine-needle aspiration. Recent advances in radiographic