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Influence of human T-lymphotropic virus type 1 coinfection on the development of hepatocellular carcinoma in patients with hepatitis C virus infection

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Abstract
Background Human T-lymphotropic virus type 1 (HTLV-1) may worsen the clinical course of hepatitis C virus (HCV) infection. The aim of this study was to investigate whether HTLV-1 coinfection influences the clinical characteristics of patients with HCV infection.
Methods This retrospective study included 523 consecutive patients from January 2001 to December 2010 with chronic liver disease due to HCV infection, in whom serum anti-HTLV-1 antibodies were examined. Among these patients, 265 were diagnosed with hepatocellular carcinoma (HCC).
Results The seroprevalence of anti-HTLV-1 antibodies was significantly higher in patients with HCC (21.1 %) than those without HCC (10.5 %, $P = 0.001$). This significant difference was observed in female patients (29.5 vs. 8.5 %, $P < 0.001$), but not in male patients (16.5 vs. 12.9 %, $P = 0.501$). In multivariate analysis, anti-HTLV-1 antibody positivity was independently associated with HCC in female patients [odds ratio (OR), 5.029; 95 % confidence interval (95 % CI), 1.760–14.369; $P = 0.003$], in addition to age (≥65 years; OR, 10.297; 95 % CI, 4.322–24.533; $P < 0.001$), platelet count (<15 × 10^4/μL; OR, 2.715; 95 % CI, 1.050–7.017; $P = 0.039$), total bilirubin (≥1 mg/dL; OR, 3.155; 95 % CI, 1.365–7.292; $P = 0.007$), and total cholesterol (≤160 mg/dL; OR, 2.916; 95 % CI, 1.341–6.342; $P = 0.007$). In contrast, HTLV-1 coinfection was not associated with HCC in male patients, although age, alcohol consumption, platelet count, and albumin were independently associated with HCC.
Conclusions HTLV-1 coinfection may contribute to the development of HCC in patients with chronic HCV infection, especially in females.

Keywords HTLV-1 · Risk factor · Hepatitis C virus · Hepatocellular carcinoma · Coinfection

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Introduction

Hepatitis C virus (HCV) has been implicated in the development of chronic hepatitis in 50–85 % of cases of acute hepatitis, with 5–25 % progressing to liver cirrhosis over 10–20 years [1–5]. HCV is also linked to the development of hepatocellular carcinoma (HCC); the risk of HCC has been estimated to be 1–8 % per year in HCV-infected individuals [6–9]. This association with HCC is

known to depend on factors such as age, sex, alcohol intake, cigarette smoking, and alanine aminotransferase (ALT) levels [10–13].

Human T-lymphotropic virus type 1 (HTLV-1) belongs to the family *Retroviridae* and infects lymphocytes, similar to human immunodeficiency virus (HIV). HTLV-1 is spread via blood transfusion, sexual or mother-to-child transmission, and results in persistent life-long infection. HTLV-1 is causally-related to adult T cell leukemia/lymphoma (ATLL), and the cumulative incidence of ATLL is estimated to be 2.1–6.9 % among HTLV-1 carriers in Japan [14–16]. In addition, persistent HTLV-1 infection was reported to be associated with myelopathy, named HTLV-1 associated/tropical spastic paraparesis (HAM/TSP) [17, 18], polymyositis, arthritis, and uveitis [19, 20]. These risks are thought to depend on immunomodulatory effects [21, 22].

Since HCV and HTLV-1 have similar routes of infection, the prevalence of HCV infection is high among HTLV-1-infected patients in endemic areas [23], which is similar to HIV/HCV coinfection. Patients with HIV/HCV coinfection have been reported to progress to liver cirrhosis 1.4–2.9 times more quickly and have poorer responses to interferon (IFN) treatment than patients with HCV infection alone [24–27]. It was also reported that HCV RNA levels were higher and that the response to IFN treatment was poorer in patients with HTLV-1/HCV coinfection compared to those with HCV infection alone [28]. However, the clinical characteristics of patients with HTLV-1/HCV coinfection have not been fully described, partly because areas hyperendemic for HTLV-1 are restricted to small geographical regions. The aim of this study was to investigate how HTLV-1 coinfection influences the characteristics of liver disease in patients with HCV infection in southwest Japan, where HTLV-1 infection is frequently observed.

Methods

Study population

In this retrospective study, we initially screened 709 anti-HCV antibody positive patients who visited the Second Department of Internal Medicine or Gastroenterological Medicine at Kagoshima University Hospital from January 2001 to December 2010. Among these patients, serum anti-HTLV-1 antibody status had not been evaluated in 157 patients, 12 patients were positive for hepatitis B surface antigen, 10 patients were negative for HCV RNA, and seven patients were considered to have other liver diseases such as autoimmune hepatitis, cholangiocellular carcinoma, or steatosis. Therefore, we included 523 patients with chronic liver disease due to HCV infection who had serum data available on anti-HTLV-1 antibody status, and compared the clinical characteristics of the HTLV-1/HCV coinfected patients and the HCV-infected patients using data at the time of the first visit to our hospital after January 2001.

This study was approved by the ethics committees of Kagoshima University Graduate School of Medical and Dental Sciences and Kagoshima University Hospital.

Follow-up study

We examined the clinical course of 523 patients with or without HCC starting with the first visit after January 2001 to the final visit before December 2010. The prognosis in patients with HTLV-1/HCV coinfection was compared to those with HCV infection alone. The follow-up period ended on December 31, 2010. We calculated the cumulative all-cause and liver-related mortality rates. In addition, the response to IFN treatment was also investigated in 123 patients treated with IFN, including IFN monotherapy and combination therapy with IFN involving peg-IFN or ribavirin. The effect of IFN was classified 6 months after the end of treatment as follows: undetectable HCV RNA was considered to be a sustained viral response (SVR), and detectable HCV RNA with or without abnormal ALT levels was considered to be a non-SVR.

Serological markers

Anti-HCV antibody titers were measured using microparticle enzyme immunoassay (AxSYM HCV, Abbott Laboratories, Abbott Park, IL, USA) until 2006, and chemiluminescent enzyme immunoassay (CLEIA, Lumipulse Presto Ortho HCV, Fujirebio Inc., Tokyo, Japan) thereafter. Anti-HTLV-1 antibody levels were examined using electrochemiluminescence immunoassay (ECLIA, Picolumi HTLV-I, Eisai Co., Tokyo, Japan) until 2006, and CLEIA (Lumipulse Presto HTLV-I, Fujirebio Inc.) thereafter. HCV viral load was quantified using a branched-chain DNA assay (Quantiplex HCV RNA 2, Daiichi-Kagaku Chemicals Co., Tokyo, Japan), a reverse transcription-polymerase chain reaction (RT-PCR, Amplicor GT HCV monitor v2.0 Original or Amplicor GT HCV monitor v2.0 High-Range, Roche Diagnostics K.K., Tokyo, Japan), or a real-time PCR assay (COBAS TaqMan HCV Auto, Roche Diagnostics K.K.). High HCV viral load was defined as $\geq$1 Meq/mL, $\geq$100 KIU/mL, or $\geq$5 logIU/mL, respectively, for these methods. HCV serotype and genotype were determined using an enzyme immunoassay (Immunocheck F-HCV Gr Kokusai, Sysmex, Hyogo, Japan) and by direct sequence analysis, respectively. HCV genotype 1b was considered serotype I, and genotypes 2a and 2b were

considered serotype II. No other HCV genotypes were detected in this study population.

Histopathology

Liver biopsy specimens were scored semiquantitatively based on the New Inuyama classification [29]. Fibrosis was scored as F0, no fibrosis; F1, portal fibrous widening; F2, portal fibrous widening with bridging fibrosis; F3, bridging fibrosis plus lobular distortion; or F4, probable or definite cirrhosis. Inflammation was graded as A0, none to minimal; A1, mild; A2, moderate; or A3, severe.

Statistical analysis

All analyses were performed using Stata version 12 (StataCorp LP, College Station, TX, USA) or Statview J 5.0 (SAS Institute Japan Ltd., Tokyo, Japan). Continuous variables were shown as medians with ranges. Between-group comparisons were performed using the Mann–Whitney *U* test for continuous variables and the χ^2 test or Fisher's test for categorical variables. Potential factors were analyzed using multiple logistic regression analysis, and odds ratios (OR), 95 % confidence intervals (95 % CI), and *P* values were recorded. Cumulative incidence curves were determined using the Kaplan–Meier method, and differences between groups were assessed using the log-rank test. *P* values less than 0.05 were considered statistically significant.

Results

Association between HTLV-1/HCV coinfection and HCC

HCC had been diagnosed in 265 of 523 patients at the beginning of follow-up. Compared to patients without HCC, age and serum levels of γ-glutamyl transpeptidase (γ-GTP), total bilirubin, creatinine, and α-fetoprotein (AFP) were significantly higher in patients with HCC, while platelet count, prothrombin time (%), and serum levels of total cholesterol, and albumin were significantly lower (Table 1). The proportion of males, cigarette smoking, alcohol consumption ≥20 g/day, and HCV serotype I were higher in patients with HCC compared to those without HCC. The rate of anti-HTLV-1 seropositivity in patients with HCC was also significantly higher than in patients without HCC [21.1 (56/265) vs. 10.5 % (27/258); $P = 0.001$] (Table 1). In addition, the rate of anti-HTLV-1 seropositivity in female patients with HCC was significantly higher than in female patients without HCC [29.5 (28/95) vs. 8.5 % (12/142); $P < 0.001$]. However, the rate of anti-HTLV-1 seropositivity in male patients with HCC was not different compared to that in male patients without HCC [16.5 (28/170) vs. 12.9 % (15/116); $P = 0.501$] (Table 1). In multiple logistic regression analysis (Table 2), age, alcohol consumption, platelet count, and γ-GTP were independently associated with HCC in all patients, and anti-HTLV-1 seropositivity tended to be associated with HCC in all patients (OR, 1.953; 95 % CI, 0.948–4.022; $P = 0.069$). In females, anti-HTLV-1 antibody positivity was independently associated with HCC in patients with HCV infection (OR, 5.029; 95 % CI, 1.760–14.369, $P = 0.003$). Age, platelet count, total bilirubin, and total cholesterol were also independently associated with HCC. In contrast, HTLV-1 coinfection was not associated with HCC in male patients in the univariate analysis, although age, alcohol consumption, platelet count, and albumin were associated with HCC in the multivariate analysis. Moreover, after 10 patients with chronic renal failure on hemodialysis were excluded from the analysis, similar results were obtained, except γ-GTP became an independent factor associated with HCC in male patients (OR, 1.858; 95 % CI, 1.000–3.451; $P < 0.05$).

