Clinical significance of *CD28* gene-related activating alterations in adult T-cell leukaemia/lymphoma

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Summary

Multiple oncogenic events are involved in the development of adult T-cell leukaemia/lymphoma (ATL). Because CD28 plays a pivotal role in T-cell activation, we focused on alterations of the *CD28* gene in ATL. We found multiple genetic abnormalities related to *CD28* among the 144 patients enrolled in the present study. These involved gene fusions with the *cytotoxic T-lymphocyte-associated antigen 4* or the *inducible T-cell co-stimulator* in 14 patients (10%), *CD28*-activating mutations in 3 (2%), and *CD28* copy number variations in 34 (24%). Patients with such *CD28* gene alterations were significantly younger than those without. In patients not receiving allogeneic haematopoietic stem cell transplantation, those with *CD28* gene alterations tended to have a worse prognosis than those without. Finally, patients with chronic or smouldering ATL subtypes with *CD28* gene alterations had a significantly worse prognosis than those without. These findings indicate that ATL, especially chronic or smouldering subtypes, have a more aggressive clinical course and are more refractory to conventional chemotherapies or mogamulizumab if they harbour *CD28* gene alterations, likely because of continuous, prolonged, and enhanced CD28 activatory signalling. Novel treatment strategies to overcome the effects of these *CD28* gene alterations are warranted.

Keywords: adult T-cell leukaemia/lymphoma, CD28, fusion, mutation, copy number variation.

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Introduction

Adult T-cell leukaemia/lymphoma (ATL) is a peripheral T-cell neoplasm caused by human T-cell lymphotropic virus type-1 (HTLV-1).[1–4] Kataoka *et al.* recently delineated the entire landscape of genetic aberrations in ATL and concluded that alterations to the T-cell receptor (TCR) and related signalling pathways resulting in cell activation were frequently observed.[5] Because antigen engagement of the TCR initiates a genetic program that results in T-cell activation, it is reasonable that these pathways might contribute to carcinogenesis. Nevertheless, TCR engagement alone is not sufficient for full activation of normal T cells, which requires a second signal, commonly via ligation of CD28.[6–8] In this context, T-cell-activating alterations to the *CD28* gene have been reported not only in ATL, but also in other peripheral T-cell neoplasms such as angioimmunoblastic T-cell lymphoma (AITL), peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS), or cutaneous T-cell lymphoma (CTCL).[9–12] These reports indicate that *CD28* gene-related activating alterations may play an important role in the pathogenesis of some mature T-cell neoplasms. The aim of the present study was to determine the clinical significance of *CD28* gene-related activating alterations in ATL.

Methods

ATL patients

The study included 144 ATL patients. Tumour samples were obtained at the time of initial presentation at the participating hospitals, and we used the clinical characteristics recorded at that time. Details are available in Data S1.[2,13–22].

Nucleic acid extraction

Details are available in Data S1.

Detection of CD28 gene alterations

The primer pairs used are shown in Figure S1 and Table SI. For positive controls for the four fusions, we synthesized their cDNAs *in vitro* (Table SII). The *CD28*-activating mutations were detected using a highly sensitive SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) (Figure S2). To investigate *CD28* copy number variation (CNV), fluorescence *in situ* hybridization (FISH) was performed. Details are available in Data S1.[5,9-11,23-27]

Detection of CCR4 gene mutations

Details are available in Data S1.[25]

Statistical analysis

The start date for assessing overall survival (OS) was the day when the tumour sample was obtained. Details are available in Data S1.[28]

Results

CD28 gene alterations in ATL patients

The ATL patients enrolled in this study included 65 men and 79 women (age range, 41–90 years; median, 64 years) (Table SIII). A multiplex reverse transcription polymerase chain reaction (RT-PCR) analysis for *CD28* fusions is shown in Figure S1B. *CD28* fusions were observed in the tumours of 14 patients (10%) (Table I). Sequences of the fusion boundary regions from a patient with inducible T-cell co-stimulator (*ICOS*) (ex1)–*CD28* (ex2), and those from another patient with cytotoxic T-lymphocyte-associated antigen 4 (*CTLA4*) (ex3)–*CD28* (ex4) are shown in Fig 1A and B, respectively. Both sequences are completely consistent with earlier reports.[9–29] *CD28*-activating mutations were present in three patients (2%). *CD28* copy number variation (CNV) was found in 34 patients (24%). Among these, three patients concurrently harboured two different types of *CD28* gene alterations. Collectively, alterations of any type involving the *CD28* gene were present in 48 patients (33%) (Table I). To illustrate the FISH analysis, *CD28:CEP2* (centromere enumeration probe for chromosome 2) signal numbers of 7:2, 6:3, 5:2, 4:2, 3:2, and 2:2 are shown in Fig 2A–F, respectively.

Clinical characteristics of ATL patients stratified by CD28 gene alterations

There were no significant differences in sex, clinical subtype, Eastern Cooperative Oncology Group (ECOG) performance status (PS), Ann Arbor stage, serum soluble interleukin-2 receptor (sIL-2R) level, serum-adjusted calcium (Ca), serum albumin (Alb), white blood cell (WBC) counts, haemoglobin (Hb), or platelet (Plt) counts between patients with or without *CD28* gene alterations. There were also no significant differences in the presence or absence of CC chemokine receptor 4 (CCR4) mutations. Patients with *CD28* gene alterations were significantly younger than those without (Table II). In relation to the types of *CD28* gene alterations, there were no significant differences in those characteristics between patients with *CD28* fusions and those without any *CD28* gene alterations. The same was true for the *CD28* mutations or CNV (Table SIV).

OS of ATL patients stratified by CD28 gene alterations

Five-year OS of all patients enrolled in the present study was 45·6% (Fig 3A), and that of 48 patients with *CD28* gene alterations and 96 patients without any alterations was 38·9% and 49·6%, respectively (not significantly different, $P = 0{\cdot}076$) (Fig 3B). Five-year OS of 14 patients with *CD28* fusions was 38·1% (Fig 3C), that of three with *CD28* mutations was 33·3% (Fig 3D), and that of 34 with *CD28* CNV was 41·6% (Fig 3E). None of these were significantly different from patients without any *CD28* gene alterations.

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Table 1. Types and frequencies of *CD28* gene alterations in ATL according to clinical subtypes.

