valuable diagnostic markers for predicting treatment efficacy and survival in MM.

It is now possible to measure some specific recurrent chromosomal changes. However, few chromosomal abnormalities are discernible in MM patients by conventional analysis because of the weak proliferation of the tumor cells (making it difficult to obtain good metaphases). Recently, the development of techniques such as double-color interphase fluorescence *in situ* hybridization (DC-FISH) may help circumvent the non-proliferative pitfall. Additionally, we established a global real-time quantitative/reverse transcription-polymerase chain reaction (RQ/RT-PCR) technique for detecting the expression of six 14q32 chromosomal translocation-associated proto-oncogenes in marrow plasma cells from MM patients [16]. This RQ/RT-PCR technique can detect transcriptional activation of 14q32-associated proto-oncogenes, is easy to perform on clinical samples, and may be more efficient and cost-effective than interphase FISH [16].

We have now extended our analysis to a consideration of the prognostic value of determining the levels of expression of 14q32-associated proto-oncogenes such as *CCND1*, *FGFR3* and *c-MAF* in 123 consecutive patients with newly-diagnosed MM. The goal of the present study was to investigate whether elevated expression of particular oncogenes is associated with prognosis and treatment efficacy in MM and to compare the global RQ/RT-PCR technique and interphase FISH in terms of clinical usefulness.

2. Materials and methods

2.1. Patients and control subjects

This study included 121 symptomatic MM and 2 plasma cell leukemia (PCL) patients, diagnosed between 1996 and 2010 at five different hospitals in Japan. Patients with other plasma cell disorders such as asymptomatic myeloma (SMM, $n=8$), monoclonal gammopathy of undetermined significance (MGUS, $n=17$), two solitary plasmacytoma, two Castleman's disease, and two primary amyloidosis were included as controls. The diagnosis and classification of MM and other plasma cell neoplasms was according to the criteria proposed by the International Myeloma Working Group (IMWG) [17]. We took the clinical characteristics at the initial diagnosis of MM/PCL for our analyses. The study was approved by the local Ethics Committee and written informed consent was obtained from all patients prior to bone marrow sampling, in accordance with the Declaration of Helsinki.

2.2. Quantification of 14q32 chromosomal translocation-associated proto-oncogene CCND1, FGFR3 and c-MAF mRNAs

Plasma cells were purified from mononuclear cells obtained from 1 to 2 mL of bone marrow aspirate by positive selection using anti-CD138 antibody-coated beads and an automatic magnetic cell sorting system (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. *CCND1*, *FGFR3* and *c-MAF* mRNA levels in purified plasma cells were determined by modified global RQ/RT-PCR as described in the Supplemental Methods. The copy number ratio of each proto-oncogene was calculated by dividing its expression level by that of *β-ACTIN*, with a ratio $\geq 10^{-2}$ being defined as positive, as in our previous study [16].

2.3. Conventional Giemsa (G)-banded karyotyping and Fluorescence in situ hybridization (FISH)

Conventional G-banded karyotyping and FISH for detecting chromosomal translocations t(11;14), t(4;14) and t(14;16) were performed by SRL Co., Ltd (Tokyo Japan) as described in the Supplemental Methods.

2.4. Statistical analysis

CCND1 mRNA levels were compared in patients with or without t(11;14) using the Mann–Whitney *U* test. Survival analyses were performed by the Kaplan–Meier method, and survival curves were compared using the log-rank and Breslow–Gehan–Wilcoxon tests. For survival analysis, the baseline was taken as the date of bone marrow collection (initial diagnosis of MM/PCL), and all patients who were still alive were censored at the date of last follow-up. Hazard ratios for overall survival (OS) and/or progression-free survival (PFS) in MM/PCL cases with or without ASCT or treatment with novel drugs in *CCND1*$^+$, *FGFR3*$^+$/*c-MAF*$^+$ and negative for all three proto-oncogenes (triple negative) groups were calculated by the Cox proportional hazards model. Univariate and multivariate analyses were performed with the Cox proportional hazard model. Multivariate analyses used variables selected by stepwise regression from any variables that were shown to be significant by univariate analysis. The clinical characteristics of *CCND1*$^+$, *FGFR3*$^+$, *c-MAF*$^+$ and triple-negative MM/PCL patients were compared by Chi-square testing. Data were analyzed with the aid of StatView software (SAS Institute, Version 5.0, Cary, NC). $P<0.05$ was considered significant.

3. Results

A total of 123 MM/PCL patients was studied, including 36 undergoing ASCT and 59 treated with novel drugs such as bortezomib, thalidomide and lenalidomide. There were 53 males and 70 females with an age range of 34–93 years (median age, 68 years). Their characteristics are summarized in Table 1.

3.1. CCND1, FGFR3 and c-MAF mRNA expression in MM/PCL patients

CCND1, *FGFR3* and *c-MAF* were expressed in 44 (36%), 28 (23%) and 16 (13%) of the 123 patients, respectively (Fig. 1). None of these three proto-oncogenes was expressed in 42 patients (34%), whereas in 7, both *FGFR3* and *c-MAF* were positive (Fig. 1). Expression of *CCND1* precluded expression of *FGFR3* and/or *c-MAF* in these patients, as previously reported [16]. *CCND1* expression was also detected in 2 of 8 SMM, 3 of 17 MGUS, and one of the two primary amyloidosis cases. It was not detected in the solitary plasmacytoma and Castleman's disease patients. *FGFR3* was expressed in 1 of 8 SMM and 1 of 17 MGUS cases, but not in solitary plasmacytoma, Castleman's disease or primary amyloidosis. Finally, *c-MAF* was not detected in any patients with these other plasma cell disorders.

3.2. Associations between CCND1, FGFR3 and c-MAF mRNA expression and 14q32 chromosomal translocations detected by FISH

The presence or absence of t(11;14) was evaluated by FISH in 31 of the 44 *CCND1*$^+$ MM/PCL patients. Thirteen (42%) had t(11;14), 12 (39%) had polysomy 11, and the other 6 (19%) were normal. Patients with *CCND1* expression and carrying t(11;14) had higher levels of *CCND1* mRNA than those with polysomy 11 or normal status (Mean ± 1SD of the copy-number ratio of *CCND1*: 124.4 ± 89.3 versus 10.6 ± 21.0, $P<0.0001$). Of the 28 *FGFR3*$^+$ MM/PCL patients, 17 were evaluated for t(4;14) by FISH; all were found to harbor t(4;14). Finally, of the 16 *c-MAF*$^+$ MM/PCL patients, 9 were evaluated for t(14;16). Five patients (56%) who were *c-MAF*$^+$/*FGFR3*$^-$ had t(14;16), whereas the remaining four had t(4;14) but not t(14;16) (Fig. 1).

Table 1
Patients' characteristics.

Total number	123
Age (year), median (range)	68 (34–93)
Sex (male/female)	53/70
M-protein	
IgG	63 (51.2%)
IgA	24 (19.5%)
BJP	27 (22.0%)
IgD	6 (4.9%)
Non secretory	3 (2.4%)
Stage (Durie–Salmon)	
I	3 (2.4%)
II	27 (22.0%)
III	93 (75.6%)
WBC ($\times 10^3/\mu L$), median (range)	5.0 (1.3–24.5)
Hb (g/dL), median (range)	9.2 (4.0–14.5)
PLT ($\times 10^3/\mu L$), median (range)	190.0 (43.0–558.0)
Ca (mg/dL), median (range)	9.9 (8.4–16.8)
TP (g/dL), median (range)	9.1 (4.5–14.3)
Albumin (g/dL), median (range)	3.5 (1.9–5.1)
Creatinine (mg/dL), median (range)	0.8 (0.3–12.7)

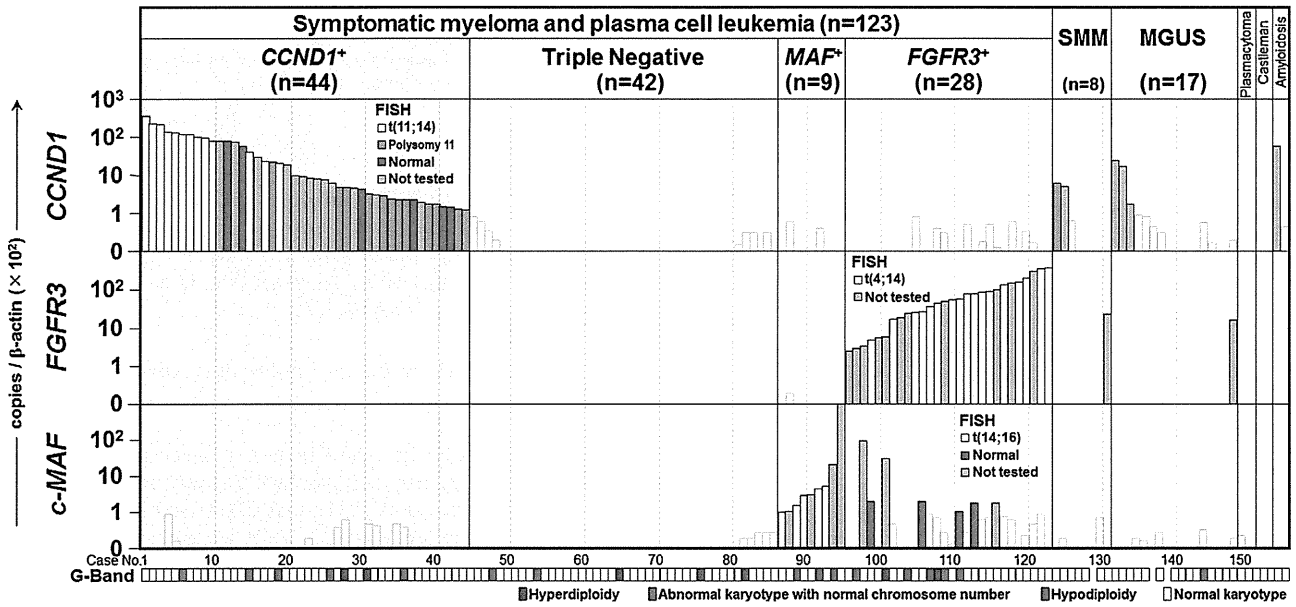

Fig. 1. Expression of *CCND1*, *FGFR3* and *c-MAF* mRNA in symptomatic myeloma (MM), plasma cell leukemia (PCL), asymptomatic (smoldering) myeloma (SMM), monoclonal gammopathy of undetermined significances (MGUS), solitary plasmacytoma, Castleman's disease, and primary amyloidosis. The copy number ratios of the proto-oncogenes associated with the 14q32 chromosomal translocations *CCND1*, *FGFR3* and *c-MAF* are shown. Samples were obtained from 123 patients with MM or PCL, 8 with SMM, 17 with MGUS, 2 with solitary plasmacytoma, 2 with Castleman's disease, and 2 with primary amyloidosis. Samples are ranked according to the level of mRNA expression of *CCND1*, *FGFR3* and *c-MAF* and according to the disease. The copy number ratio of each proto-oncogene was calculated by dividing its expression level by that of *β-ACTIN* mRNA. A copy number ratio $\geq 10^{-2}$ is defined as positive. Positivity for *CCND1* and *FGFR3* or *c-MAF* was mutually exclusive, so there are 3 patient categories; (1) *CCND1*$^+$; (2) *FGFR3*$^+$/*c-MAF*$^+$; and (3) patients without proto-oncogene expression (triple-negative). The yellow bars indicate cases confirmed to carry particular chromosomal translocations by FISH: t(11;14) for *CCND1*$^+$ cases, t(4;14) for *FGFR3*$^+$ cases, and t(14;16) for *c-MAF*$^+$ cases. The orange bars represent cases whose FISH analyses were negative for particular chromosomal translocations such as t(11;14), but showed another abnormality such as polysomy 11. The blue bars represent cases whose FISH analyses were normal. The gray bars show cases where FISH was not performed. The panels under Case No. display the results of G-band karyotyping of each case. Red panels indicate abnormal karyotypes with hyperdiploidy; gray, abnormal karyotypes but normal chromosome number; blue, abnormal karyotype with hypodiploidy; and white, normal karyotype. Abbreviations: SMM, smoldering (asymptpmatic) myeloma; and MGUS, monoclonal gammopathy of undetermined significance.

3.3. Overall survival according to CCND1, FGFR3 and c-MAF expression

The median OS time of the entire MM/PCL cohort was 4.4 years, with a median follow up of 2.0 years. The OS was significantly shorter in MM/PCL patients positive for *c-MAF* relative to those without (50% OS, 1.9 versus 5.1 years, $P=0.0028$) (Fig. 2G). In contrast, negativity or positivity for *CCND1* or *FGFR3* did not significantly influence OS (50% OS, 8.7 versus 4.1 years for *CCND1*, $P=0.2035$; and 5.1 versus 5.1 years for *FGFR3*, $P=0.1328$, respectively) (Fig. 2A and D). There were also no statistically significant differences in OS between MM/PCL patients expressing none of the three oncogenes and those expressing at least one of them (50% OS, 5.1 versus 3.8 years, $P=0.5944$) (data not shown).