Comparison of clinical characteristics in HCC patients with HTLV-1/HCV coinfection versus HCV infection alone

Table 3 shows the characteristics of the 265 HCC patients with HTLV-1/HCV coinfection or HCV infection alone. There were 170 males and 95 females, and the median age was 70 years (range, 47–88). Among the 265 patients with HCC, there were 56 (21.1 %) positive for anti-HTLV-1 antibody, comprised of 28 of 170 male patients (16.5 %) and 28 of 95 female patients (29.5 %).

Platelet count tended to be lower in patients with HTLV-1/HCV coinfection, but this difference was not significant. Serum levels of γ-GTP were significantly higher in female patients with HTLV-1/HCV coinfection. In contrast, patients with HTLV-1/HCV coinfection and HCV infection alone were similar with respect to age, ALT and AFP levels. In addition, history of IFN-based treatment and treatment outcome between HTLV-1-positive and -negative patients were not different. Furthermore, the number of tumors and maximum HCC tumor size in patients with HTLV-1/HCV coinfection were similar to those in patients with HCV infection alone.

In addition, 41 deaths occurred during the follow-up period. The median duration of follow-up was 693 days (range, 8–3,495 days). Of these 41 patients, nine patients had HTLV-1 coinfection and 32 patients had HCV infection alone. Liver-related death including HCC or liver failure occurred in 8/9 (88.9 %) patients with HTLV-1 coinfection and in 23/32 (71.9 %) patients with HCV infection without HTLV-1 infection (Table 3).

Table 1 Characteristics of patients with or without HCC

	Total			Male			Female		
	HCC (+) ($N = 265$)	HCC (−) ($N = 258$)	*P* value	HCC (+) ($N = 170$)	HCC(−) ($N = 116$)	*P* value	HCC (+) ($N = 95$)	HCC (−) ($N = 142$)	*P* value
Sex (male/female)	170/95	116/142	<0.001						
Age (years), median (range)	70 (47–88)	60 (19–82)	<0.001	69 (47–88)	59.5 (19–80)	<0.001	72 (52–86)	60.5 (24–82)	<0.001
BMI[a] (kg/m^2) (<25/≥25)	182/77	178/70	0.769	123/43	78/34	0.417	59/34	100/36	0.111
BT[a] (yes/no)	112/136	92/134	0.354	64/93	42/60	>0.999	48/43	50/74	0.074
Alcohol consumption[a] (g/day) (none/<20/≥20)	127/47/79	121/58/36	0.034	45/39/78	33/35/31	0.042	82/8/1	88/23/5	0.075
Cigarette smoking[a] (yes/no)	86/94	53/97	0.025	83/36	43/31	0.120	3/58	10/66	0.144
HCV RNA[a] (high/low)[b]	108/17	189/35	0.642	67/13	81/15	>0.999	41/4	108/20	0.323
HCV serotype[a] (I/II)	96/27	145/80	0.011	59/22	65/31	0.512	37/5	80/49	0.001
Anti-HTLV-1 (positive/negative)	56/209	27/231	0.001	28/142	15/101	0.501	28/67	12/130	<0.001
Platelet count ($\times 10^4/\mu L$), median (range)	9.8 (2.6–36.4)	15.2 (3.2–39.3)	<0.001	10.9 (2.6–33.6)	16.6 (4.4–39.3)	<0.001	8.9 (2.6–36.4)	14.2 (3.2–35.5)	<0.001
Prothrombin time (%), median (range)	80 (31–136)	93 (27–127)	<0.001	81 (31–136)	90 (27–123)	<0.001	80 (38–123)	95 (33–127)	<0.001
ALT (IU/L), median (range)	53 (12–333)	49 (6–679)	0.536	54.5 (12–321)	50.5 (6–679)	0.465	45 (13–333)	46 (11–386)	0.557
γ-GTP (IU/L), median (range)	52 (12–549)	36 (8–1,697)	<0.001	59 (12–416)	45 (12–1,697)	0.003	43 (13–549)	30 (8–314)	0.001
Total bilirubin (mg/dL), median (range)	0.9 (0.1–20.1)	0.7 (0.1–3.2)	<0.001	0.8 (0.1–20.1)	0.7 (0.1–2.6)	0.044	1.0 (0.1–4.1)	0.7 (0.1–3.2)	0.003
Total cholesterol (mg/dL), median (range)	151 (37–340)	171 (73–353)	<0.001	151 (37–340)	154 (83–245)	0.736	149 (99–246)	178 (73–353)	<0.001
Creatinine (mg/dL), median (range)	0.7 (0.3–9.83)	0.6 (0.3–12.2)	<0.001	0.8 (0.3–9.83)	0.8 (0.3–12.2)	0.695	0.6 (0.4–1.5)	0.6 (0.4–10.35)	0.054
Albumin (g/dL), median (range)	3.7 (2.2–5)	4.2 (2.2–5.1)	<0.001	3.7 (2.2–4.9)	4.2 (2.6–5.1)	<0.001	3.7 (2.4–5)	4.2 (2.2–4.9)	<0.001
AFP[a] (ng/mL), median (range)	28 (1.2–607,610.6)	5.3 (1.1–591.3)	<0.001	20.6 (1.2–607,610.6)	4.6 (1.1–497.7)	<0.001	52.8 (1.7–67,998.7)	5.5 (1.6–591.3)	<0.001

HCC hepatocellular carcinoma, *BMI* body mass index, *BT* blood transfusion, *HCV* hepatitis C virus, *anti-HTLV-1* antibodies to human T-lymphotropic virus type 1, *ALT* alanine aminotransferase, *γ-GTP* γ-glutamyl transpeptidase, *AFP* α-fetoprotein

P values were determined by the Mann–Whitney *U* test, χ^2 test, or Fisher's test

[a] Subjects with missing data were excluded

[b] Classification was stated in "Methods"

Table 2 Independent risk factors for hepatocellular carcinoma in patients with hepatitis C virus infection

	Total Odds ratio (95 % CI)	*P* value	Males Odds ratio (95 % CI)	*P* value	Females Odds ratio (95 % CI)	*P* value
Anti-HTLV-1						
Negative	1				1	
Positive	1.953 (0.948–4.022)	0.069			5.029 (1.760–14.369)	0.003
Age (years)						
<65	1		1		1	
≥65	5.219 (2.770–9.830)	<0.001	2.722 (1.504–4.924)	0.001	10.297 (4.322–24.533)	<0.001
Sex						
Female	1					
Male	1.192 (0.540–2.632)	0.664				
Alcohol consumption (g/day)						
<20	1		1			
≥20	2.296 (1.062–4.966)	0.035	2.033 (1.090–3.792)	0.026		
Cigarette smoking						
No	1					
Yes	1.751 (0.845–3.628)	0.132				
Platelet count ($\times 10^4/\mu L$)						
≥15	1		1		1	
<15	2.537 (1.333–4.830)	0.005	2.164 (1.130–4.145)	0.020	2.715 (1.050–7.017)	0.039
γ-GTP (IU/L)						
<43	1		1		1	
≥43	1.862 (1.057–3.281)	0.031	1.728 (0.943–3.167)	0.077	1.593 (0.748–3.395)	0.227
Total bilirubin (mg/dL)						
<1.0	1				1	
≥1.0	1.664 (0.866–3.198)	0.126			3.155 (1.365–7.292)	0.007
Creatinine (mg/dL)						
<0.7	1					
≥0.7	1.030(0.515–2.061)	0.934				
Total cholesterol (mg/dL)						
>160	1				1	
≤160	1.171 (0.651–2.108)	0.599			2.916 (1.341–6.342)	0.007
Albumin (g/dL)						
≥4.0	1		1		1	
<4.0	1.629 (0.860–3.085)	0.134	2.062 (1.053–4.038)	0.035	2.011 (0.889–4.549)	0.094
Prothrombin time (%)						
>90	1		1		1	
≤90	1.700 (0.881–3.279)	0.113	1.513(0.776–2.952)	0.224	1.365 (0.563–3.312)	0.491

CI confidence interval, *anti-HTLV-1* antibodies to human T-lymphotropic virus type 1, *γ-GTP* γ-glutamyl transpeptidase

P values were based on multiple logistic regression analysis

Comparison of overall survival or liver disease-related mortality in patients with HTLV-1/HCV coinfection versus HCV infection alone

In the 523 patients with or without HCC, the overall survival rate in patients with HTLV-1 coinfection was similar to that in HCV-infected patients without HTLV-1 infection (Fig. 1). In contrast, cumulative survival rates for liver-related mortality in patients with HTLV-1 coinfection was lower than in patients with only HCV infection in all patients (Fig. 2a) or in female patients (Fig. 2b), although these differences were not significant. In contrast, cumulative survival rates for liver-related mortality in male patients with HCV/HTLV-1 coinfection was similar to those in male HCV infected patients without HTLV-1 infection (Fig. 2c).

Table 3 Clinical features of patients with HCC according to anti-HTLV-1 antibody status

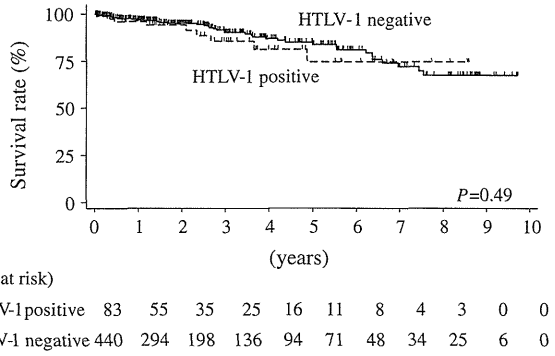
	Total		*P* value	Male		*P* value	Female		*P* value
	Anti-HTLV-1 positive ($N = 56$)	Anti-HTLV-1 negative ($N = 209$)		Anti-HTLV-1 positive ($N = 28$)	Anti-HTLV-1 negative ($N = 142$)		Anti-HTLV-1 positive ($N = 28$)	Anti-HTLV-1 negative ($N = 67$)	
Sex (male/female)	28/28	142/67	0.018						
Age (years), median (range)	71 (51–88)	70 (47–85)	0.325	68 (51–88)	69 (47–85)	0.461	74.5 (52–86)	71 (54–82)	0.092
BT[a] (yes/no)	27/24	85/112	0.269	12/11	52/82	0.256	15/13	33/30	>0.999
ALT (IU/L), median (range)	51 (12–202)	53 (13–333)	0.610	50.5 (12–162)	58.5 (15–321)	0.270	51 (15–202)	43 (13–333)	0.253
(≥31/<31)	46/10	169/40	>0.999	23/5	120/22	0.779	23/5	49/18	0.437
γ-GTP (IU/L), median (range)	52 (15–295)	52 (12–549)	0.731	51 (24–295)	63 (12–416)	0.408	53 (15–136)	36 (13–549)	0.014
(≥43/<43)	40/16	127/82	0.162	18/10	99/43	0.656	22/6	28/39	0.001
Platelet count ($\times 10^4/\mu L$), median (range)	8.9 (2.6–29.6)	10 (3–36.4)	0.081	9.55 (2.6–29.6)	10.95 (3–33.6)	0.151	8.7 (2.6–22.2)	8.9 (3.1–36.4)	0.576
(≥15/<15)	9/47	49/160	0.278	6/22	39/103	0.642	3/25	10/57	0.749
AFP[a] (ng/mL), median (range)	43.2 (1.7–607,610.6)	26.5 (1.2–161,592)	0.107	34.9 (2.5–607,610.6)	19.9 (1.2–161,592)	0.181	48 (1.7–67,998.7)	54.9 (2.4–4,214)	0.735
History of IFN-based treatment									
(yes/no)	29/27	109/100	>0.999	16/12	73/69	0.680	13/15	36/31	0.653
(treatment outcome[a], SVR/non-SVR)	2/26	11/92	0.734	1/14	8/61	>0.999	1/12	3/31	>0.999
HCC status (initial/recurrence)	39/17	151/58	0.739	20/8	99/43	>0.999	19/9	52/15	0.437
Interval from HCC diagnosis to recurrence[b] (days), median (range)	927 (234–2,314)	1,214 (27–6,120)	0.257	927 (498–1,914)	1,300 (27–6,120)	0.195	1,216 (455–2,314)	565 (518–3,225)	0.698
Method for diagnosis of HCC (angiography/histopathology/MRI or CT)	24/32/0	90/117/2	0.857	15/13/0	64/77/1	0.475	9/19/0	26/40/1	0.433
Number of HCC tumors									
(1/2/≥3)	28/13/15	107/52/50	0.900	11/8/9	68/35/39	0.706	17/5/6	39/17/11	0.677
Maximum HCC tumor size (cm)									
(>3/≤3)	11/45	43/166	>0.999	5/23	33/109	0.627	6/22	10/57	0.549
All-cause death	9	32	0.838	5	23	0.785	4	9	>0.999
Liver-related death	8 (HCC5)	23 (HCC15)	0.488	4 (HCC3)	16 (HCC10)	0.747	4 (HCC2)	7 (HCC5)	0.726