Type of gene alterations	Clinical subtype	*N* (%)				
		Acute 79 (55)	Lymphoma 41 (28)	Chronic 11 (8)	Smoldering 13 (9)	Total number (%) 144 (100)
CD28 fusions		11 (14)	2 (5)	1 (9)	1 (8)	15/144 (10)
CTLA4 (ex1)-CD28 (ex2)		0	0	0	0	0
CTLA4 (ex2)-CD28 (ex4)		0	0	0	0	0
CTLA4 (ex3)-CD28 (ex4)		1 (1)	1 (2)	0	0	2/144 (1)
ICOS (ex1)-CD28 (ex2)		10 (13)	1 (2)	1 (9)	1 (8)	13/144 (9)
CD28 mutations		2 (3)	0	0	1 (8)	3/144 (2)
F51I/V		0	0	0	1 (8)	1/144 (1)
D124V/E		2 (3)	0	0	0	2/144 (1)
T195I/L/P		0	0	0	0	0
CD28 CNV		20 (25)	12 (29)	0	2 (15)	34/144 (24)
Gain*		9 (11)	10 (24)	0	1 (8)	20/144 (14)
Amplification†		11 (14)	2 (5)	0	1 (8)	14 /144 (10)
Overall *CD28* gene alterations		31 (39)‡	13 (32)§	1 (9)	3 (23)¶	48/144 (33)

ATL, adult T-cell leukemia/lymphoma; CNV, copy number variations; CTLA4, cytotoxic T-lymphocyte associated antigen 4; ICOS, inducible T-cell co-stimulator.

*Gains were all *CD28*:*CEP2* signal number of 3:2.

†Amplifications included *CD28*:*CEP2* signal number of 7:2 in 2, 6:2 in 2, 6:3 in 1, 5:2 in 4, and 4:2 in 5 patients.

‡Two patients with acute-type harbored two different types of *CD28* gene alterations; one had a *CTLA4* (ex3)-*CD28* (ex4) fusion and a CNV gain, a second had an *ICOS* (ex1)-*CD28* (ex2) fusion and a *CD28* mutation (D124E).

§One patient with lymphoma-type had both *ICOS* (ex1)-*CD28* (ex2) and *CTLA4* (ex3)-*CD28* (ex4).

¶One patient with smoldering-type harbored two different types of *CD28* gene alterations; an *ICOS* (ex1)-*CD28* (ex2) fusion and a CNV gain.

Interaction of CD28 gene alterations with clinical subtypes in terms of OS

We investigated the interaction of *CD28* gene alterations with clinical subtypes in terms of OS. When the hazard ratio (HR) for death in patients of acute or lymphoma subtypes without *CD28* gene alterations was determined as 1·000, the HR in those with *CD28* gene alterations, and patients of chronic or smouldering subtypes with and without *CD28* gene alterations were 1·199, 0·788, and 0·072, respectively ($P_{\text{interaction}}$ = 0·316; Figure S3A).

OS of ATL patients stratified by clinical subtypes

The five-year OS of patients with acute or lymphoma subtypes was 37·6% (*n* = 120, data not shown). Of these, the five-year OS of 44 patients with *CD28* gene alterations was 35·8% and that of 76 patients without *CD28* gene alterations was 39·1% (*P* = 0·477; Fig 3F). HR for OS in patients with acute or lymphoma subtypes with *CD28* gene alterations compared with that in patients without *CD28* gene alterations was 1·197 [95% confidence interval (CI), 0·729–1·968; Figure S4A]. The five-year OS of patients with chronic or smouldering subtypes was 89·1% (*n* = 24, data not shown). In this group, the five-year OS of the four patients with *CD28* gene alterations was 75·0%, which was significantly shorter than that of the 20 without *CD28* gene alterations (92·3%, *P* = 0·023; Fig 3G). HR for OS in patients of

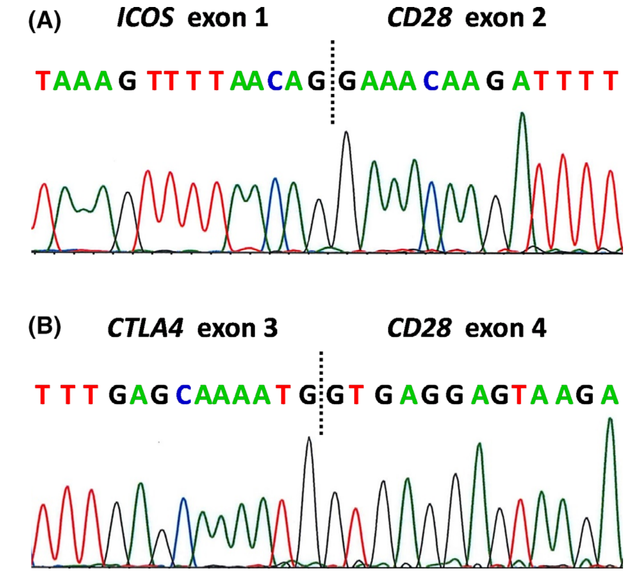

Fig 1. Sequences of the boundary regions of *CD28* gene-related fusions. Sequences of reverse transcription polymerase chain reaction (RT-PCR) products of (A) inducible T-cell co-stimulator (ICOS) (ex1)–*CD28* (ex2), and (B) cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) (ex3)–*CD28* (ex4). [Colour figure can be viewed at wileyonlinelibrary.com]

chronic or smouldering subtypes with *CD28* gene alterations compared with that without *CD28* gene alterations was 9·849 (95% CI, 0·883–109·907, Figure S4A).