3.4. Overall survival according to CCND1, FGFR3 and c-MAF expression in myeloma patients stratified by treatment with novel drugs

Survival of MM/PCL patients treated with novel agents such as bortezomib, thalidomide and lenalidomide was also analyzed. First, patients not receiving novel drugs were analyzed separately. It was found that OS was significantly shorter in MM/PCL patients positive for *FGFR3* than in those not expressing *FGFR3* (50% OS, 1.7 versus 3.2 years, $P=0.0322$) (Fig. 2E). The same was true for *c-MAF* positivity (50% OS, 0.9 versus 2.9 years, $P=0.0183$) (Fig. 2H). On the other hand, there were no significant differences in OS of patients positive or negative for *CCND1* expression (50% OS, 3.3 versus 2.3 years, $P=0.3536$) (Fig. 2B), nor between patients negative for all 3 proto-oncogenes relative to those expressing any one of them (50% OS, 3.2 versus 2.2 years, $P=0.2388$) (data not shown). Moreover, of the 59 MM/PCL patients who received a novel agent, the OS was still significantly shorter in those expressing *c-MAF* than in those not expressing it, although both were better than in patients not so treated (50% OS, 3.3 versus 5.2 years, $P=0.0094$) (Fig. 2I). However, in this case, there was no significant difference in OS between patients positive or negative for either *CCND1* or *FGFR3* expression (50% OS, not reached versus 5.1 years, $P=0.3429$, and 5.1 versus 5.2 years, $P=0.8603$, respectively) (Fig. 2C and F). Finally, there were no significant differences in OS regarding triple-negative patients versus those expressing at least one proto-oncogene (50% OS, 5.2 years versus not reached, $P=0.8472$) (data not shown).

3.5. Overall and progression-free survival after ASCT or novel drug therapy in patients stratified into the three subgroups CCND1$^+$, FGFR3$^+$ and/or c-MAF$^+$ relative to the triple-negative group

Expression of *CCND1* and *FGFR3* and/or *c-MAF* was mutually exclusive in these patients, thus defining three categories: (1) *CCND1*$^+$ patients; (2) *FGFR3*$^+$ and/or *c-MAF*$^+$ patients; and (3) patients expressing none of these three proto-oncogenes (triple-negative). We compared OS and PFS among patients receiving ASCT or conventional chemotherapy (CCT) alone in each of these three subgroups (Table 2, Fig. 3A–H). ASCT improved PFS analyzed in the entire MM/PCL cohort (Fig. 3A). Stratifying patients into the 3 subgroups showed that ASCT only improved PFS of *CCND1*$^+$ patients (Fig. 3B), but not *FGFR3*$^+$ and/or *c-MAF*$^+$ patients or triple-negative patients (Table 2, Fig. 3C and D). ASCT had no general significant OS benefit (Table 2, Fig. 3E–H). We also asked whether novel drugs influenced OS in each of the three subgroups separately (Table 2, Fig. 3J–L). In the cohort as a whole, the use of novel drugs did indeed

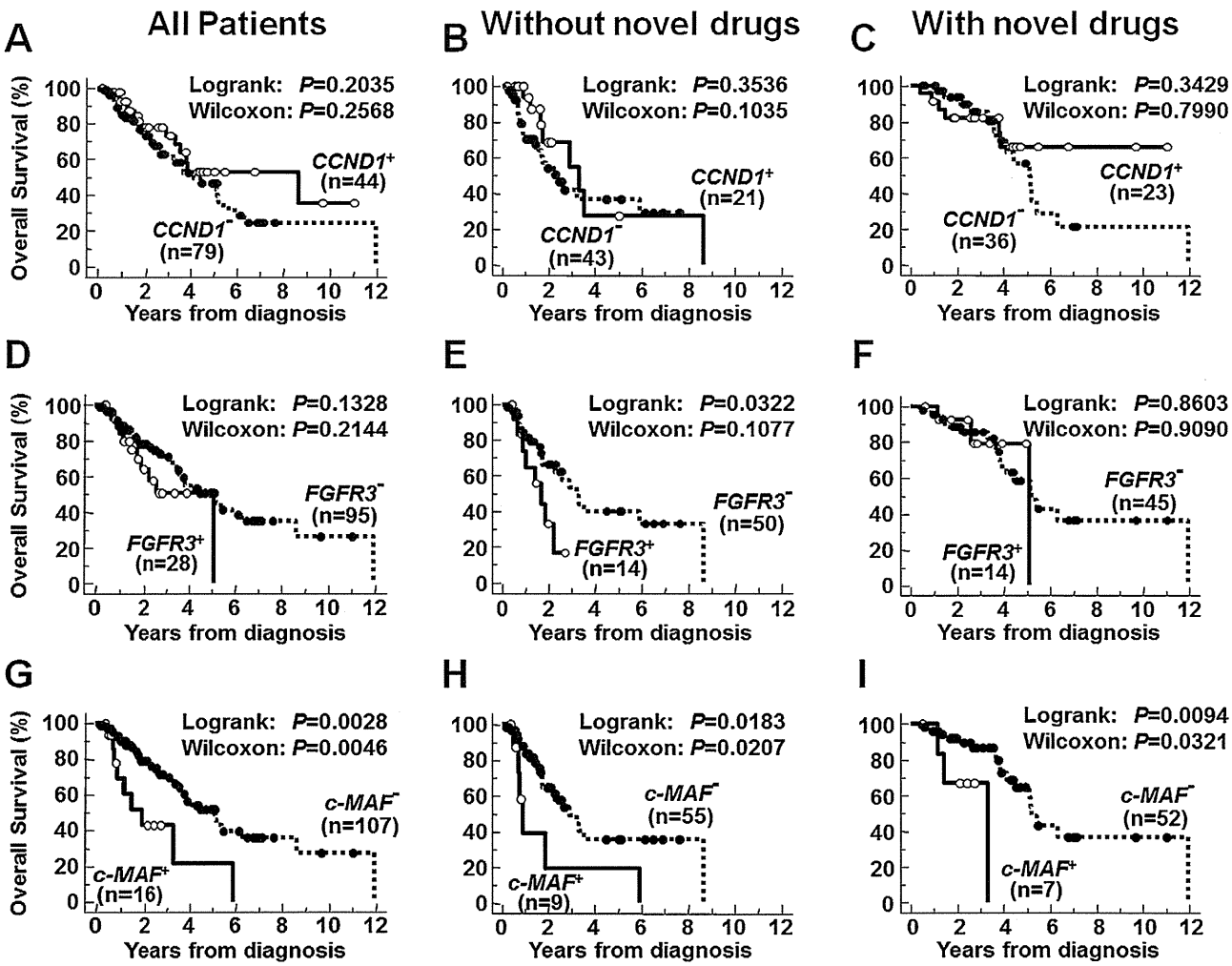

Fig. 2. Overall survival (OS) of MM patients according to the expression of *CCND1*, *FGFR3* and *c-MAF*. (A) OS curves of all patients with MM enrolled in this study according to the status of *CCND1* expression ($n = 123$). (B) OS curves of the 64 MM patients who were not treated with novel drugs, according to the status of *CCND1* expression. (C) OS curves of the 59 MM patients who were treated with novel drugs in the salvage setting according to the status of *CCND1* expression. (D) OS curves of all patients with MM enrolled in this study according to the status of *FGFR3* expression ($n = 123$). (E) OS curves of the 64 MM patients who were not treated with novel drugs, according to the status of *FGFR3* expression. (F) OS curves of the 59 MM patients who were treated with novel drugs in the salvage setting according to the status of *FGFR3* expression. (G) OS curves of all patients with MM enrolled in this study according to the status of *c-MAF* expression ($n = 123$). (H) OS curves of the 64 MM patients who were not treated with novel drugs, according to the status of *c-MAF* expression. (I) OS curves of the 59 MM patients who were treated with novel drugs according to the status of *c-MAF* expression.

improve OS (Fig. 3I). However, in the three subgroups analyzed separately, novel drugs were found to statistically significantly improve OS only of *FGFR3*$^+$ and/or *c-MAF*$^+$ patients (Fig. 3L), but not *CCND1*$^+$or triple-negative patients (Table 2, Fig. 3J–K).

Table 2
Hazard ratio and 95% CIs[a] for survivals in subgroups stratified by expression of *CCND1*, *FGFR3* and *c-MAF* mRNA.

	n	Hazard ratio (95% CI[a])	*P* value
ASCT (OS)			
CCND1$^+$	44	0.424 (0.117–1.532)	0.1906
FGFR3$^+$ and/or c-*MAF*$^+$	37	1.441 (0.478–4.344)	0.5160
Triple negative	42	0.971 (0.366–2.575)	0.9528
ASCT (PFS)			
CCND1$^+$	44	0.419 (0.185–0.950)	0.0373
FGFR3$^+$ and/or c-*MAF*$^+$	37	0.722 (0.297–1.755)	0.4723
Triple negative	42	0.449 (0.192–1.049)	0.0644
Novel drugs (OS)			
CCND1$^+$	44	0.355 (0.120–1.051)	0.0614
FGFR3$^+$ and/or c-*MAF*$^+$	37	0.116 (0.023–0.573)	0.0082
Triple negative	42	0.740 (0.298–1.838)	0.5164

[a] CI, confidence interval.

3.6. Prognostic factors for myeloma patients

Univariate Cox proportional hazards analysis identified the following unfavorable prognostic factors for OS: expression of *c-MAF*, presence of cytogenetic abnormalities by G-band karyotyping, not using novel drugs, presence of lambda-type light chain, and thrombocytopenia ($<100 \times 10^3/\mu L$) (Table 3). Multivariate analysis revealed that all of these factors except thrombocytopenia were significant independent unfavorable prognostic factors (Table 3).

3.7. Associations between 14q32 chromosomal translocation-associated proto-oncogene expression and patients' clinical characteristics

The clinical characteristics of the MM/PCL patients stratified according to their expression of *CCND1*$^+$, *FGFR3*$^+$, *c-MAF*$^+$ mRNA are summarized in Table 4. Expression of c-*MAF* was significantly associated with leukocytosis ($>10 \times 10^3/\mu L$) ($P = 0.0023$), thrombocytopenia ($<100 \times 10^3/\mu L$) ($P = 0.0337$) and a low frequency of myeloma cells expressing surface CD56 ($P = 0.0006$). None of the other clinical characteristics surveyed

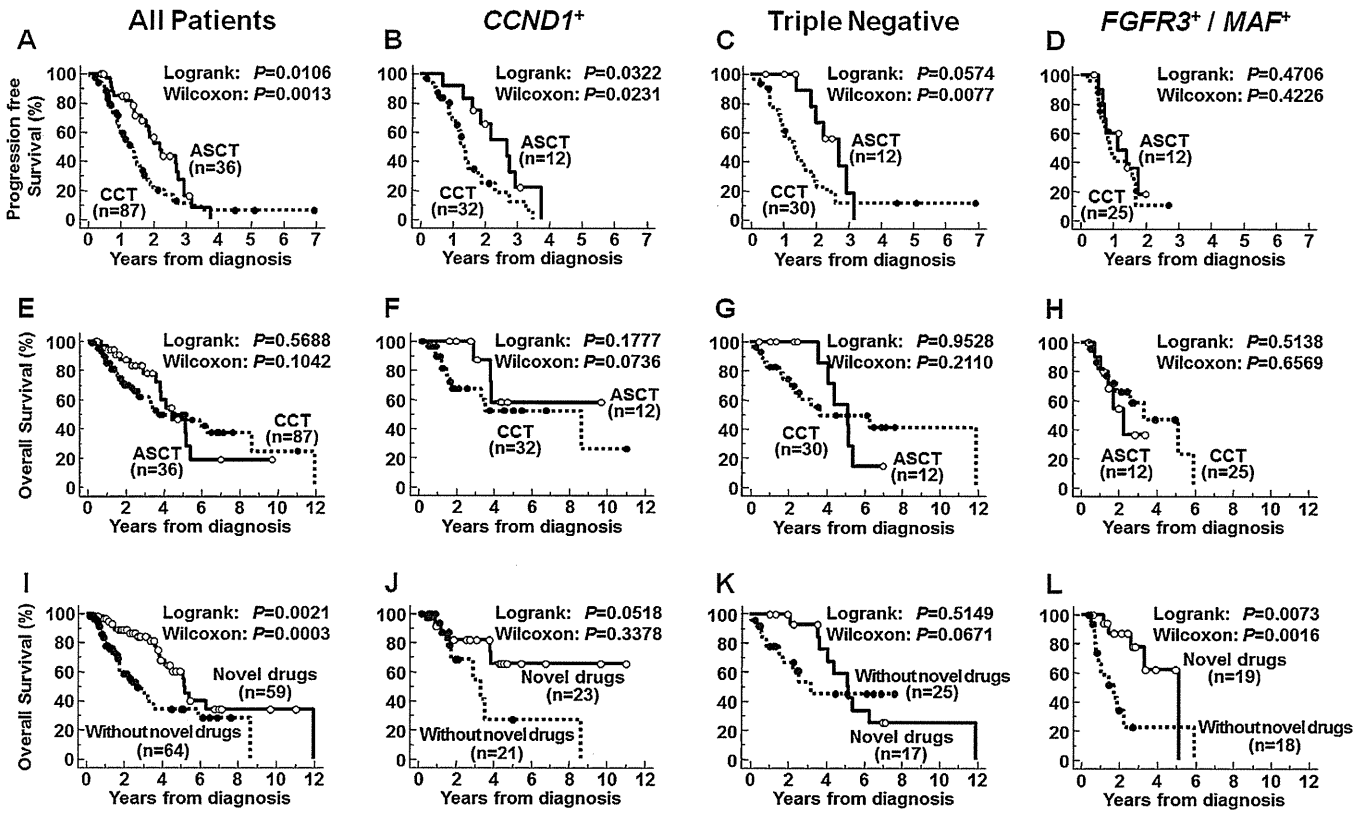

Fig. 3. Overall survival (OS) and progression-free survival (PFS) in all MM patients and in each subgroup based on the *CCND1*, *FGFR3* and/or *c-MAF* expression status and treatment with ASCT and novel drugs. (A) PFS curves of all patients with MM enrolled in this study treated with ASCT and CCT ($n = 123$). (B) PFS curves of 44 *CCND1*$^+$ MM patients treated with ASCT or CCT. (C) PFS curves of 42 *CCND1*$^-$*FGFR3*$^-$*c-MAF*$^-$ MM patients (triple-negative) treated with ASCT or CCT. (D) PFS curves of 37 *FGFR3*$^+$ and/or *c-MAF*$^+$ MM patients treated with ASCT or CCT. (E) OS curves of all patients with MM enrolled in this study treated with ASCT or CCT ($n = 123$). (F) OS curves of 44 *CCND1*$^+$ MM patients treated with ASCT or CCT. (G) OS curves of 42 *CCND1*$^-$*FGFR3*$^-$*c-MAF*$^-$ MM patients (triple-negative) treated with ASCT or CCT. (H) OS curves of 37 *FGFR3*$^+$ and/or *c-MAF*$^+$ MM patients treated with ASCT or CCT. (I) OS curves of all patients with MM enrolled in this study treated with or without novel drugs ($n = 123$). (J) OS curves of 44 *CCND1*$^+$ MM patients treated with or without novel drugs. (K) OS curves of 42 *CCND1*$^-$*FGFR3*$^-$*c-MAF*$^-$ MM patients (triple-negative) treated with or without novel drugs. (L) OS curves of 37 *FGFR3*$^+$ and/or *c-MAF*$^+$ MM patients treated with or without novel drugs. Abbreviations: ASCT, autologous stem cell transplantation; and CCT, conventional chemotherapy.