HCC hepatocellular carcinoma, *anti-HTLV-1* antibodies to human T-lymphotropic virus type 1, *BT* blood transfusion, *ALT* alanine aminotransferase, *γ-GTP* γ-glutamyl transpeptidase, *AFP* α-fetoprotein, *SVR* sustained viral response by interferon-based treatment, *MRI* magnetic resonance imaging, *CT* computed tomography

P values were determined by the Mann–Whitney *U* test, χ^2 test, or Fisher's test

[a] Subjects with missing data were excluded

[b] Period from first HCC diagnosis to the date when subjects with recurrence including repeated recurrence of HCC were enrolled in this study



(Number at risk)

HTLV-1 positive	83	55	35	25	16	11	8	4	3	0	0
HTLV-1 negative	440	294	198	136	94	71	48	34	25	6	0

Fig. 1 Kaplan–Meier curve for overall survival in all patients. *P* values were assessed using the log-rank test

Clinical characteristics of HTLV-1/HCV coinfected patients without HCC

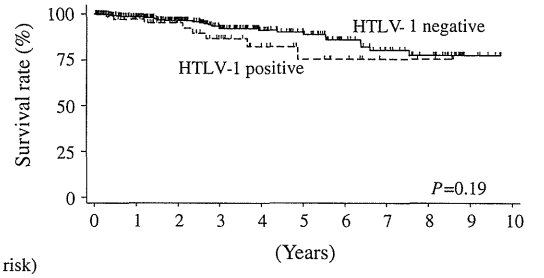
We also assessed 258 patients without HCC. In these patients, sex, age, and ALT in HTLV-1/HCV coinfected patients were similar to those in HCV-infected patients (Table 4). In addition, histological findings in the liver were available in 140 patients, and there was no difference in the degree of hepatic fibrosis and hepatitis activity between HCV-infected patients with HTLV-1 coinfection and patients with only HCV infection.

Further, we assessed the clinical characteristics according to the IFN-based treatment response (Table 5). There were 123 patients who received IFN-based treatment with data available on response, of whom 14 had HTLV-1 coinfection and 109 had HCV infection alone. The coinfected and HCV-infected patients had similar overall SVR rates after IFN-based treatment [42.9 % (6/14) vs. 42.2 % (46/109); $P > 0.999$, Table 5]. Furthermore, we assessed the efficiency of each IFN monotherapy and IFN + ribavirin (RBV) combination therapy according to anti-HTLV-1 status. The SVR rate with IFN monotherapy in patients with HTLV-1 coinfection was similar to the rate in patients with HCV infection alone [33.3 % (1/3) vs. 50 % (12/24); $P > 0.999$]. In addition, the SVR rate with IFN + RBV was similar in patients with HTLV-1 coinfection and patients with HCV infection alone [45.5 % (5/11) vs. 40 % (34/85); $P = 0.753$].

Discussion

Our study showed that among patients with chronic liver disease due to HCV infection, the prevalence of HTLV-1 coinfection was higher in patients with HCC than in patients without HCC. HTLV-1 coinfection was also independently associated with HCC in female patients, but not in male patients. HTLV-1 is a retrovirus that infects

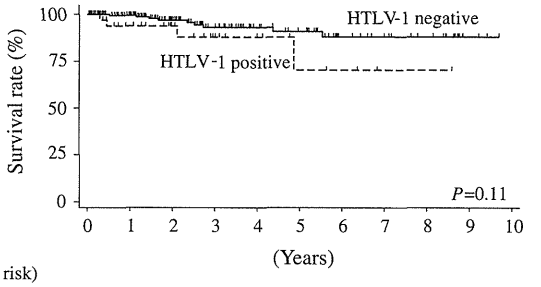
(A) All patients



(Number at risk)

HTLV-1 positive	83	55	35	25	16	11	8	4	3	0	0
HTLV-1 negative	440	294	198	136	94	71	48	34	25	6	0

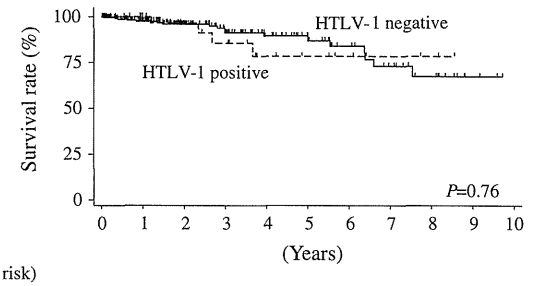
(B) Female patients



(Number at risk)

HTLV-1 positive	40	25	17	10	7	4	3	1	1	0	0
HTLV-1 negative	197	136	90	65	44	38	24	18	15	3	0

(C) Male patients



(Number at risk)

HTLV-1 positive	43	30	18	15	9	7	5	3	2	0	0
HTLV-1 negative	243	158	108	71	50	33	24	16	10	3	0

Fig. 2 Kaplan–Meier survival curve for liver disease-related mortality. **a** All patients. **b** Female patients. **c** Male patients. *P* values were assessed using the log-rank test

lymphocytes, similar to HIV. HIV coinfection is known to worsen the clinical course of HCV infection [24, 25]. Although HTLV-1 may also be a risk factor for HCC in patients with chronic HCV infection based on previous findings [30], our study provides stronger evidence with a larger number of patients with HTVL-1/HCV coinfection than in previous reports [30–32].

Our study indicates that the seroprevalence of anti-HTLV-1 antibodies in HCV-associated HCC is higher than that in HCV-associated chronic liver disease without HCC, consistent with a previous study by Okayama et al [30].

Table 4 Clinical features of patients without HCC according to anti-HTLV-1 status

	Total			Male			Female		
	Anti-HTLV-1 positive ($N = 27$)	Anti-HTLV-1 negative ($N = 231$)	*P* value	Anti-HTLV-1 positive ($N = 15$)	Anti-HTLV-1 negative ($N = 101$)	*P* value	Anti-HTLV-1 positive ($N = 12$)	Anti-HTLV-1 negative ($N = 130$)	*P* value
Sex (male/female)	15/12	101/130	0.307						
Age (years), median (range)	63 (34–75)	60 (19–82)	0.401	65 (34–75)	59 (19–80)	0.434	62 (49–69)	60 (24–82)	0.722
BT[a] (yes/no)	13/9	79/125	0.072	7/5	35/55	0.225	6/4	44/70	0.200
ALT (IU/L), median (range)	44 (13–202)	49 (6–679)	0.131	49 (13–87)	51 (6–679)	0.308	35.5 (13–202)	47 (11–386)	0.180
(≥31/<31)	20/7	187/44	0.443	12/3	83/18	0.734	8/4	104/26	0.280
γ-GTP (IU/L), median (range)	38 (9–1,697)	36 (8–314)	0.994	45.5 (14–1,697)	45 (12–239)	0.764	27 (9–58)	31 (8–314)	0.491
(≥43/<43)	10/17	92/139	0.838	7/8	53/48	0.784	3/9	39/91	>0.999
Platelet count ($\times 10^4$/μL), median (range)	18.2 (5.6–35.5)	14.6 (3.2–39.3)	0.161	18.2 (5.6–20.6)	15.9 (4.4–39.3)	0.980	19.1 (6.7–35.5)	13.4 (3.2–27.5)	0.085
(≥15/<15)	20/7	114/117	0.024	11/4	57/44	0.269	9/3	57/73	0.066
Histological findings[b,c]									
[F0/F1/F2/F3/F4]	0/9/3/4/0	5/51/42/21/5	0.731	0/2/2/2/0	3/18/15/7/2	0.465	0/7/1/2/0	2/33/27/14/3	0.305
[A0/A1/A2/A3]	0/9/7/0	0/49/67/8	0.204	0/2/4/0	0/21/20/4	0.770	0/7/3/0	0/28/47/4	0.064
HCV RNA[a] (high/low)	22/3	167/32	0.774	11/2	70/13	>0.999	11/1	97/19	0.690
HCV serotype[a] (I/II)	18/7	127/73	0.508	8/5	57/26	0.751	10/2	70/47	0.131

HCC hepatocellular carcinoma, *HCV* hepatitis C virus, *anti-HTLV-1* antibodies to human T-lymphotropic virus type 1, *BT* blood transfusion, *ALT* alanine aminotransferase, *γ-GTP* γ- glutamyl transpeptidase

[a] Subjects with missing data were excluded

[b] Subjects whose liver biopsy was performed within 1 year of the enrollment in this study were included, but subjects whose liver biopsy was performed more than 1 year before or after the enrollment in this study or subjects with missing data were excluded

[c] Classification was stated in "Methods". *P* values were determined by the Mann–Whitney *U* test, χ^2 test, or Fisher's test

Table 5 Clinical characteristics of patients who received interferon-based therapy based on virological response