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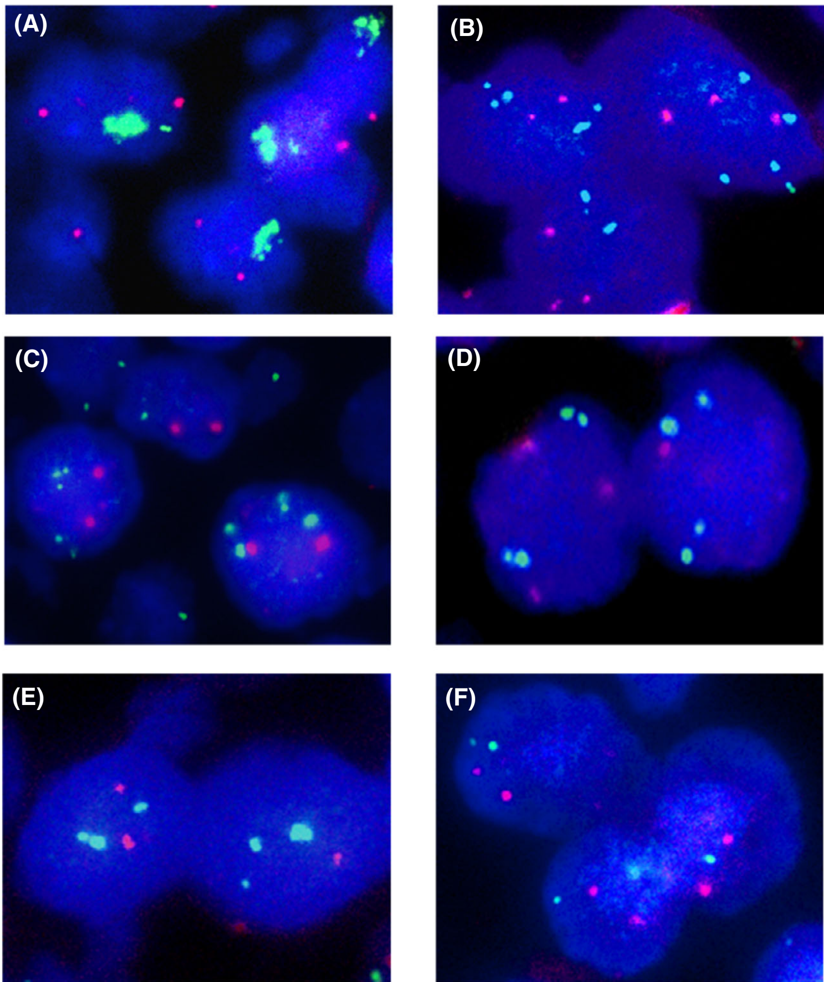

Fig 2. *CD28* copy number variation (CNV) in adult T-cell leukaemia/lymphoma (ATL) by fluorescence *in situ* hybridization (FISH). FISH analyses on FFPE sections from eight individual ATL patients. *CD28* signals on chromosome 2q33 are green, and centromeric signals of chromosome 2 are red. *CD28* signal number: centromeric signal number ratios were 7:2 (A), 6:3 (B), 5:2 (C), 4:2 (D), 3:2 (E) and 2:2 (F). [Colour figure can be viewed at wileyonlinelibrary.com]

Interaction of CD28 gene alterations in patients who received and not received allogeneic haematopoietic stem cell transplantation treatment in terms of OS

Allogeneic haematopoietic stem cell transplantation (HSCT) is generally accepted as the only curative treatment for ATL patients. However, treatment-related mortality for allogeneic HSCT is high compared with that for other treatments.[16–18] In addition, patients with *CD28* gene alterations received allogeneic HSCT significantly more frequently compared with those without *CD28* gene alterations (Table SV). Thus, we investigated the interaction of *CD28* gene alterations in terms of OS in patients who received allogeneic HSCT and in those who did not. When the HR in patients who did not receive allogeneic HSCT without *CD28* gene alterations was determined as 1·000, the HR in those with *CD28* gene alterations, and in patients who received allogeneic HSCT with and without *CD28* gene alterations, was 1·887, 1·010, and 0·825, respectively ($P_{interaction}$ = 0·241, Figure S3B).

Survival of ATL patients receiving allogeneic HSCT, stratified by CD28 gene alterations

We evaluated the impact of *CD28* gene alterations on patients receiving allogeneic HSCT, separately from that in non-transplanted patients. However, five-year survival from the day of allogeneic HSCT in all 35 transplanted patients was 50·6% (Fig 4A), and did not differ between the 18 with and the 17 without *CD28* gene alterations (47·1% and 52·4%, respectively, P = 0·748, Fig 4B). For the eight patients with *CD28* fusions, five-year survival from the day of HSCT was 37·5%, also not significantly different from that in those without *CD28* gene alterations (P = 0·461, Fig 4C). No patients with *CD28* mutations had received allogeneic HSCT. Finally, five-year survival from the day of HSCT in 11 patients with *CD28* CNV was 60·0%, again not significantly different from that of patients without *CD28* gene alterations (P = 0·672, Fig 4D). Next, we investigated survival of the patients receiving allogeneic HSCT stratified by clinical

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Table II. Characteristics of adult T-cell leukaemia/lymphoma (ATL) patients according to *CD28* gene alterations.

Characteristics	*CD28* gene alterations		*P* value
	Absence	Presence	
N (%)	96 (67)	48 (33)	
Sex			
Female	53 (55)	26 (54)	1·000
Male	43 (45)	22 (46)	
Clinical subtype			
Chronic, smouldering	20 (21)	4 (8)	0·062
Acute, lymphoma	76 (79)	44 (92)	
ECOG PS†			
0, 1	75 (79)	31 (65)	0·072
2, 3, 4	20 (21)	17 (35)	
Ann Arbor stage			
I, II	15 (16)	4 (8)	0·299
III, IV	81 (84)	44 (92)	
Serum sIL-2R (U/ml)‡			
≤20 000	61 (68)	28 (61)	0·450
>20 000	29 (32)	18 (39)	
Serum Ca (mg/dl)*,§			
≤11·0	80 (88)	39 (85)	0·603
>11·0	11 (12)	7 (15)	
Serum Alb (g/dl)¶			
≥3·5	67 (74)	31 (66)	0·429
<3·5	24 (26)	16 (33)	
Age (year)			
Mean	66	60	0·035
Median	66	61	
Range	41–90	41–84	
WBC (/μl)**			
Mean	13 153	20 570	0·634
Median	8750	8410	
Range	2800–68 400	2500–232 100	
Hb (g/l)**			
Mean	129	126	0·809
Median	130	132	
Range	79–160	88–171	
Plt ($\times 10^3$/μl)**			
Mean	229	279	0·548
Median	215	205	
Range	15–622	60–380	
CCR4 gene mutation			
Absence	65 (68)	30 (63)	0·578
Presence	31 (32)	18 (37)	

ATL, adult T-cell leukaemia/lymphoma; ECOG, Eastern Cooperative Oncology Group; PS, performance status; sIL-2R, soluble interleukin-2 receptor; Ca, calcium; Alb, albumin; WBC, white blood cell count; Hb, haemoglobin; Plt, platelet count; CCR4, CC chemokine receptor 4.