Table 3
Prognostic factors affecting overall survival in the entire MM/PCL cohort.

Variable	Univariate		Multivariate	
	Hazard ratio (95% CI[a])	*P* value	Hazard ratio (95% CI[a])	*P* value
Expression of *c-MAF*	2.914 (1.396 –6.082)	0.0044	2.717 (1.288 –5.731)	0.0087
Cytogenetic abnomalities by G-Band	2.876 (1.441 –5.742)	0.0027	3.745 (1.780 –7.879)	0.0005
Not using novel drugs	2.402 (1.350 –4.276)	0.0029	2.989 (1.634 –5.468)	0.0004
Presence of λ type light chain	1.948 (1.075 –3.528)	0.0278	2.167 (1.169 –4.018)	0.0141
PLT < $100 \times 10^3/\mu L$	2.856 (1.326 –6.155)	0.0074	–	

[a] CI, confidence interval.

were associated with expression of any of the 14q32 chromosomal translocation-associated proto-oncogenes tested here.

4. Discussion

In the present study, the expression of *CCND1*, *FGFR3* and *c-MAF* mRNAs detected by global RQ/RT-PCR was correlated with survival and treatment outcome in MM/PCL patients. *FGFR3*$^+$ MM patients not treated with any novel drugs had shorter OS than those not expressing *FGFR3*, whereas *c-MAF*$^+$ patients had worse OS than *c-MAF*$^-$ patients even when treated with novel drugs. ASCT improved PFS of *CCND1*$^+$ patients, and novel drugs extended the OS of *FGFR3*$^+$ and/or *c-MAF*$^+$ patients.

In the present study, in some *CCND1*-, *FGFR3*- or *c-MAF*-positive cases the presence or absence of 14q32 translocation corresponding to each proto-oncogene was determined by FISH. In 31 of 44 *CCND1*-positive MM cases, 13 (42%) with t(11;14), 12 (39%) with polysomy 11 and 6 (19%) with normal FISH signals were found. According to the classification system based on the gene expression profile (GEP) proposed by Arkansas University [18], t(11;14)-positive myeloma falls into two groups, CD1 or CD2, whereas polysomy 11 myeloma falls into a different HY group [18]. Thus, *CCND1*$^+$ myelomas came to be regarded as diseases with different gene expression profiles. In the 17 of 28 *FGFR3*-positive cases, it was found that all harbored t(4;14). Thus, t(4;14) seems to be essential for the expression of *FGFR3*. In the 9 of 16 *c-MAF*-positive myeloma cases, 5 were confirmed to harbor t(14;16), whereas the other 4 cases lacked t(14;16) but all carried t(4;14) and all expressed both *FGFR3* and *c-MAF* mRNA detected by global RQ/RT-PCR. These two translocations, t(14;16) and t(4;14), were mutually exclusively present in this cohort, suggesting that expression of *c-MAF* is caused by different mechanisms in patients with

Table 4
Clinical characteristics of MM/PCL patients stratified by expression of *CCND1*, *FGFR3* and c-*MAF mRNA*.

		Total (n = 123)		*CCND1*$^+$ (n = 44)		Triple Negative (n = 42)		*c-MAF*$^{+a}$ (n = 16)		*FGFR3*$^{+a}$ (n = 28)		*P* value[b]
Age (median)		68.0		69.0		67.0		66.5		67.5		
Sex	Male	53	(43%)	25	(57%)	17	(40%)	5	(31%)	9	(32%)	0.1227
	Female	70	(57%)	19	(43%)	25	(60%)	11	(69%)	19	(68%)	
M protein	IgG	63	(51%)	22	(50%)	18	(43%)	12	(75%)	15	(54%)	0.0530
	IgA	24	(20%)	5	(11%)	9	(21%)	3	(19%)	10	(36%)	
	BJP	27	(22%)	11	(25%)	12	(29%)	1	(6%)	3	(11%)	
	IgD	6	(5%)	3	(7%)	3	(7%)	0	(0%)	0	(0%)	
	Non secretory	3	(2%)	3	(7%)	0	(0%)	0	(0%)	0	(0%)	
	Kappa	74	(60%)	29	(66%)	23	(55%)	10	(62%)	16	(57%)	0.7393
	Lamda	49	(40%)	15	(34%)	19	(45%)	6	(38%)	12	(43%)	
D & S[c]	Stage I	3	(2%)	1	(2%)	1	(2%)	0	(0%)	1	(4%)	0.9369
	Stage II	27	(22%)	9	(21%)	9	(22%)	5	(31%)	8	(28%)	
	Stage III	93	(76%)	34	(77%)	32	(76%)	11	(69%)	19	(68%)	
ISS[d]	Stage I	22	(19%)	11	(26%)	9	(24%)	2	(14%)	2	(8%)	0.0605
	Stage II	47	(42%)	12	(29%)	14	(38%)	6	(43%)	18	(69%)	
	Stage III	44	(39%)	19	(45%)	14	(38%)	6	(43%)	6	(23%)	
ECOG PS[e]	2–4	27	(22%)	14	(32%)	10	(24%)	1	(6%)	3	(11%)	0.0708
	0, 1	96	(78%)	30	(68%)	32	(76%)	15	(94%)	25	(89%)	
WBC	>10,000/μL	2	(2%)	0	(0%)	0	(0%)	2	(13%)	0	(0%)	0.0023
	≤10,000/μL	121	(98%)	44	(100%)	42	(100%)	14	(87%)	28	(100%)	
Hb	<8.5 g/dL	44	(36%)	11	(25%)	20	(48%)	4	(25%)	10	(36%)	0.1326
	≥8.5 g/dL	79	(64%)	33	(75%)	22	(52%)	12	(75%)	18	(64%)	
PLT	<100 × 10^3/μL	12	(10%)	4	(9%)	2	(5%)	5	(31%)	3	(11%)	0.0337
	≥100 × 10^3/μL	111	(90%)	40	(91%)	40	(95%)	11	(69%)	25	(89%)	
Ca	>11 mg/dL	26	(21%)	14	(32%)	8	(19%)	2	(13%)	3	(11%)	0.1258
	≤11 mg/dL	97	(79%)	30	(68%)	34	(81%)	14	(87%)	25	(89%)	
Total protein	≥10.0 g/dL	39	(32%)	13	(30%)	9	(21%)	9	(56%)	11	(39%)	0.0645
	<10.0 g/dL	84	(68%)	31	(70%)	33	(79%)	7	(44%)	17	(61%)	
Albumin	<3.5 g/dL	59	(48%)	22	(50%)	16	(38%)	7	(44%)	18	(64%)	0.1872
	≥3.5 g/dL	64	(52%)	22	(50%)	26	(62%)	9	(56%)	10	(36%)	
LDH	>1.0 N	32	(26%)	10	(23%)	13	(31%)	4	(25%)	6	(22%)	0.8043
	≤1.0 N	90	(74%)	34	(77%)	29	(69%)	12	(75%)	21	(78%)	
β_2-microglobulin	≥5.5 mg/L	44	(39%)	19	(45%)	14	(38%)	6	(43%)	6	(23%)	0.3160
	<5.5 mg/L	69	(61%)	23	(55%)	23	(62%)	8	(57%)	20	(77%)	
Creatinine	>2.0 mg/dL	19	(15%)	8	(18%)	7	(17%)	1	(6%)	4	(14%)	0.7128
	≤2.0 mg/dL	104	(85%)	36	(82%)	35	(83%)	15	(94%)	24	(86%)	
G-Band	Abnormal	21	(17%)	7	(16%)	5	(12%)	4	(20%)	6	(21%)	0.5868
	Normal	102	(83%)	37	(84%)	36	(88%)	12	(80%)	21	(79%)	
PB involvement[f]	Present	26	(22%)	13	(30%)	5	(12%)	5	(31%)	4	(14%)	0.1176
	Absent	93	(78%)	30	(70%)	36	(88%)	11	(69%)	24	(86%)	
CD20	Positive	18	(17%)	10	(25%)	5	(15%)	2	(15%)	1	(4%)	0.1793
	Negative	88	(83%)	30	(75%)	29	(85%)	11	(85%)	23	(96%)	
CD56	Positive	72	(69%)	24	(63%)	24	(71%)	5	(38%)	24	(100%)	0.0006
	Negative	32	(31%)	14	(37%)	10	(29%)	8	(62%)	0	(0%)	

[a] In seven patients, both *FGFR3* and *c-MAF* were positive.
[b] *P* value were calculated by the chi-square test.
[c] Durie & Salmon stage.
[d] International staging system.
[e] Performance status proposed by Eastern Cooperative Oncology Group (ECOG).
[f] Peripheral blood involvement of myeloma cells.

t(4;14) or t(14;16). One of the mechanisms explaining the expression of *c-MAF* in *FGFR3*$^+$/*c-MAF*$^+$ cases is likely to be activation of the *MEK-ERK* pathway, since c-*MAF* transcription was activated by the *MMSET* gene through the *MEK-ERK* pathway [19,20]. New drugs targeting *CCND1* or *FGFR3* are currently under preclinical development [21–23]. Our global RQ/RT-PCR could detect expression of *CCND1*, *FGFR3* and *c-MAF* independent of the presence or absence of particular 14q32 chromosomal translocations. Thus, measuring the expression of each of these mRNAs could be a more useful tool than FISH to select which individual patients to treat with such new drugs.

Our global RQ/RT-PCR was useful for predicting survival in MM patients. Numerous studies had already reported that positivity for *FGFR3* is an unfavorable prognostic factor in MM patients who are naïve to novel drugs [7,13–15], but not in those receiving such drugs [24–28]. These results were confirmed in our study. Therefore, the use of novel drugs at least partly overcomes the negative impact on survival caused by t(4;14) [2,29,30]. The presence of t(14;16) has also been reported to be an unfavorable prognostic factor for survival in previous studies [15]. However, t(14;16) may no longer be an unfavorable prognostic factor when novel drugs are used, or if del(17) was not also present [31]. In the present study, OS was significantly shorter for *c-MAF*$^+$ relative to *c-MAF*$^-$ patients. Furthermore, *c-MAF* expression was found to be an unfavorable prognostic factor for OS regardless of the use of novel drugs. A major difference between the present and previous studies was that we regarded cases expressing *c-MAF* but without t(14;16) as *c-MAF*-positive. These cases would then be positive for t(4;14). Little is known about the clinical characteristics of *c-MAF*-positive myeloma when treated with novel drugs, and further studies are urgently required.

Our global RQ/RT-PCR also proved able to predict clinical responses and could thus help us to decide which treatments to select for individual MM patients. We evaluated the efficacy of ASCT and novel drugs in the following three subgroups: (1) *CCND1*$^+$ patients; (2) *FGFR3*$^+$/*c-MAF*$^+$ patients; and (3) patients expressing none of these 3 proto-oncogenes (triple-negative). ASCT improved PFS of patients with *CCND1*$^+$ myeloma, but not *FGFR3*$^+$/*c-MAF*$^+$ or

triple-negative patients. *CCND1* overexpression has been reported as a favorable prognostic variable for myeloma patients treated with ASCT [9]. Therefore, *CCND1*$^+$ myeloma cases would be the most promising candidates for ASCT. On the other hand, novel drugs did extend OS in the *FGFR3*$^+$/*c-MAF*$^+$ group. Bortezomib has been reported to improve the outcome for patients with t(4;14) myeloma [26], and some studies indicate the use of novel drugs could abrogate the detrimental effect of t(4;14) on survival [24–28]. In the present study, 14 of 19 *FGFR3*$^+$/*c-MAF*$^+$ cases were treated with bortezomib, which may have improved their OS. In contrast, it remains unclear whether thalidomide benefited the *FGFR3*$^+$/*c-MAF*$^+$ patients. In the MRC IX trial, patients with unfavorable cytogenetics including t(4;14) and t(14;16) randomized to thalidomide maintenance showed no prolongation of PFS and OS relative to placebo [32]. However, the use of thalidomide in a salvage setting may benefit these patients. It was recommended that *FGFR3*$^+$/*c-MAF*$^+$ cases were treated with a bortezomib-containing regimen [33]. In this respect, *FGFR3*$^+$/*c-MAF*$^+$ myeloma cases would be the most promising candidates for treatment with novel drugs, especially bortezomib. However, an appropriately stratified protocol for using novel drugs still needs to be established.