	SVR ($N = 52$)	Non-SVR ($N = 71$)	*P* value
Initial treatment/retreatment	38/14	50/21	
Sex (male/female)	24/28	25/46	0.265
Age (years), median (range)	55 (21–75)	62 (20–78)	0.002
Anti-HTLV-1 (positive/negative)	6/46	8/63	>0.999
ALT (IU/L), median (range)	50 (16–679)	51 (16–253)	0.918
(≥31/<31)	46/6	60/11	0.605
γ-GTP (IU/L), median (range)	28 (10–239)	39 (11–314)	0.023
(≥43/<43)	15/37	31/40	0.131
Platelet count ($\times 10^4$/μL), median (range)	17.3 (4.2–32)	14.0 (4.5–35.5)	0.014
(≥15/<15)	37/15	32/39	0.006
HCV RNA (high/low)[a]	39/13	65/6	0.021
HCV serotype (I/II)	23/29	52/19	0.002
Interferon (monotherapy/combination)	13/39	14/57	0.515

SVR sustained viral response, *anti-HTLV-1* antibodies to human T-lymphotropic virus type 1, *ALT* alanine aminotransferase, *γ-GTP* γ-glutamyl transpeptidase, *HCV* hepatitis C virus

P values were determined by the Mann–Whitney *U* test, χ^2 test, or Fisher's test

[a] Classification was stated in "Methods"

However, there are some conflicting results. Our study showed a strong association between the prevalence of HTLV-1 infection and HCV-associated HCC in females, but not in males, although this tendency was observed in male patients. In contrast, Okayama et al. [30] reported an association in males, but not in females, although a similar trend was observed in female patients. Their study had a smaller number of patients (127 chronic hepatitis patients

without HCC and 43 HCC patients), especially females, and the proportion of patients with HTLV-1 seropositivity was low [14.7 % (25/170)] compared to our study. In addition, potential confounders such as age and alcohol consumption were not fully considered. These differences may account for the conflicting results. A prospective study involving a larger number of patients is required.

Several factors, such as male sex, older age, excessive alcohol consumption, and cigarette smoking, have been reported to be associated with an increased risk for progression of chronic liver disease due to HCV infection [10–12]. Our study also found that alcohol consumption and age are independently associated with HCC, and HTLV-1 tended to be associated with HCC in the multivariate analysis involving all patients (Table 2). The prevalence of alcohol consumption in males was higher than in females. Male HTLV-1 carriers have been reported to have a higher risk of developing ATLL than female HTLV-1 carriers [15]. In addition, the prevalence of HTLV-1 infection is known to vary by age and sex, with higher rates associated with older age and female sex in Japan. The increased prevalence with age may be due to the accumulation of infections over the lifetime of surveyed individuals or an age-cohort effect due to declining HTLV-1 prevalence over the past few decades. The higher prevalence in females may be the result of more efficient male-to-female sexual transmission [33, 34], or differences in socio-demographic or behavioral factors. Therefore, we speculate that such background factors or additional undetermined ones may contribute to different effects of HTLV-1 coinfection in males and females, although age was not significantly different between patients with HTLV-1 coinfection and those with HCV infection alone regardless of the presence of HCC in our study population.

Stuver et al. [31] also reported that HTLV-1 coinfection did not have any measurable impact on liver cancer mortality in subjects positive for anti-HCV antibodies. In contrast, Boschi-Pinto et al. [32] reported that dual HCV and HTLV-1 infection has a synergistic effect on death from HCC. Although these reports used similar cohort populations, the results seem to be conflicting. In addition, although Arisawa et al. [35] have reported that HTLV-1 infection was not associated with an overall increased risk for cancer, HTLV-1 infection was associated with an increased risk for liver cancer, which may be explained by the confounding by HCV infection and the interaction between HTLV-1 and HCV. However, these studies did not confirm the presence of HCV RNA or viremia, which can affect the results. In our study, we confirmed the presence of viremia and this confirmation may provide stronger evidence that HTLV-1 infection affects liver cancer mortality in patients with persistent HCV infection than previous reports. The overall and liver-related survival rates in patients with HTLV-1 coinfection were similar to those in patients with HCV infection alone in our study. However, as a result, mortality, especially liver-related mortality, tended to be affected by HTLV-1 coinfection in female patients (Fig. 2b). This tendency might be affected by the high prevalence of HTLV-1 coinfection in HCC patients. Therefore, we speculate that in female patients with persistent HCV infection, HTLV-1 coinfection affects liver cancer mortality. The follow-up period was relatively short for many patients in our study, which may have affected the results; further long-term longitudinal studies are needed.

HTLV-1 mainly infects CD4+ T cells. Other cell types such as CD8+ T cells and dendritic cells (DCs) may also serve as reservoirs of HTLV-1 [36, 37]. HTLV-1 carriers are reported to have an impaired cellular immune response [38]. Purified plasmacytoid DCs (pDCs) from asymptomatic HTLV-1 carriers were found to have impairments in IFN-α production [39]. Soguero et al. [40] reported that the expression of HCV core protein in T cells induces immune dysregulation by increasing apoptosis of T lymphocytes and the development of liver damage results from recruitment of these apoptotic lymphocytes into the liver. pDCs stimulated by Toll-like receptor ligand obtained from subjects with chronic HCV infection have impaired the ability to activate naive CD4+ T cells [41]. Therefore, an impaired immune response in HTLV-1 carriers could exacerbate liver injury in patients with HCV infection, and these effects are thought to promote hepatocarcinogenesis. However, liver injury involving increased levels of ALT in patients with HTLV-1/HCV coinfection occurred at rates similar to those in patients with HCV alone, regardless of the presence of HCC in our study. In addition, HTLV-1 may affect HCV RNA levels or the effectiveness of IFN treatment for HCV clearance [28, 42], but these differences were not observed in our study. In contrast, Ioannou et al. [43] reported that low CD4+ cell count was independently associated with HCC, but not with cirrhosis in patients with HIV infection. They also suggested that although the increased prevalence of HCC among HIV-infected patients was driven primarily by the HCV epidemic, immune suppression by HIV infection is more directly relevant to hepatocarcinogenesis than uncontrolled viral replication or liver cirrhosis [43]. Furthermore, γ-GTP was independently associated with HCC in all patients, and serum levels of γ-GTP were significantly higher in female HTLV-1 coinfected patients with HCC in our study. γ-GTP has been reported to be associated with an increased risk of oxidative stress [44] and increased carcinogenesis in subjects with HCV infection [45]. Takahashi et al. [46] reported that the HTLV-1 Tax oncoprotein stimulates the production of reactive oxygen species via interactions with ubiquitin-specific protease 10 in HTLV-1-infected T cells, although

the association between this effect of HTLV-1 Tax oncoprotein and γ-GTP or hepatocyte is unclear. Therefore, we speculate that HTLV-1 infection is more directly associated with hepatocarcinogenesis through various mechanisms in patients with persistent HCV infection, as opposed to indirectly via exacerbating liver injury.

This work has several limitations. First, the study was retrospective in nature. There was insufficient data available on IFN-based treatment status. Recent advances in therapy to eradicate HCV, as well as in the understanding of genetic factors such as interleukin 28B, may affect the clinical course of HCV carriers [47, 48]. The natural history and treatment outcome in chronic hepatitis C patients with or without HTLV-1 coinfection should be further examined in a prospective study. Second, many patients in this study were referred to our hospital because of the occurrence of HCC. Therefore, the prevalence of HCC among our study population was higher than in cohort-based studies [49]. Therefore, selection bias should be considered, and a cohort study would be desirable. Third, the presence of HTLV-1 infection was defined as positive anti-HTLV-1 antibody based on CLEIA or ECLIA, but there is the possibility of false positive results. Confirmation of HTLV-1 infection by other methods such as western blot analysis is needed. However, Hanaoka et al. [50] reported that the seroprevalence rate of HTLV-1 using enzyme-linked immunosorbent assay (ELISA) and western blot analysis are similar in endemic areas. Among Jewish immigrants from Iran, 24 of 331 blood donors (7.2 %) were positive for anti-HTLV-1 antibodies by ELISA, and all these donors were also positive for anti-HTLV-1 antibodies by western blot analysis [51]. In Kagoshima Prefecture, Japan, the anti-HTLV-1 antibody positive rate as determined by CLEIA among pregnant women was 125/4,147 (3.01 %), and the true positive rate based on western blot analysis was 85 % (106/125). Furthermore, in stored serum samples available from 15 study patients, including six HCC patients and nine non-HCC patients among the 83 patients with HTLV-1 coinfection, anti-HTLV-1 antibodies (to p19, p24, p53, and gp46 of HTLV-1) were detected by western blot analysis in all samples. Although there is a possibility of false positive anti-HTLV-1 antibody determination in our study population, such cases might be rare.

In conclusion, HTLV-1 coinfection was independently associated with HCC in patients with chronic liver disease due to HCV infection. HTLV-1 infection may be associated with the development of HCC, with this effect being particularly important in females.

Conflict of interest H. Tsubouchi holds endowed faculty positions in research for HGF tissue repair and regenerative medicine, and has received funds from Eisai Co., Ltd. The other authors declare that they have no conflict of interest.

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Cancer Research

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Cancer Research

Molecular Characterization of Chronic-type Adult T-cell Leukemia/Lymphoma

Noriaki Yoshida[1,2,3], Kennosuke Karube[1], Atae Utsunomiya[4], Kunihiro Tsukasaki[5], Yoshitaka Imaizumi[5], Naoya Taira[6], Naokuni Uike[7], Akira Umino[1,8], Kotaro Arita[1,9], Miyuki Suguro[1], Shinobu Tsuzuki[1], Tomohiro Kinoshita[10], Koichi Ohshima[3], and Masao Seto[1,2,3]

Abstract

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type-1–induced neoplasm with four clinical subtypes: acute, lymphoma, chronic, and smoldering. Although the chronic type is regarded as indolent ATL, about half of the cases progress to acute-type ATL. The molecular pathogenesis of acute transformation in chronic-type ATL is only partially understood. In an effort to determine the molecular pathogeneses of ATL, and especially the molecular mechanism of acute transformation, oligo-array comparative genomic hybridization and comprehensive gene expression profiling were applied to 27 and 35 cases of chronic and acute type ATL, respectively. The genomic profile of the chronic type was nearly identical to that of acute-type ATL, although more genomic alterations characteristic of acute-type ATL were observed. Among the genomic alterations frequently observed in acute-type ATL, the loss of *CDKN2A*, which is involved in cell-cycle deregulation, was especially characteristic of acute-type ATL compared with chronic-type ATL. Furthermore, we found that genomic alteration of *CD58*, which is implicated in escape from the immunosurveillance mechanism, is more frequently observed in acute-type ATL than in the chronic-type. Interestingly, the chronic-type cases with cell-cycle deregulation and disruption of immunosurveillance mechanism were associated with earlier progression to acute-type ATL. These findings suggested that cell-cycle deregulation and the immune escape mechanism play important roles in acute transformation of the chronic type and indicated that these alterations are good predictive markers for chronic-type ATL. *Cancer Res; 74(21); 1–10. ©2014 AACR.*

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Introduction

Adult T-cell leukemia/lymphoma (ATL) is a human T-cell leukemia virus type-1 (HTLV-1)–induced neoplasm (1, 2). Four clinical subtypes of ATL have been classified on the basis of clinical manifestation: acute, lymphoma, chronic, and smoldering (3). Among these subtypes, chronic-type ATL shows characteristic manifestations such as increased abnormal lymphocytes in peripheral blood, lactate dehydrogenase (LDH) levels up to twice the normal upper limit, and absence of hypercalcemia. Chronic-type ATL is relatively rare and its frequency is estimated to be 8% to 18% of ATL cases (3). Previous reports regard the chronic type as indolent ATL compared with acute/lymphoma types, which show an aggressive clinical course (3, 4). However, a recent study of indolent ATL demonstrated that about half of the patients with chronic-type ATL progress to acute-type ATL within approximately 18 months from diagnosis and subsequent death (4). This finding suggests that patients with chronic-type ATL also had a poor prognosis. High LDH, high blood urea nitrogen, and low albumin levels have been identified as poor prognostic factors for chronic-type ATL, and patients with chronic-type ATL with these poor prognostic factors therefore need to be treated by intensive chemotherapy as in the case of patients with aggressive ATL (5).