*When serum Alb level was <4·0 g/dl, serum Ca was adjusted by the concentration of serum Alb as follows: adjusted Ca level (mg/dl) = measured Ca level (mg/dl) + [4·0−Alb level (g/dl)].

†One patient's data were unknown.

‡Eight patients' data were unknown.

§Seven patients' data were unknown.

¶Six patients' data were unknown.

**Five patients' data were unknown.

subtype. Five-year survival from the day of HSCT in patients with acute or lymphoma subtypes was 47·3% (n = 32, data not shown). Five-year survival from the day of HSCT in the 15 patients without *CD28* gene alterations was 48·6%, and 43·8% in the 17 with *CD28* gene alterations, again not significantly different (P = 0·759, Fig 4E). HR for survival in

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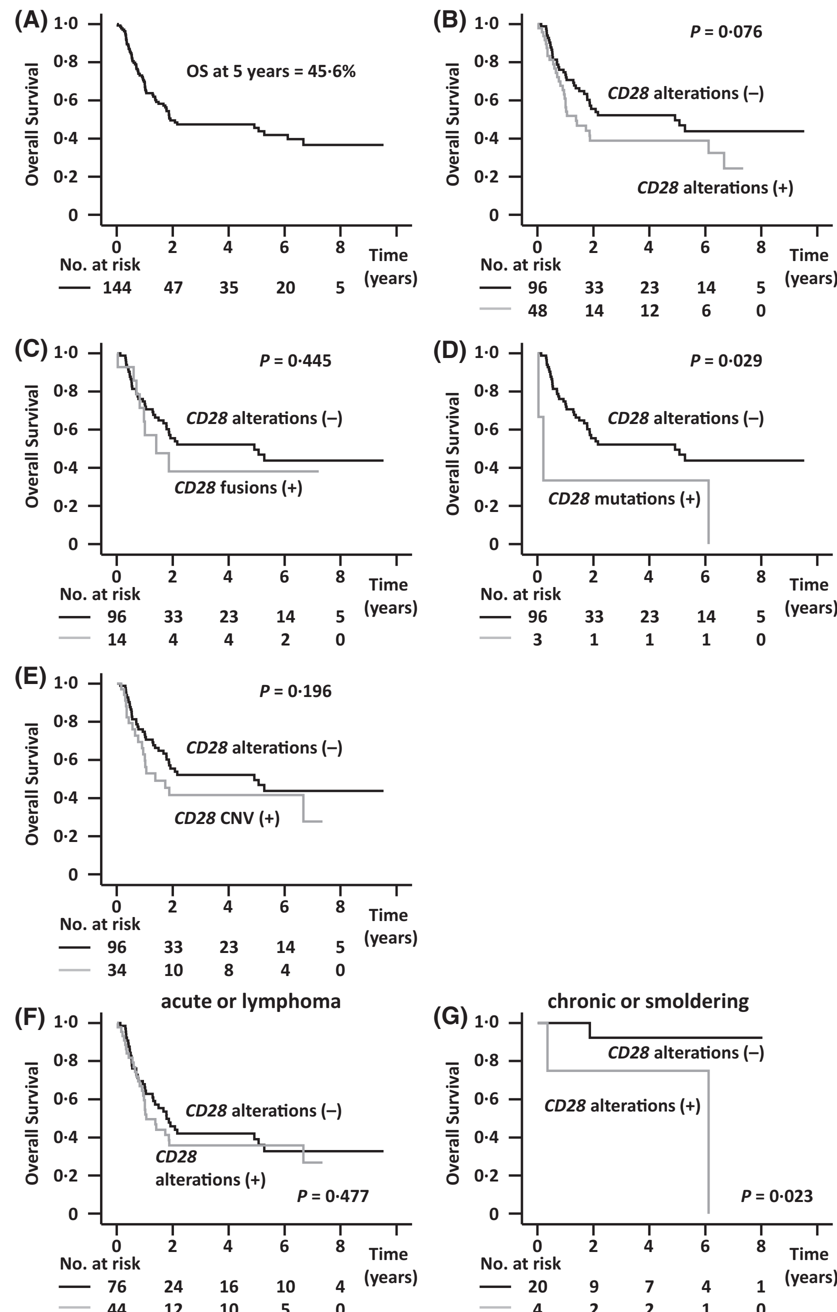

Fig 3. Overall survival (OS) of all adult T-cell leukaemia/lymphoma (ATL) patients enrolled in the study, stratified according to *CD28* gene alterations. (A) OS of all ATL patients enrolled in the study (n = 144). (B) OS according to *CD28* gene alterations. (C) OS of the 14 ATL patients with *CD28* fusions and the 96 without *CD28* gene alterations. (D) OS of the 3 ATL patients with *CD28* mutations and the 96 without *CD28* gene alterations. (E) OS of the 34 ATL patients with *CD28* CNV and the 96 without *CD28* gene alterations. (B–E) P < 0·05/4 (two-sided) was considered statistically significant after Bonferroni correction. (F) OS of ATL patients with acute or lymphoma subtypes according to *CD28* gene alterations. (G) OS of ATL patients with chronic or smouldering subtypes according to *CD28* gene alterations.

patients of acute or lymphoma subtypes with *CD28* gene alterations compared with those without *CD28* gene alterations was 1·167 (95% CI, 0·434–3·142, Figure S4B). Five-year survival from the day of HSCT in the three transplanted patients with chronic or smouldering subtypes could not be estimated. These three patients were all alive at their last follow-up. One of the three had *CD28* gene-related alterations.