There are several limitations to the present study. First, we measured only the expression of the 3 major 14q32 chromosomal translocation-associated proto-oncogenes *CCND1*, *FGFR3* and *c-MAF*. We did not investigate *CCND3* and *MAFB*, but despite the fact that the frequency of *CCND3*$^+$ and *MAFB*$^+$ myeloma cases is very low (only 2% each) [2], these translocations may have specific clinical features and could skew the results. In fact, *MAFB* onco-protein detected by immunohistochemistry was reported as a highly sensitive and specific unfavorable prognostic marker in MM [34]. According to the results of GEP, neither *CCND1*, nor *FGFR3* and *c-MAF* was detected in either *CCND3*$^+$ or *MAFB*$^+$ cases, which would therefore be assigned to the triple-negative group in the present study. A second limitation of our study was that the expression of *MMSET* was not determined and therefore we could not identify all of the t(4;14) cases, because overexpression of *FGFR3* protein occurs in only approximately 70% of patients with the t(4;14) translocation, whereas *MMSET* is overexpressed in all cases [30]. In the present study, two cases with t(4;14) were classified as triple-negative, because they were negative for *FGFR3* but positive for *MMSET*.

5. Conclusions

Global RQ/RT-PCR detecting *CCND1*, *FGFR3* and *c-MAF* will be helpful in selecting patients who will be the best candidates for treatment with the novel agents targeting *CCND1*, *FGFR3* or *c-MAF* currently under development. It is also useful for predicting OS and planning individualized treatment strategies for MM patients. Development of optimal therapies for each patient based on the expression pattern of 14q32 chromosomal translocation-associated proto-oncogenes represents an important objective in personalized treatment of MM.

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Authors' contributions

AI was the principal investigator and takes primary responsibility for the paper; AI, ET, MU, RU and SI conceived and designed the study; AI, ET, MU, HT, YA, HO, AM, TY, FM, AI, HY MR, SK, TK, SK, TI, YH, IH, HK and SI recruited the patients and acquired data; ET, MU, HI and YM performed the laboratory analysis and interpreted the data; AI and SI drafted and wrote the article and revised it critically for important intellectual content. All authors gave their final approval of the version to be submitted.

Conflict of interest

Yasufumi Matsuda is an employee of SRL Inc. The other authors report no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.09.026.

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Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Regular Article

LYMPHOID NEOPLASIA

Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Key Points

- The efficacy and safety of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for patients with adult T-cell leukemia.
- hTERT-specific T-cell receptor gene-transduced $CD8^+$ T cells lyse ATL cells, but not normal cells, both in vitro and in vivo.

Although adult T-cell leukemia (ATL) has a poor prognosis, successful allogeneic hematopoietic stem cell transplantation (allo-HSCT) in some cases suggests that a cellular immune-mediated strategy can be effective. So far, however, no effective target for anti-ATL immunotherapy has been defined. Here we demonstrated for the first time that human telomerase reverse transcriptase (hTERT) is a promising therapeutic target for ATL, and we developed a novel redirected T-cell–based immunotherapy targeting hTERT. *hTERT* messenger RNA was produced abundantly in ATL tumor cells but not in steady-state normal cells. Rearranged human leukocyte antigen-A*24:02 (HLA-A*24:02) –restricted and $hTERT_{461-469}$ nonameric peptide-specific T-cell receptor (TCR) α/β genes were cloned from our previously established cytotoxic T lymphocyte clone (K3-1) and inserted into a novel retroviral TCR expression vector encoding small interfering RNAs for endogenous *TCR* genes in redirected T cells (hTERT-*siTCR* vector). Consequently, allogeneic or autologous gene-modified $CD8^+$ T cells prepared using the hTERT-*siTCR* vector successfully killed ATL tumor cells, but not normal cells including steady-state hematopoietic progenitors, in an HLA-A*24:02-restricted manner both in vitro and in vivo. Our experimental observations support the development of a novel hTERT-targeting redirected T-cell–based adoptive immunotherapy for ATL patients, especially those for whom suitable allo-HSCT donors are lacking. (*Blood.* 2013;121(24):4894-4901)

Introduction

Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus I (HTLV-I).[1] It is estimated that there are more than 1 million HTLV-I carriers in Japan, about 5% of whom develop ATL at around 60 years of age or older.[2] Because ATL tumor cells soon acquire chemotherapy resistance and compromise host immunity against infectious pathogens, ATL has a poor prognosis.[3] Although most ATL patients are ineligible for allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of advanced age, age-related comorbidity, or lack of suitable donors,[4] the number of ATL patients who are treated successfully with allo-HSCT and achieve prolonged survival has been increasing.[5] The graft-versus-ATL effect observed in ATL patients treated successfully with allo-HSCT[5] strongly suggests that a cellular immune-mediated approach for ATL can be clinically effective. With regard to cellular immunotherapy for ATL (unlike Epstein-Barr virus [EBV]-associated malignancy[6]), targeting of antigens associated with HTLV-I (the causative virus of ATL) such as Tax[7] and HBZ[8] still remains controversial, and the recently proposed NY-ESO-1[9] (a cancer-testis antigen) still awaits clinical validation. Thus, at this time, no effective therapeutic target antigen for anti-ATL immunotherapy has been clinically defined.

Human telomerase reverse transcriptase (hTERT), which is a component of human telomerase and a catalytic subunit for telomere elongation, is activated in almost all cancer cells, including hematologic malignancies, but not in normal cells.[10] In HTLV-I–infected cells and ATL tumor cells, Tax or interleukin-2 (IL-2) signaling strongly activates the *hTERT* promoter through the nuclear factor-κB or PI3K pathway,[11-13] suggesting that expression of hTERT protein would be upregulated in ATL tumor cells. Clinical trials of anticancer immunotherapy targeting hTERT have already been conducted, and both the safety and induction of immune responses to hTERT have been reproducibly confirmed.[10,14-17] In our previous studies, we defined a [human leukocyte antigen] HLA-A*24:02-restricted $hTERT_{461-469}$ nonameric peptide (VYGFVRACL) that was capable of inducing antileukemia cytotoxic T lymphocytes (CTLs),[18] and we subsequently established a CTL clone, K3-1, specific for this

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epitope.[19] We previously conducted a phase I/II clinical trial of hTERT peptide vaccine for treatment of HLA-A*24:02$^+$ patients with lung cancer and metastatic renal cell cancer.[20] These achievements strongly encouraged us to further explore cellular immune-mediated treatment of ATL targeting hTERT. Because of concern over the potential regulatory T-cell function of ATL tumor cells,[21] in this study we focused on developing a redirected T-cell–based immunotherapy targeting hTERT rather than using an hTERT$_{461\text{-}469}$ peptide vaccine. Recently developed forms of anticancer immunotherapy using gene-modified T cells that redirect defined tumor-associated antigens have been shown to have clinical promise.[22-25] To this end, therefore, we first cloned the rearranged HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific T-cell receptor α/β (*TCR-α/β*) genes from K3-1 and inserted them into a novel *TCR* gene expression vector carrying silencers for endogenous TCRs (*siTCR* vector)[26] in redirected T cells (hTERT-*siTCR* vector). Notably, we used a souped-up second-generation 2A peptide-based *siTCR* vector that achieved an increased level of expression of the introduced TCR.[27]

In this study, we used the newly established hTERT-*siTCR* vector to examine the feasibility of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for treatment of ATL.

Patients and methods

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with EBV. ATN-1,[28] TL-Om1,[29] HUT102[29], and TL-MAT[30] were human T-cell lines established from ATL patients, and TL-Su,[31] MT-1,[32] MT-2[32], and MT-4[33] were human T-cell lines transformed by HTLV-I infection. LCLs, T2-A24,[19] K562 (American Type Culture Collection [ATCC]), and human T-cell lines (except TL-Om1), maintenance of which requires 10 U/mL recombinant human IL-2 (rhIL-2) (R&D Systems), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The HLA-A*24:02 gene-transduced K562 (K562-A24) was maintained in culture medium supplemented with 1.0 μg/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) from ATL patients and healthy donors and cord blood mononuclear cells (CBMCs) from healthy donors were isolated by density gradient centrifugation and stored in liquid nitrogen until use. All samples from ATL patients contained more than 90% ATL cells. CD4$^+$ T cells, CD14$^+$ cells from PBMCs, and CD34$^+$ cells from CBMCs were isolated by using CD4$^+$ cell-, CD14$^+$ cell-, or CD34$^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec), respectively. IL-2–dependent CD4$^+$ cell lines induced by HTLV-I infection were generated as reported previously.[8]

Cloning of full-length *TCR* α and β chain genes and construction of hTERT-*siTCR* retroviral vector

HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$ nonameric peptide (VYGFVRACL)-specific *TCR-α/β* genes were cloned from our previously established CTL clone, K3-1,[19] by using the 5′ rapid amplification of complementary DNA ends method (Clontech). The rearranged *TCR-α/β* genes of K3-1 expressed the germ line gene segments *TRAV29DV5/TRAJ34/TRAC* and *TRBV20-1/TRBJ2-1/TRBC2*, respectively. The retroviral vector expressing K3-1–derived *TCR* genes was constructed as reported previously.[26,27,34] Briefly, the constant regions of the hTERT-specific *TCR-α/β* genes were codon optimized and then integrated into a novel Splice-b2Aa-siTCR–based retroviral vector encoding small interfering RNAs that complementarily bind to the constant regions of the endogenous *TCR-α/β* genes (hTERT-*siTCR* vector).[27]

Establishment of hTERT-*siTCR*–transduced CD8$^+$ T-cell lines

Isolated CD8$^+$ T cells from PBMCs of healthy volunteers or ATL patients using CD8$^+$ cell-isolating MACS beads and stimulation with 1 μg/mL anti-CD3 monoclonal antibody (mAb; OKT-3; BioLegend) were cultured in GT-T503 (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL rhIL-2, 5 ng/mL rhIL-7 (R&D Systems), 10 ng/mL rhIL-15 (PeproTech), and 10 ng/mL rhIL-21 (Shenandoah Biotechnology). Then, CD8$^+$ T cells were transfected with the hTERT-*siTCR* retroviral vector using RetroNectin (Takara Bio) -coated plates as described previously.[34] In some experiments, because TRBV20-1 is specifically labeled with anti-Vβ2 mAb (IMGT Web resources: http://www.imgt.org/), Vβ2-positive cells among hTERT-*siTCR*–transduced CD8$^+$ T cells (hTERT-*siTCR*/CD8) were further isolated by using fluorescein isothiocyanate (FITC) –conjugated Vβ2 mAb (Beckman Coulter) and anti-FITC–conjugated MACS beads. To measure the expression levels of the introduced hTERT-specific TCR in gene-modified CD8$^+$ T cells, the cells were labeled with anti-CD8 (BD Biosciences) and anti-Vβ2 mAbs and phycoerythrin-conjugated HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or HLA-A*24:02/HIV-1 Env$_{584\text{-}592}$ (RYLRDQQLL) tetramer, as a negative control.[19] Labeled cells were analyzed by using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To expand the hTERT-*siTCR*/CD8 cells, they were stimulated weekly with mitomycin-C (Kyowa Hakko) –treated and hTERT$_{461\text{-}469}$ peptide–pulsed HLA-A*24:02$^+$ LCLs.

Cytotoxicity assays

Standard ^{51}Cr-release assays were performed as described previously.[35] Briefly, 5×10^3 unpulsed or peptide-pulsed target cells were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$; MP Bio Japan) and incubated at various ratios with effector cells in 200 μL of culture medium in 96-well round-bottomed plates. To assess HLA class I restriction, target cells were incubated with 10 μg/μL anti-HLA class I framework mAb (clone w6/32; ATCC) or a control anti-HLA -DR mAb (clone L243; ATCC) for 1 hour, then incubated with effector cells for 5 hours. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%). In some experiments, time-lapse imaging was used. Ten thousand ATL cells lentivirally gene-modified to express monomeric Azami-Green (Amalgaam) were cocultivated with 5×10^4 effector cells expressing hTERT-specific TCR (at an effector:target ratio of 5:1) for 12 hours in culture medium supplemented with 10 μg/mL propidium iodide (Sigma) to label dead cells red by using a glass dish for microscopic observation of live cells (iBIDI-dish1 Hi-Q4; Nikon). Images were acquired by using a systemic bio-imaging tool (BioStation IM; Nikon). To examine the cytotoxicity of these effector cells against early-differentiated and highly proliferating subsets of hematopoietic progenitor cells, CB-CD34$^+$ cells cultured by using a hematopoietic cell expansion medium (StemSpan CC100 and StemSpan SFEM; Stem Cell) for 7 days were subjected to flow-based cytotoxicity assay. 7-Aminoactinomycin D (7-AAD) –positive dead cells in each subset were examined by flow cytometry.

Quantitative analysis of hTERT mRNA expression

Quantitative real-time PCR (qRT-PCR) for *hTERT* messenger RNA (mRNA) was performed as described previously.[36] Briefly, after complementary DNA was synthesized, qRT-PCR for *hTERT* mRNA (NM_198253) was performed by using the QuantiTect SYBR green PCR Kit (QIAGEN) and primers as follows: forward, 5′-TTCTTGTTGGTGACACCTCACCTC-3′; reverse, 5′-CAGCCATACTCAGGGACACCTC-3′ (Takara Bio). Human hypoxanthine phosphoribosyltransferase 1 (*hHPRT1*) mRNA (NM_000194) was prepared and used as an internal control. Samples were analyzed by using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *hTERT* mRNA was corrected by reference to that of *hHPRT1* mRNA, and the amount of *hTERT* mRNA relative to that in PBMCs was calculated by the comparative threshold cycle method. K562, which strongly expresses *hTERT* mRNA, was used as an internal control.