Disruptions of *CDKN2A*, *CDKN2B*, and *TP53* have been reported as candidate genes that play important roles in acute

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transformation of chronic-type ATL (6–12). However, these acute transformation–related genetic alterations have been identified only by focusing on genes that were previously shown to be involved in tumor progression of other malignancies. Therefore, these genetic alterations may be indicative of acute transformation in some cases, although the molecular mechanism of acute transformation remains to be fully elucidated. Identification of the molecular characteristics of chronic-type ATL using unbiased and genome-wide methods can provide further insights to elucidate the acute transformation mechanisms in chronic-type ATL. However, the molecular pathogenesis of chronic-type ATL has long remained unknown due to its rarity (13).

In the present study, high-resolution oligo-array comparative genomic hybridization (aCGH) and gene expression profiling (GEP) were applied to 27 cases of chronic-type ATL in an effort to determine the molecular pathogenesis. The same approaches were used with 35 cases of acute-type ATL, and we then compared the molecular characteristics of chronic- and acute-type ATL to investigate the molecular mechanism of acute transformation.

Materials and Methods

Patient samples

We collected and analyzed 27 cases of chronic-type ATL and 35 cases of acute-type ATL (Table 1 and Supplementary Table S1 in Supplementary Data). These samples were obtained from patients at Imamura-Bunin Hospital (Kagoshima, Japan), Nagasaki University School of Medicine (Nagasaki, Japan), Heart Life Hospital (Nakagusukuson, Japan), and Kyushu Cancer Center (Fukuoka, Japan). In accordance with Shimoyama criteria, the diagnoses were made by expert hematologists (A. Utsunomiya, K. Tsukasaki, Y. Imaizumi, N. Taira, and N. Uike; ref. 3). Samples and medical records used in our study were approved by the Institute Review Board of the Aichi Cancer Center (Nagoya, Japan). Informed consent was obtained according to the Declaration of Helsinki from all patients. DNA and RNA used in this study were extracted from purified CD4-positive cells as previously reported (14). For the cumulative incidence of acute transformation, events were defined as acute transformation or any treatment for ATL.

Copy number analysis by aCGH and GEP

We performed aCGH analysis on all samples using 400K aCGH (Agilent, Cat. # G4448A; Agilent Technologies) and 44K aCGH (Agilent, Cat. # G4413A) slides (Supplementary Table S1). Thirteen acute-type cases analyzed in a previous study were included (14). Procedures for DNA digestion, labeling, hybridization, scanning, and data analyses were performed according to the manufacturer's protocols (www.agilent.com). Raw data were transferred to the Genomic Workbench v5.0 software (Agilent Technologies) for further analysis as described previously (14–16). Among these identified alterations, we focused on minimal common regions (MCR). MCRs are defined as alterations that encompass less than 3 protein-coding genes among all samples analyzed in this study (17). Copy number variations/polymorphisms (CNV) were identified using a database (HS_hg18_CNV-20120403, Agilent), which was obtained from Database of Genomic Variants (http://projects.tcag.ca/variation/) in April 2012 and then excluded from further analyses as described previously (16). We also performed aCGH analysis on matched normal DNA samples that were available and confirmed that the identified MCRs were not CNVs (Supplementary Fig. S1A).

For analysis of GEP, the Whole Human Genome 44K Oligo-microarray Kit (Agilent, Cat. # G4112F) was used for the hybridization of labeled RNA. The total RNA of 13 chronic samples and 21 acute samples was analyzed. The experimental protocol used reflected the manufacturer's protocol (www.agilent.com) as previously reported (15, 16). Using the results of GEP, gene set enrichment analysis (GSEA) was performed as previously described (15, 16, 18).

The detailed description of these analyses can be found in Supplementary Methods. The microarray data were submitted to ArrayExpress and assigned accession numbers E-MTAB-1808 (aCGH) and E-MTAB-1798 (GEP).

Mutation analyses of CD58 and β2-microglobulin

The exons 1–4 of *CD58* and 1 and 2 of *β2-microglobulin* (*B2M*), whose mutations were identified in peripheral T-cell lymphomas (PTCL; ref. 19), were amplified from gDNA using PCR. PCR primers used are detailed in the previous study (20). Twenty-six acute-type and 26 chronic-type ATL samples, for which adequate DNA was available, were analyzed. Direct sequencing of PCR products was performed through capillary electrophoresis using the ABI3100 sequencer (Applied Biosystems).

Flow cytometry

Analysis of cell surface CD58 in ATL cell lines was performed using anti-CD58 PE antibody (AlCD58, Beckman Coulter).

Table 1. Patient information at sampling

Subtype	No. of samples	Median age (range), y	Median WBC (range), u/L	Median LDH (range), IU/L	Median calcium (range), mg/dL	Median albumin (range), g/dL	Median BUN (range), mg/dL
Chronic type	27	61 (42–81)	1,1400 (6,000–22,100)	233 (155–465)	9.3 (8.4–10.2)	4.2 (3.0–4.8)	15.5 (7.4–26.4)
Acute type	35	57 (32–85)	2,1700 (4,100–224,800)	688 (203–2,223)	9.3 (7.7–17.4)	3.8 (2.6–4.5)	NA

Abbreviations: BUN, blood urea nitrogen; NA, not available; WBC, white blood cells.

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Analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo Version 7.2.4 software (TreeStar). The detailed description of these analyses can be found in Supplementary Methods.

Statistical analysis

Frequencies of genomic alterations were evaluated using Fisher exact test, and cumulative acute transformation rates were analyzed using Kaplan–Meier method.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing; ref. 21).

Results

Genomic alteration profiles of chronic- and acute-type ATL

To evaluate the genomic alterations of chronic- and acute-type ATL, aCGH was performed for 62 patient samples (27 cases of chronic-type and 35 cases of acute-type ATL; Table 1 and Supplementary Table S1). Figure 1A shows genomic alteration profiles of chronic- and acute-type ATL. We identified 362 MCRs (230 losses and 132 gains) among the alterations. These MCRs contained 1–3 protein-coding genes, which are most likely the candidate genes of the alterations (15, 17). Frequent alterations are supposed to especially contribute to the pathophysiology of the disease. MCRs that were found in