Survival of ATL patients who did not receive allogeneic HSCT stratified by CD28 gene alterations

Five-year OS of patients not receiving allogeneic HSCT was 43·6% (n = 109, Fig 5A). In this cohort, five-year OS of 30 patients with *CD28* gene alterations vs. 79 without was 32·8% vs. 48·4%, respectively, thus, the former tended to

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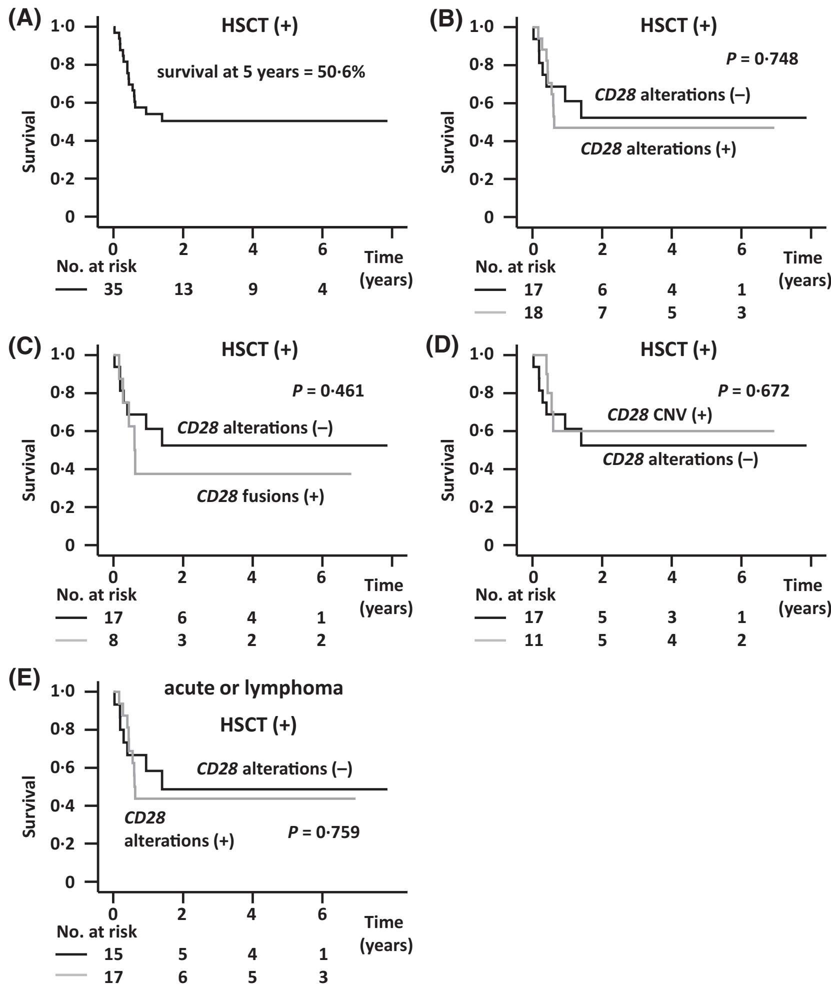

Fig 4. (A) Survival of adult T-cell leukaemia/lymphoma (ATL) patients who received allogeneic haematopoietic stem cell transplantation (HSCT), stratified according to *CD28* gene alterations. (B) Survival from the day of allogeneic HSCT according to *CD28* gene alterations. (C) Survival from the day of allogeneic HSCT of the 8 ATL patients with *CD28* fusions and the 17 without *CD28* gene alterations. (D) Survival from the day of allogeneic HSCT of the 11 ATL patients with *CD28* copy number variation (CNV) and the 17 without *CD28* gene alterations. (B–D) $P < 0{\cdot}05/3$ (two-sided) was considered statistically significant after correction. (E) Survival from the day of allogeneic HSCT in patients with acute or lymphoma subtypes stratified by *CD28* gene alterations.

have a worse prognosis than the latter ($P = 0{\cdot}024$, Fig 5B). Five-year OS of six patients with *CD28* fusions was not reached (Fig 5C), but five-year OS of three patients with *CD28* mutations was 33·3% (Fig 5D), and that of 23 patients with *CD28* CNV was 31·1% (Fig 5E). The latter two tended to have worse prognoses than those without *CD28* gene alterations ($P = 0{\cdot}044$, and $P = 0{\cdot}039$, respectively). Next, we investigated OS of the patients who did not receive allogeneic HSCT stratified according to the clinical subtypes. Of these, five-year OS of all patients with acute or lymphoma subtypes was 33·9% (data not shown). Stratifying for *CD28* gene alterations showed that five-year OS of 61 patients without such alterations was 36·5%, compared with 29·8% for 27 with such alterations. However, this difference was not significant ($P = 0{\cdot}251$, Fig 5F). HR for OS in patients with acute or lymphoma subtypes with *CD28* gene alterations compared with those without *CD28* gene alterations was 1·407 (95% CI, 0·783–2·530, Figure S4C). The five-year OS of 21 patients with chronic or smouldering subtypes, who did not receive allogeneic HSCT, was 86·8% (data not shown). In this group, five-year OS of the 18 patients without *CD28* gene alterations was 90·9%, and that of the three patients with *CD28* gene alterations was 66·7%. Despite the small number, this difference was significant ($P = 0{\cdot}010$, Fig 5G). HR for OS in patients with chronic or smouldering subtypes with *CD28* gene alterations compared with those without CD28 gene alterations was 12·688 (95% CI, 1·094–147·217, Figure S4C).

Survival of ATL patients who did not receive allogeneic HSCT, but did receive mogamulizumab, stratified by CD28 gene alterations

Five-year survival from the first dose of mogamulizumab in 53 patients who did not receive allogeneic HSCT, but were