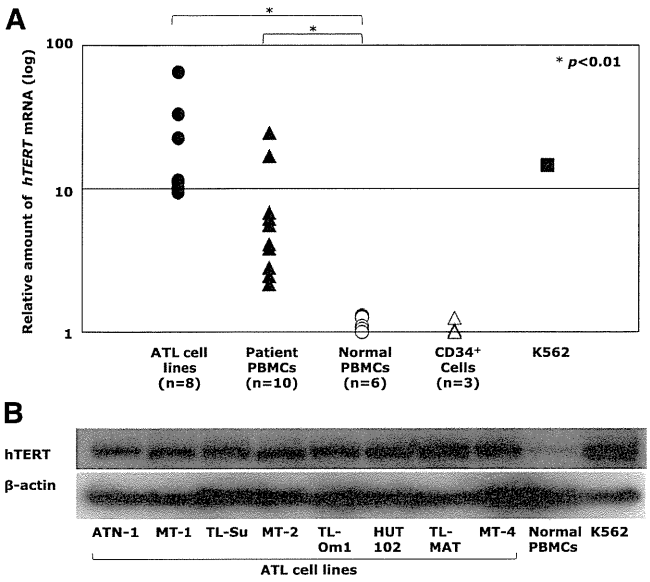

Figure 1. Abundant expression of hTERT in ATL tumor cells. (A) Expression of *hTERT* mRNA in ATL/HTLV-I infected cell lines (■), freshly isolated ATL tumor cells from patients (▲), normal PBMCs (○), and CB-CD34$^+$ cells (△) were examined by qRT-PCR. The level of *hTERT* mRNA expression in the K562 leukemia cell line (●) was used as an internal control. The expression level of *hTERT* mRNA in each sample was calculated relative to that of PBMCs. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ (mean ± standard deviation [SD]). The ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells expressed *hTERT* mRNA abundantly and significantly (*$P < .01$). (B) Expression of hTERT protein in ATL cell lines and normal PBMCs was confirmed by western blotting.

Western blotting of hTERT protein

For analysis of protein expression, western blotting was performed as described previously.[35] Briefly, cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (e-PAGEL, ATTO) and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The blots were incubated first with anti-hTERT rabbit mAb (Millipore), then with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G Ab (GE Healthcare). The probed proteins were visualized by using an enhanced chemiluminescence system (GE Healthcare). Subsequently, the blotted membranes were stripped and reprobed with anti–β-actin mouse mAb (Sigma-Aldrich) to confirm equivalent protein loading between samples.

Detection of hTERT$_{461\text{-}469}$–specific CTL precursors in the periphery of ATL patients

PBMCs from HLA-A*24:02$^+$, HLA-A*24:02$^-$ ATL patients, or HLA-A*24:02$^+$ healthy individuals were seeded in 24-well plates at 1.5 × 10^6 per well in the presence of the hTERT$_{461\text{-}469}$ peptide at a concentration of 1 μM in GT-T503 medium supplemented with 5% human serum and 10 U/mL IL-2. After culturing for 14 days, cultured PBMCs were stained with FITC-conjugated anti-CD8 mAb and HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or control tetramer at a concentration of 20 μg/mL at 4°C for 20 minutes. Subsequently, the stained cells were analyzed by flow cytometry.

IFN-γ secretion assay

hTERT-*siTCR*/CD8 or K3-1 (2 × 10^4) cells were incubated with 2 × 10^4 hTERT$_{461\text{-}469}$ peptide-pulsed (1 μM) or unpulsed K562-A24 or K562 cells for 24 hours. Interferon gamma (IFN-γ) in the culture supernatant was measured by using an enzyme-linked immunosorbent assay kit (Pierce). Enzyme-linked immunospot assays were used to detect the epitope-responsive IFN-γ production mediated by hTERT$_{461\text{-}469}$-specific CTL precursors in the periphery of ATL patients as described previously.[34]

Anti-ATL tumor effect of hTERT-*siTCR*–transduced CD8$^+$ T cells in xenografted mouse models

To assess the in vivo anti-ATL tumor effect mediated by hTERT-*siTCR*/CD8, a bioluminescence assay using a xenografted mouse model was used. First, we lentivirally generated a luciferase gene–transduced HLA-A*24:02$^+$ ATL cell line, ATN-1 (ATN-1/luc). For measurement, anesthetized xenografted mice were given an intraperitoneal injection of 2.5 mg/body VivoGlo luciferin (Caliper Life Science), and images were acquired for 5 to 10 minutes by using an AEQUORIA luminescence imaging system (Hamamatsu Photonics). The acquired photon counts were analyzed by using AQUACOSMOS software (Hamamatsu Photonics).

Six-week-old NOD/scid/γc^{null} (NOG) female mice[37] were purchased from the Central Institute for Experimental Animals and maintained in the institutional animal facility at Ehime University. All in vivo experiments were approved by the Ehime University animal care committee. For the Winn assay, 5 × 10^5 ATN-1/luc cells and 2.5 × 10^6 hTERT-*siTCR*/CD8 or non–gene-modified CD8$^+$ T cells (NGM/CD8) were subcutaneously inoculated into the abdominal wall of NOG mice that had been pretreated with 1 Gy irradiation. Thereafter, 2.5 × 10^6 effector cells of each type were administered weekly to the corresponding mice, respectively, via the tail vein for a total of 3 times. For the adoptive transfer experiments, similarly pretreated mice were intravenously inoculated with 5 × 10^5 ATN-1/luc cells. After 4 days, mice started to receive intravenously infused 5 × 10^6 hTERT-*siTCR*/CD8 or NGM/CD8, respectively, for a total of 5 times. These mice were serially monitored for tumor growth determined by photon counts acquired every 7 days until they were euthanized owing to disease progression.

Statistical analysis

The Mann-Whitney *U* test was used to assess differences between two groups; a *P* value of < .05 was considered significant.

Results

ATL tumor cells abundantly express *hTERT* mRNA and hTERT protein

The expression level of *hTERT* mRNA in the ATL/HTLV-I–infected cell line (n = 8), freshly isolated tumor cells from ATL patients (n = 10), normal PBMCs from healthy individuals (n = 6), and CD34$^+$ cells from normal CBMCs (CB-CD34$^+$) (n = 3) were measured by using the qRT-PCR method. *hTERT* mRNA expression relative to normal PBMCs was 21.3 ± 17.9 for the ATL/HTLV-I–infected cell line, 7.48 ± 6.89 for freshly isolated ATL tumor cells, and 1.10 ± 0.12 for CB-CD34$^+$ cells (mean ± standard deviation). In Figure 1A, the ATL/HTLV-I–infected cell line and freshly isolated ATL tumor cells, but not CB-CD34$^+$, abundantly produced *hTERT* mRNA in comparison with normal PBMCs, the difference being statistically significant. The *P* value was .002 for the ATL/HTLV-I–infected cell line, .001 for freshly isolated ATL tumor cells, and .243 for CB-CD34$^+$ cells. Similarly, western blotting demonstrated abundant expression of hTERT protein in the ATL tumor cells (Figure 1B).

Circulatory hTERT$_{461\text{-}469}$-specific CTL precursors were exclusively detectable in the periphery of HLA-A*24:02$^+$ ATL patients

Next, by using the tetramer assay, we examined circulatory hTERT$_{461\text{-}469}$-specific CTL precursors in PBMCs from HLA-A*24:02$^+$ ATL patients (n = 7), HLA-A*24:02$^-$ ATL patients (n = 3) before chemotherapy, and HLA-A*24:02$^+$ healthy individuals as controls (n = 6). Since freshly isolated PB lymphocytes were almost

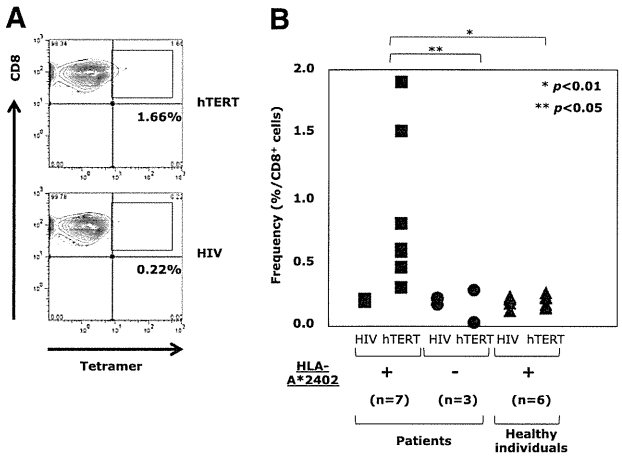

Figure 2. Detection of circulatory hTERT$_{461\text{-}469}$-specific CTL precursors in the periphery of ATL patients. (A) hTERT$_{461\text{-}469}$-specific CTL precursors in PBMCs repetitively stimulated with hTERT$_{461\text{-}469}$ peptide from HLA-A*24:02$^+$ ATL patients were detected by using HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer. A representative case is shown. HLA-A*24:02/HIV tetramer was used as a negative control. (B) In comparison with HLA-A*24:02$^-$ ATL patients (●) (n = 3) and HLA-A*24:02$^+$ healthy individuals (▲) (n = 6), the frequency of hTERT$_{461\text{-}469}$-specific CTL precursors in HLA-A*24:02$^+$ ATL patients (■) (n = 7) was significantly high (*$P < .01$; **$P < .05$). The frequency was 0.88% ± 0.55% for HLA-A*24:02$^+$ ATL patients, 0.11% ± 0.1% for HLA-A*24:02$^-$ ATL patients, and 0.2% ± 0.04% for HLA-A*24:02$^+$ healthy individuals (mean ± SD).

negative for tetramer staining, PBMCs stimulated with hTERT$_{461\text{-}469}$ peptide were analyzed. A representative example of an HLA-A*24:02$^+$ ATL patient is shown in Figure 2A. The frequencies of hTERT$_{461\text{-}469}$-specific CTL precursors in HLA-A*24:02$^+$ and HLA-A*24:02$^-$ ATL patients and HLA-A*24:02$^+$ healthy individuals are summarized in Figure 2B. hTERT$_{461\text{-}469}$-specific CTL precursors were detected at 0.88% ± 0.55% in HLA-A*24:02$^+$ ATL patients, being significantly more frequent than in HLA-A*24:02$^-$ ATL patients (0.11% ± 0.1%; $P < .05$) or HLA-A*24:02$^+$ healthy individuals (0.2% ± 0.04%; $P < .01$). These observations confirmed the presence of primed memory CD8$^+$ T cells with hTERT$_{461\text{-}469}$ epitope/HLA-A*24:02 complex (ie, that the hTERT$_{461\text{-}469}$ epitope must be naturally immunogenic) in HLA-A*24:02$^+$ ATL patients.

hTERT-*siTCR*–transduced CD8$^+$ T cells exert anti-ATL reactivity in vitro

The hTERT-*siTCR* gene was retrovirally introduced into normal CD8$^+$ T cells. Transduction efficiency determined by expression of Vβ2 on the gene-modified T cells was 85% to 95% (data not shown), and almost 50% of the transfectants were positive for HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer (Figure 3A). The cognate epitope specificity and HLA-A*24:02 restriction were examined by using standard ^{51}Cr-release assays (Figure 3B). Because expression of *hTERT* mRNA in LCLs was upregulated (supplemental Figure 2C), hTERT peptide-unpulsed HLA-A*24:02$^+$ LCLs were killed to some extent, reflecting the presence of endogenously processed hTERT (Figure 3B). Such epitope-specific cytotoxicity mediated by hTERT-*siTCR*/CD8 was obviously attenuated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 3C). The antigen sensitivity to cognate hTERT$_{461\text{-}469}$ peptide mediated by hTERT-*siTCR*/CD8 (shown in Figure 3D) was similar to that of the parental CTL clone, K3-1 (Figure 3E-F).

hTERT-*siTCR*/CD8 dose-dependently killed the HLA-A*24:02$^+$ ATL/HTLV-I–infected cell lines ATN-1, TL-Su, and MT-2, but not the HLA-A*24:02$^-$ TL-Om1, HUT102, and MT-4 (Figure 4A). Additionally, the tumoricidal effect mediated by hTERT-*siTCR*/CD8 was abrogated by anti-HLA class I mAb, but not by anti-HLA-DR mAb (Figure 4B). Furthermore, time-lapse imaging directly demonstrated this tumoricidal activity of hTERT-*siTCR*/CD8 against HLA-A*24:02$^+$ ATN-1, but not that against HLA-A*24:02$^-$ HUT102 or K562 (negative control) (supplemental Fig 1-(1)). We then examined the tumoricidal activity against freshly isolated ATL tumor cells and found that these transfectants also dose-dependently killed HLA-A*24:02$^+$, but not -A*24:02$^-$ freshly isolated ATL tumor cells (Figure 5A).