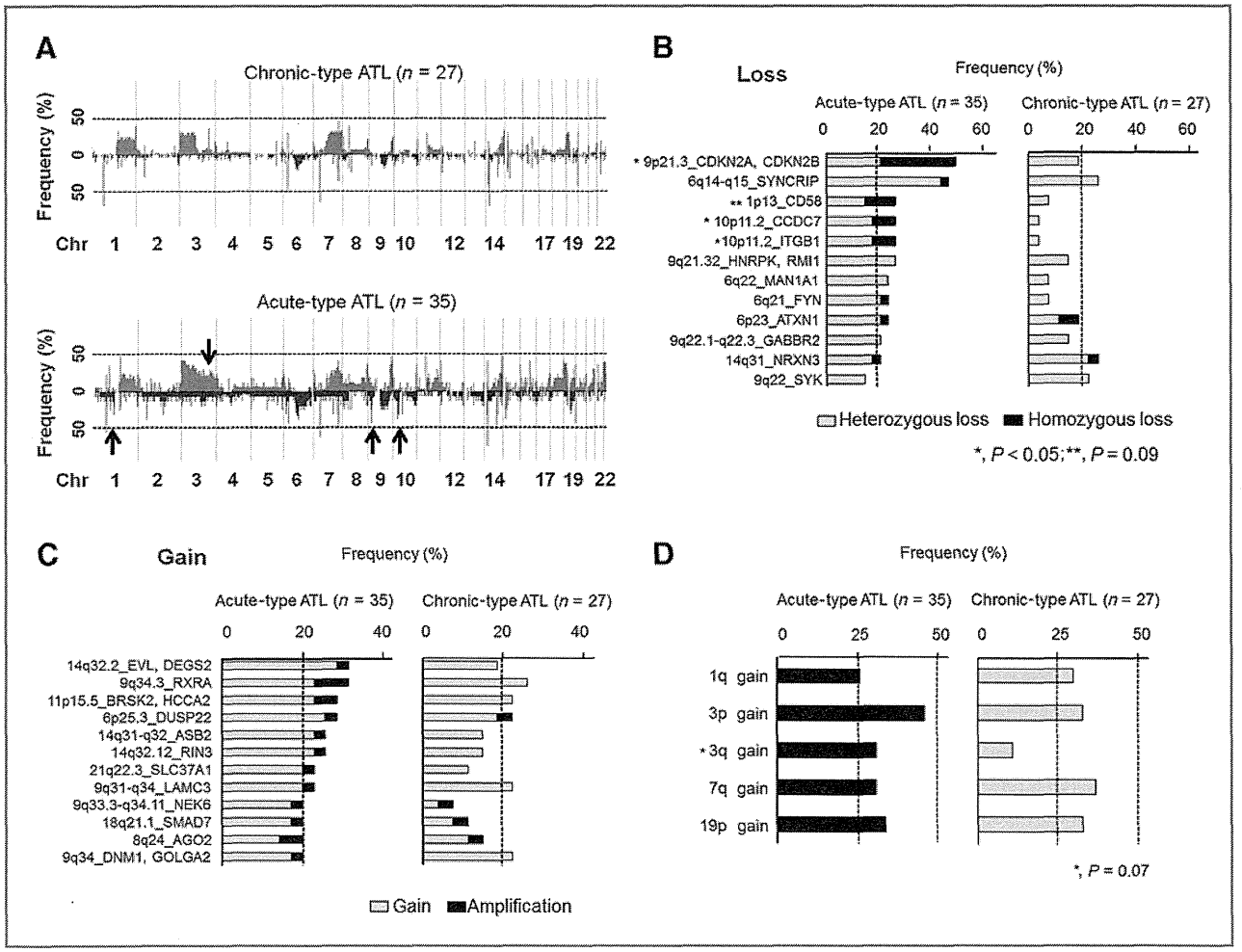

Figure 1. Genomic alteration profiles of chronic- and acute-type ATL. A, frequency of genomic alterations in chronic-type and acute-type ATL. Top, 27 cases with chronic-type ATL; bottom, 35 cases with acute-type ATL. The horizontal axis indicates each probe aligned from chromosome 1 to 22 and the short arm (p) to long arm (q). The vertical axis indicates the frequency of genomic alterations among the analyzed cases. The top area represents gain and the bottom area represents loss. Arrows represent characteristic alterations of acute-type ATL compared with chronic-type. B, MCRs encompassing 1–3 coding genes of copy number loss. MCRs found in greater than 20% of chronic-type or acute-type ATL are shown and ranked by frequency of alteration (left, acute type; right, chronic type). Among these MCRs, loss of *CDKN2A/CDKN2B* located in 9p21.3, losses of *CCDC7* and *ITGB1* located in 10p11.2 were observed more frequently in acute-type ATL. Loss of *CD58* was also found more frequently in acute type than in the chronic type (Fisher exact test; *, $P < 0.05$; **, $P = 0.09$). Frequently altered MCRs in chronic-type ATL were also recognized in the acute type. C, MCRs of copy number gain. MCRs found in greater than 20% of chronic-type or acute-type ATL are shown and ranked by frequency of alteration (left, acute type; right, chronic type). None of these MCRs were characteristic of acute-type or chronic-type ATL. D, gains of chromosomes 1q, 3p, 3q, 7q, and 19p were observed in greater than 20% of acute-type and chronic-type ATL. MCRs were not detected in any of these lesions. Gain of 3q was more frequently found in acute-type ATL than in the chronic type (*, $P = 0.07$).

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more than 20% of chronic- or acute-type ATL were therefore analyzed (Fig. 1B and C).

Genomic loss of *CDKN2A/CDKN2B* was the first most frequently altered MCR in acute-type ATL (17 of 35 cases). The second most frequently altered MCR of acute-type ATL was genomic loss of *SYNCRIP* (16 of 35 cases). On the other hand, genomic losses of *SYNCRIP* and *NRXN3* and gain of *RXRA* were most frequently altered MCRs in chronic-type ATL (7 of 27 cases). Among these identified MCRs, the losses of *CDKN2A/CDKN2B*, *CCDC7*, and *ITGB1* were significantly characteristic of acute-type ATL (Fig. 1B, $P < 0.05$). In addition, acute-type ATL tended to have a loss of *CD58* (Fig. 1B). The frequently altered MCRs in chronic-type ATL were also found in acute-type ATL (Fig. 1B and C). Gains of chromosomes 1q, 3p, 3q, 7q, and 19p were also frequently observed in acute- and chronic-type ATL, although they did not show MCRs (Fig. 1D). Among these alterations, acute-type ATL tended to have a gain of 3q ($P = 0.07$).

Frequent loss of CDKN2A/CDKN2B

Our analysis identified loss of *CDKN2A/CDKN2B* located in 9p21.3 as the most frequently and specifically altered genomic region in acute-type ATL compared with chronic-type ATL. Therefore, this loss is suggested to play an important role in the pathophysiology of acute-type ATL and acute transformation of chronic-type ATL.

Seventeen of the 35 acute-type ATL samples showed loss of 9p21.3, which was also found in 5 of the 27 chronic-type ATL samples. These losses always included *CDKN2A/CDKN2B* (Fig. 2A). Homozygous loss of *CDKN2A/CDKN2B* was observed in 10 of the 17 affected acute-type ATL samples but was never observed in chronic-type ATL. The genes whose expression was affected by copy number changes are considered candidate genes in the regions of genomic alterations (15, 22, 23). We therefore evaluated the expressions of CDKN2A and CDKN2B in acute-type and chronic-type ATL with or without loss of 9p (Fig. 2B). CDKN2A expression was much lower in acute-type ATL samples with the loss of 9p than in other samples. CDKN2B expression was not reduced in accordance with the loss of 9p. Therefore, CDKN2A is a likely candidate tumor suppressor gene located in 9p21.3.

Serial samples of a patient with chronic-type ATL showing acute transformation were analyzed in detail. The DNA and RNA samples of this patient at about 19 months before acute transformation (chronic phase, C-10) and at acute transformation (acute phase, A-15) were available. Clonality analysis of T-cell receptor gamma locus showed that clones of ATL cells at chronic and acute phases were identical to each other (Supplementary Fig. S1B). Although the chronic-phase sample showed heterozygous loss of *CDKN2A/CDKN2B*, the acute-phase sample showed homozygous loss of *CDKN2A/CDKN2B* (Fig. 2C). In addition, the expression of CDKN2A was remarkably reduced in the acute phase (Fig. 2D). Analysis of these serial samples of an identical patient also indicated that CDKN2A is the most likely candidate gene located in 9p21.3 and that the loss of *CDKN2A* is associated with acute transformation.

Frequently altered cell-cycle pathway in acute-type ATL

CDKN2A contains 2 known transcriptional variants, *INK4a* (*p16*) and *ARF* (*p14*). Both of these genes are known to be negative regulators of the cell cycle. We next evaluated the distributions of genomic alterations of *CDKN2A* with other genes that were previously reported to affect the cell cycle (Fig. 2E; ref. 24). Our analysis revealed that losses of *CDKN2A* and losses of *TP53* tended to be mutually exclusive events, and this pattern was also observed for losses of *TP53* and gains of *MDM4/RFWD2*. These alterations of cell-cycle–related genes were specifically observed in acute-type ATL compared with chronic-type ATL (80% of acute-type and 56% of chronic-type ATL, $P < 0.05$; Fig. 2F). Among chronic-type ATL cases, those with acute transformation tended to have alterations of cell-cycle–related genes (Fig. 2G). GSEA also revealed that the cell-cycle–related gene set and genes functionally associated with proliferation were significantly enriched in acute-type ATL compared with chronic-type ATL (Supplementary Fig. S1C).

These results indicated that alterations of the cell-cycle pathway, including the genomic loss of *CDKN2A*, played critical roles in the pathophysiology of acute-type ATL and acute transformation of chronic-type ATL. *In vitro* assays showed that inductions of INK4a or ARF that are encoded by *CDKN2A* caused suppression of cell proliferation, cell-cycle arrest, and apoptosis in ATL cell lines with genomic loss of 9p21.3 (Supplementary Fig. S2).

Genomic alterations of CD58 in ATL

In addition to loss of *CDKN2A/CDKN2B*, we found that losses of *CCDC7*, *ITGB1*, and *CD58* and gain of chromosome 3q were more frequently recognized in acute-type ATL than in chronic-type ATL. Alterations of cell-cycle–related genes, including *CDKN2A*, are considered important events for the transformation described above. We therefore analyzed the distributions of alterations of cell-cycle–related genes and the genes that were characteristic of acute-type ATL in each type of ATL case (Fig. 3). This analysis revealed that alterations of cell-cycle–related genes and the gene alterations characteristic of acute-type ATL mainly coexisted. A case having the loss of *CD58* or gain of 3q without alterations of cell cycle existed for each type of ATL, although all cases with losses of *ITGB1* and *CCDC7* showed the alterations of cell-cycle–related genes.

In chronic-type ATL cases without alterations of cell-cycle–related genes, a case with loss of *CD58* showed acute transformation later, although a case with gain of 3q did not exhibit the transformation without any therapy during 30 months after the diagnosis. *CD58* is a gene known to be involved in activation of natural killer (NK) cells and cytotoxic T cells (CTL; refs. 25, 26). Inactivation of *CD58* is reported to play an important role in the pathophysiology of diffuse large B-cell lymphoma (DLBCL) through the mechanism of escape from the immunosurveillance system (20). Recurrent mutation of *CD58* has also been observed recently in PTCLs (19). We therefore further analyzed *CD58* in ATL.

Analyses using aCGH revealed that 26% (9 of 35) of acute-type ATL and 7% (2 of 27) of chronic-type ATL had genomic loss of 1p13 (Figs. 1B and 4A). These losses always included *CD58* and one case showed genomic loss that only included

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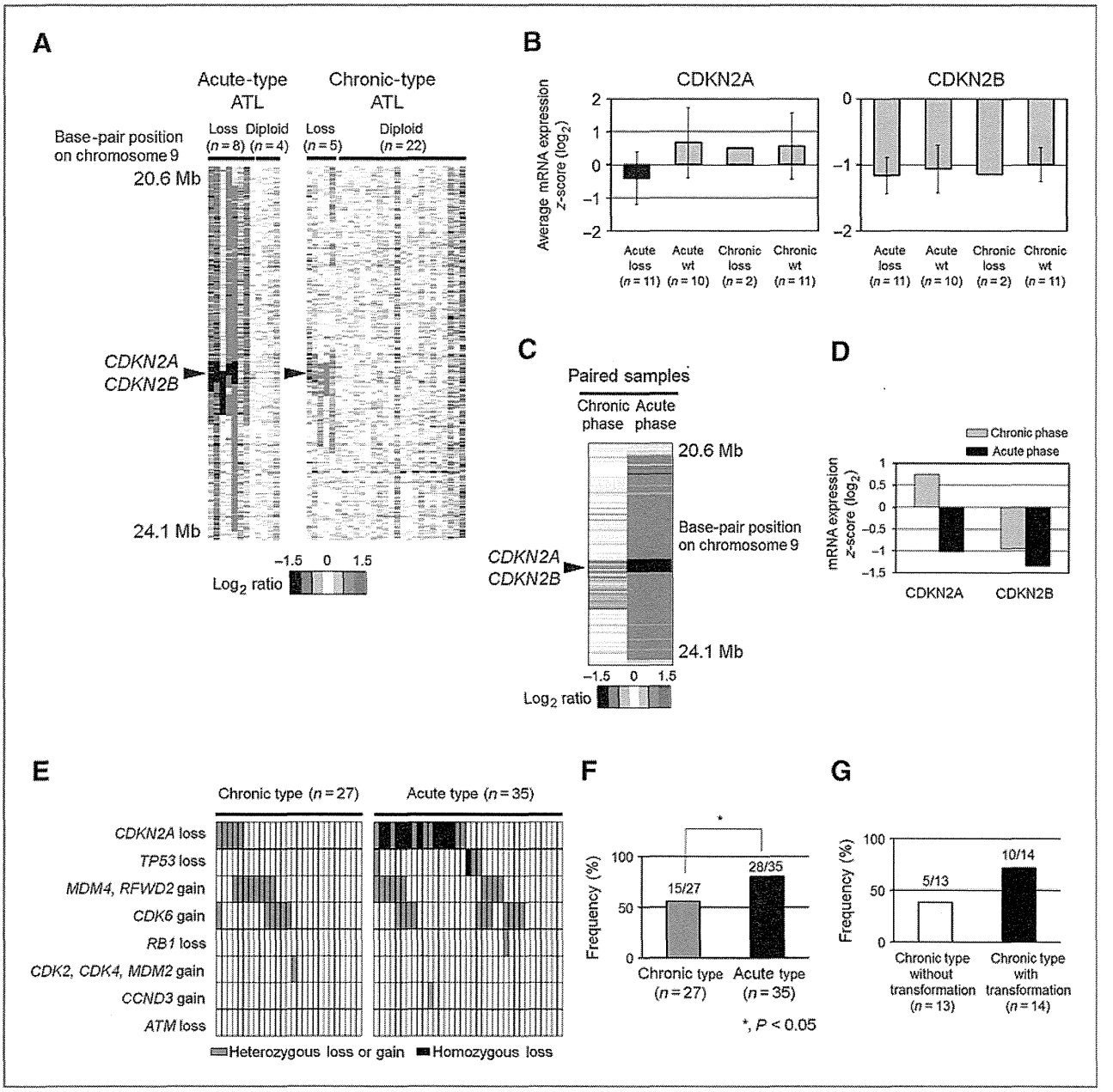