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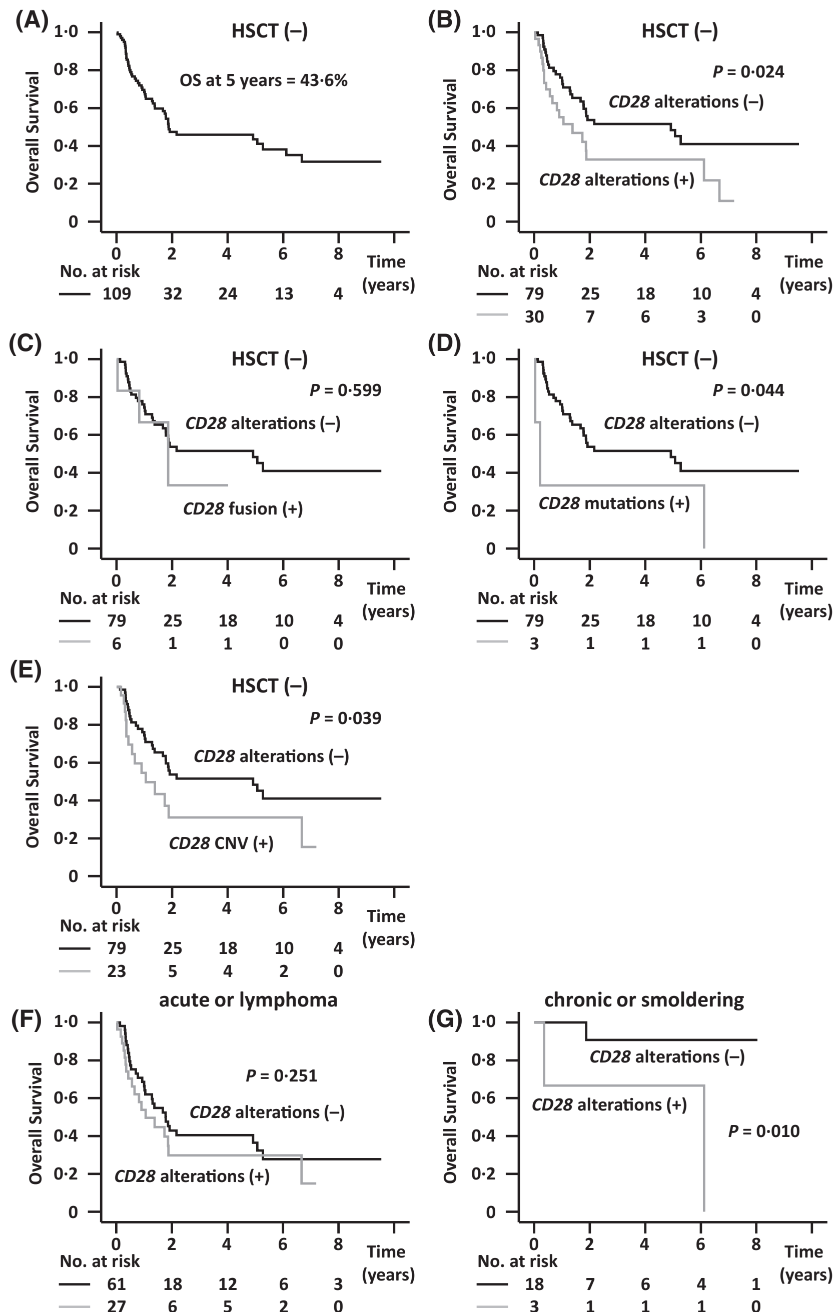

Fig 5. Overall survival (OS) of adult T-cell leukaemia/lymphoma (ATL) patients who did not receive allogeneic HSCT, stratified according to *CD28* gene alterations. (A) OS of all ATL patients who did not receive allogeneic HSCT (n = 109). (B) OS of the ATL patients who did not receive allogeneic HSCT according to *CD28* gene alterations. (C) OS of the 6 ATL patients with *CD28* fusions and the 79 without *CD28* gene alterations. (D) OS of the 3 ATL patients with *CD28* mutations and the 79 without *CD28* gene alterations. (E) OS of the 23 ATL patients with *CD28* copy number variation (CNV) and the 79 without *CD28* gene alterations. (B–E) $P < 0{\cdot}05/4$ (two-sided) was considered statistically significant after Bonferroni correction. (F) OS of the ATL patients with acute or lymphoma subtypes, who did not receive allogeneic HSCT, stratified by *CD28* gene alterations. (G) OS of the ATL patients with chronic or smouldering subtypes, who did not receive allogeneic HSCT, according to *CD28* gene alterations.

on mogamulizumab-containing regimens, was 40·0% (data not shown). In this cohort, five-year survival from the first dose of antibody in patients with *CD28* gene alterations was 34·2% (n = 17), compared to 46·5% (n = 36) in those without *CD28* gene alterations (P = 0·078; Figure S5A). Five-year survival from the first dose of antibody in patients with *CD28* gene fusions was not reached (n = 3), and was not significantly different from that in those without *CD28* gene alterations (n = 36; P = 0·931) (Figure S5B). The same was true for the single patient with *CD28* mutations who died

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3·5 years after starting mogamulizumab treatment, not different compared to survival without *CD28* gene alterations ($n = 36$, $P = 0·819$; Figure S5C). Five-year survival from the first dose of antibody in patients with *CD28* CNV was 40·1% ($n = 14$), again not significantly different from that in those without *CD28* gene alterations ($n = 36$, $P = 0·054$; Figure S5D). We next investigated survival of patients treated with mogamulizumab stratified by clinical subtype. Five-year survival from the first dose of mogamulizumab in 46 patients with acute or lymphoma subtypes was 40·4% (data not shown). In this cohort, five-year survival from the first dose of antibody in patients with *CD28* gene alterations was 32·5% ($n = 14$), compared to 43·2% ($n = 32$) in those without *CD28* gene alterations ($P = 0·203$; Figure S5E). The median survival from the first dose of mogamulizumab in seven patients with chronic or smouldering subtypes, who did not receive allogeneic HSCT, was 3·5 years (data not shown). Here also, there was no significant difference between patients with ($n = 3$) or without ($n = 4$) *CD28* gene alterations ($P = 0·320$; Figure S5F).

HSCT-censored OS of ATL patients stratified by CD28 gene alterations

We estimated the survival of all patients enrolled in the present study, from which the transplanted patients had been censored at the day of allogeneic HSCT, in order to reduce the impact of allogeneic HSCT on survival. In this way, five-year HSCT-censored survival was 46·6% (Figure S6A); survival of the 48 patients with *CD28* gene alterations and of the 96 patients without any alterations was 38·1% and 50·5%, respectively ($P = 0·063$; Figure S6B). Thus, the former tended to have a worse survival than the latter. Next, we investigated survival of the patients stratified by clinical subtype. The five-year HSCT-censored OS of patients with acute or lymphoma subtypes was 36·6% ($n = 120$, data not shown). Of these, five-year OS of the 76 patients without *CD28* gene alterations was 38·1%, and that of the 44 patients with *CD28* gene alterations 34·5%, again not significantly different ($P = 0·444$; Figure S6C). The OS of patients with chronic or smouldering subtypes was 88·1% ($n = 24$, data not shown). Of these, five-year OS of the four patients with *CD28* gene alterations was 75·0%, which was significantly less than for the 20 patients without *CD28* gene alterations (91·7%, $P = 0·012$; Figure S6D).