Conversely, as shown in Figure 5B, neither HLA-A*24:02$^+$ normal CD4$^+$ T cells (the normal counterpart of ATL tumor cells)

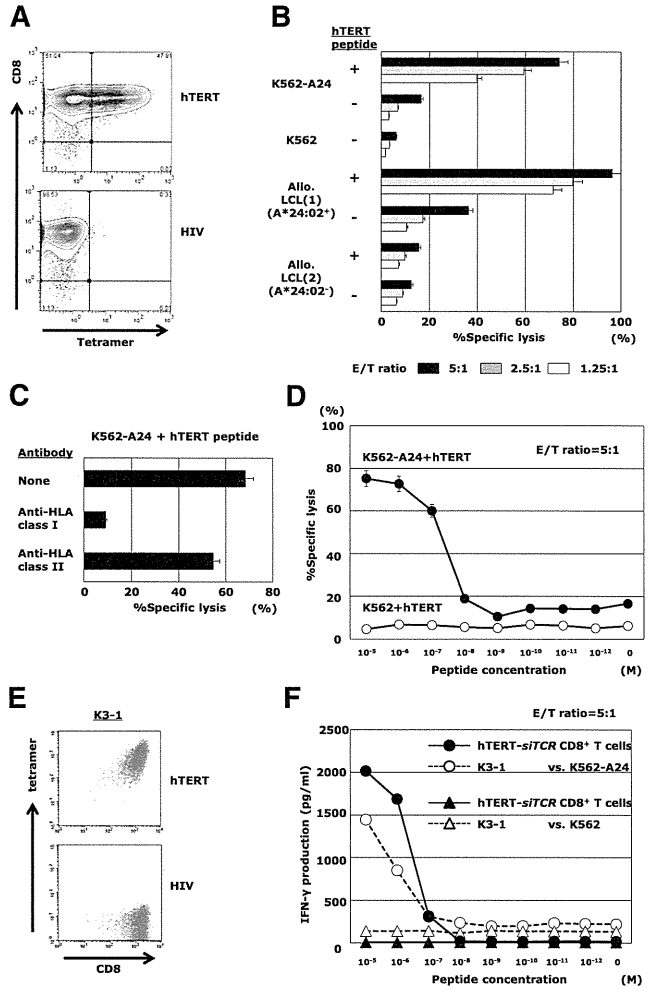

Figure 3. hTERT-*siTCR*–transduced CD8$^+$ T cells display epitope-specific responsiveness. (A) Representative flow cytometry plots showing staining of hTERT-*siTCR*–transduced CD8$^+$ T cells with HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer. HLA-A*24:02/HIV tetramer was used as a negative control. (B) ^{51}Cr-release assays were conducted by using hTERT-*siTCR*–transduced CD8$^+$ T cells with unpulsed or hTERT$_{461\text{-}469}$ peptide-loaded (1 μM) K562-A24, K562, HLA-A*24:02$^+$, or HLA-A*24:02$^-$ allogeneic B-LCLs at the indicated effector:target (E/T) ratios. (C) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against the cognate peptide-pulsed (1 μM) K562-A24 was determined by ^{51}Cr-release assays at an E/T ratio of 5:1. (D) hTERT-*siTCR*–transduced CD8$^+$ T cells were tested in ^{51}Cr release assays against K562 (negative control) and K562-A24 cells pulsed with the indicated concentrations of hTERT$_{461\text{-}469}$ peptide at an E/T ratio of 5:1. Error bars represent SDs. (E) Representative flow cytometry plots showing staining of K3-1 with the HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer (upper) and the irrelevant HLA-A*24:02/HIV-1 Env$_{584\text{-}592}$ tetramer (negative control; bottom). (F) IFN-γ production by hTERT-*siTCR*–transduced CD8$^+$ T cells was measured by using a format similar to that described for panel D. The parental K3-1 CTL clone was tested in parallel.

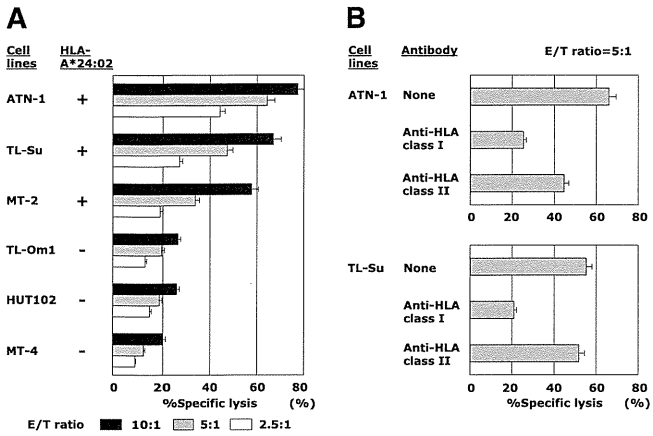

Figure 4. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATL/HTLV-I–infected cell lines. (A) Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against HLA-A*24:02$^+$ or HLA-A*24:02$^-$ ATL/HTLV-I–infected cell lines was tested in ^{51}Cr-release assays at the indicated E/T ratios. All tested ATL/HTLV-I–infected cell lines overexpressed *hTERT* mRNA and protein, as shown in Figure 1. (B) Effect of HLA class I and class II blockade on the cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells against ATN-1 and TL-Su was tested in ^{51}Cr-release assays at an E/T ratio of 5:1.

nor HLA-A*24:02$^+$ normal CB-CD34$^+$ cells as normal hematopoietic progenitor cells were killed. In the same experiment, newly established IL-2–dependent HTLV-I–infected CD4$^+$ T cells (Patient #1 and Patient #2), but not the corresponding original normal/HTLV-I$^-$ CD4$^+$ T cells (Patient #1 and Patient #2), became to some extent sensitive to the same transfectants as the level of *hTERT* mRNA expression increased (Figure 5B). This observation confirmed that not only ATL tumor cells, but also HTLV-I–infected cells from which ATL tumor cells were derived could be killed by these hTERT-specific effector cells.

Next, because the majority of ATL patients were of an advanced age and were therefore ineligible for allo-HSCT, we examined the tumoricidal activity against autologous ATL tumor cells mediated by gene-modified PB-CD8$^+$ T cells from the patient (Figure 6). Although PB-CD8$^+$ T cells from heavily pretreated ATL patients were sometimes difficult to subject to *TCR* gene modification and ex vivo expansion, hTERT-*siTCR*/CD8 cells generated from HLA-A*24:02$^+$ patients (n = 3) were able to substantially lyse autologous ATL tumor cells in proportion to the corresponding level of *hTERT* mRNA expression. Autologous CD14$^+$ PB monocytes were used as a negative control because they lacked expression of *hTERT* mRNA. These results demonstrated that hTERT-*siTCR*/CD8 cells were able to exert tumoricidal activity against ATL tumor cells through recognition of the hTERT$_{461\text{-}469}$ epitope/HLA-A*24:02 complex, which is naturally presented on the surface of ATL tumor cells.

hTERT-*siTCR*–transduced CD8$^+$ T cells display in vivo anti-ATL reactivity

In vivo anti-ATL reactivity mediated by hTERT-*siTCR*/CD8 cells was assessed by using a xenografted mouse model and bioluminescence assay. Serial bioluminescence assay images were simultaneously acquired.

In the Winn assay (Figure 7A), tumor cell growth in NOG mice treated with hTERT-*siTCR*/CD8 (n = 2) was completely inhibited for longer than 6 months. In contrast, when compared with nontreated NOG mice (n = 2) in which the inoculated ATL tumor mass rapidly enlarged, activated NGM/CD8 (n = 2) did suppress ATL tumor growth to some degree, but eventually huge tumor masses developed within 2 months. In a therapeutic adoptive transfer model (Figure 7B), the tumor cell growth in mice treated with hTERT-*siTCR*/CD8 (n = 2) was obviously suppressed within the 8-week observation period, in contrast to that in mice treated with NGM/CD8 (n = 2) and that in control mice (n = 2).

Discussion

Although ATL still has a poor prognosis, the clinical presence of the graft-versus-ATL in patients treated successfully by allo-HSCT has encouraged the search for a novel cellular immune-mediated treatment of ATL. Unlike EBV-related malignancy,[6] the feasibility of HTLV-I–associated Tax[7] and HBZ[8] proteins as therapeutic targets of anti-ATL immunotherapy still remains controversial. Therefore, in this study, we explored the feasibility of a novel therapeutic target other than one associated with HTLV-I. Consequently, we demonstrated for the first time that hTERT was a promising therapeutic target for anti-ATL adoptive immunotherapy. Freshly isolated ATL tumor cells produced *hTERT* mRNA abundantly, and HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL precursors were detected in the periphery of HLA-A*24:02$^+$ ATL patients. These findings suggested that naturally processed and presented hTERT$_{461\text{-}469}$/HLA-A*24:02 complex on the surface of ATL tumor cells was sufficiently immunogenic to be recognized by the target-specific CTLs in HLA-A*24:02$^+$ ATL patients. Additionally, *hTERT* mRNA expression in newly generated HTLV-I–infected CD4$^+$ T cells was upregulated, and these cells became sensitive to gene-modified hTERT-specific CTLs (Figure 5B). The involvement of Tax[12] and HBZ[38] in upregulation of the *hTERT* gene in

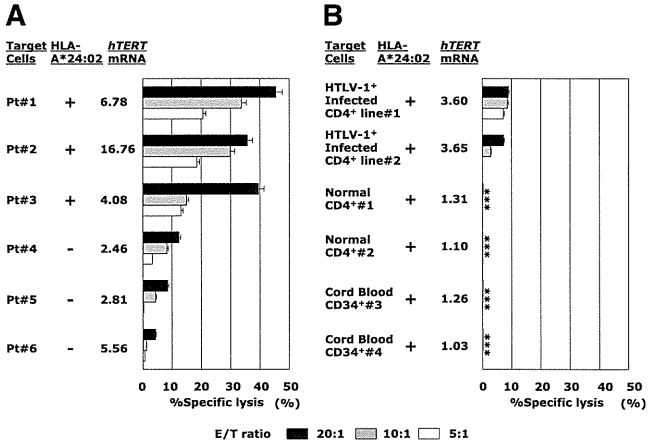

Figure 5. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated ATL cells and newly HTLV-I–infected CD4$^+$ T cells, but not normal cells, in vitro. (A) Freshly isolated HLA-A*24:02$^+$ (n = 3) or HLA-A*24:02$^-$ (n = 3) ATL tumor cells overexpressing *hTERT* mRNA were used as targets in ^{51}Cr-release assays with hTERT-*siTCR*–transduced CD8$^+$ T cells at the indicated E/T ratios. (B) The same hTERT-*siTCR*–transduced CD8$^+$ T cells used in panel A at the same E/T ratios were tested in ^{51}Cr-release assays against newly generated HLA-A*24:02$^+$ HTLV-I–infected CD4$^+$ T cells (n = 2) representing HTLV-I carrier CD4$^+$ T cells, original HLA-A*24:02$^+$ normal CD4$^+$ T cells (n = 2) representing the normal counterpart ATL tumor cells (corresponding number indicating cells from the identical donor), and HLA-A*24:02$^+$ normal CB-CD34$^+$ cells (n = 2) encompassing steady-state normal hematopoietic progenitor cells. Listed levels of expression of *hTERT* mRNA are those relative to the mean levels of expression across 6 PBMC samples from healthy donors determined by qRT-PCR and calculated by using the comparative threshold cycle method. Error bars represent SDs (* indicates less than detectable).

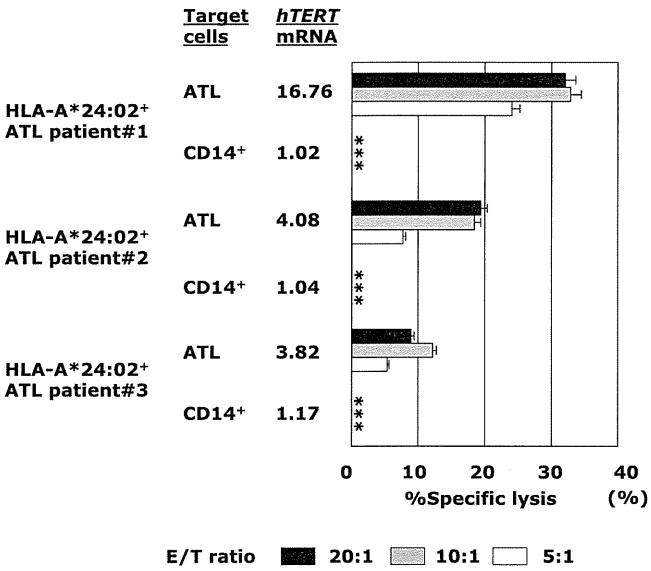

Figure 6. hTERT-*siTCR*–transduced CD8$^+$ T cells kill freshly isolated autologous ATL tumor cells on the basis of hTERT expression levels. Cytotoxic activity of hTERT-*siTCR*–transduced CD8$^+$ T cells obtained from HLA-A*24:02$^+$ ATL patients (n = 3) against autologous freshly isolated ATL tumor cells and autologous peripheral CD14$^+$ cells (negative control) was tested in ^{51}Cr-release assays at the indicated E/T ratios. *hTERT* mRNA in each patient's ATL tumor cells is listed using a format similar to that used in Figure 5. Error bars represent SDs (* indicates less than detectable).

HTLV-I–infected immortalized CD4$^+$ T cells and ATL tumor cells has been reported previously. Initially, it might seem more realistic to develop an hTERT$_{461\text{-}469}$ peptide vaccine for treatment of HLA-A*24:02$^+$ ATL patients. However, because we were concerned that CTL induction of hTERT peptide vaccine might have a tendency to be impeded by the regulatory T-cell function of ATL tumor cells,[21] we focused on developing a redirected T-cell–based adoptive immunotherapy targeting hTERT to allow administration of a number of hTERT-specific CTLs directly.