Figure 2. Loss of 9p was mainly observed in acute-type ATL and not chronic-type ATL. A, genomic alterations of chromosome 9p, including *CDKN2A/CDKN2B*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of tumor cells relative to normal controls. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the *CDKN2A/CDKN2B* locus. B, gene expression levels of CDKN2A and CDKN2B. Gene expression levels of CDKN2A and CDKN2B were analyzed in 13 chronic-type and 21 acute-type ATL cases by GEP. Average gene expressions and SDs are shown in cases grouped as indicated. CDKN2A expression was reduced only in acute-type ATL cases exhibiting loss of *CDKN2A/CDKN2B*. CDKN2B expression did not change in relation to genomic loss or subtype. Probes of A_23_P43484 (CDKN2A) and A_23_P216812 (CDKN2B) were used in experiments. C, genomic alteration of 9p in serial samples of a case with chronic type showing acute transformation. Left, a heatmap of the $\log_2$ ratio in the chronic phase; right, a heatmap of the ratio in the acute phase. The sample in the chronic phase indicates a heterozygous loss of the *CDKN2A/CDKN2B* locus and the loss changes to a homozygous loss for the sample in the acute phase. D, gene expressions of CDKN2A and CDKN2B in serial samples. CDKN2A expression was remarkably reduced in the acute phase, but CDKN2B expression was almost identical during transformation in this case. Gray, the chronic phase; black, the acute phase. E, alterations of cell-cycle–related genes in chronic-type and acute-type ATL. In the heatmap, rows correspond to the indicated alterations and columns represent individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Losses of *CDKN2A* and *TP53* tended to be mutually exclusive, and losses of *TP53* and gains of *MDM4/RFWD2* showed a similar tendency. F, alteration frequency of cell-cycle–related genes. Genetic alteration frequency of cell-cycle–related genes was significantly higher in acute-type ATL cases (80%) than in chronic-type ATL (56%; Fisher exact test; *, $P < 0.05$). The actual number of affected samples over the total number analyzed is shown at top of the figure. G, alteration frequency of cell-cycle–related genes among chronic-type ATL cases. The frequency of alterations of cell-cycle–related genes was higher in cases with later acute transformation than in cases without acute transformation.

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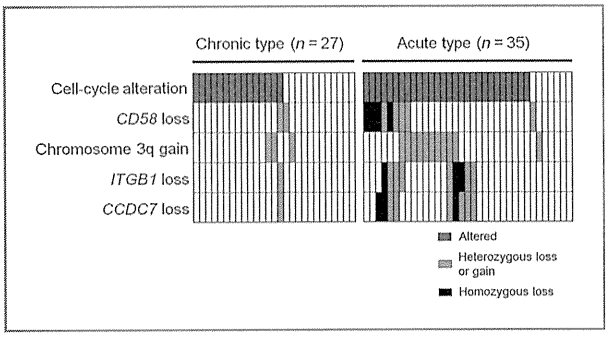

Figure 3. Distribution of genomic alterations frequently observed in acute-type ATL among ATL samples. Heatmap with rows corresponding to the indicated alterations and columns representing individual ATL cases. Gray, a heterozygous loss or gain; black, a homozygous loss. Dark gray also shows the alterations of any cell-cycle–related gene. Alterations frequently found in acute-type ATL were not mutually exclusive of the alteration of cell-cycle–related genes. Cases with losses of *ITGB1* and *CCDC7* always exhibited the alterations of cell-cycle–related genes. Most cases with loss of *CD58* or gain of 3q also exhibited the alterations of cell-cycle–related genes, but a case showing the loss of *CD58* or gain of 3q without disruption of the cell cycle existed in each type of ATL. The loss of *CD58* and gain of 3q were almost mutually exclusive, except for two cases of acute-type ATL.

CD58. Homozygous loss of *CD58* was observed only in acute-type ATL samples. Furthermore, expression of CD58 was reduced in acute-type ATL cases accompanied with the genomic loss (Fig. 4B). Flow cytometric analyses also suggested that genomic loss of *CD58* reduced the expression on the cell surfaces (Fig. 4C). Sequence analysis of *CD58* revealed a nonsense mutation in one acute-type ATL case. This mutation indicated that the 97th position of serine changed to a stop codon (p.S97X; c.290C>A; Fig. 4D). The nontumor cells of this patient showed no mutation, and we therefore regarded this mutation as a somatic mutation. One-nucleotide substitution registered as an SNP in the NCBI database (http://www.ncbi.nlm.nih.gov/gene/) was found in 7 cases (c.43A>G; rs17426456; Supplementary Table S2). Combined with the results of the genomic and mutation analyses, 29% of acute-type and 7% of chronic-type ATL had genetic alteration of *CD58*. These alterations were significantly specific to acute-type ATL compared with chronic-type ATL (Fig. 4E, $P = 0.05$).

In addition to the alteration of *CD58*, inactivation of *B2M* is also reported to play a pivotal role in the immune escape mechanism of DLBCLs (20). Among analyzed cases, only a chronic-type ATL case (C-2) had heterozygous loss of *B2M*, and this case also showed heterozygous loss of *CD58* (Supplementary Table S2). No somatic mutations of *B2M* were observed in ATL cases analyzed.

Genomic alterations predicting acute transformation of chronic-type ATL

We investigated the associations of MCRs that were characteristic of acute-type ATL and that were commonly found in more than 20% of chronic- and acute-type ATL with cumulative acute transformation rates among chronic-type ATL cases (Supplementary Table S3).

Cases exhibiting gain of *RXRA* and loss of *ITGB1*, *CCDC7*, or *CD58* were significantly associated with early progression to acute-type ATL ($P = 0.01$, 0.02, 0.02, and 0.04, respectively; Fig. 5A). Chronic-type ATL cases having the alterations of cell-cycle–related genes also tended to show early progressions to acute-type ATL ($P = 0.07$; Fig. 5B), although cases having only the loss of *CDKN2A* were not significantly associated with the progression (Supplementary Table S3). A chronic-type ATL case with losses of *ITGB1* and *CCDC7* had the alterations of cell-cycle–related genes, and we therefore analyzed the chronic-type ATL cases by the presence of alterations of *CD58* and/or cell-cycle–related genes. This analysis revealed that cases with these alterations were specifically associated with earlier progression to acute-type ATL ($P = 0.03$, Fig. 5C).

Discussion

We have studied 27 cases of chronic-type ATL and compared with 35 cases of acute-type ATL. Until now, only a few chronic-type ATL cases had been analyzed, and the molecular mechanisms of the transformation were investigated by focusing on the well-known tumor suppressor genes (*CDKN2A* and *TP53*; refs. 6–12). In contrast, our investigation comprehensively analyzed genomic profiles, and molecular aspects were analyzed using unbiased and whole-genome methods. Our study of chronic-type ATL represents the largest study to date that has analyzed the whole-genomic status of chronic-type ATL cases. We could identify characteristic molecular profile of chronic-type ATL and could demonstrate possible molecular mechanisms of acute transformation. This study suggested that alterations of cell-cycle–related genes and *CD58* are new predictive implications for chronic-type ATL (Fig. 5C).

Common genomic alterations in chronic- and acute-type ATL

Genomic alteration profiles of chronic- and acute-type ATL were found to be almost identical (Fig. 1). The number of genomic alterations was found to be higher in acute-type ATL than in the chronic-type, and the frequently altered regions of chronic-type ATL were also observed in the acute-type. Thus, chronic-type ATL might be a pre-acute form of the disease.

The common MCRs in chronic- and acute-type ATL included genes involving T-cell receptor signaling, such as *FYN* and *SYK* (27, 28). We also identified *SYNCRIP* as a common MCR in both types of ATL. *SYNCRIP* is a gene known to be involved in maturation of mRNA (29). *RXRA*, which has been reported to be implicated in colorectal carcinogenesis (30), is also frequently altered in both types of ATL. In addition, our analysis suggested that gain of *RXRA* is involved in acute transformation of chronic-type ATL because the chronic-type ATL possessing the gain of *RXRA* showed earlier progression to the acute-type. These MCRs may play important roles in the development of ATL coordinately with HTLV-1.

Deregulation of the cell-cycle pathway: an alteration related to acute transformation

Our analyses of genomic alterations revealed that no single genomic alteration seems to be responsible for the mechanism