Discussion

Multiple oncogenic events are required for the development of ATL in HTLV-1-infected cells after a long latency. Of the many genetic alterations, the present study focused on *CD28*, finding that compared with other types of peripheral T-cell neoplasms such as AITL, PTCL-NOS, and CTCL, *CD28* gene alterations are more frequent in ATL. This implies that they may play an important role in ATL tumorigenesis, just as CD28 signalling plays an important role in non-neoplastic T-cell activation.

In the entire cohort of ATL patients studied here, those with various different *CD28* gene alterations showed no statistically significant differences in OS compared to those without any *CD28* alterations. However, there was a trend for the former to have a poorer survival. For some subgroup comparisons, although not statistically significant, it was likely that of the *CD28* alterations, especially mutations contributed to the trend towards poorer survival. Notably, however, the impact of *CD28* gene alterations on OS did achieve statistical significance in patients with chronic or smouldering subtypes (but not in acute or lymphoma subtypes).

In the cohort of ATL patients receiving allogeneic HSCT, survival of those with or without *CD28* gene alterations was not significantly different. Regarding the observation that, in this cohort, the proportion of patients with CD28 gene alterations was relatively high (51%, 18/35), we feel that this is likely to reflect the younger age of the patients with these alterations.

In contrast, among patients who did not receive allogeneic HSCT, those with *CD28* gene alterations tended to have worse OS than those without. Of the different *CD28* gene alterations, patients with *CD28* mutations or *CD28* CNV had a worse prognosis relative to patients without any *CD28* gene alterations. These findings suggest that ATL with *CD28* gene alterations, especially *CD28* mutations and CNV, have a more aggressive clinical course and are refractory to conventional chemotherapies, likely because of continuous, prolonged, and enhanced CD28 signaling.[9,29-32] Notably, again, the impact of *CD28* gene alterations on OS reached statistical significance in patients with chronic or smouldering subtypes, but not in those with acute or lymphoma subtypes. In addition, patients with *CD28* gene alterations were significantly younger than those without, and this characteristic was especially noticeable in the case of *CD28* fusions, in agreement with an earlier study.[33] Thus, *CD28* gene alterations may be relatively early events among the genetic alterations required for the development of ATL. Collectively all these data suggest that, for patients with chronic or smouldering subtypes, *CD28* gene alterations are likely to be critical risk factors for progression to acute or lymphoma subtypes.

In the field of ATL treatment, several novel agents such as mogamulizumab[19–21] and lenalidomide[22] have become available in the clinic. On the other hand, the present study suggests that mogamulizumab treatment does not overcome the aggressiveness associated with *CD28* gene alterations, similar to other conventional chemotherapies. Consistent with all these findings, *CD28* gene alterations tended to be associated with worse HSCT-censored OS, and among patients with chronic or smouldering subtypes, this difference achieved statistical significance.

In conclusion, we found that *CD28* gene alterations such as fusions, mutations, and CNV were frequent in ATL

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patients. Among patients not receiving allogeneic HSCT, those with *CD28* gene alterations tended to have a worse prognosis than those without, and this difference achieved statistical significance in chronic or smouldering subtypes. These findings indicate that ATL harbouring *CD28* gene alterations, especially chronic or smouldering ATL, have a more aggressive clinical course and are refractory to conventional chemotherapies or mogamulizumab, likely because of continuous, prolonged, and enhanced CD28 signalling. Novel treatment strategies to overcome the effects of these *CD28* gene alterations are warranted.

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Author contributions

YS, TI and HIn designed the research. YS, TI, AM, MT, HIw, KY, YT, AI, SK, AU and SI performed the experiments. TI, RU and HIn analysed and interpreted data. All authors wrote and approved the manuscript.

Conflicts of interest

YS, AM, MT, HIw, YT, AI, SK and HIn have no conflicts of interest to disclose. TI received honoraria from Kyowa Kirin Co., Ltd., and Celgene K.K. KY received honoraria from Kyowa Kirin Co., Ltd., and Celgene. A.U received honoraria from Kyowa Kirin Co., Ltd., and Celgene. SI received research founding from Kyowa-Hakko Kirin, Chugai, Takeda, Ono, Celgene, Janssen, Bristol-Myers Squibb, MSD, Gilead, Abbvie, Sanofi, and Daiichi Sankyo, and honoraria from Takeda, Ono, Celgene, Janssen, Bristol-Myers Squibb, and Daiichi Sankyo. RU received research funding from Kyowa Kirin Co., Ltd., Chugai Pharmaceutical Co., Ltd., and Ono Pharmaceutical Co.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplementary methods.

Table SI. Primer sequences.

Table SII. Control cDNA sequences.

Table SIII. Characteristics of ATL patients.

Table SIV. Characteristics of ATL patients according to types of *CD28* gene alterations.

Table SV. Characteristics of ATL patients who subsequently received allogeneic HSCT or not.

Fig S1. Detection of *CD28* gene fusions.

Fig S2. Detection of *CD28* gene mutations.

Fig S3. Interaction of *CD28* gene alterations with clinical subtype and treatment.

Fig S4. Forest plots showing the effect of *CD28* gene alterations on survival in each patient category.

Fig S5. Survival of ATL patients who did not receive allogeneic HSCT, but received mogamulizumab, stratified according to *CD28* gene alterations.

Fig S6. HSCT-censored OS of all ATL patients enrolled in the study, stratified according to *CD28* gene alterations.