To this end, we cloned the full-length rearranged *TCR-α/β* genes from K3-1, the HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific CTL clone.[19] With codon optimization of the constant regions, we inserted them into our new souped-up second-generation 2A peptide-based *siTCR* vector to accomplish an increased expression level of the introduced TCR, carrying small interfering RNAs for the endogenous *TCR-α/β* genes in the redirected T cells (hTERT-*siTCR* vector).[26,27,34] The *siTCR* vector system makes it possible to simultaneously accomplish profound suppression of endogenous *TCR* genes and markedly increase the cell-surface expression of the introduced TCR, resulting in upregulated antitumor reactivity,[34] thus leading to inhibition of mispaired TCR formation between the endogenous and introduced TCR-α and -β chains, and lowering the potential risk of lethal graft-versus-host disease.[39] We found that both allogeneic and autologous gene-modified CD8$^+$ T cells using the hTERT-*siTCR* vector successfully killed ATL tumor cells both in vitro and in vivo (Figures 4-7), but not normal cells, including steady-state hematopoietic progenitor cells (Figure 5B). The introduced cytocidal activity against ATL tumor cells mediated by these gene-modified CTLs was actually accomplished through recognition of the HLA-A*24:02/hTERT$_{461\text{-}469}$ complex on the surface of ATL tumor cells (Figures 3 and 4).

Clinical studies of anticancer immunotherapy targeting hTERT have not demonstrated any significant adverse events so far.[14-17,20] However, for clinical application, because a number of activated gene-modified hTERT-specific CTLs would be administered at once, it would again be necessary to be mindful of on-target adverse events against normal tissues that constitutively express the *hTERT* gene.[10,40] Notably, any impairment of hematopoiesis would be the major concern. In this study, both allogeneic and autologous gene-modified effector CD8$^+$ T cells expressing hTERT-specific TCR from adult peripheral lymphocytes, and CB lymphocytes did not kill CB-CD34$^+$ cells representing steady-state hematopoietic progenitors (Figure 5B). By using cytokine-driven myeloid differentiation with CB-CD34$^+$ cells, gene-modified CTLs targeting hTERT showed a slight cytocidal effect against differentiated and highly proliferating subsets of CD34$^+$CD33$^+$ and CD34$^-$CD33$^+$ cells but spared CD34$^+$CD33^{dim} cells (supplemental Fig 2A). Additionally, contrary to resting CD4$^+$ cells and CD19$^+$ cells, highly mitotic polyhydroxic acid–stimulated CD4$^+$ cells and CD19$^+$ EBV LCLs became sensitive to effector CTLs because of increased expression of *hTERT* mRNA, the latter being more salient (Figure 5B and supplemental Fig 2B). Taken together, our findings suggest that gene-modified hTERT-specific CTLs will

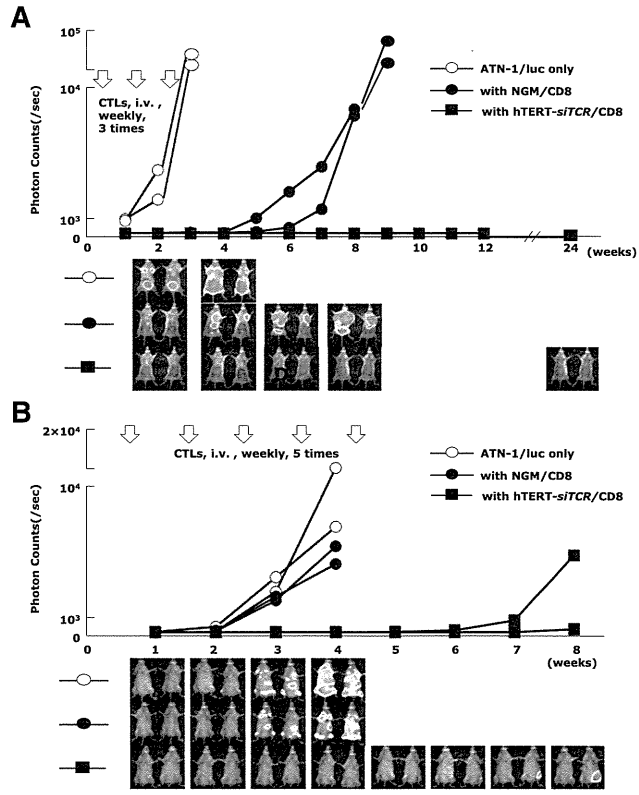

Figure 7. Anti-ATL reactivity of hTERT-*siTCR*–transduced CD8$^+$ T cells in vivo. (A) Winn assay. NOG mice were coinjected with a luciferase-transduced HLA-A*24:02$^+$ ATL cell line (ATN-1/luc) (5×10^5) and either 2.5×10^6 hTERT-*siTCR*–transduced (hTERT-*siTCR*/CD8) or NGM/CD8$^+$ T cells (n = 2 per group). Subsequently, 3 weekly infusions of the respective CD8$^+$ T-cell populations (2.5×10^6 cells per infusion) were administered intravenously (i.v.). Tumor growth was monitored every 7 days by using bioluminescence assay. Nontreated ATN-1/luc cells were similarly inoculated into NOG mice (n = 2) as a control. Although NGM/CD8 activated using OKT-3 and rhIL-2 suppressed tumor growth to some extent, hTERT-*siTCR*/CD8 durably suppressed tumor growth for longer than 6 months. (B) Therapeutic adaptive transfer model. NOG mice were intravenously inoculated with 5×10^5 ATN-1/luc cells. Four days later, intravenous administration of either 5×10^6 hTERT-*siTCR*/CD8 or NGM/CD8 (n = 2 per group) was started once a week for a total of 5 infusions. NOG mice given only ATN-1/luc cells (n = 2) were used as a control. In comparison with NGM/CD8, therapeutically infused hTERT-*siTCR*/CD8 also obviously suppressed the tumor cell growth within the 8-week observation period. Serial images of the bioluminescence assay demonstrate tumor growth in each group.

spare steady-state hematopoietic progenitor cells. However, to ensure safety, it would be better to avoid the active recovery phase of bone marrow after chemotherapy, notably under granulocyte colony-stimulating factor support, and also the acute infectious period in which immune-cell components are stimulated.

Another likely problem in clinical practice is that heavily pretreated peripheral lymphocytes from ATL patients might fail to proliferate. Proliferative activity of therapeutically infused gene-modified T cells in vivo is an important prerequisite for a successful outcome.[41] In this connection, although the control of treatment-related graft-versus-host disease still remains unsolved, use of CB lymphocytes has been investigated.[42] In this study, gene-modified CB-CD8$^+$ T cells from 2 donors successfully killed ATL tumor cells but spared autologous steady-state CB-CD34$^+$ cells (supplemental Figure 1-(2)). Compelling lack of suitable allo-HSCT donors for patients of advance age with ATL will encourage the application of CB transplantation using reduced-intensity preconditioning in the near future. Genetic redirection of CB lymphocytes using tumor antigen–specific *TCR* gene transfer will also play a considerable role.

Conversely, because hTERT is overexpressed in various kinds of cancer,[10] this approach may have widespread potential clinical application. Furthermore, the clinical availability of a new defucosylated anti-CCR4 mAb for treatment of ATL[43] can be reasonably anticipated to diminish regulatory T cells, the key player in the immunosuppressive microenvironment in patients with cancer,[44] because CCR4 is also expressed on regulatory T cells.[45] Therefore, hTERT-targeting immunotherapy after preconditioning with this anti-CCR4 mAb may become a realistically promising treatment option not only for ATL, but also for other malignancies.

In summary, using a newly established hTERT-*siTCR* vector, we have demonstrated the feasibility of anti-ATL redirected T-cell–based adoptive immunotherapy targeting hTERT, notably for patients who are ineligible for allo-HSCT. Further studies will be needed to investigate the clinical safety and utility of this novel therapy.

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Authorship

Contribution: Y.M. performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper and provided financial support; H.A., F.O., and T.O. performed the research and discussed the experimental results; T.A. interpreted the experimental results and provided financial support; T.I., S.O., J.M., K.K., and H.S. provided materials and discussed the experimental results; and M.Y. discussed and interpreted the experimental results and provided financial support.

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Bath-PUVA Therapy Decreases Infiltrating CCR4-Expressing Tumor Cells and Regulatory T Cells in Patients With Mycosis Fungoides

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Abstract

In this study, we analyzed the mechanism that bath-PUVA therapy to CCR4-expressing tumor cells and regulatory T cells (Treg) in patients with mycosis fungoides(MF). The CCR4 positive cell and Treg in patient blood and the skin were analyzed. Both type of cells decreased after bath-PUVA in the skin lesion, in contrast, bath-PUVA did not significantly change the percent circulating Treg. It suggested that bath-PUVA eliminated both pathogenetically relevant cells and Treg and systemic immunosuppression was not induced.

Background: Mycosis fungoides (MF) is a malignant lymphoma characterized by expansion of $CD4^+$ memory T-cell clones. Infiltrating cells express CCR4, which is attracted to CC chemokine ligands 17 and 22 (thymus and activation-regulated chemokine [TARC]/CCL17 and TARC/CCL22). Bath–psoralen plus ultraviolet A (PUVA) is effective against MF. In patients with psoriasis, bath-PUVA induces circulating regulatory T cells (Tregs), which suppress effector T cells. To understand the mechanisms in MF, we analyzed lesion-infiltrating cells before and after bath-PUVA therapy. **Patients and Methods:** Thirteen patients with MF (12 stage IB, 1 stage III; mean age 69.2 years, range 35-87 years; 6 men, 7 women) were recruited. **Results:** Immunohistochemical analysis revealed that lesion CCR4-positive ($CCR4^+$) cells and Tregs significantly decreased from 105.1 ± 164.8 cells/10^{-2} mm^2 to 31.4 ± 39.0 cells/10^{-2} mm^2 and from 78.1 ± 67.8 cells/10^{-2} mm^2 to 24.7 ± 25.0 cells/10^{-2} mm^2, respectively. Serum TARC levels significantly correlated with infiltrating $CD3^+$ (r = 0.997), $CCR4^+$ (r = 0.991), and forkhead box P3–positive ($Foxp3^{+)}$ cells (r = 0.843). Circulating Tregs before bath-PUVA therapy were not significantly different from those in healthy volunteers. Bath-PUVA did not significantly change the percentage of circulating Tregs. **Conclusions:** Bath-PUVA decreased $CCR4^+$ cells and Tregs in MF lesions but did not induce circulating Tregs, which might suppress effector T cells. Direct effects through skin lesions might eliminate both pathogenetically relevant cells and Tregs. Systemic immunosuppression was not induced.

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Keywords: Bath-PUVA therapy, CCR4, Mycosis fungoides, Regulatory T cell, Thymus and activation-regulated chemokine (TARC)

Introduction

Mycosis fungoides (MF) is a malignant cutaneous lymphoma with a chronic disease progression.[1] Because erythema and red plaques appear on the patient's whole body at an early stage, it is important to distinguish MF from other skin diseases. Various symptoms are associated with MF, including lymph node enlargement, skin tumors, and ulcer formation in the tumor stage. Symptom onset usually occurs in those older than 60 years of age, but the actual disease onset is earlier.[2] The histologic findings depend on the stage. In the erythema stage (stage I), the characteristic features include epidermal hyperplasia, lymphoid exocytosis, and band-like lymphoid infiltration in the

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Table 1 Patient Characteristics

Patient	Age	Sex	Disease Stage	Irradiation Frequency	Cumulative UV Doses (J/cm^2)
1	82	F	IB	46	170.4
2	68	F	III	42	146.4
3	35	F	IB	43	53.7
4	71	M	IB	20	45.0
5	87	M	IB	33	120.0
6	82	M	IB	5	8.0
7	77	M	IB	29	106.0
8	70	F	IB	37	138.0
9	83	M	IB	38	138.0
10	62	F	IB	14	29.5
11	56	M	IB	30	106.0
12	64	F	IB	42	150.0
13	62	F	IB	25	34.5
Mean ± SD	69.2 ± 14.1			31.1 ± 12.3	95.8 ± 54.5

Abbreviations: SD = standard deviation; UV = ultraviolet.