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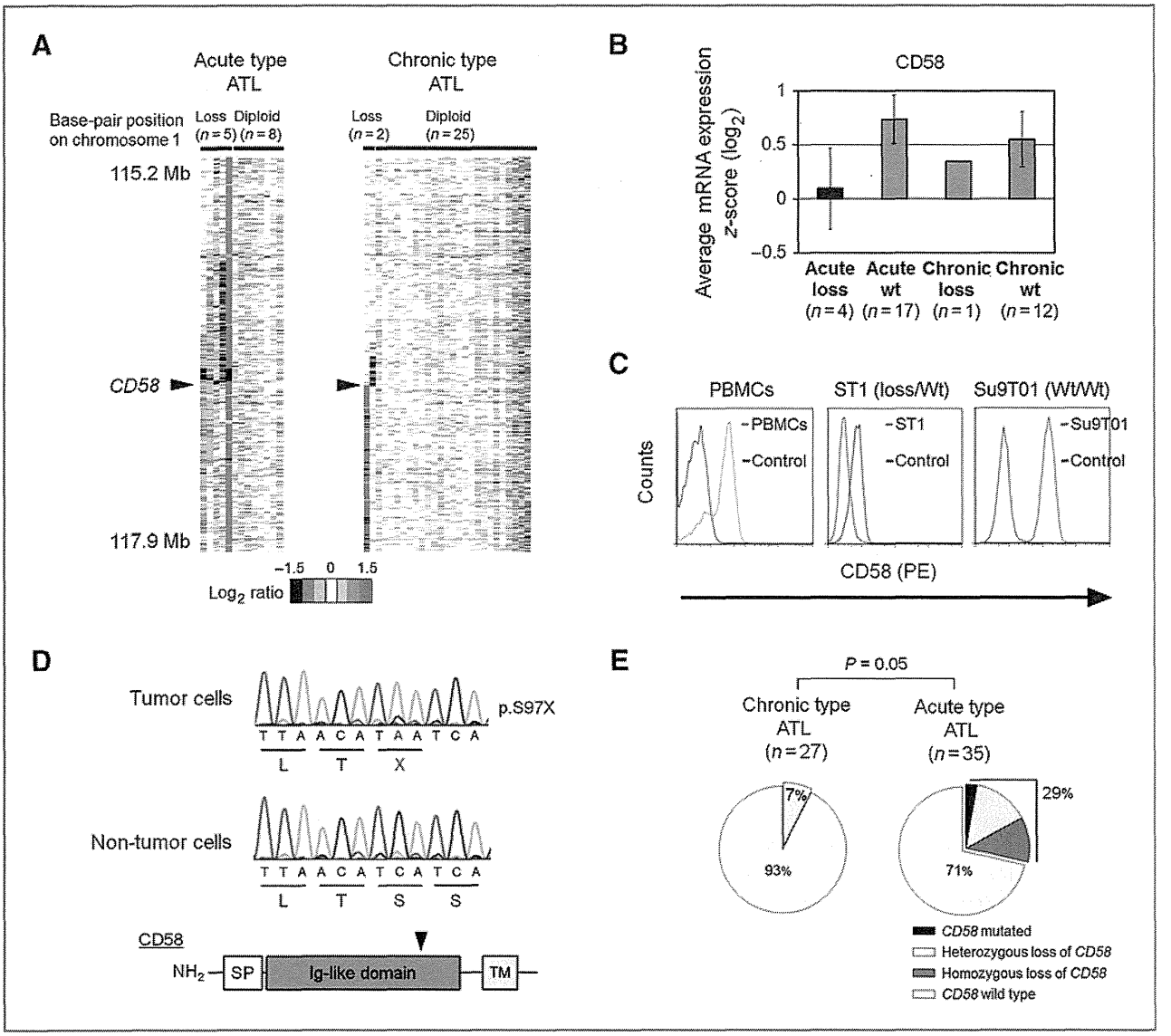

Figure 4. Alteration of *CD58* in acute transformation of chronic-type ATL. A, genomic alterations of chromosome 1p, including *CD58*. Heatmap analysis of 400K aCGH shows $\log_2$ ratios of ATL cases. White, blue, and red represent diploid, loss, and gain, respectively. Arrowhead, the *CD58* locus. B, gene expression levels of CD58. Expression was analyzed in 13 chronic-type and 21 acute-type ATL cases by GEP. Average gene expressions and SDs are shown in cases grouped as indicated. CD58 expression was reduced only in acute-type ATL cases exhibiting loss of *CD58*. Probe A_23_P138308 (CD58) was used in experiments. C, CD58 expressions on ATL cell lines and peripheral blood mononuclear cells (PBMC). Flow cytometric analysis of PBMCs from a healthy donor and two ATL cell lines for surface CD58 expression (orange line, PBMCs; blue line, ST1; red line, Su9T01). ST1 with heterozygous loss of CD58 had the low expression. The gray lines represent the cell lines with the isotype control antibody. D, DNA sequencing chromatogram of an acute-type ATL case (A-35) showing nonsense mutation in exon 2 of *CD58* (top). DNA extracted from nontumor cells (CD4-negative cells in peripheral blood of this patient) did not show the mutation (middle). Bottom, a schematic representation of the CD58 protein depicting the location of the single peptide (SP), Ig-like domain, and transmembrane domain (TM). The inverted triangle indicates the position of the mutation. E, characterization of *CD58* alteration in ATL. Seven percent of chronic-type ATL cases showed genomic loss of *CD58*, whereas 29% of acute-type ATL cases showed genomic alteration of *CD58*, with one case exhibiting mutation (Fisher exact test; $P = 0.05$).

of acute transformation, and various genomic alterations and combinations of alterations exist in this mechanism (Fig. 3). We found that deregulation of the cell cycle, including genomic loss of *CDKN2A*, might be an important event in the transformation. Genomic loss of *CDKN2A* was also reported to play a crucial role in the transformation of chronic lymphocytic leukemia known as Richter syndrome (31, 32).

Although previous studies using Southern blot analysis revealed that 11% to 17% of acute-type ATL had the homozygous loss of *CDKN2A* (7, 9), our analyses using unbiased and whole-genome methods were able to reveal the frequency of the loss in greater detail. We found that approximately 30% of acute-type ATL cases showed a homozygous loss of the *CDKN2A/CDKN2B* locus, and 50% of acute-type ATL cases

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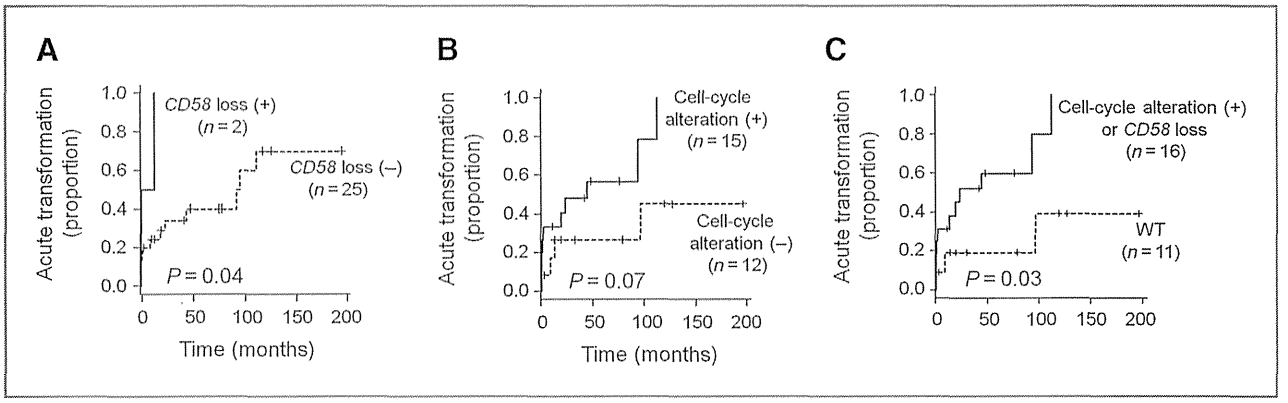

Figure 5. Genomic alterations associated with acute transformation in chronic-type ATL. A, genomic loss of *CD58* was significantly associated with earlier acute transformation ($P = 0.04$). B, chronic-type ATL cases with alterations of cell-cycle–related genes tended to exhibit earlier progression to acute-type ATL ($P = 0.07$). C, cases with either *CD58* loss or alterations of cell-cycle–related genes showed a much shorter time to acute transformation within chronic-type ATL cases ($P = 0.03$).

exhibited the homozygous or heterozygous loss of this locus. Yamagishi and colleagues used high-resolution aCGH analyses and found that this loss was frequently found in ATL samples (33). We also found that 5 of 27 chronic-type ATL cases had heterozygous loss of *CDKN2A*. Three of the 5 cases with *CDKN2A* loss progressed to the acute type, but 11 of the 22 cases without *CDKN2A* loss also showed acute transformation. Because of this finding, *CDKN2A* loss was not significantly associated with the earlier acute transformation in our study (Supplementary Table S3). Although previous studies revealed that approximately 5% of chronic-type had this loss (7, 9, 10), these previous studies did not show the cumulative acute transformation rate according to *CDKN2A* loss.

CDKN2A expression was reduced in acute-type ATL samples exhibiting genomic loss of the *CDKN2A* locus. A portion of acute-type ATL cases without the genomic loss showed a low expression level of CDKN2A, suggesting that methylation of the gene might affect the expression in these samples (11, 12). However, we consider that the genomic loss of *CDKN2A* has a greater influence on the expression of the gene than the methylation because the CDKN2A expression levels were remarkably reduced in accordance with the genomic loss (Fig. 2B and D).

Alterations of both *CDKN2A* and *TP53* were previously reported to be mutually exclusive (34), and our results showed the same trend. In addition, loss of *TP53* and gains of *MDM4*/*RFWD2* tended to be mutually exclusive in our acute-type ATL samples. Because these genes are involved in the TP53 pathway, our findings indicate that the TP53 pathway may also play a pivotal role in the pathophysiology of acute-type ATL. In fact, 80% of acute-type ATL had the alterations of cell-cycle–related genes, including *CDKN2A* and *TP53*. On the basis of this finding, we found that the alterations of cell-cycle–related genes might be predictive factors for acute transformation in chronic-type ATL cases (Fig. 5B).

Disruption of the immunosurveillance system in acute transformation of chronic-type ATL

The combined analyses of aCGH and sequencing revealed that 19% of ATL cases (7% of chronic-type and 29% of acute-type ATL) exhibited the *CD58* alteration. One acute-type ATL case showed somatic mutation, and the other cases showed genomic loss of the *CD58* locus. The alteration of *B2M* was a rare event in ATL compared with DLBCL (20). CD58 is a ligand of the CD2 receptor that is expressed on CTLs and NK cells and contributes to adhesion and activation of these cells. Previous reports showed that CTLs and NK cells could not recognize and injure target cells when treated with monoclonal CD58 antibody (35, 36). It is important to note that immune escape mechanism by CD58 inactivation was proven in DLBCL by Challa-Malladi and colleagues (20). The genomic loss and nonsense mutation of CD58 were for the first time demonstrated in ATL in this study and were suggested to be a predictive marker for acute transformation in chronic-type ATL. Therefore, the immune escape mechanism by the CD58 inactivation is likely to be involved in the pathophysiology of ATL as shown in DLBCL although detailed analysis is needed in the future.

Administration of immunosuppressive drugs to HTLV-1 carriers is currently considered a risk factor for early development of ATL (37, 38). It has been also suggested that immune escape from CTLs is induced by inactivation of the Tax protein derived from HTLV-1 in ATL (39–41). In addition, a report also suggested that immune escape from NK cells played an important role in ATL development (42). These findings suggest the presence of an immune escape mechanism in the pathophysiology of ATL. The present result regarding the significance of *CD58* alteration as a predictive factor for acute transformation in chronic-type ATL should be validated in more number of cases in the future study. Further studies are also needed regarding the protein expressions of CD58, B2M, and human leukocyte antigen class I.

In conclusion, our comparison of the molecular characteristics of chronic-type and acute-type ATL revealed that deregulation of the cell cycle and escape from the immune system are likely to be involved in acute transformation of chronic-type ATL. Development of ATL is thought to involve accumulation of several genomic alterations (43). The alterations of both pathways discovered in this study might be the late events following viral infection in the pathophysiology of ATL. These alterations could serve as biomarkers for patients with

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