References

1. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*. 1977;**50**:481–92.
2. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukemia-lymphoma. A report from the Lymphoma Study Group (1984–1987). *Br J Haematol*. 1991;**79**:428–37.
3. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer*. 2007;**7**:270–80.
4. Ishitsuka K, Tamura K. Human T-cell leukaemia virus type I and adult T-cell leukaemia-lymphoma. *Lancet Oncol*. 2014;**15**:e517–e526.
5. Kataoka K, Nagata Y, Kitanaka A, Shiraishi Y, Shimamura T, Yasunaga J, et al. Integrated molecular analysis of adult T cell leukemia/lymphoma. *Nat Genet*. 2015;**47**:1304–15.
6. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. 2013;**13**:227–42.
7. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 costimulation: from mechanism to therapy. *Immunity*. 2016;**44**:973–88.
8. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science*. 2015;**348**:56–61.
9. Ungewickell A, Bhaduri A, Rios E, Reuter J, Lee CS, Mah A, et al. Genomic analysis of mycosis fungoides and Sezary syndrome identifies recurrent alterations in TNFR2. *Nat Genet*. 2015;**47**:1056–60.
10. Vallois D, Dupuy A, Lemonnier F, Allen G, Missiaglia E, Fataccioli V, et al. RNA fusions involving *CD28* are rare in peripheral T-cell lymphomas and concentrate mainly in those derived from follicular helper T cells. *Haematologica*. 2018;**103**:e360–e363.
11. Wang L, Ni X, Covington KR, Yang BY, Shiu J, Zhang X, et al. Genomic profiling of Sezary syndrome identifies alterations of key T cell signaling and differentiation genes. *Nat Genet*. 2015;**47**:1426–34.
12. Watatani Y, Sato Y, Miyoshi H, Sakamoto K, Nishida K, Gion Y, et al. Molecular heterogeneity in peripheral T-cell lymphoma, not otherwise specified revealed by comprehensive genetic profiling. *Leukemia*. 2019;**33**:2867–83.
13. Yamada Y, Tomonaga M, Fukuda H, Hanada S, Utsunomiya A, Tara M, et al. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol*. 2001;**113**:375–82.
14. Tsukasaki K, Utsunomiya A, Fukuda H, Shibata T, Fukushima T, Takatsuka Y, et al. VCAP-AMPVECP compared with biweekly CHOP for adult

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T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *J Clin Oncol.* 2007;**25**:5458–64.

15. Cook LB, Fuji S, Hermine O, Bazarbachi A, Ramos JC, Ratner L, et al. Revised adult T-cell leukemia-lymphoma International Consensus Meeting Report. *J Clin Oncol.* 2019;**37**:677–87.
16. Ishida T, Hishizawa M, Kato K, Tanosaki R, Fukuda T, Taniguchi S, et al. Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special emphasis on preconditioning regimen: a nationwide retrospective study. *Blood*. 2012a;**120**:1734–41.
17. Ishida T, Hishizawa M, Kato K, Tanosaki R, Fukuda T, Takatsuka Y, et al. Impact of graft-versus-host disease on allogeneic hematopoietic cell transplantation for adult T cell leukemia-lymphoma focusing on preconditioning regimens: nationwide retrospective study. *Biol Blood Marrow Transplant.* 2013;**19**:1731–9.
18. Utsunomiya A. Progress in allogeneic hematopoietic cell transplantation in adult T-cell leukemia-lymphoma. *Front Microbiol.* 2019;**10**:2235.
19. Ishii T, Ishida T, Utsunomiya A, Inagaki A, Yano H, Komatsu H, et al. Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. *Clin Cancer Res.* 2010;**16**:1520–31.
20. Ishida T, Joh T, Uike N, Yamamoto K, Utsunomiya A, Yoshida S, et al. Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study. *J Clin Oncol.* 2012b;**30**:837–42.
21. Ishida T, Jo T, Takemoto S, Suzushima H, Uozumi K, Yamamoto K, et al. Dose-intensified chemotherapy alone or in combination with mogamulizumab in newly diagnosed aggressive adult T-cell leukaemialymphoma: a randomized phase II study. *Br J Haematol.* 2015;**169**:672–82.
22. Ishida T, Fujiwara H, Nosaka K, Taira N, Abe Y, Imaizumi Y, et al. Multicenter phase II study of lenalidomide in relapsed or recurrent adult T-cell leukemia/lymphoma: ATLL-002. *J Clin Oncol.* 2016;**34**:4086–93.
23. Inagaki H, Okabe M, Seto M, Nakamura S, Ueda R, Eimoto T. API2-MALT1 fusion transcripts involved in mucosa-associated lymphoid tissue lymphoma: multiplex RT-PCR detection using formalin-fixed paraffin-embedded specimens. *Am J Pathol.* 2001;**158**:699–706.
24. Nakayama T, Miyabe S, Okabe M, Sakuma H, Ijichi K, Hasegawa Y, et al. Clinicopathological significance of the CRTC3-MAML2 fusion transcript in mucoepidermoid carcinoma. *Mod Pathol.* 2009;**22**:1575–81.
25. Sakamoto Y, Ishida T, Masaki A, Murase T, Yonekura K, Tashiro Y, et al. CCR4 mutations associated with superior outcome of adult T-cell leukemia/lymphoma under mogamulizumab treatment. *Blood*. 2018;**132**:758–61.
26. Xia H, Nakayama T, Sakuma H, Yamada S, Sato F, Takino H, et al. Analysis of API2-MALT1 fusion, trisomies, and immunoglobulin VH genes in pulmonary mucosa-associated lymphoid tissue lymphoma. *Hum Pathol.* 2011;**42**:1297–304.
27. Mossafa H, Damotte D, Jenabian A, Delarue R, Vincenneau A, Amouroux I, et al. Non-Hodgkin's lymphomas with Burkitt-like cells are associated with c-Myc amplification and poor prognosis. *Leuk Lymphoma*. 2006;**47**:1885–93.
28. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Control Clin Trials.* 1996;**17**:343–6.
29. Rohr J, Guo S, Huo J, Bouska A, Lachel C, Li Y, et al. Recurrent activating mutations of CD28 in peripheral T-cell lymphomas. *Leukemia*. 2016;**30**:1062–70.
30. Gmyrek GB, Pingel J, Choi J, Green JM. Functional analysis of acquired CD28 mutations identified in cutaneous T cell lymphoma. *Cell Immunol.* 2017;**319**:28–34.
31. Choi J, Goh G, Walradt T, Hong BS, Bunick CG, Chen K, et al. Genomic landscape of cutaneous T cell lymphoma. *Nat Genet.* 2015;**47**:1011–9.
32. Vallois D, Dobay MPD, Morin RD, Lemonnier F, Missiaglia E, Juilland M, et al. Activating mutations in genes related to TCR signaling in angioimmunoblastic and other follicular helper T-cell-derived lymphomas. *Blood*. 2016;**128**:1490–502.
33. Yoshida N, Shigemori K, Donaldson N, Trevisani C, Cordero NA, Stevenson KE, et al. Genomic landscape of young ATLL patients identifies frequent targetable CD28 fusions. *Blood*. 2020;**135**:1467–71.

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