Figure 1 Immunohistochemical Analysis for Before Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. Hematoxylin and Eosin Stain (A), Anti-CD3 (B), Anti-CCR4 (C), Anti-Foxp3 (D). A Pautrier Microabscess was Observed in the Epidermis. Many CCR4$^+$ Lymphocytes Were Observed. In Contrast, There Were a Few Foxp3$^+$ Cells. Yellow Triangles Were Some of Positive Cells

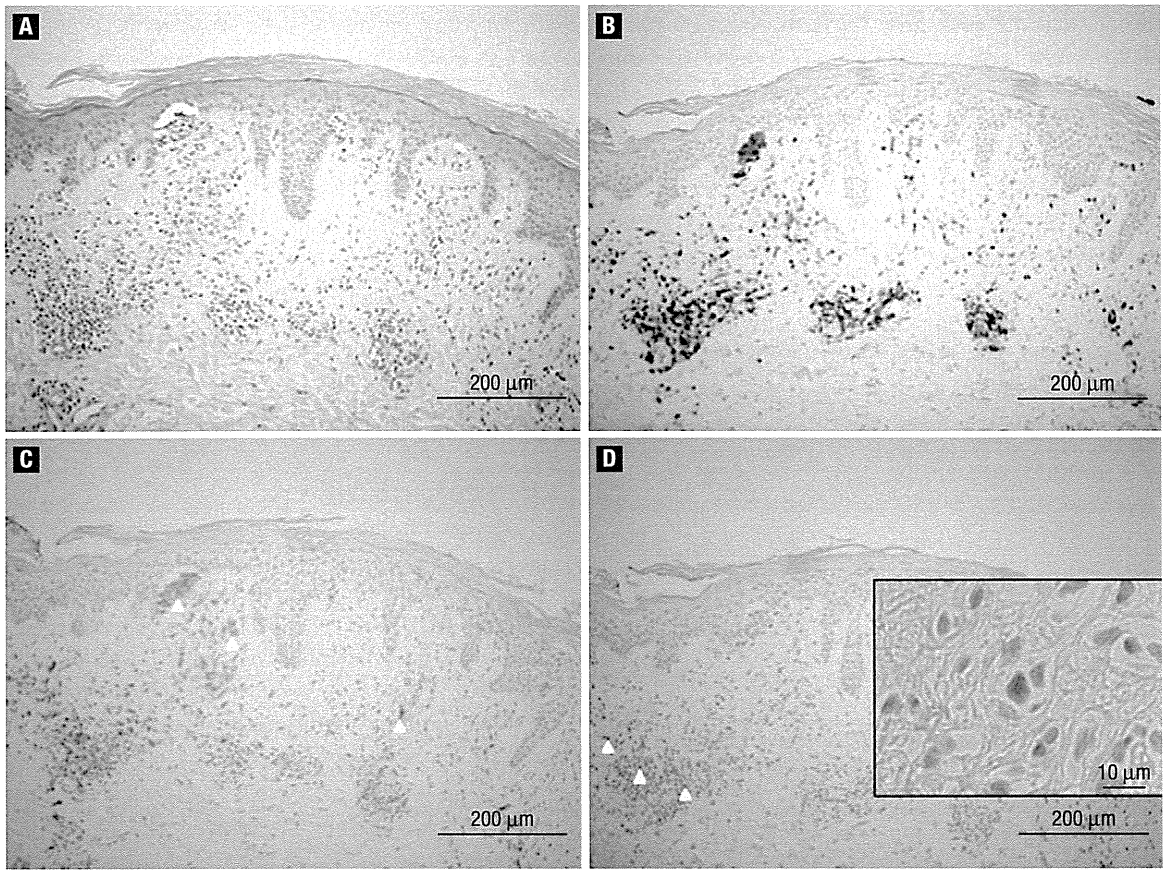
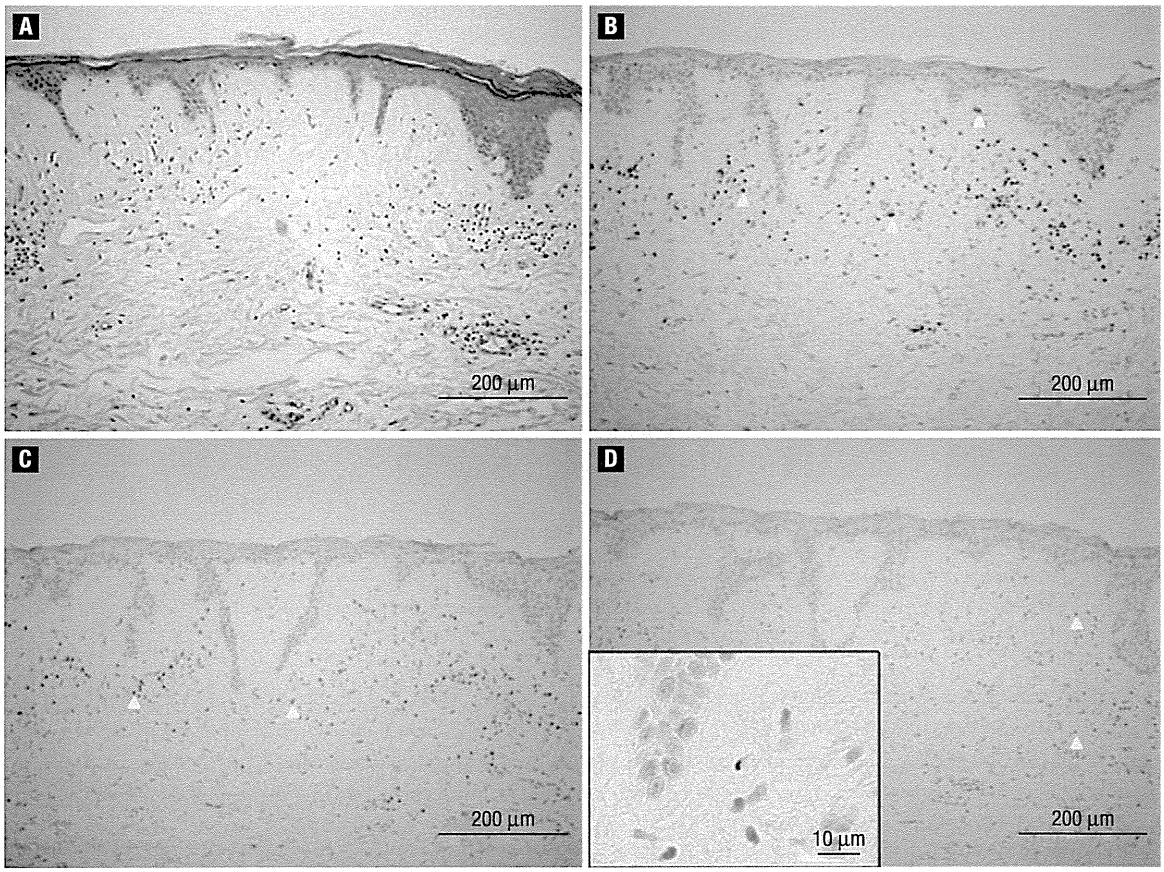


Figure 2 Immunohistochemical Analysis for After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. Hematoxylin and Eosin Stain (A), Anti-CD3 (B), Anti-CCR4 (C), and Anti-Foxp3 (D). The Number of Infiltrating Cells was Decreased. Yellow Triangles Were Some of Positive Cells



superficial dermis. In the plaque stage (stage II), Pautrier microabscesses are often observed. In the tumor stage (stage III), tumor cells infiltrate the nodular lesions and proliferate with necrosis, and then ulcers form in the tumorous lesions. In stages I and II, the 5-year survival rate is > 90%, but in stage III the rate drops to approximately 40%.[3]

In the initial stage, topical corticosteroids are used for red plaques.[4] Psoralen ultraviolet A (PUVA) or narrowband UVB is used mainly for stage I.[5,6] More severe cases require radiotherapy and chemotherapy. There are some clinical reports of bath-PUVA therapy for MF.[5] We recently reported that bath-PUVA therapy induces circulating regulatory T cells (Tregs), which suppress effector T cells such as Th17, in patients with psoriasis.[7] The underlying mechanisms of bath-PUVA therapy in MF, however, are unclear. Therefore, we analyzed cells infiltrating the lesions before and after bath-PUVA therapy. Circulating lymphocytes in the peripheral blood were also analyzed.

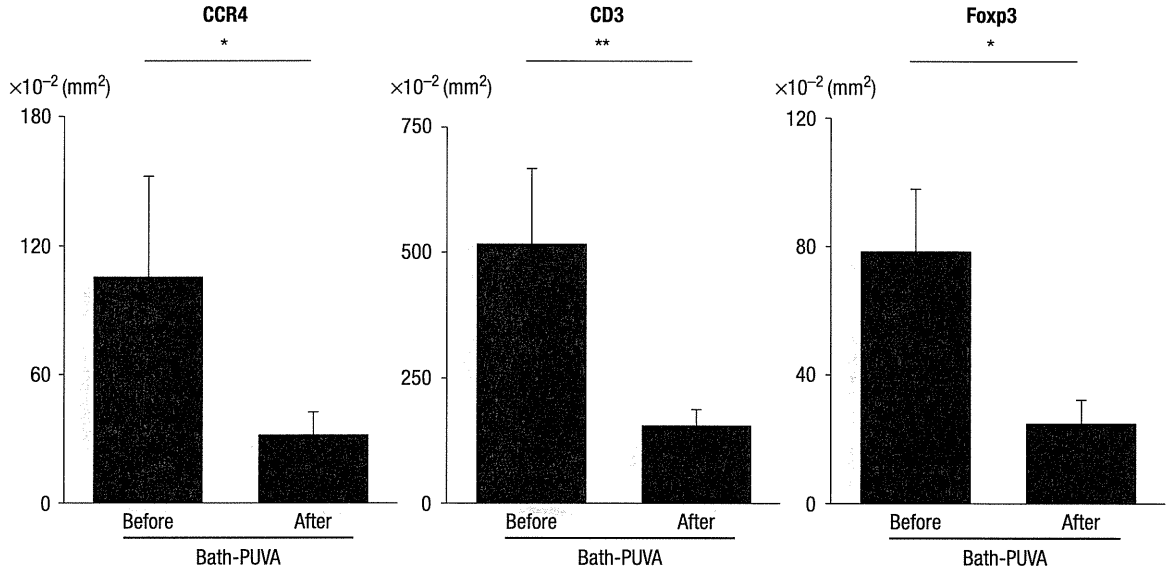
CCR4 is a chemokine receptor expressed on certain types of T-cell neoplasms, including MF and adult T-cell leukemia/lymphoma (ATLL).[8-10] Clinical development of the therapeutic humanized anti-CCR4 monoclonal antibody KW-0761 is in progress.[11] A phase I clinical trial of KW-0761 for patients with relapsed CCR4-positive ($CCR4^+$) T-cell neoplasms including MF was completed.[12] The subsequent phase II clinical trials of KW-0761 against relapsed ATLL (http://ClinicalTrials.gov Identifier: NCT00920790), untreated ATLL (NCT01173887), and relapsed peripheral T-cell lymphoma (NCT01192984) are currently being conducted in Japan. In the United States, a phase I/II clinical trial of KW-0761 against relapsed peripheral T-cell lymphoma has also been conducted (NCT00888927). In the present study, we analyzed the relationship between $CCR4^+$lymphocytes and some parameters from MF patients.

Patients and Methods

Patients

Thirteen patients of Japanese origin diagnosed with MF (mean age, 69.2 years; range, 35-87 years; 7 women and 6 men; 12 patients with stage IB and 1 patient with stage III disease) and 10 healthy controls (mean age, 31.7 years; range, 23-46 years; 6 women and 4 men) were recruited for the study. Serum-soluble interleukin-2

Figure 3 Number of Infiltrating Lymphocytes Before and After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy in Stage I MF. The Number of Lymphocytes Decreased After Bath-PUVA Therapy (*$P < .05$ by Wilcoxon signed rank test)



receptor (sIL2R) and thymus and activation-regulated chemokine (TARC)/CCL17 levels were measured in 13 and 5 patients, respectively, as part of the clinical blood examination at SRL Inc (Tokyo, Japan). Fluorescence-activated cell sorting (FACS) analysis (FACSCalibur Flow Cytometry System, Becton Dickinson, Franklin Lakes, NJ,) was applied in 6 cases. Punch biopsies of 3 or 4 mm were performed in the same lesion before and after bath-PUVA therapy. In healthy controls, only the peripheral blood was examined. The analysis was conducted with the approval from the Ethics Committee of Nagoya City University. Patient profiles are summarized in Table 1.

Bath-PUVA Therapy

Patients were placed in a 37°C bath containing 0.0001% 8-methoxypsoralen before UVA radiation treatment 5 times per week. A whole-body UVA radiation unit, the Dermaray TS (Eisai-Toshiba, Tokyo, Japan) with FLR100HBL/A/DMR fluorescence tubes, was used for UVA irradiation. The initial dose was 0.5 J/cm^2 with subsequent doses increased by increments of 0.5 J/cm^2 to a maximum dose of 4 J/cm^2. The mean number of irradiation treatments was 25.1. Mean cumulative UVA dose was 79.1 J/cm^2. The patient treatment profiles are summarized in Table 1.

Immunohistochemical Analysis

Staining for CD3, anti-CCR4 antibodies, and forkhead box P3 (Foxp3) was performed as follows. The sections were fixed with 10% neutral-buffered formalin. Formalin-fixed paraffin sections were stained with anti-CCR4 antibody (KM2160, Kyowa Hakko Kirin, Tokyo, Japan), polyclonal rabbit antihuman CD3 antibody (A0452, Dako, Carpinteria, CA), and antimouse monoclonal antibody to Foxp3 (236 A/E7, Abcam, Tokyo, Japan) following standard protocols using diaminobenzidine as the chromogen.[13] Positive cells in all sections were counted manually by 2 independent researchers (HK, CS). The sections were measured and the number of positive cells in each section was calculated.

FACS Analysis of Peripheral Blood Mononuclear Cells

Peripheral blood was obtained from patients before and after bath-PUVA therapy. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation and stained with fluorescence-conjugated antihuman CD4 (MT310; Dako A/S, Glostrup, Denmark), CD25 (ACT-1, Dako A/S), Foxp3 (PCH101, eBioscience, San Diego, CA), and the appropriate isotype control antibodies followed by FACS analysis.

Statistical Analysis

The significance of changes in the variables before and after bath-PUVA therapy was examined using the Wilcoxon signed-rank test. Correlations between 2 variables obtained from patients with MF were assessed using the Spearman rank correlation coefficient. Differences in the variables between the 2 groups were examined with the Wilcoxon rank-sum test. Statistical analyses were performed using the Pharmaco Analyst II software (Human Life, Japan).

Results

Bath-PUVA therapy was well tolerated in all patients enrolled in the study. The red plaques in all patients improved clinically after bath-PUVA therapy. The number of atypical stage IB and stage III MF tumor cells are usually minimal, so it is difficult to clearly identify the tumor cells histologically from many reactive infiltrating